Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted April – June 2023

Contents

1. Advancing the Knowledge and Treatment of Lung and Respiratory Diseases using Sirnas

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

RNA, siRNA, Respiratory disease, Nucleic acid, Nucleic acid delivery

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Small interfering RNA (siRNA), sometimes known as short interfering RNA, is a class of double-strandRNA (a nucleic acid present in all living cells) that operates within the RNA interference (RNAi) pathway. It interferes with the expression of specific genes with complementary nucleotide sequencesby degrading mRNA and preventing translation. Gene silencing by RNA interference is a rapidly growing therapeutic area for managing diseases. Despite research advances in this direction, poor cellular uptake and the stability of synthetic small interfering RNAs (siRNAs) are major impediments totheir clinical applications. The primary objective of this project licence is to develop novel delivery methods for siRNA to the respiratory tract and lung and to test novel chemical modifications that will enhance their stability and activity.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The respiratory system is persistently exposed to airborne disease-causing insults, including pollution,dust mites, viruses, bacteria, and fungi. These contagions significantly contribute to or cause commonchronic respiratory diseases, including Chronic Obstructive Pulmonary Disease (COPD), asthma and acute and chronic lung infections. Other lung diseases, for example, idiopathic pulmonary fibrosis, aremore complex in their origin, and some are genetic, such as cystic fibrosis (CF). Besides cancer and cardiovascular diseases, lung disorders are a leading cause of morbidity and death worldwide. For many disease conditions, no effective and curative treatment options are available.

An increased number of approved nucleic acid therapeutics demonstrates the potential to treat diseases by targeting their genetic blueprints. We developed an arsenal of siRNAs that can specificallytarget respiratory viruses and human genes related to respiratory diseases.

siRNA therapy requires three elements; hyper potent siRNA sequence, siRNA delivery to the target cells and stabilisation of the siRNA in vivo for a prolonged period. This project aims to address two of these crucial elements. Our first objective is to identify efficient delivery methods to the target cells andtissues. The second objective is to identify chemical modifications that will enhance siRNA stability andactivity. While basic siRNA research can be done in vitro, the delivery and stability of siRNAs can only be assessed in vivo.

The goal of this project is to develop new delivery methods and identify chemical modifications whichwill be highly relevant for any respiratory or lung-related disease, including cystic fibrosis, head and neck cancer or lung cancer. Moreover, our novel delivery methods will be applicable to other lung therapy areas which depend on the delivery of nucleic acids.

What outputs do you think you will see at the end of this project?

Nucleic acid-based therapeutics present huge potential in the treatment of pulmonary diseases rangingfrom lung cancer to asthma and chronic pulmonary diseases, which are often fatal and widely prevalent. However, the susceptibility of nucleic acids to degradation and the complex structure of the lungs retard the effective pulmonary delivery of nucleic acid drugs. Therefore, the successful clinical application of nucleic acid therapy ultimately relies on well-developed carriers and methods to ensure safety and efficacy.

The research outlined in this proposal aims to make much-needed progress in the area of nucleic aciddelivery to the lung. Upon completion of this project, we will identify (i) methods that efficiently deliver siRNAs to the lung and respiratory tract and (ii) specific modification patterns which increase siRNA efficacy in these tissues.

We have developed and tested in vitro multiple therapeutic siRNAs against viral and genetic lungdiseases. The results from this project will be integrated into these studies to generate safe and efficacious treatments for these diseases.

Publications originated from this project, and all mouse models generated will be made available to thescientific community and could be important for the scientific advancement of other researchers.

Who or what will benefit from these outputs, and how?

In the short term, the outputs of this project will provide new information that will benefit the scientificand translational community through the publication of our work.
This information will fill gaps in current knowledge and provide a basis for further investigations into thebiology of nucleic acid therapy. The delivery systems and protocols which will be developed as part of this study will have significant contributions to the field of nucleic acid-based therapeutics for pulmonary diseases. Moreover, it will provide guidance for the delivery of nucleic acids to other organsand tissues.

The mouse models generated will provide powerful tools for investigating siRNA function, dynamicsand activity in the lung. They will also serve as a tool for testing novel delivery strategies to the lung.

Nucleic acid delivery is the main barrier, holding the field of nucleic acid therapy from moving faster tothe clinic. In the mid-term, our research strategy will enable a faster translation of siRNAs from the bench to human clinical trials. Our project will provide significant evidence regarding the efficacy and safety of the siRNAs and delivery vehicles in mice and, as a result, increase confidence in their success in human subjects. Benefits at this stage will be seen by the translational research scientific community and clinical scientists.

Our long-term aim for this project is to enable the successful use of siRNAs in clinical practice in the treatment of pulmonary diseases, from viral to chronic genetic diseases. While this project is focusedon developing efficient, long-lasting and safe delivery methods, we are already testing our siRNAs efficacy in other relevant rodent and non-rodent disease models (in collaboration with academic institutes and CROs). The combination of these studies will allow us to bring safe and long-lasting siRNA therapy to the clinic.

How will you look to maximise the outputs of this work?

To maximise the output of this work, we are already collaborating with multiple companies and academic labs. For the development of nanoparticle-based delivery, we are collaborating with multiple establishments in the UK and US. For the development of mouse models to monitor siRNA delivery to the lung, we are collaborating with an additional US establishment. Similarly, in collaboration with othercompanies and research labs, we are developing a high-throughput method to identify chemical modifications which will increase siRNA stability and efficacy. The information from this project will be regularly shared with our partners to support their research.

Maximising the output of this work will be achieved through open communication and sharing of ourprogress throughout this project with academics, clinical, and lay audiences using a variety of platforms, such as conferences, publications and our website.

All scientific results will be published in open-access journals in a timely manner according to thecompany policy to ensure that the information presented is widely available.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

This project aims to develop safe, effective, and long-lasting delivery of nucleic acid to the lung. To fullyunderstand the activity, durability, and safety of nucleic acid in the respiratory system, one needs an invivo model that encapsulates the tissue's complexity, cellular interaction, and microenvironment.

Currently, there are no models that can mimic the systemic vasculature or interaction between circulation and organs of a human in vitro or in silico. Live animals and tissue are necessary to predict what specific test articles do and which tissues they distribute to in a live system. While using cell linesand other in vitro assays reduces our use of animals, it cannot answer all the questions that can be addressed by in vivo rodents where the biological systems are similar to those of humans.

Rodent models are the most widely used in vivo models in research. Rodents are considered to be theleast sentient species that still demonstrate pharmacokinetic and pharmacodynamic characteristics similar to humans, given that we share >95% of the same genetic background (https://www.jax.org/genetics-and-healthcare/genetics-and-genomics/why-mouse-genetics).

The scientific community has a range of techniques that enable manipulation of the mouse genome,allowing us to generate models with which we can answer specific key questions. The models described throughout this project represent optimal models for the study of nucleic acid activity and durability while reducing the number of animals used and minimising their suffering.

The studies outlined within this project will involve the use of adult mice in which the respiratory systemis fully developed.

Typically, what will be done to an animal used in your project?

This project will use traditional inbred wild-type strains such as Balb/c and C57bl6 or genetically modified altered mice. GEMM animals will be used to test siRNA activity against a reporter marker such as Luciferase expressing mice or the Antares model, which expresses a nano-luciferase proteinfused to an orange fluorescent protein.

Breeding of genetically altered animals by natural breeding behaviour. These mice are genetically modified to express a reporter gene in specific cells or the whole respiratory system. The expression ofthese reporters is not expected to have a harmful phenotype or adverse effects. When CRE (Carbapenem-resistant Enterobacteriaceae; bacterial strains which are resistant to some antibiotics), expression is required, we will use one of two methods:

Induction using a gene inducer (e.g tamoxifen) administered by drinking water, food or injection,or

Delivery of CRE mRNA using one of the methods described below.

Pulmonary delivery of mRNA or siRNA with or without the delivery vehicle will be performed using oneof the following well-established nasal methods:

Intranasal (IN) delivery using light anaesthesia.

Intratracheal delivery (IT) using light anaesthesia.

Inhalation using a Nebuliser

Animals expressing a reporter gene will be monitored for its expression post siRNA (against the reporter gene) delivery. This will be done using a non-invasive imaging method, which requires light anaesthesia and injection of a substance to enable visualisation of the target tissues. This will be performed on a weekly/twice weekly basis until the expression of the reporter gene is recovered to itsbase level before treatment.

What are the expected impacts and/or adverse effects for the animals during your project?

Many of the animals used in this project are not expected to experience suffering. The reporter genes we chose to use were already tested in vivo and found to be safe. In collaboration with different CROs,we tested both the heterozygote and homozygous models and the expression of the Antares gene in the respiratory tract. These studies showed no adverse effects.

Repeat nasal delivery can lead to a weight loss of up to 20%. In rare cases, the administration might result in mild clinical signs such as laboured breathing, reduced activity, lethargy and hunched posture.All animals will be monitored closely during the study for these effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The minority of animals used within this project (~33%) will experience a moderate level of severity.The remaining animals (~66%) will be expected to be in the mild or subthreshold category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At this stage, achieving our goals of developing nucleic acid therapy for pulmonary diseases requirestesting our delivery methods in vivo to assess the efficacy and stability of the nucleic acid in the respiratory system.

Moreover, animals are required to achieve our aim as regulatory and research bodies require a preclinical assessment of potential therapies in animal models before their translation to the humanclinic.

In spite of this, we will continue to use tissue culture methods and machine learning tools to optimisethe selection of RNA molecules and their chemical modifications and to optimise the performance ofour delivery vehicle. This will allow us to optimise the selection of RNA molecules, production of the delivery vehicle and the complexation procedure. Together these steps will minimise the number of animals used in testing the potential conditions to those that have shown the most promising effect.

Which non-animal alternatives did you consider for use in this project?

In the careful design of a research project, we considered the use of methods that would minimise theneeds of animals, such as 3D air-liquid interface (ALI) organoids or lung on a chip. However, such systems do not always model the complex nature of the lungs or the biology of the respiratory system.

Where possible, we will use alternative research approaches that do not involve the use of animals. Inparticular, we will use in vitro methods such as ALI organoids from healthy and patient cells or primaryairway cells to screen for the delivery and efficacy of RNA molecules.

Why were they not suitable?

Although the use of cell cultural methods will enable us to reduce the use of animals, they cannot fullyreplace them as they do not model the complexity of the organs and tissues. Organoids, cell culture, and primary airway cells lack many of the features that accompany the human respiratory system, such as a movement of air, cilia movement, the multilayer multicellular tissue or the presence of immune cells. The fact that we aim to develop efficient and safe delivery to the respiratory system requires models which encapsulate its complexity and microenvironment. This is the only system whichwill allow us to test the dynamic of action and long-term effect of RNA molecules in the lung. Moreover,regulatory bodies require the preclinical assessment of any new modality prior to translation to human trials. It is, therefore, critical that we use the best models and methods for this research.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each of our mouse model experiments has a careful statistical design that is aimed at minimising the use of animals while ensuring robust and meaningful statistical endpoints. These animal numbers areselected in collaboration with highly qualified statisticians and are based on our experience from the last years with lung delivery in mouse models.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

In designing the experiments described in this project, we aimed as much as possible to reduce, replace and refine our use of animal models. In order to achieve this, whenever possible, we will use cell culture methods to replace or reduce the number of animals used in studies. For example, in trialsof new sequences or new chemical modifications, we will first determine the efficacy and safety of these molecules in primary airway cells, cell lines generated from animal models and in 3D ALI organoids. Similarly, new batches of our delivery vehicle will be first tested in vitro for delivery efficiency. These will be performed using a series of stringent downstream analysis methods. These invitro results will help indicate how and whether a mouse study should be performed.

Where mouse model experiments are required, we have consulted an experienced in vivo statistician to design our studies in a way that allows us to minimise the use of animals whilst achieving the maximum amount of data in maintaining reliable statistical endpoints. These calculations took into account the fact that siRNA inhibition on gene expression varies between different siRNAs and can be between 30-70%. We will continuously update the number of animals required for each study based onthe accumulating data.

In many of our animal models, we have included the use of a bioluminescent reporter (a gene thatmakes the respiratory system cells glow) to enable the visualisation of the cells receiving the RNAmolecules. This will allow us to follow up on the activity of our siRNA molecules over time in live animals. As a result, we will be able to reduce the number of animals required to achieve both theefficacy and stability of the RNA.

We have also worked closely with a senior biostatistician to develop and implement an experimentaldesign that enables the adjustment of mouse numbers used in a particular group, according to the latest information.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To minimise the number of animals required in our project, we ensure the use of the most up-to-date laboratory methods for processing materials collected from animals used in this licence. By doing this,we are often able to achieve the maximum amount of data from smaller samples. In this way, we can split samples collected from one mouse into downstream applications. For example, one piece of tissue can be used to generate frozen samples for molecular analyses and fix material for histology.

By employing standardised SOPs for our experimental design, particularly in our novel studies, we canuse data from control animals in one study as controls in others and thereby reducing the number of animals required in this project. Where needed, pilot studies will be performed, which will inform the design of subsequent studies, potentially enabling the reduction of numbers.

Where possible, our genetically engineered mouse strains will be maintained as homozygous in orderto reduce the breeding needed to generate mice of the required genotype and to optimise the efficiency of offspring carrying the appropriate alleles for the experimental purposes.

As part of a good standard of practice, each experiment will be preceded by the writing of an experimental study protocol which will outline our objectives and methodology and will

includestatistical endpoints.

All of this will ensure that we are using the optimal number of animals to achieve the aims of this project.

To further reduce the number of mice used in this licence, we will implement two strategies:

Use the surplus of GEM mice available from the breeding protocol in protocol 3 in preference tobuying additional wild-type mice.

In some situations, we will reduce the number of breeding steps to omit crossing mice with strainsexpressing CRE by administering Nanoformulated CRE mRNA.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the only species that are employed in our protocols. We will use inbred wildtype mice to studythe delivery and activity of different nucleic acid molecules (such as siRNA and mRNA) and geneticallyengineered mouse models (GEMMs) that express a bioluminescence reporter gene to study the delivery and activity of siRNAs. In this project, we will use these strains to investigate nasal delivery of nucleic acids to the respiratory tract and explore nucleic acid therapy for pulmonary diseases.

Nucleic acid administration will be done intranasally, intratracheally or using a nebuliser. Between administration events and post-administration, mice will be maintained under standard conditions untila specific time, at which point material will be collected and analysed as previously described. These methods are all designed to result in no more than a temporary discomfort to the mice.

Our GEMMs utilise a bioluminescent reporter gene that enables real-time monitoring of gene expression and siRNA activity in live mice. This allows us to reduce the number of animals required forour studies and to assess the expression of the reporter gene using a method that limits the suffering of the mice.

We will minimise suffering by adhering to the best practice guidance, currently the NCRI guidelines, forthe welfare and use of animals in research. Every protocol proposed in this licence is the most refined for the purpose and designed to cause the minimum distress and suffering to the animals.

Why can’t you use animals that are less sentient?

Mice are the most relevant species with the least sentience that we can use to carry out

the research proposed in this project. Similar to the human organ, the mouse lung is subdivided into lobes of lung parenchyma containing a branching bronchial tree and is vascularised by the pulmonary circulation originating from the right ventricle. The extensive published knowledge and the array of techniques thatenable manipulation of the mouse genome allow us access to genetically engineered animals in which we can explore the effect of our siRNAs in specific models.

The advancement of knowledge and development of concepts to improve human and animal health and well-being requires the use of live animals. Exhaustive literature searches of the pulmonary disease field show that the mouse is the most accurate model for their study. Therefore, testing our delivery system in this model will be crucial for its ability to be used in disease models and later on inthe clinic.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Our procedures will be regularly assessed throughout the project to ensure that they are being performed in the most refined manner. Refinements will be routinely made over the course of the project through constant advice from veterinary and husbandry staff. For example, we will work closelywith the NVS to look for less aversive inhalation anaesthesia to reduce animal stress.

During the last year, in collaboration with CROs, we made several refinements to our procedures to ensure that we are using the most refined method for the study and reduce suffering to the mouse. Forexample, we optimised the maximal volume delivered IN, IT and using a nebuliser and refined the administration methods. This resulted in reduced weight loss post-administration.

We aim in this licence to determine administration regimens that reduce exposure to anaesthesia butthat result in better drug delivery to the respiratory system.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Work in this licence will be undertaken in accordance with the guidelines published by the NC3Rs,which are updated regularly with the best current practice.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will follow updates provided by the National Centre for the 3Rs through receipt of electronic andprinted media and by attending the Cancer Institute AWERB committee meetings.

Through this project, we will:

- Communicate constantly with veterinary, NACWOs and husbandry staff
- Receive updates from the NC3R
- We may attend NC3R and scientific conferences

These approaches will allow us to be aware of the most recent advances in the field and will enable usto implement these into our research in a timely manner.

2. Brain Inhibition in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Neurotransmitter receptors, Brain inhibition, Neurophysiology, Neuropharmacology, Neurology

Animal types	Life stages
Mice	juvenile, adult, neonate
Rats	pregnant, neonate, juvenile, adult
Xenopus laevis	embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To understand the role(s) of proteins, brain cells and their electrical circuits which are responsible forcontrolling communication between various brain regions. Maintaining a proper balance in this communication is important for normal healthy brain physiology, but when it becomes dysfunctional and imbalanced it is often linked to neurological disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our work allows us to study the properties of key proteins called receptors that reside on the surface membranes of brain (nerve) cells, which are fundamental to brain physiology. These receptors are thegatekeepers for the activity and information transfer that occurs between all parts of the brain and ultimately controls human behaviour. There are many different families of these receptors, many of which we study.

Paramount amongst these are those that control the correct balance of activity which occurs between specialised nerve cells also called neurons. A subset of these neurons are excitatory, and crucially theiractivity is controlled by another subset called inhibitory neurons.

The receptors which reside in all these brain cell types are closely associated with diseases such asanxiety and stress, fear, depression, epilepsy, and intellectual disability. These are widespread conditions which affect not just the individuals who suffer them, but they also have societal impact. Many of these diseases manifest because of genetic abnormalities in the receptors we study (i.e. through genetic mutations which generate dysfunctional versions of the same protein).

Our work aims to increase our understanding of how these receptors operate in neurons to permitnormal behaviour, and to rationalise how their dysfunction causes disease.

What outputs do you think you will see at the end of this project?

Our studies aim to increase understanding and knowledge of brain function that can inform and be used by other university researchers (academics), the pharmaceutical sector (industry), and medical practitioners (clinicians) to pursue related studies, and to provide breakthrough drug candidates to explore new disease therapies. For example, in one major continuing research aim, we have discovered, mapped and exploited a binding site within a specific receptor type, which interacts with naturally produced brain molecules to increase the physiological impact of that receptor in the brain. Inso doing, we have made great strides in understanding how the receptor’s physiological function contributes to the relief of anxiety, stress and epilepsy in individuals. We are currently using this information to explore atomic level interactions between novel, related molecules and receptors. This research, and concurrent work exploring Down Syndrome, will provide therapeutic opportunities for treating these debilitating diseases, and co-morbid conditions such as anxiety and depression.

Who or what will benefit from these outputs, and how?

The most essential output from our studies will be the dissemination of a greater understanding and more detailed knowledge to related scientists, with the view that this will amplify its impact in the longerterm, and also reach out to a wider community. This will primarily be through the publication of academic papers, but also via lecture and seminar presentations designed to engage not only academics, but also industry, school students and the lay public to ensure maximum outreach. Equally,the interdisciplinary nature of our research methods, practised in a higher education environment, allows us to make a significant contribution to the training of future research scientists.

Collaboration with academic and pharmaceutical chemists is currently enabling improved drug selectivity among the classes of molecules that we study. Our long-term goal is to improve therapeuticchoices, for example, in cognitive enhancers (so called 'smart drugs'),

general anaesthetics (used for surgery) and anxiolytics (for anxiety relief).

The mouse colonies we use in our research, are genetically altered to provide disease models whichsimulate Down Syndrome, epilepsy or anxiety, are often studied in conjunction with clinical data fromindividuals suffering these conditions. From this liaison we are able to provide clinical guidance as tothe benefit of using existing drugs to combat specific diseases.

How will you look to maximise the outputs of this work?

In order to provide wider benefit from the outcomes of our research, we collaborate closely with a number of pharmaceutical companies on concurrent projects with the aim of improving drug effectiveness. In fact, the dedicated aim of this work is to enable improved therapeutic benefit by increasing the ability of these drugs to interact with their intended protein targets in the brain. In thelonger term we hope our research will facilitate the development of our discoveries into the clinic.

These projects, and others, all produce data which has been, and will continue to be, widely published in highly-respected scientific journals. This ensures that we are able to share our knowledge advances,novel methodologies and mouse disease models more widely in order to maximise their impact, both locally and world-wide, so that others may benefit from their application as research tools and drug leads.

Species and numbers of animals expected to be used

- Mice: 13400
- Rats: 730
- Xenopus laevis: 20

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use both mice and rats, and occasionally, Xenopus laevis frogs. Mice (used from neonate to adult ages) are a widely used laboratory resource for experimental biological science. There is an extensive database of available information on this species including physiology, brain structure and behaviour. They also breed reliably and frequently, with consistent litter numbers, and are also amenable to genetic manipulation, which is essential for the generation of our models of specific diseases, such as Down Syndrome. As suggested, mice have also been very well documented and quantified in terms of their behavioural traits, thus making them amenable to our behavioural experimental testing for stress, anxiety, fear and related disorders. An important class of molecules westudy, which are made in the brain, are strongly affected by the levels of circulating sex hormones, so there is a strong bias in our work towards using only male mice, as this obviates complications from the oestrous cycle of females.

Rats are used in embryonic and/or early postnatal forms to provide tissue for the long-term culturing ofbrain cells, as this age of tissue is sufficiently adaptable to survive these procedures, and provides reliable and reproducible material on which to perform

experimental manipulations.

Both mammalian species we use are sufficiently closely related to humans in terms of their physiological and genetic similarities, as to represent a good basis for our studies which require us tomutate genes to generate disease models.

Xenopus laevis frogs are used for the provision of their egg cells (oocytes). This is a useful resource for 'difficult-to-express' receptors we may need to study prior to using mammalian species and neuronsfor extended studies.

Typically, what will be done to an animal used in your project?

There are several tiers of use for our animals. We have, over many years, established viable mousemodels which emulate certain disease states (e.g. anxiety, epilepsy, and cognitive ability (i.e. Down Syndrome)). These mice are used in ongoing breeding programmes, with periodic backcrossing (genetic background refreshing) , over the long-term (typically up to six generations) to minimise genetic drift.

Following breeding, mice will diverge in their use. A proportion (~65%) will be used in the short term toprovide tissue, most often in the form of thin brain slices or parts of the brain are used to culture specific types of neuronal cells. This tissue is used to study the electrical activity of the brain cells (using a technique called 'electrophysiology'), to label native proteins with fluorescent probes ('cellimaging'), or to undertake biochemical manipulations.

Other cohorts of mice (~35%) will be subjected to surgical procedures to inject substances into thebrain ('craniotomy'), such as viruses to manipulate their genetic profiles, or to understand brain cellconnectivity, or to introduce drugs which have intended targets in the brain. These injections are performed before the same animal is subjected to either a behavioural test (i.e. to establish if it is anxious), or its brain tissue is collected to perform electrophysiological (or related) studies. This typically occurs 1-4 weeks after the initial injection.

These same initial surgical and injection procedures will also allow us to control the activity of a specific subset of brain cells, so that we can understand if some are linked to certain behaviours. This will additionally involve the surgical implantation of a light-emitting electrode/probe which remains permanently fixed to the skull of the animal. It is used to activate other proteins we will have introducedinto the brain (by surgical injection or genetic manipulation), and will occur 2-3 weeks after implantationof the device - this technique is called 'optogenetics'. Alternatively, we will activate other introduced proteins using specific chemicals ('chemogenetics'), or we will insert a device to wirelessly record 'brainwave' activity (the 'EEG') in a mouse disease model. For many of the above manipulations, mice will be left to recover for 1-2 weeks, before we will perform any further experimentation, after which the mouse will either be culled or used for the collection of brain material.

A minority of mice (<5%) may also undergo procedures to induce either short-term (acute) or long-term (chronic) epilepsy through the injection of substances, into the body cavity, which promote thiscondition. This may involve repeated injections to achieve this condition, and further injections to subdue and maintain the condition over a period of approximately two weeks (typically).

Mice will also be injected with therapeutic and experimental doses of drugs (into either the

brain or body cavity) to affect behaviour associated with a disease model, or to cause behavioural change innormal mice. Some of these mice will be assessed using one, or no more than two, of a range of behavioural tests.

Rats will be used mainly for the provision of tissue for culturing brain cells in vitro. Pregnant femaleswill be culled when the foetuses are at embryonic day 18 for this purpose. The foetuses are culled immediately and humanely upon removal and the tissue used thereafter.

Xenopus laevis frogs will only be used for the provision of oocytes for the injection of genetic material.The female frog is immersed in general anaesthetic for this, prior to culling.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect significantly adverse effects for the majority of our animals.

A cohort of our current mouse disease models exhibit distinctive but mild behavioural traits, which caninclude anxiety, a heightened response to stressful stimuli, cognitive impairment and mild forms of epilepsy. Because these mice are genetically modified, these traits will persist until the animal is usedin an experiment or for tissue collection. Our animal models for epilepsy do not have spontaneous seizures but rather are more susceptible to a seizure, but this still requires initiation, usually by injection of a triggering chemical. Very occasionally some mice will be underweight.

Mice that are subject to injection into the brain will experience recovery from general anaesthesia andexposure to pain control measures required as a result of their surgery. Generally, animals resume their normal behaviour within days of such surgery, even when carrying an implanted probe or EEG monitor.

From previous experience using viruses to inject foreign genetic material (using viral vectors), the outcome of this protocol only has subtle, or very often no, consequences for animal behaviour. This isespecially so for those modifications which require additionally either light or chemical stimulation to change the activity of brain cells.

Epilepsy induction through body cavity injection of drugs, usually manifests as isolated seizure eventson the day of the procedure. This is mitigated through the injection of clinically used anti-epileptic drugs, so that seizures are reduced in their intensity and duration. The mild nature of the seizure is apparent only from the brain activity (EEG) profile, rather than from a change in behaviour. The EEG seizure event is also only transient (i.e. for a few seconds), and the wireless monitoring of the EEG is maintained for no more than 2 weeks in these animals.

In the rare event that epilepsy induction causes severe seizures (as outlined in the detail of ourprocedures), the animal will be immediately terminated.

Overall we do not expect significant adverse effects from our animal cohorts and experimentalinterventions.

Our use of rats (for the culturing of brain cells) and Xenopus (for oocyte extraction and use), proceedas terminal events, so there are no recovery issues from surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The severity outcome for our mouse disease models breeding is mild, as there are no discernible changes in their behaviour, litter numbers or long-term outcomes compared to wild type littermates.

Surgical procedures such as virus injection and EEG implantation are classified as moderate in termsof severity. Beyond the immediate impact of surgery (over 1-2 days), the majority of animals are expected to not be in pain or distress.

Substance administration is classified as mild. Apart from situations where we will perform multiplesubsequent administration, animals are expected to experience a moderate level of severity.

Chemically-induced epilepsy mouse models are considered to be 'moderate'. This applies to allanimals undergoing this procedure.

Behavioural tests are also considered to be moderate, though in reality most of the proposed tests areinnocuous, with the exception of tests such as fear conditioning, which involves several trials over subsequent days (up to five). This test would represent up to 10% of all animals subjected to a behaviour test.

Nearly all of our rats are only used for tissue collection so experience only 'mild' severity.

In summary, our estimates of severity are: mice (85% mild, 15% moderate); rats (100% mild);Xenopus (100% mild).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To examine our hypotheses around specific neurophysiology and neurological disorders, it is necessary to study our proteins and receptors of interest in the nerve cells in which they exist, and of course these can only be derived from the brain. Neural tissue derived from small rodents provides thebasis from which we can assess the impact of these proteins on the behaviour of the whole animal.

There are currently no other tissue- or cell-based models that can emulate nerve cells, the proteins therein and the specific ways in which they communicate. This is especially relevant if we wish to applyour findings to diseases which are common to the brains of

animals and humans.

We need to use Xenopus oocytes to study some membrane-bound proteins, because the secondarycells (see below) we would otherwise use, are unable to express some proteins for study that are derived from larger genetic material.

Which non-animal alternatives did you consider for use in this project?

We extensively use so-called 'secondary' cell lines from commercial tissue libraries (e.g. ATCC (www.atcc.org)) for much of our work. These secondary cells have been immortalised (the original cellsdivide and can be used for extended periods), and are used to reconstitute receptor proteins for study. They continually grow in culture conditions providing a very convenient and minimal impact source of tissue. We use these cells for study as they allow us to isolate our proteins of interest from the influence of more complex nerve cells in which they normally reside. This has great benefit for understanding the effects of disease-causing genetic mutations, for example. These same cells are therefore very useful to examine and triage modifications made to proteins/receptors before they enter an animal for more complex assessment in situ.

We are also making increased use of induced Pluripotent Stem Cells (iPSCs) derived from human tissue obtained from consenting individuals who suffer syndromes relevant to our line of study. More recently we have been making use of human brain organoids that originate from similar stem cells, buthave been grown over longer time periods to better emulate intact brain tissue. However, the methodology for generating stem cells is still largely developmental, so is less reliable than our use ofanimal-derived tissue.

Why were they not suitable?

There are currently no other tissue- or cell-based systems that can usefully emulate real nerve cells, alltheir associated proteins and the way they interact. This is especially relevant if we wish to apply our findings to diseases which are common to the brains of animals and humans. The development of 'engineered' animal or human tissue for the type of biological research envisaged in this project, although very useful to a point, falls significantly short of what can be achieved with animal models. Forinstance, our experiments with human organoids only minimally emulate some of the physiological signatures of animal-derived cells, and as such may mask some of the effects that may be crucial to our understanding of the role of a protein in a nerve cell population.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on our extensive experience of working with animals to generate statisticallyrobust data over the last 25 years in this field of research. This incorporates observations and discussions made with other laboratories undertaking similar studies, and also by comparison with similar studies that have been published in

peer-reviewed journals, and in the process of publishing our own studies, receiving scrutiny from journal editorial statisticians.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The first step in the design of all of our experiments is for us to ask how much initial characterisation of a protein modification we have made can be undertaken in immortal secondary cells. We aim to take this step as far as we possibly can as the information in these triage experiments dictates whether a protein modification is taken forward into an animal model. To help with this we have made use of the NC3R's experimental design assistant (EDA) tool which has proved effective for our animal behavioural experiment design. EDA is somewhat less powerful for in vitro experimental design (i.e. obtaining material for culturing nerve cells or for slicing whole brains for experimentation). For this, we use our previous experimental design experience, bearing in mind the type of analysis to be performedto reliably provide statistically distinguishable data. This approach is supplemented by the use of powercalculations, which has proved especially relevant when there is any ambiguity in the numbers of animals to provide for cultured cells and brain slices that are required for necessary statistical power.

By following these steps, we ensure that we do not over-use our animal resources.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Gender - We study naturally-occurring molecules in the brain which are derived from sex and stress hormones. These molecules are called neurosteroids and they can radically affect animal behaviour. Low levels of neurosteroids are critical in mediating stress and anxiety behaviours (in normal rodents and our mouse models). This means that female mice, which regularly undergo the oestrous cycle, experience natural fluctuations in their circulating levels of sex hormones and also therefore in their levels of neurosteroids; this can affect the numbers and types of our study receptors in the brain, bothof which can impact on behaviour (Maguire et al 2005 Nature Neurosci 8, 797-804). Thus, using female mice in our studies on stress and anxiety would be prone to unpredictable results. Additionally,the importance of neurosteroids in modulating the epileptic state of an animal is well documented (Biagini et al 2010 Curr Opin Neurol. 23, 170–176), so also for this reason our studies on epilepsy (either as mouse models or by seizure induction via drug injection) can only be reliably performed on male mice. However, for cell culture and our work on Down syndrome, we include female mice as partof our study regime, and intend to increase their use in future.

Breeding - In terms of optimising the breeding and maintenance of our mouse colonies, we have usedresources such as those provided on the Jackson lab website (https://www.jax.org/jax-mice-and- services/customer-support/technical-support/breeding-and-husbandry-support/colony-planning). As part of this, we bring in new parent strains on which our mouse models were founded, in order to regularly backcross colonies to ensure good genetic penetrance of the model's mutation and reduce genetic drift. This reflects in healthier and larger litters and so helps reduce our breeding numbers.

Experimental bias - To improve our statistical robustness , improve randomisation and reduce our experimental bias, as far as is possible, we ensure an experimentalist is blinded to the identity of the animal they are using (i.e. a wild type or homozygote for a disease model). This often involves many lab members in a selection regime and largely

applies to behavioural experiments, but is increasinglybeing used for mice used for tissue provision (e.g. brain slices).
Drug dosing - If we are performing experiments monitoring the behavioural response of a mouse to theinjection of a drug, then our prior use of secondary cell-based experiments, along with researched literature, generally ensures that we know the single most effective dose of that drug to give. This avoids repeated injections for dose optimisation (which would involve a separate animal for each new dose).

Cryopreservation - We currently have four mouse models cryopreserved. Two are from a recently completed study, the other two are still used in a current study. This provides many benefits (as statedby NC3Rs) but primarily allows us to be flexible in keeping current colonies no larger than is essential.Additionally, we can share these strains with colleagues in the same research field to prevent replication (e.g. through EMMA, the European Mouse Mutant Archive).

Genotyping - We undertake this for nearly 80-90% of our mouse colonies (parents and litters) toreduce unnecessary breeding, to allow experimental blinding, and to reduce genetic drift.

Tissue sharing - We routinely share animal tissue between members of our laboratory and those in other local laboratories. For example, in the collection and use of oocytes, we are part of a 'Xenopusconsortium' which informs all members about oocyte availability. Oocytes from a single frog are plentiful and this prevents wastage. Feedback within the group reports on the quality of oocytes. This enables a judgement to be made as to whether a new protein modification, which may not be workingwell, is due to poor quality oocytes, or problems related to the genetic manipulation. Where possible, we also endeavour to share collected brain tissue from rodents, depending on demand (within or outside the lab) and tissue type. We expect to continue and extend this sharing practice in the future.

Power calculations - Where necessary, we use power calculations to estimate how many animals arerequired to ensure that the effect we see is statistically relevant. These calculations are either performed manually or by using G*Power (https://stats.oarc.ucla.edu/other/gpower/).

Micro-electrode arrays (MEA) - These are electrode matrices in a tissue culture plate which record electrical activity from a collection of nerve cells which have been seeded onto them from a single animal's brain tissue. Data from the MEA can complement data obtained from brain slices so, whereappropriate, it can represent a significant way to reduce animal use, and we hope to develop this further.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Point mutant 'knock-in' mouse models. These target specific murine genes related to our target proteins (primarily the GABA type A receptor (GABA-AR)). These models incorporate a single amino acid exchange in the final protein which is expressed in an animal's brain. It is designed to remove the receptor's sensitivity to the endogenous neurosteroid molecules we study. As a result of this change, bred homozygote animals are more susceptible to anxiety, stress and hyperexcitability. However, theseare subtle changes so homozygote animals behave as a wild-type animal until subjected to an appropriate challenge/test, and are not considered to be suffering any long-term harm. We will apply electrophysiological, behavioural and brain viral injection methods to all these models.

Gene dosage mouse models of Down Syndrome (DS). Mouse models reproduce the duplication of genes on human chromosome 21 (= mouse chromosome 16) which occurs in DS. Hence the 'dose' ofall of the genes on chromosome 16 is increased in the animal because each is duplicated. The model manifests some mild cognitive impairments (only revealed when challenged/tested). Hydrocephaly is present in <5% of the offspring, which are not used but culled immediately. Our collaborators have extensive experience in breeding and maintaining these and other mouse strains which contain different numbers of duplicated genes on chromosome 16. Because we will not be breeding these mice, but only maintaining those which are healthy, we do not expect them to experience distress as aresult of their genetic modification. These models are subjected to electrophysiological, transcriptomic(to reveal changes in gene activity in single cells), imaging and behavioural analyses.

Use of secondary cell lines. As indicated previously these are very useful because they allow pilot studies to be undertaken on our proteins and drugs before they are introduced into animals. The advantages are manifold in terms of reducing animal use and adverse effects from substance injection.

Tissue collection. This is performed under either terminal or non-terminal anaesthesia so causes noharm to the animals. All appropriate measures (righting / pinch reflex, eyelid brush) are used to ensureappropriate anaesthesia for non-terminal procedures. There is no recovery as animals are humanely culled as soon as anaesthesia is established. We also collect tissue from new-born animals (P1-14) -- because these are less sentient and our target receptors for study are modulated by general anaesthetics we perform decapitation without anaesthesia as detailed in Protocol 2. We also collecttissue from foetuses (embryonic day 14 - 21, E14-21) where the parent is killed via schedule 1 and the foetuses are decapitated.

Surgery. This relates to virus or substance injection into the brain, cannula insertion or EEG electrode placement onto the skull. These procedures have the potential to cause harm but pre- operative procedures are scrutinised by our animal facility to minimise suffering or lasting harm. The refinements we have made to these are mentioned in the Refinements section below (and the detail isin the related Protocol). This includes extensive post-operative monitoring for abnormal behaviour andpain relief as needed. Distressed animals are culled.

Epilepsy induction and monitoring. Induction for this procedure uses an increasing dosing regimen over the course of 30-60 minutes so that we can terminate the dosing when the first seizure occurs. Atthis point, any further seizures are prevented by injecting a pre-established dose of an anti-epileptic drug. This prevents the animals suffering any further physical seizures and limits epileptic activity to a wirelessly monitored EEG signature only,

which is less harmful. The EEG monitoring occurs over a period of two weeks, during which time animals are monitored for distress every day (often twice a day).
Behaviour. The majority of these tests are not harmful, but may induce stress because they involve anew environment, or a more invasive intervention such as a mild electric shock. The parameters for these tests have been established over many years of use by other research groups, using either peer-reviewed publications or through consultation with experienced behavioural labs.

Why can't you use animals that are less sentient?

In many of our tissue collection procedures we do use less sentient species. For example, we use Xenopus frogs for harvesting oocytes to express complex proteins to perform electrophysiological experiments. We also use foetal mice to culture brain cells (over extended periods) for study, and occasionally young postnatal (P1-4) rodents, both of which are more immature and less sentient. We also anaesthetise (either terminal or non-terminal) juvenile rodents in other tissue collection procedures. In terms of the scope of our research in the lab, these procedures represent 70-80% of theexperimental procedures we undertake.

However, our aim in using our mouse models is to understand key aspects of behaviour and brain communication, and disease-related abnormalities which manifest, so that they can be compared tothe human conditions. Immature, less sentient or anaesthetised animals do not lend themselves to understanding communication patterns between complex brain structures, nor do they allow us to emulate and observe complex behavioural signatures which are only found in sentient, higher order animals. Our ultimate goal is to try and understand human disease conditions more completely, thus the closer we can get to emulating the complex traits of humans, the more valid will be our projectionsfrom experimental observations to the human condition.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

There are several ways in which we can look to refine our procedures.

Animal handling & behavioural experiments: to minimise stress we use the hand-cup or tunnel method to transfer animals between cages / environments / experimental apparatus, and keep siblings together in the home cage as much as possible. We practice pre-experimental habituation as it significantly reduces the stress animals experience. This is especially important in our studies as someof our mouse models are genetically altered to have a lowered threshold for stress or epilepsy induction. We regularly review our procedures among ourselves in this respect and liaise with other BSU user groups / laboratories to improve this. Occasionally we require to singly house animals for certain behavioural tests (i.e. the 'preference test') - this requires up to five days of habituation followedby testing, which can last up to two weeks. Single housing is necessary because in the Preference testanimals are given a choice of fluids to drink and the amount imbibed of each, and the animal's weight, needs to be closely monitored for each individual. We currently do not have access to split cages (where two animals can be separated but in the same cage) to undertake these sorts of experiments. Where appropriate, we will pre-expose an animal to other behavioural apparatus (such as the light/dark box, elevated plus maze, mild electric shock chamber), in order that this initial stressor has minimal impact.

Surgery: all our surgical techniques are scrutinised by the BSU staff prior to

implementation and for perioperative care and pain management, and the associated record keeping we adhere to fosters avery proactive and animal-centric welfare regime. Surgical procedures are carried out in anaesthetised animals accordingly to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) and all animals are expected to have a rapid and unremarkable recovery from the anaesthesia, and are returned to their home cages and siblings as soon as fully recovered. In the uncommon event of failing to do so, or presenting any signs of distress and pain that cannot be remedied promptly following NVS advice, animals will be humanely killed by a Schedule 1 method. If minimally post-operative complications or discomfort occur, enhanced monitoring and care is provided until the animal fully recovers.

Furthermore, for viral injections, we always undertake a pilot study when using new variants (or novel injectable compounds). This is to ascertain optimal viral titres (injection volumes) and transduction (expression of protein) times, as every new virus works with a different efficiency. This means we can optimise (and shorten) the latency between surgery and the experiment if necessary, eg. in case thereis discomfort for the animal. Drug injection, or cannula insertion, is performed under general anaesthesia and animals returned to their home cages and siblings as soon as fully recovered post- surgery.

Epilepsy induction: we have adopted our protocol following discussions with our colleagues. They haveover many years refined their epilepsy induction procedures. These minimise the dosing required for epilepsy induction and also the time period over which any animal suffers with seizure events, before their cessation by drugs, or by schedule 1 methods. Seizure events are monitored continuously by wireless EEG recording. Because the procedure involves multiple intraperitoneal (IP) injections, we use a new hypodermic for each, and different injection sites for induction (up to five) and seizure cessation. We use multiple induction injections to allow us to cumulate the dose to seizure threshold, a procedure that causes considerably less harm. Because we wish to establish status-epilepticus (by EEG signal), we monitor animals rigorously after induction - this amounts to multiple checks (up to 4) on welfare for the first 2 days after induction, and everyday thereafter during the course of the experiment.

We undergo regular training to hone our procedural skills, which reflects on much-reduced post- operative complications, and therefore minimises the timeline to the ultimate ‘use’ of the animal and itswelfare costs. In all of our previous Project licences (PPL), we have rarely experienced any complications to the welfare of animals in our care.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

The BSU website publishes and updates regular ‘Policies and guidance’ notifications for BSU users toengage with for guidance on all aspects of animal welfare and experiment design.

In addition, we always seek advice from relevant guiding principles for good practice, for exampleLASA, which has an extensive selection of publications made available for researchers: https://www.lasa.co.uk/current_publications/

Many other Universities are equally proactive in publishing guidance on animal welfare issues, whichwe receive as part of regular eToC notifications relating to these topics.

Equally, we continually make extensive use of the published literature in our field with regard to ourexperiments and their practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a very proactive Biological Services Unit (BSU) in this respect, who host regular workshops and events relating to animal use, in which BSU users are encouraged to participate (e.g. through reporting (posters/talks) their own experiences of improving 3Rs as applied to their own work). We findthis a very useful resource as it is a continually updating process and interactive.

Our BSU staff are also good communicators of improvements in 3R-related knowledge on a day-to-daybasis, and we are often provided with good advice or recommendations which we have implemented, or allowed the BSU staff to do on our behalf. These mostly relate to improved breeding practices and experimental design. We also have a very approachable and knowledgeable NC3Rs representative who is very active nationally, and is very able in providing up-to-date information in this regard.

Our liaisons and collaborations with other groups who use the BSU, and who also participate in theprotocols under our PPL, are also a good source to impart fresh ideas about improvements over experimental design and 3Rs, which we have adopted.

3. Cilia Diversity in Development and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cilia, Genetic disease, Development, Therapeutics, Genome editing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project aims to understand the structural and functional diversity of mammalian cilia- small hair- like projections from the surface of most of our cells. Cilia play important signaling and sometimes motile roles. They are essential for embryonic development and are also required postnatally, for howwe see, hear, smell, breath, excrete and reproduce. However, human disease genetics tells us not allcilia are created equal- our lab aims to understand why.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mutations in over 200 genes that affect cilia structure and/or function result in a growing list of over 40conditions termed ciliopathies. They are associated with a spectrum of diseases, ranging from lethal multiorgan syndromes to non-syndromic forms like retinal

dystrophy, which impact only a specific organ. The molecular basis for the clinical heterogeneity observed amongst patients with ciliopathies remains unclear. This is in part because of the structural and functional diversity of mammalian cilia, which is still largely unexplored. These gaps in our understanding impact genetic diagnosis, clinical management and the development of therapeutics for the ciliopathies.

What outputs do you think you will see at the end of this project?

This project will provide knowledge- it will improve our understanding of cilia diversity and how this contributes to health and disease. It will lay the groundwork for potential therapeutic interventions for cilia diseases. It will provide resources- we build important in vivo reporters and biosensors towards understanding cilia diversity for the community, as well as new mouse models of human disease to understand cilia dysfunction in disease relevant tissues. All work will be shared and promoted via publication in peer-reviewed journals and presentations at national and international conferences. Itwill be also communicated via interactions with key stakeholders including the general public, industry, or with patient groups.

Who or what will benefit from these outputs, and how?

By building a fundamental understanding of cilia diversity in human health, we can start to ask why different cilia types are sensitive to disease-causing mutations to better understand genotype- phenotype correlations and underlying mechanisms of disease. Our work will benefit research scientists, including cell and developmental biologists, but also rare disease clinicians to help with diagnosis, prognosis and management of ciliopathy patients. At the same time, our technology and approach will benefit industry with rare disease interests, particularly our studies to investigate whetherdisease phenotypes are reversible with genome therapies. Patients, their families and patient advocacy groups in the short-term benefit from our work in understanding their diseases as well as helping shape the nature of our research questions. Longer term our work will lead towards the development of effective therapeutics to halt or reverse the symptoms of cilia-related disease, whichwill benefit patients and families.

How will you look to maximise the outputs of this work?

We will aim to publish datasets of all of our studies regardless of outcomes. We will also attempt to maximise outcomes of our research through publication as well as presentations in national and international conferences. All results will be shared in open-access journals, and animal models and datasets made available to the community upon publication. All animal model details will be submittedto an online searchable database (MGI: Mouse Genome Informatics) linked to the publication to encourage wider and accessible use. We work closely in collaboration with many cilia and human disease genetics groups from across the world. The PI is an executive committee and founding member of the UK Cilia Network and co-lead on the MRC Mouse Genetics Network ‘Congenital anomalies’ cluster, such that we are highly entrenched in revelant UK academic, clinical and industry research spheres.

Species and numbers of animals expected to be used

- Mice: 15100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Animal models provide a great deal of information on the causes and progression of congenital disorders, such as the ciliopathies. In particular, genetic mutants often phenocopy human conditionsand lead us toward molecular events underlying developmental processes that are affected. These kinds of developmental studies need to be performed in vertebrates where analogous genes are implicated in the development of analogous organs and structures.

Mice provide a good, genetically manipulable model of organ development and function, like the airways or eye affected in ciliopathies. With genome editing, genetic modification of mice is precise andefficient, allowing quick analysis of a gene/process during development. As we can breed together strains of mice carrying different mutations, we can study complex genetic interactions in biological processes, such as airway development and homeostasis.

Many of the genes implicated in the ciliopathies play multiple roles in different tissues during prenatal and postnatal development; thus, these genes are difficult to study in humans, even in stem cell 'disease-in-a-dish' models. In this research, our goal is to make precisely-engineered mouse models ofpatient variants, which will help us to replicate complex interactions disrupted during early life, across multiple organ systems. Moreover, novel mouse models will also allow us to monitor disease progression later in life and serve as platforms for developing much needed therapeutic interventions.

Typically, what will be done to an animal used in your project?

The vast majority of the mice on this license will not undergo any licensed procedure (e.g. injection or surgery) as in general most of our analysis is done once the mouse has been humanely culled (either by extracting and imaging selected tissues, obtaining protein, RNA or DNA samples, or taking organs for ex vivo culture of cells such as mouse tracheal epithelial cells). A small proportion (roughly ~10%)will undergo a licensed procedure, which can include injection or eye phenotyping- such as electroretinogram (ERG) which involves anaesthetising the animal and placing electrodes on the eye to monitor vision.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the disease models we study are recessive, meaning that within a litter from a heterozygous intercross only a quarter of pups are expected to display a harmful phenotype, which can result in embryonic or perinatal lethality. The presence or absence, rate and severity of harmful phenotypes canbe modulated by the genetic background of the crosses involved. Harmful phenotypes we have observed in our mutants include hydrocephaly (swelling of the brain ventricles), runting and death.
When we know or suspect that a mutation will cause a potentially harmful phenotype the litters are closely monitored by an experienced researcher or technician. The humane endpoints will be constantly refined accordingly to prevent the harmful phenotype causing any unnecessary suffering.

Some animals will undergo procedures which may cause mild, or very occasionally (<5%) moderate suffering, for example including ocular pain after sub-retinal or intrvitreal injection, or ocular imaging. We will limit such effects by applying topical anaesthetics prior to and after such procedures. Some of the animals undergoing procedures may be neonates, in which case steps to reduce cannibalism suchas rubbing pups with bedding/maternal urine will be employed. For procedures which involve general anaesthesia, recovery will be monitored, and aided by heatpads and cleaning bedding after 2-3h.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The majority of our animals will be maintained on a Mild Breeding Protocol (Protocol 1). A proportion(~30%) will be maintained on a Moderate Breeding Protocol (Protocol 2), although past records suggest only a very small percentage (<5%) will actually experience moderate harm. A small number(~15%) will undergo Mild procedures including injection or phenotyping under anaesthetic. Very few <5% will undergo moderate procedures.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We will use mouse models of human disease candidates in this license. This project aims to understand the diversity in cilia structure and function between different tissues of the body and howand why this diversity is maintained, in order to inform on development of potential therapeutic strategies to treat diseases caused by cilia dysfunction.

This complexity requires the use of animal models, preferably mammalian, to understand disease mechanisms and develop possible therapeutic strategies. While we continue to use cell-based modelswhere possible, many of our research questions cannot be properly addressed without the use of animals. Addressing the diversity of physiological signals received by different cilia in cell line conditions is not possible, which is why this project focuses on in vivo profiling to understand cilial disease.

Mice provide a good, genetically manipulable model of organ development and function, and are good preclinical models of disease for the human ciliopathies. With genome editing, genetic modification of mice is precise and efficient, allowing quick analysis of a gene/process during development. As we canbreed together strains of mice carrying different mutations, we can study complex genetic interactions in biological processes, such as airway development and homeostasis.

Which non-animal alternatives did you consider for use in this project?
Human airway cells: In parallel to our mouse studies, we use commercially and patient-derived human nasal brush epithelial cultures. Human airway epithelial cells are cultured at an air-liquid interface (ALI) to produce a pseudostratified epithelium that recapitulates many of the features of thehuman airways.

Mammalian cell lines: We also use established human, mouse and canine cell-lines with primary ciliasuch as hTERT-RPE, IMCD3, MDCK.

Pluripotent cells: Mouse and human pluripotent stem cells offer the ability generate organoids fortissues through time and ressource intensive differentation protocols to study eye, lung or kidney biology.

Summary: We will continue to use all current in vitro approaches to complement our in vivo work where possible and are always open to try new methods that may enhance our studies and replace theneed for animals.

Why were they not suitable?

Human airway cells: These are limited primary resources harvested from healthy donors or patients,which can only make airway cultures.

Mammalian cell lines: Some but not all commonly used tissue culture cell lines are ciliated. However,they are highly removed from the cell types they may have originated from. For example, our field commonly used hTERT RPE-1 cells which are human retinal pigmented epithelial cells in origin.
However, they are no longer pigmented, nor do they recapitulate many of the markers of RPE in vivo. Human tracheal cell lines are also available however they have lost the ability to ciliate and thus do notrecapitulate the biology we wish to study. Importantly, although these complement our mouse work, wecannot achieve our aims to understand the diversity in cilia structure and function between different tissues of the body and how and why this diversity is maintained, to inform on development of potentialtherapeutic strategies to treat diseases caused by cilia dysfunction, using solely these culture models models.

Pluripotent cells: Similarly robust and reproducible differentiation protocols to late stages of organogenesis for lung, eye and kidney remain a challenge for non-specialist labs. For the eye, late differentiating photoreceptors with a fully functional connecting cilium are still not possible with currentprotocols.

Summary: While we continue to use cell-based models where possible, many of our research questions cannot be properly addressed without the use of animals. We do cell biology on an organismal scale- it cannot be done with cell lines or organoids alone. Understanding the structuraland functional differences between cilia types in different tissues at different developmental stagesrequires the use of an animal model. With our unique understanding of cell-type, and cilia-type differences in terms of sensitivity to mutations, we will be uniquely poised to attempt to turn these processes back on and reverse disease in our pre-clinical models.

Reduction

Explain how the numbers of animals for this project were determined. Describe

steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We expect to use a similar number of mice to previous years and so have based it on previous records. The numbers required for upkeep and desired out-breeding of lines are derived from estimates provided by the animal facility. To stay updated on optimized experimental design, we willregularly seek guidance from biostatistical experts employed by our establishments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

I, and the users on the license have completed a Research Optimisation Course offered by our establishment. This course covers rigorous design, conduct, analysis and reporting of research usinganimals. We also use the Experimental Design Assistant from the UK-based scientific organization that works nationally and internationally towards ways to replace, refine and reduce the use of animalsin research and testing, termed **NC3R**s, to design our studies effectively, using the minimal number of mice necessary to answer the questions we pose. All experiments will be conducted according to the **ARRIVE** (Animal Research: Reporting of In Vivo Experiments) guidelines.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will implement efficient breeding procedures and will additionally perform well-designed pilot studies whenever possible to assess the potential for publishable results. We will perform computational modelling and analysis of datasets to maximize the information extracted. The number of animals required for the project will be kept to a minimum through the implementation of good husbandry practice. Furthermore, we will consult with a statistician to ensure that appropriate numbersof animals in groups of sufficient size are used in our experiments, giving enough power to draw conclusions from before the experiments begin. Experimental design aspects such as randomisation and blinded scoring will be used to avoid experimental bias. Lastly, mouse models will only be generated for genes where no existing mouse models have been generated or the disease-causing potential is not well understood.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to achieve our project aims, we will carry out in vitro work in primary cells, vibratome sectionsand/or organoids grown from animals mutant in one or more of these candidate genes. These tissuesand cells help us define cellular and molecular pathways in vitro. Our work will also require experiments in vivo in genetically modified mice to understand the systemic effect of dysfunction and the effect on cell signaling through to organ physiology and overall health.

The species of choice for these studies is mice. Mice provide an outstanding model to understand gene function in health and disease, with many protocols and resources in place for their maintenance and welfare (see https://www.nc3rs.org.uk/3rs-resources/housing-and-husbandry/rodents). In accordance with these guidelines, our staff, both researchers and the support staff within the animal facilities, are extremely experienced with best procedures for breeding, maintenance and welfare of our colonies. Animals are kept in a well-resourced and well-equipped modern facility, using best practice systems for obtaining and maintaining the health of our stock lines. Where more refined approaches become available during the course of this licence either through personal communication,publications or veterinary advice, we will investigate their use following discussion with the NVS and Home Office Inspector.

Why can’t you use animals that are less sentient?

As our goal is to understand cilia diversity in mammals and many cell types of interest like cholangiocytes or photoreceptors develop perinatally, this cannot be achieved in a less sentientspecies or earlier time points. Most of our phenotyping work is on animals culled by Schedule 1methods, or under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The precise adverse effects of genetic alterations are not known, although can some times be predicted. For each line, we will make a detailed phenotypic assessment for inclusion in the mouse passport and continually reassess phenotypes with changing genetic backgrounds, age etc. A few of our lines, homozygous mutants are deemed as “moderate”- eg. *Tubb4b*, *Zmynd10* and *Pcm1* mutants are subviable due to hydrocephaly. A subset of these will be extremely runted with pronounced doming of the skull, whilst others will only be distinguishable by genotype, particularly on an outbred background. In order to minimize the phenotypes, lines will be maintained as heterozygote x wild type crosses for breeding purposes, and for experimental heterozygote x heterozygote crosses, litters will be closely monitored by experienced researchers or technicians for the development of phenotypes well defined on an animal passport. Animals exhibiting signs of distress will be swiftly culled by schedule 1 measures. In order to bypass this weaning lethality, we are looking to adopt methods to alleviate the central nervous system (CNS) phenotype of the mutants resulting in the early lethality, by generating a mutant with an exclusively respiratory and fertility phenotype, having no/minimal impair onthe welfare of the animal. This could involve injection of anti-inflammatory substances to alleviate the brain inflammation due to hydrocephaly or by transgenically reinstating expression of the mutated gene(stable or virally mediated), driven only in the brain to rescue the cilia immotility phenotype. As part of the National Mouse Genetics Network ‘Congenital Anomalies’ cluster, we are working to improve automated live monitoring of early life in our animal models, which will help us understand the consequences of these genetic mutations during the critical perinatal period. This is directly relevant to the human conditions studied, and will greatly refine our use of mouse models.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rswebsite (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locallyand provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (**NC3Rs**).

4. Mechanisms Controlling Cell Division and Cell Identity During Development, Reprogramming and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cell division, Cell identity, Development, Reprogramming, Frog

Animal types	Life stages
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the rules that cells use to control division and to specialize into particular types, such as nerve or muscle cells, during development and in response to reprogrammingand disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One of the most important decisions a cell makes is to choose its fate. All aspects of a cell are definedby its identity: its shape and size, its migration and choice of neighbours, when it divides, and its abilityto do specialized functions. For example, muscle cells are specialized for contraction, neurons for electrical activity, and red blood cells to carry oxygen.

Establishing and then maintaining the correct cell identity throughout a lifetime is fundamental for health. In several diseases, such as cancers and Parkinson’s disease,

mature cells have difficulty maintaining their specification. Most cells gain their identity during development and understandingthese rules should help us to uncover how disruption of a cell's identity can cause disease. The knowledge from this work may also help to advance regenerative therapies that engineer cells to restore a normal function in the body, for example to replace the special nerve cells that are lost inParkinson's disease.

What outputs do you think you will see at the end of this project?

This project is expected to discover novel fundamental insights into how cells control their identity. Thiswill be during normal development of the embryo, and when cells are challenged to alter their fate during reprogramming or in diseases such as cancer. We will report our findings in publications in peer-reviewed scientific journals, and at scientific conferences.

Who or what will benefit from these outputs, and how?

In the short/medium term, our research and data are of potential interest to other scientists studyingsimilar topics, such as developmental biology, mechanisms controlling gene expression, and reprogramming cells.

In the longer term, we anticipate that our work may be useful to researchers studying diseases. For example, understanding how cell identity can contribute to diseases may help in the discovery of new treatments. Understanding the rules of cell identity should also help scientists to develop techniques to effectively reprogram cells into specific types. Such cells could then be used in cell therapy, a promising treatment in which new healthy cells are used to replace diseased or damaged ones.

How will you look to maximise the outputs of this work?

We value collaboration with other scientists and research groups, as a means of sharing skills and discussing ideas. For example, we will continue to collaborate with experts in reprogramming usingfrogs.

Our findings will be published open access so that they are available to all at no cost. If appropriate,we will make our manuscripts available on a preprint server such as bioRxiv. We will consider publishing unsuccessful approaches, for example as supplementary information in our manuscripts.The project may generate large datasets, for example from sequencing experiments, and we will deposit these in publicly accessible repositories for use by other scientists.

Species and numbers of animals expected to be used

- Xenopus laevis: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use adult Xenopus, the clawed frog, and their embryos at early stages before they can

feed independently. Adult females are used to obtain oocytes (immature eggs) and, after hormone-inducedovulation, eggs. The eggs can then be fertilized in vitro (i.e. in a culture dish or tube) to generate the embryos.

Frog embryos are an ideal model to answer our questions about how cells control when they divide and how they choose to mature into specialised types of cell. Practically, amphibian embryos have several advantageous features, such as external development and large size; they are robust, easy toculture and are amenable to various experimental manipulations. There are Xenopus Resource Centres that facilitate the production and distribution of genetically altered animals that we can use to address our aims. Moreover, there is extensive knowledge of Xenopus development, which we can use to understand our discoveries.

Reprogramming by transplanting nuclei into frog eggs or oocytes is a well-established procedure. We use this technique to investigate the mechanisms that allow or prevent the fate of a cell to be changed.

Finally, we use frog eggs/oocytes or embryos to make extracts that allow us to study the molecularmachinery that controls cell division and cell fate decisions.

Typically, what will be done to an animal used in your project?

This is the typical procedure that will be done to a frog in this project: An adult female frog is injected with hormones to induce it to lay eggs. The female is injected under the skin at the base of the back into a lymph sac, which helps to spread the hormone throughout the body. Typically, females are giventwo hormone injections. The first is to encourage the female to produce a high number of mature eggs.The second injection, given approx. 2 to 14 days later, is to make the female lay the eggs. The female is placed in a salt water solution to lay the eggs, typically for 2 to 8 hours. This salt water mimics the condition inside the female's body to keep the laid eggs 'fresh' so we can use them for several hours after laying. Females are then returned to tank water. After a rest of no less than 3 months, females can undergo this procedure again.

What are the expected impacts and/or adverse effects for the animals during your project?

The female is expected to experience transient mild discomfort caused by the injection.

Rarely, in fewer than 2% of procedures, females do not lay their eggs properly and become bloatedand lethargic. These animals will be killed unless there are signs of recovery within 24 hours.
Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

- Species: Xenopus Expected severity: mild
- Proportion of animals: 100%

What will happen to animals at the end of this project?

- Killed

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of an organism from a single cell is an orchestration of biological processes that varyintricately across space and time. This complex process cannot be replicated by cells in culture, and sowe need to use embryos. The large size and rapid external development of frog embryos allows us to study cell fate most easily and efficiently. Moreover, many mechanisms controlling development in frogare conserved in mammals.

We need to use oocytes/eggs for our reprogramming experiments, for example to investigate the features and factors that allow the identity of a cell to be changed. There is currently no alternativetechnique that will allow us to directly answer some of the questions in our project.

Which non-animal alternatives did you consider for use in this project?

We considered using tissue culture cells as a non-animal alternative.

Why were they not suitable?

This project will also involve complementary tissue culture experiments, for example using stem cells.We can only use tissue culture cells to address some of our questions because cells grown in dishes cannot recapitulate the development of an embryo nor provide the reprogramming environment of ooctyes and eggs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will need to use in this project based on our plannedwork and our previous use of frogs for similar experiments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Female frogs can lay hundreds of eggs per ovulation. We keep females in a salt solution during theday as they are laying the eggs. This means we can collect large numbers of eggs, for example to prepare egg extracts. It also allows us to continue to use eggs

throughout the day, for example to generate small batches of embryos as they are needed.

We re-use females for superovulation multiple times, as is standard practice in the Xenopus research community to reduce the number of animals used. We do not set an arbitrary maximum number of times we superovulate (administer hormones to stimulate egg production) each individual female. The eggs need to be of sufficient quality to be useful in our experiments. So, we monitor the quality of eggsproduced to identify females that are ‘good layers’ suitable for re-use.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

The quality and yield of eggs varies between individual superovulated females. When possible, weshare ‘good quality’ eggs with other researchers.

The double ovulation procedure (in which an individual female lays eggs twice with a 7-14 day rest between each ovulation) is being used more commonly in the Xenopus research community to reducethe number of animals. We will trial double ovulation, if appropriate for our experimental needs and if the procedure does not increase the harm each female experiences.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use an amphibian model Xenopus, the clawed frog, in this project. Some of the frogs may begenetically altered, but the genetic modification is not expected to cause any harm.

We inject adult females with hormones to induce them to lay eggs. We may induce ovulation in the same female on multiple occasions. This is similar to what occurs in the wild, where Xenopus femalesovulate multiple times in a season.

The eggs and immature embryos we will use in our experiments are not protected animals (under the Animals (Scientific Procedures) Act). They will be used in several methods. Early stage embryos, produced by in vitro fertilization of the eggs, can be manipulated using micro-dissection and micro- injection of various reagents, for example to change the expression of specific genes. Oocytes / eggs can be injected with nuclei to assess reprogramming. Eggs /embryos can be crushed to make extracts.

Why can’t you use animals that are less sentient?

The majority of our experiments use eggs and immature frog embryos. We are using frogs

because they an excellent model for vertebrate embryo development and the large size of the embryos allows us to easily micro-manipulate them. We cannot use less sentient models, such as Drosophila, as theydo not have these advantages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will consult our specialist Xenopus NACWO who is proactive about improving the welfare of our Xenopus colonies. We will continue to follow improvements in colony care, for example individual frogsare photographed for identification, which is a minimally invasive ID method and improves clinical monitoring. Animals are kept in tanks with dark backgrounds and refuge tubes, which reduces stress. We have observed that females maintained in such enriched environments produce better quality eggs. We will source our animals from in-house breeding when possible. This reduces the need to import animals from other facilities, which not only removes the stress associated with transport but also reduces the risk of introducing pathogens into the colony.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow guidelines for planning and reporting research involving animals, for example the checklist in PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) and ARRIVE (Animal Research: Reporting of In Vivo Experiments).https://norecopa.no/prepare; https://arriveguidelines.org/.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We discuss best practice with colleagues in the Xenopus research community and with the Chief Aquatic Technician at our facility. We will consult and consider guidance from the Named Animal Careand Welfare Officer and the Named Veterinary Surgeon. We will also refer to material provided on theNC3Rs website (https://www.nc3rs.org.uk/3rs-resources). I am currently on the European Xenopus Resource Steering Committee where new developments in frog husbandry are often discussed.

5. Mechanisms Underpinning Normal Heart Development and Congenital Heart Defects

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cardiovascular, Heart, Development, Congenital Heart Defect, Mechanism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To identify the processes that underpin normal heart development, and to understand how these aredisrupted to result in common heart defects found in newborn babies.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Congenital heart defects (those seen at birth) are very common, affecting almost 1 in 100 babies born in the UK. Many of these are life threatening and require surgery, sometimes multiple times, in order tocorrect them. These surgical procedures are not a cure, however, and in many cases the patient will have lifelong health problems and often early death. Although we now have a basic understanding of how the normal heart and blood vessels develop, we still lack the details about how specific parts form,and how these processes

are regulated. We know even less about how these normal processes are disrupted to result in heart defects in newborn babies. It is only by gaining this understanding that we can design strategies for preventing these life-limiting malformations.

What outputs do you think you will see at the end of this project?

A large amount of new information will come from the planned studies.

We anticipate a number of publications coming from this research that will help to move forward the scientific field as a whole. We will present our findings at conferences and to the public, where possible, in order to help them understand this serious problem and the importance of medical research in this area. In the longer term, this research may lead to better diagnostic testing for inherited heart abnormalities and potentially even to treatments that prevent or reduce the severity ofsome defects.

Who or what will benefit from these outputs, and how?

Our studies aim to understand how the heart develops and how this goes wrong in some newborn babies. In the short term, our work will be the starting point for studies that use (rare) human embryonic tissue - to confirm that the data we obtain from studies in mice are relevant to humans. Itwill also enable genetic studies that aim to understand why some heart problems are inherited.

In the longer term, if we could identify more genes and/or processes that, when disrupted, cause heart defects, that would be a major step forward to preventing them. Once it is known that a specific gene isresponsible for a heart problem in a baby, then their family can be counselled about the likelihood of the same thing happening again in another baby. It can also lead to the identification of more minor problems in the parents or other family members, that can help protect them from developing symptoms and becoming ill themselves. In the long run we hope that we will be able to developtreatments that would reduce the risk or the severity of heart problems in babies.

How will you look to maximise the outputs of this work?

We will spread new knowledge as rapidly as possible via publication of our work and participation at scientific conferences. We will collaborate with other researchers to take forward our studies as quicklyas possible. We are strong supporters of open science and will continue to share positive and negativefindings as widely as possible. We will also make efforts to engage with the public (in schools, open days etc) so that a wider audience understands the purpose and outputs of our research.

Species and numbers of animals expected to be used

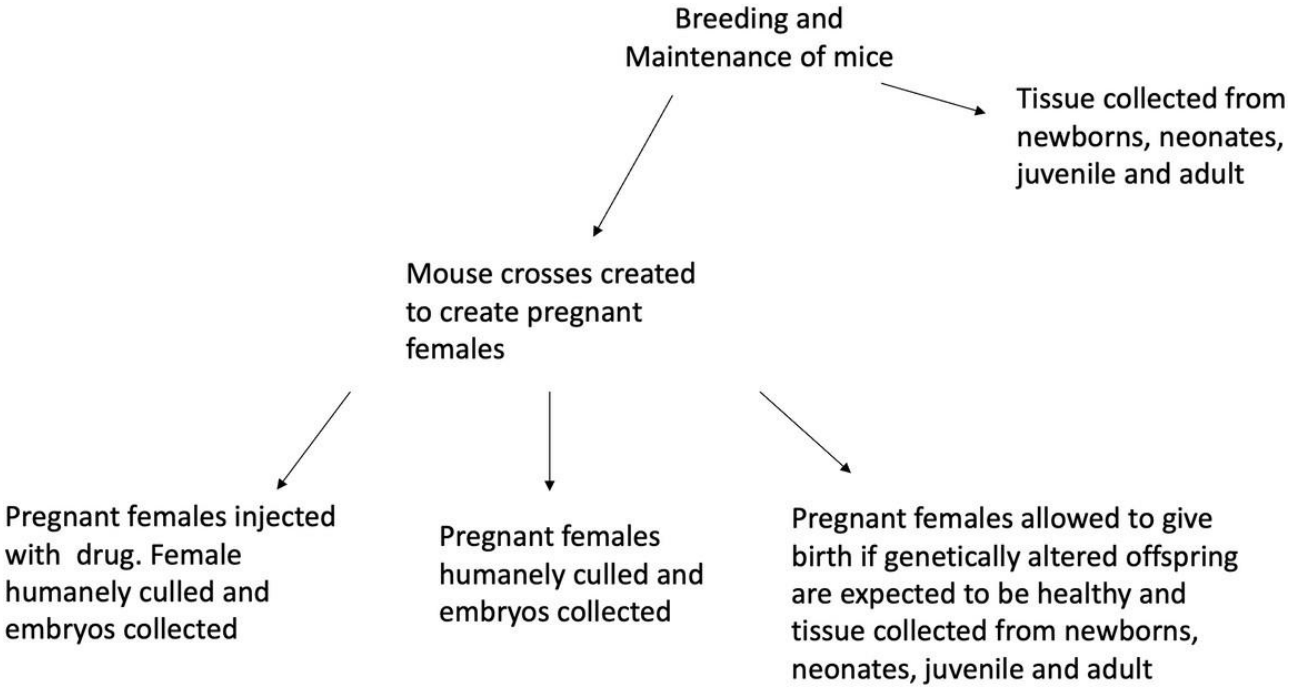
- Mice: 16000 adults, 500 neonates and 3000 fetal animals

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice throughout this project, many of which are genetically manipulated. These are usually analysed as embryos, many before they have a well-developed nervous system and before it is thought that they are able to experience pain or distress. We use these stages of developmental because this is when heart and blood vessel development is occurring and when heart defects arise.Throughout the 5 years of the project we will use up to 16,000 adult animals, although most of thesewill be used only for breeding purposes.



We will also use up to 3000 fetuses (from later in gestation) and 500 newborn pups.

Typically, what will be done to an animal used in your project?

The vast majority of our planned studies are simple breeding experiments with genetically modified mice. These mice, that themselves have no (or very limited) physical abnormalities, undergo timed matings and the offspring are collected at specific timepoints during development - this involves humane culling of the mother to obtain the embryos/fetuses. We will also cull newborn, juvenile andadult mice to collect tissues for analysis - again using a humane method.

In some cases we will expose the animals to drugs (usually by injection) either to understand aspects of normal development or, potentially, to see if this makes the heart defects worse or better. These willbe short term experiments (over only a few hours or days).

What are the expected impacts and/or adverse effects for the animals during your project?

Genetically modified mice (that themselves have very limited or no abnormalities) will be bred togetherand then embryos or fetuses collected at key time points during their development in order to collect their tissues for analysis. As these animals are collected before birth they will not develop any adverseeffects. Some animals will be allowed to be born and develop to adulthood but in most situations theseanimals should not develop obvious disease or distress. At the end of all the experiments the animals are killed by a humane method. In some cases we will inject pregnant mothers with drugs but these

should not cause them any harm and the mothers will be humanely killed after a few hours and the embryos/fetuses collected for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Breeding and maintenance (mild): 13000 adults Breeding and maintenance (moderate): 3000 adults

Collection of samples (moderate): 1500 adults (these are also included within the breeding andmaintenance numbers), 500 neonates and 3000 fetal forms

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For the study of three-dimensional form, as in the development of the functional heart, animals have tobe used as these processes cannot be mimicked by studies using cell culture (in a dish). Similarly, in order to study how genes and other factors (such as blood flow through the heart) interact to cause heart defects and related disease, it is also necessary to use live animals as these interactions, and the effects they have on the architecture of a given tissue, cannot be mimicked in cell culture.

Which non-animal alternatives did you consider for use in this project?

In the past we have used embryonic isolated cells for some studies but these are not useful when weare trying to understand interactions between different cell types or three dimensional form. We also use human embryonic material where this is available and is more directly relevant. Mouse is only used as a model system when these other models are not suitable.

Why were they not suitable?

Isolated cells are not useful for the study of three-dimensional form, which now makes up the vast majority of our research. Moreover, development is regulated by many different types of cells communicating with each other, which is extremely difficult to model in tissue culture. Thus, to understand these aspects of development and disease, we need a mammalian model. Mouse is most useful because its heart and blood vessels are very similar to human, and lots of genetically modified animals exist that help us to understand the role of specific genes. Human embryos are very useful forverification studies but cannot be used experimentally.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals is based on our experience of the minimum number required for each experiment to produce statistically reproducible and reliable outcomes. This is based on more than 25 years of similar studies. Our work involves analysing normal heart and blood vessel development and defects in these structures caused by abnormalities in specific genes, so a suitable number of mutant embryos need to be collected for analysis to confirm that the results are reproducibleand to determine how frequently they occur. We will also collect embryos that will be used to isolate tissue to use in experimental techniques. In these cases, 3 to 8 biological replicates (experimental and control groups) are required to enable us to distinguish differences between groups not caused by underlying biological variability.

We have consulted with the Animal Welfare and Ethical Review Body (AWERB) statistician and colonymanager in estimating these numbers.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

As a general rule, only mouse lines that are in use or expected to be used in the near future will bemaintained. We carefully design the experiments (based on our previous experience), taking into account the requirement for the optimal number of replicates to produce reliable results. Statisticaladvice is sought at this stage whenever the need arises.

The outcome of many experiments will be visual (for example a different distribution of a protein) ratherthan numerical. In these circumstances, we routinely examine 5-10 embryos (experimental and control) in each group to ensure reproducibility of the findings. Where possible, this data is complemented with numerical analyses. We have used this approach successfully in several publishedstudies.

To minimise variability throughout our studies, all our mutant and transgenic mouse lines aremaintained on the same mouse strain (C57Bl/6), where possible.

Group sizes are constantly being re-evaluated and updated when necessary. When using new strains,or combinations of currently used strains, small numbers of animals will initially be analysed to check for the expected outcomes. This will ensure that large numbers of mice that do not fit our theories will not be produced and will also inform the outcome of subsequent experiments. The development of animal alternatives, such as cell culture models, will be used when appropriate and may lead to the reduction of animal numbers in future experiments by allowing us to address specific scientific questions that do not rely on whole animals. Where appropriate we will use tools such as the Experimental Design Assistant to support our programme of work.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We carry out pilot studies following the experimental design phase to help us predict how many animals will be needed to obtain reliable and reproducible data. Statistical advice is sought at thisstage whenever the need arises.
All of our studies are planned so that we can use the tissues for a range of experiments. For example,offspring from a single litter may be distributed between several experiments, as may tissue from an individual embryo or adult heart. We will conserve tissues by storing as wax-embedded or frozen samples, from experimental animals and extra control embryos, so they can be preserved and used over long periods without needing to produce new animals for experiments. This will maximise outputsfrom animal procedures and minimise numbers of animals used.

We will only use animals up to their optimal breeding age, and not beyond, and we will not breed animals that display unexpected background-specific abnormalities that are harmful. To avoid problemswith inbreeding we will backcross with the animals regularly (i.e. breed the genetically modified animalswith purchased animals of the background strain) and will genetically screen the animals (SNP analysis) to confirm that inbreeding has not occurred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the best-established animal model for heart and blood vessel developmental studies becausethe heart is structurally very similar to the human heart and because their genes can be modified. In the majority of cases, animals are collected as embryos, before feeling or sensation develops. We will be using genetically manipulated animals throughout these studies. In some cases these will be well characterised. In others (for example new models), we will characterise the heart and blood vessels aspart of our studies. All animals will be monitored on a daily basis to ensure they are in good health. The majority of mice used for investigation in this project will be collected prior to birth as embryos andfetuses for analysis. Thus, these animals will not go on to develop any clinical effects that might occur in postnatal life. Thus, clinical effects are kept to the absolute minimum necessary for the project.

We will minimise suffering in adult animals wherever possible, for example by avoiding repeated injections. We also monitor our genetically modified lines to ensure that no unexpected/undesirablefeatures occur (e.g. overgrowth of teeth, brain swelling), not linked to the genetic manipulation, are bred out of the lines.

Why can’t you use animals that are less sentient?

We use animals at the earliest life stage possible to address our experimental questions -

this is both biologically and ethically desirable. Thus, many embryos are collected before 2/3 of the way through pregnancy. We use less sentient animals (zebrafish) when these are biologically appropriate although these cannot be used to accurately model heart defects where mice are the most appropriate model asthey closely mimic human heart development. Most of our studies are simple breeding experiments and tissues are collected from culled animals (schedule 1 method).

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

All animals, regardless of the line used, will be checked regularly and supportive care will be provided to minimise distress or suffering and to improve welfare. Most of our experiments will involve the collection of embryos and fetal forms which will minimise the welfare costs to All animals, regardless ofthe line used, will be checked regularly and supportive care will be provided to minimise distress or suffering and to improve welfare. Most of our experiments will involve the collection of embryos and fetuses which will minimise the welfare costs to the animals.

The majority of the mouse lines we use are physically normal, healthy and viable and we keep lines that are least impacted by their genetic modifications. For the breeding protocols, genetically modifiedmice will be mated with wild-type mice to avoid producing animals that will die on the day of birth fromcardiovascular defects. Two strains (eNOS null and Wnt1-Cre) may experience sudden death. Thesestrains will be kept at reduced numbers, monitored closely and killed at an earlier time point than the other strains (at less than 26 weeks). The cause of death in each case is unknown but if observationsare made that could mitigate the severity (e.g. seizures are observed) then this strain could be movedto a less severe protocol.

In the case of the Wnt1-Cre line, we have recently imported an alternative line (Wnt1-Cre2). Once wehave validated the Wnt1-Cre2 line and shown that it is suitable for our studies, we will use this as an alternative and stop maintaining the original Wnt1-Cre colony.

ApoE null mice develop atherosclerotic plaques if maintained on a high fat diet. However, we are notinterested in this phenotype and so will maintain the animals on normal chow which will minimise thedevelopment of atherosclerosis.

For any other lines that may present with welfare issues, health monitoring will be carried out regularlyand data recorded, for example, weight, activity, appearance, and body condition. If a welfare risk is identified, the impact will be minimised by working with the Veterinary team to administer appropriate treatment.
To demonstrate proactive management and care for animal welfare, we will limit inbreeding in ourstrains and therefore increase reproducibility by:

Cryo-preserving (i.e. deep freezing of eggs or sperm for future use) new and non-commercial strainsas soon as practicably possible after arrival. This will mean that small colonies can be maintained without worrying they will be lost.

Perform SNP analysis for new non-commercial strains at arrival, before each backcross and whencryopreserving

Backcross each strain after 5 generations (for colonies of 1-6 breeding pairs) or 10 generations (forcolonies with 6-10 breeding pairs)

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Alongside the guidelines listed below, I will also adhere to local AWERB standards for research animals, and where appropriate, support the development of new local standards for refinementsdiscovered during the project licence.

Code of Practice for Housing and Care of Animals Bred, Supplied or used for scientific purposes

LASA Guidelines

RSPCA Animals in Science guidelines
UFAW Guidelines and Publications

NC3R's and Procedures with Care

I will consult with the Colony Manager to review genetic health, breeding practices and overallcolony health and management at regular intervals.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

All members of our research team regularly attend seminars and meetings held by our animal facility.We also have regular (3 monthly) meetings with the animal care staff to discuss any issues or developments in the field.

The local animal support and Veterinary team regularly inform and suggest improvements related toanimal work, including recent studies involving reduction, refinement and replacement. This also includes information from external resources including (but not limited to): collaborators, peers, conferences, lab animal and animal welfare bodies.

For the 1st, 3rd and 5th year review of the project licence, I will provide updates on implementation or considerations for reduction, refinement and replacement that has occurred during the previous period,alongside a review of the linked training plan. This will be in collaboration with the animal support and Veterinary team, with a particular focus on refinements.

6. Modulation of Inflammatory and Immunological Processes to Prevent and Treat Pathologies and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Inflammation, Immune, Disease, Modulators

Animal types	Life stages
Mice	Adult
Rats	Adult

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to manipulate the immune system using novel therapeutic approaches to interrupt, reduce and prevent the immunological and inflammatory cascades that ultimately lead to thedevelopment of disease and disorders.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite progress in the discovery and development of treatments, there remains a significant unmetclinical need in the treatment and prevention of diseases and disorders that involve inappropriate responses and dysfunction of the inflammatory response and immune system.

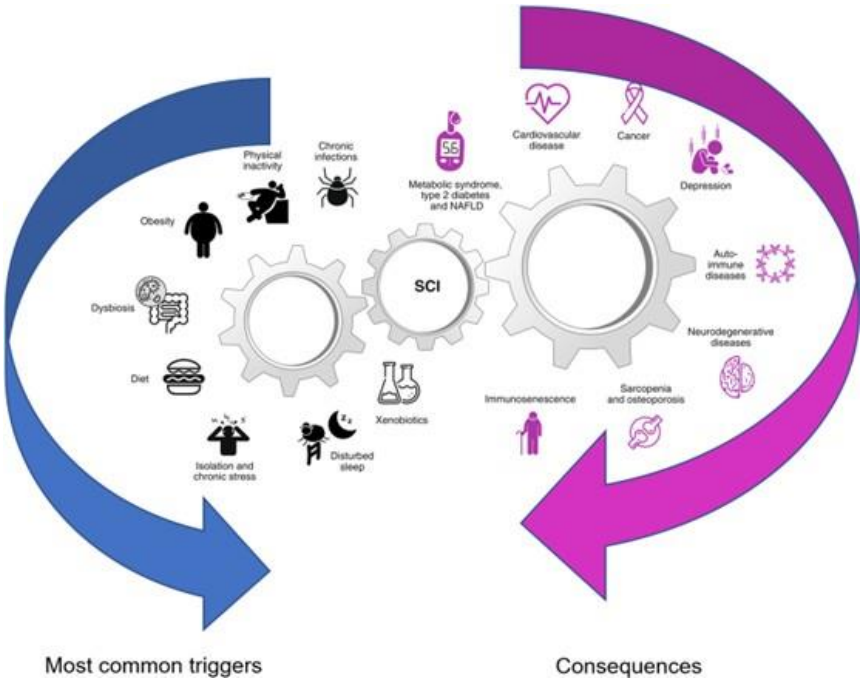
The immune system is a conserved system which has evolved to protect the body against infections. Itis also integral in a wide range of processes within the body. For example, during infections the immune response identifies the pathogen and efficiently and effectively removes it from the body.

Simultaneously the immune response initiates wound healing processes to keep tissue damage to a minimum. The immune cascade triggered in response to identification of a pathogen, foreign body ordamage to a barrier e.g. the skin, is highly regulated. Failure in the regulation of these processes andpathways is an underlying factor contributing to the development of inflammatory diseases and disorders, such as auto immune or fibrotic diseases, but also cancers, sepsis, allergies, pain, obesityand physical impairments. Some, if not all chronic diseases can also be associated with depression. Comorbidities are often present and greatly contribute to the burden of disease, healthcare utilisationand an impairment of the quality of life.

Chronic inflammatory diseases have been recognised as the most significant cause of death in the world today, with more than 50% of all deaths being attributed to inflammation-related diseases suchas ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease (NAFLD), autoimmune and neurodegenerative conditions. There is a high societal and economic burden associated with chronic inflammation alone. For example, within the EU, it was reported in 2015 that diseases of the respiratory system accounted for 8.5% of all deaths. Whilst in the UK it was 14.1%. In the EU it is estimated that 0.3% of the populationsuffer from IBD, equalling 2.5-3 million individuals. Approximately 1.6million Americans currently sufferfrom IBD with as many as 70,000 new cases a year. Whilst in Asia in incidence of IBD is also on the rise.

Overall, the estimated prevalence of chronic inflammatory disease in Western society is 5% to 7% ofthe population.

Several causes of low-grade systemic chronic inflammation (SCI) and their consequences have beenidentified.



What outputs do you think you will see at the end of this project?

This licence supports several scientific methods to enable investigation of the inflammatory and immunological systems underlying many diseases. The experiments performed will provide evaluationof novel therapeutics or biologics which are targeted at both the inflammatory and immune systems.

They will also generate essential information, such as biomarker identification, to support scientific programs aimed at identifying and understanding new ways of modulating and ultimately treating diseases and disorders associated with immune/inflammatory system dysregulation and dysfunction.

We will generate data on the effectiveness of potential new treatments in comparison to other treatments and determine how they might distribute throughout the body in the presence or absence ofan activated inflammatory/immune response. This will aid the selection of potential new treatments andthe data will contribute to programmes aimed at addressing unmet clinical needs in a range of disease types to benefit patient health.

Who or what will benefit from these outputs, and how?

Short-term. Our customers will initially benefit from the outputs generated. Data produced will enableinformed decisions on test items that may have potential utility in the clinic. The data will also help reduce the number of inappropriate test items progressing into more detailed, larger scale animal studies, therefore reducing the number of animals used going forward.

Long-term. Following on from the initial screening the best test item candidates may eventually providepatients with novel treatments for diseases and disorders that have an immunological and or an inflammatory aspect to them. New treatments will enable better symptom management and aid in the reduction of disease burden.

How will you look to maximise the outputs of this work?

Although our work is customer confidential, we have a commitment to the dissemination of informationinto the scientific community and findings will be made available to other scientists through publicationin peer-reviewed journals and presentations at scientific conferences and meetings.

Our customers will use the knowledge and data produced under this authority to inform project progression. This may involve dissemination of information to investors, developmental partners andpublication where appropriate. The outputs will also be used to guide further development within projects, both in-house and externally, that are focused in relevant disease areas.

Species and numbers of animals expected to be used

- Mice: 20000
- Rats: 16000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats and mice are the lowest sentient animals to be used under this authority. The inflammatory/immune systems are very similar to that of man in their complexity and responsiveness toitems the body perceives as a threat to its internal state of balance and comprises both the innate and adaptive immune systems present in man making the systems and their responses translatable toman.

Typically, what will be done to an animal used in your project?

These studies will involve animals challenged with non-lethal doses of substances to elicit an immuneresponse. The test substance, challenges and stimulants will be administered through various routes and by various dose regimens which will be selected based on published research articles, prior experience, and customer needs. Blood sampling may also be carried out to provide samples for PK. Studies will not last more than 4 weeks in any of the models.

Each experimental model will be monitored daily following intervention and animals will be assessed for any signs of distress with supportive measures provided. Procedures will be undertaken using the most appropriate anaesthetic where required and analgesia will be given. The mode of substance administration will be chosen to cause the least harm and distress to the animals. Any new substancesor route of administration will be tested in a small pilot study and the animals monitored daily for signs of distress. Humane endpoints will be strictly adhered to.

Induced systemic inflammation. These models can be used to induce a generalised inflammation whenthe challenge is dosed intravenously (IV), intraperitoneally (IP) or subcutaneously (SC) in an animal.

The inflammatory response induced by a direct challenge may be measured over time peripherally in the blood via repeat blood sampling (needle stick in the rat/tail cut in the mouse for repeat sampling) orby terminal blood collection to measure levels of inflammatory biomarkers in the blood and tissue suchas, but not limited to, cytokines.

Primed systemic inflammation. During the afferent phase the animals are typically immunised by subcutaneous (s.c) or intraperitoneal (i.p.) injection with a specific hapten or antigen in its chemically reactive state and emulsified with an adjuvant. This initial sensitisation primes the memory T helper cells within the body. When the animals are then challenged later with the same antigen, the efferent phase, typically initiated 5-12 days after sensitisation, occurs. A hallmark response is elicited by the repeat exposure to the same antigen which includes induration, swelling and monocytic infiltration intothe site of the injection within 24 to 72 hours. This reaction has been shown to be dependent on the presence of memory T helper cells. Assessment types for this response are ear thickness measurement using callipers, circulating or tissue levels biomarkers, cellular infiltrate analysis in tissues and blood collections for inflammatory biomarkers and test/reference items for PK.

At a pre-determined timepoint prior to the induction of inflammation or the efferent phase, a test item orreference item/substance may be dosed to the animal. Typically induced systemic inflammation modelsare acute and have a duration of 24hours after pro-inflammatory challenge, whilst primed systemic inflammation models have a duration of 6-15 days.

Local inflammation models. Alternatively pro-inflammatory agents may be dosed locally to induce amore specific localised inflammation such as directly to the lung via inhalation (IN) or intratracheal instillation (IT) or other structures such as the paw or a joint via direct injection to the area.

Oedema models. These models are widely used to assess acute localised inflammatory responses without causing unnecessary systemic effects associated with general inflammation. Injection of a pro-inflammatory agent into the sub plantar surface of the paw or the hock joint induces a biphasic oedema. The first phase observed approximately 1h following dose, is related to the release of histamine, serotonin, bradykinin, and to a lesser extent prostaglandins produced by cyclooxygenase enzymes (COX), whereas the delayed phase, second phase (after 24 h) is attributed to neutrophil infiltration, and the continuing of the prostaglandin generation. Release of the neutrophil-derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines are also involved in the delayed phase of induced acute inflammation. During the second phase, after 24 h, oedema is more pronounced, presenting a clear dose-response relationship and peaking at 72 h after injection.

Histological analysisof the subplantar area 4 h after pro-inflammatory agent reveals a diffuse cellular infiltrate with predominance of neutrophils. Between 48 and 72 h, an intense accumulation of macrophages, eosinophils and lymphocytes is observed, together with a great increase in the number of circulating leukocytes and platelets. Administration of the pro-inflammatory agent is carried out under general anaesthesia for restraint purposes. The degree of inflammation in the foot or hock is then measured using callipers or an alternative non-invasive method, a plethysmometer following injection of saline or challenge agent. Each animal will only have one paw or hock used to minimise discomfort and an assessment of mobility will be carried following injection and during the study period to assess for lameness.

Terminal samples may be collected. Blood samples may be collected by tail cut in mice or

needlestick in the rat for PK analysis to help build a PK/PD profile. Test/reference substances/items will be dosed to the animal at a predetermined time prior to induction of inflammation. Typical model duration is up to7 days.

Air pouch models. The air pouch is an excellent model to study localised inflammation without causing unnecessary systemic effects associated with general inflammation models or local pain direct caused by injection into joints. It can be used to study the components of both acute and chronic inflammation,the resolution of the inflammatory response and potential therapeutic targets. The model is initially generated under inhalation anaesthesia. Under general anaesthesia sterile air is subcutaneously (SC) injected to form a subcutaneous pouch. Following initial formation of the air pouch top ups of sterile arerequired to ensure the integrity of the pouch. The animals are re-anaesthetised during this process for restraint purposes and to reduce the risk of damage to the air pouch and to prevent discomfort to the animal. This procedure induces the proliferation of cells that stratify on the surface of the cavity to forma cellular lining consisting of macrophages and fibroblasts, which is morphologically similar to the synovial cavity. Later, local inflammation can be induced by injection of a pro-inflammatory agent (e.g. LPS, zymosan or bradykinin) into the air pouch. Stimulation of the surface cells produces an inflammatory response and the pouch serves as a reservoir of locally accumulated cells and mediatorsthat can be easily measured in the fluid. The pouch exudate will be harvested by lavage following schedule 1. These endpoints can be quantified and used to determine the degree of inflammation, resolution of inflammation, or anti-inflammatory activity of drugs. Multiple parameters from a single animal can be used to evaluate the extent of the inflammation and the effect of a given test substance, including but not limited to: histopathologic analysis, cytokine/chemokine analysis, biochemical analysis, pharmacokinetic/pharmacodynamic (PK/PD) blood collections and immunohistochemistry.

Terminal samples may be collected for PK analysis to help build a PK/PD profile.

Test/reference substances/items will be dosed to the animal at a predetermined time prior to inductionof inflammation. Typical model duration is 6-10 days.

What are the expected impacts and/or adverse effects for the animals during your project?

All expected impacts or adverse effects will be monitored carefully by a licensed member of our team.

The maximum severity limit in this licence is moderate. Possible adverse effects include irritation fromlocal injection, pain from localised inflammation, weight loss or mobility problems. Any animal showingdeviation from normal behaviour as judged by; body weight, body condition, general and coat appearance, gait or behaviour will be monitored. Mash may be given to encourage eating and preventdehydration. We do also use temporary anaesthesia that puts the animals to sleep for a few minutes -this can be done if injection will occur in a sensitive part of the skin such as the foot. Therefore, we minimise any pain or discomfort that may be generated from the injection.

Before pain and inflammation exceed a moderate severity level, animals will be killed to prevent anyon-going pain or suffering. All animals will be killed at the end of the protocol.

Drug administration

Unless otherwise specified, the administration of substances will be undertaken using a combination ofvolumes, routes and frequencies that of themselves will result in no more than transient discomfort andno lasting harm. If any of these procedures result in or induce evidence of suffering in an animal that isgreater than mild and transient or in any way compromises normal behaviour the animal will be killed by a Schedule 1 method unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions (such as providing wet mash, additional warmth or topical treatments). Enhanced monitoring and care will be instituted as advised bythe NVS or NACWO until the animal fully recovers. If there is not a rapid improvement within the working day, the animal will be killed by a Schedule 1 method.

Novel test substances and reference items (e.g.clinically effective treatments) may result in transientbehaviour changes following their administration. A number of potential clinical signs (but not an exhaustive list) are indicated below. Estimated frequencies and humane endpoints are listed in the protocol steps:

Diarrhoea

Dyspnoea (Abnormal respiratory movements, laboured breathing, gasping or panting)

Hunched appearance

Piloerection (Raising of the hair coat)

Ptosis (Eyelid drooping)

Changes in body temperature

Red Tears (chromodacryorrhea)

Scratching/local irritation

Weight loss; reduction in weight gain; reduction in food and water intake

In addition to observations for specific symptoms, the general overall condition of animals will be assessed on a daily basis (i.e., post-surgery or during chronic drug administration studies) since nonspecific loss of condition is an adverse event. Overall condition will be assessed, for example, bychanges in activity, feeding and drinking, demeanour, behaviour, coat condition and body weight (where measured). Animals in ‘poor’ condition will be killed. Assessment of condition will usually include a combination of information based on body weight, coat quality, body condition scoring, activity and food and water intake.

General anaesthesia

There is potential for pain or death if an inappropriate level of anaesthetic is used. Anaesthetic concentration will be carefully controlled and the level of consciousness of the animal will be continually monitored (following the guidance of the NVS and using indices such as flinching on light pinching of the rear paw and depth and frequency of breathing). Animals will be killed by a Schedule 1method on failure to maintain or control anaesthetic depth. (0.01-0.5%)

Expected severity categories and the proportion of animals in each category, per

species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Approximately 20% of rats and 20% of mice are likely to experience mild levels of severity due theiruse as baseline vehicle treated animals where no adverse effects are expected, however repeated blood sampling may be required.

The administration of challenge and test substances and withdrawal of body fluids will be undertakenusing a combination of volume, route and frequency that of themselves will result in no more than transient discomfort and no lasting harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because of the complexity of the immune and inflammatory responses to an infection/challenge, it is necessary for us to use animal models. This is because immune responses are carefully orchestratedin an intact animal in ways that cannot be recapitulated using non-animal alternatives.

Which non-animal alternatives did you consider for use in this project?

In vitro assays, cell lines, analysis of blood from human volunteers and human tissue.

Why were they not suitable?

Currently no in vitro assays can recapture the diversity of the immune and inflammatory response.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated numbers based on the anticipated number of experiments, the number of experimental groups and the number of animals in each group. Based on published studies and prior experience we have a good idea of the variation we expect to see in our

results. With variability taken into account, advice from professional statisticians and with reference to published relevant studies, wehave accounted for a sample size of between 6-12 animals in each group to be studied for each experiment planned.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Literature reviews were undertaken to help establish the standard group size and study design currently in use in industry and academia for this area of research. Also having worked in this area previously I am aware of the possible variability within a study cohort and that to establish a measurable, reproducible response over background noise it may be necessary to use the higher nnumbers (10-12) per group to achieve this dependant on the model being utilised.

To validate a study, it is necessary to have both a positive and negative control and establish the upperand lower limits of the system within each study. This is highly likely to vary across studies and between batches of challenge agents due to differences in potency between batches. We will review batches of challenge agents, prior to initiating further studies using a new batch, to minimise inter batch variability and ensure data are as consistent as possible within this area of research.

Furthermore, we will consult the NC3Rs experimental design assistant allowing us to calculate theappropriate sample size based on each experiment. This will ensure that we are using the fewest amount of animals and that each animal is being utilised to its full potential.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Pilot studies will be carried out to establish responses to challenge agents. These studies will be usedto both ensure the response is of a suitable magnitude that can be manipulated but not of such a magnitude that it forces the immune system into overdrive and also that challenges do not cause unnecessary pain, suffering, distress, or lasting harm. These studies will be guided by the literature and previous experience of the team. Following the establishment of the models we will continually review the response variability within groups such as the controls and standard items to assess if we are able to keep the statistical power of the studies whilst reducing the number of animals required in these groups.

Tissues/samples that may be collected will be shared across the teams and specialities to help reducethe number of animals used overall.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm

to the animals.

Our objective is to provide models of inflammatory disorders that will allow the pharmaceutical and biotechnology industries to test novel and existing drug candidates for use in human diseases. In vivomodels are combined with advanced in vitro assays that allow us to study the effects of the test substances on inflammation driven by the immune system.

We use rats and mice as the choice of animals in all our studies as the main components of their immune system are shared by humans; this is essential where immune responses as opposed to thefunction of individual genes is being studied and thus will produce satisfactory results.

Immune modulation: To functionally investigate immune responses we need to experimentally perturb immune system functioning. There are 2 main approaches. Firstly, using a systemic challenge where the animal is given a known immune modulator. Secondly, using a local challenge to a designated area(e.g. ear, paw, lung). The lowest dose of immune modulator that leads to moderate, reproducible inflammation will be used.

Administration of substances. We will use the smallest needle available and the most appropriate dosevolume according to published guidelines. Where injections may cause transient pain, such as the ear,paw, or pouch formation, inhalation anaesthesia will be used to minimise the impact on the animals.

The absorption of some substances is affected by the presence of food so may need to be dosed in afasted animal for pharmacokinetic reasons. We do not intend using fasting as a standard dosing condition, only where data suggest it's an optimal condition for the test substance of interest.

We will ensure that the animals suffer as little as possible. For example, we will ensure that restrainersare cleaned prior use to avoid inducing extra stress in the animals caused by prior experiments. The equipment used to restrain the animals are designed well to hold animal comfortably. Tail vein dilation is stimulated a warming chamber to increase the likelihood of getting the injection correctly placed first time; thus, reducing the stress for the animal. Similar methods are used whilst blood sampling. In addition, whilst blood sampling the smallest amount of blood will be collected. Substances that will be injected to either accelerate or reduce the inflammatory process will have been tested previously in different animals/humans or in-vitro. A thorough literature review will be undertaken prior to establishingthe models.

In all protocols we will aim to utilise approaches that minimise the animals' potential suffering. The likelihood of adverse responses will be minimised for agents by choice of dose rationale informed byprior in-house pilot investigations and in vitro studies and experience from collaborators or publisheddata.

Why can’t you use animals that are less sentient?

Mice and rats are the lowest vertebrate groups on which well characterised and minimal severity inflammation and immunology models have been developed. Their mammalian bodies are similar tohumans in that they have a complex and interactive immune and inflammatory system which can provide a good basis for predicting how, for example, a novel test substance will react inside the human body and interact with the system under investigation.

Animals at a more immature life stage will not have a fully developed immune system present.

It is possible to carry out some models in the immune/inflammation space under general anaesthesia.However, these are usually more severe models, terminal models or require the collection of more invasive samples throughout the study

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

To minimise harm to animals, especially those on procedures with which we have less experience, animals will be monitored regularly for routine signs of ill health or distress. Anaesthetics will be usedas appropriate to the procedure being undertaken and advice from local veterinary surgeons will be sought in any situation where animals are showing unpredictable signs of ill health or suffering.

Any further refinements that can be implemented over the course of this project will be put in placefollowing consultation with the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS).

Vehicle, pro-inflammatory agents or test/reference items will be administered as single or repeated (including in combination) administrations by the oral (by gavage), sc, ip and/or iv routes as detailed in the experimental protocols. They will always be given by the least severe route and administration will be performed by highly skilled staff, using appropriate dosing techniques:

(e.g.,http://www.procedureswithcare.org.uk/administration%20-of-substances/) and dose volumes (LASA guidelines) to minimise any stress and discomfort to the animals. Appropriately sized needles will be used, and a separate needle will be used for each animal for sc, ip and iv injection to maximise welfareand reduce the chance of inter-animal infections (https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care). Oral administration to rats will normally be by gavage using flexible catheters tominimise oesophageal trauma. Short dosing needles are typically used for mice which are likely to bite the flexible tubing. Following oral dosing, animals will be observed and killed by a Schedule 1 method ifdisplaying any signs of respiratory distress (lung-dosing). However, the incidence of this is rare (<1%). Any tissue damage or discomfort due to multiple subcutaneous, intraperitoneal or intravenous injections will be minimised by using appropriate sterile techniques, solutions at suitable pH and different sites of administration.

Where prior knowledge exists regarding administration of the test compound in vivo this will be used tooptimise the choice of doses and route of administration wherever possible.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

https://www.nc3rs.org.uk/news/re-use-needles-indicator-culture-care.

LASA Good Practice Guidelines/NC3Rs Blood sample volume Guide. Ullman-Cullere and Foltz LabAnimal Sci. 49(3) 1999 p. 319-323.

NC3R's - Species specific Grimace scale for pain.

Refining procedures for the administration of substances. Morton et al. Lab Animals (2001) 35, 1-41

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We receive the 3R's monthly updates and have access to the 3R's website plus we have a local 3Rsrepresentative.

Where we wish to implement an advance, depending on the nature of the advance we may carry outan in-house study to evaluate any effects on the scientific readouts prior to use on customer studies.

Where an advance is considered small such as the change in recommended needle size for aninjection route this would be implemented without the need for an in-house study.

7. Molecular Basis of Meiotic Recombination and its Impact on Fertility

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Fertility, Chromosome Pairing, Cell Division, Egg, Sperm

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

This project aims to understand how maternal and paternal chromosomes - the DNA structures that carry the genetic information in every cell of the body - pair and exchange material (recombine) duringthe cell division that produces eggs and sperm in mammals (meiosis), and to determine the impact of this process on fertility.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The cell division that produces eggs and sperm (called meiosis) is fundamental for healthy sexual reproduction in mammals. In sex cells, maternal and paternal chromosomes must pair and exchange genetic material (recombine) to maintain a correct number while dividing, which ensures successful meiosis and normal fertility. However the mechanisms that control this process remain poorly understood. This project will characterise in detail existing and novel regulators of each of these stepsto understand how they impact meiosis and fertility levels in the mouse.

Errors in meiosis result in miscarriage, birth defects, and infertility in humans. Infertility affects 1 in 6 couples worldwide. Despite recent advances in the treatment of male infertility in particular, the exactsdefects involved remain largely unknown.

The results from this project in mice will be directly relevant to human fertility, and indeed infertility. Findings may therefore assist diagnosis and counselling in patients with fertility issues, and open new treatment options.

What outputs do you think you will see at the end of this project?

This project will greatly advance our knowledge and understanding of the molecules involved in meiosis (the cell division that generates sperm and eggs), and how they impact fertility in mammals. These experiments conducted in the mouse will provide a detailed characterisation of the role of bothknown (but poorly understood), and novel (yet to be discovered) protein factors that regulate this fundamental process.

We expect the findings will be published in high ranking scientific journals, and will support successful applications for further funding. All new computer-based methodologies developed, as well as the datagenerated from the animals used during the course of this project will be extremely valuable and become available to the wider scientific community for use in other types of analysis that may advancefurther research in this field.

Who or what will benefit from these outputs, and how?

As meiosis (the cell division that produces eggs and sperm) is highly conserved across mammals, we anticipate the findings will have an immediate impact and advance both scientific and medical researchinto human fertility.

In the long term, the molecules identified and characterised in these studies in mice will provide new fertility candidate genes for screening which may help diagnosis and counselling in couples with fertilityissues, and open new treatment options for patients with infertility.

How will you look to maximise the outputs of this work?

This project is established in close collaboration with three other groups locally, each bringing specificskills and expertise. This enables an efficient and coordinated analysis of the mice studied, and the integration of the findings into a highly informative view not otherwise possible. The experimental strategies will be continually reviewed, and revised if necessary against the objectives, as a collaborative discussion. The findings from this work will be published (including all data generated made publicly available), and regularly presented at relevant conferences in the field, providing additional collaborative opportunities that may advance further the progress of the scientific discoveries.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Meiosis, the specialised cell division that produces sperm and eggs, occurs exclusively in cells of themale and female reproductive tissues, respectively. To characterise the protein factors involved, andstudy their role in maintaining normal fertility in mammals, we chose the mouse as our experimental animal model.

The laboratory mouse is the most widely used animal model in research to study key biological processes in human health and disease. Due to similar genetics and biology between mice and humans, genetic manipulation in the mouse can be used to identify disease mechanisms and develop treatments for humans. Importantly, infertility naturally occurs in the male offspring of crosses between certain mouse species; these represent natural (non genetically modified) fertility models to study. In addition, sites where genetic material was swapped between maternal and paternal chromosomes (thestructures that carry the genetic information in all cells of the body) are easy to detect in these crosses. Finally, a number of mouse mutants already exist for key molecules involved in meiosis; this provides agreat resource for this project. For all these reasons combined, the mouse represents the most appropriate model for this research and its relevance to infertility in humans.

Meiosis occurs at specific times in males and females during their lives. In females it begins in the mother's womb, in the ovary of the foetus, and pauses at birth, resuming later at puberty to complete at the time of sperm fertilisation; in males it starts in the testis around 10 days of age, and carries on intoadulthood. We will therefore use mice ranging from foetal to adult stages.

Typically, what will be done to an animal used in your project?

The animals will only be used for breeding and maintenance of the lines of interest to:

- assess fertility in live animals (through breeding)
- provide reproductive tissues for downstream analysis of meiosis

What are the expected impacts and/or adverse effects for the animals during your project?

The genetic modifications engineered during this project are not expected to cause any harm to theanimals besides a reduction in fertility (ranging from a partial to a complete loss).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mild severity: 100% of all mice

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Meiosis is a cell division that occurs exclusively in sex cells of the male and female reproductive systems. While we will be using non animal, easy to expand, cancer cells to study certain properties ofthe molecules involved in this process, these systems lack the specialised cell environment only present in sex cells. It is extremely difficult, however, to maintain sex cells alive and functional outside the animal to carry out experiments. At present, meiosis can therefore only be studied in the whole organism by analysing reproductive tissues of animals from relevant strains. In addition, this project requires live animals to investigate the impact on fertility of certain genetic modifications, by monitoring breeding performance to quantify fertility levels.

Which non-animal alternatives did you consider for use in this project?

Cancer cell systems: before moving to animal studies, if appropriate, we will first test the relevance of molecules of interest in cancer cell systems where they can be artificially expressed and some aspects of their function studied. In addition, such system has the advantage of high expression (in contrast to typically very low levels in the living animal), and more complex, quicker gene manipulation than possible in the mouse. The findings from these studies can then guide and complement experiments in animals, directly impacting animal numbers down. In some instances, theymay even preclude animal strain development if the function of the molecules tested do not prove to berelevant.

Sex cell systems: a number of studies have demonstrated the benefit of using mouse embryonic (stem) cells to characterise some aspects of meiosis, and we are keen to try and implement these more relevant cell systems for functional studies in the future. Recently, this cell type was successfully turned into sex cells using a specialised cocktail of factors. If feasible, we would consider implementingthis methodology in the future to replace some of the animal-based studies. This alternative, however, is likely to be technically challenging with limited success. This would have to be weighed against the level of replacement and reduction in animal use this approach would potentially enable so as not to compromise the progress of the research. Trials would first be required to determine the feasibility of this methodology in our hands.

Why were they not suitable?

We have successfully used in the past and intend to continue to use as much as possible non animalcancer cell systems where key molecules can be artificially expressed and some aspects of their function studied. However, as mentioned earlier, these systems lack the specialised factors only expressed in the male and female reproductive tissues, and cannot fully replace (only contribute to areduction in animal numbers) the studies in the whole body where the correct cell environment is present.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates are primarily based on the actual number of animals used on 2 previous Licences withsimilar animal experiments, taking into consideration:

- established mouse lines of interest already breeding on the current Licence
- new genetically altered mouse lines for genes of interest that will be generated during the courseof the project.

On average, we used 1000 mice per year over 5 years for maintenance of mouse colonies and experimental animals, all lines combined (average of 5 lines being studied). Based on an average of 8lines in breeding, we estimate we will use a total of 8000 mice over 5 years to complete this project.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Wherever possible, if appropriate, the molecules of interest will be tested first in cancer cell systems before moving to animal studies. We have successfully used and will continue to use cancer cell lineswherever possible to help inform and refine the animal studies, therefore working towards a reductionof the number of animals needed for experimentation. However, these cell systems lack the specialised environment only present in sex cells, and therefore remain limited for functional studies.
During the experimental design, we searched and will continually review available cryobanks and databases, including MGI (http://www.informatics.jax.org), the international mouse knockout consortium (http://www.knockoutmouse.org), and the DNA Archive for ENU chemical mutagenesis screens (http://www.har.mrc.ac.uk/services/archiving-distribution/enu-dna-archive) for existing mouse lines of relevant gene mutants and fertility models. Every care will be taken to avoid duplicating animalresources.

All new analysis downstream of animal tissue collection will be first trialled and optimised in pilot experiments on a small group of animals to estimate the minimum number to be used to achieve theresults.

Once determined, we will carefully control the breeding to avoid excessive and unnecessaryproduction of animals.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Breeding: breeding of all colonies will be reviewed every 2 weeks with the animal care staff toproduce the numbers required and, if possible, only the genetic modification desired, avoiding excessive and unnecessary production of animals.

Sharing of tissues: we will aim to share the tissues from the same animal between multiple analyses. When new opportunities arise, pilot experiments will be conducted to assess the feasibility ofthese approaches.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most widely used animal model in research to study key processes in human healthand disease. Due to similar genetics and biology between mice and humans, genetic manipulation in the mouse can be used to identify disease mechanisms and develop treatments for humans.

Based on these principles, we have chosen to use the mouse as our experimental model to studymeiosis (the cell division that produces eggs and sperm), and its role in fertility in mammals.

Importantly, infertility naturally occurs in the male offspring between very distinct mouse species, providing natural models of infertility. In these, and in other fertile crosses between distinct mouse strains, sites where some genetic material was swapped between maternal and paternal chromosomes (the structures that carry the genetic information in all cells of the body) are easy to detect. These mice are therefore very informative strains in which to introduce genetic modifications ofinterest to test their impact on meiosis and fertility.

During this project, we will characterise existing and newly generated mouse mutants for key genes of interest expressed in reproductive tissues. No harmful consequence is expected from the genetic modifications studied in these animals besides an impact on fertility (ranging from a partial to a complete loss). This project only requires breeding and maintenance of these mouse models to assessfertility in live animals, and provide reproductive tissues for analysis of:

- fertility levels by measuring the weight of testes and ovaries, and counting eggs and sperm
- pairing of chromosomes by antibody staining of sex cells
- binding of key molecules on chromosomes
- changes in expression of key genes during meiosis

Why can’t you use animals that are less sentient?

The mouse is the most appropriate animal model to study the cell division that generates eggs and sperm, and characterise the key molecules involved in this process for the reasons outlined above. Dictated by the biology of the process we are studying, we will use mice ranging from foetal to adultage. Like in humans, this cell division occurs at specific times during the development of male and female mice: in females it begins in the foetal ovary and arrests at birth, resuming later at puberty tocomplete during egg

fertilisation by sperm; in males it starts in the testis around 10 days of age, andcarries on into adulthood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

No harmful consequence is expected from the genetic modifications introduced in these animalsbesides an impact on fertility (ranging from partial to complete loss).

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow the published guidelines for best practice “Refinement and reduction in the production ofgenetically modified mice, Laboratory Animals Vol 37, Supp 1, July 2003”.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

The licence holder and all animal users involved in the project are regularly updated on advances inthe 3Rs via the following routes:

termly 3Rs newsletters published by the 3Rs sub-committee of the AWERB (Animal Welfare and Ethical Review Board) at the establishment are circulated to all animal users: they provide an update on the 3Rs advances in many areas of research and give specific examples of good practice and successful implementation in specific areas of research across the establishment. Useful links to other3Rs resources and upcoming 3Rs workshop events (both internal and external) are also advertised here.

Animal Welfare Meetings for animal facility users (3 times/year): the Regional Programme Manager for the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animalsin Research) and NACWO (Named Animal Care and Welfare Officer), are presenting examples of successful trials and new implementations of the 3Rs from projects within the establishment (also published in the 3Rs newsletter). Useful links to 3Rs resources and upcoming 3Rs workshop events (both internal and external) are also circulated during these meetings.

discussion with the Regional Programme Manager for the NC3Rs: opportunities for the implementation of a recent advance in the 3Rs within or outside the establishment, or the developmentof a novel approach can be explored in more detail.

N3CR website: www.nc3rs.org.uk.

8. Molecular Switches in Inflammation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Inflammation, Arthritis, Colitis, Immune Cells, Molecular Switch

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify key molecular regulators of myeloid cells in inflammation. This research will involve human and mouse studies which, taken together, should define the functions of the identified regulators in inflammatory disorders. In the short-term it will provide the scientific rationale for pre-clinical validation studies and in the longer term for clinical trials of strategies designedto interfere with these regulators.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sustained inflammation is an underlying factor in a wide range of diseases, including rheumatoid arthritis (RA), inflammatory bowel disease (IBD), vasculitis, atherosclerosis, diabetes and certain typesof cancer. Despite progress in understanding how they develop and what components of the immune system may be involved, much remains unknown. Although inflammation is a widespread occurrence during many common diseases the

range of treatment options for patients with chronic inflammatory diseases is very limited. People with inflammatory diseases who are obese tend to have more active disease, more pain and worse overall health. For example, obese people with RA are less responsive to biologics and traditional anti-rheumatic drugs. This phenomenon is linked to the fat depot being infiltrated by active immune cells constantly releasing inflammatory mediators that cause inflammation,which are already overactive in RA. Both bioactive lipids of Western diet and gut dysbiosis, e.g. an altered composition of intestinal microbial populations, are thought to provide continuous immunological stimulation contributing to the development of immune response anomalies in numerous inflammatory disorders. Antibiotic treatment and /or germ-free housing have a profound effect on disease development mice, with both protective and pro-pathogenic outcomes noted depending on the context. Therefore, it is important to gain a greater understanding of the molecular mechanisms underlying these processes in order to design novel treatment strategies for acute and chronic inflammatory disorders such as RA and IBD as well as during infections.

What outputs do you think you will see at the end of this project?

We have made considerable contributions to the understanding of the molecular events involved in theinflammatory response and have led to the identification of potential targets for the development of novel anti-inflammatory therapeutic interventions. Applying a similar strategy during this project, it is highly likely that we will identify novel potential anti-inflammatory drug targets with this approach. We expect to analyse up to 5- 10 new targets during the course of this programme. The proposed study will increase our knowledge of myeloid cell function in tissue, at norm and during inflammation. We will generate new mouse strains, new knowledge and new technologies that will be of benefit to the scientific community.

Who or what will benefit from these outputs, and how?

In the short term, our research will promote the new field of specific white blood cell (i.e. neutrophil, monocyte) adaptation to tissue microenvironment during inflammation and will help to define the role ofthese particular white blood cells in chronic inflammatory disorders.

In the medium-term, the identified key regulators of effector myeloid cell function can be used as biomarkers in future studies of human pathologies. The patients with chronic inflammatory disorderswill benefit from better molecular phenotyping of their conditions and thus more tailored treatment regime.

Our research on the impact of microbiome and diets will highlight more specific sites for interference and is likely to be of interest to pharma as well as companies working with nutraceuticals. The patientswith inflammatory disorders may benefit from advice on diet and the development of more holistic treatment based on a combination of therapeutics and diet.

In the long-term, we will facilitate the development of new anti-inflammatory drugs based on the targetsidentified within this study. We will use our established pharmaceutical framework to further the development of therapeutics targeting key inflammatory molecules identified during the course of this programme. This will lead to new patent applications and benefit players in the industry and private sector, as they will be able to capitalise on our prior discovery to bring about new drugs for chronic inflammatory and autoimmune diseases.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in peer-reviewed journals andpresentations at scientific conferences and meetings. The transgenic animals developed will be valuable to other scientists interested in the development of anti-inflammatory therapies.

We will conduct collaborative studies with clinical colleagues that would allow us to translate ourfindings to human pathologies.

Species and numbers of animals expected to be used

- Mice: 22,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We choose to work with mice, due to the availability of well-established models of inflammatory diseases in this species, and the fact that they have ~70% similarity to humans, which gives us a goodstarting point for translating findings to the clinic. We will use both wildtype and genetically altered mice, which are either global or myeloid-specific knockouts of regulators of interest, or exhibit fluorescence in cell types of interest, so that we can track immune responses. For our models, adultmice will be used, to allow time for their immune system to develop and mature before we induce inflammation.

Typically, what will be done to an animal used in your project?

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where administration is required for prolonged periods, animals will be surgically implanted with slow-releasedevices such as a mini-pump. These animals will experience some discomfort after surgery and somemild to moderate pain which will be treated with analgesics.

Animals may undergo changes in diet, which are not expected to cause distress but may sometimesresult in obesity.

Animals may experience blood sampling, which will cause mild and transient discomfort.

Animals may undergo multiple instances of gait analysis testing (where they are placed on a pressure pad for a few minutes). This may be mildly and transiently distressing but will not cause them any pain.

Animals may undergo non-invasive imaging, such as bio luminescence or micro-CT under anaesthesiato immobilise the animal while an image is being taken. The imaging process will take a maximum of 30 minutes. The animals will be aware of the anaesthetic being administered but will experience only mild distress.

Animals may undergo intravital imaging. This is undertaken under non-recovery anaesthesia, where the animals will only be aware of the anaesthetic being administered and may experience mild distressbut no pain.

Animals may undergo bone marrow irradiation and reconstitution (where applicable), which can causeradiation sickness, which usually stabilises within 3 days of the procedure. The animals may be givenantibiotics to prevent infections because their immune system will be low, as well as soft food, floor- level water and extra bedding to support thermoregulation.

Animals may develop arthritic pain and swelling in their joints, with symptoms lasting typically for up to 14 days. Animals will still be able to move around, climb, eat and drink, but their joints may feel sore indoing so. They will develop arthritis following injections into the joint and/or base of the tail, which will occur under anaesthesia, so the animals will only be aware of the anaesthetic being administered and may experience mild distress.

Animals may experience flu symptoms following nasal administration of virus, which may lead to breathing difficulties. It is likely that the animals will show signs of illness, much like humans, which should resolve after 10 days – symptoms may include reduced food and water intake, listlessness, weight loss, shivering. Animals will be provided with extra bedding to help with thermoregulation, andsoft food and floor-level water to help them recover.

Animals may develop colitis, with symptoms including loose faeces, lasting for up to three weeks. They may feel some swelling of their abdomen, feel dehydrated or experience weight loss, so they will be carefully monitored and given access to soft food and floor-level water to ease their symptoms. They will develop colitis following oral administration of bacteria into their stomach and/or by injectionof antibody, which may cause them to experience mild distress. In order to monitor colitis symptoms,animals may be given a colonoscopy to take intestine biopsies – this will occur under anaesthesia.

These animals will experience some discomfort after surgery and some mild to moderate pain whichwill be treated with analgesics.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may be administered substances such as tamoxifen by oral gavage or injection in order to modulate expression levels of a transgene. This may cause the animals to lose their appetite, resultingin transient weight loss, which will be monitored throughout the dosing period. There may also be soreness at the site of injection, due to multiple administrations.

Animals may be subjected to bone marrow irradiation, which can cause radiation sickness. The animals may exhibit a transient weight loss approximately 7-10 days following irradiation due to loss ofappetite and diarrhoea, which usually stabilises within 3 days. They may also exhibit a mild hunched posture. The animals may be given antibiotics to prevent infections, as well as soft food, floor-level water and extra bedding to support thermoregulation.

Animals may be infected with influenza virus or RSV, which can lead to signs of illness for up to 10days following intranasal administration. There is a small chance that animals may experience breathing difficulties as a result of administration of the virus. The virus

symptoms may include listlessness, reduced food and water intake, and weight loss of up to 15%; however, animals are expected to regain normal behaviour and restore their body weight within 10 days post-infection.

Animals will be given soft food, floor-level water and extra bedding to support thermoregulation.

Animals may develop arthritis symptoms, caused by joint and/or subcutaneous injections of adjuvant/antigen under anaesthesia to stimulate an inflammatory immune response directed at the knee/paw joints. Symptoms of the arthritis models mimic human disease – swelling and pain of thejoints, leading to reduced mobility and discomfort, and ultimately cartilage damage if the disease is allowed to progress. Typically the animals will experience swelling of the joints for up to 14 days.

Some experiments may continue for up to 12 weeks in order to study resolution of arthritis, during which time the swelling and inflammation will decrease but the mice will still experience reduced mobility and some discomfort due to cartilage damage in their joints. The subcutaneous injection sitesmay experience some soreness and ulceration due to the irritant nature of the adjuvant used. Ulcers can be treated with barrier cream to soothe and encourage healing. Animals will be given soft food, floor-level water and extra bedding as required.

Animals may develop colitis for up to 3 weeks following oral administration of bacteria or compounds inthe drinking water and/or intraperitoneal injection. Symptoms may include temporary weight loss and loose faeces. Animals will be given floor-level water to prevent dehydration. In some instances, anal inflammation and rectal bleeding may occur. In order to monitor colitis symptoms, animals may be given a colonoscopy to take biopsies – one colonoscopy can be given each week, and a maximum of two biopsies taken on each occasion (maximum 6 times). This procedure should last 5-15 minutes.

There is a small chance (<1%) that the colon could be damaged by the endoscope or that rectalbleeding or abdominal swelling could occur, so the animals will be carefully monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

43% Sub-threshold, 24% Mild, 33% Moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The steps leading to the development of the state of chronic inflammation are poorly understood and itis likely that multiple physiological processes are involved, including proliferation of precursor cells, activation of lymphocytes and recruitment of inflammatory cells. This and modelling the effects of new therapies inevitably involves the use of whole organisms and, in particular, the use of animal models ofinflammation. As we are unable to induce inflammation in the clinical setting, mice and with ~70% similarity to the human and well-established models of inflammatory diseases of interest, make an ideal model for our work. Mice are preferable to rats because of the greater availability of reagents (e.g. monoclonal antibodies; genetically modified strains) specific for this species.

Which non-animal alternatives did you consider for use in this project?

We considered and use human cells to address some aspects of the project, such as questions relating to the mechanisms of action of compounds targeting the molecular regulators identified, or therole of inflammation in cell activation.

We also use newly developed cell lines, that allow for in vitro differentiation of immature cells from bone marrow into mature white blood cells (neutrophil and macrophage) in the blood to address basicquestions on their biology.

Why were they not suitable?

Isolated human cells can not fully reproduce the interaction between various cells in tissue, the scaffoldthe tissue makes or the impact of systemic manipulations on local responses and vice versa.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number is based on our historical data and familiarity with the protocols included in therenewal of this licence. We estimate the experimental group size for each protocol to detect the expected difference between groups (approximately 6-8 mice per group is usually sufficient). We alsouse our annual returns to consider how many mice are required for our breeding programmes.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

To ensure that the minimum number of animals will be used in this project, we will control the variationintroduced by experimental layout, e.g. animals of the same sex, similar age, weight, fed on the samediet, provided with the same bedding. To remove any bias, we will consider running independent replicas, randomising and blinding the animals between the subject and control groups. We will make appropriate arrangements to randomly assign animals to experimental groups and blind studies. The design of experimental layout will

be adequate to the hypothesis tested and based on the results of pilot experiment(s) with the help of the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where possible littermate controls will be used to further increase the sensitivity of our read-outs, untreated control groups may also be shared between treatment groups. In addition, untreated controlgroups will be used as a source of tissues for analysis of expression of pro-inflammatory mediators.

We will also maximize the use of harvested tissues and cells. For example, immune cells isolated frominflamed tissue from a genetically modified animal may be used as a source of immune cells for multiple in vitro experiments (genomics, FACS-based characterisation, migration). Cells and tissues may be shared by multiple researchers.

In preference to adoptive transfer studies, we will utilize genetically modified animals with deletion of agiven gene only in specific cell types, significantly reducing further the numbers of animals required formechanistic studies. Experiments will be planned so they can be published in accordance with the NC3Rs' ARRIVE guidelines.

We also pay a considerable attention to our mouse colony management. We keep a careful documentation of the number and type of breeders to help organize the colony and ensure no unnecessary breeding is carried out. Animal requirements are reviewed on a weekly basis. We use homozygous genetically altered and wild type breeder pairs, which have been generated from originalheterozygote breeder pair and hosted in the same environment. We re-set these pairs on a regular basis. Embryos of strains not currently in use are cryopreserved.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen models which have already been refined to minimise the number of immunisations and the severity level of adjuvant. They are the least severe and cause the minimum amount of distress . For example, between the two models of arthritis, such as antigen induced arthritis (AIA) andcollagen induced arthritis (CIA), the AIA model will be used in preference where possible; as it gives rise to a greater incidence and less variability; and is less severe. The CIA model will only be used when necessary to replicate the human disease pathogenesis more closely. For our colitis studies we will use a strain of helicobacter that is widely utilised as a model system, and therefore the protocol and disease timecourse is well established. We will preferentially choose acute models of inflammation such as the air-pouch model or LPS-induced lung injury, where the inflammation is contained to a single compartment to answer our scientific questions

where possible, as opposed to systemic models to ensure we cause the minimum amount of distress.

We pay careful attention to animal husbandry and provide environmental enrichment and co-housingto avoid social isolation. Where possible we will use genetically altered mice where the genes of interest are either expressed or deleted in specific cells or tissues types rather than in the whole organism to minimise potential harm.

Why can't you use animals that are less sentient?

We cannot use fish, flies, or worms, as we need mammals with a fully functional immune system to address the questions we would like to answer. We cannot use terminally anaesthetised animals as weneed to induce the disease and allow it to progress.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Wherever possible we will administer substances for experimental purposes in food or drinking water, instead of injections. Where daily administration cannot be done in food or water the use of a surgicallyimplanted miniature infusion pump will be considered over injection routes. If injection routes are necessary, we will choose the route with minimal harm but will still enable the maximum delivery to thetissue target.

Where knockout of regulators is required, conditional knockout in myeloid cells is preferable to globalknockout, to focus the reduction of expression in our immune compartment of interest and minimize off-target effects. Moreover, expression levels can be controlled by administration of drugs such astamoxifen in some mouse strains, which minimises the need for crossing mice.

The use of adjuvants is necessary in some of our models, and we will use those adjuvants that cause the least severe response. Where we need to use an adjuvant that will cause a more severe response,we will deliver the dose at two sites to reduce the risk of inflammation at a single injection site.

For bone marrow transfer experiments, irradiation will be given in 2 separate doses - at least 4 hoursand no more than 24 hours apart - to minimise side effects. We will also provide antibiotics prophylactically to prevent infection as advised by NVS.

In models where anti-inflammatories cannot be given because their anti-inflammatory activities tend toinhibit the induction and progression of the disease (e.g. arthritis); additional husbandry support, such as easier access to food and water (e.g. food pellets and water gel packs placed at floor level), and/orsupplemental bedding will be provided during the acute inflammatory stage of disease in all animals. In consultation with NVS, opioid analgesics e.g. buprenorphine may be provided if required. In modelsof colitis, mashed food will be provided to aid recovery.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow PREPARE and ARRIVE guidelines of best practice when designing and reportingexperiments under this licence. The LASA guidelines will also be consulted.

How will you stay informed about advances in the 3Rs, and implement these

advanceseffectively, during the project?

We will attend welfare meetings and 3R's meetings held at the establishment, and sign up to the NC3R's website updates. We will interact with the regional NC3R's manager, as well as the NamedInformation Officer, and disseminate acquired information in our group lab meetings.

9. Monitoring and Supplementation of Minerals in Ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
  - Improvement of the welfare of animals or of the production conditions for animalsreared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man oranimals

Key words

Supplementation, Mineral, Grazing, Ruminant, Trace Element

Animal types	Life stages
Cattle	neonate, juvenile, adult, pregnant, aged
Sheep	neonate, juvenile, adult, pregnant, aged
Goats	neonate, juvenile, adult, pregnant, aged

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To develop strategies and supplements to improve management of mineral status in cattle, sheep andgoats.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that

accrue after the project has finished.

Why is it important to undertake this work?

The project aims to develop new supplements and optimise strategies to fully utilise current supplements to improve the mineral status of grazing sheep, cattle and goats. Grazing ruminants are completely dependent on the mineral composition of the grass/plant in the pasture. In most grazing situations animals are not fed in the same way as housed animals but instead forage their own diet from the pasture. If the grazing does not supply optimum minerals for optimal health and/or production then supplementation is likely to be required. Indirect supplements include free access powders, blocks and licks all of which are subject to variable intakes and rely on nutritional wisdom on the part ofthe animal (which tends to only occur at deficiency levels resulting in abnormal appetites called pica). Direct to animal supplements include injections (not many available), drenches (orally delivered like a spoon full of medicine) and boluses (large tablet like, which are administered orally and will remain within the rumen (large fermentation stomach) and slowly dissolve, releasing minerals over a long timeperiod (months rather than weeks or days)).

Drenches have previously been shown to have a variable response, this depends on which element is being supplemented. Cobalt drenches have been found to be effective for a very short time, if at all, and hence boluses can be better for this mineral, whereas selenium via drench can give a longer response meeting the animal's selenium requirements.

Ruminants as grazing animals are reliant on the pasture they consume, multispecies swards which often include deeper rooting grasses and herbs which as well as being more drought resistant and flood tolerant potentially have a better mineral profile. Trees are also a good source of fodder in drought situations and the growing of trees may alter the grass underneath through soil structure andshade improving mineral composition of this grazing area. Ruminants can selectively graze so to fullyevaluate these novel grasses and tree fodder approaches grazing trials need to conducted to show animal mineral status responses. Amongst the trees, willow in particular has elevated cobalt and zincenabling it to be utilised as a biosupplement potentially replacing the use of inorganic (carcinogenic) compounds in feed mills.

What outputs do you think you will see at the end of this project?

The outputs of the work in this license will lead to the development of of new products and/or strategiesto get the best utilisation of supplements and strategies. This will include developing best methods of utilising tree fodder for ruminant production.

The outputs will include scientific publications but will also include the release of research briefings viaorganisations such as the Woodland Trust. Knowledge transfer to the end user is key for this type of work so outputs will also be via the agricultural (Farmers weekly, Farmers Guardian) and veterinary (Vet Times, Vet Record etc) press. There maybe some publication of results via social media platforms, especially through partner organisations and the use of consumer-friendly infographics.

Who or what will benefit from these outputs, and how?

The farmers, the animals, the livestock feed and supplement industry, and the veterinary industry will all benefit from the greater understanding and/or a more optimised approach

to using the supplementsand strategies available, which may include the development of new supplements or evaluation and improvement of the current ones.

Whilst all work which increases the optimisation of production will enhance the environment, there arefurther environmental benefits from the use of trees to provide fodder and mineral supplementation.

Some tree species have been shown to reduce the urine green house gas emissions of livestock feeding on them, and as trees themselves can act as effective carbon sinks an increased tree growthon agricultural land as a fodder source will also be beneficial environmentally. The work here will support more planting and will look to guide planting strategy in order to be able to get the best livestock utilisation.

How will you look to maximise the outputs of this work?

We look to collaborate extensively whether this is with industrial partners, charities such as the woodland trust, agricultural levy boards, veterinary practices/organisations. We would look to utilisethe agricultural and veterinary press, as well as social media for dissemination of results and the benefits of these results on animal health, welfare, productivity and the environment.

It is important to show results when things do not work and in light of this have recently published anarticle demonstrating the inability of an *in vitro* approach for determining rumen interactions.

Species and numbers of animals expected to be used

- Cattle: 180
- Sheep: 300
- Goats: 90

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project is using the species for the benefit of the species in question and not using the sheep, goats or cattle as a model for another species. The outputs of this work are relevant to ruminants in the UK predominantly sheep and cattle as these are most commonly farmed. This project will use theruminant species and life stage appropriate to the output for example the effect of browsing willow on the growth of lambs will use growing lambs, effects of supplementation on lamb numbers would use ewes being mated. The use of supplements or altered forage is not intended to be detrimental to animal health and performance; it is intended that these experiments will serve to enhance health and welfare of the individuals used in the trials. The mineral levels available would be in ranges available in the natural and commercial situation.

Typically, what will be done to an animal used in your project?

Predominantly the project will take blood samples to monitor mineral status, although there

is also thepotential to collect faeces and rumen fluid which can be useful to determine the mineral intake or supplement release particularly of long-term rumen residing mineral boluses. Neonatal and pre- ruminant juvenile sampling will only be blood to monitor placental/lactational transfer to the offspring from maternal alterations (eg changed grazing or supplementation)

The duration of each individual experiment will be appropriate to the experimental aim and will vary. Some supplements are designed to provide a mineral release over a long time period, rumen bolusesare often targeted to last 6 months, whereas the effect of grazing willow on the cobalt status (vitamin B12) can be seen over a matter of 2 weeks. An experiment to determine the duration of effective supplementation may require more samples and instead of than using a set time period (eg every 2 weeks) a varied timescale may be used (eg monthly until 3 months, then fortnightly and then weekly around the expected end of supplement duration- to pin-point the end of the effect). With longer trials itis often possible to reach a suitable result on a shorter timeframe, eg if a supplement has an expected duration of 6 months but the 4 month sampling indicates the supplement has run out then the samplingcan stop.

Animals at the end of the experiments on farm will either be released back to the farm stock (recordedand not available to use again under license on other trials) or if at the appropriate life-stage (prime production or cull) may be sent to slaughter at an abattoir as the animals will remain fit for human consumption.

What are the expected impacts and/or adverse effects for the animals during your project?

The major procedure used throughout the trials is blood sampling, this will have a short instant pain from the needle and potentially some local bruising. The effects of this are no worse than a blood sample collected for a diagnostic reason. If carried out, faecal and rumen sampling are behaviourallyinvasive and the most likely adverse effect would be local bruising. Training and refinement of techniques will be undertaken to try and minimise any discomfort from these procedures. Weight willbe monitored on trials using the methods regularly used as normal management practice and trials stopped if there is weight loss which is likely to compromise animal welfare.
To minimise effects on behaviour and to reduce stress we try as much as is possible to minimiseadditional handling events and will work with animals when they are being routinely gathered andhandled for normal farm work.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The severity should be mild for all animals under this project.

What will happen to animals at the end of this project?

- Killed
- Kept Alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ruminants are complex, the very nature of their fermentative, microbiota-filled rumen which utilises around 8,000 bacterial species as well as protozoa and fungi means that it cannot be effectively replicated without using the intended species. In addition to its complexity, ruminants have regulatorymechanisms in place which can prevent uptake of minerals if their status is already within ‘adequate’.Due to the unique environment created by the rumen there are also many interactions which occur between minerals, and between minerals and other dietary components, which prevent any other model being used.

Ruminants also will have large intakes of forage, either from grazing or via conserved feed. Whilst it ispossible to analyse conserved forage it is still inherently variable and intakes and interactions cannot be reliably estimated. Grazing is even more variable and there is a greater likelihood of selective grazing meaning that the diet offered is not the one consumed. Even in more controlled feeding situations the diet formulated will often not match the diet offered or the one consumed and analytical techniques are also unable to give a reflection of the bioavailability of the element to the animal. Directevaluation of animal indicators are therefore required to be able to reliably indicate mineral status from different forage systems (different pasture types, or use of trees for forage) or from different mineral supplements and strategies.

Which non-animal alternatives did you consider for use in this project?

For some of the supplement development work, especially boluses then in vitro dissolution can be undertaken. This can give an idea of the release profile. These would be used for an initial screeningand selection process but are dependent on rumen buffer pH, and stir rate and are non-continuous systems.

Laboratory analysis of the mineral composition of feed components can be undertaken and used toestimate effects.

Why were they not suitable?

In vitro dissolution whilst appropriate as a pre in vivo screen does not truly evaluate bolus dissolutionas the rumen is a complex environment subject to variable pH, bacterial population, rumen contractions and continuous turnover (in/out) of digesta. They also do not allow determination of bioavailable release (ie is the released mineral absorbed and is available for use by the animals).

Laboratory analysis will ignore selective grazing behaviour/feed intake as well as intake from non-direct dietary sources (grazing hedges, soil consumption*).

* soil consumption can make up to 25% of a sheep's dry matter intake, higher with shorter grazing andin very wet or very dry situations.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Through experience and some trial work to determine parameter variance we have found that typicallyrequire 8-10 animals per group for blood indicators of nutritional status. The exact numbers will more depend on exactly which trials are to be run and the immediate trial hypothesis. Previous trial work has typically used between 20 (2 groups) and 100 (10 groups).

So for the animals in the project we have estimated an average of 3 groups of 10 per trial and then:Cattle - 3 years of cattle trials with 2 trials per year = 3x2x3x10 = 180
Sheep - 5 years of sheep trials with 2 trials per year = 5x2x3x10 = 300Goat - 3 years of goat trials with 1 trials per year = 3x1x3x10 = 90
What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have previously carried out trial work to determine the variability of the parameters that we utilise todetermine mineral status and applied power calculations which indicate for research relevant powers (a little higher than diagnostic power) to achieve a significant result (scientifically normally a probability of $P<0.05$). This has give group numbers typically in the range of 8-10. If certain experimental designsare used, then cross over of factors etc will allow a reduction in numbers. Typically for this we would also review the trial set up using the resource equation (Learnt as part of module 5 training in experimental design).

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

To optimise the animal use, spare samples will often be taken and stored and then subsequently usedto extend/expand the trial beyond it's initial aims and objectives, for example in trials evaluating effectsof tree fodder on zinc and cobalt status then other element status such as iodine and copper may alsobe evaluated on stored samples.

If a trial involves slaughter then additional tissue will be covered if possible and retained.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The license protocols are for monitoring. The supplements and strategies under investigation are designed to improve the health and productivity of the species and life-stage which is being trialled, although there will be some relevance of the outcome to other species and/or life-stage. Neonatal andpre-ruminant juvenile will only be used to monitor the effect of maternal changes in strategy or supplementation (Blood only, no faeces or rumen content samples). The project is about optimising animal experience and the sampling techniques used are minimally invasive and should only give minimal short term pain/discomfort with no lasting harm to the animal.

Why can't you use animals that are less sentient?

Ruminants are complex, the very nature of their fermentative, microbiota-filled rumen which utilises around 8,000 bacterial species as well as protozoa and fungi means that it cannot be effectively replicated without using the intended species. In addition to its complexity, ruminants have regulatorymechanisms in place which can prevent uptake of minerals if their status is already within 'adequate'.Due to the unique environment created by the rumen there are also many interactions which occur between minerals, and between minerals and other dietary components, which prevent any other model being used.

The animals need to be behaving normally, eg showing the same natural selection within grazing asthe normal animal. Different ages may act differently so the most appropriate life-stage for the question being asked will be used. The age and life-stage used will be the most appropriate, we arenot looking to directly model different species/life-stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We have post procedure protocols in place to monitor health and welfare. The licensees are trainedand experienced in the procedures undertaken.

When working on farm, we attempt, if possible, to match the normal farm handling times so our workwill not add additional gathers and handling away from the normal kept environment.

For urine collection which can be achieved by free catch or urethral stimulation (stroking are under vulva externally to induce a urination) in cattle was more tricky in sheep.Sheep are more difficult to free catch due to the size and do not respond to urethral stimulation so previously would have requiredcannulation. However, we have developed a non-invasive alternative in sheep, which uses a prolapseharness to hold a collection bag over the vulva whilst the sheep is able to continue with its natural behaviour.

Liver samples are also a useful indicator of mineral status and whilst they can be collected moderatelyeasily via liver biopsy, this is a surgical procedure likely of moderate severity. We have successfully over a number of years utilised collection of liver tissue recovered at commercial slaughter. This may require collection over a number of different sampling days from a great number in total to get the timescale that would be achieved from repeated liver biopsies on the same animals to show change inliver mineral status.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will work within the scope of the ARRIVE guidelines (Animal Research: Reporting of In

Vivo Experiments). These are a checklist of recommendations to improve the reporting of research involving animals – maximising the quality and reliability of published research, and enabling others tobetter scrutinise, evaluate and reproduce it.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Information of advances in 3Rs including 3R conferences are circulated via internal distribution lists.There are also advances in 3Rs in scientific papers and the scientific and veterinary magazines. In addition, there are the licensing support pathways with NACWO, NVS and even inspector inputs.

10. Physical and Functional Effects of the Sex Chromosomes in Reproduction, Development and Human Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Infertility, Reproduction, Early Embryo Development, Male-Female Differences, Stem Cells

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Our research focusses on sex chromosome functions in the germ line, and their role in infertility. Weare interested to understand how the identities of male and female germ cells are established duringearly embryonic development.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Males and females differ due to the complement of sex chromosomes that each carry. Males are XY and females are XX. Previous studies from my group have shown that mismatch between sex chromosome complement, and phenotypic sex leads to infertility (e.g. Klinefelter syndrome (males withan extra X; XXY) and Turner syndrome (females with one X; XO)). My research group has a focus on determining how differences in the identity of male and female germ cells is established during their development, and understanding how mismatch between sex chromosome complement and phenotypic sex

leads to infertility.

What outputs do you think you will see at the end of this project?

We aim to gain advancement into understanding the origins of infertility. Specifically, outputs will focuson how the X / Y chromosomes are involved in processes that govern normal and impaired fertility.

Anticipated results at the end of the project will include new experimental data and novel findings, which will be disseminated via publication and communication. Publications will include peer-reviewedarticles and reviews in scientific journals, and posters as conferences. We will share our findings oral presentations at meetings in the United Kingdom (UK) and internationally. Communicating our research will improve data sharing and dissemination, as well as sharing of reagents that can potentially be accessed by the worldwide research community.

Who or what will benefit from these outputs, and how?

The research proposed is aimed at providing answers to basic biological questions regarding germ celldevelopment, and as such will benefit the global research community including developmental / reproductive biologists, biomedicine researchers, clinical researchers and physicians both in the UnitedKingdom and internationally. Academics with a basic interest in the biology of germ cells / testis / ovarywill be the primary beneficiaries. However, the project will be of significant interest to a much wider set of scientific fields, including stem cell biology (e.g. ES cells, spermatogonial stem cells), X chromosome regulation and regenerative medicine. Clinical disciplines that could benefit from this research include endocrinologists (e.g. testis development), specialists in reproductive medicine /andrology and oncologists (e.g. fertility preservation).

We will adopt a comprehensive plan to disseminate our research findings to the widest possible audience and stakeholders. This will include regular presentations of our research findings in meeting presentations at UK and international meetings and workshops in the testis biology and reproductive physiology. We will strive to publish our research in leading journals and will continue to update the field with review articles. As part of our focus on disseminating research progress, we will also highlightimportant research advances through the social media. Key scientific developments would be publicized through the press office and public-engagement networks with the research sponsor.

Skills training and development of the post-doctoral fellows and PhDs employed as part of the projectwill be prioritized. This will include training and career support under my supervision, including formal training modules and continued professional development in sustainable / transferable skills provided.Group members will be encouraged to participate in research forums, and there is an array of diverselearning and development opportunities more widely available more widely. A key goal would be to nurture the development of future researchers in reproductive biology and regenerative medicine.

How will you look to maximise the outputs of this work?

The proposed research will be based on our unique insights into germ cell biology, sex chromosomesand sexual dimorphism, to answer basic mechanistic questions regarding the nature of sex chromosome functions in germ cells, which is a particular strength. This approach will be useful in developing new therapies that target germ cell loss resulting from cell-intrinsic / epigenetic effects in germline stem cells.

In keeping with commitments to openness in academic research, we will deposit data, resources and methodology (e.g. publications, data and models) in public open-access repositories. In particular, large next-generation sequencing datasets will be deposited in established repositories (e.g. EuropeanNucleotide Archive), to serve as part of a comprehensive record of the world's nucleotide sequencing information, covering raw sequencing data, sequence assembly information and functional annotation.

We will promote our links with clinicians and researchers at UK Higher Education Institutions, clinical Royal Colleges and National Health Service leaders to foster research connections in the future. We will continue to build on our existing collaborations to ensure that results generated are discussed. I willendeavour to establish new research collaborations to integrate / expand research networks, aimed at bringing together leading researchers in fields highlighted earlier. These new research links will be aimed at sustaining our research endeavour, enabling translational applications of our research into future clinical cell-based therapies. As part of our commitment to broad public engagement in science, we will engage positively with charities, social enterprises and community engagement networks (e.g. schools, public libraries).

Academic impact: The project will produce new information / knowledge that will be of interest and benefit to academic researchers, clinicians, the public as well as commercial entities globally. The knowledge accumulated will benefit diverse sectors of the research community with interests in germline stem cells (spermatogonial stem cells), regenerative medicine (spermatogonial preservation),testis biology, developmental genetics, sex chromosomes functions and infertility.

We will seek to disseminate our research findings using a variety of communication media and outreach efforts in order to reach the widest possible audience and to achieve maximum impact. This will include presentation of our research as oral communications and posters at national / internationalmeetings and workshops in the fields of germ cells, testis biology, endocrinology and oncology (i.e. direct data sharing of findings). The project will also produce new information that will be disseminatedthrough high quality peer-reviewed publications and reagents that can be accessed by the worldwide research community. Datasets and technologies generated will be made available in open access andfree public databases. Sharing of research developments, reagents and methodological progress will be promoted using innovative methods to achieve high impact.

Impact on Communication and Engagement: A comprehensive public engagement plan will be enactedto effectively communicate research developments from the group to the wider public. This will involve the use of social media (e.g. lab Twitter account and using interactive media) as well as the host institution's active press office and outreach programmes (e.g. KCL open days). The PI will engage clinicians and clinical academic networks to disseminate research (e.g. Royal College of Obstetricians and Gynaecologists, British Fertility Society, European Society of Human Reproduction and Embryology). Public engagement will also be encouraged with staff members on the Fellowship to interact and promote communication with young people (e.g. science festivals, and though participation in interactive media platforms), raise awareness and communicate with broad areas of thecommunity which will promote equality and diversity.

Species and numbers of animals expected to be used

- Mice: 13100

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The laboratory mouse is a well accepted and highly characterized animal model to understand human conditions. Previous studies from us and others have utilized mouse models to study sex chromosomefunctions in development and in disease. Specific advantages of using the mouse include the availability of high-quality genome with extensive structural annotation for the genes / exons, promoters, enhancers, genetic tractability of specific inbred strains. The mouse is further amenable to the introduction and propagation of highly targetted genetic changes through careful and controlled breeding. The laboratory mouse can be relatively easily housed within an animal unit, where variationsdue to temperature, light, water, feed, etc. are strictly monitored and controlled. Mice reach maturity to breed within weeks of birth (6 weeks for females and 8 weeks for male mice), and have multiple births in litters, thereby permitting the study of control and experimental animals within the same litter.

Together these factors reduce the time required to perform high-quality experiments.

Germline development occurs over a protracted time frame and all these ages will be incorporated inour experiments in order to obtain a holistic understanding. Hence the project will involve analyses performed at a variety of life-stages including preimplantation embryonic stages (E0 - E3.5), post- implantation embryos (E4.5 - E19.5), early post-natal stages (P0 - P21), juvenile and adult mouse stages in both males and females.

Typically, what will be done to an animal used in your project?

The plan of work would involve the following Protocols:

Breeding and maintenance of genetically altered (GA) mice.

We would expect the bulk of the proposed work to relate to this.

Genetically altered mouse colonies will be established or maintained on this protocol either as a resultof being recovered by embryo transfer or being purchased from an approved supplier to expand and distribute within the establishment for research purposes. The most efficient colony management techniques will be used, and accurate breeding and welfare records will be maintained to monitor the condition of the colony.

Mouse superovulation.

Mice may be administered substances to induce superovulation to harvest oocytes, embryos, andblastocysts. Females may be naturally timed-mated to generate embryos.

Phenotype analysis of mice, long term monitoring and provision of tissue.

Mice may be administered substances to induce or repress gene expression, to label dividing cells or proteins, for later data analysis, or to remove germ cells. Occasionally we

may need to image animals.Mouse material may also be harvested following perfusion fixation under terminal anaesthesia.

Occasionally it may be necessary to humanely kill pregnant females, after which late stage embryoswill be delivered by Caesarean section and fostered.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding and maintenance of genetically altered (GA) mice.

Animals under this protocol are not expected to exhibit any harmful phenotype. Although some animalsmay have the potential to develop a harmful phenotype (e.g. tumours, neurological signs) after a certain age. In all cases, these will be killed before reaching that age and before onset of clinical signs,unless moved on to another protocol for a specific purpose (continued use).

Animals with altered immune status will be maintained in a barrier environment, thereby minimizing thelikelihood of compromising health.

Animals exhibiting any unexpected harmful phenotypes will be killed (Schedule 1), or in the case of individual animals of particular scientific interest, advice will be sought promptly from the local HomeOffice Inspector.

Any animal will be immediately humanely killed if it shows signs of suffering that is greater than minorand transient or in any way compromises normal behaviour

Mouse superovulation.

Animals under this protocol are not expected to exhibit any harmful phenotype. Superovulation procedures are expected to result in no more than transient discomfort and no lasting harm. Female mice will be of an appropriate size, in particular if they are to be mated, and over vigorous males will bereplaced.

Any animal showing any deviation from normal health or wellbeing will be killed immediately by aSchedule 1 method.

Phenotype analysis of mice, long term monitoring and provision of tissue.

The majority of animals are not expected to show signs of adverse effects that impact materially ontheir general well-being.

In all cases, the general health and condition of an animal will remain the overriding determinant.

While it is not possible to fully predict the nature or severity of any potential defect and for all types ofmice, careful monitoring for possible side effects will be instituted. Assessment will be made of pain and distress, as measured by normal and provoked behaviour; movement; physical signs such as altered respiration rate; animal posture (huddling or hunching) skin and coat changes such as piloerection or over-grooming; reduced appetite or inactivity; changes to body weight, food consumption; inflammation of injection sites; and comments on the animal’s general appearance.
If these signs persist for 24hr, mice will be culled immediately. Body weight loss of 10%

over a 24hrperiod or loss of 15% of body weight (compared to age-matched controls) that persists for 48hrs willalso result in animals being killed. Animals that display more serious clinical signs such as marked piloerection, persistently hunched posture and unresponsive behaviour to extraneous activity or provocation, and/or clinical signs of suffering like persistently laboured respiration (dyspnoea), or persistent diarrhoea or dyspnoea will be culled immediately.

Where the immune status of the animals might compromise health, they will be maintained in a barrierenvironment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Breeding and maintenance of genetically altered (GA) mice.

- mild (100%)

Mouse superovulation

- mild (100%)

Phenotype analysis of mice, long term monitoring and provision of tissue.

- mild (85%)
- Moderate (15%)

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept Alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To form properly, germ cells require multi-way *in vivo* interaction with other cell types in the gonad, as well as changing levels of hormones provided via the bloodstream. Our studies require the use of animals, because currently it is not possible to make germ cells (i.e. eggs and sperm) *in vitro* and studythese effects on the organism. Furthermore, pathways that control egg and sperm development are complex and require two-way interactions with supporting cells within the intact gonadal environment. Our experiments frequently involve assessing the effects of genetic alterations in mice that already have abnormalities in sex chromosomes and / or other genetic alterations, and the resulting unique combinations can only be created through breeding. Animals are therefore required for these experiments. Notably, most analyses that we perform in laboratory mice are carried

out on material obtained after schedule 1 killing, which is in itself unregulated. Nevertheless, one of the aims in the lab would be to generate germ cells *in vitro* in the laboratory, inorder to replace the use of animals in the longer term. The application of high-throughput next- generation sequencing analysis of gene expression patterns in developing germ cells by us and by others will be important in developing such systems.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are currently not available for use for all aspects of this project on sex chromosome functions and fertility. We rely on the use of live animals for our research. However, partof this project will involve the development of potential non-animal alternatives, e.g. in vitro model of gametogenesis. We also use bioinformatics and computational approaches wherever relevant.

Why were they not suitable?

Suitable options are not currently available.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is difficult to estimate precisely, as the the nature of the work requires that we will need to introduce multiple genetic alterations into the same mouse line. This means that many mice are needed to generate the desired genotype. Nevertheless, we take several steps to reduce overallanimal numbers.

As a guide to estimate animal numbers required, at a minimum we will need to assay a number of genotypes across multiple embryonic / postnatal timepoints, and each of which will need to be performed in triplicate (minimum) for statistical accuracy. The number of genetic alterations, timepointsand generations can mean that a single experiment will involve a minimum of 160 animals. Up to 10,000 animals will be used in protocol 1 to accommodate the generation of mouse lines with complex genetics. Protocol 2 will use up to 100 animals, and Protocol 3 will use up to 3,000 animals to accommodate approximately 15 experiments.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

A number of approaches are used to reduce animal numbers. Experimental design: Genes that we think are important for the processes we are studying (e.g. infertility, germ-cells, X-reactivation) are carefully chosen based on rigorous analysis of our ongoing studies, published literature and datasets generated by us and by other scientists (e.g. genome / transcriptome / proteome). This vastly reducesthe number of “false-leads”. We have started using the NC3R Experimental Design Assistant (EDA) forexperimental planning. We will seek to use it further in our studies where possible. . Genetically alteredanimals

are only created if they are not available from existing sources. If this is the case, the genetically altered animals are created in-house by highly trained personnel, and are usually maintained as small colonies.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Experimental protocols: We maintain clear and detailed experimental protocols that ensure uniformityin the outcome and success of animal experiments, thereby minimizing the numbers of experimental repeats.

Experimental planning / coordination: We plan our experiments so that each animal provides the maximum amount of material for analysis, and that tissue harvested post-mortem from a single animal can be stored and repeatedly reused in different experiments. This approach, together with statisticalapproaches, means we use few animals to address a specific scientific question.

Where genetically altered animals are required, we will first ascertain whether these or substitute models are available at other institutions. If these are not available, then the required models are created in-house, where highly trained personnel are able to generate genetically altered models usingthe minimum number of embryos / foster mothers. Where possible, we test the function of constructs used for the generation of genetically altered mice *in vitro*, before we proceed to *in vivo* experiments.
Where genetically altered animals are required, we first ascertain whether these or substitute modelsare available at other institutions. If this is not the case, the requisite models are created in-house, where highly trained personnel are able to generate genetically altered models using the minimum number of embryos / foster mothers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is a well-accepted and highly characterized animal model to understand human conditions.

Advantages of using the mouse would include the availability of high-quality genome with extensive structural annotation for the genes / exons, promoters, enhancers, genetic tractability of specific inbredstrains, and amenability to the introduction and propagation of highly targetted mutations through careful and controlled breeding. The laboratory mouse can be relatively easily housed within an animal unit, where variations due to temperature, light, water, feed, etc. are strictly monitored and controlled. Mice reach maturity to breed within weeks of birth (6 weeks for females and 8 weeks for male mice), and have multiple births in litters, thus permitting the controls and experimental animals being present within the same litter. Together these factors reduce the time required to perform high- quality

experiments.

The nature of the work requires that we will need to introduce multiple genetic alterations into the samemouse line. This means that many mice are needed to generate the desired genotype. Nevertheless, we take several steps to reduce overall animal numbers. Although most animals will only be subject to breeding and maintenance and the overall effects of pain / suffering is minimized. For protocol 3, most animals are not expected to suffer adverse effects that will affect their well-being. The administration ofsubstances will be kept to a minimum, and in the case of tamoxifen, the intraperitoneal route will only be used when scientifically justified to minimize potential gastrointestinal effects.

Why can’t you use animals that are less sentient?

Our work focusses on understanding the mammalian sex chromosomes, hence less sentient models(e.g. C. elegans or zebrafish) will not be suitable or representative in this regard. Furthermore, the mouse serves as the best mammalian alternative to performing natural breeding and the study of effects at all life course stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

We will refine our procedures using non-aversive mouse handling methods, minimize the use of GAmice, and adhere to LASA guidance (Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals).

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow up-to-date guidance in keeping with the Animals (Scientific Procedures Act 1986, best- practice guidance from the NC3Rs for project licence applicants (https://www.nc3rs.org.uk/3rs- resources/3rs-advice-project-licence-applicants), colony management (https://www.nc3rs.org.uk/3rs- resources/breeding-and-colony-management/colony-management-best-practice) and other applicableguidance (e.g. Refining procedures for the administration of substances (https://doi.org/10.1258/0023677011911345) to keep up-to-date.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

My group will keep abreast with advances in the 3Rs using the extensive e-resource library available inthe National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs;https://www.nc3rs.org.uk/) and the Jackson Laboratory (www.jax.org). We further receive monthly newsletter updates from the NC3Rs.

I will engage in regular discussions with the Named Persons and animal technicians at their institutionsto review current approaches and whether there are any new 3Rs opportunities, and be involved in institutional 3Rs symposia. We will also aim to discuss the 3Rs at conferences and meetings.

11. Production and Maintenance of GM Biomedical Research Fish Lines

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Genetic Modification, Breeding, Production, Husbandry, Cryopreservation

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile
Medaka (Oryzias latipes)	adult, embryo, neonate, juvenile

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The primary purpose of this project licence is to provide a service production licence under which it willbe possible to create, breed and maintain aquatic species, i.e. zebrafish and Medaka, which will be genetically modified. Utilising such animals, and predominantly their embryos (<120hpf), will permit studies to be undertaken in diverse biomedical fields, such as cancer, cardiovascular development, regeneration, drug-induced liver injury, resolution of inflammation, fat disposition and skeletal development.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The emergence of zebrafish as a pre-eminent vertebrate biomedical research model continues to grow, whose value in research has increased exponentially over the years and whose use will only continue to increase. It is however worth noting that other GM fish lines are now coming into prominence. This service/production licence is in support of biomedical research projects, specificallythe generation or maintenance, as well as the

preservation, of genetically modified (GM) fish lines.
As zebrafish genes, organs and tissues share many similarities with humans, what we learn in zebrafish and other GM fish lines, helps our understanding of how the genes work in humans. Many ofthe changes to genes that cause thousands of different human disorders remain poorly understood. Bystudying similar genetic changes in zebrafish or other fish species we can begin to answer which genes are important or not in human genetic disorders.

This service/production licence will ensure our highly skilled and experienced animal technicians provide the full husbandry care, together with the highest welfare standards, to these genetically modified fish lines to be studied by researchers at our research institute. These experiments will allowthe study of mechanisms of development and disease to inform our understanding and treatment of anumber of human diseases and conditions. Those researchers working with these GM fish lines will receive training and support from dedicated staff who are qualified in animal welfare, husbandry and service support, with the emphasise on welfare to ensure the fish are kept in the best condition.

What outputs do you think you will see at the end of this project?

This licence exists to support research into the fundamental mechanisms of human disorders.

In the short term: Generating new genetically modified zebrafish, which are subsequently available toall researchers as tools for investigating human genetic disorders.

In the longer term: Helping to understand disorders and supporting the development of methods tomanage and treat human disease.

The benefits of this service licence will be supporting research into the fundamental mechanisms of human biology and disease. This is worthwhile because it provides the basis for our understanding ofhuman and animal life, as well as potential therapies.

The scientific output from the work performed under this licence has been and will continue to bepublished in peer reviewed, high impact, leading journals.

Furthermore, in the short term: having a dedicated number of staff under a service/production licencepermits a high level of training and skill development in the maintenance of GM fish lines. Plus in the long term, having those dedicated number of staff under a service/production licence, who are responsible for the husbandry and welfare of the various fish lines, will permit the focus of the researcher to be conserved on their science.

As a vertebrate system, GM fish models complement the rodent model systems, for the study of genesand environmental factors that contribute to human disease. The genetic relationship between zebrafish and humans has been clearly highlighted in published work. This invaluably provides the basis for our understanding of human and animal life, as well as for the development of therapeutic potential to manage and treat human disease.

The Aquatic Facilities manager is also ideally placed to provide researchers within the University, and the wider scientific community, new transgenic and mutant lines by offering advice on established and new technologies available to the zebrafish community. The facility manager is able to co-ordinate imports and exports of GM fish lines, in with

standardised protocols, e.g. IATA regulations and guidelines for transporting live fish. This will allow the dissemination of best practice to facility Users. Such new technologies, which are becoming more commonplace, include CRISPR/Cas9 technologies,with which it is now possible to efficiently generate heritable mutations in GM fish lines against targeted genes.

The Aquatic Facilities are part of the establishment Zebrafish community, as well as the UK and ZHA (Europe) Zebrafish Network, which allows the quick and easy sharing of all resources and information.As facility manager I am always mindful of newly published literature on zebrafish husbandry and diseases and ensure that this is disseminated to facility staff, including zebrafish husbandry/biosecurityand stock management. Our dedicated aquatic facilities staff are able to support research groups, through their knowledge of animal welfare and husbandry, which would be difficult for individual labs to maintain.

Overall therefore the benefits of retaining this PPL are three-fold, i.e. to provide a platform on whichpilot data can be generated using non-protected stages (i.e. <120hpf), to enhance and ensure bestwelfare practice and thirdly to maintain standards through dedicated staffing.

Any scientific output from the work performed under this PPL will continue to be published in peerreviewed journals

Who or what will benefit from these outputs, and how?

The continuing long-term aim is to facilitate and produce a high quality service and support to scientistsof the establishment who study mechanisms of human disease, using these aquatic model species.

The purpose therefore of this project licence renewal application is to continue to provide a service/production license that is available to create, breed and maintain mutant and transgeniczebrafish or Medaka in the pursuit of discoveries to resolve human genetic disorders.

Dedicated staffing will provide full husbandry care, in addition to which experimental training and support. This project licence renewal is in support of existing licences, together with potential new facility Users, although this licence does not permit any specific scientific studies on protected stages.Presently we are aiming to provide an aquatic species service for scientists who study mechanisms ofhuman disease. Currently (2023) this facility has a number of PIs leading zebrafish related projects.
Under the current service/production PPL, there are currently seven research groups using the breeding and maintenance of GM zebrafish protocol, some of whom have imported lines under thislicence.

Such utilisation of this Service/Production licence is only expected to continue, especially for new Users of GM fish model systems. The Service/Production licence does not allow for the provision of scientific protocols on protected stages. Nonetheless, there are also a significant number of prospective scientists who have expressed a wish to use these model species. The vast majority of studies undertaken are with non-protected stages, i.e. <120hpf. Of the scientific PPLs the research themes undertaken include inflammation, cardiovascular/kidney/liver disease, cardiac and CNS regeneration, adipogenesis, neuroregeneration and development, brain injury and cancer models, plusMedaka osteogenesis. With the incorporation of CRISPR/Cas9 GM application, the number of GM

fishlines available are expected to increase significantly.
This Service licence will also allow the facilitation of import/exports of zebrafish to/from this Schedule 2designated facility. Co-ordinating the import/exports will allow careful monitoring of the animals, which,after screening, may be released from quarantine and transferred to the clean aquaria and the appropriate PPL.

The facility has two NACWOs and as such we must also ensure the health and welfare of the GM fishspecies. Any consultation by the PPL holder/facility manager and NACWO on scientific projects will always be in respect of A(SP)A'86 and the 3Rs; close liaison and the continued good working relationship with an establishment NVS will be maintained to ensure no conflict of interest.

There is a demand for transgene design and construction services, especially for busy clinical researchers that do not have a well-established molecular biology laboratory and who wish to expandtheir science to include zebrafish transgenic lines and cryopreservation. This facility current houses a number of zebrafish lines, such valuable lines may be cryopreserved in the future, at the PPL holder'srequest.

Improvements in zebrafish husbandry are constantly being sought and applied, as well as the continued professional development of staff. The aquatic facilities staff, involved in service provision, are of a high calibre and their skills are relevant and up-to-date, ensuring minimal number of animals are required to generate new GM lines and that the most efficient husbandry practices are adhered to.

How will you look to maximise the outputs of this work?

There is an effective establishment zebrafish community and therefore information and resources are communicated and shared. Similarly, this community facilitates the establishment of new collaborativestudies, which can be outside the establishment.

The published works generated from the scientific studies undertaken within this facility, some supported by this PPL on non-protected stages, will continue to be published in high-impact, peer reviewed journals. The current licence has held GM animals that have provided non-protected stagesfor scientific studies, therefore contributing to 12 scientific publications.

This facility and its dedicated staff are registered with the Zebrafish Husbandry Association (and by extension the UK zebrafish community), as well as a network of UK zebrafish facility managers, whichfurther permits effective communication, albeit related to animal husbandry and welfare.

Good colony management, with good care and welfare, are paramount and this is disseminated to allPIL responsible persons and PPL holders. This is made easier through our use of the stock management database which has now been successfully rolled out.

We have close communication with other large research facilities and therefore further information andresources are shared. As a designated Home Office breeding establishment, plus a registered Aquaculture Production Business with the Fisheries Health Inspectorate, we are able to import and export fish lines all over the world.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 25,400Medaka (Oryzias latipes): 4,300

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Within the establishment there is increased recognition of the benefits of zebrafish as a model organism, especially with the consideration of the 3Rs, therefore it is believed that there will be increased demand for the production of GM lines, as well as their preservation, as part of the 3Rwelfare considerations.

Zebrafish is a unique vertebrate model system that can provide valuable insight into human geneticsand biology for several reasons:

The establishment of the zebrafish genome project. The Human genome shows at least 70% ofhuman genes have at least one orthologous gene in zebrafish. As the genomes of zebrafish and humans share significant similarity [http://www.ensembl.org/Danio_rerio/Info/Index] this allows comparative genomic analysis. The Genome Reference Consortium now oversees the Danio reriosequencing project.

There are strong genetic and cellular parallels to be drawn between zebrafish and mammalianbiological systems, thus providing an ideal platform to study integrative physiology.

Genetic technologies continue to develop, which expand the potential for the use of aquatic species in biomedical research. Furthermore, through our research of drug-induced liver injury, we canacknowledge the suitability of zebrafish for drug screening, especially in non-protected stages.

Available are large numbers of synchronous and rapidly developing, optically transparent, embryos, with ex-utero fertilization and development. This allows the examination of early developmental abnormalities.

Large numbers of embryos permit rigorous and robust statistical analysis.

Embryos are freely permeable to soluble drugs and vital dye staining, however if required embryos (<120hpf) can be also be microinjected.

Fish embryos are directly and readily amenable to well-established genetic manipulation.

Relatively low cost maintenance and smaller facilities are required than other model species.

There is now a significant amount of data and information available online and/or in scientificpublications to support zebrafish research.

As a vertebrate model species the zebrafish has a number of distinct and unique qualities, including its ability to survive without a cardiovascular system in early development, thus allowing thestudy of embryonic-lethal genetic abnormalities and phenotypes.

The ability of the zebrafish to regenerate all organs also makes it unique as a model species.

This Service/Production licence will not only provide a centralised and co-ordinated service for established zebrafish PPL and PIL holders but also aid the development of a new zebrafish researchers providing access to zebrafish to undertake studies to generate pilot data for grants, albeiton zebrafish embryos when non-protected stages. Furthermore, with the establishment of alternative GM fish lines, e.g. Medaka, there will be a continued need for a Service/Production licence until new research groups become established.

Maintaining this Service/Production licence, with its dedicated staff provides significant benefit to the scientists as the appropriate high-standards of husbandry is always upheld, welfare of all stocks are effectively monitored and support is provided to the scientists. Overall there are significant benefits tosuch governance, especially in regard to the 3Rs.

It is acknowledged that where animal use continues to be necessary, every effort must be made to minimise the numbers of animals used and to improve their welfare, for example the use of cryopreservation to preserve lines. Zebrafish are a parallel and complimentary model system – with information being traded between model species, i.e., zebrafish and rodents. Furthermore, this modelspecies complies with the concept of integrating the 3Rs into scientific research.

Within the establishment there is increased recognition of the benefits of zebrafish as a model organism, especially with the consideration of the 3Rs, therefore it is believed that there will be increased demand for the production of GM lines, as well as their preservation, as part of the 3Rwelfare considerations.

The Aquatic Facilities have a dedicated team of six full-time technical staff. The facility supports numerous research groups (study areas including development of the CNS, cancer, cardiovascular development, regeneration, drug-induced liver injury, resolution of inflammation, adipogenesis and bone development) whose success is exemplified by the number of high-impact journal publicationsavailable and media coverage.

Typically, what will be done to an animal used in your project?

The vast majority of animals on this licence will not have any procedures performed and they will bebreed by natural behaviour. All aquatic species will be housed in expertly maintained aquaria.

Where the fish is genetically modified and requires genotyping, which overall will be a minority ofanimals, to genotype these animals a small piece of caudal fin will be removed by scalpel under anaesthetic; this fin tissue will regenerate. Alternatively skin swabs may be taken to undertake genotyping or animals may be fin clipped at <120hpf for genotyping.

The production of new GM lines, whether transgenic or mutant, will only be raised and maintained ifthere is a useful phenotype/genotype and will not exceed the perceived severity limit.

For cryopreservation, a very small number of male and female adult fish will be gently squeezed by trained staff so that sperm may be extracted and cryopreserved and the extracted eggs utilised for IVF.

A very small minority of animals may require gene activation or be non-invasively imaged

byanaesthetising them and briefly imaging them under a microscope to confirm phenotypes.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals held under licence on the main breeding and maintenance (B&M) protocol (maximum mild severity limit), will live out their life breeding through natural behaviour; no significant impairment andtherefore have no significant adverse effects.

Animals held under licence on the second breeding and maintenance protocol (maximum moderate severity limit), will similarly live out their life breeding through natural behaviour and therefore it is notanticipated that there will be any significant adverse short-term effects, however there may be, as yetunknown, cumulative or long-term mild effects of the genetic modification, which will be carefully monitored.

The production of GM lines, initiated at the one-cell stage, will only carry forward animals that, beyondfirst-feeding, have as a maximum a mild phenotype, i.e. transgenic lines or if mutants maintained as heterozygotes under this moderate protocol. It is anticipated that these animals, once an established line, will live out their life breeding through natural behaviour and therefore have no adverse effects;
animals will be transferred from the production protocol to the appropriate breeding and maintenanceprotocol.

For the cryopreservation, only trained staff will gently squeeze adult fish to release the gametes, thiswill be done under anaesthetic and if no gametes are immediately released no further attempt will bemade. Fish may be single-housed to permit welfare monitoring. Therefore, at a maximum a mild severity limit is anticipated, although there are no significant impairments and consequently no significant adverse effects.

All GM fish, at the end of a study or at the end of their permitted maximum healthy lifespan, will behumanely euthanised in accordance with the approved licenced method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Consequently, it is anticipated, based on previous experience, 85% of animals may experience subthreshold severity, 14% may experience mild severity. 1% may experience moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?
Unfortunately, there are no alternative in-vitro model systems that can be used for the studies that haveused this license. However, amongst all the currently used model organisms in biomedical scientific research, we have chosen a lower vertebrate model species compared with mammals, GM fish are considered less sentient than mammalian model species. Every effort will be employed to understand gene function from published literature however where an in-vivo model is required to be provided we will employ GM aquatic models.

Information from shared genomic databases, allow the exchange of data between a wide-variety of species, including invertebrate and vertebrate models. Nonetheless, GM fish are vertebrate model systems, albeit less sentient than mammalian models, which still draw closer comparison to humans inbiomedical research.

The study of genes and disease aims to model human disease processes as accurately as possible.Due to the complex interactions of tissues and genetic systems, and because humans cannot be manipulated genetically for research purposes, a whole animal model is required. Zebrafish are now arecognized suitable model to study human disease. Nevertheless, the PREPARE guidelines are considered.

Animals, naturally bred under this licence, may provide non-protected stages, i.e. <120hpf, to otherstudies; the vast majority of research is conducted on non-protected stages.

Which non-animal alternatives did you consider for use in this project?

In accordance with the 3Rs principle cell-culture based in-vitro models have been used where they have successfully contributed to publication of studies. However, to fully understand the contribution ofgenetics and tissue response an in-vivo model is required; here GM aquatic species, as non-protectedstages, will have a significant and important role.

Why were they not suitable?

Genetic disorders with genetic and/or pharmacological intervention are dependent on complex interactions between genes, cells, tissues and organs in living animals. This can only be achieved to avery limited degree with monoculture cell- or organoid-cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous experience and record keeping has ensured that animals held under this production/service licence are closely monitored, with encouragement to those holding lines under licence that they hold fewer, heathier, and more productive lines. Furthermore, our databases of animal use, whether stock management or annual Return of Procedures,

and forecast research projects, provide an insight to theanimal numbers required.
We have highly skilled and dedicated staff that are continuously focused on improving their skill set torefine welfare, husbandry and experimental procedures and handling. Together with the incorporationof a new stock management database will ensure overbreeding and maintenance of aged stocks do not occur. Dedicated staff and a coordinated central management of the aquatic facilities will ensure the appropriate support for any new users of these model systems.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Research groups, are aware of the in-house Research Optimisation Course through AWERB notification as well as the NC3Rs, Experimental Design Assistant tool. The facility manager will beaware of proposed projects, which will use GM fish and therefore can provide appropriate advice.

Researchers, holding animals under this production/service licence, are closely monitored through theuse of our database, with encouragement to hold fewer, heathier and more productive lines. Animals,bred under this licence, may provide non-protected stages, i.e. before first-feeding, to other studies, with the research being conducted on these non-protected stages.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Animals held by research groups under this production/service licence, are closely monitored, withencouragement to hold fewer, heathier and more productive lines.

Before a new genetic line is made we make sure it does not already exist somewhere in the world, thus when importing the line(s) we maintain the genetic integrity of the original genetic background andline.

Our database, permits close scrutiny of the lines held under licence, as well as wild-type populations,thereby reducing waste.

If a genetic line is not being used, we have the capacity to cryopreserve the sperm and keep it until theline is again required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

GM fish lines will be used to investigate a wide range of biomedical conditions and subsequently seektreatments for these conditions. However, the vast majority of these GM

fish lines will be used for natural breeding to produce embryos, i.e. non-protected stages, which could be used in biomedical studies; this natural breeding process is very unlikely to register as a severity level.

Unlike other vertebrate model systems used in the laboratory, GM fish embryos/larvae, as non- protected stages, are externally fertilized, and are transparent during early embryogenesis; the optically clear embryos/larvae provide an unprecedented opportunity for in-vivo live imaging studies. This allows for the exquisite detail of cell and tissue development to be visualized in a living vertebrate animal, something that is not possible in other vertebrates. Furthermore, zebrafish is a lower vertebratespecies for biomedical research compared with mammals.

The zebrafish and Medaka are very amenable for genetic modifications, i.e. the generation of a new transgenic fish strain in a number of months. The number of CRISPR/cas9 mutant fish is increasing very rapidly and these are freely available to all researchers, with various specific online databases detailing their characteristics. Gene knock-out or knock-in, by site-specific CRISPR/cas9, provides thepotential to specifically target any gene of interest.

Possible adverse effects will depend on the nature of the genetic material, its site of insertion, and levelof expression. Embryonic mortality may occur and there may be unpredictable effects caused by interference with expression of endogenous genes or inappropriate expression of the transgene. Fish will be monitored frequently for signs of ill-health, and animals exhibiting any unexpected abnormal phenotypes will be sacrificed by a Schedule 1 method. Our database, permits close scrutiny of the lines held under licence, as well as wild-type populations, thereby also ensuring that only healthy, appropriately aged animals, will be maintained.

All fin clipping for genotyping will be conducted under an appropriate general anaesthetic and with theadvice of the NVS, the use of analgesia. Adverse effects from a genetic mutation will be minimized bymaintaining the fish as heterozygous lines if possible, and/or rearing fish in areas of reduced daily stress and noise.

To further reduce pain and thereby stress, embryonic fin clip (<5dpf in zebrafish) can be conducted on mutants for genotyping. Alternatively, a skin-swab technique may be employed for genotyping, this is non-invasive but still requires anaesthesia and fish handling. Developments in such techniques are notonly a refinement but the employment of such techniques will reduce the numbers of fish raised beyond 5dpf. Pain will be controlled by using anaesthesia and analgesia where required.

We have highly skilled and dedicated staff that are continuously focused on improving their skill set torefine welfare, husbandry and experimental procedures and handling. Together with the incorporationof a new stock management database will ensure overbreeding and maintenance of aged stocks do not occur. Dedicated staff and a coordinated central management of the aquatic facilities will ensure the appropriate support for any facility users of these model systems.

Why can’t you use animals that are less sentient?

Unfortunately, there are no alternative in-vitro model systems that can be provided and used for thestudies that have used this license. However, amongst all the currently used model organisms in biomedical scientific research, we have chosen a lower vertebrate model species compared with mammals, zebrafish are considered less sentient than

mammalian model species.

Animals held under this Service/Production licence by Researchers are closely monitored, with encouragement to hold fewer, heathier and more productive lines. Animals, naturally bred under this licence, may provide non-protected stages, i.e. <120hpf, to other studies, with the biomedical researchonly being conducted on non-protected stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

In the past we have undertaken husbandry trials to improve the husbandry best practice and are evermindful to ensure welfare best practice is implemented. The facility NACWO regularly monitors all fishstocks, including those held under this licence, reporting issues to the responsible person, the PPLh and the NVS. Monitoring fish stocks through a database also ensures that aged stocks are kept appropriate, in compliance with PPL protocols.

The vast majority of studies undertaken within this facility and conducted on non-protected embryonicstages, nonetheless that maintenance of the parental stocks needs to be of the highest standard to ensure that this provides the best quality material for the actual science. As the parental fish are bredfor the offspring through natural behaviour, the most likely experience for the adult fish would be sub-threshold.

This licence will provide a centralised and co-ordinated service for the importation and exportation of GM zebrafish lines; this maintains the health status of the facility. Information provided by scientist onnew GA lines is reviewed by NACWO/NVS as part of the import request. The information is held centrally which is accessible to animal care staff. It will also provide a centralised and co-ordinated service for the husbandry and welfare of the WT and GM zebrafish lines held in these facilities.

Best practice will also include pain management, which will utilise anaesthesia during genotyping by fin-clipping and appropriate analgesia. Users will be encouraged to use, which will include training, alternative methods, i.e. skin surface swab and or fin-clip of embryos <120hpf. Adverse effects from agenetic mutation will be minimized by maintaining the fish as heterozygous lines if possible, and/or rearing fish in areas of reduced daily stress and noise.

Posted, in strategic places throughout the facility are posters for fish body condition scoring, welfareassessments , pain and distress scores sheet and sign of ill health.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

RSPCA Guidance on the housing and care of zebrafish (2010)

The Laboratory Zebrafish (Laboratory Animal Pocket Reference) (2011)FELASA Zebrafish Housing and Husbandry Recommendations (2019)
CCAC Guidelines Zebrafish and other small warm-water laboratory fish (2020)

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Together we are registered with the Zebrafish Husbandry Association and attend meetings, following which we disseminate the information and best practice. Furthermore I

have contributed to the RSPCAFish welfare (2020) meeting and attended the Focus on Fish (2022) meeting, again disseminating the information and best practice following the meeting. Our NTCO and NIO also keep us updated with 3Rs, UAR and HO related information, which can be disseminated.

I attend and have run national NACWO meetings and Zebrafish Facility managers meetings (UK) to share and communicate best practice. Over the years I have attend numerous workshops relating tobest welfare practice.

The Zebrafish Information Database (www.zfin.org), The EU ZF-HEALTH - Zebrafish Regulomics for Human Health (http://zf-health.org/) or European Zebrafish Resource Centre (EZRC) (http://www.itg.kit.edu/ezrc), together with the published literature, are important resources that will be used to identify when zebrafish mutants or transgenic lines already exist, thereby reducing the numberof lines that need to be generated and maintaining the genetic integrity of the line(s).

Improvements in zebrafish husbandry are constantly being sought and applied, as well as the continued professional development of staff. The aquatic facilities staff, involved in service provision, are of a high calibre and their skills are relevant and up-to-date, ensuring minimal number of animals are required to generate new GM lines and that the most efficient husbandry practices are adhered to.I receive zebrafish journals and scan the published literature regularly so that may be discussed with staff, as well as encourage staff on education to incorporate such information.

This Service/Production licence will not only provide a centralised and co-ordinated service for established zebrafish PPL and PIL holders but also aid the development of a new zebrafish researchers providing access to zebrafish so that the principals of the 3Rs are adhered to, as well asbest welfare practice.

Subsequently, my experience with zebrafish, coupled with my direct line management of the dedicatedaquatic technical staff at our Aquatic Facilities, as well as my liaison with academic staff who wish to use zebrafish as a model species under A(SP)A ideally places me as the person to disseminate the welfare related information and best practice, plus maintain highest standards. Best practice is communicated to facility researchers through a series of quarterly forum meetings.

12. Production of Pregnant Time Mated Rabbits

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Pregnancy, Toxicology, Prevention, Avoidance, Diagnosis

Animal types	Life stages
Rabbits	Adult

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To supply high quality pregnant time-mated rabbits to pharmaceutical companies, CROs or other research establishments in the UK to enable them to carry out safety assessment and other studies fornew medicines and other chemicals.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Rabbits time-mated under this licence will go on to studies at other establishments to assess toxicological effects on pregnant animals including the embryonic and foetal development of the offspring. This will include the preclinical safety evaluation of pharmaceutical, agricultural and chemicalmaterials as well as avoidance, prevention,

diagnosis and treatment of disease, ultimately leading to the development of new and/or improved medicines.

What outputs do you think you will see at the end of this project?

The provision of a successfully pregnant rabbit supplied for the next stage of research

Who or what will benefit from these outputs, and how?

Feedback from successful studies inputs into breeding production in our colony.

How will you look to maximise the outputs of this work?

Feedback on pregnancy success rates will improve reduction of the number of time-mated rabbitsrequired for studies.

Species and numbers of animals expected to be used

- Rabbits: 18,000

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Female rabbits are used at a minimum of 16 weeks of age, once they reach sexual maturity.

The choice of model for reproductive toxicology studies is governed by regulatory requirements (e.g. ICH guideline S5). The rabbit is the most commonly accepted second (non-rodent) mammalian speciesused.

ICH S5 states: In embryotoxicity studies only, a second mammalian species traditionally has been required, the rabbit being the preferred choice as a "non-rodent". Reasons for using rabbits in embryotoxicity studies include the extensive background knowledge that has accumulated, as well asavailability and practicality.

Typically, what will be done to an animal used in your project?

Normally two Animal Technicians will oversee the injection of luteinising hormone. One technician willhold the rabbit securely or may towel wrap the rabbit, if required, with the eyes covered to minimise stress to the animal but the ear exteriorised to allow ease of access to the marginal ear vein. Handlingin this way provides security for both the rabbit and licensee, thus minimising the stress of the procedure to the animal.

Prior to the injection, the ear will be wiped with an antiseptic solution and if required anaesthetic creammay be used, if an animal is overly sensitive, before the procedure to reduce distress and discomfort. The animals are then placed back into their original housing and receive environmental enrichment and a 'treat' as standard. They will be monitored regularly by an experienced member of staff until despatch.

What are the expected impacts and/or adverse effects for the animals during your project?

This procedure is not expected to cause more than transient discomfort and no lasting harm.

Manual pressure is applied over the injection site to prevent bleeding, if bleeding continues surgicalglue may be applied to aid haemostasis. Infection at the injection site is not expected as aseptic technique is used.

Should there be a second failure to gain intravenous access, the marginal ear vein of the other ear is used. No more than two attempts in the first ear and only one attempt in the second ear may be made,giving a maximum of three attempts in total.

If three attempts have been made unsuccessfully, the mated rabbit will then be placed into and remainin the breeding colony.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

This licence has a mild severity and no adverse effects are expected.

What will happen to animals at the end of this project?

- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The animals supplied under this project licence are generally required for regulatory toxicology studieson pregnant animals, including the embryonic and foetal development of the offspring.

The choice of rabbit for use on this licence is governed by the industry regulatory requirements eg ICHguideline S5 and is the preferred non rodent mammalian choice. The rabbit has many advantages as anon-rodent and second model for assessing the effects of toxic agents on fertility, developmental toxicity and teratology. Rabbit extraembryonic membranes closely resemble humans and differences in maternal embryonic exchange contribute to understanding mechanisms of action for developmental toxicants.

Which non-animal alternatives did you consider for use in this project?

None. This project requires the use of the rabbit.

Why were they not suitable?

Currently the research carried out at the receiving establishments cannot be replaced by non-animalmethods

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This figure is based on the number of time mated rabbits produced over the life of the last licence andthe committed orders received for 2023 and onwards.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The number of animals anticipated over the life of the licence is based on previous customer demand.Time mating only takes place once the completed customer justification has been received and approved by the PPLh, NACWO or authorised person.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Monitoring litter production and utilising best performing stud males will increase success rate forcustomer studies potentially reducing the number required per study.

Performance of stud males will be monitored and any male found to be overly aggressive with the females at the time of mating are removed from the colony. Examples would be pulling fur excessivelyor causing injury to the female.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

The only animal model that will be used under this licence is the rabbit. Historically rabbits have been handled by scruff lift and support of the body/hind quarters. We have been working both in-house andwith other establishments on ways to improve both the handling

and housing of our rabbits. We now endeavour to remove rabbits from their home cage by gently guiding the rabbit towards the front of thecage and then scooping a hand under the chest and supporting the hind quarters to lift the animal towards the technician's chest, providing security and reducing stress.

Since 2021 we have provided hay to all animals within the barrier. This has been possible as we havesourced irradiated hay with a certificate of analysis to ensure biosecurity of the barrier.

We now provide a 'treat' to the rabbit on return to the home cage, following the mating and LHprocedure.

Why can't you use animals that are less sentient?

Use of rabbits is considered the least sentient mammalian species required under developmentaltoxicity legislation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In-house colony management and breeding records, including stud male performance, regular reviewof diet, environmental enrichment, housing.

Discussions with recipients of the time mated rabbits regarding percentage success rates, with theview to reduce the number of rabbits required for these procedures.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We have considerable experience established over many years in the breeding of high health status rabbits. Over this time, we have continually refined our handling, enrichment and breeding techniques.The supply of high quality time mated rabbits is essential for scientific research by our customers.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Information from organisations such as the NC3Rs, industry meetings and scientific papers.

There are regular meetings in which improvements, especially those concerning handling techniques,are discussed. In addition, networking with peers and colleagues build relationships and help to shareinformation.

13. Refining the Tagging of Wild Fish

Project duration

5 years 0 months

Project purpose

- Basic Research
- Research aimed at preserving the species of animal subjected to regulated procedures aspart of the programme of work

Key words

Fish welfare, Refinement, Biologging, Fish Anaesthesia, Fish Analgesia

Animal types	Life stages
Perciformes or salmoniformes	juvenile, adult

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Quantify the efficacy of techniques used to attach data recording tags to fish (termed "biologging") to improve welfare and minimise bias introduced by these processes when used in wild settings. Quantifyand offer improvements (refinement) on life support techniques (such as gill irrigation) and pain management techniques used during tag attachment procedures.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improved knowledge of the responses of fish to tagging gained from this project will help promotebetter practice and higher fish welfare from an empirical evidence base.

What outputs do you think you will see at the end of this project?

Peer-reviewed scientific publications addressing evidence-based refinements of tagging

procedures that lead to improvements in fish welfare (e.g. handling fish out of water, darting fishand the role of local anaesthesia in wild scientific studies)

Infographics (i.e. posters) of the findings for sharing with the research community

A workshop at a relevant conference that will focus on improving welfare in wild fish biologgingstudies

Development of an Early Research Career scientist in the field of fish biologging and welfare

Who or what will benefit from these outputs, and how?

Improved welfare for fish: In both the short (<5 years) and long term (>5 years), the project will promote improved fish welfare during and following electronic tagging procedures (e.g. decreasingpost-tagging recovery times of wild fish).

Improved data and practices for the scientific community: more robust science (i.e. reduced and betterunderstood biases introduced from handling and tagging procedures) over the long term with reduced uncertainty and provide lasting improvements in field techniques. At least 45,000 animals over of 30 years period have undergone aquatic biologging procedures (Hussey et al. (2016) Science). In our research group alone, we perform licensed procedures on approx. 50-100 animals per year, many of which would benefit from improvements to be investigated in this PPL.

Improved knowledge for relevant stakeholders: We will share the findings of this project with the NC3Rs, institutional AWERBs (Animal Welfare Ethical Review Body), regional AWERB Hubs, and toHome Office ASRU (Animals in Science Regulation Unit), which will enable them to direct all new applicants to the relevant findings. The sharing of findings will benefit research groups and industry suppliers who manufacture darts and tags as well as research groups globally working on fish to encourage them to refine / incorporate findings into their own practices.

How will you look to maximise the outputs of this work?

To maximise our outputs, we will collaborate and share findings with research organisations regionally,nationally and globally. We have approached several research groups and industry suppliers who manufacture darts and biologging tags to discuss how findings from the research could be shared through their networks. We propose a workshop at the annual conference of the Society of Experimental Biology that will focus on improving welfare in fish biologging studies. We will also attendNC3Rs events and present the findings from the research once they are peer-reviewed. Our research outputs will be made easily accessible to all relevant researchers and organisations worldwide on our research group website. We will incorporate findings from this research into our own ongoing work licensed under other project licenses through amendment requests.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use marine fish or marine-adapted species as surrogates for wild fish frequentlysubjected to electronic tagging studies in the wild. Adult or large juvenile fish with suitable body features (e.g. muscle and scale morphology) are most likely to be selected as model species.

Typically, what will be done to an animal used in your project?

Fish will be used either in the artificial gill irrigation or external tagging/local anaesthetic protocol.

Efficacy of artificial gill irrigation:

Fish will be cannulated following an initial acclimitisation period. After cannulation, fish will be rested for up to 72 hours prior to experimental procedures. Individual cannulated fish will be taken from their holding tank and placed onto a moistened pad. Fish will be exposed to either 100% artificial gill irrigation, 50% artificial gill irrigation or no irrigation for up to a maximum of 5 minutes. Repeat blood samples will be taken during this time (up to a maximum of 5 samples). During this period a monitor may be attached to the fish, which displays heart rate and/or rhythm.Fish will subsequently be single housed for up to 36 hours post-procedure to assess their behaviour, then returned to the group holding tank and humanely culled 7 days after the procedure.

Wound healing responses to external tagging procedures and the role of local anaesthesia:

Individual fish are taken from their holding tank and placed onto a moistened pad with water pumped into their mouth across their gills for a maximum of 5 minutes. To replicate current wild tagging practices, where analgesia is commonly not used, two treatment groups of fish will not recieve pain relief prior to darting. As such, fish may have pain relief applied and/or be darted. Blood samples may be taken during this time. Fish will subsequently be single housed for up to 36 hours to assess their behaviour and then returned to their group. They will be humanely culledat 30, 90 or 150 days after the procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

This project seeks to quantify the magnitude and frequency of adverse effects arising from gill irrigationand external tagging procedures commonly used in the wild. Potential adverse effects may extend to pain from handling, stress, temporary loss of balance, loss of condition, lethargy, or abnormal behaviours (e.g. rubbing on tank sides, reduced feeding, colour change) or increased risk of wound sites being targeted by other fish. These effects are predicted to be short term; however, the project may highlight that there are chronic effects from tagging procedures on the fish (e.g. delayed wound healing, abnormal behaviours), which is in part one of the main objectives of the work (i.e. to establish whether external tagging wild fish is really a mild procedure as often cited). Adverse effects may also represent bleeding or infection at the site of dart insertion, or in the mouth/gill area where artificial irrigation equipment may have caused minor tissue

damage.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Moderate severity protocols will be experienced by 81% of fish, with remaining fish (19%) experiencingmild severity protocols.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Only rudimentary knowledge exists on how fish experience acute or chronic post-release pain or infection from tagging activities once they have been released to the wild. There is no alternative to inform future refinements on welfare of fish carrying electronic tags without experimenting with tagging procedures on whole animals. Animals in a research aquaria environment will be used in this project assurrogates for wild animals. The information to be gained will include observations of wound repair and tissue healing and incorporate behavioural observation, as such, living whole organisms need to beused.

Which non-animal alternatives did you consider for use in this project?

Computer tissue modelling of fish muscles darted for the attachment of biologging devices.

Why were they not suitable?

We would not be able to accurately predict behaviour post-tagging, nor would we be able to estimate fish oxygenation (and other blood chemistry parameters indicating physiological stress) using gill irrigation techniques without experimentation in whole living animals to obtain holistic organism level responses (i.e. physiology and behaviour). Although the hydrodynamic effects (e.g. lift and drag) produced by an externally mounted tag can be modelled, these models do not enable a simultaneousunderstanding of the healing process of the muscle to be quantified. We have insufficient data on thehealing assays of fish instrumented with different dart types, thus a computer-based model is not suitable at this time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

The number of animals to be used was estimated from a comprehensive literature search of relevantscientific material to obtain knowledge on response means and variance along with the use of statistical power calculations and the NC3Rs Experimental Design Assistant.

Pilot studies: 2 pilot studies (18 fish)

Pilot study for the efficacy of artificial gill irrigation experiment (6 fish, 3 perciformes and 3 salmoniformes)

Pilot study for the tagging and pain relief experiment (12 fish, 6 perciformes and 6 salmoniformes)

Efficacy of artificial gill irrigation experiment: 3 experimental groups, 24 fish (8 fish for each group)

Fully irrigated (100% flow; gill opercula are flared, but not over-extended)

Partially irrigated (50% flow)

No irrigation (0% flow; air exposure without artificial irrigation)

Tagging and pain relief experiment: 5 experimental groups, 120 fish (4 treatment groups and 1 controlgroups; 3 timesteps, 8 fish for each group and timestep)

Dart type A with pain relief

Dart type A without pain relief

Dart type B with pain relief

Dart type B without pain relief

Control without pain relief

All fish are randomly assigned to 1 of 3 wound recovery groups (i.e. 30, 90 or 150 days), fish are followed through time to track wound healing processes, all fish are terminated at the specified numberof days dependent on the group they are assigned to. Each wound recovery group will have a similar number of individuals to compare histopathology (study of the fish tissues) and behaviour over time.

The duration of these wound recovery groups has been determined from observations of tag sheddingby wild tracked fish. The time points reflect key inflections of unexplained tag loss, which this project has been designed to better comprehend.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The experimental protocols have been designed using the NC3R's Experimental Design

Assistant. ThePREPARE and NORECOPA guidelines have been reviewed and incorporated into the experimental design where appropriate. Reporting of results in publications will adopt the ARRIVE guidelines.

When considering variability in the key responses to darting (e.g. muscle damage, skin damage and scarring, death of the muscle tissue surrounding the darted area), administered pain relief (e.g. behavioural assessments of fish such as isolating from other fish within the same tank or rubbing against tank walls), and gill irrigation (laboratory blood assessments for measuring oxygen concentration in the blood), meaningful data will likely be gathered from a sample size of 8 individualsper experimental group.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Optimising the number of animals to be used will be informed by pilot studies and by remaining appraised of scientific work published in relevant peer-reviewed scientific journals, by remaining engaged with the NC3Rs and relevant community groups focusing on animal welfare (e.g. RSPCA,LASA), by sharing our efforts and seeking relevant collaborations across the animal biologging community.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will assess the methods commonly used in wild fish biologging, with the objective ofmaking recommendations for refinements.

The common method of externally tagging wild fish does not include the administration of pain relief. As such, to replicate this within the experimental design used in the PPL, the starting points of some treatment groups do not necessarily cause the least pain, suffering, distress or lasting harms, they instead represent the status quo of techniques used globally to tag fish (e.g. no use of analgesia). Weseek to create empirical data through structured experimentation to demonstrate where refinements inwild settings (such as delivery of pain relief) can be delivered and welfare improved around these established methods.

In the field of wild biologging we have determined that darting, pain relief and gill irrigation are threeareas that are data deficient with respect to their welfare impacts and hence offer the greatest opportunities for refinement.

The most refined species for the project will be determined from two candidate species using a pilot study to: i) assess the simplicity/practicality of sourcing and quality/condition of supplied animals, ii) establish suitability for the experimental design that necessitates long-term holding of some individuals, iii) behavioural interactions between individuals of

the same species, iv) appropriate body size range, v) suitability for observing experimental effects based on study design given this replicates practices onlarge fish in the wild, vi) practicalities of cannulation, cannula maintenance and housing requirements of cannulated fish, vii) establish practical range of water flow rates for artificial irrigation of individuals ofgiven body size range, viii) establish a protocol for repeated blood sampling of conscious fish (e.g. use of anticoagulants), and to determine minimum number of samples required (for blood gas analysis), and xi) external scale morphology and underlying muscle block anatomy concerning darting. Both species are commonly used in laboratory aquaria settings.

Why can't you use animals that are less sentient?

There is no other way to mimic the behaviour of a live fish that has a dart inserted into the muscle with or without the use of pain relief (i.e. local anaesthesia) and observe any lasting or chronic harm withoutusing sentient fish. In addition, understanding the healing of a fish dorsal muscle over time is not possible in species that are less sentient or on terminally anaesthetised fish, as the healing process will not be able to mirror those of a live fish. The use of immature life stages (before the point of independent feeding) will not be possible as the fish will be physically too small to handle and conduct the experimental darting procedures.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

The purpose of the study is to directly experiment with procedure refinements and to assess their efficacy for improving welfare. Fish behaviour will be closely recorded before and after procedures using direct visual observation and by camera systems to quantitatively gather continuous data. Thesedata will meet the requirements of the scientific questions of the projects but will also be used to assess (and address) aspects such as stress which could be caused by human disturbance in the aquaria environment and contingent harm. Examples of minimising welfare costs to the fish during procedures, include providing animals with eye coverings to reduce visual stimuli and cushioned/moist surfaces to reduce any friction to their bodies during procedures, which may damage their mucus-skin barrier.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Evidence of best practice has been gathered from peer-reviewed literature, NC3Rs, engagement withother industry-relevant professionals and from Named Persons, including NVS and NACWOs and veterinary surgeons with expertise in fish.

In addition, following guidelines available from the Laboratory Animal Science Association (LASA) andNorecopa have been incorporated where relevant:

Refining procedures for the administration of substances: report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement

A guide to defining and implementing protocols for the welfare assessment of laboratory animals:eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement

CCAC Guidelines on the care and use of fish in research, teaching and testing

An overview of existing guidelines for handling, bleeding, administration and identification techniques in fish

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

This project is funded by the NC3R's, as such we will strive to attend relevant conferences (e.g. NC3Rs, LASA, RSPCA) and talks as well as frequently review new literature for emerging advances inthe 3Rs that can be effectively implemented into our project plans.

14. Regulation of Basement Membrane Function in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Kidney Disease, Basement Membrane, Glomerulus, Alport Syndrome, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, pregnant

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The overall focus of this PPL is to define the mechanisms that regulate the function of basementmembranes in health and disease with a primary focus on kidney physiology and disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Basement membranes are sheet-like structures which line most tissues in the body and

separate different types of cells. Basement membranes help to maintain the shape of tissues in the body and regulate different cell functions. We found that basement membrane defects in mouse kidneys occur before there is evidence of kidney disease and we propose that these early defects start a process thatleads to kidney scarring. Diseases that cause kidney scarring can lead to chronic kidney disease, which affects 10% of the world population and there are no curative treatments. When kidneys fail, renal replacement therapy with dialysis or transplantation is necessary but costs are escalating and replacement therapies are not universally accessible. Strategies to improve early detection of chronic kidney disease and targeted therapy to prevent disease progression would have significant impact on improving human health. We aim to investigate how basement membranes are maintained in health and affected in disease. The primary focus will be on the kidney, but we also aim to study other tissuesand organs to understand overall basement membrane regulation. We will conduct studies in human cells in culture, where we can investigate how basement membrane components are produced, but weare not able to properly test their function. Since it is necessary to understand how basement membranes function in the body, we will conduct *in vivo* studies in parallel using mouse and zebrafish.

What outputs do you think you will see at the end of this project?

We will disseminate our research findings in a variety of ways to reach broad audiences. For academicaudiences we will publish in peer reviewed journals and to preprint servers to enable rapid access to our results. For clinical, academic, and commercial audiences, we will present our work at relevant conferences, both in person and with virtual presentation. We have established an online resource for basement membrane research, and we will continue to maintain this resource and post relevant findings from our studies. For patient and public audiences, we will maintain our strong connections with patient organisations and present at our public programmes events at the establishment.

Who or what will benefit from these outputs, and how?

This research programme aims to identify basic mechanisms of basement membrane regulation. It is anticipated that in the medium term, this will enable the identification of early biomarkers of basement membrane damage, which will eventually contribute to the development of new therapies and enable early intervention to rescue basement membrane damage in multiple tissues and organs. This could impact the early detection and treatment of diseases that are associated with basement membrane damage, such as genetic and acquired kidney disease.

The knowledge and understanding generated by this work will benefit kidney researchers and will also have wider relevance for researchers studyingbasement membranes in other tissues. The work will also benefit clinicians by improving understandingof basic science, and it will ultimately benefit patients with kidney and other diseases characterised by basement membrane defects. It will also have impact on commercial organisations seeking to develop therapies for kidney disease and other researchers working in this field over the next 5 years.

The genetically altered mice we use in our experiments have a knockout of the Col4a5 gene. Defects in the COL4A5 gene are the most common cause of Alport syndrome, which causes kidney failure anddeafness. Using our mouse model to study how this defect in Col4a5 causes disease, and to test possible therapies, has the potential to improve the detection and treatment of patients with Alport syndrome.

How will you look to maximise the outputs of this work?

We have a good record of publication and aim to publish all research that could be of benefit to others.To increase the impact of our research we collaborate with groups across the world and we will continue to maintain these international collaborations. We will also publish negative results as these are important for progression in research. These can be published in a range of journals as short data notes or datasets.

Species and numbers of animals expected to be used

- Mice: 1,500
- Zebra fish (Danio rerio): 3,000

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In humans it is not possible to perform the mechanistic studies or the in depth analyses of disease tissue that I propose in this application. Mice and zebrafish will therefore be used in these studies as they are the lowest species in which suitable models of kidney disease exist. The genetically modifiedmodels across the lifecycle that we will use recapitulate many features of the human equivalents and therefore will provide valuable insights into mechanisms of basement membrane disruption during kidney disease. We have established new human cell systems including kidney organoids to investigate our research questions. These new tools will reduce our use of animals in this programmeof work.

Typically, what will be done to an animal used in your project?

Mice:

The severity of kidney disease can be measured by testing the urine and the blood of mice, and so ineach study we will take samples from the mice to measure kidney function. Blood pressure is also affected by kidney function, so this will be measured either by a tail cuff, or by surgically inserting a small probe into the neck of the mouse while it is anaesthetised. In some mice kidney injury will be induced by removing kidney tissue surgically while the mice are anaesthetised, or by administering substances to cause injury. We can then study the changes that have occurred in the injured kidneysversus healthy control mice, which will help us to understand which therapies and treatments might work to prevent, slow down, or treat the kidney damage. We will also test therapies and use imaging techniques to visualise the changes in tissues. In experiments when the interventions are mild or moderate, we will follow the progress of mice. Should signs of ill health become apparent, the animalwill be killed by a humane method.

Zebrafish:

We will genetically modify zebrafish so that they express genes which are 1) relevant to kidney diseases and 2) allow us to measure kidney function. We do this by injecting the zebrafish with chemicals and proteins, or by performing surgeries on the zebrafish.

Injections and surgeries are conducted while the fish are anaesthetised so that they cannot feel any pain. When we edit genes, wealso add fluorescent proteins which allow us to visualise what has changed in the kidney or the basement membranes of the zebrafish. Once the gene editing is complete, we can breed these genetically altered zebrafish and use the offspring to test potential new therapies which may treat kidney disease and basement membrane dysfunction. Any signs of ill-health in the zebrafish will resultin the fish being humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Since we are investigating early stages following injury or disease onset the animals will not havesevere phenotypes, however some of the procedures we carry out can cause pain, discomfort or stress to the animals.

Mice:

Almost all of the mice we use in our studies will experience ear-punching, where a small amount oftissue is clipped from the ear. This procedure causes momentary pain and discomfort, but has not been shown to have any long-term effects on animal wellbeing.

Surgery to remove kidney tissue or to place a probe that measures blood pressure will cause short- lived post-operative pain and discomfort, but once healed should not have adverse effects on the mice.The removal of kidney tissue will affect kidney function clinically but will be asymptomatic to the mice. Some mice will have a telemetry device surgically implanted to allow blood pressure measurements.

These mice will experience post-operative pain and discomfort which will be minimised as much aspossible using pain relief.

Mice administered drugs via oral gavage will experience mild stress and discomfort during the administration procedure, but they are able to quickly recover. For urine collections, mice are housedseparately from their cage mates for up to one hour. During this time, they will experience mild stressdue to isolation, though this is resolved once the animals are returned to their home cage.

Fish:

Fish will be anaesthetised prior to receiving injections or undergoing imaging sessions. The fish mayexperience mild stress due to the addition of the anaesthetic agent to the water, but are expected tomake a full recovery. Fish will also be anaesthetised prior to surgery, which may cause mild stress. Analgesia is used peri-operatively. Sterile technique will be used to minimise the chance of infectionwhich could cause pain.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The project has a ‘Moderate’ severity level.Mice:
Subthreshold 50%, Mild 30%, Moderate 20%, Severe 0%Zebrafish:
Subthreshold 50%, Mild 30%, Moderate 20%, Severe 0%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim to determine whether therapies can prevent or reduce the effects of diseases associated with abnormal basement membrane function. We have experience of using human cells to study mechanisms of disease. In addition to standard cell culture, we have also developed organoid cultures as a more complex culture system, which mimics an in vivo environment more closely than standard cell culture. Although these studies are improving options for replacing animal studies, they still have limitations. Therefore, for our research to have greater impact on human health, it is necessary to use animal models for realistic preclinical experiments. The administration of treatments to whole animals will ensure that we can detect any side effects on other organs. The use of zebrafish will allow us to perform early screening experiments in embryos and therefore replace the need for some experimentsin older fish or in mice.

Which non-animal alternatives did you consider for use in this project?

Where possible, cell culture models using human embryonic kidney (HEK) cells and kidney organoidswill be used in studies as an alternative to animals.

Why were they not suitable?

Cell culture models are a tool we use alongside our animal models, however cell culture models aloneprovide limited insights into mechanisms of kidney disease and response to therapies. Cell culture models enable us to study cell interactions and basement membrane production, but animal models are required to study the effects of therapies on organ function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the above numbers based on our work over the past 5 years and the anticipatedincrease in our work.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Sources of variability will be considered at all stages of the experimental design. For mice we will consider the genetic background and sex of the animal carefully when designing the experiment and choose animals that are appropriate to address the specific research question for a particular experiment. We will also consider the variability of experimental observers and where possible will allocate one observer to each animal experiment. We have experience of using the NC3Rs Experimental Design Assistant and we review the relevant literature to find data on effect sizes of interventions. To minimise the numbers of animals in our experiments we use a factorial experimentaldesign approach with replication of optimised conditions.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

We will regularly review our breeding strategies to ensure efficiency and we will consider strategies to reduce the number of animals overall by good experimental design. To do this systematically we have established a monthly animal research group meeting in my group. We also routinely perform pilot studies when we are using testing a new intervention. An initial sighting/pilot study with a small numberof mice per cohort (<5) will enable estimates of variability, interaction effects and main effects that will be used in a subsequent formal statistical power calculations to determine sample sizes for contrasts of interest. Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with statistical consultants. Important experimental results will be repeated or validated via an alternative follow-up experiment to minimise the likelihood of spurious nonreplicable results. We will continue to share tissue with other research groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

At present, the mouse represents the best or most refined species with which to test the efficacy of new therapies for basement membrane-associated kidney disease. It has a kidney of similar structureand anatomical complexity to human organs. However, our inclusion of zebrafish studies will allow usto refine the number of studies in mice and the nematode C. elegans is also a suitable in vivo model for studying basement membranes. Our experiments are proposed in mice during development and after birth when they will be closely monitored. Particular attention will be paid to their weights and behaviour. Should these parameters deviate markedly and/or persistently from normal, mice will be humanely killed. For all experiments in animals, we will use good experimental conduct with the appropriate use of peri-operative analgesia for surgical interventions and the appropriate species- specific management of animals during and post anaesthesia.

Why can’t you use animals that are less sentient?

Where the scientific aim can be achieved using less sentient animals, such as zebrafish embryosinstead of zebrafish, the less sentient animals will be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

If we make changes to procedures we will ensure that these are approved for implementation and thenwe will increase monitoring, post-operative care, pain management, and training of animals as appropriate.

Currently, the refinement procedures we have in place include: Use of peri-operative analgesics, frequent observations during post-operative periods, rotation of blood sampling/injection sites, alteringcage enrichment tools (i.e. using tubes with larger entrance holes) to reduce risk of skin catching/rubbing post-operatively, and coating gavage tubes in sucrose solution.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

There are no best practice guidelines for the use of animals in kidney research, however within an international group hosted by the International Society of Nephrology, I am preparing guidelines for theuse of animals in kidney research which uses the PREPARE guidelines as reference. We expect to publish the guidance document in 2023.

For general best practice we will follow the Working Group refinement guidelines, 2001.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will attend seminars hosted within our establishment and interact with the international researchcommunity and the published literature to keep up to date with practices that could improve our application of the 3Rs. We will regularly review updates in our monthly animal research group meetings.

15. Responses of the Cardiovascular System to Trace Amines

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cardiovascular Disease, Trace amines, Blood pressure, Sepsis

Animal types	Life stages
Rats	juvenile, adult

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Trace amines and related substances occur in the body naturally, in our diets and are used as therapeutics or as recreational drugs. The aim of this project is to understand the effects of these substances upon the heart and blood vessels of the cardiovascular system and how they affect bloodpressure. We will determine the mechanisms and processes involved in the responses of the cardiovascular system. Our overall goal is to determine whether trace amines have a role in the treatment of the cardiovascular consequences of sepsis.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Knowing the effects of trace amines or related substances on the heart and circulation and the mechanism of action is essential in understanding their potential beneficial or harmful

effects in humans and animals. It will also provide a basis for developing new applications for the treatment and management of human and animal disease. One potential target that has been identified is the emergency lifesaving treatment of the cardiovascular collapse in sepsis. Currently, there is no clinicallyeffective way of supporting the circulation during the collapse of blood pressure caused by sepsis.

What outputs do you think you will see at the end of this project?

Trace amines include therapeutics such as nasal decongestants, dietary materials such as tyramine incheeses, and recreational drugs such as amphetamines (ecstasy). Our study will provide new knowledge on how these agents affect the intact cardiovascular system, the mechanisms involved andhow these effects may be ameliorated or utilized for therapeutic benefit to humans and animals.
Potential new applications, such as treatment of sepsis may also be a favourable outcome. Results willbe published and disseminated by presentation to the scientific community at appropriate domestic and international conferences.

Who or what will benefit from these outputs, and how?

Medical professionals, patients, domestic and farm animals, and the pharmaceutical industry will benefit both in the short-term while the project is still running and in the long-term after its completion or extension. Health-care professionals will be armed with improved knowledge on how to use trace amine-based therapeutics. Patients will benefit from improved clinical outcomes in the use of these. Inthe longer term the pharmaceutical industry could benefit commercially from developments of novel molecules based on trace amine pharmacology.

How will you look to maximise the outputs of this work?

The outputs of this work, and therefore of the animals to be used, will be maximised by several approaches that have been features of work from these laboratories for many years. Every experiment will be designed to generate as much information as possible. Thus, experiments or approaches that might be deemed unsuccessful from the perspective of the project’s aims can be modified in the light ofthe data generated in order to provide insights into alternative approaches. This progressive development would be published to prevent others using to follow a potentially unproductive approach. A further approach to maximising outputs is to examine more than one question in the same experiment with the same animal.

Species and numbers of animals expected to be used

- Rats: 1500

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

A major output of this work is the measurement of blood pressure and heart rate and how they are affected by the substances of interest. Small adult laboratory animals are

therefore ideal for this purpose, since we can record blood pressure continuously over a period of a few hours and administerthe substances while monitoring.

Typically, what will be done to an animal used in your project?

Animals will be anaesthetised so that cannulas can be inserted into veins for drug administration andinto arteries for continuous recording of blood pressure and heart rate. Blood samples may be withdrawn for analysis. The duration of anaesthesia will not exceed 6 hours, at the end of which, an overdose of anaesthetic will be administered to terminate the experiment without recovery.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will experience mild discomfort during handling and the initial administration of anaesthetic.Thereafter they will not be aware of any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Non-recovery

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Blood pressure is a multifactorial consequence of the close interaction between the vasculature and pumping effect of the heart. Changes in blood pressure can therefore only be monitored in the intact individual. Human volunteers could be used, but the properties of some of the materials to be used arenot sufficiently understood for human administration yet. Furthermore, our experiments need to be performed on healthy normal individuals without interference from any medication and with controlled diet - these conditions are extremely difficult to achieve in human volunteers.

Which non-animal alternatives did you consider for use in this project?

We have considered human tissues and lower non-animal life-forms such as bacteria and cell culturemethodologies. None of these alternatives are suitable for achieving the project aims.

Why were they not suitable?

We have used human isolated blood vessels in preliminary studies to show the effects of trace amineson arterial and venous isolated sections of vasculature. They were suitable for this purpose but had limitations with regard to the present project in that they cannot reveal their actions on the blood pressure and the cardiovascular system as a whole. This can only be achieved in an intact animal.

Other lower non-animal life-forms are not relevant to the questions posed by this project as they do nothave an intact cardiovascular system equivalent to that of mammals. Cell culture techniques are not appropriate for studying the interaction of a system as complicated and as many different components as the cardiovascular system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

From the variances of blood pressure and heart rate responses to trace amines in anaesthetized rats, groups of 6 animals will be required to detect a significant 15% difference between control and test groups. The final estimate of numbers of each species is based on group sizes of six and the predictednumber of groups needed to address the questions posed by this project.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We have carefully designed our experiments to get the maximum information possible from any individual animal. An example of this is our experimental design that allows us to address two separateaims thereby reducing the total number of animals required for the project overall. We will only use thenumbers of animals required to make our studies statistically valid.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

As mentioned above, careful experimental design can address more than one question in the same experiment. One of these experiments could take the form of a pilot study, providing valuable information for the design of the final experiment in later studies. This approach reduces the overall numbers of animals needed. We will offer any tissue that is not required for the project to the wider scientific community through collaboration outside our institution or within using the Tick@Lab system3rs blackboard for advertising available tissue.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

All animals will be terminally anaesthetised. They will only experience short-lived mild discomfort oninitial handling and administration of the anaesthetic

Why can’t you use animals that are less sentient?

Adult animals that have a well developed intact functioning cardiovascular system are required for theproposed project. They will be terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

These are non-recovery experiments. Refinement of the post-anaesthetic procedures will have noimpact upon animal welfare. Prior handling of animals will reduce anxiety during anaesthetic administration.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Animals in Science Regulation Unit (ASRU) Guidance and Regulatory Advice will be referred to for themost up-to-date guidance on refinement.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our institution provides a regular e-mail update on all 3Rs matters and new initiatives to Licensees.

16. Sensory Processing in Teleost Fish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Zebrafish, Vision, Neuron, Synapse, Danionella Cerebrum

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, adult, neonate, juvenile
Danionella cerebrum	embryo, adult, juvenile, neonate

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

Our aim is to understand how nerve cells transmit information at their specialised connections calledsynapses. The specific context in which we study this question is the visual systems of zebrafish (Danio rerio) and its close relative Danionella cerebrum (DC). To understand how synapses in the retina and brain of zebrafish and DC transmit and process sensory information it will be necessary tovisualise patterns of neural and synaptic activity as the fish respond to visual stimuli.

Our general project plan is to make genetically altered zebrafish/DC expressing fluorescent proteins that emit light when the synaptic connections are activated. This will allow us to investigate the function of synapses between different types of neuron in the retina as they respond to defined visualstimuli. We will also investigate the activity of neurons that provide the final output from the retina to the higher visual centres in the brain.

Comparing the results from zebrafish to those from DC will furhter allow us to build towards a better understanding of how neral circuits evolve. While the two species are closely related, and they even share part of their habitat in south east Asia, they do live in different visual environments. Zebrafish dwell near the surface, where there is ample light, while DC live further down, close to the riverbed, where stirred up debris provide shelter but also make for a more "cloudy" visual experience. How haveDC's visual circuit adapted,

relative to zebrafish, to deal with this type of turbid environment?

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We are pursuing this project because relatively little is known about the mechanisms by which neuronstransmit sensory information across synapses, and how they evolve. The mechanisms controlling neurotransmitter release at the synapse play a key role in determining what we can and cannot see and we need to understand these processes as they operate in the intact retinal circuit.

This project will advance science in several ways. First, we will improve our understanding of the processes which transfer signals between nerve cells. Defects in these processes have been associated with important diseases, such as Alzheimer's and Parkinson's. Second, we will improveour understanding of the way visual signals are processed in the brain.

What outputs do you think you will see at the end of this project?

We will improve our knowledge of vision alongside a more general gain in understanding of howsynapses, neurons and their networks "achieve computation".

Better understanding "how the brain works" is perhaps one of the most important scientific endeavoursof our generation. Here, few neuronal circuits offer a more direct window into this greater vision than those that make up the vertebrate retina.

First, the computational purpose of the retina is readily defined. Second, unlike for most other complex neuronal circuits, the input (light) can be closely controlled, while the output and any intermediate stepscan be probed using existing technologies.

Accordingly, specifically in the retina, we can study how computation happens at a synaptic, neuronal, and network level, probing step-by-step how the incoming message is changed to ultimately allow animals to see. Third, in zebrafish and its close relative danionella cerebrum (DC), unlike e.g. in mammalian retinas, all this is possible in the live animal. Fourth, since the fundamental blueprint of the vertebrate retina is ancient, it is shared by all vertebrates, from lampreys to humans.

Accordingly, shared insights gained from studying different species readily translate across the entire lineage, while understanding any of their systematic differences is informative about how they involve.

Together, the vertebrate retina therefore offers probably one of the best chances right now to trulymake inroads into our understanding of how neuronal networks "function", and how they evolve.

Who or what will benefit from these outputs, and how?

A greater understanding of how neuronal circuits in general function, and how they evolve.

How will you look to maximise the outputs of this work?

We will intimately collaborate with leading experts in the field of vision research and systems neurosicience to maximise the impact of our work. Any new insights gained, including any negativeresults, will be widely disseminated at conferences and via peer reviewed publications.

Species and numbers of animals expected to be used

- Other fish: No answer provided
- Zebra fish (Danio rerio): 50000

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use larval and juvenile zebrafish (up to 21 dpf) as well as Danionella cerebrum of any age including adult. It will be important to work on animals over this age range becasue visual functions(and dysfunctions) may vary with age.

The choice of working on zebrafish and danionells cerebrum is motivated by several factors:

Currently uniquely in these two closely related species, it is possible to noninvasively image theactivity of retinal neurons in vivo. This is made possible by their small size and partly transparent bodies Zebrafish, and more recently also Danionella, offer the possibility for relatively straightforward genetic manipulation, e.g. to target an activity biosensor specifically to a particular class of neuron.This is very powerful when aiming to understand how each given class of neuron contributes to thefunction of the network as a whole.

The fundamental blueprint of the vertebrate retina is ancient and first appeared in our earliest chorade ancestors. As a consequence, the basic make up of the retina is shared between all vertebrates, from lamprey to humans. Accordingly, by studying zebrafish and danionella, two relately "simple" vertebrate species, we can nontheless readily translate key insights into other visual systems,including our own.

Typically, what will be done to an animal used in your project?

Procedures to be applied involve breeing and genetic manipulation of zebrafish / Danionella cerebrum(including the use of viruses and fin biopsy), as well as imaging in vivo (including injection and paralysis). Concretely, the latter means that animals will be immobilised, primarily by embedding themin low melting point agarose. As needed (e.g. to measure activity in the eye, the eye needs to be still),the animal will be furhter stabilised by way of inject neuromuscular blocking agents (NMBs) into the eye muscles.

What are the expected impacts and/or adverse effects for the animals during your project?

No common adverse effects are anticipated from breeding and transgenesis, or for in vivo

imaging in the absence of additional injections (see above). Where there is need to furhter immobilise part of the body using injection of NMB agents experiments are classed as moderate as the injection itself and theeffects of the NMB may cause transient discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

The highest severity category of all procedures was judged "moderate" (in vivo imaging). The vastmajority of animals will be used for breeding / transgenesis only, which is judged as subthreshold.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For our in vivo work, we have no alternative but to use the intact retinal circuit or the whole zebrafish/Danionella brain. This is the essence of our approach: to use the actual, unperturbed, neuralcircuit. There is no substitute for the retina or brain to understand how the retina or brain works.

Cultures of neurons cannot see and are not connected in the way the visual system is in the intact animal. We believe that the fish visual system represents an excellent alternative to mammals for thestudy of many aspects of nervous system function that are conserved among vertebrates.

Which non-animal alternatives did you consider for use in this project?

It might be possible to study small parts of the general question (such as the basic mechanisms by which synapses work) using neurons in culture. But this is not the major question that this project setsout to answer.

Why were they not suitable?

Cultures of neurons cannot see and are not connected in the way the visual systems are in the intactanimal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot

studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate requiring and average of 1000 Danionellas and 10,000 zebrafish per year, over the 5-yearproject duration. These numbers are based on the following considerations:

On average, we will have 10 scientists working on zebrafish, and 1 scientist on Danionella, over thefull duration of the project. Each of these scientists will require an average of ~1,000 animals per annum.

In all cases, the vast majority (~80-90%) of these animals will be exclusively used for breeding andtransgenesis. Only 10-20% (100-200 animals), per scientist and year, will be used for imaging. This corresponds to <1 animal per working day.

In general, number of animals are estimated as follows:

Many experiments will be observational rather than involve a specific experimental manipulation. For instance, we will observe patterns of synaptic activity in the retina in response to different types of visual stimuli. The major objective will be to ensure that the observations are reproducible and reliable,which can usually be achieved with 5-10 complete experiments, each on a different animal, repeating the same stimulus. Some experiments will involve an experimental manipulation, such as alterations in the amino-acid sequence of a protein. Control animals will be wild-type fish that do not contain the mutation. The number of experiments that it will be necessary to carry out to understand the role of themutated protein will depend on the stimuli delivered and the obviousness of the effects. In general, we will carry out experiments on 3-5 heterozygous animals to see if the manipulation has an obvious effect. If it does, we will carry out the minimum number of further experiments necessary to understand the role of this protein (possibly as few as 3-5 more). If the effects are more subtle, we will make a judgement at this early stage as to whether it is scientifically important to carry out further experiments to establish whether there is statistically significant effects of the manipulation.

Our most effective experiments consist of comparing experimental animals with controls that have been treated in exactly the same way except for the critical variable, but, when we can, we use positivecontrols as well as negative controls. We collect data from at least ten test and control cases for each specific experiment and frequently more where the data comes easily. It is unusual for us to collect more than 20 cases for particular experiments because we generally design our experiments to see major qualitative effects and do not generally deal with effects that deal with minor trends or slight differences. Moreover, in many cases we can share controls between experiments, thus further reducing the number of animals required.

We generally use simple parametric comparisons of experimental and control groups where the data are normally distributed and non-parametric tests when the data is not normally distributed. We usually run experiments in block design, and generate standard errors as well as standard deviations.Occasionally, when there is more than one variable in the block matrix, we use multiple analysis of variance tests.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

When collecting tissue of one type from adults, care is taken to also collect other types of

tissue fromthe same animal (e.g. both eyes and the brain) thus minimising the number of adults used. Animals used for in vivo experiments will be killed by Schedule 1 procedures and the retina of these animals used for experiments in vitro whenever possible.

For in vivo imaging work, typically already a single animals allows recording the activity of 100s or sometimes 1,000s of neurons at once. Accordingly, and as elaborated in the above section, the number of animals required to gather very substantial datasets, with correspondingly high statisticalpower, is relatively small.

Where appropriate, power calculations will be used to estimate the appropriate numbers of animals onthe basis of expected variability, and anticipated effect sizes. Where possible, we will use within- subject comparisons to increase the statistical power of the experiments, and to minimise the numbersof animals that are used (e.g. when injecting a drug into the eye). Cryopreservation will be used to preserve important lines and remove the necessity to hold stock for extended periods.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

For breeding and transgenesis, we always screen offspring before the age of independent feeding(<5 dpf), and only positive embryos are raised.

During imaging, we always aim to maximise the amound of data that can be obtained from a given animal within a recording session. For example, time permitting, we will present different sets of visualstimuli to the same animal, that serve data collection for multiple scientific projects.

In general, we will always perform pilot recordings for any new projects before committing to runninga full experimental series.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish and Danionella are currently unique amongst vertebrate model system in that they allow fullynoninvasive in vivo moitoring of their retinal circuits. This is made possible by their small size and limited pigmentation which together offer optical access. Currently, there is no alternative vertebrate model system available that allows similarly comprehensive experimental access.

They will be bred from adults kept in well-maintained aquaria and when used for experiments they willusually be under terminal anaesthesia. For embryos at motile stages of development before they areprotected by the Act, we still use anaesthetic for all operations and embryonic surgery. MS222 (also known as tricaine) is currently the

anaesthetic of choice but we will also investigate the utility of etomidate, which has been reported to produce less obvious aversive effects. The anaesthetic regimen is similar for fish used after they are protected by the Act: the anaesthetic is added (with antibiotics) to the sterile water bathing the fish at appropriate concentrations (2 parts per thousand for MS222). The animals quickly become insensible, and so analgesics are not used.

Why can't you use animals that are less sentient?

We have to use a vertebrate species because the structural plan of the visual system is very differentin invertebrates.

Within the vertebrates, teleost fish like zebrafish are probably the least sentient clade suitable to thistype of work. More ancient lineages (e.g. cartilagenous fish / jawless fish) are generally not experimentally amenable in the same was as zebrafish or danionella are (e.g. no routine genetic access), and they are moreover generally substantially larger and more difficult to keep and breed incaptivity. Accordingly, within the vertebrates, where insights gained can directly translate to humans due to their circuit similarities, teleost fish are arguably the least sentient choice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

For embryos at motile stages of development before they are protected by the Act, we still use anaesthetic for all operations and embryonic surgery. MS222 (also known as tricaine) is currently theanaesthetic of choice but we will also investigate the utility of etomidate, which has been reported to produce less obvious aversive effects.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow "Responsibility in the use of animals in bioscience research", produced by the NationalCentre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will stay informed by:

Using the National Centre for Replacement, Refinement and Reduction (NC3Rs) as source of help,especially regular e-mail bulletins (NC3Rs News) that are forwarded to us by our NACWO.

Good communication with staff in the Animal facility staff who attend industry conferences and are inregular contact with staff in other facilities in the UK, as well as our Home Office Inspector and Named Veterinary Surgeon.

Meetings of Animal facility staff and users.

Implementation of advances will occur in consultation with our NACWO and Named VeterinarySurgeon. All members of the lab working under this license will be involved.

17. The Development of In Vivo Expressed Biologics as a Platform for Novel Therapeutic Agents

Project duration

5 years 0 months

Project purpose

- Basic research
  - Avoidance, Prevention, Diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

DNA, RNA, Delivery, Molecular switches, AAV

Animal types	Life stages
Mice	adult

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

We are interested in developing new technologies that can enable and assist delivery of therapeutics(medicines that heal disease), encoded in DNA or RNA molecules either alone or within viral or non- viral particles, with precise control over when and where it is expressed (turned on) in patients. This work aims to increase our understanding of the duration of expression and activity of the delivered transgene (artificial gene), cell or organ targeting, host responses, and how to precisely control therapeutic activity using molecular switches.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Due to population growth and increased longevity, the total number of deaths attributable to non- communicable diseases (NCDs); diseases that are not caused by an infectious agent, has risen over the last 20 years. Cancer, cardiovascular disease, diabetes and

chronic respiratory diseases killed approximately 33.2 million people worldwide in 2019, a 28% increase compared to 2000. NCDs continued to be leading causes of ill health worldwide and were responsible for seven of 10 premature deaths in 2019 (from WHO World health statistics report 2022). Therefore there is a clear need to pioneer new medical treatments and technologies to address this increasing disease burden. This work will aim to develop several innovative drug delivery platforms by overcoming the existing technological hurdles or otherwise improving the expression, targeting, immunogenicity (the degree to which the immune system recognises it) or control of the expressed DNA or RNA molecules. This could enable us to produce new medicines that are more effective, longer lasting and with fewer side effects than is currently available.

What outputs do you think you will see at the end of this project?

The primary output of this work will be a greater scientific understanding of how to achieve peak performance with new therapeutic delivery technologies in vivo (in a living organism). This could include expression parameters (how much and how long the therapy is present in the organism, or pharmacokinetics), how to target these molecules to a particular cell or organ, how to dampen immuneresponses, or how best to control the activity of these molecules in vivo. This will inform specific drug projects that are aiming to produce new medicines for patients. Where possible this work will be published in scientific journals, presented at conferences, or used in the creation of patents.

Who or what will benefit from these outputs, and how?

In the short term the work will show us which parameters, vector (the vessel that carries the gene) designs or molecular sequences create the best possible performance in vivo for novel therapeutic drug delivery platforms. In the medium term this will allow us to choose the best designs or methods totake into full pre-clinical (before testing in people) and clinical (testing in people) drug development in order to create novel or improved medicines. In the long term this will benefit patients in multiple therapy areas including cancer, lung disease, heart disease, brain disorders, or infectious disease, andmany others. Furthermore the papers published from this work will enlighten and benefit the entire scientific and medical community working on similar technologies.

How will you look to maximise the outputs of this work?

This work is a collaboration of many internal teams, working on different aspects of these technologies,and we frequently meet to discuss results, and how to link our discoveries and learnings across the teams. We also have collaborations with academic labs and technology providers, broadening our scope further. Where possible we will publish the knowledge gained to the wider scientific or medical community, as papers or as presentations at conferences.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the preferred species for our studies because they are small and easily-handled mammals with a highly characterised and well-defined biology. This makes them an excellent model for this work,as they will model the expression, immunogenicity and targeting of our technologies very well. They can also be genetically altered in order to better study or reproduce human disease, or allow us to assess our technology in a more human-like context. For instance, there are several mouse strains that are modified to not mount an immune response to human cells, which allows us to implant human tumour cells or even human cell therapies without fear of these being rejected by the mouse host. We don't require juvenile or aged mice, as mice of adult age are perfectly adequate for this work.

Typically, what will be done to an animal used in your project?

Animals will be bought from suppliers and transported to the facility at around 8 to 12 weeks of age. They will then be moved into cages in groups of 3-5 animals, with constant access to food and water, and lots of enrichment including multiple types of nesting and/or bedding material, plastic tunnels, pyramid houses, and wooden chew sticks. Both male and female mice will be used for this work, with the sexes kept apart in their groups of 3-5 animals. We expect female mice to remain in these group sizes without incident, but at times males may need to be housed separately to avoid fighting. Once on study animals will typically experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes, for example via intravenous (into a vein) or intramuscular (into muscle) injection, with electroporation (the application of a short pulse of electric current) for the latter. When mice are given intravenous injections they will be restrained fully awake ina clear plastic tube, and typically mice will receive only one intravenous injection in their lifetime. For intramuscular injections the mice will be briefly anaesthetised (put to sleep) during the procedure (for around 5-10 minutes), and will typically receive only one intramuscular injection in their lifetime. Most other injections will be given while the animal is restrained by ‘scruffing’, such as subcutaneous (underthe skin) or intraperitoneal (within the peritoneal cavity) injections or oral dosing. When these administration methods are used animals will typically receive 5-10 total doses in their lifetime from one of these routes only. Occasionally animals will be implanted with tumour cells via a single subcutaneous (under the skin) injection while under anaesthesia (duration of around 5-10 minutes), and this will be accompanied by microchipping for identification (also applied subcutaneously). After the first procedure mice will typically remain on study for approximately 3 to 6 weeks. During this period, serial (one after the other) blood samples may be taken via the tail vein (typically 2-3 per animal), or the mice may occasionally be anaesthetised for around 20 minutes for the purposes of imaging (typically 1-2 times in a lifetime). In nearly all cases animals will either have blood samples taken or be imaged, and not both, as generally only one of these methods will be appropriate to monitor the expression of the test article in each case. Rarely, mice will undergo changes in diet whichare not expected to cause distress, but may result in weight loss due to unpalatability. At the end of thestudy mice will be humanely killed, and tissues may be taken for ex vivo (outside of the living body) analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are expected to experience only mild, transient pain and no lasting harm from administration of substances in most cases. The use of anaesthesia when dosing the animals will be used when appropriate to further reduce the pain the animal will feel.

Occasionally animals may exhibit signs of ill health following administration of substances, such as piloerection (bristling of fur), inactivity, or hunching. Mice that show one or more of these signs will be given supportive treatment such as warming or supplying extra bedding or enrichment, and if signs do not resolve the animals will be culled before the limits are exceeded. Unexpected weight loss, another sign of ill health, may also be observed occasionally. Weight loss will be measured against the highest weight recorded for each animal, and in most cases mice will be given diet supplements to help the weight loss recover before limits are reached. As the substances that will be tested on this project are designed to be as harmlessas possible for the recipient, the majority of animals (60%) will be studied on a mild protocol, which hasa low limit on the duration of signs of ill health (less than 24 hours) and weight loss (10%). The remaining animals (40%) will be studied on a moderate protocol, which has a slightly higher limit on theduration of signs of ill health (less than 48 hours) and weight loss (15%). In the case of tumour studies there will be additional limits on tumour volume and tumour condition that will ensure animals are not experiencing discomfort or signs of ill health for a period greater than 48 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

Mice: 60% mild, 40% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While a range of in vitro (in a test tube, culture dish, or elsewhere outside a living organism) assays willbe performed by our research team to triage (decide the order of) molecules or vectors prior to testing in vivo, animal models are needed because in vitro or in silico (modelled in a computer) systems cannot fully capture the complex spatial-temporal (over time and space) pathways that will determine the pharmacokinetics (amount of the compound present in the body over time), pharmacodynamics (the effect of the compound on cells in the body) and biodistribution (where in the body the compound goes) of these novel therapeutic technologies or substances.

Which non-animal alternatives did you consider for use in this project?

We considered the use of human and/or mouse cells in culture either as single layers grown on a dish,or as organoids (3D cell culture) suspended in media. We also considered the use of computer modelling (i.e. in silico) technologies.

Why were they not suitable?

There are fundamental limitations to the in vitro culture of cells or organoids for this work. For instance there is no way to model the biodistribution at a systemic level (i.e. which organ does the substance goto), nor the complete host immune response, as those complex systems are not currently possible to adequately reproduce in culture. Similarly, while in silico technologies are continuously improving, it is yet to get close to capturing the full complexity of a biological organism like a mouse or human.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on previous in vivo work performed by the relevant teams on other licences in the past, combined with a projection of the number of studies that are planned to be run over the next five years, and the approximate mouse numbers required in each study. Approximate numbers are based on recommendations from in-house statisticians on what would be appropriate powering for the studies I have run over the past 18 months on other institutional licences, or studies that are planned inthe future.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

The most appropriate models and experimental design were selected only following a review of all in vitro and in silico analysis and the scientific literature. Furthermore, consulting with statisticians in the study design phase is a requirement for compliance with Company Global Policy on Good Statistical Practice (GSP). For example we will always use the minimum number of animals required to achieve asignificant biological effect as determined by a power analysis for every study. Study designs will also include positive and negative controls, where relevant. This statistical approach ensures sufficient datais obtained from each study, negating wasteful repeats, but also preventing superfluous treatment groups or excessive group sizes. Randomisation will be incorporated into studies, based on either body weight or tumour size, and blinding (an experimental design where the user is unaware whether an experimental group was given a treatment or a control substance to avoid user bias) will be performed where possible, especially on samples collected for ex vivo analysis.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

This work will make extensive use of pilot studies, as most of the test substances will be by definition novel, as they concern novel technologies or modalities (method of treatment). Thus pilots will be run routinely to discriminate new vector designs or sequence modifications with small group sizes intendedto give an initial steer. Only when results from those pilots are positive will larger studies be designed in order to refine the data that can be obtained. For pharmacokinetic and some pharmacodynamic studies, animal numbers will be minimised through the use of serial sampling wherever possible.

Biodistribution or tumour studies will make use of in-life imaging of fluorescent (glows a bright colour under certain light) or bioluminescent (emits a bright light like a glow-worm or firefly) reporters when appropriate, allowing several data points to be obtained from a single animal throughout the experiment. To further reduce animal use, when endpoint is reached we will take and appropriately store numerous tissues in order to perform extensive ex vivo analysis. This will include splitting tissuesinto several parts in order to fulfil multiple assays, such as fixing for IHC (immunohistochemistry) analysis, or freezing for DNA/RNA extraction.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emergingrefinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the preferred species for our studies because they are small and easily-handled mammals with a highly characterised and well-defined biology. The majority of studies will use wild-type (not genetically modified) mice, but the genetically altered mice we do use will be immunodeficient (lack parts of the immune system) strains such as SCID (Severe Combined Immunodeficiency) mice that show no significant physiological abnormalities and do not exceed mild severity. Most studies will not induce disease, except for the minority of studies that involve the establishment of subcutaneous tumours. For the typical study the number of procedures will be minimal with a single injection of the test article, followed by up to 6 tail bleeds or imaging under anaesthetic. This serial bleeding or imagingto measure pharmacokinetic (PK) or biodistribution respectively will reduce the numbers of animals required per study. Where tumour cells are implanted into the mice this procedure will be performed under anaesthetic to minimise pain or suffering. Pilot or tolerability studies will be carried out for new models or test agents respectively to minimise the number of animals impacted by any unexpected adverse effects.

Why can’t you use animals that are less sentient?

The mouse is among the least sentient mammalian species that is available for routine in vivo work. Non mammalian species, i.e. flies or zebrafish, would be too distant from the human for the results to be usefully translated into patient use. For example, similar tissue types and tissue structure, a similar circulatory (blood) system, and a similar immune system, are required for the results of the in vivo workto be translatable into the human context. This work cannot generally be performed on terminally anaesthetised animals, as the studies need to last for several days or weeks for us to study the pharmacokinetic (PK), pharmacodynamic (PD) or biodistribution of these test articles over time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

When planning experiments I will consult relevant published guidelines, including the PREPARE guidelines checklist. I will also check the NC3R online resource library for the

most up to date information on refined procedures, including dosing or sampling techniques. After transportation from the supplier to the facility, animals are given an acclimatisation period of at least one week prior to being placed on study. Tumours are measured with callipers but also monitored for changes in appearance, such as redness or scabbing, to flag potential issues before they arise. Tumour-bearing mice will be assessed using the body condition scale where body weight monitoring may not be sufficient. All mice will be assessed according to the grimace scale where appropriate. Animals are group housed in cages of 2-5 mice depending on weight and sex, and we avoid lone housing unless absolutely unavoidable (i.e. fighting in the group). Animals will be given lots of enrichment as standard,and extra bedding can be provided following procedures. Our technicians are all experienced users of animals and use non-aversive handling techniques. When adverse effects or weight loss is recorded inany of the studies, the observation of the mice will increase to monitor any progression of the pain and suffering of the animals. This will ensure acceptable severity parameters are not exceeded. Post-mortem examinations will be performed to investigate unexpected deaths. Where relevant, tumours willbe studied at the earliest stage of growth compatible with a meaningful result to the experiment.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

Unless otherwise justified studies will be carried out to best practice as described in Workman et al(2010). Other published best practice guidance that is familiar within the establishment are the Handbook of Laboratory Animal Management and Welfare (Wolfenson & Lloyd) and LASA best practice guidelines. I will also refer to the PREPARE and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

I will stay in touch with my local in vivo network of companies, universities and animal facilities, that have regular meetings. I will regularly check information on the NC3Rs website, sign up to the NC3Rsnewsletter, and will attend regional 3Rs symposia. I will also establish regular contact with my named persons in the animal establishment (NVS, NACWO and NIO). Advances in the 3Rs will be incorporated into my studies at the design stage by informing the scientists designing the studies on this project, and at the implementation stage by informing the technicians carrying out the hands-on invivo work. This will be communicated at regular team meetings within the in vivo team.

18. Tolerability and Pk Profiling Of Substances

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aimsmentioned in paragraph (b)

Key words

Pharmacokinetics, Pharmacodynamics, Metabolism, Tolerability

Animal types	Life stages
Mice	juvenile, adult, aged
Guinea pigs	adult
Rats	adult, juvenile
Hamsters (Syrian) (Mesocricetus auratus)	adult
Cotton rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

To enable the discovery and development of new medicines it is necessary to understand how theyinteract with the body. This licence will help characterise how potential medicines are absorbed into,transported around, then removed from the body and if they cause any clear adverse effects.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite the advances in medical treatments that have been made, there is still massive unmet medicalneed, with diseases such as cancers, arthritis, infectious diseases, heart disease, chronic pain, Alzheimer's and many others still having no effective treatments. This results in suffering and deaths occurring that may be preventable if a treatment can be found.

To help the development of new medicines it is key to understand that the substance being investigated can be absorbed by the body, that is reaches the tissues where it needs to provide an effect, that it is present in those tissues for long enough to have a chance of improving the course ofthe disease and that the way the body processes the substance would not be expected to cause clinical problems (pharmacokinetics). It is also important to assess how the body changes the substance (metabolism), as the changes may reduce the effectiveness or increase the toxicity of thepotential medicine.

Although normally undertaken under separate licence authority, whilst performing these studies with minimal impact on the animal it will be possible to obtain additional samples that provide informationon how the substance is affecting the disease process of interest (pharmacodynamics).

Many aspects of the behaviour of a substance can be predicted by computer modelling approaches,and further understood by studying its physical properties and behaviour in cell-based experiments. These results allow the intial development of the substance by for example changing its structure orthe material used to dissolve it. However these approaches currently are not fully predictive of how the substance will behave in, andbe changed by, the body, with all the organ complex systems interacting. The use of animal models helps provide this understanding before taking only the very best substances forward to testing in human clinical trials.

A separate need for investigating the substance in an animal model is to ensure that the subsequent studies undertaken to understand the effectiveness of the substance in affecting disease processes in animal models are properly designed with respect to dosing the substance. For example when triallingpotential new antibiotics it can be crucial to check that sufficient levels of the substance to provide bacterial killing reach the infected organ.

The tolerability studies performed on this licence are normally the very first time a substance will be administered into a living vertebrate animal. These studies check for any obvious adverse reactions toensure that a very early decision can be made on continuing to work with a substance whilst using a very small number of animals.

What outputs do you think you will see at the end of this project?

The studies performed under this licence will contribute to the early stages of drug developmentprojects

This will build on studies performed without using animals where potentially useful new medicines areproduced and refined prior to testing in animals.

The animal studies performed under this licence will provide information on how a potential medicine interacts in a complex organism with similar biological processes to humans.

This will help scientists tounderstand how to improve their substance, provide evidence of suitable absorption and spread within the body so the potential medicine can be progressed towards testing its ability to affect disease processes in animal models, the safety assessment of the substance and ultimately human trials.
Where significant results are not subject to confidentiality agreements they will be communicated morewidely at scientific meetings or published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

In the short term clients, project teams and funding bodies will be able to make progression decisionsto be able to better target their limited financial resources on to the substances with the highest likelihood of clinical success.

In the medium term stopping the progression of projects with little chance of success (either due to tolerability issues or lack of reaching the target tissue) will also mean that animals will not be used insafety assessment and human volunteers will not be put at risk in clinical trials.

In the long term the successful identification of potential medicine will result in reductions in patientmortality, improved clinical outcomes (including shorter hospital stays) and reduced societal costs.

How will you look to maximise the outputs of this work?

Where there may be broader interest in an animal modelling approach, and if the studies are not subject to confidentiality agreements, these will be published or shared at relevant conferences. Refinements to techniques will be shared with others working in the field via individual contacts madeby the establishment’s Named Persons

Clients and collaborating partners will be encouraged to publish all results in journals or share atrelevant conferences.

Species and numbers of animals expected to be used

- Mice: 8000
- Guinea pigs: 100
- Rats: 6000
- Hamsters (Syrian) (Mesocricetus auratus): 100
- Other rodents: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These animal studies serve as a bridge between computer or cell-based experiments and human clinical trials. The similarity and differences of the rodent models used compared to higher species used for safety assessment and humans are well (although not absolutely) understood, allowing modelling approaches based on the rodent data to determine intial dose rates for safety assessmentstudies and human clinical trials

In the majority of cases adult animals will be used as these are representative of the majority of thepatient population. Rarely, where a specific potential medicine focusses on the paediatric or aged population, juvenile, neonatal or aged animals may be used.

Most animals used in this project will be rats and mice (occasionally including genetically altered animals) as these have been demonstrated to provide information that enables decisions to be madeon the progression of potential medicines in a wide variety of therapeutic areas.

Using genetically altered animals allows the investigation of drug human-specific targets andtreatments for these targets.

Other rodent species will be used where specific information on the substance is required in a speciesthat is planned to be used for testing its effectiveness as a potential medicine, or if it has previously been identified in, for example, cell-based assays that the rat or mouse is not a suitable species to be used.

Typically, what will be done to an animal used in your project?

In a tolerability study small numbers of animals will be given substances at dose administration rates similar to those that will be required for initial pharmacokinetic or drug efficacy studies. The route of administration will generally be injection into blood vessels, under the skin or into the abdomen or delivered directly into the stomach via a stomach tube. Normally a single dose will be administered, butin some studies there will be repeated administrations. The animals will then be regularly observed forup to 24 hours to identify any symptoms that may mean the substance is unsuitable for further studies. Depending on the project requirement, there may be higher or lower doses administered to subsequent animals until a clear understanding of the tolerability is established.

Routine pharmacokinetic and metabolism studies are normally conducted in rats and mice, as thesespecies have been identified as being suitable models to provide data for further studies in higher species and man.

A typical study in the rat would involve the surgical implantation of a catheter into the jugular vein, which allows high quality samples to be taken without disturbing the animals within the cage. Followingrecovery for several days, administration of a substance is then performed, generally by injection into blood vessels in one group and delivered directly into the stomach of a second group to enable comparisons to be made between these two common routes of administration. Normally only a single dose will be administered by each route, however in some studies there will be repeated administrations. At time points relevant to the proposed clinical use of the substance samples of bloodwill be withdrawn from the jugular vein catheter (normally up to 8 samples in 24 hours). In some casesthe two routes of administration may be performed in the same animal several days apart to be able tocompare the response within the same individual.

For routine pharmacokinetic studies in mice, a typical study would involve the administration of a substance generally by injection into blood vessels in one group of mice and delivered directly into thestomach of a second group to enable comparisons to be made between these two common routes of administration. Normally only a single dose will be administered by each route, however in some studies there will be repeated administrations. At time points relevant to the proposed clinical use of the substance microsamples of blood will be withdrawn from the tail vein (normally up to 8 samples in 24

hours). If it is critical to understand the level of the substance in a particular tissue it may be necessary to euthanise mice at each timepoint to sample the tissues for analysis.

Where animals have undergone jugular vein cannulation surgery, and the Named Veterinary Surgeonis happy that it is appropriate from severity, scientific and animal health aspects, the animals may be used for further studies. Once recovery from surgery is complete, the limited disturbance to the animals for the pharmacokinetic studies mean this approach reduces the overall suffering involved byreducing the numbers of animals undergoing surgery.

To understand how the substance is removed from the body it may be necessary to place the animalsby themselves for up to 24 hours in a cage with a grid floor over an apparatus that separately collectsthe urine and faeces (a metabolic cage), which helps identify whether the kidneys or liver are involvedin this process.

When it is important to investigate metabolism (how the body alters and eliminates the substance) studies, it may be necessary to sample bile (produced by the liver) by placing a cannula into the bileduct. This is normally performed in the rat as the mouse duct is very difficult to cannulate due to the size of the animal. Following surgery the animal is allowed to recover, after which substance isadministered and bile is collected for up to 24 hours.

To enable studies to be performed on animal tissues, it is often necessary to use freshly prepared (where immortal cell cultures are not suitable or available and commercially available tissue may notbe suitable due to delays in delivery reducing effectiveness). In this case animals are anaesthetisedand blood collected from the heart, until the heart stops. The animal is then euthanised, and other relevant tissues (normally liver, heart and muscles) are taken for use in the test system.

What are the expected impacts and/or adverse effects for the animals during your project?

In tolerability studies it is theoretically possible to see a wide and unpredictable range of impacts due tothis being the first administration to an animal, however screening in non-animal systems prior to first dose in animals means severe adverse effects are very rarely seen. Typically at the doses and routes being used the animal may exhibit subdued behaviour, pain at injection site, altered breathing patterns,weight loss (normally caused by loss of appetite). These are generally short term effects as most substances are cleared from the body within a few hours, but may be present for the duration of the study (24 hours in most cases) and will not result in euthanasia if considered of mild severity.

Similar mild adverse effects may be seen following substance administration in the other studies performed under this licence, and will be controlled by careful choice of dose level, and the applicationof humane end points if they are greater than expected.

Administration of substances and blood sampling from the tail vein causes brief stress and pain due tohandling and needle insertion. These are controlled by skilled handling and minimising the numbers of administration and sampling events.

Surgical cannulation of blood vessels or bile duct will cause pain that is controlled by the use of pain-relieving drugs: generally this lasts for 48 hours, but the animals are closely monitored in case furtherdoses are required.

Being placed in a metabolic cage for collection of urine and faces is mildly stressful but will

be limitedto a maximum of 24 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice - 50% Mild and 50% Moderate
Rats - 100% Moderate
Hamsters - 20% Mild and 80% Moderate
Cotton rat - 20% Mild and 80% Moderate
Guinea pig - 20% Mild and 80% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the initial studies performed to understand the pharmacokinetics and metabolism of potentialmedicines now take place using computer simulations, assessments of the physical properties of the substance, then in enzyme- and cell-based experiments. In particular human cell-based approachesto understand the range of metabolites being produced radically reduces the number of studies requiring bile cannulation. Using the results generated by these methods many substances are not taken forward into animal experiments.

However, even with these advances, non-animal approaches still do not allow the interactions of thesubstance with the body to be assessed in a way that reflects the full complexity of an integrated mammalian system.

As part of bridging from testing of the efficacy of a substance to safety assessment studies bile cannulation studies may occasionally be required to understand the animal species that are mostrepresentative of the human responses to enable the best choice of species.

This project aims to provide the data from a complex, integrated organism to allow decisions to bemade on whether to progress potential medicines to the next stage of development.

Which non-animal alternatives did you consider for use in this project?

Some or all of computer modelling, physical property assessment and enzyme- and cell-based approaches will be used prior to undertaking studies on protected animals to minimise the number ofstudies and potential impact on the animals.

Prior to performing animal studies clients will be asked to provide information on the work undertaken with approaches not using protected animals and an outline of literature

reviewed searching for alternative approaches. It would be expected that non-animal approaches will have been undertaken tohave confidence that the potential medicine is likely to have suitable pharmacokinetics or metabolism in the animal model. These tests will reduce the numbers of experiments performed and increase the likelihood of those completed to deliver meaningful results.

Why were they not suitable?

There are no non-animal alternatives that can currently replicate the full complexity of absorption,distribution and metabolism of a substance in the mammalian body. Now and for the foreseeablefuture there will need to be animal experiments performed to perform initial investigations into pharmacokinetics as part of the process of bridging from non-animals studies to clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers are based upon the number of animals used in the preceding project licence, which reflect the scientific and commercial demand for assessment of pharmacokinetics of novel medicines, with an adjustment to reflect that studies using hamsters, cotton rats and guinea pigs havesignificantly reduced.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

Consultation with biostatisticians has indicated that 3 animals per group gives an effective assessmentof inter-animal variability whilst still providing decision making data. Lower numbers are considered inappropriate due to inadequate understanding of variability in responses.

The extensive use of modelling within projects teams has minimised initial pharmacokinetic studies to a single administration via at most two routes of administration (intravenous and oral): this allows decisions on progression to be made and studies investigating the effectiveness of the substance to beaccurately designed.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where appropriate and accepted by the client, the use of cross-over designs (when investigating thesame substance) or re-use of surgically prepared animals (to investigate different substances) will reduce the number of animals undergoing surgical procedures.

Cassette dosing studies (where several low doses of compounds are administered simultaneously,thereby reducing the number of animals used) may be performed if drug-drug interactions are not anticipated: this will enable multiple compounds to be screened

simultaneously.

When animals are euthanised to provide tissues, other users within the company are offered theremaining tissues to ensure maximal usage.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered animals that demonstrate no or only Mild signs will be used.

Jugular vein cannulated rats allow for central blood samples (considered to be the most appropriate samples) to be taken with minimal disturbance to the rat. The use of skin buttons for exteriorisation inlonger term animals, although requiring more invasive surgery than the use of exteriorised catheters with a pin port, does allow for group housing. Use of buprenorphine jelly for analgesia, with regular clinical assessments to determine whether additional doses are required minimises the post-surgical pain.

For non-surgical pharmacokinetic models, the use of microsampling methods together with removal of scab rather than repeat insertion of needles reduces the pain associated with sampling. Exposure to awarm environment to ensure dilation of the tail veins is reduced to the minimum required to help reduce the stress associated with this procedure.

The bile cannulation model used for the majority of procedures recirculates the bile back into the intestine in between collection periods, allowing normal intestinal function whilst full recovery from thesurgery takes place (with pain relief as above).

Why can’t you use animals that are less sentient?

Currently pharmacokinetic modelling in non-mammalian systems such as zebrafish or drosophila doesnot provide information that can be utilised for progressing to humans without the need for mammalianstudies. These studies are useful only when working on the species in question, and would simply represent an additional step in the process rather than a decision making study.

These simple animals do not provide sufficient data to enable the development of a thorough understanding of the effects of the substance in a fully-integrated organism, which allows the complexinteraction of the potential medicine with many body systems to be assessed prior to moving on the human clinical studies.

Most studies will be investigating the progression of process that occur over many hours so the use ofterminal anaesthesia is not appropriate in most cases.

How will you refine the procedures you're using to minimise the welfare costs

(harms) for theanimals?

On site, working proactively with the AWERB, NVS and NACWO, there is a culture of constant improvement to animal care, control of adverse effects, performance of procedures and study design.

Scoring systems are used to identify early intervention and end points in studies, pain-relieving drugsare used when there is concern an animal is suffering, and monitoring is performed as often as required, including throughout the night.

Further refinement to technical procedures and housing are implemented when they are shown to bebeneficial for the animals and will not reduce the quality of the scientific outputs.

When a refinement to an established model is identified from the scientific literature and proposed for use under this licence, a small number of pilot studies will be performed using standard compounds to ensure the model delivers high quality scientific data whilst allowing the development of adverse eventcontrols and scoring systems that may be able to be used to reduce the severity experienced by the animals.

When placed in metabolic cages, enrichment items (such as shelters) that do not interfere with samplecollection will be placed in the cage.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

In addition to Home Office and EU guidance documents, relevant best practice guidance will be sourced from the NC3Rs (e.g. ARRIVE Guidelines, blood sampling, experimental design), NORECOPA(e.g. PREPARE guidelines), LASA (e.g. blood sampling, drug administration, aseptic surgery) RSPCA and model-specific publications

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

AWERB and the Named Persons routinely circulate information about the 3Rs and identify opportunities for enhancements during their routine rounds.

On a monthly basis the PPL Holder receives and reviews automated literature alerts on animal modelsand journals relevant to the project licence.

When a new animal model is proposed, a thorough literature review is performed to determine themost scientifically relevant approach whilst causing the least harm, and a new automated alert generated.

In addition to conference attendance, webinars and discussion groups are participated in by the PPLHolder and scientific staff.

19. Understanding the Role of the Tumour Microenvironment in Cancer Pathogenesis and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Cancer, Immune, Stroma, Lymph node, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

With this project we aim to understand the mechanisms by which non-cancerous cells of the tumour microenvironment support cancer growth and metastasis, using the knowledge gained to identify newtargets and approaches to better treat cancer

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancers have developed many ways in which to prevent our defences, that is our immune system, from destroying a tumour. A tumour is much more complex than a collection of cancer cells. Many other cell types make up the tumour – these cells support the tumour in many ways and are known asthe stroma. Stromal cells may help a tumour by acting on our immune system to prevent it from working properly, or by attracting bad immune cells.

New therapies that ‘kick-start’ our immune system back into action are proving promising, but these stillonly work on some patients in a few cancers. Thus there remains a critical need to develop new targetsand platforms to treat cancer, alone or in combination with current therapies. But, to do this successfully, we need to first understand how the tumour microenvironment supports disease.

What outputs do you think you will see at the end of this project?

From this project our primary benefit will be the increased understanding of how supporting cells foundin a tumour act, specifically how they work to switch off the immune system.

We will generate data that describes new mechanisms or cell types, and interactions that support disease. These will be published in journals and shared at scientific meeting. Datasets will be madefreely available for other scientists to use and benefit from. We hope that some of the interactions identified can be targeted- and may result in the development of new agents or repurposes of drugsthat are already in use for other conditions.

Who or what will benefit from these outputs, and how?

These findings will provide immediate benefit to the research community, sharing potential mechanisms and features that could be used for development of new or improved treatments which use our immune system to target a tumour. New mouse models and experimental systems developed will offer potential to benefit other scientists, and the knowledge we gain will begin to benefit clinicians,Biotech industries with drug discovery pathways, hopefully being initial steps towards the patient benefiting.

How will you look to maximise the outputs of this work?

To maximise outputs we will collaborate with scientists in the community. We also design projects thatrun in parallel, so that one mouse can help multiple projects and we can therefore maximise the valuable information gain from an experiment, reducing the number of animals involved. We will disseminate the knowledge we gain at scientific conferences and through publication in journals. We will share advances and how we work to achieve our goals with the public; primarily at public engagement events such as science festivals and Pint of Science events

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need a model with a complex, functional immune system as is found in humans, and which is lacking in lesser species. Thus, adult mice represent the least complex system that possesses theparameters needed to yield meaningful data translatable to human

disease.

Typically, what will be done to an animal used in your project?

Most animals under the authority of this PPL will develop tumours that have been induced by a variety of methods such as cell implantation, inducing genes in genetically altered animals, using chemical carcinogens or using viral vectors. Tumour growth will be measured and blood samples may be taken. Implanted tumours will grow rapidly (typically within 3 weeks), but tumours that develop in genetically modified can take longer, up to 5 months. The spread of tumours to different sites around the body, is asign of poor prognosis, but we still don't know how to stop it. Some mice will therefore develop secondary tumours. This may be achieved by injection of tumour cells into the blood, or by removal of the primary tumour. This will be needed to look for e.g. lymph node metastasis in cases where tumoursgrow too fast and too large before metastatic tumour can grow.

In tumours and metastases we will use methods to help us understand how stromal populations and interactions contribute to tumour growth; tumour-associated tissues may be imaged on a microscope and substances such as labelled cells, blocking antibodies, molecular inhibitors or cell tracers may beadministered by a range of routes such as intratumoural or intraperitoneal injection. Healthy mice without tumours may also undergo similar procedures to help us understand how a tumour changesnormal tissues and stroma to help disease development.

As the stroma may impact how effectively therapies work, mice with tumours may also receive cancertreatments such as immunotherapy, hyperthermia or radiotherapy. This will enable us to understand which supporting cells contribute to resistance and the mechanisms they use, helping us to identify ways that we may boost therapy response.

What are the expected impacts and/or adverse effects for the animals during your project?

In the majority of cases, tumours will not significantly impact animal welfare or normal behaviour and will be classified as mild. Transient discomfort may be experienced following procedures such as injection. Rarely, mice may develop more clinical signs such as tumour ulceration or weight loss. If these are seen, mice will be killed immediately to limit any suffering experienced. However, in the vast majority of cases, our endpoints occur before any of these adverse effects have the chance to developthus most animals be experience mild severity in their lifetime, and will not exceed a moderate severitylimit.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

In our previous PPL, >98% of animals experienced a maximum severity of mild. We expect this trend to continue with 98% of animals experiencing either sub-threshold or mild severity. A maximum of 2%may experience clinical signs and moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tumours contain tumour cells, but they are in fact much more complex systems including fibroblasts, blood endothelial cells, lymphatic endothelial cells, pericytes, adipocytes, smooth muscle cells, fibroblast and immune cell populations all of which interact, adapting as a tumour develops and evolves. We work routinely with in vitro models, making them as complicated and accurate as we canto generate as much information as possible with regards to the tumour microenvironment. The modelsystems we use aim to complement and help design mouse experiments appropriately. They help to identify key populations to investigate in mice, doses, potential responses to therapy before using mice. They also critical for us to home in on specific mechanisms following data generated in mice. However, the complexity of complete tissues and a changing tumour environment cannot be fully recreated in a plastic culture vessel. To do this we require a living system with aspects of the immunesystem comparable with humans. Such a system does not exist in non-protected alternatives.

Which non-animal alternatives did you consider for use in this project?

Within the field of cancer research, in vitro assays including co-culture approaches and 3D models such as organoids are widely used. We implement these in our studies, incorporating stromal and immune components. In contrast, having performed literature searches and meeting with collaborators,in vitro approaches to study of metastatic disease are limited. We are developing a microfluidic model of the lymph node with engineering collaborators. While this is addressing a gap in immunology research it can and will be applied to address mechanisms of initiation and establishment of lymph node metastases. We have also considered in silico computational models, and performed preliminary studies with collaborators.

Why were they not suitable?

While such models are extremely useful, and we continue to use them, tumours are highly complexand dynamic; cells continually enter and leave the tumour, and because of our circulatory system, chemicals continually enter and leave a tumour by blood and lymphatic systems.

We cannot yet fully recreate the complexity of complete tissues and a changing tumour environment ina plastic culture vessel.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

We have estimated numbers based on our experience, and the numbers used in previous PPLs whichform the foundation of this PPL. Our previous PPLs and propsed work follow a similar reserach focus and exprimental approach. The numbers required in this PPL reflect this.

We will continue to use similar GA lines in this PPL. We have used actual numbers from our previousPPL breeding protocols to detemine what is required for 5 years on the new PPL.

Similarly with experimental mice; the work outlined in this PPL is a direct follow on from our previous PPL. We continue to utilize tumour models, examining the role of stromal and immune components inpathogeneisis, and identifying ways to perturb these interactions . From our previous experiments we have determined that in some cases up 11 mice per group is required to meet statistical power. Treatment and control groups must also be incoporated into experimental design. We have used actualnumbers from our previous PPL experimental protocols to determine numbers required.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

To help reduce numbers we take several approaches. 1) We consult the NC3Rs website and tools contained within. 2) We plan to also use wildtype littermates of genetically modified animals, reducingsurplus. 3) We design projects that run in parallel, to maximise the data we can obtain from a single animal. For example, we have projects that examine events at the tumour that run alongside projects that aim to understand events at the tumour draining lymph node. This way, one mouse is used in twodistinct projects. 4) We utilise additional tissues from experimental cadavers to perform further in vitroexperiments that help us gain more information, or optimise conditions.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

As described above, we utilize wildtype of "wrong genotype" littermates in our work, and we design projects to run in parallel to maximize the data generated from a single mice. We also share tissues from experimental animals i.e. we frequently share spleens, mammary tissues and skin with other researchers to avoid them having to request animals solely for tissue isolation. When introducing a new protocol or agent, we perform small pilot studies as advised by Workman et al., 2010 to ensure itssafety and dose.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We need a model with a complex, functional immune system as is found in humans, and

which islacking in lesser species. Thus, mice represent the least complex system that possesses the parameters needed to yield meaningful data translatable to human disease.

We have extensive experience in mouse tumour models and work closely with staff at the animal facility. We have refined models so that animals largely develop superficial tumours and experience very few clinical signs. This means our experiments end before adverse effects can develop. As animals are monitored very closely, by well trained staff, any welfare concerns raised can be dealt withrapidly. Mice are also housed in social groups and are given multiple bedding types for nesting, chew sticks, fun tubes and platforms to provide a rich living environment.

Why can’t you use animals that are less sentient?

To be able to study the tumour microenvironment adequately we need to use animals with a fully developed immune system, which can only be achieved at maturity. Less sentient species do not contain the diversity of cell types to sufficiently recreate the human tumour environment. We are unable to perform our studies in animals that are terminally anaesthetised due to the duration neededfor tumours to develop, or treatments to take effect.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

When preforming regulated procedures, we utilize sites that aim to minimize any impact on animal welfare. We induce tumours at superficial sites, such as the ear, and subcutaneously in the back or flank which are less likely to cause the adverse effects expected in the equivalent internal tumour models, e.g. lung cancer or ovarian cancer. We have experienced that tumours at such sites allow mice to continue social and exploratory behaviour (e.g. climbing upside down from food racks). We also will administer substances using the most refined route, which minimized discomfort or change of adverse effects. For example instead of foot pad injections, we will perform less intrusive subcutaneous injection of lower limbs that permit the same experimental outcome with less discomfort.

Non-invasive imaging inherently provides the potential of longitudinal imaging which provides substantial amounts of data from one individual over the course of disease development and/ortherapy response.

Animals are imaged or receive magnetic hyperthermia under general anaesthesia which reducesanimal stress during sessions.

Animals that have undergone regulated procedures will receive enhanced monitoring. Mice may receive enhanced post-operative care with soft bedding, mash and pain management (analgesia) toenhance recovery.

Once received, mice are returned to their social groups and are given multiple bedding types fornesting, chew sticks, fun tubes and platforms to provide a rich living environment.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We have and continue to follow Workman et al, Br J Cancer 2010, alongside guidance documents fromthe BVAAWF/FRAME/RSPCA/UFAW Joint Working Group (Morton et al , 2001) and the LASA Guiding Principles for Preparing and Undertaking Aseptic Surgery

(2010).

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We receive the NC3R'S newsletter, which contains the latest updates, and will attend relevant seminars to keep abreast of new/improved methods. We have and continue to perform regular literature searches alongside discussions with colleagues and collaborators. From these, we aim to refine our approaches and reduce mouse numbers through improved experimental design and new experimental approaches. To implement advances effectively we will maintain a close relationship withresearch colleagues, animal technicians, NACWOs and NTCO within the facility.

20. Molecular Switches in Inflammation - 2022

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants

Key words

Inflammation, Arthritis, Colitis, Immune Cells, Molecular Switch

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify key molecular regulators of myeloid cells in inflammation. This research will involve human and mouse studies which, taken together, should define the functions of the identified regulators in inflammatory disorders. In the short-term it will provide the scientific rationale for pre-clinical validation studies and in the longer term for clinical trials of strategies designedto interfere with these regulators.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Sustained inflammation is an underlying factor in a wide range of diseases, including rheumatoid arthritis (RA), inflammatory bowel disease (IBD), vasculitis, atherosclerosis, diabetes and certain typesof cancer. Despite progress in understanding how they develop and what components of the immune system may be involved, much remains unknown. Although inflammation is a widespread occurrence during many common diseases the

range of treatment options for patients with chronic inflammatory diseases is very limited. People with inflammatory diseases who are obese tend to have more active disease, more pain and worse overall health. For example, obese people with RA are less responsive to biologics and traditional anti-rheumatic drugs. This phenomenon is linked to the fat depot being infiltrated by active immune cells constantly releasing inflammatory mediators that cause inflammation,which are already overactive in RA. Both bioactive lipids of Western diet and gut dysbiosis, e.g. an altered composition of intestinal microbial populations, are thought to provide continuous immunological stimulation contributing to the development of immune response anomalies in numerous inflammatory disorders. Antibiotic treatment and /or germ-free housing have a profound effect on disease development mice, with both protective and pro-pathogenic outcomes noted depending on the context. Therefore, it is important to gain a greater understanding of the molecular mechanisms underlying these processes in order to design novel treatment strategies for acute and chronic inflammatory disorders such as RA and IBD as well as during infections.

What outputs do you think you will see at the end of this project?

We have made considerable contributions to the understanding of the molecular events involved in theinflammatory response and have led to the identification of potential targets for the development of novel anti-inflammatory therapeutic interventions. Applying a similar strategy during this project, it is highly likely that we will identify novel potential anti-inflammatory drug targets with this approach. We expect to analyse up to 5- 10 new targets during the course of this programme. The proposed study will increase our knowledge of myeloid cell function in tissue, at norm and during inflammation. We will generate new mouse strains, new knowledge and new technologies that will be of benefit to the scientific community.

Who or what will benefit from these outputs, and how?

In the short term, our research will promote the new field of specific white blood cell (i.e. neutrophil, monocyte) adaptation to tissue microenvironment during inflammation and will help to define the role ofthese particular white blood cells in chronic inflammatory disorders.

In the medium-term, the identified key regulators of effector myeloid cell function can be used as biomarkers in future studies of human pathologies. The patients with chronic inflammatory disorderswill benefit from better molecular phenotyping of their conditions and thus more tailored treatment regime.

Our research on the impact of microbiome and diets will highlight more specific sites for interference and is likely to be of interest to pharma as well as companies working with nutraceuticals. The patientswith inflammatory disorders may benefit from advice on diet and the development of more holistic treatment based on a combination of therapeutics and diet.

In the long-term, we will facilitate the development of new anti-inflammatory drugs based on the targetsidentified within this study. We will use our established pharmaceutical framework to further the development of therapeutics targeting key inflammatory molecules identified during the course of this programme. This will lead to new patent applications and benefit players in the industry and private sector, as they will be able to capitalise on our prior discovery to bring about new drugs for chronic inflammatory and autoimmune diseases.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in peer-reviewed journals andpresentations at scientific conferences and meetings. The transgenic animals developed will be valuable to other scientists interested in the development of anti-inflammatory therapies.

We will conduct collaborative studies with clinical colleagues that would allow us to translate ourfindings to human pathologies.

Species and numbers of animals expected to be used

- Mice: 22,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We choose to work with mice, due to the availability of well-established models of inflammatory diseases in this species, and the fact that they have ~70% similarity to humans, which gives us a goodstarting point for translating findings to the clinic. We will use both wildtype and genetically altered mice, which are either global or myeloid-specific knockouts of regulators of interest, or exhibit fluorescence in cell types of interest, so that we can track immune responses. For our models, adultmice will be used, to allow time for their immune system to develop and mature before we induce inflammation.

Typically, what will be done to an animal used in your project?

Typically, animals will experience mild, transient pain and no lasting harm from administration of substances by injection using standard routes (intravenous, subcutaneous, intraperitoneal). Where administration is required for prolonged periods, animals will be surgically implanted with slow-releasedevices such as a mini-pump. These animals will experience some discomfort after surgery and somemild to moderate pain which will be treated with analgesics.

Animals may undergo changes in diet, which are not expected to cause distress but may sometimesresult in obesity.

Animals may experience blood sampling, which will cause mild and transient discomfort.

Animals may undergo multiple instances of gait analysis testing (where they are placed on a pressure pad for a few minutes). This may be mildly and transiently distressing but will not cause them any pain.

Animals may undergo non-invasive imaging, such as bio luminescence or micro-CT under anaesthesiato immobilise the animal while an image is being taken. The imaging process will take a maximum of 30 minutes. The animals will be aware of the anaesthetic being administered but will experience only mild distress.

Animals may undergo intravital imaging. This is undertaken under non-recovery anaesthesia, where the animals will only be aware of the anaesthetic being administered and may experience mild distressbut no pain.

Animals may undergo bone marrow irradiation and reconstitution (where applicable), which can causeradiation sickness, which usually stabilises within 3 days of the procedure. The animals may be givenantibiotics to prevent infections because their immune system will be low, as well as soft food, floor- level water and extra bedding to support thermoregulation.

Animals may develop arthritic pain and swelling in their joints, with symptoms lasting typically for up to 14 days. Animals will still be able to move around, climb, eat and drink, but their joints may feel sore indoing so. They will develop arthritis following injections into the joint and/or base of the tail, which will occur under anaesthesia, so the animals will only be aware of the anaesthetic being administered and may experience mild distress.

Animals may experience flu symptoms following nasal administration of virus, which may lead to breathing difficulties. It is likely that the animals will show signs of illness, much like humans, which should resolve after 10 days – symptoms may include reduced food and water intake, listlessness, weight loss, shivering. Animals will be provided with extra bedding to help with thermoregulation, andsoft food and floor-level water to help them recover.

Animals may develop colitis, with symptoms including loose faeces, lasting for up to three weeks. They may feel some swelling of their abdomen, feel dehydrated or experience weight loss, so they will be carefully monitored and given access to soft food and floor-level water to ease their symptoms. They will develop colitis following oral administration of bacteria into their stomach and/or by injectionof antibody, which may cause them to experience mild distress. In order to monitor colitis symptoms,animals may be given a colonoscopy to take intestine biopsies – this will occur under anaesthesia.

These animals will experience some discomfort after surgery and some mild to moderate pain whichwill be treated with analgesics.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may be administered substances such as tamoxifen by oral gavage or injection in order to modulate expression levels of a transgene. This may cause the animals to lose their appetite, resultingin transient weight loss, which will be monitored throughout the dosing period. There may also be soreness at the site of injection, due to multiple administrations.

Animals may be subjected to bone marrow irradiation, which can cause radiation sickness. The animals may exhibit a transient weight loss approximately 7-10 days following irradiation due to loss ofappetite and diarrhoea, which usually stabilises within 3 days. They may also exhibit a mild hunched posture. The animals may be given antibiotics to prevent infections, as well as soft food, floor-level water and extra bedding to support thermoregulation.

Animals may be infected with influenza virus or RSV, which can lead to signs of illness for up to 10days following intranasal administration. There is a small chance that animals may experience breathing difficulties as a result of administration of the virus. The virus

symptoms may include listlessness, reduced food and water intake, and weight loss of up to 15%; however, animals are expected to regain normal behaviour and restore their body weight within 10 days post-infection.
Animals will be given soft food, floor-level water and extra bedding to support thermoregulation.

Animals may develop arthritis symptoms, caused by joint and/or subcutaneous injections of adjuvant/antigen under anaesthesia to stimulate an inflammatory immune response directed at the knee/paw joints. Symptoms of the arthritis models mimic human disease – swelling and pain of thejoints, leading to reduced mobility and discomfort, and ultimately cartilage damage if the disease is allowed to progress. Typically the animals will experience swelling of the joints for up to 14 days.

Some experiments may continue for up to 12 weeks in order to study resolution of arthritis, during which time the swelling and inflammation will decrease but the mice will still experience reduced mobility and some discomfort due to cartilage damage in their joints. The subcutaneous injection sitesmay experience some soreness and ulceration due to the irritant nature of the adjuvant used. Ulcers can be treated with barrier cream to soothe and encourage healing. Animals will be given soft food, floor-level water and extra bedding as required.

Animals may develop colitis for up to 3 weeks following oral administration of bacteria or compounds inthe drinking water and/or intraperitoneal injection. Symptoms may include temporary weight loss and loose faeces. Animals will be given floor-level water to prevent dehydration. In some instances, anal inflammation and rectal bleeding may occur. In order to monitor colitis symptoms, animals may be given a colonoscopy to take biopsies – one colonoscopy can be given each week, and a maximum of two biopsies taken on each occasion (maximum 6 times). This procedure should last 5-15 minutes.

There is a small chance (<1%) that the colon could be damaged by the endoscope or that rectalbleeding or abdominal swelling could occur, so the animals will be carefully monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

43% Sub-threshold, 24% Mild, 33% Moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The steps leading to the development of the state of chronic inflammation are poorly understood and itis likely that multiple physiological processes are involved, including proliferation of precursor cells, activation of lymphocytes and recruitment of inflammatory cells. This and modelling the effects of new therapies inevitably involves the use of whole organisms and, in particular, the use of animal models ofinflammation. As we are unable to induce inflammation in the clinical setting, mice and with ~70% similarity to the human and well-established models of inflammatory diseases of interest, make an ideal model for our work. Mice are preferable to rats because of the greater availability of reagents (e.g. monoclonal antibodies; genetically modified strains) specific for this species.

Which non-animal alternatives did you consider for use in this project?

We considered and use human cells to address some aspects of the project, such as questions relating to the mechanisms of action of compounds targeting the molecular regulators identified, or therole of inflammation in cell activation.

We also use newly developed cell lines, that allow for in vitro differentiation of immature cells from bone marrow into mature white blood cells (neutrophil and macrophage) in the blood to address basicquestions on their biology.

Why were they not suitable?

Isolated human cells can not fully reproduce the interaction between various cells in tissue, the scaffoldthe tissue makes or the impact of systemic manipulations on local responses and vice versa.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number is based on our historical data and familiarity with the protocols included in therenewal of this licence. We estimate the experimental group size for each protocol to detect the expected difference between groups (approximately 6-8 mice per group is usually sufficient). We alsouse our annual returns to consider how many mice are required for our breeding programmes.

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

To ensure that the minimum number of animals will be used in this project, we will control the variationintroduced by experimental layout, e.g. animals of the same sex, similar age, weight, fed on the samediet, provided with the same bedding. To remove any bias, we will consider running independent replicas, randomising and blinding the animals between the subject and control groups. We will make appropriate arrangements to randomly assign animals to experimental groups and blind studies. The design of experimental layout will

be adequate to the hypothesis tested and based on the results of pilot experiment(s) with the help of the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Where possible littermate controls will be used to further increase the sensitivity of our read-outs, untreated control groups may also be shared between treatment groups. In addition, untreated controlgroups will be used as a source of tissues for analysis of expression of pro-inflammatory mediators.
We will also maximize the use of harvested tissues and cells. For example, immune cells isolated frominflamed tissue from a genetically modified animal may be used as a source of immune cells for multiple in vitro experiments (genomics, FACS-based characterisation, migration). Cells and tissues may be shared by multiple researchers.

In preference to adoptive transfer studies, we will utilize genetically modified animals with deletion of agiven gene only in specific cell types, significantly reducing further the numbers of animals required formechanistic studies. Experiments will be planned so they can be published in accordance with the NC3Rs' ARRIVE guidelines.

We also pay a considerable attention to our mouse colony management. We keep a careful documentation of the number and type of breeders to help organize the colony and ensure no unnecessary breeding is carried out. Animal requirements are reviewed on a weekly basis. We use homozygous genetically altered and wild type breeder pairs, which have been generated from originalheterozygote breeder pair and hosted in the same environment. We re-set these pairs on a regular basis. Embryos of strains not currently in use are cryopreserved.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen models which have already been refined to minimise the number of immunisations and the severity level of adjuvant. They are the least severe and cause the minimum amount of distress . For example, between the two models of arthritis, such as antigen induced arthritis (AIA) andcollagen induced arthritis (CIA), the AIA model will be used in preference where possible; as it gives rise to a greater incidence and less variability; and is less severe. The CIA model will only be used when necessary to replicate the human disease pathogenesis more closely. For our colitis studies we will use a strain of helicobacter that is widely utilised as a model system, and therefore the protocol and disease timecourse is well established. We will preferentially choose acute models of inflammation such as the air-pouch model or LPS-induced lung injury, where the inflammation is contained to a single compartment to answer our scientific questions where possible, as opposed to systemic models to ensure we cause the minimum amount of distress.

We pay careful attention to animal husbandry and provide environmental enrichment and co-housingto avoid social isolation. Where possible we will use genetically altered mice where the genes of interest are either expressed or deleted in specific cells or tissues types rather than in the whole organism to minimise potential harm.

Why can't you use animals that are less sentient?

We cannot use fish, flies, or worms, as we need mammals with a fully functional immune system to address the questions we would like to answer. We cannot use terminally anaesthetised animals as weneed to induce the disease and allow it to progress.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Wherever possible we will administer substances for experimental purposes in food or drinking water, instead of injections. Where daily administration cannot be done in food or water the use of a surgicallyimplanted miniature infusion pump will be considered over injection routes. If injection routes are necessary, we will choose the route with minimal harm but will still enable the maximum delivery to thetissue target.

Where knockout of regulators is required, conditional knockout in myeloid cells is preferable to globalknockout, to focus the reduction of expression in our immune compartment of interest and minimize off-target effects. Moreover, expression levels can be controlled by administration of drugs such astamoxifen in some mouse strains, which minimises the need for crossing mice.

The use of adjuvants is necessary in some of our models, and we will use those adjuvants that cause the least severe response. Where we need to use an adjuvant that will cause a more severe response,we will deliver the dose at two sites to reduce the risk of inflammation at a single injection site.

For bone marrow transfer experiments, irradiation will be given in 2 separate doses - at least 4 hoursand no more than 24 hours apart - to minimise side effects. We will also provide antibiotics prophylactically to prevent infection as advised by NVS.

In models where anti-inflammatories cannot be given because their anti-inflammatory activities tend toinhibit the induction and progression of the disease (e.g. arthritis); additional husbandry support, such as easier access to food and water (e.g. food pellets and water gel packs placed at floor level), and/orsupplemental bedding will be provided during the acute inflammatory stage of disease in all animals. In consultation with NVS, opioid analgesics e.g. buprenorphine may be provided if required. In modelsof colitis, mashed food will be provided to aid recovery.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We will follow PREPARE and ARRIVE guidelines of best practice when designing and reportingexperiments under this licence. The LASA guidelines will also be consulted.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We will attend welfare meetings and 3R's meetings held at the establishment, and sign up to the NC3R's website updates. We will interact with the regional NC3R's manager, as well as the NamedInformation Officer, and disseminate acquired information in our group lab meetings.

21. Neural Plasticity and Cognition in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, ortheir effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

Learning & Memory, Emotion, Neural Plasticity, Limbic System, Animal Models

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective Assessment

The Secretary of State has determined that a retrospective assessment of this licence is notrequired.

Objectives and Benefits

Description of the projects objectives, for example the scientific unknowns or clinical orscientific needs it's addressing.

What's the aim of this project?

The primary aim of this program of work is to identify how neural plasticity contributes to cognition andemotion in mammals. The secondary aim is to discover how changes in cognition and emotion as a result of deficits and/or abnormalities in neural plasticity, result in neuropsychiatric and neurodegenerative disorders, including schizophrenia, anxiety, depression and Alzheimer’s Disease.

Potential benefits likely to derive from the project, for example how science might be advancedor how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Plasticity describes the changes in the nervous system that result from experience. This can involve changes in the strength of the synaptic connections (chemical junctions) between nerve cells in the brain, changes in the structure of the neurons and their support cells, or even the generation of new neurons in some brain regions. Plasticity is thought to play an important role in learning and memory, and deficits in plasticity (too much or too little) lie at the heart of various neuropsychiatric and neurodegenerative disorders (e.g. schizophrenia, anxiety depression, Alzheimer's Disease). Not surprisingly, therefore, neural plasticity has been a major target for trying to develop new treatments forthese brain disorders. However, there has been very limited success so far. This likely reflects that there are multiple different flavours of plasticity and our failure to understand fully the role of these different flavours of plasticity in different aspects of cognition and emotion. The outcome of this project will be a better understanding of the role of different kinds of plasticity in different kinds of learning and memory, and in adaptive behaviour more generally. It is essential to better understand the functional significance of plasticity in different parts of the brain if we are to understand and treat these different diseases. Ultimately this could lead to the development of new drugs or treatment strategies, or a better utilisation of existing treatments.

What outputs do you think you will see at the end of this project?

The output of this programme of work will be:

Scientific data (e.g. in terms of behavioural performance and recordings of neural activity)

New scientific knowledge (e.g. regarding the role of different forms of neural plasticity in cognition andemotion)

New behavioural assays for assessing cognitive function and emotion in rodents.

Publications in peer-reviewed journals

Who or what will benefit from these outputs, and how?

The following groups may benefit from our programme of work:

Short-term: our colleagues and collaborators working on related projects will benefit from the scientificknowledge that we will generate which will help them interpret their own data.

Medium-term: other researchers in both academia and industry will benefit from this knowledge in anypeer-reviewed publications. We have a long and extensive track record of collaborating with various drug companies.

Long-term: ultimately a better understanding of the neural plasticity mechanisms that underlie different aspects of cognitive and emotional processing in the mammalian brain, and why these processes become dysfunctional in neuropsychiatric and neurological disorders, will help in developing new/bettertreatment approaches, and better targeting of existing approaches to specific sub-groups of patients.

How will you look to maximise the outputs of this work?

We will maximise the output of this work through a number of different routes:

Collaborators: We have a long and extensive history of collaborating widely within our institution, aswell as nationally and internationally. Our results and the scientific knowledge generated will have astrong bearing on these collaborative research projects as well as the independent research of our collaborators. Our strong track record of collaborating with industrial partners means that we have been well placed to influence their research programs.

Publishing and Presentations: In addition to publishing our work in respected, peer-reviewed journals,we will present the work as oral and/or poster presentations at national and international conferences.

Publishing null results: We will endeavour to design our studies in such a way that null results will be publishable (e.g. including positive control groups, validation measures of experimental manipulations,triangulation to determine generality of findings).

Data sharing: Wherever possible we will happily share our data (behavioural measures and recordingsof neural activity) with other researchers.

Teaching: We will also include our recent research in our undergraduate and graduate teaching(lectures, seminars, tutorials), thus hopefully influencing the next generation of researchers.

Species and numbers of animals expected to be used

- Mice: 19500
- Rats: 2500

Predicted Harms

Typical procedures done to animals, for example injections or surgical procedures, includingduration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We work on rats and mice because they are the lowest vertebrate group whose brains reasonably resembles human brains. In many experiments we will use genetically modified rodents. This provides a really good way to selectively manipulate the function of key plasticity proteins in the brain (and evenin specific cell types in specific brain areas) in order to assess their functions. It also provides a way to mimic mutations that are found in patients in our mice or rats such that we can then study the effects ofthe mutation on behaviour and neural activity and plasticity. Most of our behavioural experiments will be performed in adult animals as behavioural performance tends to be more stable at this stage of life.

Typically, what will be done to an animal used in your project?

The experiments will involve testing the ability of rats and mice to perform behavioural tasks in which they have to learn and remember crucial information such as where a food reward is or how to escapefrom a pool of water. The animals will readily learn what to do to get a tasty food reward or how to climb out of the water. We will also assess anxiety, for example, by asking the animal whether it wantsto explore a new place or remain in a safe location, or using mild footshock.

We will examine the effects of brain lesions, drug treatments, various genetic manipulations, and sleepdeprivation on these behaviours. We will also record different signals of brain activity while the animalsperform behavioural tasks (e.g. levels of neurotransmitters, electrical activity of neurons, measures of activity based on blood flow which resemble fMRI in humans). Recording of brain signals involves intracranial implantation of microelectrodes and could involve single housing of the animals. A number of our experiments therefore involve brain surgery (e.g. to make a brain lesion or insert a recording electrode or inject a virus to alter the genetics of neurons in the brain). Importantly, the brain manipulations we perform do not affect basic sensory or motor performance. Basic home cage behaviours are unaffected and an observer would be unable to differentiate between the experimental animals and the controls based on their home cage behaviours. In some of experiments drugs will be given by systemic injections. Sleep deprivation involves a prolonging of the time awake by exposing the animals to lots of novel objects which they keep exploring.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of our behavioural experiments would not be stressful for the animals and could even be considered environmental enrichment. Animals are motivated to perform our behavioural tasks by testing at a time just before they would expect to be fed their meal, and thus at a time when they are hungry or thirsty. There would be brief periods of mild distress during tests of anxiety (e.g. after a mildfootshock), but this is short-lasting.

There may also be transient pain and discomfort after brain surgeries but animals are given analgesicdrugs to cover the period when any pain would be expected. Injection of drugs will cause only minor discomfort at the time of injection and some of the drugs that we use might result in abnormal behaviours (e.g. increased or decreased levels of exploration, hallucinations). Animals will be humanely killed at the end of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animaltype)?

56% of mice and 48% of rats would experience sub-threshold procedures30% of mice and 17% of rats would experience mild procedures
14% of mice and 35% of rats would experience moderate procedures

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you haveconsidered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to show that a particular brain region, or a particular neurochemical, neurotransmitter or receptor, is important for the brain to work properly, it is necessary to remove or silence that bit of brain, or remove or block the neurotransmitter from working. This is not ethical (or practical) in humans.Likewise, in order to understand how the brain works we need to be able to measure the activity of the neurons in the brain and measure the levels of the different neurotransmitters. Again, this is not possible in humans. Computer simulations of the brain actually rely on the information that we will provide and so cannot replace the work that we do.

Which non-animal alternatives did you consider for use in this project?

We have made use of in silico techniques and computational models for data analysis which help us togenerate hypothesis which can be used to assist in designing our experiments.

Why were they not suitable?

We study behaviour in the vast majority of our work, and it is not possible to replicate this in ex vivo orin vitro experiments. Computational models for brain function are limited in their use and are generally constructed from information provided by data collected in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that havebeen taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of animals we will use is based on our prior experience (> 30 years).

What steps did you take during the experimental design phase to reduce the number of animalsbeing used in this project?

We will minimize the numbers of animals used by making both the behavioural tests and the experimental manipulations (e.g. lesions, genetic modifications) as accurate and sensitive as possible.For example, over many years we have developed and modified our behavioural tasks such that they are better able to detect subtle changes in brain function. Furthermore, we have refined our experimental manipulations (e.g. by using more sophisticated mutant mice) so that any changes in behaviour are more readily attributable to a specific aspect of brain function.

In many experiments we will use what are called "within-subjects designs" in which the same animal might receive both the treatment condition and the control condition (e.g. on one day the animal mightget the drug but then on the next day it will receive the control solution but lacking the drug). This reduces the number of animals required and also reduces the amount of variability in the experiment overall which then, in turn, further reduces the number of animals that we need.

In many of our experiments we will collect both behavioural data and recordings of brain activity. We are therefore able to gather lots of information from the same animal, reducing the numbers of animalsthat we need overall.

What measures, apart from good experimental design, will you use to optimise the number ofanimals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will bepaid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

We will conduct pilot studies to ascertain whether experiments are feasible and to optimise the way theexperiment is conducted.

We will use computer modelling to help us get the maximum information from the analyses of our data.

Wherever possible, we will share tissue with our colleagues. For example, we can provide ex vivobrain tissue to collaborators for in vitro electrophysiological or biochemical experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfarecosts (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these modelsand methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to study how the brain works we need to be able to record the activity of its neurons and interfere with its normal function. This requires using techniques which disrupt brain function in differentways (e.g. selective brain lesions which remove a particular brain region, drugs which block specific receptors in the brain, or genetic mutations which alter brain function), or implanting electrodes or sensors which measure brain activity. We will use genetically altered mice and rats that have mutationswhich allow us to manipulate brain function in very specific ways or which mimic human neurodegenerative and neuropsychiatric disorders. We will also use animals that have undergone manipulations of the brain or mimic brain disorders by either surgery or drug administration. The methods we use cause only minimal and short-lasting pain or discomfort. Behavioural testing is likely to cause only transit and mild discomfort as we aim to study behaviour under normal conditions.

Why can’t you use animals that are less sentient?

We can't use less sentient animals such as flies, worms or fish because their brains are not similarenough to human brains, and they exhibit few of the same behaviours as humans.

Furthermore, we cannot study behaviour in anaesthetised animals. We need to study awake animalsthat are capable of sensing their environment and moving normally.

How will you refine the procedures you're using to minimise the welfare costs (harms) for theanimals?

Operations on the brain are done very carefully and in state-of-the-art surgical theatres, and the animals are given pain killers after the operations until they have fully recovered. They are also given highly palatable food to help them recover. The animals are monitored very carefully for at least 7 daysafter any surgery. Soon after the operations you would not be able to tell the difference between the experimental animals and controls in terms of the way they behave in their home cages. Similarly, the genetically modified mice are virtually indistinguishable from normal mice when viewed in their home cages. It is only with the sophisticated tests of learning and memory that you can begin to tell them apart. Animals are monitored very carefully during our behavioural tests. Indeed, many of our behavioural experiments involve us watching the animals perform. The animals will get gradual exposure to handling and behavioural training prior to most tests in order to increase the reliability ofthe tests.

What published best practice guidance will you follow to ensure experiments are conducted inthe most refined way?

We follow best practice guidelines issued from the NC3Rs (see above) and Laboratory Animal ScienceAssociation (LASA). We will follow ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

We stay informed about advances in the 3Rs through regular attendance at local 3Rs meetings. Weregularly liaise with the regional NC3Rs manager and the Named Information Officer, and we keep alook out for latest information on the NC3Rs website.

22. Neural Basis of Behaviour and Cognition in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuron, Brain, Cognition, Autism Spectrum Disorder, Neurodevelopmental Disorder

Animal types	Life stages
Rats	embryo, neonate, juvenile, adult, pregnant
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The first aim of the project is to understand the brain mechanisms involved in cognitive functions such as learning and memory, spatial navigation, decision making and social cognition. The second aim is to understand how and why these are altered in neurodevelopmental disorders such as autism and intellectual disability.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Human neurodevelopmental and mental health disorders result in alterations in many aspects of cognition, including learning and memory, navigation, decision making, and

social cognition. Investigating how the neural circuits of a healthy brain support these cognitive processes will not only provide a better understanding about the biological basis of these fundamental cognitive processes, but will also provide a basis on which to build an understanding of how the brain is altered in disease states. Studying how these circuits and mechanisms are altered in models of neurodevelopmental disorders will help us to understand how and why cognition is affected, and may allow us to identify new approaches for treatment. This is important, as it is estimated that 3-4% of the population have a neurodevelopmental disorder (Emerson, 2012), but current treatments tend to focus on managing symptoms rather than reversing or preventing the emergence of the core deficits.

What outputs do you think you will see at the end of this project?

The results of this work will lead to:

- greater fundamental understanding of how the brain supports cognitive processes including memory, spatial navigation, decision making and social cognition;

- an improved understanding of how these processes and underpinning mechanisms are altered in neurodevelopmental disorders, which may in turn lead to the identification of viable targets and effective time windows for treatment strategies.

Who or what will benefit from these outputs, and how?

The outputs of this research will directly and immediately benefit the global research community studying the neural basis of cognition and how it is impacted in conditions such as autism and intellectual disability.

In the medium term, we hope that it will benefit patients and their families impacted by such conditions, through greater understanding and eventually the development of new therapeutic approaches.

How will you look to maximise the outputs of this work?

- We aim to publish our work in open access research journals.
- We aim to make all completed datasets available to the scientific community
- We will communicate with members of the scientific community through collaborations and at relevant conferences.

Species and numbers of animals expected to be used

- Rats: 7250
- Mice: 1100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use rats and mice. We use these animals as models for several reasons:

The cognitive functions we wish to understand, and their underlying neural circuitry are generally well conserved across mammalian species. Therefore, while details may differ in humans, the general circuits and mechanisms are likely to be similar. By making advances first in rodents, it is then much easier to address whether similar mechanisms arise in the human brain.

While similar questions could be addressed in primates, it is ethically preferable to use less sentient species for experimentation where possible.

It is not possible to use simpler established models, such as flies or worms, as these species do not use same cognitive functions and mechanisms.

A remarkable array of experimental tools is available for neuroscience experiments with rats and mice. This enables more rapid progress than would be possible using other similar species.

Several rat and mouse models of known genetic causes of neurodevelopmental disorders have been developed, which we will use to understand alterations in cognition and the underpinning neural circuits.

The project will use both juvenile and adult animals. We seek to understand cognitive function in the mature brain, as well as how cognition develops postnatally as animal mature from juveniles to adults, and how this development is altered in neurodevelopmental disorders.

Typically, what will be done to an animal used in your project?

The project will typically involve two different types of experiment:

To study how cognition develops during juvenile development to adulthood, and how it is affected in rat models of neurodevelopmental conditions such as autism and intellectual disability, animals will be trained and tested on one or more cognitive tasks over a period of 1 week to 3 months. In some studies, they will be administered compounds (in their food or via injections) to test whether these compounds can correct or prevent the emergence of any cognitive deficits. At the end of the experiment, the animals will be killed humanely, and brain tissue may be taken for further analysis.

To investigate information processing by neurons that underpins memory, spatial navigation, decision making and social cognition, and how this differs in rat models of neurodevelopmental conditions such as autism and intellectual disability, a typical experiment will involve recording and manipulation of the activity of neurons as animals perform cognitive tests. The recordings of neural activity will tell us what cognitive processes the neuron represents. The manipulations will enable us to test contributions of neurons to performance on the cognitive tasks. This experiment requires surgical procedures to implant electrodes or optical devices into the animal's brain which are used to readout activity of nerve cells in the brain. In some cases, another surgery is required to inject substances into the brain to alter neuronal function. All of the surgical procedures are carried out under general anaesthesia and analgesia, and typically have duration of between 1 and 4 hours. At the end of the experiment, the animals will be killed humanely, and brain tissue may be taken for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The major expected impacts arise from experiments requiring surgery in order to inject substances into the brain and to implant devices for recording from the brain. These may include transient pain and weight loss, which will be mitigated with pain relieving medication and supportive care. These procedures are carried out with refined surgical protocols and are not typically associated with other adverse effects. They are not expected to cause more than minor and transient distress or suffering to the animals.

Animals may be housed singly where there is a concern of aggressive behaviour or increased likelihood of damage to implanted devices, for example for electrophysiology or optical experiments. Single housing is thought to be stressful to rodents and will only occur when scientifically justified. In such cases, the duration will be limited to the minimum period necessary and all cages will be enriched with objects for exploration and play. These methods have been shown to reduce stress in rodents.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity is not expected to be classified above a moderate level. This applies to all animals used for the project.

We expect that for 40-50% of animals the severity will be mild or subthreshold since they are only used for breeding, behavioural testing and/or administration of compounds followed by humane killing for tissues. The remaining 50-60% will experience moderate suffering as they will have surgical procedures as described above.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We want to understand how cells in the brain mediate its cognitive functions. Because these functions of the brain rely on sensory input and manifest as outputs that change behaviour, we can only study how they work using live animals that are able to carry our measurable behaviours.

Which non-animal alternatives did you consider for use in this project?

Computational models Human brain imaging studies Cell culture systems.

Why were they not suitable?

Computational models are useful as part of an overall refined approach, and can be used

to simulate aspects of brain function and to make predictions about how biological substrates can implement cognitive functions. However, it is not possible to use them as alternatives to answer the questions we aim to address. Essentially, the models don't yet explain the cognitive functions we wish to understand. Development of better models requires data that can only be obtained with experiments in which animals use the cognitive functions we aim to understand.

Human brain imaging studies are also useful as part of an overall refined approach, and one that we have a track record in using. However, while functional brain imaging in humans can point us towards brain areas and circuits that may be involved in cognitive processes, and can also help identify which brain regions and circuits may be affected in brain disorders, they have two main weaknesses. First, they provide only an indirect measure of neuronal activity, and cannot distinguish between excitatory and inhibitory signals or different cell types. Second, they provide only correlational data. That is, they can show which brain regions are active during specific processes, but not whether the brain activity causes or is required for the cognitive processes. Animal models allow direct recording of neural activity during cognition, identification of specific cells in specific circuits, and the ability to make interventions that address causal questions.

Cell culture systems do not have the organisation of real brains, do not process sensory inputs and do not execute behaviours. They therefore can not be used as alternatives to answer the questions we aim to address.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of animal numbers takes account of the experimental goals for the period of the project, together with the number of PIL holders who will be working on the project (and hence, the rate at which the work can be done). The numbers take into consideration the animals required for experimentation (including both experimental and control groups as appropriate), as well as the numbers required for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

It is essential that the number of animals we use allows us to collect data that are robust. We have many years of experience in performing behavioural and neural recording experiments using animals, and this allows us to make predictions, using statistical analysis based on previous data and the expectation of effect size, about the anticipated effect size we are likely to see and how variable that effect will be. Using this information we can estimate the numbers of experiments we need to carry out.

Good experimental design means that we assess drug effects alongside non-drug/vehicle treatments and that we compare genetically modified animals with littermate wild type animals as appropriate.

For example:

- during behavioural training and testing, and where the task allows, multiple trials and/or sessions are performed. This reduces the overall variability due to confounding variables, and thus decrease the number of animals needed.

- when assessing the development of cognition across development, where possible we use tasks that are based on natural/spontaneous behaviours rather than tasks that require extensive training. This means that we can assess the same animals at multiple time points across development (using a within-subjects design) rather than using a new group of animals at each developmental timepoint.

- when assessing the effects of neuroactive compounds on cognition and its underpinning mechanisms, where possible we use within-subjects designs such that we can compare the effects of the experimental compound and vehicle/control condition is the same animals. In experiments taking this approach the order of treatment (experimental vs control) would be counterbalanced between animals.

- Appropriate statistical testing will be performed (e.g. GLMM), and where relevant we assess the variability accounted for by fixed factors (such as genotype, time point, and drug condition) as well as the variability that can be ascribed to random factors such as litter, cage, cohort etc to ensure that our results are robust.

- All PIL holders working under the authority of this PPL will be asked to take the local statistics course that covers rigorous experimental design, research conduct, analysis and the reporting of animals in research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies on test subjects. Before performing experiments involving surgeries, a small number of animals will be used to confirm and test that the surgical procedure yields the desired scientific outcome (e.g. stereotaxic coordinates, expression of transgenes from viral vectors).

Sharing of tissue. When possible, brain tissue may be shared between experimenters for in vitro and histological experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Both rats and mice will be used under this licence. Both species are genetically very similar

to humans, and have anatomical organisation of neural circuits underlying cognition that are similar to humans (unlike invertebrate or other non-mammalian model systems). As the work involves understanding circuit mechanisms of cognition, common anatomical organisation is crucial, as well as the ability to exhibit a range of cognitive abilities exhibited by humans such as episodic-like and everyday memory, spatial navigation, decision making and cognitive flexibility, and social behaviours and cognition.

Rat: The rat is the species of choice for most of the experiments in the proposed programme of work. We use these rather than mice because, in certain cases, we can study more sophisticated cognitive abilities such as one-trial paired associate learning and strategy switching. Moreover, rats have a much richer repertoire of natural social behaviours than mice, making them more suitable for test of social behaviour and cognition. In the past, we have generally used outbred Lister-hooded rats; they are easily handled and have excellent vision. We also use some transgenic and knock-out rats (as well as genetically modified mice). Importantly, in choosing the strain background for recent lines of our neurodevelopmental work, the Long-Evans strain was chosen (e.g. the FMR1 KO rat model of Fragile X Syndrome). This is because they are widely used in the United States (market forces) but also, like Lister-Hooded, they have excellent vision. These two lines of rats are the most refined for the intended purpose as the animals can successfully learn the tasks we seek to study and there is a valuable literature of past work that (a) we do not need to replicate; and (b) serves to guide future work.

Mouse: In a subset of experiments investigating the neural circuits underlying spatial cognition we will use mice instead of rats. Although their cognitive flexibility is not as great as rats (which means they cannot successfully learn some of the more complex and demanding cognitive tasks), they show the same complement of spatially modulated neurons as rats, and are proficient at a subset of the spatial and memory tasks. The rationale for their use in some experiments is that the mouse is particularly amenable to genetic and molecular manipulations that provide refined approaches to manipulate specific neurons and circuits, e.g. via cell- and circuit specific Cre-Lox procedures and/or targeted viruses.

Why can’t you use animals that are less sentient?

We want to understand cognitive functions such as episodic memory, spatial navigation, decision making and social cognition. These functions are either absent in less sentient animals or are implemented through mechanisms that diverge substantially from those used by mammals including humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will adopt a number of refinements that minimise welfare costs, which by increasing the precision of experimental tests and manipulations will lead to clearer and increasingly specific answers to the questions we aim to address:

Many of the behavioural tasks we use capitalise on the natural behaviours or the animals (e.g. exploration tasks) or are positively reinforced (e.g with palatable foods); these pose substantially less stress to the animals than tasks that are negatively reinforced (such as those that induce fear memories associated with shock). The only task that may be considered aversive at the outset is the water maze, but measurements of the stress hormone corticosterone indicate rapid habituation of any stress response in this task.

Animals will be group housed wherever possible, with enrichment provided in their cages. In animals that have been subject to surgery for the implantation of recording/stimulation/drug infusion devices, it is sometimes necessary to house animals singly to avoid contact between animals causing damage to the ‘head-cap’ used for these. To mitigate against this, we have developed a mesh barrier that can be used to divide a large cage into two smaller compartments. This allows animals to interact with one another through the mesh, which increases social stimulation. This can be used wherever possible for animals that are small enough such that the footprint of the "half" cage is sufficient to allow them to move around freely and exhibit normal home cage behaviours.

Where possible and relevant, we will make use of silicon probes for recording neural activity from large populations of neurons in vivo. Silicon probes allow recording from larger populations of neurons at a time than using single electrodes or tetrodes, which means that more data can be collected from an individual animal over a shorter period of time, reducing the cost to the individual animal.

When monitoring neural activity using miniature microscopes in behaving rodents, we will make use of a new protective headcap which we have recently developed. This reduces the risk that the miniature microscope will be damaged or come loose, which ensures better quality and longer lasting recordings from each animal (meaning fewer animals need to be used, and less variable data quality). It also reduces the risk that animals would need to be culled before the planned end of the experiment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow all best practice guidance provided by our institution. We will also follow procedures that we and others have published that establish refined methods for the specific experimental questions we aim to address.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have excellent institutional support and training to stay informed about and implement advances in the 3Rs. The Home Office Liaison Contact (HOLC) circulates HO guidance notes and welfare organization’s newsletters through emails. The Named Veterinary Surgeon (NVS) team and full time Named Training and Competency Officer (NTCO) work together with PIs to refine procedures and disseminate best practice ideas.

23. Neural Mechanisms of Learning Predictions

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neural Circuits, Neurons, Learning, Neurodevelopmental Disorder, Brains

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the brain mechanisms underlying the learning of sensory predictions, and how this process is different in neurodevelopmental disorders like autism spectrum disorder.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The ability to predict our sensory inputs allows us to distinguish sensations that are caused by our own movements and sensations that are caused by the environment. How the brain learns to do this is not yet known. Based on the symptoms, it is thought that a disruption in this learning process gives rise to the features of certain neurological disorders, including autism spectrum disorder and psychosis. To understand the causes of these disorders, we must study the process of predictive learning both in healthy brains and in the brains of animal models of these disorders. The new understanding resulting from this work is expected to help identify potential therapy targets in the future.

What outputs do you think you will see at the end of this project?

The results of this work are expected to generate a better understanding the neural mechanisms underlying learning in the healthy brain and in animal models of neurodevelopmental disorders like autism. This new knowledge will be output in the form of preprints, peer-reviewed publications, and public datasets.

Who or what will benefit from these outputs, and how?

Outputs relating to the neural mechanisms of learning in the healthy brain will build on the foundation of knowledge about how the brain learns, eventually enabling us to build a mechanistic model of this process. The primary benefit will be to basic research. Outputs relating to models of neurodevelopmental disorders will provide new understanding of learning dysfunction in these disorders. These results could provide new neural targets for therapies in the future and guide future research into the neural causes of these disorders.

How will you look to maximise the outputs of this work?

All scientific output arising from the project will be disseminated first as preprints, then as open access publications, freely available online. All datasets and analysis code used in these preprints and publications will also be uploaded to a public repository, where other researchers can freely access and reuse the data. We aim to publish all results and datasets regardless of the result.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use neonate, juvenile, and adult mice for this research for a number of reasons. First, the cerebral cortex is the primary brain region that we will study as it is involved in neural learning and in neurodevelopmental disorders. This is primarily a mammalian brain region. Second, there are many established genetic mouse models for neurodevelopmental disorders, and for targeting specific genetic neuron types. Finally, the mouse shows complex behaviour which will allow us to study the process of learning at the behavioural level.

Typically, what will be done to an animal used in your project?

Animals will undergo a combination of surgery (including substance injection into the brain and implantation of recording devices), behavioural training and assessment, neural recordings, and euthanasia. Durations of the experiments will range from acute (less than 24 hours) to chronic (A maximum of 4 months).

What are the expected impacts and/or adverse effects for the animals during your project?

The possible adverse effects resulting from the procedures are primarily death under

anaesthesia (during survival surgeries), transient pain following survival surgeries (which will be mitigated with analgesic regimes), and transient, limited weight loss in the case of water restriction for behavioural tasks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are considered to be moderate for the majority of procedures (two-thirds), with the remainder a mixture of mild, non-recovery and subthreshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The natural process of learning involves many regions of the brain working together in a complex manner during behaviour. This is particularly true for the type of learning we are studying - the predictions of sensory inputs during behaviour. Due to our need for the whole brain to be intact, and for studying learning processes during natural behaviour, there is no option other than to utilise live animals.

Which non-animal alternatives did you consider for use in this project?

In vitro tissue preparations and computational models.

Why were they not suitable?

Since we are studying how the brain learns to predict the sensations caused by behaviour, we must study intact animals that are engaged in behaviour. In addition, in vitro tissue involves the severing of long range brain connections that we believe are important for the learning process. Computational models of the brain are only accurate when they are based on physiological data. There are significant gaps in knowledge that currently limits the use of these models in understanding natural learning processes in health and disorder. In addition, such models cannot generate new physiological data.
However, we aim to use the results generated by this project to advance computational models of learning, which will potentially help guide and reduce the number of experiments needed in the future. In addition, we will explore generating in vitro methods to study the forms of plasticity we discover.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design

studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of animals needed based on the number of animals required for robust statistics in previous publications, as well as usage information from other license holders at the establishment. Information from the animal facility, and from previous experience in managing mouse lines, was used to estimate the number of animals required for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use pilot studies in the case of exploratory experiments that will provide an idea of effect sizes and optimal cohort sizes. Studies and publication practices will follow ARRIVE guidelines. Animals and brain tissue will be shared across coordinated experiments as far as possible. The technologies used, such as electrophysiological recordings, offer exceedingly high signal to noise ratio, and high temporal resolution data. This enables information-rich data that is highly interpretable, and reduces the number of animals needed to obtain reliable estimates of neural activity.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To optimise the number of experimental animals needed, pilot studies will be used in exploratory experiments before expanding to larger cohort sizes, in particular where there is no indication from previous literature on the expected effect sizes. In cases where the data obtained from an experiment is nested (i.e., large numbers of measures, like neural recordings, coming from the same animal, and therefore not independent), we will utilise hierarchical bootstrapping to optimise the power of the statistics without needing to increase the number of animals used. To reduce the number of animals used for breeding and maintenance of genetically altered mouse lines, we are planning to explore switching to recent alternative virus vector technologies for targeting genetic neuronal populations that do not require a mouse line.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this study we will utilise mice. Mice are the most appropriate species for this study for a number of reasons: 1) The mouse has the capacity for readily learning complex behaviours. 2) The mechanisms of learning, such as synaptic plasticity, are likely

conserved between mice and humans. 3) There are many genetic strains in mice that allow us to target specific neural populations, as well as strains that emulate the genetic alterations underlying autism spectrum disorders. 4) Many state-of-the-art neural recording and manipulation techniques have been developed in mice.

We will use a combination of experimental techniques including optical and electrophysiological methods to record from genetically defined populations of neurons in the brain, and behavioural training and analysis. Many of these techniques require recovery surgery, due to the necessity of recording neuronal activity during behaviour.

Why can’t you use animals that are less sentient?

Species that are less sentient cannot be utilised for our research question, because the cerebral cortex, which is a primary site of sensorimotor learning and strongly implicated in autism spectrum disorder, is primarily a mammalian structure.

While we will use methods under terminal anaesthesia when the research question allows it, terminal anaesthesia cannot be used in the majority of our experiments. This is due to the fact that the process of sensorimotor learning takes place during active behaviour. In addition, the physiological states of the cerebral cortex and of neuromodulatory systems under anaesthesia are fundamentally different compared to wakefulness. Therefore, to study the mechanisms of learning, we must primarily utilise awake, behaving mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice undergoing chronic procedures will be habituated and familiarised to the experimenter to limit stress. Studies involving surgical or invasive methods will adopt appropriate pain management regimes and post-operative care, as directed by the NVS. Researchers will carefully monitor their study animals at minimum daily, and all study animals undergoing surgery or water restriction will be scored on the 'Study Animal Score Sheet', adapted from the post-surgical and water-restriction score sheets recommended by the NC3Rs-convened ‘Refinements to rodent head fixation and fluid/food control for neuroscience’ . Animals undergoing behavioural procedures will be trained in a gradual step-wise manner designed to cause minimum distress, and take place in quiet, dedicated rooms that minimise animal stress. Water-restriction will be avoided wherever behavioural experiments are possible and high-throughput enough without the need of water-related motivation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidelines provided by the extensive resource libraries and published guidelines on the NC3Rs website and the LASA website. Since this project relies heavily on head- fixation procedures, and, to a lesser extent, fluid control in mice for behavioural purposes, we will follow the practices advised in the published paper 'Refinements to rodent head fixation and fluid/food control for neuroscience' written by an expert working group convened by NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend informative seminars and events provided within the establishment and

locally, and events provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). We will regularly check the NC3Rs and LASA websites for opportunities to stay informed and up-to-date on animal welfare matters.

24. Parasite Life Cycles

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cryptosporidium, Diarrhoea, Drug discovery, Parasitology, Transmission

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to improve the understanding of the basic biology of *Cryptosporidium* parasites and their interaction with animals that they infect, which include humans. We also aim to test new medicines that are being designed to treat the disease in humans.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diarrhoea is a major cause of death (10%) and illness in young children worldwide. Diarrhoea can be caused by a range of different diseases, including viruses, bacteria, or parasites. The Global Enteric Multicenter Study (GEMS) investigated which diseases cause deadly diarrhoea. GEMS revealed that *Cryptosporidium* is the second leading cause of deadly diarrhoea in young children worldwide.
Rotavirus is a leading cause of diarrhoea in young children; children in the UK receive a vaccine early in life to protect them from this disease. Unfortunately, there is no vaccine for

cryptosporidiosis.

There is also no effective treatment for cryptosporidiosis. Through this work, we will test new treatments for cryptosporidisios. Mice are used to model human *Cryptosporidium* infections. Determining how well a new medicine works in an infected mice is an essential step in the process to create a new medicine for humans.

Cryptosporidium is a microorganism that infects the intestine. It has a parasitic life style and can only live and grow in the guts of a human or animal. It scavenges food from the intestines and can harm the guts. This often leads to disease. We have only a rudimentary understanding of the biology of this parasite. With the advent of gene editing, we can study how *Cryptosporidiu*m infects the guts. By improving our understanding of the parasite, we will gain insight into how to treat and prevent infections.

What outputs do you think you will see at the end of this project?

We expect to publish several research articles about our discoveries concerning *Cryptosporidium*
biology, host-parasite interaction, and drug discovery.

We expect to advance new drugs (candidate anti-cryptosporidial compounds) from our collaborators closer to clinical development.

We will present our findings at international scientific conferences.

We will engage with the public to share our discoveries.

Who or what will benefit from these outputs, and how?

In the short-term, these outputs will benefit other researchers, including parasitologists and drug discovery scientists. In the medium-term these outputs will benefit a wider scope of researchers, potentially those that study clinical cryptosporidiosis and those who study gut-microbe interactions. In the longer term these outputs may impact pre-clinical and clinical development of therapeutics for treatment of cryptosporidiosis.

How will you look to maximise the outputs of this work?

When possible, we will share data, compounds we make or use, and methods with other researchers prior to publication. All datasets and publications will be open access so that they can be accessed by other researchers and members of the public. We will publish articles where the methods are described in detail.

We will provide advice and training to other research groups as requested. This will ensure that our experimental approaches are robust and reproducible.

We will use the information we gain into the basic biology of *Cryptosporidiu*m (for example, how the parasite causes disease and how it interacts with the host) through the help of our collaborators (who are developing new types of drugs and looking at how different compounds kill the parasite) to develop new therapeutics to treat cryptosporidiosis.

Species and numbers of animals expected to be used

- Mice: 9750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There is no simple method to grow *Cryptosporidium* in the laboratory. Therefore, *Cryptosporidium* research is dependent on the use of animals to grow (propagate) the parasite and for observation of the entire life cycle (how it grows, reproduces and spreads). Large animal models can be used to propagate *Cryptosporidium*: calves, sheep, and some piglets (which have known gut microbes).

However, these animals experience more severe adverse effects (diarrhoea, dehydration) than mice, have more variable responses, require specialised equipment and training that is difficult to access, and are prohibitively expensive. In contrast, adult mice are considered one of the best animal models for *Cryptosporidium* infection and study. This is because these mouse models have much more predictable and reproducible outcomes and do not experience as severe welfare outcomes, and so are considered the standard to study *Cryptosporidium*.

Cryptosporidium parvum (which is a clinically relevant species of parasite because it affects both humans and farm animals) does not naturally infect mice but does infect immunocompromised mice (these are mice that have been developed to have a weaker immune system than wild mice). We use mice that have had their genes altered so that they are immunocompromised. These mice look normal, breed normally and do not have any welfare costs associated with the genetic changes during day-to- day living – they are just able to become infected with the parasite we are interested in. Cryptosporidiosis patients themselves are immunocompromised (young children with undeveloped immune systems, or adults immune-compromised due to advanced HIV or immune suppressive therapies). There are two types of mouse models of cryptosporidiosis, one where there is a short term (“acute”) infection and one where there is a longer term (chronic) infection. The two different models give different information about the parasite and so we use both of them.

As well as researching *Cryptosporidium* that is clinically relevant to people and livestock in immunocompromised mice, to investigate host-parasite interactions in a natural setting, we use mice that have a normal immune system. These mice can be infected with mouse-specific species of *Cryptosporidium* (for example *C. tyzzeri*, which is not clinically relevant) to let use find out how the parasite interacts with and adapts to the body’s defences.

Typically, what will be done to an animal used in your project?

Mice are infected orally with genetically modified *Cryptosporidium*. We do this by either giving the mice a known number of parasites by stomach tube (“gavage”), which is a very brief procedure that doesn’t seem to cause the mice anything other than possibly slight discomfort at the time of dosing, or we house mice with an animal that is infected and is shedding parasites in its faeces so the cage mates pick up the parasite from the environment (which is how infection happens naturally as *Cryptosporidium* parasites are shed in the faeces). Samples are collected by picking up faecal pellets from the bottom of the cage floor. Faecal samples are analysed and *Cryptosporidium* is separated from the

faeces so that we can do various types of analysis in the laboratory. As part of our analysis, we look at the levels of infection levels present. This is usually done from the faeces, but sometimes we use a special type of imaging system to work out the levels of infection in the animal itself. To do this, we anaesthetise the mice and inject it with a compound that makes the parasite luminesce. The imager lets us visualise the parasites in the living mouse non-invasively (a bit like when a person has an MRI or CT scan). We can repeat the imaging several times without harming the mouse and this gives us information on how the infection changes over time. Imaging gives us information we can't get in other ways whilst letting us use as few mice as possible .

Mice may be treated with compounds that we wish to test to see if they can kill the parasite or reduce its numbers. Sometimes, we will take small blood samples (taken from a vein in the tail via a small pin-prick) to let us measure the levels of the compound in the blood, as this is important for knowing how much and how often a drug would need to be given in the clinical situation.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice may be genetically modified. These genetic modifications are not expected to effect animal welfare.

In most cases, the infection with *Cryptosporidium* is asymptomatic and the animal appears fit and well throughout the study. In some instances, infection may result in weight loss (this is fairly common) and behaviour changes such as loss of appetite or the animal looking "hunched" (we don't see this very often).

We have a very large amount of experience with cryptosporidiosis mouse models and we recognise patterns in infection level, behaviour, weight loss, and body condition score that lead to changes in animal welfare. If we see clinical signs that make us concerned that the animal may be becoming unwell, we increase levels of monitoring. There are limits in place for weight loss or loss of body condition and for how ill the animal can get and we always try to intervene before these humane end points are reached.
Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

During our previous licence, we developed experience using these mouse models of cryptosporidiosis. We observed approximately 25% of animals on this protocol experience moderate actual severity (based on reaching a predefined weight loss, although often these animal don't show other clinical signs). The remaining 75% of animals experience mild actual severity (low level or no weight loss and no other clinical signs).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no simple way to continuously grow Cryptosporidium in the lab. The most successful in vitro continuous culture systems are intestinal organoids (3D cell systems that mimic the cells in the gut). Unfortunately, as well as being complex and expensive, these systems are currently small and can't be expanded for the amount of work we do. They do not generate the large numbers of Cryptosporidium parasites required for downstream laboratory experiments.

Therefore, we have to use mice to grow and keep colonies of genetically modified Cryptosporidium parasites. We also require animal models to observe infection patterns because the way the host's immune system responds to the parasite is critical in the response to infections and there isn't currently any way to look at this without a living animal, although we hope that organoids might be useful for some aspects of this soon.

Which non-animal alternatives did you consider for use in this project?

Our group has an on-going collaborative project to develop human intestinal organoid models for continuous culture of Cryptosporidium. As above, this culture system is unlikely to replace mice used to generate large numbers of genetically modified Cryptosporidium for downstream laboratory work, but we will use it when it is possible to do so.

However, as our project advances we may be able to use the system to conduct preliminary studies using co-culture of Cryptosporidium with human intestinal organoids. This may be used for exploration of host-parasite interaction or for testing new potential drugs (candidate therapeutics). Preliminary data will be used to direct further in vivo studies, potentially reducing the numbers of animals used.

Why were they not suitable?

In vitro culture methods do not generate large numbers of genetically modified *Cryptosporidium* required for downstream laboratory work.

Mouse models are also required to study host-parasite interaction. This is complex and cannot be recapitulated sufficiently *in vitro*.

Mouse models are also an essential "filtering step" to advance candidate therapeutics for further evaluation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Because there is no simple way to continuously grow *Cryptosporidium* in the lab, we have to use mice to grow and keep colonies of genetically modified *Cryptosporidium* parasites.

We also use mouse models to study host-parasite interaction, infection patterns, and to test candidate therapeutic compounds.

Objective: Generate transgenic Cryptosporidium strains for downstream analysis in the laboratory. (3,000 mice on protocol 2)

The *Cryptosporidium* oocyst is the transmissible form of the parasite and is a hardy, shell-like structure that protects parasites while they are in the environment. During my previous licence we generate the first oocyst wall proteome, providing a list of genes involved in parasite transmission. To date we have characterised <20% of the genes that we hypothesise are involved in parasite transmission. There is much still to discover. For each transmission-related gene involved, we need to make several different parasite strains to investigate the role of this gene in parasite biology. We anticipate studying 10 transmission-related genes during this new licence.

10 transmission-related genes x 4 strains each = 40 new strains of *Cryptosporidium*

2 mice to generate a strain + 73 mice to generate enough parasites for downstream analysis in the laboratory = 75 mice per new strain of *Cryptosporidium*

40 new strains x 75 mice = 3,000 mice total on protocol 2

Objective: Define the function of genes in parasite transmission. (3,000 mice on protocol 2)

We are developing the next generation of genetic tools to study the function of transmission-related genes in more sophisticated ways. This involves inducible conditional expression systems and *in vivo* imaging of *Cryptosporidium.*

10 transmission-related genes x 4 strains each = 40 new strains of *Cryptosporidium*

40 new strains x 75 mice to investigate the function of the gene in transmission *in vivo* = 3,000 mice total on protocol 2

Objective: Identify genes required for host-parasite interaction. (950 mice on protocol 2)

Recently a screen of host genes required for *Cryptosporidium* infeciton was performed: Gibson AR, Sateriale A, Dumaine JE, Engiles JB, Pardy RD, Gullicksrud JA, O'Dea KM, Doench JG, Beiting DP, Hunter CA, Striepen B. A genetic screen identifies a protective type III interferon response to *Cryptosporidium* that requires TLR3 dependent recognition. PLoS Pathog. 2022 May 18;18(5):e1010003. doi: 10.1371/journal.ppat.1010003. PMID: 35584177; PMCID: PMC9154123.

Few of the genes identified in this screen have been characterised further. We anticipate investigating 5 host genes during this licence.

This involves making new strains of *Cryptosporidium*. These strains will be both reporter strains, and strains genetically modified for *Cryptosporidium* proteins that interact with the host gene:

5 host genes x 3 *Cryptosporidium* strains = 15 new *Cryptosporidium* strains

2 mice to generate a strain + 40 mice to generate enough parasites for downstream analysis = 42 mice per new strain of *Cryptosporidium*

15 strains x 42 mice per strain = 630 mice total

630 mice +70 mice for contingency = 700 mice total on protocol 2 We will also using mice that have genetically altered for these host genes:

5 host genes x 50 mice with genetic modifications related to the host gene = 250 mice on protocol 2

Objective: Test new candidate therapeutics for treating cryptosporidiosis. (1000 mice on protocol 3; 750 mice on protocol 4)

We anticipate testing 25 new candidate therapeutic compounds during this licence. When testing a new candidate therapeutic, we will start with the acute model of cryptosporidiosis (protocol 3) because the infection is very robust.

An initial study to determine if the compound has an any efficacy requires the following groups: a vehicle control (4 mice), treatment with a control compound that clears the infection (4 mice), and experimental treatment (4 mice per candidate therapeutic). For these initial efficacy experiments we require a minimum of 12 animals.

We expect that half of the candidate therapeutics will require follow-up work. Specifically, we will determine efficacy at a range of doses (typically 2-3) of the candidate therapeutic. These follow-up experiments can be expected to require a minimum of 20 animals (includes controls).

Once the lowest effective dose is identified, a final experiment to replicate the results and ensure reproducibility is required. This will require at minimum 12 mice (3 groups with 4 mice each: vehicle, control compound, minimum dose of experimental compound).

25 compounds for initial efficacy testing x 12 mice = 300 mice

12 compounds for follow-up studies x 20 mice for dosing range experiment = 240 mice 12 compounds for reproducibility experiments x 12 mice = 144 mice
316 mice for contingency (in case we test more than 25 compounds, or if more follow-up experiments are required)

300 + 240 + 144 +316 = 1000 mice on protocol 3

Compounds that demonstrate efficacy in the acute model of cryptosporidiosis (protocol 3) will also be tested using the chronic model of cryptosporidiosis (protocol 4). This model is more variable and so is typically performed once efficacy is demonstrated in the acute model. Due to the variability of this model, twice as many animals will be initially enrolled in this study. Mice that are not infected at high enough levels will be moved to protocol 2.

12 compounds for follow-up studies x 40 mice for dosing range experiment = 480 mice 12 compounds for reproducibility experiments x 12 mice = 144 mice
126 mice for contingency (in case we test more than 25 compounds, or if more follow-up experiments are required)
480 + 144 + 126 = 750 mice on protocol 4

Objective: Describe the mode of action of new anti-cryptosporidial therapeutics.
(1,050 mice on protocol 2)

We collaborate with the Mode of Action group at the University of Dundee to identify the target of anti- cryptosporidial compounds. Most anti-cryptosporidial compounds originate from phenotypic screens, and how they kill *Cryptosporidium* parasites is completely unknown. We have adopted technology for target identification for *Cryptosporidium*. We are now ready to deploy this technology to compounds with unknown targets and unknown modes of action. These target identification experiments do not require transgenic parasites.

However, once the target is identified, we require transgenic parasites to confirm on-target activity of the compound and to provide a deep understanding of the target-compound relationship. This greatly advances the impact of the drug discovery programme.

We anticipate working with 5 drug targets during this licence. Each target requires generation and propagation of 5 new strains of *Cryptosporidium*:

5 targets x 5 strains per target = 25 new *Cryptosporidium* strains

2 mice to generate a strain + 40 mice to generate enough parasites for downstream analysis = 42 mice per strain

25 strains x 42 mice per strain = 1,050 mice total

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have found that 2 mice (rather than 4) is sufficient to generate a new strain of genetically modified *Cryptosporidium*. This effectively reduces the number of mice we use for this purpose by 50%. We have not observed any changes to animal welfare associated with this reduction.

For production of genetically modified *Cryptosporidium*, we have decreased the number of animals in our cages from 6, to 4 or less animals. We can collect roughly the same amount of material from smaller group of mice due to our refined fecal collection protocols/SOPs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When we need to produce a large number of organisms we cull the animals at peak infection (we have previously determined the optimal infection level) and collect the contents of the small intestine. We can then purify a large number of organisms from the fecal samples shed at peak infection and from the contents of the small intestine. This approach minimises the length of study time and thus the overall severity that the animals experience, while maximising the scientific outputs.
We keep extensive records about infection patterns. Based on previous data, we plan studies to use the smallest number of animals required for the scientific purpose.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative

care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the acute and chronic mouse models of cryptosporidiosis (immune compromised mice). These models will allow us to efficiently propagate genetically modified *Cryptosporidium* for downstream laboratory analysis. These models will also allow us to observe infection patterns and test candidate anti-cryptosporidial therapeutics.

We will use natural mouse models of cryptosporidiosis (immune competent mice) to investigate host- parasite interaction.

Mouse cryptosporidiosis models are well established in the field, have predictable outcomes, and experience less suffering compared with other animal models of cryptosporidiosis (for example calves experience dehydration, diarrhoea, etc).

Why can’t you use animals that are less sentient?

Mice are the lowest animal that is capable of being infected with species of *Cryptosporidium* that are clinically relevant.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over the course of our last licence, we have refined our use of these models. To maximise amount of *Cryptosporidium* produced in this model and to minimise the amount of time animals experience discomfort, we often cull mice at peak infection. We closely track infection, cull mice at peak infection, and harvest intestinal tissue and collect the contents of the intestinal tissue. This greatly increases our yield of Cryptosporidium and limits the amount of time mice experience higher levels of infection and also means we use as few mice as possible.

We also have removed the need for surgical infection. Previously, parasites were directly implanted into the small intestine to avoid passage through the acidic environment of the stomach. By pre- treating mice with an oral solution that has a basic pH (is alkaline), we can neutralise the stomach acid. Then when we deliver an oral dose of parasites, the parasites survive transit through the stomach and survive until they reach the small intestine and can establish infection.

We have also established a monitoring schedule and welfare scoring system that allows us to intervene before animals experience adverse effects and well before humane endpoints are reached. We established this with input from the NVS and NACWO. We also provide high-calorie nutritional supplements to help prevent animals losing weight and so to mitigate against adverse effects.

We will continue to implement refinements to these models. We frequently discuss mouse models with other research groups employing these models and share methods for improving animal welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to information and guidance published on the appropriate NC3Rs website link:

Baumans V, Brain PF, Brugére H, Clausing P, Jeneskog T, and Perretta G (1994). Pain and distress in laboratory rodents and lagomorphs: Report of the Federation of European Laboratory Animal Science Associations (FELASA)

Working Group on Pain and Distress accepted by the FELASA Board of Management November 1992. Lab Animals 28(2), 97-112Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM and van de Vorstenbosch C (2001). A good practice guide to the administration of substances and removal of blood, including routes and volumes. J. Applied Tox., 21 (1), 15-23

Morton, DB and Hau J (2011) Chapter 18: Welfare assessment and humane endpoints. In Handbook of Laboratory Animal Science, 3rd Edition, Volume 1 Essential Principles and Practices. Hau J and Schapiro, SJ (eds), CRC Press LLC, USA, pp 535-572

Institute for Laboratory Animal Research (ILAR) (2008) Recognition and Alleviation of Distress in Laboratory Animals. Washington, DC: National Academies Press Institute for Laboratory Animal.

Research (ILAR) (2009) Recognition and Alleviation of Pain in Laboratory Animals. Washington, DC: National Academies Press. See http://dels.nas.edu/animal_pain/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Members of the group receive the e-mail newsletter from the NC3Rs (National Centre for Refinement, reduction and replacement) as well as information from the local animal users' group about best practices regarding the 3Rs. We regularly check the NC3Rs website for updated information and ideas to implement the 3Rs in our work. We have detailed group discussions about how to specifically implement the 3Rs in our work, including designing studies to maximise scientific output while reducing, refining, and replacing animals were possible. We attend local training and seek out advice from the Named Veterinary Surgeon (NVS) and the Named Animals Care and Welfare Officer (NACWO).

25. Preventing Contagious Ovine Digital Dermatitis: Characterisation of Bacterial Pathogens, Exploring Disease Transmission and Assessment of Diagnostic Tests

Project duration

5 years 0 months

Project purpose

Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Sheep, Lameness, Diagnosis, Transmission, Infection

Animal types	Life stages
Sheep	juvenile, adult, pregnant, aged
Cattle	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this study is to describe the bacterial pathogens associated with contagious ovine digital dermatitis in sheep; examine how the disease might spread within and between farms; and assess the vailidity of diagnostic tests for the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Contagious ovine digital dermatitis (CODD) is an infectious and severe cause of sheep lameness. It affects approximately half of UK sheep flocks, and is emerging globally. Due

to its' severity, CODD has a substantial and highly visible impact on sheep welfare. The economic consequences, and high levels of antibiotic use associated with disease control, negatively affect sustainability. In the 2021 Ruminant Health and Welfare Group survey of UK livestock farmers, CODD was the second top priority endemic disease for the livestock industry; whilst in a recent survey of UK vets and farmers, improved understanding of disease transmission and improved prevention methods were their top CODD research priorities.

What outputs do you think you will see at the end of this project?

The outputs will be new information on the causes, transmission and diagnosis of contagious ovine digital dermatitis in sheep.

Who or what will benefit from these outputs, and how?

By the end of the project we hope to have more fully understand the causes of CODD, how it it is spread within and between farms and assessed the validity of new diagnostic tests . This information can be used to inform early treatment interventions and help prevent disease spread between species and within and between farms . These will benefit sheep and cattle welfare, farm sustainability, farmers, veterinary surgeons, and other researchers in the field. In addition, the knowledge gained from the project will directly inform new treatment and vaccination strategies that are planned for cattle and sheep.

How will you look to maximise the outputs of this work?

The findings of the project will be communicated to sheep farmers and veterinarians through open access scientific publications, open access data sets and knowledge transfer materials and webinars.

Species and numbers of animals expected to be used

- Cattle: 100
- Sheep: 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying cattle and sheep of all life stages (apart from neonatal) as the diseases can affect animals of any life stage from juvenile onwards. By studying animals on farms we will ensure our findings will be relevant to the farm situation.

Typically, what will be done to an animal used in your project?

The sheep will be examined by a veterinary surgeon, have a blood sample and bacteriology samples collected; and thermal and photographic images taken . Any lame sheep will be treated as per the farmer's usual veterinary prescribed protocol. This procedure will typically take 15-30 minutes.

If there are any lame cattle on the farm they will also be examined by a veterinary surgeon. If they are diagnosed with bovine digital dermatitis they will have bacteriology samples collected. Any lame cattle will be treated as per the farmer's usual veterinary prescribed protocol. This procedure will typically take 15-30 minutes.

Each animal will remain on their farm of orgin at all times, be handled by trained and experienced staff, and will only experience this procedure once.

What are the expected impacts and/or adverse effects for the animals during your project?

The cattle and sheep will experience brief pain or discomfort during the sampling which will last 15-30 minutes. After the animals have been examined they will be given relevant treatment for their condition as prescribed by their own veterinary surgeon

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity is mild for all animals

What will happen to animals at the end of this project?

- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We wish to take bacteriology and blood tests samples from farm animals affected by contagious ovine digital dermatitis and bovine digital dermatitis and also sample their farm environment. It is important to do this in naturally infected sheep and in the farm environment so that the findings of the study are relevant to the real world situation.

Which non-animal alternatives did you consider for use in this project?
There are no non-animal alternatives to this.

Why were they not suitable?

There are no non-animal alternatives to this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals used in the study are based on advice from a statistician and experience gained from our previous studies on contagious ovine digital dermatitis in sheep and digital dermatitis in cattle. We estimate 10 animals per foot disease type per farm. For sheep this means 10 healthy control animals , 10 affected by interdigital dermatitis, 10 affected by footrot and 10 affected by CODD. For cattle we will require a maximum of 10 cattle digital dermatitis cases from farms that have sheep and cattle. We will study disease on 10 farms affected by CODD in sheep (case farms) and 10 farms unaffected by CODD in sheep (control farms).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used online sample size calculators (www.open epi.com) and advice from a statistician. We also referred to previous published research and our experience of the different disease presentations we see on farms.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For some parts of this project we have archived tissues samples we are able to use to answer some of the research questions. We will continually monitor the study as it progress to ensure only required number of samples are collected.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will study disease in naturally occurring affected sheep and cattle. We will collect the minimum number of samples and minimally invasive samples from these sheep and cattle. Sampling will be brief (approximately 15-30 minutes). Local anaesthetic and pain relief will be used where appropriate. All diseased animals will receive appropriate treatment for the disease. All animals will remain on the farm of origin.

Why can’t you use animals that are less sentient?

There are no animal models for this disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will study disease in naturally occurring affected sheep and cattle. We will collect the minimum number of samples and minimally invasive samples from these sheep and cattle. Sampling will be brief (approximately 15-30 minutes). Local anaesthetic and pain relief will be used where appropriate. All diseased animals will receive appropriate treatment for the disease. All animals will remain on the farm of origin.

The study will be supervised at all times by an experienced farm animal veterinary surgeon.

All staff involved in farm sampling will be experienced in handling animals and trained personal licence holders.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE guidelines will be used for study design and reporting https://arriveguidelines.org/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project licence holder will stay informed about advances in 3R's through engagement with the National Centre for Replacement Reduction and Refinement of Animals in Research Website and through seminars and information disseminated through the research institution where the project licence is held.

26. Role of Innate Immune Cells in Initiation of Inflammatory Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

macrophages, inflammation, inflammasome, innate sensing and signalling

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this research is to understand how innate immune cells such as macrophages initiate the inflammatory response, and when does this response lead to the inflammatory disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The purpose of the inflammatory response is to restore tissue homeostasis. In infection, inflammation aims to eliminate the insult and induce protective immunity, while inflammation triggered by non- infectious activators aims to limit the damage and enable tissue repair. If inflammatory response is excessive, mistargeted or prolonged, it drives the development of inflammatory diseases, such as colitis or arthritis, which present a major

health, social and economic burden.

What outputs do you think you will see at the end of this project?

The key short-term benefit of this proposal is to generate new knowledge about how inflammatory responses are initiated. Therefore, in first instance, scientific community will have the most benefit of the work proposed in this application.

Because we also propose to validate our key findings using mouse model of inflammatory bowel disease (IBD) and arthritis, the long-term benefit of this work is potential translation of the new knowledge into the therapy development.

Finally, because we will also validate key findings in an acute model of peritoneal inflammation and systemic infection, another long-term benefit of this work is the translation of the key findings to other inflammatory diseases as well.

Who or what will benefit from these outputs, and how?

In short-term, scientific community will have the most benefit of the new knowledge generated in the proposed study. In long term, patients with inflammatory diseases, such as IBD or arthritis, will benefit as well.

Short-term benefits will be achievable in the 5 years of the duration of this project. Long-term benefits, (translation to potential therapies) will require 10 -20 years.

How will you look to maximise the outputs of this work?

Outputs of this study will be disseminated through publication and presentations at national and international meetings, as we have done in the past.

Species and numbers of animals expected to be used

- Mice: 22,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the lowest vertebrate groups on which well-established models of inflammatory diseases of interest have been developed. Furthermore, they show high homology to human immune system.

Lower organisms do not have an adaptive immune system, and many do not have joints and therefore cannot be used for our studies. Finally, there are more available reagents (e.g. monoclonal antibodies, genetically modified strains) in mice then in other species required for this project. Mice also offer unique genetic modifications generated by others or us, which are essential for the work proposed. To ensure proper development of the immune system, life stage used in all experimental protocols is adult.

Typically, what will be done to an animal used in your project?

Breeding and maintenance. Some animals will be used for breeding and maintenance of genetically modified lines.

In vivo infection and immunisation experiments. We will be administering, via injection or oral routes, infectious agents such as Listeria monocytogenes or Candida albicans, or non-infectious agents, that result in the activation of the innate immune system and acute inflammation.

In vivo colitis experiments. We will be administering, via injection or oral routes, infectious agents such as Citrobacter Rodentium or Salmonella Typhimurium, or non-infectious agents, that result in the activation of the innate immune system and gut inflammation.

In vivo arthritis experiments. We will be administering, via injection, preformed auto-antibodies or antigens that will induce auto-antibodies, that result in the activation of the innate immune system and chronic joint inflammation.

The mice will be humanely killed at the end of the experiment or if humane end points have been reached.

What are the expected impacts and/or adverse effects for the animals during your project?

Breeding and maintenance. Mice are not expected to experience clinical adverse effects.

In vivo infection and immunisation experiments. Infections are non-lethal, but mice typically develop symptoms such as reduced grooming and motility, reduced food and water intake and weight loss. Mice will be humanely killed if the clinical effects cannot be ameliorated.

In vivo colitis experiments. These mice will typically develop temporary weight loss, intestinal inflammation and diarrhea that is either self-limiting or resolves spontaneously. Experiments will be terminated, and mice will be humanely killed if the clinical effects cannot be ameliorated.

In vivo arthritis experiments. Mice will experience swelling of the joints, reduced movement and pain, which will be controlled by the provision of food pellets and water gel packs placed at floor level, and/or supplemental bedding and by the administration of adequate analgesia. Mice will be humanely killed if the clinical effects cannot be ameliorated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity of the experimental protocols in this project is 'moderate'. 35% of mice will be on sub-threshold, 30% on mild, 35% on moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand how inflammatory mediators and signalling pathways affect inflammatory disease progression, particularly in chronic inflammation where multiple organs and cell types are involved, where cell-cell communication and the 3D tissue architecture are important, studies require the use of whole organisms and cannot be to date replicated in vitro. These studies also require the use of animal models of inflammation and the use of genetically altered animals, such as animals lacking key inflammatory sensors and mediators. Mice are the lowest vertebrate groups on which well-established models of inflammatory diseases of interest have been developed. Furthermore, they show high homology to human immune system. Finally, there are more available reagents (e.g. monoclonal antibodies, genetically modified strains) in mice then in other species required for this project. Mice also offer unique genetic modifications generated by others or us, which are essential for the work proposed.

Which non-animal alternatives did you consider for use in this project?

To identify relevant inflammatory mediators and pathways we will wherever possible use in vitro experiments or ex vivo models to replace or complement in vivo studies. We will also perform in vitro work on primary human cells to select those inflammatory pathways and mediators that are conserved between mice and humans for our future studies.

Why were they not suitable?

Lower organisms do not have an adaptive immune system, and many do not have knee joints and therefore cannot be used for our studies. Finally, there are more available reagents (e.g. monoclonal antibodies, genetically modified strains) in mice then in other species required for this project. Mice also offer unique genetic modifications generated by others or us, which are essential for the work proposed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Using our own historical data combined with good experimental design and statistical calculations, we estimated animal numbers required to reach our objectives. We have also included numbers to allow for the growth in researchers and in the number of experiments that are planned.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Appropriate use of statistics and careful experimental design. When performing new assays, we will use pilot experiments to determine intra and inter group variations. From there, main statistical principles governing the design of an animal experiment will guide (1) defining an experimental unit; (2) controlling variation; (3) designing experimental layout and (4) calculating the sample size.

Randomisation and blinding. To minimize the bias in in vitro experiments we will blind the genotype of the mice until the end of the experiment. To minimize cage effect and the effect of micro flora on in vivo experiments we will cohouse non genetically altered and genetically altered mice and randomly assign mice to treatment conditions.

Because males and females are cohoused separately, we will also be able to evaluate the effect of sex on the phenotype. For histology scoring we will blind slides and have two independent people from the laboratory of our collaborators score them. For complex experiments, we may use the Experimental Design Assistant tool from NC3R.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Maximize information obtained for each experiment. Immune cells isolated from secondary lymphoid organs or organs isolated from a genetically modified animal may be used as a source of immune cells for in vivo and in vitro experiments. Cells and tissues may be shared by multiple researchers or frozen for future analysis.

Mouse colony management. Following published guidelines in best practice for the management of breeders, we will keep careful documentation of the numbers and use electronic database that will provide breeding data to inform the breeding calculations to ensure that we only breed animals for which there is a scientific need. We will also cryopreserve lines not immediately required.

Use of in vitro and ex vivo models. Whenever possible, we will use cells and tissues from untreated mice for in vitro assays. For example, we will routinely obtain bone marrow from control animals from our own work or from other investigators after humane killing to obtain precursors and differentiate into dendritic cells and macrophages for vitro assays.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Inflammatory disease models (immunization, infection, colitis, arthritis) have been chosen as most relevant for our objectives because innate inflammatory pathways that our group studies in macrophages have been linked to those diseases through human studies and through previous published work in mice. We have generated or obtained mice deficient in these key signalling molecules, which will allow us to assess their contribution to the

disease development and potential use as therapeutic targets in vivo. We have then chosen models of inflammatory disease that can be induced by chemical methods removing the need to perform surgery or breed animals with these responses keeping pain, suffering or lasting harm to a minimum and over a shorter period. We have also chosen models which closely mimic features of human disease in the treated animals, and that have already been refined in the literature to minimize the number of experimental treatments and the severity level. We are also collaborating with experts in the field to acquire new expertize in the most refined methods for models of immunization, infection, colitis and arthritis.

Why can't you use animals that are less sentient?

Mice are the lowest vertebrate groups on which well-established models of inflammatory diseases of interest have been developed. And there are more available reagents for mice (e.g. monoclonal antibodies, genetically modified strains needed for this project) then other species. Lower organisms do not have an adaptive immune system, many do not have knee joints, and therefore cannot be used for our studies. We need to use live animals over time, as our disease models often involve resolution and recovery phase as well, and can not be recapitulated in terminally anesthetized animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will make every effort to minimize the number of procedures per animal. We will pay careful attention to animal husbandry and provide environmental enrichment and co-housing to avoid social isolation. We are already using non-aversive method of handling, such as tunnel handling. When possible, we will use mice where gene is deleted only in one cell type, or only transiently, rather than in whole organism so that the harms are limited to only the experimental phase. For all dosing, the smallest needle diameter which allows rapid injection of the substance will be used, the smallest possible volume of substance will be administered, and aseptic technique will be used.

When possible, we will use the least painful injection method. For instance, we have switched from foot-pad to sub-cutaneous injection under the skin next to the hock, which is less painful for the mice as they do not have to walk on the injected, swollen area. Where required (e.g. in arthritis experiments), analgesia will be administered from the expected time of disease onset.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the latest scientific literature (Pubmed) and scientific guidelines (LASA guidelines, NC3R guidelines, ARRIVE guidelines) to ensure experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow the latest scientific literature and also get informed about the latest guidance from NC3R (e.g. by attending internal welfare meetings, the annual 3R's seminar, liaison with the Named Information officer and the regional 3R's manager.) to ensure experiments are conducted in the most refined way. We will continue to collaborate with experts in the field to acquire new expertise and training in the most refined methods for our disease

models (immunization, infection, colitis and arthritis).

27. Role of the Brain in the Regulation of Body Metabolism

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

food intake, obesity, hunger, brain, body metabolism

Animal types	Life stages
Mice	adult, juvenile, neonate, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This work aims at investigating the mechanisms utilised by the brain to regulate hunger, and how obesity may arise when these mechanisms are disrupted.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Food intake is clearly critical for health and survival, yet we do not fully understand how the brain regulates this behaviour. Moreover, excessive food intake often leads to health complications such as obesity and diabetes, as evidenced by the current obesity crisis that many societies are currently experiencing, and while brain dysfunction seems to contribute

towards the development of such complications, we do not know the details of how that may happen. Thus, this work is important to help us understand some of the brain mechanisms that govern healthy eating behaviour, and how these mechanisms may lead to disease when they fail.

What outputs do you think you will see at the end of this project?

This is a basic science project, which means that our main objective is to increase our knowledge. In consequence, the outputs that we can expect are those related to the dissemination of this new information, such as (freely-accessible) publications, and participation in scientific conferences and science-related public events.

Who or what will benefit from these outputs, and how?

The short-term benefit of this project will mainly be seen in the academic environment: this project will have an immediate impact among colleagues interested in studying brain control of eating behaviour, and in particular those studying how brain cells integrate different sources of information in order to maintain energy balance. In the medium and longer terms, this work will add to our knowledge of how metabolic diseases such as obesity and diabetes originate, and it may contribute towards a better understanding and management of such diseases.

How will you look to maximise the outputs of this work?

We will publish our results, regardless of whether the findings align or not with our initial hypotheses. Moreover, we will disseminate these results in freely-accessible formats (for example, bioRxiv) to ensure that anyone interested have access. We also aim at collaborating with colleagues in the field, and share openly our results with them by participating in scientific meetings and similar exchanges.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

At the core of this project is the ability to observe and manipulate specific cells in the brain in order to investigate the links between these cells and metabolism regulation. It is not possible (or ethical) to do this in humans; thus, we need to use an experimental model, and mice are ideal because their physiology, like that of other mammals, is very similar to human physiology, especially when it comes to food intake. Humans share over 85% of their genome with rodents. In addition, there is already a very large body of knowledge acquired using mice, and that is an advantage because we can add to that body of knowledge instead of starting from zero (as it would be the case if we were to use alternative animal models).

Typically, what will be done to an animal used in your project?

We will use genetically-modified mice, bred and maintained at our animal facilities. We will perform brain surgeries to inject a very small volume of substances into the brain in order to label or tag dedicated groups of cells in this organ. Often, cannulae will also be implanted into the brain during the procedure.

The mice will be observed and tested to evaluate some aspects of their metabolism. For example, we will measure food and liquid intake and preference, body weight, nutrient levels in the blood, or physical activity in these mice. We may in some cases modify their diet to measure the effects of diet composition on metabolism. We may combine some of these behavioural observations with measurements of cell activity in the brain, or with cell activity manipulation, which will be possible thanks to the surgical procedures described above. These behavioural/metabolic experiments will be conducted for up to three months.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgical intervention tends to cause a degree of pain, which we will manage with pain relief. The animals fully recover from the brain surgery and anaesthesia within 4-6 days; no changes in behaviour or physiology are anticipated from the surgical intervention.

After the animals recover from surgery we will test several metabolic parameters. Some of these tests will include changes in the diet and/or manipulation of some cells in the brain which may lead to gain or loss of body weight. We also expect to see alterations in feeding patterns (e.g. eating at times of day when mice do not normally eat). These changes may last for the full duration of the experimental tests.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity expected for mice that undergo surgery (20%) is moderate. Severity for all other mice (80%) is expected to be mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims at investigating how the brain coordinates behaviour (food intake) and metabolism (body weight) to maintain health. It is not possible to assess these links using isolated cell lines or computer models, as neither are capable of simulating or replicating the physiological environment.

Which non-animal alternatives did you consider for use in this project?

In principle, it should be possible to gain insight into complex mammalian behaviours using computer models specifically constructed for this purpose; however, no such model exists yet.

Why were they not suitable?

We do not yet have computer models sophisticated enough to be able to simulate complex mammalian behaviours and metabolism. Moreover, to be plausible, computer models must be informed by the rules of biology: we do need to gather biological knowledge via experimentation if we ever want to build useful computer models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required for the experiments was calculated based on past experience, as I have already done work similar to the one described in this project. The number of animals needed for breeding purposes was calculated with the help of the staff at our animal breeding facility, and are also based on experience and best practices.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I designed experiments with the aid of statistical tools (R software, https://www.r-project.org/, with package pwr, https://cran.r-project.org/package=pwr) to estimate the minimal useful number of animals. Also, to minimise the number of animals needed while maximising statistical validity, whenever possible the animals will be their own controls.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding practices will be as efficient as practically possible in order to minimise the number of experimental animals. We will also use tissue taken post-mortem for ex vivo/in vitro analysis as often as possible; this is to maximise the amount of useful experimental data obtained from each animal. In addition, pilot studies will be conducted if necessary to ensure that we can plan experiments in the best possible way.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime

of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice, both wild-type and transgenic. None of our transgenic models are any different physiologically or behaviourally from wild-type mice; thus, the genotype will not cause any suffering, distress, or harm.

Our methods involve brain surgery. Such surgical intervention is the only method currently available to achieve the labelling and manipulation of brain cells required. Thus, while no known alternatives to this method exist, we will ensure that our technique is as refined as possible, as explained below.

In addition to surgery, our methods also involve behavioural analysis in our mice. These methods are based on observing natural behaviours (e.g. food intake) and are thus unlikely to cause anything more than transient distress from e.g. handling. Some of these mice will have an implant in their head, used to manipulate or monitor brain cell activity. There are no known alternatives to using these implants, but they are not painful and do not cause any harm.

We will administer drugs by injection. This is the most effective way to give a drug to mice; despite the needle, this method minimises handling and ensures the correct drug dosage. In some cases we will take blood samples to measure e.g. glucose levels. This again is unavoidable; no alternatives exist for measuring metabolites in blood or plasma. The expected distress is only transient.

Some interventions are expected to cause obesity in our experimental animals, but this is not expected to be severe: the degree of obesity will not cause pain or suffering.

Why can’t you use animals that are less sentient?

We want to study brain involvement in behaviour and metabolism in mammals. This is only possible with freely-moving, awake animals; terminally-anaesthetised animals are not an option.

Non-mammalian species do not share the brain structures that are relevant for food intake regulation in mammals. These structures are an important part of the object of study in this project. This makes it impossible to use other, less sentient species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use tubes to handle animals.

Animals that undergo surgical procedures are likely to experience a degree of post-operative pain or discomfort. This will be managed with appropriate perioperative care including aseptic technique and pain relief. All animals are expected to fully recover from surgery within a few days. Following the surgery, the animals will be checked on a daily basis to ascertain their recovery. Additional post- operative care will involve placing wet food on the floor, to facilitate food consumption.

For our behavioural experiments, we will habituate the animals to their new, temporary environment (e.g. the cages where food intake is measured). We will also handle these animals regularly to habituate them to the interaction with the experimenter, and we will use tube handling and cupping to minimise distress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For general guidance, we will consult the resources page on the N3CRs website (https://www.nc3rs.org.uk/3rs-resources) and the related videos available from https://researchanimaltraining.com/article-categories/procedures-with-care/.

For surgery, we will follow the Guiding Principles for Preparing for and Undertaking Aseptic Surgery, published by the Laboratory Animal Science Association (LASA), http://www.lasa.co.uk/publications/.

For planning and reporting experiments, we will follow the ARRIVE (https://arriveguidelines.org/) and the PREPARE (https://norecopa.no/prepare) Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will make sure that I and my team keep up-to-date by following the Latest News on the NC3Rs website (https://nc3rs.org.uk). Any advances relevant to the project will be discussed with the Named Persons at our University in order to implement these promptly and effectively.

28. Self-Renewal Regulation in Human Hemopoietic Stem Cells

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Human haematopoietic stem cells, Gene Regulation, Stem Cell self-renewal, Stem cell niche, Organoids

Animal types	Life stages
Mice	embryo, juvenile, adult, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Haematopoietic stem cells (HSC) are a life-saving therapy for leukaemia and have recently found broadened applications to gene and cell therapy, against other cancers, genetic, autoimmune, and infectious diseases. Nonetheless, practical limitations render these therapies unfeasible or unsuccessful for many patients.

To overcome these limitations, we aim to identify the molecular determinants for human HSC function. By targeting these factors, we seek to understand the stemness network of HSC and to improve human HSCs culture in a dish.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project will focus on improving generation and maintenance of HSC through regulation of gene expression and modulation of the stem cell microenvironment. Control over the self-renewal machinery will constitute an essential step for generating patient-specific HSC. By gaining a better understanding of HSC biology, our studies will contribute to the development of successful novel HSC therapies.

Ultimately, our findings will expand the knowledge of the self-renewal process in HSC and to provide new insights into the molecular determinants of stem cell function, with implications for other stem cell fields and cancer biology.

What outputs do you think you will see at the end of this project?

The aim of this project is to identify novel molecular determinants of human hematopoietic stem cell (HSC) nature and evaluate their impact on their maintenance and function through regulation of gene expression. These findings will be constantly disseminated in scientific conferences and ultimately will give rise to scientific publications. I expect the output of this project to contribute to the stem cell as well as to gene therapy fields, therefore providing new opportunities for further collaborations.

Who or what will benefit from these outputs, and how?

The demand of haematopoietic stem cells for transplantation in leukaemic patients, as well as for blood, autoimmune and infectious disease is constantly increasing. Yet, technical hurdles of expanding HSCs for allotransplantation as well as personalized gene-therapy limited their applications.

Our findings will contribute to the stem cell field in short term, by improving the expansion of HSC in culture for further functional screening.

As long-lasting goal, we aim to recapitulate the HSC maturation in 3D-culture, which will open new avenues for the biomedical and bioengineering research for stem cell culture, and ultimately for novel HSC therapies.

How will you look to maximise the outputs of this work?

During the 5 years of the project, all scientists involved will disseminate their research findings in regular institute-based meetings, as well as in national and international conference. As member of the ISSCR (International Society for Stem Cell Research) and ISEH (International Society of Experimental Haematology), I annually attend and present my scientific findings at both conferences and intend to continue to do so. I also plan to exchange ongoing results with our national and international collaborators on regular basis. Where possible, scientific manuscripts will be submitted for open access in pre-print repository, allowing their dissemination within the scientific community

Species and numbers of animals expected to be used

- Mice: We estimate to use 1000 mice over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures,

including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

We will use established immunodeficient mouse lines that are permissive to human cell transplantation and engraftment. Transplantation of human haematopoietic stem and progenitor cells in immunodeficient mice represents the gold standard to test the function of HSC.

For testing the functionality of HSC in transplantation assay, engraftment ability will be evaluated in young to adult mice.

Typically, what will be done to an animal used in your project?

For all the studies within this project, immunodeficient mice will be used as recipient of human haematopoietic stem cells, which will be typically delivered through injection the bloodstream, either in the tail vein or in the retro-orbital sinus. Animals of the desired immunodeficient genotype will be either bred in house or purchased at the desired age (6-10 weeks, as this is optimal for evaluating human engraftment up to 24 weeks post-transplantation) for usage in the experimental protocols as needed. For some proof-of-concepts experiments HSC will be delivered by in utero injection (into the foetal liver) or by implanting human ossicle (human bone-like structures grown under the mouse skin) subcutaneously or by implanting 3D structures / organoids under the kidney capsule or subcutaneously.

In all cases, to monitor the transplantation outcome (engraftment), blood or bone marrow will be collected at regular time intervals (not more frequent than every 6 weeks). To evaluate and quantify the engraftment ability of the transplanted cells, the recipient mice will be humanly killed, typically after 24 weeks for organ collection, and tissue will undergo molecular characterization and single cell analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The mice will receive intravenous injection of purified human cells, which is typically associated to no adverse effect. Injection in the retro-orbital sinus is associated to no adverse effect and justified by the proven superiority of this technique as an administration route of normal/non-cancer cells, particularly in the setting of bone-marrow transplant. As the cells injected are normal (non-transformed) primary cells, the reconstitution of the hematopoietic system is expected to be uneventful. The possibility of the insurgence of graft-vs-host disease will be minimized by excluding T cells from the transplanted material through FACS sorting. In certain cases, pre-conditioning of the mouse with sub-lethal irradiation or chemotherapy (busulfan) may be needed prior to the engraftment. This procedure is expected to generate a small degree of radiation or chemotherapy-associated toxicity which is expected to resolve in 7-10 days (moderate severity). In this case weight, hydration and overall aspect will be monitored closely and the mice will be sacrificed if the appropriate endpoints reached. Mice will undergo bone marrow aspiration (performed under general anaesthesia through needle insertion in the femur), and blood collection at different time during the experiment (spaced out by at least 6 weeks).

To facilitate human blood stem cells engraftment in a human environment, some mice will be inoculated with human bone marrow mesenchymal stem cells under the back skin. These cells will grow and form a 3D structure that resembles a small human bone with a bone marrow cavity (ossicle), recapitulating the natural human HSC niche. The procedure

is not associated with any adverse events for the mouse.
To investigate the maturation of HSC during embryonic development, embryos will be transplanted with human HSC by injection into the foetal liver. This procedure is not associated with adverse effect, will be performed under anaesthesia, and in case of post-operation complications (infections which will result in wound dehiscence) the pregnant mouse will be humanly killed.

To evaluate the functionality of HSC-organoids, these 3D-structure will be implanted under the kidney capsule, a standard procedure for implanting tissues / organoids to facilitate the vascularisation of the transplanted element. This procedure (performed under general anaesthesia) does not produce adverse effect. Both surgeries will follow the recommended general constrains, e.g. peri and post- operation analgesia will be provided and not fully recovered animals within 24 hours post-surgery will be humanly killed.

At the end of all adoptive transfer experiments, set typically at 24 weeks, the mice will be humanly killed with a schedule 1 method and the tissues will be collected post-mortem for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% of the animals will be used for intravenous transplantation (mild severity); 10% of the animals will be used for intravenous transplantation after conditioning (moderate severity), 10% for breeding and maintenance (mild severity), 10% for kidney subcapsular implantation (moderate severity), 10% for in utero foetal liver transplantation (moderate severity) and 10% for humanized scaffold implantation (moderate severity).

All transplanted animals will be monitored for engraftment ability, by sampling blood (mild severity) or bone marrow (moderate severity) at interval spacing at least 6 weeks and will be humanly killed typically within 24 weeks post-transplantation.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The main function of blood stem cell is to populate the hematopoietic system (blood and immune reconstitution) for a lifetime, and this effect can only be observed and studied in living organism. We will use genetic altered immunodeficient mice, as they are the only mice in which transplantation of human cells can be performed. Using mouse models as recipient in transplantation assays is required to demonstrate the engraftment ability of human haematopoietic stem cells.

Which non-animal alternatives did you consider for use in this project?

We considered to use in vitro culture of HSC, and we are currently using in vitro alternatives when possible.
We considered to use in vitro protocols for HSC differentiation from iPSC cells, which we are currently optimising.

Why were they not suitable?

To date, options to maintain HSC in culture without perturbing their self-renewal property are technically limited. Using the current protocols is not possible to investigate the mechanism of the stemness of HSC to obtain a functional readout as the engraftment ability, for which immunodeficient mice are required.

The stem cell research field is actively and extensively investigating how to generate functional HSC from iPSC. However, these attempts so far failed to establish a working protocol.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We carefully revised the research projects, currently funded or pending approval, which we aim to conduct in the next 5 years. As the immunodeficient mice will be obtained by inbreeding (no genotyping assessment required), we will be able to use 100% of the progeny. According to the expectation of every single experiment, we calculate the exact number of animals we need to analyse, using, when appropriate, software such as the NC3Rs' Experimental Design Assistant.

For this calculation we consider technical and biological replicates: for example, for testing two experimental conditions (test and control) of engineered HSCs, we count a transplantation in at least 3 recipients (technical replicates), for 3 independent times (biological replicates) per condition, for a total of 18 mice.

We considered that not all tested conditions will provide an improvement in HSC function; in these cases, no secondary transplant will be performed. We aim to coordinate multiple experiments with the same control condition.

On the calculated number, we added 10% for counting of rare loss of animals during maintenance or mild procedure, given the immunodeficient background of the mice.

We added 15% of animals for procedure classified as moderate. Upon that, 10% additional animals were also counted in the case of embryonic injection, as it is not possible to predict the litter size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Most of the protocols (established and novel ones) for engineering human HSC in vitro will require less than 6 weeks. As the most recurrent experiment include transplantation of these cells in 8 weeks mouse recipient, we will plan the preparation of human cells to transplant after a litter will be born and close to weaning, aiming not to waste any animal.

As we aim to investigate the mechanistic function of several genes, we will pool experiment which share the control group.

Barcoding the HSC as well as the HSC-organoids will allow to implant multiple elements per mouse recipient in the humanized scaffold implantation or renal subcapsular transplantation procedures, respectively.

The most effective technique to transplant human non-cancerogenic HSC intravenously, retro-orbital injection under general anaesthesia will be performed whenever possible to reduce the number of animals in each experimental group. This will help reducing the number of animals that will need to undergo the procedures and then removed from the experiment due to procedural failure.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When possible, we will purchase mice from an established breeder. This strategy will avoid both unnecessary breeding or separating breeders for long time, resulting in male breeders' isolation. As the strains are immunodeficient, intermittent breeding with wild-type or other strains is not feasible.

Regarding the breeding and maintenance of mouse strains which are not commercially available, if possible, we will outsource the breeding to an in-house breeding facility. This will allow to share the strain with other users without unnecessary breeding. Should this option be unavailable, we will minimize the breeding by synchronizing the experiments with the breeding performances: after weaning the litters, we will scale accordingly the experiment to be performed.

For timed breeding, required for the in-utero transplantation procedure, an intermittent breeding will be set using the same male breeders between time mating and the breeding for maintain the strain. This will avoid constant breeding, therefore improving breeding performance as well as reducing the breeders number requirement.

By implanting multiple humanized scaffolds (subcutaneously) or HSC-organoids (renal subcapsular transplantation) we will drastically reduce the number of mice undergoing surgery procedure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetic altered immunodeficient mice as the optimal model in which transplantation of human cells can be assayed.
Using mouse models as recipient in transplantation assays is required to demonstrate the engraftment ability of human haematopoietic stem cells.

Why can't you use animals that are less sentient?

We aim to assess the engraftment ability of human HSC (non-cancerogenic) minimizing the age of the animals involved. Typically, it will be span a 36 week timeframe: we aim to transplant mice within 12 weeks of age, and to analyse the outcome of the transplant 24 weeks after. As the readout for human HSC function is the long-term engraftment, it is required to use a living animal model for the indicated time span.

Regarding the transplantation in utero, animals will be analysed at 24 weeks of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Optimized husbandry conditions will be employed (e.g., acclimatising animals to restraint tube in the home cage or proper amount of bedding material to avoid frequent change of the home cage).

Animals used in this protocol are not expected to exhibit any significant harmful phenotype although, due to an impaired immune system, they may be susceptible to infections not affecting normal mice. Mice will be kept in pathogen free status within barrier systems to protect them from intercurrent infections.

Animals undergoing surgeries will be provided with post-operative supportive care (e.g., providing analgesic, facilitating access to food providing foodgel and hydrogel on the cage floor, providing hydration if needed, ...).

For the breeding, there will be no need to determine the genetic status, avoiding tissue biopsy.

For the adoptive transfer, retro-orbital injection of non-cancerogenic cells is considered, given the proven superiority of this technique as an administration route of cells, particularly in the setting of bone-marrow transplant. In this setting, compared to caudal vein injection, retro-orbital injection has been associated with reduced of stress for the anesthetized animal.

Following transplantation, to minimise pain and distress, ear punch will be carried to identify animals. When possible, a previous barcoding of the human cells will be performed; in this case, no ear punching will be required.

To follow the engraftment, blood and bone marrow sampling will be performed. The interval between sampling will be maximized to reduce the frequency of this procedure, in order to reduce the distress and pain.

We aim to use a novel immunodeficient strain (NSGW41), which allows transplantation of human cells without conditioning (myeloablation by radiation or busulfan treatment).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For general guidelines (husbandry, basic procedures for substance administration, intravenous cell delivery, blood sampling, ...), guidelines from nc3rs.org.uk will be followed.

For surgical procedures, LASA guidelines for the application of aseptic techniques during surgical procedures will be followed.

For implantation under the renal capsule, the following published protocol will be followed:

Renal Subcapsular Transplantation of PSC-Derived Kidney Organoids Induces Neo-vasculogenesis and Significant Glomerular and Tubular Maturation In Vivo (2018)

https://doi.org/10.1016/j.stemcr.2018.01.041

Mouse Surgery – Transplantation of human thymus into the kidney capsule of NSG mice (2021) dx.doi.org/10.17504/protocols.io.bvuen6te
A Method for Murine Islet Isolation and Subcapsular Kidney Transplantation (2011) http://www.jove.com/video/2096
For the implantation of humanized scaffold:

Engineered humanized bone organs maintain human hematopoiesis in vivo (2018) https://doi.org/10.1016/j.exphem.2018.01.004
reviewed in: Engineering human hematopoietic environments through ossicle and bioreactor technologies exploitation (2021)

https://doi.org/10.1016/j.exphem.2020.11.008 For the intrauterin injection:
A mouse model of in utero transplantation (2011) https://www.jove.com/v/2303/a-mouse-model-of-in-utero-transplantation
Systemic multilineage engraftment in mice after in utero transplantation with human hematopoietic stem cells (2018)

DOI 10.1182/bloodadvances.2017011585 For the retro-orbital injection:
Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice.

DOI 10.1177/0023677214567915

Busulfan Administration Flexibility Increases the Applicability of Scid Repopulating Cell Assay in NSG Mouse Model DOI 10.1371/journal.pone.0074361

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will constantly look for novel mouse models to minimize our animal use either in number of animals or in the time of analysis. It might include novel animal models as well as novel in vitro protocols. All users involved in this project will be strongly recommended to get informed with the 3R resource library in the nc3rs.org.uk and tutorials in the affiliated websites (EDA, ARRIVE…). To stay informed beyond the guidance websites (e.g.,

nc3rs.org.uk, arriveguidelines.org, etc), I will communicate with the animal facility operators to get updated of new procedures / husbandry workflows, as well as with the scientific community (with scientific collaborators and in scientific conferences / symposia / meetings where unpublished results and methods could be discussed). Moreover, I will keep updated with the literature online regarding the mouse models / alternative models I could use.

29. Skeletal Homeostasis, Remodelling and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Arthritis, injury, skeletal joints, repair, therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this research is to obtain new knowledge on the mechanisms of skeletal joint maintenance and repair, and how this is affected in arthritis, with the ultimate aim to develop new therapies for skeletal joint injury and arthritis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Arthritis is a leading cause of disability, affecting over 9 million people in the UK, and its total costs have been estimated to be over £30 billion annually in the UK. The high prevalence and heavy impact on working capacity make arthritis a huge problem for the society. Cartilage breakdown is the irreversible outcome of nearly all forms of arthritis. For osteoarthritis, the most common joint disease affecting over 8 million people in the UK,

current treatment options are limited to symptom relief with pain-killers and to prosthetic joint replacement in end-stage disease. At present, we do not have any means to slow down or prevent cartilage breakdown in arthritis or to regenerate damaged cartilage.

What outputs do you think you will see at the end of this project?

This project is expected to generate new information about the mechanisms of skeletal joint maintenance and repair, and how this is affected in arthritis. We anticipate that this will uncover new therapeutic targets to slow down or halt progression of joint damage in arthritis and promote repair of damaged joints. The findings from this project will be published in scientific journals and presented at scientific conferences.

Who or what will benefit from these outputs, and how?

In the short term (<5 years), other researchers in academia and industry will benefit from the new information obtained in this project. In the longer term (>5 years), patients with joint injury or arthritis will benefit from new therapies that are developed based on the information obtained. In addition, horses and some breeds of dogs suffer from disabling cartilage loss and arthritis. Thus cartilage repair is also eagerly pursued in veterinary medicine.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through collaboration with scientists at other institutions, dissemination of findings via publications in scientific journals and presentations at scientific conferences, and via engagement and collaboration with industry. We will also share information regarding unsuccessful approaches via collaborative networks and inclusion in scientific publications, where appropriate.

Species and numbers of animals expected to be used

- Mice: An estimated 3000 mice will be used in this project.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice in this project because they have skeletal joints that are similar in structure and function to humans. In addition, they develop different forms of arthritis, either spontaneously or after experimental induction, that resemble the types of arthritis seen in humans. Furthermore, studies using mice have previously led to the successful development of new therapies for arthritis in humans, for example anti-tumour necrosis factor (anti-TNF) therapy for inflammatory arthritis. For research into the mechanisms of arthritis and joint damage and repair, we will focus on the adult life stage, as arthritis mostly affects adult humans. To understand how arthritis may be linked to the way the skeletal joints develop in the embryo and during skeletal growth in childhood, we will also study skeletal joints in embryos and young mice.

Typically, what will be done to an animal used in your project?

Typically, a small tissue sample is taken from the ear and a small blood sample obtained from the tail vein for genetic and blood cell analysis. Then, one type of injury or arthritis is induced in one knee of the mouse on one occasion. This will be done either via a surgical procedure to induce an injury or osteoarthritis, or via injections of an immunogen in the skin followed one week later by an injection into one knee to induce inflammatory arthritis. These procedures will all be performed under general anaesthesia. In addition, either before or after injury or arthritis induction, substances may be administered to the mice either systemically (orally or via injection), or by injection into the knee under general anaesthesia. Mice will typically be kept for around 1 week after injury or arthritis induction, or up to 12 weeks to study cartilage repair or osteoarthritis.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice are expected to show transient weight loss within the first 3 days after injury or arthritis induction, or after administration of certain substances, such as tamoxifen.

After injury or arthritis induction, some mice may experience pain in the affected knee, resulting in modest alterations in their gait to reduce weight-bearing on the affected hindlimb, which is expected to resolve within 3 days. Mice receive analgesia and are expected to use all four limbs and to move around freely after recovery from anaesthesia.

A small number of mice may experience patellar dislocation and lameness following surgery. Mice showing non-weightbearing lameness will be humanely killed.

Following surgical induction of arthritis, mice may develop pain in the affected knee as arthritis progresses, typically from about 8-10 weeks after surgery until the end of the experiment (up to 12 weeks after surgery), which may result in modest alterations to their gait to reduce weight-bearing on the affected hindlimb.

Injections may cause transient discomfort at the injection site, which is expected to last for minutes.

Following injection of an immunogen in the skin, inflammation at the site of injection is common, and scabs often form but these are typically dry and transient. In a small number of mice, this can progress to ulceration. This is expected to improve within 3 days and resolve within 1 week.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

85% of mice on this project are expected to have a mild severity, and 15% of mice a moderate severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The processes of joint damage, repair and arthritis are complex and involve the entire joint. Laboratory experiments using cells or tissues alone are not sufficient to fully understand the mechanisms involved.

Which non-animal alternatives did you consider for use in this project?

We use cells and tissues from human donors for experiments when suitable. No other non-animal alternatives could be used to study skeletal joints.

Why were they not suitable?

Human cells and tissues can be used to study specific aspects, but there is as yet no suitable laboratory model of a skeletal joint that can mimic all the complex processes involved in joint repair or arthritis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is estimated based on almost 15 years of experience, as Project Licence holder, of carrying out transgenic mouse breeding and the types of experiments that will be performed in this project. Data from previous experiments, either our own or those from our network of collaborators, is used to inform the number of mice needed for experiments. The models that will be used are reliable and well established in our laboratory. In addition, we use randomisation protocols, and analyse the contralateral knee in our injury and arthritis models as internal control when scientifically suitable, to minimise the influence of confounding factors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each experiment is carefully planned to ensure the use of the model, strain, sex and age that best addresses the scientific question and gives the most robust and reliable outcome with the lowest inter- individual variability.

Data from previous experiments, either our own or those from our network of collaborators, is used to inform the number of mice needed for experiments, and biostatistical input is sought as needed, to minimise wastage of mice.

We have protocols in place for randomisation and blinding to minimise the impact of confounding factors and ensure reliability of findings.

We use mono-injury or mono-arthritis models, i.e. in which only one knee is affected, and analyse the unaffected knee as internal control. This minimises the influence of inter-individual variability and confounding factors, and reduces the number of mice needed.

We do not routinely include sham-operated or vehicle-injected controls. These are only included if essential to achieve a specific research objective, e.g. to assess the efficacy of a drug compared to vehicle.

We prospectively identify mice suitable for inclusion in experiments by analysis of a small blood sample to increase reliability of outputs and thereby reduce the number of mice needed for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We ensure efficient breeding through active management of mouse colonies and forward planning to tie in with experimental demand. For complex breeding schemes, we perform computer modelling to select the breeding scheme that yields the highest expected proportion of mice with the required genotypes in the fewest generations. In addition, we consolidate maintenance of genetically altered mouse strains by maintaining multiple genetic alterations within the same colony when possible, thus minimising the number of mouse colonies to be maintained, and the number of breeding steps needed to generate mice for experiments.

We perform pilot studies when there is no existing data available to inform best experimental design, for example to determine the optimal dose and frequency of administration of a substance. In addition, we employ a group-sequential design whereby smaller cohorts of mice undergo the same procedures on separate occasions, and data is combined for analysis. This helps to minimise excess breeding and to validate reproducibility of findings across multiple cohorts of mice. In addition, where appropriate, interim data analysis is used to inform number of mice needed.

We share mouse breeding and share tissues with collaborators, including collaborators at other institutions, when practically possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice with genetic alterations that allow to identify and trace different cells,

using for example fluorescent labels. These genetic alterations are not known or expected to impact on the welfare of the animals.

We will use a surgical model of injury known as the joint surface injury (JSI) model. We create a narrow injury in an area of the joint that is minimally weight-bearing. The injury is shallow and limited to the surface of the joint, to minimise pain caused by the injury. Only one knee will be operated to minimise pain, suffering, and distress.

We will use a surgical osteoarthritis model known as the destabilisation of the medial meniscus (DMM) model. This is the most widely used and validated model of osteoarthritis in mice that shows disease progression that is comparable to the human disease. There are currently no non-surgical models that reliably induce arthritis that resembles osteoarthritis in humans. Only one knee will be operated to minimise pain, suffering, and distress.

We will use a model of inflammatory arthritis called antigen-induced arthritis (AIA). This model of inflammatory arthritis only affects one knee, thus minimising pain, suffering and distress compared to other models of inflammatory arthritis in which multiple joints are affected. In addition, we use the minimum number of procedures needed to reliably induce arthritis and avoid using an extra immune- boosting step that is used in some studies and leads to more severe arthritis.

Why can’t you use animals that are less sentient?

Skeletal joints only exist in vertebrates. Arthritis primarily affects adults, and the response to injury is known to be different between immature and mature mice. The response to injury or mechanisms of arthritis need to be studied in living animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For surgical models, we use refined surgical procedures to minimise complications and harm to animals. This includes use of aseptic technique, keeping surgical incisions to a minimum, and use of effective suturing technique to minimise risk of accidental loosening of sutures post-surgery.

Mice will receive peri- and post-operative analgesia to manage pain, will be provided with mashed soft food on the floor of the cage, and extra bedding. Increased monitoring of animals post-operatively and following arthritis induction will ensure that any animals that require extra care are promptly identified.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Home Office guidance, for example regarding best practice for aseptic technique (LASA Guidelines 2010), injection technique (guidelines from the Joint Working Group on Refinement published in Morton et al., Lab Anim 2001;35(1):1-41), or mouse handling technique. We also follow ARRIVE guidelines and best practice for arthritis models based on scientific literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through the NC3Rs website, regular discussions with named veterinary surgeons (NVS) and named animal care and welfare officers (NACWOs), and attendance of meetings focussed on advancing the 3Rs.

30. Axon Degeneration and Repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Axon degeneration, Motor neuron disease, Alzheimer's disease, Parkinson's disease, drug targets

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to identify genes that influence the survival, degeneration and repair of nerves and understand their roles in human disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be

short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are very few disease-modifying treatments for neurodegenerative diseases (progressive degenerative diseases of the nervous system such as those mentioned below). A disease-modifying treatment is one that halts, or even reverses the course of the disease, in contrast to most current treatments which block only the symptoms. Neurodegenerative diseases include common disorders such as Alzheimer's disease, Parkinson's disease, peripheral neuropathies (common pain disorders associated diabetes, cancer chemotherapy and genetic conditions) and glaucoma, some moderately common ones such as motor neuron disease and multiple sclerosis, and many rare disorders such as hereditary spastic paraplegia (a type of motor neuron disease characterised more by spasticity than paralysis) and spinal muscular atrophy (a childhood onset, very severe form of motor neuron disease). Around half the population suffer one or more of these disorders at some point in their lives. Some molecular mechanisms recur in many of them so blocking such a mechanism could have profound implications for healthcare. One such mechanism, into which our research has played a leading role, is known as 'programmed axon death'. This leads to loss of axons (nerve fibres) with consequences including pain, paralysis, spasticity, blindness and memory loss, and it is triggered by a wide range of causes. Blocking programmed axon death is protective in animal models of disease, and in some circumstances rescues nerves for the entire lifetime of a mouse. We have pioneered understanding of its roles in human disease and here we aim to find more disorders to which it contributes. This will enable drug companies aiming to prevent programmed axon death to direct clinical trials to disorders in which they are most likely to work.

What outputs do you think you will see at the end of this project?

Our plan is to publish research papers with new information on which human diseases involve programmed axon death and which specific people are most at risk. We may also carry out testing in cell culture of drugs that subsequently enter clinical trials.

Who or what will benefit from these outputs, and how?

Ultimately, it is patients with neurodegenerative diseases, and their families, who will benefit because some of these diseases will be prevented, slowed down or even reversed. Individual people in families known to be at risk will also be able to determine their genetic makeup, if they wish, in order to understand their personal risk and any lifestyle modifications they may wish to make as a consequence. In the more immediate future, as well as longer-term, pharmaceutical companies and their employees and owners will benefit from commercial use of the knowledge we generate. Most immediately, our outputs benefit the research community by advancing their research and careers, and they benefit the funders of this research by helping them achieve their aims of advancing knowledge and medicine.

How will you look to maximise the outputs of this work?

Our papers are all 'open access' (not behind any paywall) to maximise their accessibility to readers. This will include publication of unsuccessful approaches to avoid unnecessary duplication by other researchers. We also regularly discuss our research results with other scientists, both in academia and in the Pharma industry, to maximise its uptake in future

research. This includes activities such as conference presentations, seminars and collaborations.

Species and numbers of animals expected to be used

- Mice: 25,000
- Zebra fish (Danio rerio): 4,050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice because they have an approximately 1:1 correlation of their genes with those of humans, because their genome (their complete genetic material, or DNA) is fully sequenced and well- characterised, and because there are existing genetically-altered strains of mice that are appropriate for our research. We use newborn mice or embryos because these enable us to grow neurons in cell culture effectively (such methods do not work well with older cells). However, this requires us to have an older stock of mice to breed, both to obtain newborn mice for these cells and to maintain the genetic lines.

We use zebrafish embryos before five days post-fertilisation because the ability to render these fish transparent makes them highly useful for imaging and characterising the growth, survival and repair of nerves. This too requires us to maintain a stock of older zebrafish in order to breed them and they become protected animals.

Typically, what will be done to an animal used in your project?

Adult mice with harmless genetic alterations will be mated to generate newborn mice, or embryos. Humane methods of killing will be used to obtain embryonic or newborn mouse tissue for cell culture.

Zebrafish with harmless genetic alterations will be used for breeding to obtain embryos for nonregulated procedures before reaching the free-feeding stage.

Zebrafish gametes may be collected using a mild procedure involving gentle pressure or stroking, causing only transient discomfort.

What are the expected impacts and/or adverse effects for the animals during your project?

There will be no adverse impacts of the experiments themselves, apart from transient discomfort during biopsy or gamete collection. Health problems from non project-related causes will be assessed and if necessary the animal humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities in mice are subthreshold (90%) and mild (10%). The expected severities in zebrafish are subthreshold (60%) and mild (40%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are modelling normal biology and disease processes and this requires the use of living cells. Sometimes we need these to have a specific genetic makeup that is present in genetically altered mouse strains, and always we need the cells to closely resemble those in the living animal.

Which non-animal alternatives did you consider for use in this project?

We have already moved all of our ongoing experimental work to mouse neurons in culture and tissue obtained from humanely-killed mice. We have also considered (and increasingly use - see below) both human stem cell (iPSC)-derived neurons and cell lines.

Why were they not suitable?

We are in the process of moving all our research away from animals to focus it exclusively on iPSC (stem cell)-derived neurons and human genetics. However, optimising these techniques, in particular adapting them for the higher throughput we can already attain in mouse neurons in cell culture, takes time, and ongoing projects that were begun using mouse cells in culture must continue to use them because to change the experimental system in mid-project would introduce unquantifiable variables that make accurate interpretation of the data impossible. Cell lines are not useful for most of our work because cell lines acquire major genetic artefacts (known as 'chromosomal abnormalities') when propagated in culture for long periods. We do use them for some work that is not substantially affected by this but they are not a solution for most of our work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A typical experiment using cells in culture derived from mice requires a group size of up to 10 for statistical comparisons and may have three variables we are testing, so it requires 30

newborn or embryonic mice. The number of experiments is in practice limited by people's time and resources (equipment, space, funding) because there is always a logical next question to ask, even within this specific area of research. Thus, the following calculation is based on what is a feasible level of throughput for the time and resources we have. We do around three experiments per week, so with 30 newborn or embryonic mice per experiment this brings the total to 90 per week, giving a theoretical total of 23,400 (90 mice x 52 weeks x 5 years) in five years if the work was continual. In practice, however, holidays, sickness, and the time needed to interpret data and design new experiments will lower this number, while the need to keep additional adult mice to generate newborn and embryonic mice and maintain the strains raise it. Thus, the estimate of 25,000 is based on this starting point of 23,400 which is then modified by both of these effects.

Similarly, a single experiment using zebrafish typically requires up to 10 fish per group and has three variables, so 30 fish. The frequency of these experiments is lower, around one every two weeks. Thus, over 5 years we require 30 x 52/2 x 5 = 3900 zebrafish. After similar adjustments to above for holidays, sickness, planning/interpretation time, and adult breeding, the estimated number is 4,050.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We minimise experimental variables, which would otherwise increase the group sizes needed to obtain statistically significant results, by using standardised protocols and performing experiments in parallel whenever possible. We only retain the required number of adults to ensure maintenance of the strain and the ability to breed enough young animals for our work. We also follow guidance on the NC3Rs website https://nc3rs.org.uk/3rs-advice-project-licence-applicants-reduction and the PREPARE guidelines https://norecopa.no/PREPARE.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We breed homozygous lines where possible (lines where all offspring are identical to the parents), to avoid excess generation of animals that will not contribute to our conclusions. Occasionally, breeding of homozygous lines would involve adverse effects on health so as a Refinement measure to prevent this, we breed those specific animals as hybrids instead, which do not have this problem. We regularly use pilot studies to optimise experimental design and identify potential problems, before deciding whether to scale up a particular line of work. We select our experiments carefully based on sound knowledge of the relevant scientific literature and extensive internal and external discussion before proceeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All our experimental work is in cell culture or zebrafish embryos. Thus, apart from the need for breeding and maintenance of mice and zebrafish to generate these, we have replaced animal experiments entirely in our work. Apart from minimal tissue biopsies to determine genetic makeup of individual animals, there will be no pain, suffering, distress or lasting harm resulting from the project.

Why can't you use animals that are less sentient?

We do use immature life stages and humane methods of killing. We cannot use less sentient species because we need to use mammals for a 1:1 relationship with human genes, and vertebrates to retain representation of peripheral and central nervous system. There are fruit fly geneticists working in this research field, and this is a very powerful experimental system, but the results need to be validated in vertebrates and in mammals. Thus, we collaborate with several such groups to do this. There would be no point for us to duplicate their work, especially when we have less expertise in those methods, while no-one does the necessary validation in vertebrates and mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will carry out all our experimental work in cell culture or in zebrafish embryos. Where it is necessary to breed a genetic alteration that is harmful in homozygotes in order to generate cells for culture, we will maintain the line using heterozygotes (hybrids) where the alteration is not harmful. For the regulated procedures of adult breeding and maintenance needed to produce these embryos and young animals, we use enriched environments, optimal handling methods and minimise single housing of animals to limit stress

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review the NC3Rs website for this guidance https://www.nc3rs.org.uk/3rs-resources, the most relevant sections of which pertain to mouse https://nc3rs.org.uk/3rs-resources/breeding-and- colony-management and zebrafish breeding and maintenance: https://nc3rs.org.uk/3rs- resources/zebrafish-welfare. We also consult specific guidance or position papers from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We routinely attend conferences and discuss with other scientists at seminars and in collaborations. We will also consult with the NVS and NACWO for new advances, and journals and web resources such as ATLA (Alternatives to Laboratory Animals) Journal: https://journals.sagepub.com/home/atla and the LASA Guidelines: https://www.lasa.co.uk/current_publications/. As we have shown, when a new 3Rs method becomes available (e.g., hiPSC-derived neurons), we use it.

31. An In Vivo Intraluminal Intestinal Injection Model

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

oral delivery, biopharmaceuticals, in vitro/in vivo correlation, intestinal biology

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project uses a novel method of directly injecting test materials into the intestinal lumen of anesthetized rats to examine the potential for these agents to increase the oral bioavailability of poorly absorbed biopharmaceuticals (e.g., protein and peptide therapeutics). The aim of this work is to identify successful oral uptake strategies of these biopharmaceuticals that can lead to transformative changes in healthcare.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Any new approach/material to enhance the oral uptake of a biopharmaceutical is initially screened using a variety of in vitro models, such as human intestinal epithelia cells (e.g., Caco-2) grown as polarized monolayers on semi-permeable filters. Such studies provide a critical first screen to ensure that any approach/material is feasible and non-toxic to the cells

comprising the epithelial barrier. The next, essential, step to assess the potential of a new approach/material requires more complex conditions present in the intact intestine in vivo. This step considers complexities of the gastrointestinal environment at the site of intestinal absorption that cannot be readily produced in in vitro models: digestive events, mucus, muscle contractions of the intestinal wall, etc. It can also examine events associated with the direct delivery of an absorbed biopharmaceutical following uptake into the hepatic portal venous system. Promising outcomes in this in vivo step provide a critical proof of mechanism assessment essential for clinical translation.

Biopharmaceuticals are typically labile (digested) in the stomach. This instability makes it impossible to test these approaches/materials by oral gavage, the technique commonly used to assess stable small molecule drug candidates. This protocol mimics how novel uptake enhancing approaches/materials would function as they are released from an oral dosage form dissolving in the small intestine. This in vivo protocol overcomes this challenge through the direct injection of test materials into a segment of the small intestine of a rat under terminal anaesthesia. Data collected over the first 180 min following administration of test approaches/materials is sufficient to determine their safety and efficacy. Thus, this model provides data essential for assessing the feasibility of a new delivery approach/material in an in vivo model where the animal never regains consciousness. Undertaking this work is important in that it provides an in vivo method with minimal animal stress, but which provides valuable proof of mechanism outcomes.

What outputs do you think you will see at the end of this project?

During the past five years, this in vivo method has provided critical data to validate two approaches/materials to improve oral biopharmaceutical uptake. In the case of the permeable inhibitor of phosphatase (PIP) peptide technology for the delivery of peptide therapeutics, three peer-reviewed publications have provided critical validation for a new start-up company using. In the case of bacterial toxin-based oral protein delivery technology, four additional peer-reviewed publications were made possible by data generated using this protocol, helping move a molecule using that technology through a successful Phase 2 study.

Based upon these successes, we believe that similar outputs will be achieved in the next five years through extensions of new approaches/materials that are now being tested in vitro. Though work funded by pharmaceutical companies, this protocol, will would specifically address the feasibility of intestinal uptake of biopharmaceuticals that have been approved for use in the areas of diabetes, colitis, and short-stature children. During discussions to set up this funding, individuals from that company commented that this model had been established at their animal research unit, where they were impressed with its reproducibility and correlation with higher species (dog, pig) outcomes. They came to us to run these studies, not because the model was difficult or could not be reproduced, but because of man-power constraints caused by internal obligations with other pipeline programs. This information highlights the fact that this model is being tested by others, with its acceptance and successful use in at least one pharmaceutical company that we know of at this time.

Who or what will benefit from these outputs, and how?

Almost all biopharmaceuticals are currently be given by injection. Oral delivery of these agents would completely change the treatment paradigm for patients, not only improving convenience and compliance, but improving safety and efficacy in certain cases. This will result in tremendous benefits for patients through a paradigm shift in the pharmaceutical

industry and government healthcare strategies. Outputs from this work has already brought the first oral protein therapeutic (interleukin-10) to Phase 3 testing. One of the most important functions of this work was to measure and visualize the extent of uptake of interleukin-10 across the intestinal mucosa, information that was used to help select the amount of material put in an oral dosage form for first-in-man studies. This selection was important to ensure patients were not under- or over-dosed. Thus, in the immediate future, this work can be considered as a valuable, even essential, step in the clinical translation of any protein therapeutic being considered for oral administration. In a longer-term view, the work has identified a path for validation of agents and guidance for dose requitements at the early stages of clinical development.

How will you look to maximise the outputs of this work?

Outputs from this protocol have already been identified as valuable contributions in publications and supporting clinical strategies for the oral delivery of multiple biopharmaceuticals. Viability for these approaches/materials to enhance oral delivery has already supported the establishment of a publicly traded company and has provided the basis for establishment of an early-stage biotechnology spinout company. We will continue to maximize the value of these outputs through dissemination by peer- reviewed publications, presentations at scientific conferences and academic institutions, and interactions with pharmaceutical companies that will be instrumental for clinical translation.

Species and numbers of animals expected to be used

- Rats: 625 animals.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This model involves the introduction of a test approach/material, using a fine-gauge needle, into an exposed segment of rat small intestine to test its ability to enhance the uptake of a biopharmaceutical across the mucosal epithelium and into the body. Blood samples are used to obtained pharmacokinetic (PK) profile information to assess the extent of this uptake and intestinal tissue samples are collected at study termination to examine the nature of the trans-epithelial pathway accessed by the biopharmaceutical during its entry into the body. As these uptake events are relatively rapid (on the order of 15-45 min), a complete understanding of the uptake events and their implications to further refine the delivery approach can be achieved in ~180 min. For this reason, we use rats that can be placed under anaesthesia, manipulated for the introduction of test articles with all the required blood samples, and killed using a schedule I euthanasia method without recovery from anaesthesia: a non- recovery protocol.

There are several reasons that we have chosen young (6-8 weeks), healthy rats for this work. They represent the lowest species with physiological and anatomical properties of the gastrointestinal tract and systemic criteria required to obtain data relevant for subsequent clinical translation using the minimal number of animals. 1) Structural and functional properties of the adult rat small intestine are comparable to human. Unlike the mouse, the volume of a test material that can be delivered to the rat intestine will not over-extend the physical capacity of the rat small intestine, ensuring that non- physiological stress on this

tissue does not occur. 2) The surface areas of rat jejunal and ileal small intestine segments are sufficiently large to allow for introduction of the same test article at multiple locations to obtain a time course of tissue events using a single animal. These same small intestinal segments in a mouse are too small to allow this to be performed and multiple animals would be required to collect the same time course information. 3) A single rat is sufficient to obtain the total volume of blood required to construct a serum PK profile for a test material. By comparison, multiple mice would be required to collect the blood required to construct this same PK profile. 4) The use of rats expands the repertoire of reagents that can be used to characterize the outcomes of test articles administered using this protocol. When characterizing the route taken by a biopharmaceutical across the small intestinal epithelium induced by a test approach/material, specific reagents are often used to identify cellular elements that represent guideposts for cellular pathways. As these reagents are often monoclonal antibodies generated in mice, they are not useful in studies involving mice.

Typically, what will be done to an animal used in your project?

An overview of the steps involved in the intraluminal injection (ILI) model are as follows. A rat is collected from their cage, weighed, transferred to procedure room, and placed in an anaesthesia induction box. Once the anaesthetic has taken effect, the animal is transferred to a heated pad and fitted with a nose cone to maintain continued anaesthesia. Upon verification that the animal is still deeply anesthetized, the abdominal area of the animal is trimmed close to the skin using clippers and cleaned with alcohol. A midline incision beneath the sternum of approximately 3 centimetres is made to access abdominal cavity contents and a segment of the small intestine in the mid-jejunal region is extracted. This segment is positioned on pads moistened with saline placed adjacent to the incision site and the exposed small intestine is examined to select a region or regions to be used for test article administration. An indelible marking pen is used to tag the mesentery adjacent to the site where a test article injection will be made. Following test article injection, the exposed segment of small intestine is placed back into the abdominal cavity and the incision site covered and maintained moistened for the duration of the study. The extent of introduced biopharmaceutical reaching the systemic circulation is determined from blood drawn through either a jugular or tail vein. Upon completion of these blood draws, the protocol is terminated with a schedule 1 killing of the animal.

To determine the relative bioavailability of a molecule injected into the intestine from the protocol described above, a pharmacokinetic profile is acquired following the intravenous or subcutaneous injection of that compound. In this case, a rat is collected from their cage, weighed, transferred to procedure room, and placed in an anaesthesia induction box. Once the anaesthetic has taken effect, the animal is transferred to a heated pad and fitted with a nose cone to maintain continued anaesthesia. Upon verification that the animal is still deeply anesthetized, the test compound is administered through an IV infusion or a subcutaneous injection. The time-concentration profile of an administered compound in the systemic circulation is determined from blood drawn through either a jugular or tail vein. Upon completion of these blood draws, the protocol is terminated with a schedule 1 killing of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

This non-recovery protocol is designed to remove as many potential concerns of negative impacts and/or adverse effects on the animals used as possible. Animals, however, could experience stress during handling prior to the onset of anaesthesia. Personnel performing these studies are experienced in handling rats to limit such stress. It is also possible that an

animal could start to recover from anaesthesia before study completion. Personnel performing these studies continually check animal response to verify maintenance of anaesthesia, have a back-up system to administer anaesthetic gas readily available if necessary and can rapidly terminate the animal by a schedule 1 killing if required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All animals used in this protocol will be maintained under anaesthesia throughout the entire procedure and killed prior to recovery from anaesthesia: non-recovery.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The intestinal mucosa and portal venous drainage to the liver are critical biological barriers that must be overcome for a biopharmaceutical to reach the systemic circulation. Complexities of these barriers cannot be readily or accurately re-created in vitro. An in vivo model to examine the ability of approaches/materials to overcome these barriers is needed. We have established a series of in vitro tools to initially screen approaches/materials for actions and safety. This in vivo model is needed to test those approaches/materials that warrant such studies based upon sufficiently promising in vitro outcomes.

Which non-animal alternatives did you consider for use in this project?

We have considered in vitro systems to model intestinal mucus, more complex forms of the epithelial barrier and hepatic portal circulation. Many of these models require collection of tissues from animals, such as rats, that would limit the proposed benefit of reducing animal use. Further, we do not believe these in vitro models can provide information that would replace the subsequent testing in this in vivo intraluminal injection model for the selection of safe and effective approaches/materials for the oral delivery of biopharmaceuticals.

While there are no current plans to use SyRF(https://www.nc3rs.org.uk/camarades-nc3rs-systematic- review-facility-syrf) to perform a systematic review and meta-analysis of animal studies, this platform may be used if sufficient data is acquired to make this analysis valuable.

Why were they not suitable?

We have considered ex vivo and in vitro models to examine the impact of mucus at the apical surface of intestinal epithelial cells. Isolated intestinal tissue can either lose its mucus layer or produce an artificially high level of surface mucus by non-physiological goblet cell

activation (Brownlee et al., 2007 Action of reactive oxygen species on colonic mucus secretions. Free Radic Biol Med. 43(5):800-8).

While certain human intestinal cell lines (e.g., HT29) can produce mucus, the properties of this mucus do not recreate the complexities of this barrier observed in vivo (Navabi, et al., 2013 Gastrointestinal Cell Lines Form Polarized Epithelia with an Adherent Mucus Layer when Cultured in Semi-Wet Interfaces with Mechanical Stimulation. PLoS One 8(7): e68761).

We have considered using isolated rat intestinal tissue ex vivo to model the epithelial barrier. Isolated intestinal epithelia lose cell viability within 30-45 min of removal from the animal (Roskott et al., 2010 Reduced ischemia-reoxygenation injury in rat intestine after luminal preservation with a tailored solution. Transplantation 90(6):622-9). This does not allow adequate time for novel approaches/materials to function by their proposed mechanism(s). Further, available information suggests that using Caco-2 monolayers in vitro is essentially equivalent to the ex vivo tissue transport studies (Rubas et al., 1996 Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. J Pharm Sci. 85(2):165-9). Since we already screen our candidate test agents using Caco-2 monolayers, performing the ex vivo tissue transport studies would be redundant and a waste of animals.

Following intestinal uptake, an absorbed biopharmaceutical must pass a gauntlet of cellular filters presented by the portal venous endothelium and various cell types of the liver. We were unable to identify an in vitro model of the portal vein endothelium. Hepatocyte systems have been used in vitro to examine protein and peptide drug metabolism after systemic injection (Zvereva et al., 2016 Comparison of various in vitro model systems of the metabolism of synthetic doping peptides: Proteolytic enzymes, human blood serum, liver and kidney microsomes and liver S9 fraction. J Proteomics 149:85-97). Such studies model these materials entering the liver through the hepatic artery. We could not find any in vitro model that correlated with events when a biopharmaceutical enters the liver via the portal vein.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each year, we plan to test ~100 formulation/material approaches using our lab-based methods of assessment that do not involve animals. From these, we have found that approximately 30-50% (assume 40%) can be predicted to show sufficient promise and have a presumed mechanism of action that can be tested in hypothesis-driven experiments in this animal model. Of these initial 40 formulation/material approaches tested in animals, roughly 50% will be considered for additional testing to ensure reproducibly. We initially thought that this would require five animals but have found that we can obtain robust data with just three animals. So, 40 animals for the initial testing, plus 20 approaches/materials tested (typically) twice more to obtain statistically valuable information to determine the efficiency of the drug being tested to reach the blood circulating through the body. Thus, 40 + 40 = 80 animals.

A few of these approaches/materials will be considered sufficiently promising to justify determining the efficiency of this oral delivery relative to how these drugs are currently given: injection directly into the body. This requires three animals for each biopharmaceutical being considered. We anticipate six different biopharmaceuticals to be tested in this way (18 animals) per year. We further anticipate 15 animals being used to examine cellular processes and tissue time course events to address specific hypothesis-driven questions involving the fate and local impact in the intestine of an absorbed biopharmaceutical. From all these activities combined, we arrive at ~125 animals when adding 12 animals for unexpected repeats due to technical or analytical issues (80 + 18 + 15 + 12 = 125) per year.

We have approached the issue of minimizing the number of animals used in several ways:

1. Careful selection of only a few transport enhancing methods for in vivo testing. Rigorous in vitro and cell-based assays allow for rejection of molecules do to overt cytotoxic effects prior to in vivo testing.

2. The in vivo method of direct injection of a transport enhancing agent and a biopharmaceutical into the intestinal lumen addresses the uncertainty of these materials effectively reaching a location of the intestine following oral gavage.

3. The direct injection approach permits isolation of the application site, and rapid determination of safety and efficacy, reducing the number of animals required to obtain unambiguous results.

4. We use inbred stains of animals to reduce animal-to-animal variability.

5. Studies are blinded to ensure that unbiased outcomes are obtained.

6. A single animal can be used for multiple (up to eight) intraluminal injections when examining the time course of intestinal uptake events, reducing the number of animals used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

As noted above, we initially thought an N = 5 was required to obtain statistically relevant outcomes, but we have achieved such reproducibility that N = 3 for testing these formulation/material approaches is sufficient. Additionally, we have shown that multiple intestinal injections can be performed in the same animal by coordinating the timing of these administrations without compromising the quality of the data obtained. This has led to a reduction the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In all cases, a very specific hypothesis will be tested regarding a test formulation or delivery enhancing strategy. This will minimize the number of animals to be used. Learnings from each experiment will be examined to determine if additional studies are required BEFORE the initiation of subsequent animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures,

to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have selected a model that minimizes animal suffering while still allowing for the acquisition of data required to make these studies successful and the outcomes unambiguous. All experiments will be conducted under terminal anaesthesia. The model involves anaesthesia induction and maintenance using a gaseous anaesthetic throughout the study protocol. Thus, all studies will be non-recovery and performed aseptically as per LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery:

http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf)

Anaesthesia – The protocol uses inhalation anaesthesia. This ensures that all steps, such as intestinal injections and blood draws do not result in pain or stress. After induction of anaesthesia and prior to injection, fur covering the abdomen is clipped to provide a clear view of the surface anatomy selected for the incision site to gain access to the intestinal abdominal contents.

Blood sampling - We have improved the timing of how blood samples are collected to further improve the quality of the PK data obtained. This has allowed us to continue using rats rather than requiring a higher species. All procedures are carried out under general gaseous anaesthetic. We will follow the NC3R guidelines for blood sampling (https://www.nc3rs.org.uk/3rs-resources/blood-sampling).

Why can’t you use animals that are less sentient?

Complexities of the small intestinal anatomy and physiology in mammals that regulate the uptake of biopharmaceuticals do not appear to be similar in non-mammalian species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have optimized the timing of sample collection to maximize quality of the data obtained. This has allowed us to continue using rats, not requiring a higher species. All procedures are carried out under general anaesthetic in a non-recovery protocol that eliminates concerns for post-operative care and pain management. We had initially considered also using mice for these studies. As noted above, using rats rather than mice has reduced the number of animals used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The intraluminal injection (ILI) model described in this protocol is novel, being described in the literature through our own publications. We have taken learnings from the extensive literature focusing on the administration of biopharmaceuticals through other routes of injection.

D.B. Morton, M. Jennings, A. Buckwell, R. Ewbank, C. Godfrey, B. Holgate, I. Inglis, R. James, C. Page, I. Sharman, R. Verschoyle, L. Westall, and A. B. Wilson 2000 Refining procedures for the administration of substances. Laboratory Animals, 35(1): 1-41.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are engaged with events regarding animal care and use provided through organizations across the UK. These provide current advances in 3R ideas and processes that might be incorporated into this ILI protocol.

32. Analysing Gene Function in Xenopus Development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cell migration, cancer, gene regulation, malformations, cranofacial defects

Animal types	Life stages
Xenopus laevis	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how the vertebrate nervous system is formed, using frog embryos as our model system. Development of the nervous system results from tissue interactions that begin during the first 24 hours of frog development. These tissue interactions continue throughout development, to generate the many different regions and cell types of the adult nervous system.

We will have two aims in this project:

1. To study the cell interactions and to identify the genes that collaborate to generate the nervous system. Defects in the development of the nervous system are amongst the most common causes of congenital abnormalities in the neural tube.

2. To study the genes that regulate migration of the cells that form the peripheral nervous system (such as that lining the gut). Migration of these cells shares many similarities with migrating cancer cells and may provide insights into the latter process.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The project will increase our understanding of fundamental questions of how anatomy is built during embryonic development. A good understanding of normal development can help us understand how defects arise that lead to congenital abnormalities. Specifically, this project will study the cells that form the face of vertebrates, and it will have a direct impact in understanding craniofacial malformations.

Since embryonic development is similar in all vertebrates, the knowledge gained in this project will be applicable to humans. The project will also help our understanding of diseases such as cancer and may therefore provide future health benefits.

Part of this project will study the migratory behaviour of neural crest cells, both in vivo and in vitro, that form the peripheral nervous system of vertebrates, as well as parts of the face and neck. It is now clear that many aspects of their migratory behaviour is shared with other migratory cells, including metastasising cancer cells. This project will therefore inform studies on migration of cancer cells that may lead to long-term benefits in cancer treatment.

What outputs do you think you will see at the end of this project?

- This work is expected to provide new information how neural crest cell migration is controlled during development. Specifically, we will explore the role of proinflammatory factors controlling neural crest migration; we will use Xenopus embryos to test the hypothesis that cleft palate/lip is trigged by environmental factors that induce a p roinflammatory response in the neural crest cells

- The primary expected benefit is the publication of new scientific knowledge about development of the nervous system and the migration of neural crest cells, which could have a direct impact on healthpolicies, as it could show the role of environmental factors in clef palate/lip.

Who or what will benefit from these outputs, and how?

A short-term and medium-term benefit of this project is that it will increase our understanding of fundamental questions in vertebrate embryology, concerning the migration of the neural crest, and its failure as the cause of craniofacial malformations. The data generated will provide the basis of scientific publications that will be of great value to the wider scientific community, as all our publications will be full open access. In the long-term, they will help us understand developmental abnormalities in humans, such as oral clefts, one of the most common malformations in humans Congenital defects are common in humans, affecting 3-4% of new-borns, and result from defects in the normal developmental process. In most instances the causes are unknown.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings.

Species and numbers of animals expected to be used

- Xenopus laevis: 400 Xenopus leavis (females)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project uses embryos of the amphibian Xenopus lavis to study how cells acquire different developmental fates and how cell migration is regulated. Studies on amphibians have played key roles in our current understanding of both problems. For example, amphibians were used to first demonstrate a process known as neural induction, whereby signals from the mesoderm instruct ectodermal cells to form the nervous system. Studies on the South African clawed frog, Xenopus laevis, identified the key signalling molecules involved in this process.

- Xenopus laevis provides several key advantages for these studies, including:
- large numbers of synchronously developing embryos that are available at all stages of embryonic development
- large embryos that are amenable to micromanipulation, allowing groups of cells to be transplanted into different embryos
- large cells are easily injected with reagents that either increase or decrease gene activity
- cells are easily isolated and cultured in vitro
- developmental mechanisms that are largely conserved amongst all vertebrates embryos, including human embryos.
- Our studies will involve the use of embryos at stages that they are not believed to be sentients or suffer pain

Typically, what will be done to an animal used in your project?

Adult females will undergo superovulation on up to 12 occasions. This procedure involves restraining the Xenopus and one hormone is injected into the abdomen followed by a second injection three days later, both injections are of mild severity. A few hours later eggs are collected (by massaging the abdomen). Only adult females will undergo regulated procedures under this project licence, and female can lay more than a thousand eggs a few hours after she has been injected with hormone, which provides enough fertilised eggs for the experiments of several researchers. We co-ordinate our experiments to make best use of the embryos resulting from each ovulation. The procedure can be performed many times on the same female with equal success with no harm to the female. Since the severity of the injection procedure is mild, and females rarely show adverse effects, but if that happens animals will not be re-used.

What are the expected impacts and/or adverse effects for the animals during your project?

The injection procedure causes no more than mild stress and animals show no long-term adverse effects. Handling is kept to a minimum and animals are returned to the holding tanks within 48 hours of them being removed (once they have stopped laying eggs). Only healthy animals will be reused in this procedure, which can be determined by their general appearance. Healthy females have large round abdomens, whereas unhealthy frogs may

also develop rough and red skin; these frogs are not used for hormone injection. Sick animals receive veterinary care and increased monitoring, and the cause of the ill health will be identified and treated, however, if necessary, animals will be killed by schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity is expected for all animals. These effects are usually reversed within a few days, but animals are first isolated and treated as advised by a veterinary surgeon if they persist. They are killed by an appropriate humane method if advised by the NVS.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Embryonic development is a complex process that requires multiple tissue interactions. These cannot yet be reproduced using cultured cells, we therefore require female frogs to provide the embryos needed for this project.

Which non-animal alternatives did you consider for use in this project?

An alternative could be the use of cells cultured in vitro to reproduce the developmental process of formation of the nervous system and neural crest migration during head development.

Development of computational models of neural crest migration, where the effects of internal of external variables could be tested in silico

Why were they not suitable?

Formation of the nervous system and head development are highly complex process that so far has not been possible to reproduce with cells cultured in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Female frogs can lay thousands of eggs, which are sufficient for many experiments. However, there is a high variability in the number and quality of the eggs produced by each female. We therefore will use 6 frogs per week and organise our work schedules so that up to 7 scientists may use these eggs.

Sharing resources minimises the number of procedures that we need to perform.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Sharing the number of animals among different researchers and experiments minimises the number of procedures that we need to perform.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Sharing the number of animal among different reserachers and experiments minimises the number of procedures that we need to perform.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Xenopus is our animal model of choice because it has numerous advantages over other model vertebrates (such as the mouse and chick). They produce larger numbers of eggs than mouse and chick and can be observed more easily at all stages of development. To obtain the embryos females need to be injected with hormones that will induce ovulation. Over the years we have tested different injection regimes (different hormones, doses, time of injection, etc), so we have found the optimal conditions to produce the largest number of embryos (diminishing the number of animals needed) with the lowest harm to the animals. We have also improved the housing by introducing environment enrichment (PVC tubes) that mimic the natural condition where Xenopus live. Animals displaying adverse effects receive veterinary care and if necessary, killed by terminal anaesthesia.

Why can’t you use animals that are less sentient?

All the experiments in this project will be performed in immature life stages (early embryos that are not sentient); however, we need mature females of Xenopus laevis to obtain large number of embryos

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The injection procedure causes no more than mild stress in the majority of cases and animals show no long-term adverse effects. Handling is kept to a minimum and animals are returned to the holding tanks as soon as possible. Animals are monitored daily for any sign

of a health issue (skin rough or red, lethargy, etc). In addition, animals are weight before and after injection (at several time point) to monitor any effect on their health.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published guidelines including the ones issued by NC3Rs_ https://www.rspca.org.uk/webContent/staticImages/Downloads/GuidanceXenopusLaevisReport.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow the recommendations published by NC3Rs and implement them when possible and by sharing information between the researchers in the field. In addition, we will follow the work and activities of the European Xenopus Resource Centre (EXRC).

33. Assessment of the Efficacy of Novel Drugs to Treat Worms that Infect the Farm and Domestic Animals

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Anti-parasitic, Parasite, Worms, De-wormer

Animal types	Life stages
Mice	adult, juvenile
Gerbils	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the efficacy of novel drugs to treat intestinal worms that infect farm and domestic animals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasites in the intestinal tract, often called ‘worms’ are a major cause of disease and production loss in livestock and domestic animals. Infections frequently cause significant economic loss and impact on animal welfare. In addition to the impact on animal health and production, control measures are costly and often time-consuming. A major concern is the development of resistance by worms to the anti- parasitic agents (dewormers) administered.

Anti-parasitic resistance is the genetic ability of parasites to survive treatment with an anti-parasitic drug that was generally effective against those parasites in the past. After an animal is treated with an antiparasitic drug, the susceptible parasites die and the resistant parasites survive to pass on resistance genes to their offspring. Researchers and veterinarians have documented an increasing level of antiparasitic resistance in grazing species, such as cattle, small ruminants (sheep and goats), and horses, both within UK and globally.

Planned preventative programs are necessary to minimize the risks of parasitic disease outbreaks and sub-clinical (invisible) losses of animal production, and to ensure the most efficient use of control chemicals. Integrated parasite management programs aim to provide optimal parasite control for the minimal use of chemicals by integrating pre-emptive treatments, parasite monitoring schedules and non-chemical strategies such as nutrition, genetics and pasture management.

Even with best practices anti-parasitic resistance can't be stopped. Parasites will continue to evolve and develop resistance. Due to the development of resistance, there is an ongoing need to develop novel antiparasitic agents that can be added to the armamentarium to reduce the health and economic impacts of worm infestation. In this program of work we will be testing the efficacy of novel antiparasitic agents against some of the common worms that infect farm and domestic animals.

What outputs do you think you will see at the end of this project?

The likely outputs of the project will be the identification and ranking of lead candidate drugs for the treatment of gastrointestinal parasites in livestock and domestic animals. In addition, these candidates will have been assessed for tolerability in mice and gerbils which will act as a basis for future detailed toxicology and safety studies which are required before the drugs are used clinically. Based on the tolerability and efficacy data, calculations will be made to predict the likely dosing regimens required for livestock and domestic animals.

Successful drug candidates will progress to trials in larger animals (under separate licence authority), If successful in these trials the drugs would be added to the anti-parasitic armamentarium and hence improve animal welfare and provide alternative options when the parasites are resistant to currently available drugs.

Data generated will be used to file patents (IP) on the novel drug candidates and published as conference posters or manuscripts (including drug structures) when IP has been secured.

Mice are the least sentient species that is likely to mirror adverse effects that might occur in gerbils.

Who or what will benefit from these outputs, and how?

In the short term, the benefits would be identification of novel anti-parasitic agents with potent efficacy in animal models leading to the generation of new IP and publication of the data.

In the longer term the most important impact would be improvement of animal health and welfare, as the drugs discovered would be effective against parasites that are resistant to the current anti-parasitic drugs. There would also be significant benefits for farmers and owners of livestock who would have additional options for pre-emptive treatment or therapy against intestinal parasites.

The sponsor pharmaceutical company would also benefit due to holding novel IP. Subsequent to successful clinical trials commercial sales of the novel drug would also benefit the pharmaceutical company. As importantly the outcomes of this program would also advance the scientific understanding of anti-parasitic drugs and would potentially open-up new avenues of research leading to novel clinical products.

How will you look to maximise the outputs of this work?

The protocols in this application will mainly be used to provide a service to pharmaceutical and biotech companies specializing in animal healthcare as such any refinements generated during the project will benefit all sponsors. In addition, any 3Rs benefits identified in the study will be widely disseminated at conferences or publications.

Data generated in the models will be used to generate IP which will be available to the scientific community. Following securing IP data will be published as posters or manuscripts.

Species and numbers of animals expected to be used

- Mice: 600
- Gerbils: 1200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult and juvenile mice (6-10 weeks old) and gerbils will be used in this project. These animal species and life stage have been selected as there is a large body of scientific data available using these species for the infection models we plan to run. The models have been validated at multiple centres and have proven to provide good translation to larger animals and successful clinical programs. As there is a considerable body of data available we already understand any side effects of infection and also the duration of the models that will be required to generate robust data.

Selection of the toxicology species for these studies requires consideration of scientific, ethical and practical factors. These include comparisons (similarities and differences) between various species including potential tissue targets impacted by toxic effects and the absorption, distribution and excretion of the test compound. In addition, it is essential to be able to recognize minor acute clinical responses in the test species.

In terms of similarities between species mice and gerbils do broadly have similar anatomy with no major diversity in potential off-target organs that could suffer adverse events.

Whilst mice are not the most common rodent species there is a great deal of scientific data on the use of mice in toxicology studies (Prior H et al, Justification for species selection for pharmaceutical toxicity studies, Toxicology Research, Volume 9, Issue 6, December 2020, Pages 758–770) but this is not the case for gerbils, which leads to some risk in missing minor clinical signs (in addition there is no standardized ‘grimace’ scoring sheet for gerbils which are commonly used to monitor the condition of mice).

Due to these considerations, mice are the preferred species for screening for tolerability of compounds for subsequent efficacy studies in gerbils.

Typically, what will be done to an animal used in your project?

There are two types of protocol in this application, the first protocol will assess the tolerability of novel drug candidates and the second will assess the efficacy of well tolerated drugs in an infection model.

During the tolerability protocol, mice will be given a single dose of the candidate drug. In most cases this drug will be administered orally by gavage but occasionally treatment might be subcutaneous or transdermal onto the back. For oral treatment. mice will be restrained by hand and drug administered through a cannula/feeding tube inserted through the mouth into the stomach. For subcutaneous treatment, mice will be restrained and drug administered into the fat layer at the back of the neck. For transdermal treatment, mice will first have an area of the back shaved followed by topical application. Following treatment, mice will be observed through the cage for 24 hours, animals will be monitored closely for 2 hours at least post dosing, with another review at 4-6 hours post dosing then frequently observed following this. 24 hours post dose animals will be humanely killed.

During the efficacy protocol, gerbils will be subjected to a single infective dose with one or more worm species administered by oral gavage. 6 days later they will be subjected to treatment by the novel drug, a positive control drug, or negative control vehicles either orally by gavage but occasionally treatment might be subcutaneous or transdermal onto the back. For oral treatment, gerbils will be restrained by hand and drug administered through a cannula/feeding tube inserted through the mouth into the stomach. For subcutaneous treatment, gerbils will be restrained and drug administered into the fat layer at the back of the neck. For transdermal treatment, gerbils will first have an area of the back shaved followed by topical application. Following treatment, gerbils will be observed through the cage, gerbils will be monitored closely for 2 hours at least post dosing, with another review at 4-6 hours post dosing then frequently observed following this. Between 48-60 hours post treatment, gerbils will have food removed. Whilst it is possible to identify and quantify parasites in fed animals. food removal reduces the volume of GI contents making identification and counting of gastro-intestinal parasites more accurate and allows overall reduction in group sizes as variance should be reduced. 12 hours after food removal animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

In the tolerability protocol, mice will suffer a small degree of stress due to restraint and administration of the treatment. The procedure should be completed in a few seconds before mice are returned to their home cage. In the majority of cases there should be no adverse effects of the treatment administered. In a small number of cases, mild gastrointestinal disturbances might occur (either diarrhoea or constipation) and may result in abdominal discomfort such as hunching seen. In very rare cases adverse events might occur outside the gastrointestinal tract leading to some mild illness including ruffled coat and reduction in interaction with cage mates.

In the efficacy study, gerbils will suffer a small degree of transient stress due to restraint during administration of parasite larva and the treatment. The procedures should be completed in a few seconds before gerbils are returned to their home cage. Infection with the parasites should only cause very mild (mostly sub-clinical) effects, these include slight

reductions in weight gain or reduction in food intake in some animals. Administration of test agents, should not cause any adverse effects as they will already have been screened in mice. Withdrawal of food for 12 hours will cause some degree of stress for the gerbils but food removal will be timed to coincide with their resting phase to minimize this.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In the mouse tolerability more than 90% of mice should experience mild severity. Up to 10% of mice might suffer moderate severity but this would be of short duration as mice would be euthanized as soon as moderate adverse effects were observed.

In the gerbil efficacy study more than 90% of gerbils should experience mild severity. Up to 10% of gerbils might suffer moderate severity due to a combination of being handled on multiple occasions, the infection procedure, treatment plus some mild adverse effects following infection and treatment.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This area of pharmaceutical research is very fortunate to benefit from excellent models in the early stages of development that do not require animals and significantly de-risk any further studies.

Unfortunately whilst the 'test-tube' models are able to de-risk programs at some point they need to be tested in animal models. Parasites mature through various larval stages once inside the gastro- intestinal tract of animals and during these stages tightly attach to the walls of the upper and lower intestines. This close attachment to the gut walls can also protect the worms from exposure to anti- parasitic drugs as they often attach to deep folds in the tissue and this type of 'sanctuary site' can not be fully modelled in the test tube. In addition the gastro-intestinal tract acts as a pipeline and if the treatment is given by mouth the drug passes through this tube and parasites might only be exposed to the drug briefly and hence might escape the treatment. All test drugs will have been tested for safety in a variety of cell and enzyme tests but it is not possible to test against all of the types of tissue in the gastrointestinal tract plus it is not possible to fully simulate the interaction of the test drug with the trillions of microbes in the gut. For these reasons it is also important to screen the drugs in animals to show that are well tolerated.

Which non-animal alternatives did you consider for use in this project?

Prior to initiating any animal experiments, ant test drugs will have successfully completed a wide variety of non-animal tests. These tests include proof that the drug can inhibit an essential process in the parasite by shutting down certain metabolic pathways.

The drugs will also have been tested against larval stages of the target parasites, specifically the larval stages that is associated with transmission of infection.

Where the test drug has been isolated from a natural product and the drug target is unknown, it is possible to use a small free-living worm called C. elegans to identify the target using well described gene knockout technologies.

It is also possible to screen drugs in sophisticated cell cultures. In these tissue cultures clumps of mammalian cells call organoids are grown into structures simulating whole animal organs that can be infected with parasites.

Why were they not suitable?

In this project C. elegans are unlikely to be used as the drug target is known.

Whilst screening in mammalian organoids is feasible for some parasites they have not been validated for all the parasites required in this project. In addition, organoids are unable to replicate the complex structure of whole animal gastrointestinal tracts and they also are grown in the absence of bacteria which brings into question the validity of the data generated.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In this application we have two protocols.

The first protocol uses mice to test that the candidate drugs are tolerated in animals. The study has been designed to be the minimal feasible size to generate useful data. To minimize the risk of observing severe adverse effects two dose levels will be tested, firstly a low dose and only if this is tolerated a higher dose that is likely to be required in parasite treatment models. Four animals will be used to test each dose level, two male and two female. It could be argued that a single mouse per sex per group would be sufficient but there is an increased risk that borderline adverse effects could be missed using single animals plus there might be a sex bias.

The second protocol uses gerbils to test the anti-parasite efficacy of test compounds. The study has been designed to compare the efficacy of the test compound compared to no-treatment and also the standard of care treatment. As most compounds will be tested following oral treatment, there is a requirement for the test drugs to be suspended in a suitable formulation and there is a risk that the test formulation could also impact on the parasite population in the gastrointestinal tract. Therefore an efficacy model will include 4 groups of animals, one group untreated following infection, one group treated with

formulation vehicle, one group treated with the test compound and one group treated with the standard of care treatment. The group size in these studies will be 5 gerbils per group, meaning a minimum study size of 20 gerbils if only one test compound is assessed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For the tolerability study in mice, we believe that two animals per sex is the minimum feasible number (four aimals only) as there are well documented sex differences in tolerability. The study design uses 'fresh animals' for each dose level rather than escalating the dose in each animal as we believe that multiple rounds of treatment would increase the overall severity of the protocol.

Gerbils are the selected host species for worm infections as the model is already validated and has been used in successful drug development programs. In addition, it is possible to infected different regions of the GI tract with different parasites leading to an overall reduction in numbers of animals used in the program. For the efficacy study, where feasible, we will infect the animals with more than one type of worm simultaneously. This is feasible and has been previously validated as the selected worms infect different regions of the gastrointestinal tract (it is not advisable to co-infect parasites that infect the same section of the GI tract as they will compete for nutrients etc that might compromise the data). Combining two or three parasites in a single model will reduce the total number of animals required to fully assess the test compound for efficacy in the stomach, upper GI tract and lower GI tract. In these studies there is some inter-model variability and some intra-animal variability. Inter- model variability is due to subtle changes in gerbils used but more importantly the stock of parasite larva used to infect the gerbils. Due to this variability we need to include a positive and negative control in each experiment, in addition it is possible that the formulation used to suspend the test drug might impact on the parasite burden so we also need to include a formulation vehicle control. As the test drugs used in this experiment will be used for the first time in animals the inter-animal variability is unknown so it is difficult to reliably calculate groups sizes. Based on published data on variability of burdens in untreated gerbils and gerbils treated with standard of care drugs plus the need to demonstrate statistically significant reductions in burdens treated with test compounds 4-6 animals will be initially required per group. Dependent of the early data generated, these group sizes will be modified to minimize the number of animals whilst maintaining statistical robustness.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All animals used will be from commercial breeders and will use a mix of males and female animals to reduce wastage at the supplier.

We have carefully considered using pilot studies, but due to the small group sizes used in this study there is no real advantage in using pilot studies and the likely outcome would be a larger number of animals used.

Where possible, we will perform efficacy tests on multiple compounds simultaneously to increase the ratio of test groups to control groups. There will be a limit of 3-4 test compound groups per study based on technical limitations of running larger studies.

As previously mentioned, where feasible we will infect the gerbils with more than one type of worm simultaneously. This is feasible and has been previously validated as the selected worms infect different regions of the gastrointestinal tract (it is not advisable to co-infect

parasites that infect the same section of the GI tract as they will compete for nutrients etc that might compromise the data). Combining two or three parasites in a single model will reduce the total number of gerbils required to fully assess the test compound for efficacy in the stomach, upper GI tract and lower GI tract.

Where appropriate, clinical materials (including worms and sections of the GI tract and general tissues) will be retained for subsequent analysis of histopathological changes in the host and development of resistance in the worms.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this licence we are using two stages to test the efficacy of novel drugs to treat worm infestations. In the first stage we will be testing the drugs for adverse effects on mice. The test compounds will have successfully completed a range of in vitro tests and in silico analysis which indicate they will likely to be safe to use. The duration of the study is the minimum feasible that provides a robust tolerability assessment. Mice will be killed in case of unexpected adverse related to the novel treatments.

Historically many potential animal hosts have been tested for suitability as models of gastrointestinal worm infections. Based on a wealth of published data gerbils are the least sentient species in which robust infections can be established. Models in gerbils respond to treatment with standards of care in a predictable fashion based on PKPD modelling. In addition, drug discovery programs using gerbils as hosts have resulted in novel clinical drugs entering the clinic. The adverse effects of infection are very minor and significant clinical deterioration is very unlikely. The sequence of events in the model, will be infection, followed by observation for 6 days, treatment with vehicle, standard of care or novel entity with observation for 2 days, food withdrawal for 12 hours then humane killing, leading to a total duration of under 9 days, The duration of the model is minimised and is only sufficient to allow establishment of infection (and maturation of worm larvae), determination of efficacy of the drug and clearance of dead worms (it would be very difficult to distinguish live and dead worms in gastro- intestinal contents or faeces). Novel drugs used in the model will already have been screen for tolerability in mice and so should be safe to use.

A key question when designing this program of work was the species of animals to be used in the initial screening fir tolerability and also the most appropriate species for the efficacy studies. Selection of the most appropriate toxicology species for these studies required consideration of scientific, ethical and practical factors. These included comparisons (similarities and differences) between various species including potential tissue targets impacted by toxic effects and the absorption, distribution and excretion of the test compound. In addition, it is essential to be able to recognize minor acute clinical reactions in the test species.

In terms of similarities between species mice and gerbils do broadly have similar anatomy with no major differences in potential off-target organs that could suffer adverse events.

There is almost no data available on screening of tolerability of novel test drugs in gerbils and recognition of minor adverse events will likely be more difficult due to less experience of this type of test in the species, we therefore propose to screen for tolerability in mice,

Whilst mice are not the most common rodent species there is a great deal of scientific data on the use of mice in toxicology studies (Prior H et al, Justification for species selection for pharmaceutical toxicity studies, Toxicology Research, Volume 9, Issue 6, December 2020, Pages 758–770) but this is not the case for gerbils, which leads to some risk in missing minor clinical signs (in addition there is no standardized 'grimace' scoring sheet for gerbils which are commonly used to monitor the condition of mice).

Due to these considerations, mice are the preferred species for screening for tolerability of compounds for subsequent efficacy studies in gerbils.

Why can't you use animals that are less sentient?

Non-mammalian animals are limited in their use because they either do not have sufficiently complex bodies to reliably predict adverse effects in higher species. For similar reasons, we are unable to use embryos or very young animals as observation of the potential range of adverse events is unreliable.

Mammals need to be used for the following reasons:

1) For tolerability/adverse events- the organisation of the biological system for the metabolism of the drugs and the expression of its toxicity. It is critical to understand if we are going to see toxicity in organs such as the kidney, liver or cardio toxicity. Insects are organised in a different way.

With the infection with worms, there are different maturation stages and there is need to have a host compatible with conditions appropriate for larval development.

Mice are the least sentient animals that can be used to assess the tolerability of novel test compounds. Animals need to be awake and mobile to allow for the observation of adverse effects. The mice will be juvenile (6-10 weeks old) as this age group have been shown to be sensitive to adverse events that impact weigh change (such as weight loss if food intake is decreased).

Gerbils are the least sentient animal host that has validated data available as a host for gastro- intestinal worm infections. Whilst it might be possible to use mice, historic data generated is less robust, so would require larger group sizes and the data generated would be less reliable. Due to the duration and the requirement for normal eating and drinking it is not possible to use terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored closely for 2 hours at least post dosing, with another review at 4-6 hours post dosing then frequently observed following this. The humane endpoints in the licence will be closely followed to monitor potential adverse events such as abnormal/ laboured breathing, severe diarrhoea, piloerection, changes in mobility to ensure consistent data records. If adverse events are observed the animals will be humanely killed.

Following each model, the clinical course of the animals will be discussed by the research team to ensure and lessons learned will be applied to future models.

As mice will only be restrained on a single occasion and administered the test treatment during that restraint, training is unlikely to be beneficial.

Dependent on the responses of gerbils to restraint, infection and treatment, training of the animals will be reviewed but due to the small number of procedures is unlikely to be beneficial.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE guidelines have been used from the outset of this program. The PREPARE Guidelines Checklist has been completed prior to the application.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an ongoing program of reviewing publications and attending conferences that address the 3Rs. We have also signed up for the NC3Rs newsletter and are in contact the NC3Rs program manager and attend regional, national and international 3Rs symposia.

34. Bat Responses to Global Change

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Bats, Wildlife conservation, Molecular Ecology, Climate change, Artificial lighting

Animal types	Life stages
Bats (Barbastella barbastellus, Eptesicus serotinus, Myotis alcathoe, Myotis bechsteinii, Myotis brandtii, Myotis daubentonii, Myotis emarginatus, Myotis myotis, Myotis mystacinus, Myotis nattereri, Nyctalus leisleri, Nyctalus noctule, Pipistrellus pipistrellus, Pipistrellus pygmaeus, Pipistrellus nathusii, Pipistrellus kuhlii, Plecotus auritus, Plecotus austriacus, Rhinolophus ferrumequinum, and Rhinolophus hipposideros)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to assess threats to British bat populations under human-made environmental changes and how these threats affect their conservation status, using genomic tools.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be

short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Bats are of high conservation concern in Britain and across Europe, but the conservation status of many bats is unknown. To inform accurate conservation assessments and plan more effective conservation strategies, we need to understand how bats cope with and are sensitive to human-made environmental changes. Anecdotal evidence suggests British bat populations have suffered major declines in the past few centuries because of persecution and habitat loss, but empirical evidence of these declines is lacking. Genomic approaches can be applied to identify historic changes in bat population sizes, and therefore help set appropriate targets for species recovery. Genomic approaches can also be used to identify the mechanisms driving how bats respond to environmental changes, which will help guide how we manage bat populations in the future.

What outputs do you think you will see at the end of this project?

All bat species are protected by law in the UK. Yet, we know very little about the population sizes of most British bats and many bat populations are still declining. A major focus of this project is to provide the evidence needed to support bat conservation management through better understanding the factors responsible for bat population declines.

This project will directly benefit the conservation of British bats by providing vital missing information on their population status, conservation requirements and sensitivity to human-made environmental changes. This project will identify the conditions necessary to sustain thriving bat populations and provide evidence of historic bat population sizes. This will help identify targets for species recovery and inform the work of Natural England and conservation organisations. This project will also provide the needed information to predict future threats to British bat populations.

This project will increase our understanding of wildlife responses to climate and land-use changes and of how future changes may affect genetic patterns in animal populations. Finally, this project will answer fundamental scientific questions about mechanisms of responses to the environment and environmental change, as well as the biology of the bat species and their interactions with the environment.

Who or what will benefit from these outputs, and how?

The primary beneficiaries of this project are British bats and bat conservation. The objectives of this project have been developed through consultations with conservation managers and governmental nature conservation bodies to address evidence needs to support the conservation of British bats. Outcomes of the project will be used to provide evidence to Non-Governmental Organisations (e.g. the Bat Conservation Trust) and governmental Nature Conservation Organisations (e.g. Natural England, Natural Resources Wales, Nature Scot) about the management approaches needed to sustain or enhance British bat populations. Evidence provided by this project of historic bat population sizes and factors contributing to bat population declines will be shared with Natural England to inform the conservation status assessment for the studied bat species and identify targets for species recovery.

Throughout the life of this project, data produced will be presented at national and international scientific conferences and published in academic journals. The new information

will provide a new understanding of how animals are responding to environmental changes. Understanding how human- made environmental changes affect the ability of organisms to adapt is a major scientific challenge for biologists, ecologists, conservation scientists, molecular and evolutionary biologists. The assessment of the bat responses to environmental changes and how these responses may affect bat populations will be instrumental for informing environmental policy aimed at managing biodiversity under future global change.

We will make remaining DNA or RNA available to other researchers to reduce the need to obtain tissue samples from animals.

How will you look to maximise the outputs of this work?

Findings will be made available to other scientists through publication in open-access journals and presentations at scientific conferences and meetings. Findings will be communicated to policy makers and conservation managers through lay reports and via our collaborations with bat conservation organisations and the Eurobats Agreement for the conservation of European bats.

We will make remaining DNA or RNA available to other researchers through the CryoArks biobank initiative that aims to enhance diversity, visibility, and availability of zoological samples in the UK for research and conservation purposes.

Species and numbers of animals expected to be used

- 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because the aim of this project is to understand the conservation needs of British bats and their responses to human-made environmental changes, it is not possible to do this work without the capture, sampling and releasing back to the wild of the bats we are studying. To minimise distress and impacts on the population, we do not sample pregnant females. We will commonly sample bats from or near their summer roosts, which mainly include adult females and their juveniles. Because adult males are rarely found in the known summer roosts, we will need to sample juvenile males to get information on impacts of environmental changes on the whole population, both males and females.

Typically, what will be done to an animal used in your project?

Bats will be captured from roosts or field sites, either from their undisturbed natural environment or following environmental manipulation, such as the introduction of artificial lighting. After capture, bats will be held in clean individual cotton bags. They will then be examined to determine their health, age and reproductive condition, and measured. Heavily pregnant females will be immediately released.

We will take a small tissue sample from the bat's wings and occasionally also a small blood sample. The bat's health will be re-assessed prior to release back to the wild.

On occasion, if the colony will be resampled as part of a long-term study, a ring will be placed on the bat's forearm for future identification.

What are the expected impacts and/or adverse effects for the animals during your project?

Bats may experience momentary distress when being captured, and will therefore be kept in cotton bags for at least 5 minutes before entering into procedures, which calms them down.

All bats that are not heavily pregnant will undergo the wing biopsy procedures. Blood samples will only be taken occasionally for specific studies of changes in gene expression in response to environmental changes (<40% of the total number of bats will undergo this procedure). Only adult bats and bats within the healthy weight range for the species will undergo blood sampling procedures.

Bats will experience momentary pain when we take small tissue samples from their wings. When taking a small blood sample from bats they may occasionally experience temporary excessive bleeding that is quickly stopped by applying pressure with a finger.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild (100%)

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because this project specifically studies wild animals in their natural environment to understand the conservation needs of British bats and their responses to human-made environmental changes, it is not possible to do this work without the capture, sampling and releasing back to the wild of the bats we are studying.

Which non-animal alternatives did you consider for use in this project?

I already use computer models to project potential changes in the distribution of suitable conditions for bats under climate change. However, these models cannot tell us how bat populations are responding to environmental changes and which populations are likely to be most sensitive.

The are no non-animal alternative to study the genetic responses and genetic conservation status of bat populations. We are currently testing the efficacy of using oral swabs and faecal droppings to obtain good quality DNA for bat genomic studies, but quality so far is insufficient.

Why were they not suitable?

The are no non-animal alternative to study the genetic responses and genetic conservation status of bat populations. While DNA can be extracted from non-invasive faecal samples or samples obtained from oral swabs, the amount and quality of the bat DNA retrieved from these samples is insufficient for genomic studies. Therefore, it cannot be used to study adaptations to the environment and environmental changes, which require good quality data from across the animal's genome, or give broad enough genomic representation to compare historic changes in the genetic makeup of the bats. Moreover, faecal material for genomic studies needs to be collected directly from the individual captured bat, which need to be placed in a cotton bag for up to an hour to obtain sufficient quantities of faecal material. Hence the time involved in collecting faecal samples is much longer than that required when using a regulated procedure (approximately a minute), and as a result imposes more stress on the animal. In addition, not all animals defecate, and therefore it is more challenging to obtain sufficient samples from each sampling site to carry out the analysis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample size estimates are based on my experience of studying and publishing bat genetic and genomic studies in the past >10 years. I also have in-depth knowledge of statistical design. I will keep the proposed sample sizes under constant review during the project. The sample sizes will also be agreed by Natural England which has statutory responsibility for safeguarding the conservation status of the species concerned.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All efforts have been made to reduce the number of individual bats sampled during this project. I reviewed the scientific literature and best practice guidelines for the study of bats to ensure the minimum number of bats are used, while including a sufficient number of bats to represent the genetic variation of the population and to measure changes in population size and genetic adaptations (10-20 bats per population, depending on the colony size). For the lighting experiments we use the minimum number of sites to obtain sufficient power to detect an effect, based on our experience of carrying out similar experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continually revise the number of animals we plan to use for each study based on the results of previous studies and technological advances.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild bats caught in their natural environment to collect non-lethal small tissue (wing biopsies) and blood samples for genetic studies. The sampling techniques used are the least severe way of obtaining reliable, high-quality sources of genetic material for our analysis. These techniques have been used extensively in previous studies without affecting the long-term wellbeing of the bats. Our methods ensure that any discomfort experienced by the bats will be temporary, minimal and last for a short period before the bats are released back into the wild.

We are only collecting non-lethal samples for genetic analysis. Instead of killing the animals to obtain different tissue samples, as is commonly done in gene expression studies, we are studying gene expression patterns in the blood (which can be obtain without killing the animals) as a proxy for responses across the animal's body.

Why can't you use animals that are less sentient?

This project specifically studies the response of bats to environmental change to understand their conservation needs. We can only obtain this information from studying bats. The methods we use do not involve killing the animals, while sampling immature life stages will involve killing the embryo and subjecting the mother to much higher levels of and longer lasting pain, suffering and distress than the procedures that we carry out to obtain genetic samples.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All methods used have been refined to minimise animal suffering and to consider animal welfare. Bats will be captured by hand or using hand nets from bat boxes/roosts in buildings, netted using mist nets or captured in harp traps in field sites. These are all standard techniques and Natural England's Good Practice guidelines will be followed. The capture of bats is authorised separately by licences from the relevant Statutory Nature Conservation Organisations (e.g. Natural England), and only persons authorised on those licences to capture bats will undertake this work. Harp traps are considered to be a less stressful capture method than mist nets, as the animals do not become entangled, and are provided with a roosting opportunity in the capture bag. However, harp traps only cover a small space, and therefore are only suitable when flight paths are known and in denser forests. Mist nets can cover a larger area, and are therefore more suitable in more open spaces or when flight paths are unknown. Therefore, the combination of mist nets and harp traps is needed to capture different bat species under different conditions. To minimise distress during capture, nets will be checked regularly (every 10-20 minutes, depending on activity levels and temperature) to ensure bats do not get entangled, and only experienced people that are able to remove bats quickly and gently from mist nets will use this capture method.

Lighting experiments will only be carried out outside the roost, 2.5 m away from the roost exit, to minimise disturbance to the roost itself. Moreover, lighting experiments will last for a maximum of two consecutive nights and only for three hours after sunset to ensure that in case some bats have not left the roost due to the light outside the roost they still have enough time to forage once the lights are turned off.

To minimise disturbance to the bat populations, bats will not be sampled during the hibernation season when they are most sensitive. Heavily pregnant bats will be released immediately after undergoing a visual health assessment without being used in procedures to minimise harm to their foetuses.

Lactating bats will be prioritised to be used first in procedures so that they can be released within 1 hour to return to their young. Lactating bats leave the roost for foraging and tend to return to the roost every few hours to feed their young.

Taking small wing samples is currently the least severe way of obtaining reliable, high-quality sources of genetic material because it does not involve killing the animal, as has been common practice in genetic studies in the past. Similarly, instead of killing the animals to obtain internal tissue samples, as is still commonly done in studies of gene expression (the process by which the instructions in our DNA are converted into proteins), we are studying gene expression patterns using small blood samples to give a representation of responses across the animal's body.

Bats will be monitored during and after the procedures and released back to the wild as soon as possible to minimise distress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Bat Worker Manual' published by the Joint Nature Conservation Committee (https://data.jncc.gov.uk/data/e5888ae1-3306-4f17-9441-51a5f4dc416a/Batwork-manual-3rd-edn.pdf), as well as guidelines published by the Bat Conservation Trust and the IUCN bat species specialist group.

For ecological experimental design we follow: Gotelli NJ and Ellison AM (2013) A Primer of Ecological Statistics, 2nd edition. Sinaeur.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date by reading scientific articles and attending conferences. I will also liaise frequently with the named veterinary surgeons and named animal care and welfare officer.

35. Cellular and Network Mechanisms of Defensive Behaviours

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Brain, Computation, Neuron, Behaviour, Threat

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how neurons in the brain represent threatening stimuli and coordinate defensive behaviour. We will record and manipulate neuronal activity in behaving animals exposed to threat, and investigate what are the properties at the network and single neuron level that control the conversion of threat information into defensive actions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The main benefit that will arise from this project is knowledge about how the brain computes the decision to engage in defensive behaviour, and more generally about how the brain processes information. Gaining knowledge about these processes in the healthy brain is an essential prerequisite for understanding what goes wrong in the diseased brain, such as in anxiety or post-traumatic stress disorders. This project will also increase our knowledge of mouse animal behaviour, and in particular will identify stressor stimuli and behavioural signs

of stress, which can be used to refine future animal experiments. Moreover, during the project we will develop new tools for data acquisition and analysis, these and the data will be made freely available and will be of interest to scientists in many different disciplines (neuroscience, mathematics, clinicians, artificial intelligence and machine learning, psychology).

What outputs do you think you will see at the end of this project?

1. Knowledge gained about how the brain computes and executes the decision to engage in defensive behaviour from information taken from the outside world. Gaining knowledge about these processes in the healthy brain is an essential prerequisite for understanding what goes wrong in the diseased brain, such as in anxiety or post-traumatic stress disorders.

2. Knowledge gained about mouse behaviour, as well as new sets of behavioural parameters that can be used as a measure of mouse stress and anxiety.

3. International scientific publications on the new knowledge gained.

4. Large amounts of behavioural and physiology data.

5. Technical advances in cutting-edge technique such as high-resolution behavioural monitoring, high-density electrical recordings and imaging in freely moving animals, and as well as new data analysis and modelling tools, and new software for data acquisition and analysis.

6. New vectors for genetic manipulation of specific types of neurons.

Who or what will benefit from these outputs, and how?

1. The wider neuroscience community will directly benefit from the large amounts of behavioural and physiology data generated by the experiments, as well as from the technical advances and knowledge gained.

2. The general population will indirectly benefit from the knowledge gained. Elucidating the mechanisms of defensive behaviours will promote the development of strategies for managing maladaptive states such as anxiety and post-traumatic stress disorder. Anxiety is present in the general population being often associated with substance abuse, and it is a major symptom of highly prevalent psychiatric and cognitive medical disorders. By contributing to the understanding of the mechanisms by which the brain converts sensory input into defensive behaviour and a state of fear, this project will increase the public awareness of the biological basis of anxiety, and in particular of why anxiety levels vary between individuals and are dependent on the environment and the individual’s past history.

3. Researchers and other professionals with an interest in animal welfare will benefit from the knowledge gained on mouse behaviour, in particular for identifying in a quantitative manner signs and symptoms of stress and anxiety.

How will you look to maximise the outputs of this work?

1. In addition to international scientific publications, knowledge gained by this research will be communicated through scientific talks.

2. Knowledge gained will be directly disseminated with the public through public engagement talks and events, in particular with the local schools and community.

3. All data will be made freely available to the scientific community through dedicated internet- based repositories.

4. All technical advances will be made freely available to the scientific community through deposition of material and methods in appropriate repositories.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The experiments in this proposal aim to improve our understanding of the mechanisms of neuronal computations underlying defensive behaviours in the mammalian brain. This requires studying the intact network as well as investigating how changes to network function change defensive behaviour, and it is therefore impossible to avoid the use of animals for addressing these questions.

Rodents are probably the lowest species for which direct comparisons can be made with the structure and functioning of the human brain, and mice are the species of choice in most areas of biomedical research because of the ease of access to a vast library of existing genetically modified strains, not available for other species. Importantly, the defensive system is similar between mice and humans, and therefore the results of this project will give fundamental insight into the function of the human brain without having to make use of higher mammals.

The use of genetically modified strains that allow targeting of genetically identified populations of neurons is essential for dissecting the individual components of neural circuits participating in information processing. In addition, there is a vast amount of information on the neuron physiology and synaptic transmission of mice, as well as on the anatomy of circuits controlling defensive behaviours, which allows highly refined experimental design.

We will use mostly adult mice because they express a diverse range of defensive behaviours and because that can rapidly change their defensive strategy in an experience dependent manner, two properties that are critical for reaching our scientific objectives. In <5% of experiments we will use neonate/juvenile mice to compare their behavioural and neural data with the adult stage and use this comparison as a strategy for identifying neural mechanisms of defensive behavioural that are only present in adults.

Typically, what will be done to an animal used in your project?

All experiments will be done using mice, and the estimation for animal numbers is based on our previous project licence. We will use a total of 5000 animals, and we anticipate that half of all animals will undergo surgery, with 80% undergoing behavioural experiments. More

than half of the animals will come from genetically modified lines (60%) and the remainder will be wild-type mice.

What are the expected impacts and/or adverse effects for the animals during your project?

The main procedures used in the project require a surgical procedure to gain access to the brain, and adverse effects will mostly result from post-operative complications. Recording and activity manipulation procedures are expected to have very minor adverse effects, repeated exposure to threatening stimuli may increase generalised anxiety. At the end of experiments, or if mice show signs of ill health, distress or suffering, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Post-operative animals may reach moderate severity levels in some cases for a short period of time.

The recording and activity manipulation procedures are expected to have very minor adverse effects, resulting in a mild severity.

During the remainder of the procedures the animals are expected to experience no or very minor adverse effects.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The experiments in this proposal aim to improve our understanding of the mechanisms of neuronal computations underlying defensive behaviours in the mammalian brain. The main aim is to identify the cellular and neural network mechanisms that underlie threat recognition and initiation of defensive action, which requires studying the intact network as well as investigating how changes to network function change defensive behaviour. It is therefore impossible to avoid the use of animals for addressing these questions.

Which non-animal alternatives did you consider for use in this project?

We have considered techniques such as primary neuronal cultures and organoids for investigating neural mechanisms.

Why were they not suitable?

Currently available non-animal alternatives are unfortunately inappropriate because the culturing procedure alters the organisation of the network, and crucially, precludes behavioural assessments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals was estimated based on the experience of our group performing similar experiments and procedures over the last 10 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The overall experimental design relies on using the same animal for performing experiments and controls, as well as for gathering in vitro and in vivo data. This approach reduces the number of animals required because it prevents duplication of procedures for experiments and controls, and because it reduces variability thereby increasing statistical sensitivity. Similarly, by recording repeatedly from the same animals in longitudinal studies, we can obtain more valuable data about the dynamics of neuronal processes from individuals, and increase the statistical power of each experiment, thereby again reducing the number of animals needed, compared to single time-point experiments that require data comparison from large numbers of experimental subjects.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The statistical power of each experiment will be increased by measuring neuronal activity using different state-of-the-art methods simultaneously. In particular, large scale recording methods such as high-density silicone probes allow recordings of several hundred neurons at the same time, thereby reducing the number of animals required by at least 10-fold when compared with traditional methods. Sophisticated statistical and data analysis will be done in collaboration with computational scientists within the establishment, to extract the maximal amount of information from a particular experiment.

Furthermore, the use of techniques such as viral transfection for genetic manipulations minimises the number of animals necessary for research, as it alleviates the need to make transgenic mice expressing or lacking candidate genes, which requires the breeding of many generations of mice.

Importantly, data and findings at all stages of the project will be supplemented with computational modelling, which will further reduce animal use. All experimental procedures are well established in the laboratory and have been used successfully during the previous project licence, which reduces experimental errors and thus animal numbers. Pilot studies involving small numbers of animals will precede definitive experiments so that unpredicted adverse effects and difficulties with experimental assessment can be recognized and corrected early on. These pilot studies will also allow assessment of variations in

experimental outcomes that will lead to improved strategies for minimizing variability. To maximize the data generated from a single animal, different procedures will be done sequentially and contribute to more than one key objective.

The breeding strategy for genetically altered animals will be dependent on breeding performance of individual lines, litter sizes and demand. Where possible, lines will be maintained in a homozygous state, thereby obviating the generation of excess offspring with inappropriate genotypes. When heterozygous breeding is required, wild-type littermates will be used for control experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All experiments will be done in mice. We will use this animal model because it is the lowest species with defensive behaviours and brain structures directly comparable to humans. Two key advantages of this model are 1) the potential for using transgenic animals, which allow highly refined experimental designs that target genetically identified populations of neurons; 2) a vast body of literature on mouse behaviour and the underlying neurobiology, which again allow for highly refined experiments.

Throughout the project the main methods are stereotaxic surgeries, behavioural assays, electrical and optical recordings of neural activity, chemical and optical manipulations of neural activity, and in vitro electrophysiological recordings. These methods are designed to enable maximum information extracted with as minimal pain and distress of the animals as possible. Some of the most important refinements in these methods include the use of high-density silicon probes that allow recording the activity of hundreds of neurons in a single animal. Importantly, techniques used for chronically implanting these probes function in a way that the animals continue to display the entire repertoire of natural behaviours.

Experiments in awake animals will only be performed if the animals are stress-free and experience no visible discomfort. Manipulations of neural activity are genetically targeted to specifical neural cell types, which minimises potential distress. We have also refined stereotaxic surgery procedures in general, including devising precise stereotaxic coordinates that minimise injection volumes and off- target effects. Surgical procedures will be done with appropriate anaesthesia and analgesia. Several datasets will come from in vitro studies in acute brain slices, which we have extensively refined by increasing tissue viability and maximising the number of slices obtained from each animal.

Why can’t you use animals that are less sentient?

A key goal of this research is to ultimately map the findings in our animal models to the neurobiology of the human brain. Less sentient, non-mammalian, species do not readily allow this because their brain structure is vastly different from the human. Also, the defensive behavioural repertoire of less sentient species is considerably poorer, which precludes investigation of higher cognitive functions, such as using spatial knowledge to select and

execute defensive actions in a flexible manner, a cornerstone of our research programme. The experimental plan includes the use of animals that have been terminally anaesthetised. However, anaesthesia precludes the display of defensive behaviours, and therefore experiments in awake animals are necessary for linking neural mechanisms to behaviour.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When performing in vivo recordings under anaesthesia we will ensure that the animals are sufficiently deeply anaesthetised using standard procedures (e.g. pedal withdrawal, tail and ear pinch reflexes, rate, depth and pattern of respiration).

Experiments requiring surgery with recovery will only be used where it is absolutely necessary and we will use adequate analgesia during any surgery and recovery periods to minimize pain that could be experienced by the animals due to surgical manipulations. Analgesia with be given for a minimum period of 48 hours after surgery. For procedures in awake head-fixed mice, great care will be taken to habituate the animals to the experimental setup to ensure they are stress-free.

When performing genetic perturbations, these will be mostly limited to strains where the genetic status and phenotypes are known, and in most cases we will use acute perturbations with viral vector delivered stereotaxically and inducible genotypes, to maximize the specificity of the phenotype and reduce the likelihood of generating severe phenotypes. When using pharmacological agents, dose- response curves will be generated in vitro to guide in vivo application and minimize side effects.

Experiments conducted in the home cage environment represent a significant refinement as they minimize disturbance to the animals (e.g.: moving the animal out of the cage into an experimental arena located in different room) and allow for continuous, longitudinal monitoring of animal behaviour and welfare. This ability not only reduces experimental confounds and increases statistical power, but also provides a unique opportunity for mapping the consequences of experimental manipulations on animal welfare.

Computer modelling will be used intensively as part of this study, which is extremely helpful for designing experiments and maximizing the generation of statistically powerful data.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Refinements to rodent head fixation and fluid/food control for neuroscience. Barkus C et al., J Neurosci Methods. 2022. Nov 1; 381:109795

Guiding Principles for Preparing for and Undertaking Aseptic Surgery. Jennings, M. and Berdoy, M., 2010. A Report by the LASA Education, Training and Ethics Section.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date with 3R developments through the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), including recommendations for best practices in neuroscience rodent experiments. I will further be informed by the local 3Rs group at the establishment as well as animal facility staff and Named Persons, who I will work closely with to continuously implement 3Rs advances.

36. Contribution of Inflammation and Other Host Factors to Tumour Development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

zebrafish, cancer, inflammation, genetically modified, tumourigenesis

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Elucidating the mechanisms regulating tumour initiation and progression with a focus on the role of tumour - host interactions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a leading cause of death worldwide (WHO cancer key facts) accounting for nearly 1 in 6 deaths. Our understanding of cancer biology in later stages of disease have led to many successful therapeutic interventions over the last 50 years. It has become apparent

that many cancers can be cured if detected early. However, there is still a lack of sufficient knowledge regarding the initiation stage of cancer development, which is key to devising efficient strategies for cancer early detection and prevention.

What outputs do you think you will see at the end of this project?

Our work will advance our understanding of cancer development, in particular the earliest molecular and cellular event during tumour initiation. The output of the project will be disseminated openly through journal publications, data deposition in public databases, and presentations at scientific conferences. New knowledge gained from this project will guide future development of cancer prevention and early detection strategies.

Who or what will benefit from these outputs, and how?

Short term - Novel transgenic animals produced through this project will benefit other researchers using zebrafish models to study human disease and developmental processes. Mid term - new mechanisms identified through this project would benefit other researchers and the Biomedicine Industry in the field of cancer biology and cancer prevention. Long term - new knowledge would eventually benefit human patients and society by reducing the cancer disease burden.

How will you look to maximise the outputs of this work?

All research output from this project will be published in open access journals. All data will be deposited in public databases. We also pledge to publish negative data in open access journals and/or BioRxiv to ensure maximum knowledge dissemination. All transgenic zebrafish lines will be shared with collaborators before publication, and these are normally done by presenting early data in research conferences to attract interest from other researchers.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 25500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to address our questions in a living organism which will provide information that is directly relevant to the development of human cancer. Zebrafish develop cancers that are similar to human cancers and 84% of human disease related genes include oncogenes have homologues in zebrafish. Compared to other mammalian models, zebrafish larvae are transparent enabling in vivo live imaging studies of cell-cell interaction which is ideal for research into tumour cell and host immune cell interaction and how such interaction shapes the developmental trajectory of the tumour. During this project, we will maintain adult zebrafish for breeding purposes and we will generate more transgenic stains. Most of our experiments will be carried out using larvae under 5 days old which is before first- feeding and are therefore considered non-protected stages.

Typically, what will be done to an animal used in your project?

We will cross adult Genetically Modified (GM) zebrafish to generate embryos and we will use larvae to perform live imaging studies or we will fix larvae tissue to undertake various staining protocols for histology analysis. We will monitor fish that have the potential to develop tumour using a non-invasive imaging approach to investigate the tumourigenesis process and cell-cell interactions. We will transfer human cells with tumour development potential into zebrafish larvae to investigate their interaction with cells in the local microenvironment.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of our transgenic fish, when bred together to produce compound transgenic fish might have the potential to develop tumour(s) within a few months. We would only perform the tumourigenesis experiment(s) and assess the tumour formation when absolutely necessary for the project. We would only use a small number of fish for this purpose. If fish were to develop tumours we will limit their progression such as observation of abnormal movement and limiting tumour size. As soon as abnormal behaviour were observed or the tumour size reached the permitted size limit, the fish would be culled using schedule 1 killing procedure for histology analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for tumour formation is moderate, and this would only represent a small propotion of our animal usage (<20% in total). Most of our fish usage will be breeding and maintenance of GM zebrafish lines, which is mild. We will have also have a small number of animals used for generating new transgenic lines or tissue specific loss of function for candidate genes using genetic modification technologies, which are also mild. (in total 80%) Significant of proportion of our experiments will use pre-feeding larvae, which is deemed subthreshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals because we are unable to address our questions in any non-animal system and consider it directly relevant to human disease. We need to study the function of different host cell types and genetic mutations and potential drugs usage in the context of a living organism. We use larvae (< 5dpf) where possible, but the final stage of tumourigenesis occurs in adult fish and in some cases we would need to obtain evidence of changes in

tumourigenesis rate to demonstrate how mechanisms that we uncovered in larval models would influence tumourigenesis.

Which non-animal alternatives did you consider for use in this project?

There are co-culture systems that can be used to study immune cell influence on cancer development.

Why were they not suitable?

My lab is working with the initial stages of tumourigenesis and we wish to investigate how host innate immune cells as well as healthy neighbouring cells interact with emerging preneoplastic cells. Due to the complexity of many cell types present in vivo, currently this is not feasible to model using an in vitro simple co-culture system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We ensure that the minimum numbers of animals are used by planning our experiments ahead of time and using proper statistical methods, using the fewest animal numbers needed for reliable results, and where possible using fish in the embryonic stages. We will use optimized protocols for generating genetically modified zebrafish to reduce the number of animals needed to maintain the genetic mutation. We will also use Crispant or F0 gene knockout fish in our experiments to reduce the requirement of generating mutant lines.

To estimate maximum number of fish to be used: for breeding and maintenance we typically require 50 fish per strain per year to produce embryos for experiments. Currently we have over 60 transgenic/mutant lines that are maintained each year, we estimate for the renewed licence we will require 68X50X5=17000 for maintenance. Animals required for additional protocols are mainly larvae and juvenile, the number was estimated according to typical number of animals required for each statistically meaningful experiment, which typically require 15 - 30 per group across 3 biological repeats. But sometimes group size might exceed 60 to achieve statistically meaningful results. We took 30 as an estimated group size and 4 experimental conditions for a standard experiment design. To test 25 candidate genes/pathways in our project within next 5 years, we estimate that we will require 8500 fish for experimentation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will perform pilot experiment with small numbers of fish to allow power calculation. We will use NC3R's Experimental Design Assistant to plan any experiment using adult fish. All trainees working under this license will attend our Establishment's Research Optimisation Course.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of our mechanistic experiments will be carried out at the larval stage <5dpf. Reducing the numbers of adult zebrafish that are not transgenic (wild-type) from transgenic breeding pairs can be minimized by selecting for fluorescent fish early in embryogenesis (before day5). We will work with in house statisticians to ensure that the appropriate numbers of animals are used. We have access to zebrafish with specific genetic modifications, reducing the numbers of animals that need to be generated. Two International stock centres freely distribute zebrafish genetic lines, in the USA and the Europe, as well as other designated breeding establishments. Collaborators also share genetic lines and resources.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish are vertebrates and share many genetics, physiological and pathological features with mammals. Much of our work with zebrafish is in the embryonic form, a developmental stage prior to free feeding (and therefore considered non-protected stages under ASPA).

Zebrafish develop cancers, and the histopathological features of these cancers are highly similar to human cancers, and therefore relevant to our study of human disease. Animals that are less similar to humans (e.g. flies or worms) are not vertebrates, and do not develop cancers with similar features to humans. Zebrafish symptoms develop at a rate where they can be identified and the animals culled before they exceed the moderate severity level.

We use non-invasive imaging approaches to investigate cell-cell interaction during preneoplastic cell initiation and tumour development. Non-invasive imaging is only possible in zebrafish due to the translucency of larvae and near translucency of juvenil fish. We will minimize suffering by ensuring that the zebrafish procedures are done under anaesthetic when possible or by providing analgesia.

We will use chemical treatments to interrogate certain protein function, which would reduce the need for generate additional genetic modified zebrafish. For chemical treatments for adult and larval forms, we will start with published data or the lowest determined dose, to ensure we use the lowest possible treatment dose. We will also select chemicals that have the least obvious side-effects in zebrafish embryos before treating adult zebrafish. We have expert aquatics staff that monitor the fish daily, who, together with the Responsible Person(s), will look for signs of abnormal swimming or eating behaviour that might be associated with distress or welfare issues. Posted, in strategic places throughout the Aquatics facility are posters for fish body condition scoring (DOI: 10.30802/AALAS-JAALAS-18- 000045), welfare assessments (DOI: 10.1089/zeb.2021.0021), pain and distress scores sheet (DOI: 10.1177/0023677216670686) and sign of ill health (reproduced from Arkofsky 2002 by the RSPCA in https://norecopa.no/no/textbase/guidance-on-the-housing-and-care-of-zebrafish-danio-rerio).

Why can’t you use animals that are less sentient?

Most of our experiments use larvae under 5 days before free feeding and adult animal are used for producing embryos. However, experiments conducted in larvae < 5dpf cannot reveal the true tumourigenesis potential, therefore, a small number of zebrafish older than 5 days and adults will need to be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimize suffering by ensuring that the zebrafish procedures are done under anaesthetic when possible and use analgesia. For chemical treatments for adult and larval forms, we will start with the lowest dose, to ensure we use the lowest possible treatment dose. We will also select chemicals that have the least obvious side-effects in zebrafish embryos before treating adult zebrafish. We have expert aquatics staff that monitor the fish daily, and look for signs of abnormal swimming or eating behaviour that might be associated with distress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. We will used the NC3Rs Experimental Design Assistant to assist the experiment design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am following the new research in my field closely through literature and going to research conferences, I will implement new techniques that have a 3Rs impact in my project.

37. DMPK Studies in The Drug Discovery of NCE's

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

DMPK, Drug discovery, Pharmacokinetics, Drug screening, ADMET

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The work carried out under authority of this licence aims to provide a service that will aid the drug discovery and development process for a wide range of therapeutic areas using established and refined Drug Metabolism and Pharmacokinetic (DMPK) Studies in pre-clinical models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The process of discovering, testing, and approving a drug for use in man can take a long time, anywhere between 10 and 20 years depending on the medical need, and as a result

involves a huge financial investment by companies in the pharmaceutical industry. Therefore, decreasing both the time and the cost of developing new drugs has the potential benefit of saving many lives in the long term.

Computational modelling of drugs via Artificial Intelligence (AI), Machine Learning (ML), and Deep Learning (DL) technologies (Vemula et al, 2022) and the increasing use of Zebra fish are helping to speed up the initial screening process for New Chemical Entities (NCE's) and this step is followed by in-depth in-vitro analysis of the NCE's of interest. However, there is still a need for the pre-clinical assessment of the NCE's that may become future drugs to assess whether they have suitable pharmacokinetic (PK)/pharmacodynamic(PD)/metabolic profiles for use in man as the PKPD profile can to a large extent govern the efficacy and safety of drugs (Singh, 2006).

What outputs do you think you will see at the end of this project?

As a Contract Research Organisation (CRO), the main output from this project licence will be to provide a service to generate high quality DMPK data using established protocols for our clients/sponsors that will aid the progression of NCE's along the drug discovery/development cascade for a wide range of therapeutic areas. We anticipate that, in addition to the more obvious translational benefits to drug discovery and development for our clients or by collaborating with academic partners, the work under authority of this licence will also help to provide insights into the basic mechanism of actions of drugs in-vivo and therefore aid the advancement of scientific knowledge for our clients/partners that have required a service for generating DMPK data.

Who or what will benefit from these outputs, and how?

The benefit from the output from this project licence will cover the short term to long term development of novel therapeutics for use in humans, including those suffering from unmet medical needs. Although it is unlikely that in the timescale of this project licence that a NCE for which we provide DMPK service work will be brought to market as a drug, our aim is still that by aiding our clients with their drug discovery cascade that in the long term we can help them to develop NCE's that have the potential to be medicinal drugs of the future. In the short to medium term, within the timeframe of this licence, our expertise in established DMPK studies will therefore aid in the design and development of robust novel therapeutics as candidates on the basis of providing very high quality pre-clinical and informative data within our aim of the reduction, replacement and refinement of the animals that are utilised in drug discovery process with the aim to generate and PK/PD data for the eventual use of NCE's in humans.

How will you look to maximise the outputs of this work?

With the confidential early phase/development nature of the service work it is expected that even though there will be limited opportunities for publishing DMPK data that is part of the confidential early stages in drug discovery, we will make every effort to partner with our clients to publish relevant data output whenever possible for the wider scientific community. However, since the establishment of the company our collaborations with academia have resulted in many publications and it expected that will continue in the future from work carried out under authority of this licence. In any case and whenever possible, our aim would be that from our service work we would look to present short communications or posters at dedicated meetings highlighting any developments in methodologies related to the 3R's.

Species and numbers of animals expected to be used

- Mice: 18450
- Rats: 12550
- Guinea pigs: 160

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The studies to be carried under authority of this licence will use young adult/adult rodent models that will have a fully developed biology using those strains of mice, rats and guinea pigs appropriate for the study requirements. The choice of these animals for pre-clinical DMPK studies is based on the availability of a vast amount of historical data which allows for a direct comparison within a Company's drug development pipeline. These models have been refined to use the least number of animals and giving the most amount of data from the animals.

These animals also have been shown to suitable for use because of the in-house experience in our staff with the husbandry, dosing and sampling required which ensures that the studies are carried out to a high standard with the practical minimum level of stress to the animals.

Typically, what will be done to an animal used in your project?

The protocol for a study will determine what is done to the animals. Typically, the animals will undergo dosing via a host of routes followed by sampling of blood and/or tissues and/or dialysis samples for the determination of the PK parameters of the test compounds. Routinely, these studies last between 8 and 24hrs, and in some studies these animals may be surgically prepared but only when scientifically justifiable (i.e. for robustness, study length, accuracy, frequency and sample volumes etc) and would use established robust surgical procedures and protocols. There may also be some studies where there is a requirement to use preconditioned animals with reversible pathological conditions (i.e. diabetes mellitus) that may impact the PKPD of an NCE. These animals would be sourced from external suppliers or if they are not readily available then they would be preconditioned/pre-treated to induce the reversible pathological condition in-house prior to use on a DMPK study.

Additionally, there is also a requirement for biospecimen/matrix samples from naïve animals that will be specifically sacrificed to provide the control matrix, such as plasma and/or brain, for the analysis of samples which have been collected in DMPK studies.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the studies carried out under this licence will use sub-optimal dose levels which will be enough to allow for the determination of the required PK parameters with very low incidences of expected adverse effects. However, there does remain the chance that there may be an unexpected affects post-dose but from our experience these effects would be transient and followed by full recovery to the pre-dose state of the animal. Using historical in-house data, the adverse effects (less than 1%) include may include pain/inflammation at the site of injection or post-dose changes in behaviour. In longer term studies there may be

some transient weight loss post-surgery or dosing, however our experience has shown that the animals make a full recovery in these incidences.

As best practice, interventional steps will be taken to address any observed adverse effects that impact would impact on animal welfare and the outcome of the study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that the animals will experience mild or moderate severity of pain/discomfort which would be dependent primarily on whether the animals have required surgical preparation and/or preconditioning/pre-treatment prior to use on a study. The major proportion historically has primarily been of mild severity (~70%) with the remaining of moderate severity (~30%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The entire biological system contributes towards the overall ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) characteristics for any NCE while in-vitro models that are used in ADME studies are only useful in looking at particular aspects of the overall process, such as using species specific liver microsomes to measure parameters such as drug clearance/metabolism.

Currently, you cannot mimic for example the liver blood flow or cell-cell interactions in vitro for taking ADMET measurements that can then give the definitive PK/PD for a NCE. Also, for drug tolerability, bio-distribution and dose level determination that would be required for downstream NCE studies the whole organism system is the only suitable model.

Which non-animal alternatives did you consider for use in this project?

It is integral in our approach of providing a service in drug discovery and development, to replace the use of animals for achieving the objectives in this licence and reduce the need for performing in-vivo studies. We continuously look for novel non-animal alternatives, both in-vitro (cell-based) and in-silico (computational) studies. Presently, we employ a number of established assays to pre-screen any potential new drug if the client has not carried out these assays prior to undertaking their request for our services in-vivo.

Examples of these assays include metabolism studies using cryopreserved liver microsomes/hepatocytes, blood/plasma/tissue stability and/or partitioning assays, blood/plasma/tissue binding assays and assessing BBB (Blood-Brain Barrier) permeability using the MDCK (Madin-Darby Canine Kidney) Transwell assays, including the cells

transfected with the drug efflux protein P-gp (P- glycoprotein) that can play an important role in drug permeability into cells.

Why were they not suitable?

In-vitro systems currently lack the complexity required to precisely model pharmacokinetic and pharmacodynamic parameters that are part of in-vivo models. Additionally, it is a requirement to have 'proof-of-concept' in-vivo data prior to further pre-clinical and clinical regulatory studies and therefore, at the moment, non-animal alternatives are not yet suitable and in-vivo studies remain essential for DMPK studies for any new drug candidate.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based on our historical workload, during which we have used protocols utilising the minimum number of animals possible, and then extrapolated to cover for the increasing number of studies expected during the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The estimated number of animals to be used is based on our extensive experience of designing and running studies over the last 18 years.

The minimum number of animals to be used per study is based on expected inter-animal variability and the sensitivity/reproducibility of the procedures to be undertaken so as not to risk the validity of the study. We will seek statistical advice as and when required for any bespoke studies to ensure the minimum number of animals are used to give scientifically valid data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The studies will be optimised so that number of animals used will give the maximum amount of scientifically valid data and therefore minimise the overall use of animals during the drug development process for our clients.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are the research species of choice for drug discovery and development due to their size and the availability of extensive rodent literature data using validated methods that has provided much of our knowledge in DMPK. The choice of animals is also dictated by the governmental regulatory bodies as they demand that prior to first-in-man studies DMPK is provided in two rodent models (from those as requested for use in this licence) and a non-rodent species to ensure that the drug is suitable for human use. Even though regulatory work is currently beyond the scope of this licence, the rodent DMPK data that will be generated under the protocols on this licence can inform the decision-making process and may in some cases be submitted as part of a regulatory process. An example of this may be the generation of screening PK/PD data of NCE's that will form the basis for a regulated GLP (Good Laboratory Practice) study on a potential medicinal drug.

Why can't you use animals that are less sentient?

The animals to be used under this licence are the least sentient that can be used to achieve the required outcomes from regulated procedures performed under this licence. Also, the use of young adult/adult mice, guinea pigs and rats as the gold standard for in-vivo DMPK studies along with the wealth of historical data that has been translated to humans, supports their continued use in future studies. Moreover, their use in pre-clinical DMPK studies has been well documented and validated in the scientific literature and in the pharmaceutical industry over many years across different therapeutic area. These species biologically replicate the complex interactions of the human body for all aspects of the ADME process which cannot be clearly observed in less sentient or at immature life stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The overall premise of this license is that the most refined, most relevant and least invasive methods will be used at each stage of a study for the administration of NCE's and the sampling procedures. We expect the greatest number of compounds to be tested in models with minimum burden on the animals (duration of study, stimulus required for experimental window and end-point). When the experimental question justifies additional burden to the animal (i.e. surgery, single housing or chronic dosing) we always aim to keep such burden to a minimum level and all protocol specific monitoring, post-operative care, pain management will be in line with established in-house guidance and best practice guidelines (i.e. Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery) so as to minimise any welfare costs to the animals that will be used in the regulated procedure.

We also continually review our in-house procedures and each of the Project Licences within the company learn from each other with respect to procedures and refinements. Our NACWO, NVS and NIO also attend regular national meetings and provide us with feedback on learnings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All in-vivo experiments will be carried out so they can be reported in line with the NC3Rs ARRIVE 2.0 Guidelines. Regular literature reviews will be conducted to ensure our work aligns with best practice for the duration of this licence.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The 3R's is an agenda item during all of our quarterly scheduled AWERB (Animal Welfare and Ethics Review Body) meetings. Additionally, regular discussions will be held with the NACWO (Named Animal Care and Welfare Officer), NIO (Named Information Officer) and NVS (Named Veterinary Surgeon) in addition to receiving regular updates from the NC3R's and other suitable international authorities

38. Early Life Origins of Heart Disease In Mammals

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

fetus, cardiovascular, heart, circulation, maternal

Animal types	Life stages
Sheep	embryo, pregnant, adult, neonate, juvenile
Rats	embryo, pregnant, adult, neonate, juvenile, aged
Mice	embryo, pregnant, adult, neonate, juvenile, aged

Retrospective assessment

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine some of the mechanisms via which adverse conditions during pregnancy or early postnatal life trigger an increased risk of heart disease in mammals

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heart disease is one of the greatest killers in the world, causing one in four deaths in the UK today. Therefore, understanding mechanisms that contribute to the risk of cardiovascular disease is important to design interventional therapies. While our genetic makeup interacts with lifestyle factors, such as smoking, an unhealthy diet, or a sedentary life to influence the risk of heart disease, strong evidence suggests that adverse conditions during pregnancy

or early postnatal life are just as, if not more important in determining cardiovascular risk in the adult offspring. This concept of the 'Developmental Origins of Health and Disease' has brought attention to the study of complicated pregnancy, and to consider intervention strategies to reduce the cardiovascular burden as early as possible during development, even in pregnancy. Thus, it is important to undertake this work to understand how suboptimal conditions during development may influence the cardiovascular risk in progeny.

Understanding mechanisms will shed light into designing interventional therapies. The data generated has therefore the potential to hasten translation to rational interventions not only to treat mother but also her offspring. This will help reduce the vast burden of pregnancy-induced origins of cardiovascular disease, thereby having a major clinical, economic, and societal impact on cardiovascular health.

What outputs do you think you will see at the end of this project?

The outputs that we will see at the end of the project will be:

1. Data relating to cardiovascular function, cardiovascular morphology (structure) and underlying molecular pathways in pregnant animals and their fetal, newborn or juvenile/adult offspring exposed to control or adverse pregnancy (e.g. lower than normal oxygenation or hypoxia, obesogenic diet, altered ambient temperature) with and without treatment (e.g. antioxidants). Data relating to cardiovascular function will include measurements of arterial blood pressure, heart and blood flow measured in circulations of interest during basal and stimulated conditions. Data relating to changes in cardiovascular structure may include alterations in the thickness of the walls in the heart chambers and in different vessels (e.g. aorta). Data relating to molecular pathways will include the measurement of signalling mechanisms at the level of the gene and the expression of important proteins.

2. Data relating to cardiovascular function, cardiovascular morphology (structure) and underlying molecular pathways in first and second generation animals raised from control or adverse pregnancy (e.g. lower than normal oxygenation or hypoxia, obesogenic diet, altered ambient temperature) with and without treatment (e.g. antioxidants);

3. Data relating to cardiovascular function, cardiovascular morphology (structure) and underlying molecular pathways in post-partum females exposed to control or adverse pregnancy (e.g. lower than normal oxygenation or hypoxia, obesogenic diet, altered ambient temperature) with and without treatment (e.g. antioxidants);

4. Publications as abstract, papers, reviews and book chapters of Points 1-3;

5. Dissemination of unsuccessful approaches or findings.

Who or what will benefit from these outputs, and how?

In the short-term term (1-3 years), the data outputs of this project licence (e.g. changes in arterial blood pressure, heart rate, cardiac function) will inform us how adverse conditions during early life may increase the risk of cardiovascular dysfunction later on in the adult offspring, as well as in the pregnant and post-partum mother. For instance, is the heart affected more than the vasculature? Which is worse and which may contribute more to triggering cardiovascular disease?

In the medium-term (3-5 years), the data outputs of this project licence will tell us which are the areas to focus on, and what similarities and differences there are between species. The

idea is then to create a layered approach of understanding on the effects of adverse conditions during development on the cardiovascular system across the life course in different species. The work will generate collaborations seeking to address questions with diferent expertise, usually involving work ex vivo in tissues generated from this project. Therefore, other scientists will also benefit from the work.

Combined, all outputs will help to better translate our findings to the human clinical situation. For example, knowledge of common effects of an adverse condition in early life on the heart of the mouse, rat and sheep may precipitate human benefit by designing a treatment with protection across species and thereby likely to be as efficient in humans.

How will you look to maximise the outputs of this work?

The outputs of this work, including the dissemination of unsuccessful approaches or findings, will be maximised at several levels:

Scientific advancement and collaboration: In the longer term (>4 years), the data will benefit the design of therapies in higher vertebrate models of adverse pregnancy with a view to human translation and the design of clinical trials. This will be achieved via collaboration with experts in different fields.

Therefore, the proposed work in this new project licence may hasten translation to relatively simple but novel human clinical interventions to not only treat the mother, but also her progeny. This will contribute to a reduction in the burden of developmental origins of heart disease, thereby having a positive clinical, economic and societal impact on health.

Dissemination of new knowledge. Other pathways to further increase impact will include contacting the funders and the institution's communications office to alert them of the potential influence for human health of the scientific findings. This will lead to press releases, which will be supported by radio and television interviews. In addition, the data will benefit the design of cures to protect the health of the unborn child. The proposed research is therefore likely to be of significant interest and benefit not only to researchers carrying out similar or related research in the field, but also to national and international researchers in other disciplines, such as biochemistry, pharmacology and nanotechnology, as well as cross-disciplinary teams in the pharmaceutical industry. To deliver translational benefit to the nation’s health, wealth and culture we will adopt a number of strategies such as seeking patent protection for any new therapies or diagnostic biomarkers revealed by the research as well as actively engage with the commercial pharmaceutical and healthcare sectors to exploit our research at the earliest opportunity.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 4000
- Sheep: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Types of animals

Working with different mammalian species provides an integrative approach to understanding the problem using each animal species for its own particular strength.

The work with sheep has several advantages. First, the cardiovascular development is similar between sheep and humans, enhancing the clinical translation of the work. Secondly, the sheep is the only established animal model in which the mother and the fetus can be surgically prepared under general anaesthesia with catheters, probes and electrodes to record alterations in physiology in long- term preparations following full post-surgical and anaesthetic recovery. Similar insight does not exist for any other species.

Similarly, the work with rats and mice has several advantages. First, both rats and mice are litter- bearing, meaning that different pups from the same litter can be used for different outcome variables, reducing the number of pregnancies needed (1 pup for studies in vivo (in the living organism), 1 pup for studies ex vivo (after death) in isolated organs, such as the heart preparation, 1 pup for fixation for subsequent histology (using a microscope) studies, 1 pup for freezing of isolated organs for subsequent molecular studies). Secondly, litter-bearing animals means that we can compare outcomes in male and female pups from the same litter, again reducing the number of pregnancies needed. Studies controlling for the effects of sex of the offspring on outcome variables are highly encouraged by the Home Office and grant-awarding bodies. Thirdly, rats and mice have shorter lifespans, facilitating studies across generations in a reasonable time-frame, for example during the tenure of a Project Licence or a PhD.

Work with rats offer an important advantage over mice. Their larger size means that more detailed experiments can be done in the adult offspring in the living animal. This includes, for instance, cardiovascular recording during basal conditions and in response to treatment, e.g. drugs administered. In turn, work with mice offer some important advantage over rats. Previous work in our laboratories and by others have used mice preferentially rather than rats for several studies of adverse pregnancy, for instance studies of maternal obesity during pregnancy. Using mice for some studies in this project licence means that we can compare data generated to a much greater body of existing genetic tools and literature.

Choice of life stages

The studies focus on pregnancy, fetal, newborn and adult offspring as the project is designed to determine the effects of adverse conditions during pregnancy on the cardiovascular system of progeny across the life-course, from the fetal stage through to the adult offspring. The studies also focus on the pregnant mother and the post-partum mother, as there is evidence that adverse pregnancy conditions, such as obesity, can also increase the risk of heart disease in the mother not only during, but also long after pregnancy.

Typically, what will be done to an animal used in your project?

The studies focus on pregnancy, fetal, newborn and adult periods. This is because the project is designed to determine the effects of adverse conditions before and during pregnancy on the cardiovascular system of progeny across the life-course, from the fetal stage through to the adult offspring. The studies also focus on the mother during pregnancy and after birth. This is because evidence suggests that adverse conditions during pregnancy can also affect the maternal cardiovascular health during pregnancy and long after birth.

To study the mother during pregnancy or in the post-partum period, or to study the offspring in the fetal, newborn or juvenile/adult period, typically a pregnancy will be exposed to control (e.g. normal air or normoxia with or without a treatment, such as antioxidants, or glucocorticoids or stem cells or miRNAs) or challenged conditions (e.g. lower than normal oxygenation or hypoxia with or without a treatment, such as antioxidants, or glucocorticoids or stem cells or miRNAs) during the whole or part of the pregnancy (e.g. the last third of gestation). Alternatively, a pregnancy may be exposed to control ambient temperature (with or without a treatment, such as antioxidants, or glucocorticoids or stem cells or miRNAs) or challenged conditions (high ambient tempreature with or without a treatment, such as antioxidants, or glucocorticoids or stem cells or miRNAs) during the whole or part of the pregnancy (e.g. the last third of gestation). In all cases above, the majority of the studies will be ex vivo (after death), i.e. in tissues isolated from animals following Schedule 1 killing. A minority of studies will investigate the function of the heart and circulation under terminal or recoverable anaesthesia following surgery (e.g studies using sheep). Under terminal or recoverable anaesthesia, an animal at the appropriate stage of the lifecourse (e.g. mother during pregnancy or in the post-partum period, or offspring in the fetal (sheep only), newborn (sheep only) or juvenile/adult period) will be surgically prepared with catheters, electrodes and probes to record cardiovascular function in the living organism. Under terminal anaesthesia, typically 1 experiment will be performed in any one animal, lasting approximately 5 hours. For animals undergoing recoverable anaesthesia procedures, there will be typically 1 exposure to recoverable anaesthesia. Then, following 1-5 days of post-surgical recovery, typically there will be 1 experiment performed on any one day, lasting approximately 5 hours. Typically, there will be 4 of these daily experiments following exposure to one recoverable anaesthetic, typically with 1-2 rest days in between experimental days.

Similarly, to study the effect of obesity during pregnancy on the maternal and offspring physiology, non pregnant animmals will be fed a control or an obesogenic diet (typically for 2-3 months in sheep or 3-6 months in rodents) prior to conception. Control or obese pregnancies may then be treated (e.g. typically in the last third of gestation with vehicle or antioxidants, or glucocorticoids or stem cells or miRNAs). As above, the majority of the studies in the mother, fetus or adult offspring will be ex vivo (after death), i.e. in tissues isolated from animals following Schedule 1 killing. A minority of studies will investigate the function of the heart and circulation under terminal or recoverable anaesthesia following surgery (e.g studies using sheep). Under terminal or recoverable anaesthesia, an animal at the appropriate stage of the lifecourse (e.g. mother during pregnancy or in the post-partum period, or offspring in the fetal (sheep only), newborn (sheep only) or juvenile/adult period) will be surgically prepared with catheters, electrodes and probes to record cardiovascular function in the living organism. Under terminal anaesthesia, typically 1 experiment will be performed in any one animal, lasting approximately 5 hours. For animals undergoing recoverable anaesthesia procedures, there will be typically 1 exposure to recoverable anaesthesia. Then, following 1-5 days of post-surgical recovery, typically there will be 1 experiment performed on any one day, lasting approximately 5 hours. Typically, there will be 4 of these daily experiments following exposure to one recoverable anaesthetic, typically with 1-2 rest days in between experimental days.

What are the expected impacts and/or adverse effects for the animals during your project?

It is well established that pregnancy complicated by adverse developmental conditions (e.g. lower than normal oxygenation, or maternal obesity, or exposure to glucocorticoids, or exposure to high ambient temperature) can reduce fetal growth and survival. Our own experience is that relative to controls, adverse pregnancy can reduce fetal growth by 25-

30% in 80% of fetuses. It is also well established that relative to controls, fetuses from adverse pregnancies are much more vulnerable to acute challenges in late gestation, such as compression of the umbilical cord modelling prolonged labour.

Clinically, the reason for this is completely unknown and trying to find out is one of the main points of this project. As it happens clinically in humans, we expect that a large proportion of fetuses from adverse pregnancy (80%) will not survive an acute challenge (e.g. umbilical cord compression) in utero. If fetuses from adverse pregnancy are allowed to deliver naturally, we expect some of them not to survive the birth process (20%). Relative to newborns from control pregnancies, surviving newborns of adverse pregnancy show a ca. 25% reduction in body weight and reduced growth rates until adulthood. Relative to offspring from control pregnancies, we expect that those from adverse pregnancy will be more vulnerable to acute challenges in the juvenile or adult period, and a minority of them (10%) may not survive experimentation in adulthood. Why adult offspring from adverse pregnancy are at increased risk during acute challenges is again one of the main questions that this project is aiming to address.

Administration of substances in sheep can result in (on occasion) preterm delivery/abortion. Therefore, we expect that administration of some substances (e.g. glucocorticoids, antioxidants, stem cells, miRNAs or vehicle) during pregnancy in all species studied may similarly induce preterm delivery/abortion in 10% of animals over the life of the project licence. It is also possible that administration of substances may induce inflammation, swelling and infection in 10% of animals studies at any stage of the lifecourse, over the life of the project licence.

While under terminal anaesthesia, some protocols may require surgical implantation of probes or occluders, or the application of vessel occlusion. Some of these procedures may lead to unexpected bleeding. If more than 10% of the estimated blood volume of the animal is lost, then the animal will be killed by a Schedule 1 method.

A minority of experiments in the newborn, juvenile or adult offspring require surgery under recoverable anaesthesia. All surgical procedures will be carried according to the Home Office Minimum Standards for Aseptic Surgery. Through previous work, we have gained significant experience with experimentation on rats and sheep following surgery. Pain killers will be administered as required, judging from the animal behaviour. In some animals, arterial catheters will be placed which allow for blood sampling. The health of the animal can then be monitored through measurements of blood gases. For example, we will be able to ask if oxygen levels in blood are normal? We expect most of the animals to survive, but there is an increased risk of death (20% over the life of the project licence) during or following surgery under any type of anaesthesia. In preparations, where the fetus is surgically prepared with significant instrumentation (e.g. catheters, probes, eletrodes, occluders) there is a greater chance of accidental occlusion of the umbilical cord and fetal death (20% over the life of the project licence). Pregnant animals undergoing recoverable anaesthesia can also present with abdominal hernias (10% over the life of the project licence). To minimise this possibility, animals are dressed in tubigrip around their abdomen to increase support.

Some protocols require study of the animal after nerve transection or removal of an endocrine (hormonal) organ (e.g. removal of the nerve that is attached to the carotid body or removal of the adrenal glands). Denervation of the carotid bodies does not produce any resultant harm. In fact, carotid body denervation is currently being trialled in human patients to treat hypertension. In contrast, removal of the adrenal glands can trigger adverse effects on blood volume and blood pressure over prolonged periods of time. Adrenal insufficiency in man and other animals is known as Addison’s disease and these individuals through

hormone insufficiency can develop low blood pressure over a period of months. To minimise this possibility, no post-natal animal will be studied and kept for longer than 1 month after surgical removal of the adrenal glands.

In the event of post-operative complications, such as a catheter being removed, animals will be killed unless such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence (bursting open), uninfected wounds may be re-closed on one occasion.

We estimate that most animals will recover well from surgery under recoverable anaesthesia. However, we expect that 10% of animals will not recover well. In all cases, the animal's food intake and ability to pass faeces will be closely monitored at least twice daily. Following NVS advice, any animal that does not show appropriate food intake, defecation, blood gases and blood glucose concentration for >72h will be killed by a Schedule 1 method or earlier if its condition deteriorates before this point.

For studies involving a scan of the heart in the conscious animal, restraint of the animal is necessary. Usually, we use the same person for restraining any one animal, so the animal gets used to the environment, and we minimise the duration of restraint, typically 30 minutes. Combined, these strategies minimise the animal's discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Species: Sheep
- Mild: 80%
- Moderate: 20%
- Species:
- Rat Mild: 90%
- Moderate: 10%
- Species:
- Mice Mild: 90%
- Moderate: 10%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In some experiments, we must use whole animals or organs isolated from animals because function, for instance in the cardiovascular system, is regulated by complex networks, none of which have been reconstituted completely in computer models. The overall system is not well enough understood to make mathematical modelling useful.

Which non-animal alternatives did you consider for use in this project?

There are no suitable non-animal alternatives to use in this project for the majority of the work. A small component of the work could be achieved by investigating isolated organs and tissues after death, from non-regulated investigation in PM tissue/organs. It may be possible in future to extend some of the work by using cell lines to identify possible signalling pathways and to test candidate therapies.

Why were they not suitable?

For the majority of the work, no non-animal alternatives are suitable to use in this project as none can model the effects of adverse conditions during embryonic/fetal development in programming an increased risk of cardiovascular disease in the adult progeny, or in the post-partum mother.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals that we will use in this licence from experienced use of animals, as detailed in more than one retrospective review using these species.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental design was created with the NC3R's Experimental Design Assistant to ensure reproducibility using the least number of animals to satisfy statistical power for anyone measurable output.

To ensure the minimum number of animals is used in this project to address all objectives, we have considered the choice of species very carefully. Sheep are used in some experiments because it is the only established animal model in which the mother and the fetus can both be surgically prepared under general anaesthesia with catheters, probes and electrodes to record alterations in physiology in long- term preparations following full post-surgical and anaesthetic recovery. Similar insight does not exist for any other species. However, we will reduce the number of experiments where surgically prepared animal preparations are required, and the majority of the work will be done in tissues isolated from animals after death. A large component of the work will be done using rats and mice. These species are litter-bearing, meaning that different pups from the same litter can be used for different outcome variables, reducing the number of pregnancies needed (1 pup for studies in vivo (in the living organism), 1 pup for studies ex vivo (after death) in isolated organs, such as the heart preparation, 1 pup for fixation for subsequent histology (using a microscope) studies, 1 pup for freezing of isolated organs for subsequent molecular studies). Secondly, litter-bearing animals means that we can compare outcomes in male and female pups from the same litter, again reducing the number of pregnancies needed. Studies controlling for the effects of sex of the offspring on outcome variables are highly encouraged

by the Home Office and grant-awarding bodies. Therefore, this significantly contributes to the 3Rs principle of reduction as enshrined in EU Directive 2010/63.

Where relevant, multiple experimental designs will be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. Therefore, in the same animal, there may be studies in the living organism as well as in isolated organs after death. For most experiments, the study design will adopt methods of analysis previously published extensively by our group, which compares 4 groups: control and experimental groups with and without a treatment or intervention. For example, outcomes from normoxic (normal air) or hypoxic (lower than normal oxygenation) pregnancies with and without treatment with an antioxidant. Control groups treated and untreated are necessary, as the treatment may affect normal and complicated pregnancies differentially.

Sex differences are an important consideration in the risk of developing cardiovascular disease. Therefore, assuming a 1:1 ratio of males to females, the number of animals required per outcome variable will be at least doubled to be able to address sex differences. In such cases, statistical analysis able to compare three factors comparing treatment, intervention and sex will be adopted (e.g. a Generalised Mixed Linear Model; SPSS).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will optimise the number of animals used in this project from 1) pilot data and 2) by using multiple data obtained from the same animal. For instance, we will obtain data from the living organism as well as from tissues isolated from them after death. In addition, we routinely share tissues generated from projects for collaborative studies by other investigators. About 20% of our publication output is derived from such collaborative studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project has been designed such that the majority of the work can be achieved by investigating isolated organs and tissues. For example, after death, the function of the isolated heart and vessels can still be investigated, as well as experiments at the level of the cell and molecule. Comparatively smaller components of the work will involve studying whole living animals under terminal anaesthesia, or conscious animals which have been surgically prepared under general anaesthesia. In some cases, it is necessary to study conscious animals, as anaesthesia can impair normal cardiovascular function.

Experiments will only be performed following appropriate post-surgical recovery. We will keep suffering to the minimum by using procedures with the least possible severity, and by subsequent monitoring with veterinary advice.

Why can’t you use animals that are less sentient?

Other projects under licence are studying the effects of adverse developmental conditions in less sentient species, using birds, to isolate the effects of challenges on the embryo independent of effects on the mother and/or placenta. However, to increase the clinical translation of the work, one has to extend those studies using mammals, which is the purpose of this project.

The development of the cardiovascular system in other less sentient species, for instance reptiles, amphibians, worms or flies is very different than in humans, as these species are ectothermic (creatures that must rely on the temperature of their physical environment to regulate their internal body temperature), in addition to not having a placenta. The regulatory mechanisms of cardiovascular function in such species are not well understood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

With experience from previous work, we have streamlined and refined surgical procedures that reduce bleeding, shorten anaesthetic exposure and improve port-surgical recovery. For example, most surgeries under recoverable anaesthesia are completed within 2.5 hours. We have found that exposure to recoverable anaesthesia for <3h significantly accelerates post-surgical recovery minimising harms.

We will refine protocols by ensuring that all surgical procedures are carried at least to the Home Office Minimum Standards for Aseptic Surgery. In addition, any animal exposed to surgery under recoverable anaesthesia will be observed and scored for any signs of pain, administered pain killers and antibiotics, appropriate for the species and life-stage, to minimise discomfort and infection. We will explore palatable substances for voluntary pain treatment rather than injection such as flavoured paste or apples (sheep). This will minimise possible resultant harms and maximise physiological outcome.

In some animals, arterial catheters will be placed which allow for blood sampling. The health of the animal can then be monitored through measurement of blood gases and acid/base status on a daily basis, maximising monitoring, post-operative care and pain management.

The animal's appetite and general demeanour will be noted. The rectal temperature will be taken to determine changes in body core temperature. Alternative food may be provided (e.g. offering grass to sheep to encourage eating). The animals wil, be weighed routinely to ensure appropriate body weight is maintained.

Further refinement such as the use of group housing, sedation, acclimatisation to handling and environmental enrichment will be implemented to ensure the animals are less stressed and well cared for. The animal's quality of bedding will be reviewed routinely. We will continue our practice to consult with the NACWO to balance the harms and improve the welfare of the species we are using.

A component of the work involves protocols in sheep. Whenver possible, sheep will be housed as flocks in barns and paddocks with access to hay and grass. The duration of time that sheep will spend indoors in a laboratory environment is always minimised.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, we follow the NC3Rs' ARRIVE guidelines, the LASA guidelines and the PREPARE guidelines.

For example, we will refer to the latest edition of the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017, at the time of preparing this project licence application.

The Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines issued by NORECOPA (https://norecopa.no/prepare) covers all stages of quality assurance, from the management of an animal facility or population to the individual procedures which form part of a study.

We will refer to specific guidance or position papers from the Laboratory Animal Science Association, (LASA) https://www.lasa.co.uk/current_publications/. For example: Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T (2018) PREPARE: guidelines for planning animal research and testing. Lab Animal 52(2): 135-141. doi: 10.1177/0023677217724823.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Project licence holders are ultimately responsible for implementing the 3Rs within their work. Therefore, the project licence holder will have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3Rs opportunities.

We will use of other resources, such as Norecopa https://norecopa.no/databases-guidelines. Regular consideration and reflection of the latest practical guidance from Laboratory Animal Science Association (LASA) will provide additional sources of new recommendations and advances in animal techniques.

39. Enhancing Control of Poultry Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Chicken, Parasites, Bacteria, Microbiota, Vaccines

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	juvenile, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project is intended to generate new data and knowledge about (1) fundamental biology and genetics of coccidial parasites and intestinal bacteria, (2) host immune responses, and (3) the interactions and co-interactions of parasites and bacteria with the chicken.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious diseases of chickens can compromise meat and egg production, cause severe welfare problems, and be a source of human zoonotic disease. There is a requirement for new approaches to control pathogens that infect chickens for several reasons including pathogen resistance to antimicrobials, legislative restrictions on the use of some in-feed

drugs, and consumer concerns about chemical residues in meat. New types of control (i.e. vaccines and alternative compounds such as plant extracts) will be required, supplemented by breeding of poultry with increased resistance to infection, improved immunological responses to vaccines and optimised interactions with gut bacteria. This project is intended to address fundamental issues related to control of parasites with the greatest impact on broiler chickens (Eimeria, cause of the disease coccidiosis) and their interactions with bacteria. The project is organized into seven work packages (WP).

WP1: Identification of target antigens for recombinant vaccine development

A panel of proteins has been identified as candidates for use in new anti-Eimeria vaccines, but to date none have reached the market. While experimental vaccine performance has been promising, reducing parasite replication and host pathology, improving performance and welfare, these prototype vaccines are not currently competitive with drugs used in routine prophylaxis. Building on the vaccine candidates that have already been identified and will be tested further in WP2, we will continue to identify and test the vaccine potential of new candidates using our established protocols, bioinformatics and cell biology of host-pathogen interactions. This work will include studies of basic parasite biology to identify essential proteins and processes, including genetic manipulation of laboratory parasite lines.

WP2: Development of vectored vaccines for poultry

With a need to vaccinate large numbers of animals against multiple diseases the poultry industry leads the way in take-up of new vaccines, including live replicating and killed recombinant ‘vector’ vaccines. Many vectors have been tested experimentally but commercial usage is limited. There is a need for vaccination strategies that are non-invasive, appropriate for mass administration and have capacity to induce long-lasting protection against a range of different pathogens. We aim to continue development of novel vaccination strategies including use of transgenic parasites and killed yeast lines to deliver vaccines via the oral route. Parasites produced here will also be used in in vitro studies wherever possible to accelerate vaccine development while reducing the use of live chickens.

WP3: Validation of novel anticoccidial feed or water additives

As demands for alternatives to anticoccidial drugs intensify interest in feed or water additives such as botanicals, botanical extracts, enzymes and other molecules is increasing. We currently use an in vitro model as a screening tool to identify candidates, but final efficacy will be verified using chickens reared under field conditions.

WP4: Understanding Eimeria population biology

The widespread occurrence of anticoccidial drug resistance combined with strong legislative and consumer driven requirements for reduced antimicrobial use in livestock production has increased demand for vaccines to protect against Eimeria. Seven Eimeria species have long been recognized to infect chickens; however recent detection of three new Eimeria species circulating in chickens across much of the southern hemisphere that can escape from current vaccines has revealed unexpected levels of complexity. The consequences of vaccine breakthrough by cryptic parasites can include very high prevalence of sub-clinical infection as well as outbreaks of clinical disease. The occurrence, characteristics and genetic flexibility of Eimeria field populations is unclear. We will employ new sequencing technologies and traditional parasitology to improve Eimeria genome resources and characterise Eimeria field populations, exploring the impact of drug or vaccine selection and mixed infections to improve control of disease.

WP5: The genetic basis of susceptibility/resistance to coccidiosis

Genetic resistance of chickens to infectious disease is well documented, including coccidiosis caused by Eimeria. Understanding genetic resistance provides a possible means to breed flocks that are naturally protected against disease. We aim to explore resistance/susceptibility traits during infection including body weight gain, intestinal structure, circulating immune cells and proteins and parasite replication, plus chicken activity and behaviour to identify genetic, biological and visual markers that can be used in selective breeding and routine husbandry of chickens to improve resistance to disease.

WP6: Understanding the consequences of pathogen interaction and impact on gut bacterial populations

Eimeria infection of chickens can alter, and be influenced by, the composition of the intestinal microflora, in some examples modifying colonisation of specific pathogens such as Campylobacter jejuni. We aim to use next-generation sequencing techniques to further define these interactions to (i) improve poultry health in terms of altered colonisation and/or pathogenesis of bacterial pathogens, (ii) reduce the risk of zoonotic transmission to humans through the food chain, (iii) explore the host genetic contribution to variation, and (iv) evaluate interactions in the presence of known anticoccidials or alternative additives.

WP7: Explore the use of embryonated chicken eggs as replacements for hatched chicks in studies with Eimeria

A small number of lines of Eimeria have previously been adapted to replicate in embryonated chicken eggs. We aim to resurrect this technique and explore its use to replace some studies with hatched chicks.

What outputs do you think you will see at the end of this project?

The work will generate new data and knowledge about (1) fundamental biology and genetics of coccidial parasites and intestinal bacteria, (2) protective host immune responses, and (3) the interactions of parasites and bacteria with each other and the chicken. These outputs are worthwhile in their own right because they contribute to understanding of the pathology and impact of serious diseases. They are also essential for more targeted specific objectives:

Identifying Eimeria antigens that induce immune protection or play substantial roles in the biology of the parasite is critical for downstream development of new vaccines that remove the need to produce live parasites for use in current vaccine formulations, and to reduce routine drug use.

Validating the beneficial effects of candidate botanicals, botanical extracts, enzymes and other molecules identified using in vitro tests as candidates to improve control of Eimeria can improve the welfare of farmed chickens and reduce routine drug use.

Progress towards **new vaccine vectors** that can be used to immunise against multiple pathogens, including species of Eimeria in addition to viruses and/or bacteria, and can be administered safely (orally) from day of hatch.

Improving understanding of Eimeria parasite population structure will be important to optimise future vaccination efficacy and safeguard vaccine longevity, while also facilitating reduced use of drugs in poultry production.

Breeding for disease resistance is an additive approach that requires understanding of host genetics linked to resistance and improved responses to vaccination. Mapping sequences within the chicken genome linked to resistance/vaccine responsiveness will provide genetic and phenotypic biomarkers that can facilitate downstream development of tools to estimate disease susceptibility and inform future breeding strategies.

Understanding the contribution of Eimeria to colonisation of chickens by bacterial pathogens such as Campylobacter species can improve control of zoonotic disease.

Understanding interactions with gut bacterial populations can improve poultry health, welfare and productivity.

Using embryonic chicks to work with Eimeria offers opportunities to replace use of hatched chicks and access some parasite lifecycle stages with improved efficiency.

Who or what will benefit from these outputs, and how?

New vaccine vectors offer several benefits to:

- poultry, including fewer vaccinations/injections; better disease protection; less handling; wider uptake of vaccines
- farmers, reducing costs of pathogen control, improving ease of vaccine or feed additive administration
- animal health companies, maintaining a competitive position in markets for control of coccidiosis
- consumers, reducing the cost and improving availability of poultry products
- the environment, reducing use of antimicrobial and anticoccidial drugs.

Commercial development of new anticoccidial vaccines based on vectored proteins would have many benefits including use of none or fewer birds for production of the parasites used in existing live vaccines, cheaper vaccines, and wider uptake. The UK leads the world in manufacture of live attenuated coccidiosis vaccines but there are major issues associated with the need to incorporate many ‘lines’ of parasites in order to induce immune protection. Figures from industry indicate that more than 2 billion doses of Paracox are sold each year requiring sacrifice of >250K birds for parasite production. A prototype multi-valent vaccine will ensure that the UK animal health industry has a solid foundation from which to retain a leading position on coccidiosis control, contributing to overall wealth creation. Inclusion of vaccine antigens protective against other pathogens such as Campylobacter jejuni can improve health and welfare further, while reducing the number of vaccinations required.

Consideration of new and emerging parasite types may be essential in the absence of drug-based prophylaxis.

Identification of markers that can be used in selective breeding of chickens that are more naturally resistant to Eimeria, or respond better to vaccination, offer benefits to poultry, poultry consumers and producers, and the environment, as outlined above. Reducing the occurrence of ill health in chickens will lower the overall cost of poultry products, benefiting consumers as well as production and distribution networks.

Understanding interactions between Eimeria and bacterial zoonoses can improve poultry product quality, reducing risks to consumers and increasing confidence in food supply. Understanding interactions with the 'healthy' gut bacteria found in chicken intestines can improve chicken health, welfare and productivity. Improved broiler chicken gut health is expected to lower demand for antimicrobial intervention, reducing drug use in livestock

production. Lower antimicrobial consumption will reduce selection for antimicrobial resistance in enteric and environmental microbial populations, and reduce antimicrobial flow into environments around chicken production systems.

Indirect benefits include staff and students working on the project who will receive training in laboratory and simulated farm level settings, including a range of protocols that can only be applied with live animals and can also be used to answer a variety of experimental questions beyond the remit of this work. The national and international scientific community will benefit from improved understanding of Eimeria, their interactions with bacteria, and provision of improved vaccine vectors for poultry.

How will you look to maximise the outputs of this work?

All data produced from these studies will be published in Gold Open Access peer reviewed journals, as mandated and supported by the funding bodies. In addition to data, protocols and standards developed or applied will be described, providing resources and benchmarks for comparative studies. Data such as DNA or RNA sequences (e.g. defining bacterial populations or genes that are active) will be submitted to open repositories, specifically the European Nucleotide Archive (ENA), linked to the DNA Data Bank of Japan (DDJB) and GenBank. Published studies will include results of null or unassociated measures to share awareness. Results will be shared with peer audiences through national and international conferences (e.g. British and World Veterinary Poultry Association meetings, British Society for Parasitology).

Results and progress will also be reported in industry journals and magazines, as well as live events such as the Pig and Poultry Show, to ensure dissemination to relevant target audiences.

A series of collaborations with partners in industry and academia will enhance outputs for the work, including links to researchers in Asia, Africa and Central/South America, as well as Europe and North America.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Chickens will be used throughout these studies, recognising that they are the target animal and not just a model. A range of chicken types will be used, including (i) specific pathogen free (SPF) chickens to permit optimal parasite production and accurate assessment of measures with minimal background variation, (ii) commercial broiler and layer chickens, representing target populations and providing real- life examples that can be reared under simulated industry conditions, and (iii) genetic knockout (KO) chicken lines lacking specific immune functions to assess the impact of the host immune response on parasite replication, pathogenicity and vaccine response. Chicks will typically be received at day of hatch for vaccination and equivalent characterisation studies, or 2-3 weeks of age for parasite

production and selection studies. Studies with embryonated chicken eggs will conclude prior to hatching.

Chicks will typically be used up to six weeks of age (maximum of ten weeks old), recognising that coccidiosis is primarily a problem during the early phase of chicken growth.

Typically, what will be done to an animal used in your project?

Experiments will usually fall into one of four types, with some variation around specific procedures.

1. Parasite amplification, selection or characterisation

SPF chickens will typically be used for these studies, although a range of inbred or genetically modified chickens may be used (e.g. the range available at the National Avian Research Facility [NARF]).

Chicks will usually arrive between two and three weeks of age and undergo a minimum seven day settling in period. Once settled, chicks will receive a parasite dose (usually by oral inoculation, mimicking the natural route of infection). Doses are carefully managed based on previous experience to prevent the occurrence of clinical disease. Chicks will usually be kept in groups in cages with wire gridded floors and a range of environmental enrichments. Primary outputs include recovery of fresh parasites from faecal material collected below the wire gridded floors or from tissues collected post- mortem. Supplementary steps may include the addition of selection during parasite replication (e.g. dietary drugs, previous controlled infection), and measures of performance (e.g. body weight gain), parasitology (e.g. faecal excretion of parasite 'eggs' – known as oocysts), immunology (e.g. blood collection from the wing vein), gut integrity (e.g. faecal or blood markers), and pathology (e.g. lesion scoring) during or after infection. Measures that are being assessed for potential to replace invasive procedures or improve endpoint precision include use of accelerometers to measure chicken movement. Chickens in this type of experiment will usually be kept for seven to ten days after parasite inoculation, undergoing a single procedure (oral dosing) or two if blood is collected.

2. Efficacy assessment of novel anticoccidial controls (experimental vaccines, candidate feed additives)

A wide range of chicken types will be used for these studies. SPF, inbred or genetically modified chickens will be used to assess immune responses and responses to infection or vaccination with limited host genetic variation, permitting increased reproducibility and small group sizes. Commercial broiler and layer chickens will be used as representatives of the target populations, providing data that is directly relevant to field populations. Chicks will usually arrive within one day of hatch and be accommodated in groups in floor pens or cages following industry standard practices. Chickens may be accommodated at commercial stocking densities to mimic field conditions. Most chicks will undergo a minimum seven day settling period unless a commercial vaccination strategy is followed, in which case vaccination/feed additives may be administered at any time after arrival. Vaccines/feed additives will usually be delivered by oral inoculation, but for vaccines alternatives include administration into muscle or under the skin. Chicks will be infected by oral inoculation, mimicking the natural route of infection, and doses will be carefully managed based on previous experience to prevent the occurrence of clinical disease. A range of measures will be used to define the effects of parasite infection and/or vaccination including measures of performance, parasitology, immunology, gut integrity, and pathology during or after infection, as described above. Chickens in this type of experiment will usually be kept for

five to seven weeks, including periods of vaccination or additive supplementation, infection and recovery. The number of procedures will vary depending on the protocol (e.g. regularity of repeat vaccination or blood collection), but rarely exceeds five per bird.

3. Parasite interaction with host microbiota

In most studies commercial broiler or layer chickens will be used as representatives of the target populations, providing data that is directly relevant to field populations. SPF, inbred or genetically modified chickens may be used to assess responses with reduced host genetic variation, permitting increased reproducibility and small group sizes. Chicks will usually arrive within one day of hatch and be accommodated in groups in floor pens or cages following industry standard practices. Chickens may be accommodated at commercial stocking densities to mimic field conditions. Most chicks will undergo a minimum seven day settling period unless a commercial vaccination strategy is followed, in which case vaccination may be administered at any time after arrival. Chicks will be infected with

Eimeria by oral inoculation, mimicking the natural route of infection, and doses will be carefully managed based on previous experience to prevent the occurrence of clinical disease. Co-infections with bacteria such as Campylobacter coli or C. jejuni may be initiated by separate oral inoculation. A range of measures will be used to define the effects of parasite infection and/or vaccination including measures of performance, parasitology, immunology, gut integrity, and pathology during or after infection, as described above. Variation in specific bacterial colonisation and shedding, or in composition of bacterial populations in the gut, will be quantified by microbial culture and molecular techniques from faeces or tissue/intestinal samples collected post-mortem. Chickens in this type of experiment will usually be kept for up to four or seven weeks, depending on age at arrival. The number of procedures will vary depending on the protocol (e.g. regularity of repeat inoculation or blood collection), but rarely exceeds five per bird.

4. Parasite inoculation into embryonated chicken eggs

Embryonated chicken eggs will receive up to two injections containing antibiotics and purified parasites to grow egg-adapted parasite lines. Eggs will be incubated at 41oC to support parasite development.

Embryos will be harvested for purification of parasites to study parasite cell biology. Each study is expected to last seven to ten days and chicks will not be hatched.

What are the expected impacts and/or adverse effects for the animals during your project?

Oral inoculation is straightforward in chickens and very well tolerated. Parasite doses are carefully managed based on previous experience to prevent the occurrence of clinical disease. Clinical signs are non-specific but include ruffling of feathers, paleness of comb and wattles, consistently closed eyes, wet droppings, diarrhoea and/or bloody faeces, or reluctance to move. Chickens exhibiting two or more of these signs will be removed and euthanasia will not be delayed for any experimental or procedural reason. Chickens will also be removed if a single sign persists for > 24 hours. Bacteria inoculated in co-infection studies are well defined strains known to be non-pathogenic for chickens.

Blood collection from the wing vein can result in a localised haematoma but these are tolerated well. The risk is far lower than sampling from the jugular vein and consequences usually resolve within 24 hours.

Other adverse effects include conditions common during commercial chicken production including lameness and feather plucking. Regular inspections and good husbandry will be used to minimise the occurrence of these effects.

Injection of embryonated eggs is routine in the poultry industry during in ovo vaccination and is well tolerated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The impacts and adverse effects of the procedures described here are expected to be mild for most individuals. However, a moderate severity is indicated in recognition that host susceptibility can vary considerably, especially in hybrid commercial chickens, possibly reaching ~5%.

Industry data indicate that 10-30% of pedigree and commercial broiler chickens will experience enteric dysbiosis by five weeks of age when reared under standard commercial conditions in the absence of antimicrobial prophylaxis. Chickens that experience dysbiosis will be removed from the study, not retained and treated.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Eimeria parasites do not grow productively in cell culture and can only be obtained from infection of live animals. Similarly, studies of anti-parasite control and host-pathogen interactions require use of live animals to assess effects on parasite growth and consequences of infection. Over the past two decades our lab has significantly improved propagation of Eimeria in cell culture, and we routinely use immortalised cell lines for the study of early invasion events in the parasite's lifecycle and effects of potential interventions, significantly reducing the use of chickens. We will continue to use cell culture whenever possible, but studies beyond the first step in parasite replication cannot be supported in this manner. We will also continue to test new methods for full propagation of the parasite life cycle in cell culture or tissue explant systems. However, currently the only way to amplify parasites, perform genetic crosses, maintain pure strains, study pathology and host immunity, generate novel transgenic lines of parasite, or evaluate vaccine efficacy is to carry out infections of animals.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives for Eimeria replication are not currently available. Compartmental models such as fermenters (gut lumen models), organoids and tissue explants represent incomplete systems and are not currently fit for purpose. The topic has been reviewed during

preparation of this application (e.g. targeted searches of the published literature via PubMed, Web of Science and Google Scholar).

Progress with explants and organoids has been made in recent years (e.g. publications including Nash et al, doi: 10.1038/s42003-021-01901-z), but lack the ability to replicate interactions between host, environment and gut bacteria.

Considerable progress has been made in cell culture of the initial invasion and early development steps for one Eimeria species that infects chickens, however, the system is not productive (i.e. does not complete the lifecycle) and is not suitable for the other Eimeria species that infect chickens.

Further, parasites to be used in these limited cell culture studies can only be produced by prior replication in chickens.

Why were they not suitable?

Non-animal alternatives cannot currently be used to re-create the complex interaction between parasite and host and cannot be used to reproduce Eimeria. Similarly, interactions with the environment, host immune response and gut bacteria cannot be replicated.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals requested has been estimated based on our own and other published experience of studies with Eimeria species parasites in a range of inbred, broiler and layer commercial chicken systems. Data defining the variation expected within experimental groups and the magnitude of responses to treatments such as prior exposure or vaccine application are used in power calculations to identify optimal group sizes with sufficient statistical power using the minimum number of animals. Input figures vary considerably between chicken types (e.g. inbred SPF chicken lines to outbred commercial layer lines), parasite species (e.g. caecal dwelling versus small intestinal species) and even some parasite strains. Further details are available in the relevant protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will follow ARRIVE guidelines for experimental reporting and study design to ensure the most relevant, reduced and transparent experimental design. We will also apply community consensus guidelines when available for reporting study design to ensure all sampling is fit for purpose (e.g. adapting the human microbiome STORMS checklist to poultry; Mirzayi et al., 2021, doi: 10.1038/s41591-021-01552-x).

For parasite amplification infectious doses are calculated to yield high numbers of progeny without inducing clinical signs based on our experience and taking into account replicative potential and known pathogenicity. For all parasites the doses and numbers of animals are calculated from theoretical and actual data. For each study groups of 5-20 animals are

typically used, and to minimise the overall numbers the research group operates a pooled resource so that each batch is utilised with minimal wastage. The number of animals used in studies for parasite production is determined based upon the number of parasites required for downstream applications (e.g. purification of genomic DNA for genome sequencing, protein for mass spectrometry, selection of transgenic sub-populations, use for cell culture studies), not statistical comparison between groups.

For comparative studies such as definition of vaccine or feed additive efficacy, immune responses, cross-protection between Eimeria species and impact on gut bacterial populations, animal numbers are the minimum required based on experience supported by statistical power calculations (at 5% significance, 80% power). For example, immunisation/challenge experiments are carried out using the minimum number of animals required for statistically significant results identified by power calculations (usually n=5-10, dependent on the trait and the parasite species). Data such as parasite replication are usually analysed using a parametric method, while measures such as rating of pathology require non- parametric analysis.

For research on alternative additives every candidate will be tested using our in vitro model before moving to trials in experimental animals to reduce the number of studies and experimental groups. We have estimated that use of this model as pre-screening for novel additives can help to reduce the use of chickens by at least 30%.

Our recent development of oral dosing using a non-toxic fluorescent dye as a quantifiable measure of gastrointestinal damage is intended to permit repeat sampling from living birds, potentially replacing terminal measures such as intestinal lesion scoring. Piloted on an existing licence, this strategy will be developed further here and can reduce the number of chickens required in an experiment since it will not be necessary to cull multiple birds to assess different timepoints. The quality of the data will also be improved when repeat measures are made from the same individuals, reducing background variation.

Previous relevant innovations include development of molecular tools to quantify parasite replication, replacing traditional parasite counting by microscopy. Application of the assay has permitted reduction of group sizes by up to 50% in some studies.

Testing Eimeria amplification in embryonated chicken eggs may offer opportunities for reduction in future studies if background variation and inefficiencies in harvesting some parasite lifecycle stages from hatched chicks can be reduced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The studies proposed here have been based on current commercial knowledge, extensive experimental data collected over more than 20 years of work, and consideration of the published literature. Background data defining the level of variation expected in measures of infection, parasite replication, pathology and performance, including the influence of distinct host breeds/lines and parasite species/types, are used in power calculations as outlined in each protocol. Samples collected from these studies will be blinded for laboratory analyses wherever possible. Tissues and data will be shared with other projects within the group and made available to others within the wider community to maximise their use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Chickens are the natural hosts of the Eimeria species to be used and the data generated is of direct relevance for development of improved control strategies.

Methods for infection of chickens with Eimeria have been refined over many years and are carried out with a minimum of stress to the animals. We minimise animal suffering by thorough monitoring, well defined end points and carefully controlling doses of parasites. For the vast majority (~95% of animals used) it is expected that suffering will be within a mild severity band; we require a moderate severity limit because we cannot rule out rare occasions where animals may show clinical signs of coccidiosis. The inclusion of a behavioural measure of chicken health (using accelerometers to measure movement) adds a new quantitative trait that can be assessed without a terminal or invasive procedure and offers opportunities for repeated measures. Further, data produced using accelerometers may be appropriate for use as a new clinical end point, permitting intervention before pathology-based measures become apparent.

Other procedures such as oral inoculation, blood sampling from the wing vein rather than the jugular, and vaccination by injection into muscle or under the skin are well established and selected to minimise the need for (more) invasive procedures. For example, final blood sampling will be undertaken immediately post-mortem whenever possible rather than from live birds to minimise the number of procedures per individual.

Chickens will be housed in the RVC Animal Welfare Barn (AWB) facility that was refurbished in 2018. Chicken environments are enriched with perches and materials for activities such as reflective discs, balls or cable ties to peck including caged and commercial conditions studies.

Why can’t you use animals that are less sentient?

The study of Eimeria in chickens cannot be accurately replicated in any other less sentient animal. Each Eimeria species is specific to an animal host species, therefore, the species of interest to combat chicken coccidiosis can only be studied in chickens, infections cannot take place in any other host species.

The use of embryonated eggs is planned to replace some chicken use for studies with specific parasite lines and lifecycle stages, but is not currently applicable for non-adapted lines (i.e. most current parasite lines, all field isolates).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Chickens will be habituated to experimental staff from arrival, including routine ‘pen walk throughs’ that will be used to detect chickens experiencing the effects of Eimeria infection.

Based on experience with defined doses of the Eimeria species that infect chickens it is clear when the consequences of infection are most likely to first occur (e.g. ~112-130 hours

for Eimeria tenella). We increase monitoring during periods of elevated risk, acting to remove individuals if they approach protocol endpoints.

Research staff involved in any experiment will be trained by researchers experienced in each procedure and assessed by the Named Veterinary Surgeon (NVS), Named Training Competency Officer and/or other competent personnel following established practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow expert guidance on husbandry from industry and veterinary practitioners, benefitting from interactions with the British and World Veterinary Poultry Associations to ensure that best practices are always followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant and all team members who will work on the project frequently interact with the NC3Rs, checking the website regularly and attending seminars and webinars when they occur. The host organisation works with a NC3R Regional Programme Manager, providing opportunities for periodic training in 3R related topics and one-to-one meetings for advice. The host organisation is also very active in dissemination of 3Rs relevant news, providing training and updates via a newsletter, emails and online notifications.

40. Evaluating the Roles of Immune Modulators on Anti-Tumour Immune Responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Immunotherapy, Immunity, Immune cells

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the roles and effects of immune modulators and regulators of immune cells (eg. tumor-reactive T cells), in mediating tumour control and promoting anti-tumor immunity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We recently identified and generated in vitro tumour-reactive T cells from several cancer patients. Utilising in vitro immune functional approaches and analyses, we have discovered several unique anti- tumor immune-associated modulators and regulators of human immune cells (eg. tumour-reactive T cells), that are shown to promote better or worse anti-tumor immune activities, at least, in in vitro setting. Moving forward, this in vivo work is important to build upon the preliminary data established in our in vitro system, and is useful to enable physiological observation of the potential impacts on mitigation of tumour growth and protective anti-tumour immunity, in a more complex organism compared to in vitro system.

What outputs do you think you will see at the end of this project?

- Publications of research data
- New information on the physiological relevance of identified immune modulators
- New information on products' efficacy and safety

Who or what will benefit from these outputs, and how?

In the short-term, these outputs will provide essential new knowledge on less-studied tumor-associated immune modulators and regulators, in a more complex tumour micro-environmental conditions. In the long-term, these outputs will also provide pre-clinical understanding on the safety and efficacy of the modulators-derived products for potential new design of cancer immunotherapy strategies.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximised by dissemination of new knowledge through publications of successful and unsuccessful approaches to the work in question, as well as providing new inputs in designing better cancer immunotherapy strategies.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using genetically altered mice that are immunocompromised because we plan to humanised the immune system with implanted human cells. The immunocompromised model is needed to prevent any potential non-humanised-associated confounding factors in the mitigation of human tumour growth and anti-tumour adaptive immunity.

We are using adult animals over juveniles because we want to use an animal life stage model that have a well develop physiological system, and to avoid any potential animal growth-related confounding factors from affecting the outcome of the work.

Typically, what will be done to an animal used in your project?

The animals in this project will typically be xenografted with tumour cells to induce tumour growth, on a single occasion. They may then be given other cells which may accelerate or decelerate the growth of the tumour. Substances may also be given which may similarly, accelerate or decelerate the immune activities and growth of the tumour. We may use imaging techniques to measure and view the tumour as it grows, and to track the migration of the cells we have injected, which is performed under general anaesthesia. Blood samples may also be taken at allotted time points to assess the number of cells, tumour load, cytokines levels and substances levels in the blood. Typically, the maximum number of procedures the animal will undergo is between 8 to 15, with a typical experiment lasting up to 35 days.

What are the expected impacts and/or adverse effects for the animals during your project?

During the lifespan of the experiment, animal would experience mild transient pain during injections, as well as the growth of xenografted tumour. The estimated duration of xenografted tumour is up to 35 days, unless the tumor first reaches the permitted maximal size prior to reaching the 35 days. No weight loss or long-lasting abnormal behaviour are expected during the course of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold: 58%, mild: 29%, moderate 13%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed to provide physiological evidence on the immune activity against tumours within a more complex biological system, and to verify the efficacy of the immune modulator treatments. In contrast, in vitro system might not provide in-depth information on the efficacy aspects of these treatments. The efficacy data should be more easily obtained, observed and identified using a more complex organism such as mice.

Which non-animal alternatives did you consider for use in this project?

In vitro model (eg. T cells and autologous cancer cells system)

Why were they not suitable?

The in vitro system could not provide necessary information about the efficacy aspects and physiological evidence of immune activity in a more complex microenvironment. The use of

more complex organism model such as mice, would enable us to identify the immune efficacy of these treatments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation was done based on an experimental design phase and optimisation performed in our previous work with university collaborators, and previous literature searches on in vivo mice studies on immuno-oncology.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We initially utilised the NC3R's experimental design assistant to determine the potential minimum number of animals that can be used per experiment (that can achieve maximal data output) before performed an optimisation step as done in our recent work, and previous literature searches on in vivo mice studies on immuno-oncology.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Continuous measures will be carried out to further optimise future experiments. Breeding colonies will be managed in line with best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future plans on breeding animals and in assisting and maintaining a suitable colony size, to ensure only those animals needed for experiments are produced. We will also use and share tissues whenever possible, especially during optimisation of works outside of mice models. Additionally, continuous use of in vitro studies will be performed before carrying out animal works to ensure the cells and compounds that will be used in animal works underwent an initial in vitro pre-animal assessment for suitability, functional efficacy and viability. We will continuously use the NC3R's experimental design assistant to help determine the optimal number of animals to be used, and conducting initial pilot studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using immunocompromised mice that will be injected with tumour cells via subcutaneous route because this route of administration is the most suitable for the measuring of tumor growth, by the use of non-invasive methods such as precision measurement callipers and MRI scans.

The use of immunocompromised mice compared to wild-type mice prevent any potential non- humanised-associated confounding factors in the mitigation of human tumour growth and anti-tumour adaptive immunity.

Immune cells and compounds will be introduced into the mice primarily via intravenous injection as it is the most relevant (especially in the assessment of targeted movement or migration of the immune cells and compounds towards the tumor), and similar approach to the injection of cells or compounds in clinical setting.

Why can’t you use animals that are less sentient?

We will be using mice because they have the most similar and relevant anatomy and physiology to humans, in comparison to other organisms (eg. Flies or fish), apart from non-human primates.

We cannot use immature life stage animal because their body are not fully developed, and the treatments on these immature animals might cause more severe harm to the animals during their developmental stage of normal growth. In addition, immature life stage animals do not have fully developed immune system, including innate immunity, which are required for general survival of the animal.

Animals that have been terminally anaesthetised are not useful for this project because their tissue environment has ceased to function properly and cannot provide the nutrients and necessary viability factors for the implanted tumour cells and immune cells to function properly.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will continuously refine our procedures, including by using the acclimatising period of the animals to give familiarity during animal handling, and in increasing and consistent monitoring of animals that have undergone procedures. Initial optimisation and pilot experiment will be done, especially as to ensure any pain management regimens could be in place for post-procedure care, including continouously getting in touch with local NVS and NACWO for animal welfare advices. Continuous seeking of advice on better refinement of experimental designs will be done throughout the project from the local NVS, NACWO, NTCO and local university research collaborators. We will also continuously be using the NC3R’s experiment design assistant to continuously refine the use of animals for the experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay informed and follow the refinement recommendations as suggested by the NC3R’s portal website, the advices from the Strategic Planning of Research Programmes of the FRAME (Researching Alternative for Animal Testing) website and guideline, as well as the LASA, ARRIVE and PREPARE guidelines, which will be regularly read through the Norecopa portal website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will be attending the institutes termly welfare meeting and other internal 3R's meetings. We will also have access to the NC3R's regional manager and the establishment's Named Information Officer to get update on the advances of 3Rs and advice and recommendation. Additionally, we will stay informed about the advances in 3Rs by continuously checking the NC3R's portal website, as well as joining and keeping up to date with the mailing lists of organisations that discusses on topics regarding comparative medicine and the use of laboratory animals, such as the CompMed and TransgenicList.

41. Functional Genetics of Lung Squamous Cell Carcinoma

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Lung cancer, Human bronchial epithelial cells, Genetic drivers, Inter-patient heterogeneity, Premalignant lesions

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to investigate the contribution of the most frequently altered genes to the progression of lung squamous cell carcinoma (LUSC) and their role in inter-patient differences.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite current promising targeted treatments and immunotherapies, lung cancer (LC) patient outcomes have barely improved over several decades, and LC remains the most frequent cause of cancer-related deaths in the UK (35,500 deaths per year). Lung squamous

cell carcinoma (LUSC) is an aggressive type of LC that originates in bronchial basal cells (a subset of bronchial epithelial cells) with limited therapeutic options.

No successful targeted therapies have been used in LUSC. Apart from chemotherapy, only immunotherapies result in marginal improvement of survival in LUSC patients. Furthermore, the Lung Cancer Matrix Trial, a large, personalized medicine trial has not shown encouraging results in LUSC. Early detection is currently the most effective tool to prevent deaths by LUSC. Screening programs by X-ray imaging in high-risk populations (mainly smokers) have overwhelmingly confirmed this benefit. However, 40% of patients diagnosed with early-stage disease still die within 5 years, the rate of false positives is high (48-96%) due to non-cancerous changes and CT-scans fail to detect preinvasive lesions. These precancerous bronchial lesions show high-risk of malignant progression but can be easily removed with minimally invasive procedures.

Hence, preventing deaths in LUSC patients requires improving therapeutic modalities and early detection methods. These improvements depend heavily on more ambitious, innovative, and patient- relevant preclinical models that recapitulate the developmental stages of LUSC progression as well as the intra-tumour and inter-patient heterogeneities so frequent in this disease. However, existing LUSC models do not recapitulate those complexities, and this is a barrier to reverse the dismal landscape of LUSC. With this project, we intend build these much needed new models of LUSC in order to increase the repertoire of models available for the scientific community and accelerate the development of new therapies and methods of early detection.

What outputs do you think you will see at the end of this project?

The outputs from this project will determine how the most important genetic and epigenetic alterations observed in LUSC drive the development of this disease.

- A group of LUSC models, bearing multiple combinations of genetic alteration in genes that are important for LUSC progression, that will recapitulate the genetic heterogeneity observed in patients.

- By interrogating the individual and combined effect of the most important genetic alterations in LUSC using these models, we will model the heterogeneity that we observe in patients and the role of each gene in LUSC progression. These group of models will enable us to carry out preclinical research that is more patient-relevant.

- A comprehensive dataset on the function of genetic events in determining the transition from a normal lung tissue into a cancer. This will include the individual contribution and/or cooperation between pathways and smoking history driving LUSC progression, revealing potential therapeutic strategies such as co-inhibition of pathways.

- Association of genetic alterations with transcriptional signatures, specifically with expression of known therapeutic targets and other actionable downstream genes suggesting new tumour vulnerabilities

- Role of the KMT2D, a protein that modifies the structure of the genome, in LUSC progression.

Who or what will benefit from these outputs, and how?

The main beneficiaries after the completion of the project will be other cancer biologists as they will have a new human model of lung cancer progression that did not exist so far. In the longer term (3-5 years), new therapeutic modalities and/or early detection strategies will be developed using the new human models of LUSC in our laboratory or other laboratories. This will ultimately result in changes in cancer medicine and patient benefit. We anticipate that patients won't be able to benefit from this work within the time frame of this project licence but might benefit in the next 10 years. Patient benefit might result from the identification of new early detection biomarkers, identification of synthetic lethalities as the basis for new treatments and identification of new actionable therapeutic targets.

How will you look to maximise the outputs of this work?

Our findings will be made available to other scientists through collaborations, publication in high quality journals and presentations at scientific conferences and meetings. The establishment has a policy of ensuring that all publications generated are available on free access to all.

Species and numbers of animals expected to be used

- Mice: 1056

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cancer development is dependent not only on the changes occurring within the transformed cells, but also on the interactions of the cells with their neighbouring non-cancer cells. Apart from being less comparable to humans, less sentient model systems (fish, invertebrates) do not have lungs. Mice also have higher levels of conservation in nucleotide and amino acid sequences. This is important as we intend to investigate genes whose functions are frequently not conserved in less sentient models.

Although the most sophisticated non-animal models can provide valuable information about cancer progression, they provide limited information about the local environmental factors influencing cancer evolution. Also, certain hallmarks of cancer, such as spread of cancer and blood vessel formation, are difficult to study in vitro (cell culture). Therefore, mouse models are important for studying LUSC. Mouse models have been engineered to develop tissue-specific cancers, which accurately mimic their human counterparts, and have potential applications to test the effectiveness of novel cancer therapeutics. Alternatively, genetic manipulation of the LUSC cells-of-origin ex-vivo for implantation in syngeneic mice is an alternative to germline genetic engineering that can reduce the numbers of mice required for breeding. Moreover, lung anatomy and histology in non-protected species and less sentient species is dramatically different when compared to humans, so we would be unable to use them for animal models. Embryonic stages would not provide us with a sufficient window to follow tumour development and besides it is not feasible to perform the desired interventions in embryos (such as orthotopic implantation of genetically modified bronchial epithelial cells). Therefore, adult mice are to be used.

Typically, what will be done to an animal used in your project?

Genetically altered mice that do not have a fully functional immune system will be used to investigate the role of certain specific molecular pathways in the induction, progression and metastatic potential of lung squamous cell carcinoma cells. This defective immune system does not reject cells from human origin when implanted in the mouse. Although the immune system is known to influence multiple steps of the carcinogenesis process, we will use immunocompromised mice as they still recapitulate other components of the tumour microenvironment.

The mice will have tumours grown in them initiated by the implantation/injection of human cancer cells, including cell lines and genetically modified human bronchial epithelial cells (HBECs). We will use injection under the skin (subcutaneous) as the preferred modality of transplantation as it has been used before and causes the lowest level of harm. However, if cells do not grow under the skin, we will try transplantation in the lung (orthotopic), and kidney capsule injection in parallel. Both modalities are more likely to cause more harm. However, in the orthotopic implantation cells find the lung microenvironment, which is similar to the human lung and therefore they are likely to grow better. The kidney capsule has been shown to be the most permissive site of implantation. The experience with HBECs implantation is limited. For this reason, we will try the least harmful procedure (subcutaneous) first but if unsuccessful we will have to resort to orthotopic and kidney capsule. The spread of these cells (metastasis) cannot be excluded, but we do not know the eventual locations until we complete the first study. Implantation into the lung will require anesthesia and causing lung injury, and kidney capsule will require surgery under anesthesia.

To monitor the tumour growth, a combination of methods will be used: the use of callipers (to measure subcutaneous implanted tumours) and in-vivo whole mouse imaging under light general anaesthesia will be performed on a number of occasions to monitor tumour growth. On occasions it will also be necessary to inject, by one of several possible different routes, chemical agents that will allow better imaging of the internal tumours. The majority of animals are not expected to show signs of adverse effects that impact on their general well-being. Very rarely the severity of these signs may be such that the humane end points may be reached, and the mice culled humanely. The majority of the procedures will result in no more than transient discomfort and no lasting harm.

When the humane end-points are reached, mice will be killed using Schedule 1 methods.

What are the expected impacts and/or adverse effects for the animals during your project?

The impact of tumourigenic mutations is not expected to cause any adverse effects per se as the mutations will be carried out ex-vivo.

Implantation of cells in the kidney capsule and in the lung will be carried out under general anaesthesia and are likely to cause some discomfort during the process of anaesthesia and during recovery time

Tumour growth is expected to cause weight loss if the implanted cells cause cachexia or by symptoms associated to cancer progression (such as metastasis).

Injections of agents for tumour imaging would only cause very transient discomfort.

After surgical procedures (kidney capsule implantation) we will monitor mice for signs of pain and administer effective pain relief for as long as it is required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

If subcutaneous transplantation is the selected modality, all mice are expected experience mild symptoms only as a result of the discomfort caused by the injection before they are humanely killed. It is possible that there could be a fraction of mice with metastatic tumours. The preliminary experiments will be used to estimate the frequency of metastasis.

Both lung-injury and intratracheal implantation will be carried out under general recovery anaesthesia. Mice are expected to experience moderate symptoms as a result of these procedures. Since intratracheal procedures could cause adverse effects in mice, optimisation experiment will be carried out to use the conditions that cause the least adverse effects. Should these adverse happen, mice will be killed before reaching the humane endpoints.

Kidney capsule implantation of mice will require surgery and this will be carried out under general recovery anaesthesia for the operation and receive pain killer post-operatively until pain subsides.

Mice that undergo intratracheal or kidney capsule implantation will have repeated brief anaesthesia for the purposes of imaging the internal tumours. The expected severity caused by these procedures (intratracheal or kidney capsule implantation) is expected to be moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While valuable studies of human cancer are performed using tumour material and cell lines derived from both mice and human samples, the mechanistic understanding of cancer pathogenesis and its dynamics from tumour initiation to metastasis requires use of living animals (in vivo). In particular, cancer development and spread involves a plethora of interactions between cancer cells and their surrounding host and their behavior is governed by multiple signals originating from both their immediate neighbors and from distant tissues.

Mouse models of cancer (genetically engineered and implantation models) can mimic their human counterparts more accurately than any other in-vitro and 3D models and have potential applications to test the effectiveness of novel cancer therapeutics.

Which non-animal alternatives did you consider for use in this project?

We will be using cell culture modalities that recapitulate the three-dimensional architecture of tumours (organoids) to investigate certain cellular effects of the genetic manipulations, such as proliferation, epithelial homeostasis and interactions of cancer cells with the

surrounding non-cancer cells, such as fibroblasts. Use of animals will be minimised by making use of cell culture systems that recapitulate the natural architecture of the lung tissue (organotypic cultures). This modality is useful to investigate simple heterotypic interactions but fail to recapitulate the multiple components of the tumour vicinity.

Why were they not suitable?

The study of cells in culture (in vitro) provides us with clues on the mechanisms of cellular processes in a simple and valuable context, which allows the establishment of hypotheses regarding the function of cells in a living animal. However, these systems do not recapitulate the complex cellular interactions described above.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The first aim of the project will be to investigate 9 different combinations of mutant cancer genes in human bronchial epithelial cells from 6 donors (3 smokers and 3 non-smokers). Our results using organoids have shown that a combination of five mutations induce changes in the cells that indicate tumourigenesis. Therefore, we intend to use this combination of mutations as a positive control, and cells without mutations as a negative control to investigate the number of cells required to observe tumour growth under the skin and to investigate differences between males and females. If we do not observe tumour growth, we will proceed to repeat the same experiments using kidney capsule and intratracheal transplantation. As a result. These preliminary experiments will require 60 mice if subcutaneous implantation produces tumour growth, and 180 if we need to explore the other two routes. Once the number of cells to observe tumour growth has been determined, we'll carry out the experiments. If differences between males and females are detected, we will carry out using both sexes, if not, we will use females to reduce the number of mice and optimise cage usage. Considering 5 mouse per mutant, we will need 270 mice if we only use one sex and 540 mice if we use both sexes. Therefore, we'll need a maximum of 840 mice for this aim: 720 mice for the main experiment plus a 20% of mice for unexpected repeats. With this strategy, we'll first identify the site in which the mutant cells manifest the most aggressive behavior, the number of cells needed to cause tumours in mice and differences between sexes.

We are also interested in investigating a gene, KMT2D, responsible for producing a protein involved in DNA packaging. For the experiments with cell lines, we intend to use two KMT2D mutant cell lines with and without restoration of KMT2D function and two KMT2D wild type cell lines, with and without inactivation of the KMT2D gene. This experimental design produces 8 experimental groups. We will first run a pilot experiment with unmodified cell lines to test the minimal number of cells required to cause tumours in 80% of mice, and differences between sexes. Three cell dilutions will be used for each cell line and 5 animals of each sex will be injected subcutaneously. For this pilot experiment 120 mice (10 mice x 4 cell lines x 3 dilutions) will be needed. If sex differences in tumour growth are observed, we'll use 10 mice (5 of each sex) per experimental group. If no differences are detected, we'll use only 5 females per experimental group. Therefore, 80 mice (8 experimental groups x 10

mice per group) plus a 20% for repeats (96) will be the maximum number of mice used to complete the experiment, and 216 (120 mice for pilot experiments + 96 for the main experiment) will be the maximum number of mice needed to complete this aim.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Use of animals will be minimised by (i) making use of in vitro model systems wherever scientifically justified, (ii) use of in vivo bioimaging to follow disease development and response in real time (rather than culling cohorts of mice at defined time points), (iii) use of implantation models (xenografts) instead of genetically engineered mice (GEMMs) to avoid breeding programs that result in increased numbers of mice (iv) the use of pilot experiments to test for the extent of an expected phenotype prior to a full scale confirmatory experiment (thus avoiding full scale experiments that may lack sufficient statistical power).

Experiments will be appropriately controlled, and mice of the same age and sex used to reduce the variability of results and to produce highly consistent data. The proposed experimental designs and methods of analysis of the results will follow statistical guidelines and involve discussion with our bioinformatician scientist to provide sufficiently powered studies, minimizing the number of animals used in each experiment. The design of individual experiments will generally involve factorial designs, which maximise the information obtained from the minimum resource.

We will be conducting and recording our experiments to be able to publish our results following the ARRIVE [https://www.nc3rs.org.uk/arrive-guidelines] and PREPARE guidelines [https://norecopa.no/prepare], and will use randomisation, blinding etc. where appropriate so as to minimise biases. Furthermore, additional resources may be used to aid experimental design such as the NC3Rs experimental design assistant tool

(https://www.nc3rs.org.uk/experimental-design-assistant-eda).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of animals will be minimised by (i) making use of in vitro model systems wherever scientifically justified, (ii) use of in vivo bioimaging to follow disease development and response in real time (rather than culling cohorts of mice at defined time points), (iii) use of implantation models (xenografts) instead of GEMMs to avoid breeding programs that result in increased numbers of mice (iv) the use of pilot experiments to test for the extent of an expected phenotype prior to a full scale confirmatory experiment (thus avoiding full scale experiments that may lack sufficient statistical power).

Experiments will be appropriately controlled, and mice of the same age used to reduce the variability of results originated in heterogeneous mouse genetic background and to produce highly consistent data. Wherever possible and appropriate, a single group of animals will serve as a control for duplicate experimental group. The proposed experimental designs and methods of analysis of the results will follow statistical guidelines and involve discussion with our bioinformatician scientist to provide sufficiently powered studies, minimizing the number of animals used in each experiment. The design of individual experiments will generally involve factorial designs, which maximise the information obtained from the minimum resource.

Pilot studies will be performed if applicable and, after analysis of the results, group sizes for subsequent experiments will be determined based upon these data. As far as possible,

multiple parameters will be evaluated in a single mouse. Live imaging of the same animal at multiple time points also greatly reduces the numbers required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been chosen for the study because they represent the least sentient species from which meaningful experimental data can be generated, while exhibiting considerable genetic and biological similarities to humans. Other less sentient non-mammalian species, such as zebrafish and frogs, have anatomically and histologically different respiratory system than Homo sapiens.

The conditions under which the experimental animals are kept and used for the proposed procedures are designed for the least possible disruption of natural behaviour and quality of life. We constantly work to improve husbandry and procedures to minimize actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable. As detailed in each protocol, there is provision for the appropriate anaesthetic and analgesic regimes as well as appropriate culling methods following approved guidelines on administration of substances to mice.

Additionally, we will use immunocompromised animals, to permit human tumour cell transplantation without rejection by the host species immune system, and so far, mice is the only species with immunocompromised strains.

Why can’t you use animals that are less sentient?

Only a mammalian lung cancer model system has the potential to accurately mimic both the anatomy and complex cell biology, including certain microenvironmental interactions. Furthermore, there is considerable experience in the wider scientific community regarding the use of mice as a model system for human malignancies and many reagents exist for the phenotypic characterisation of mouse and human cells.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The conditions under which the experimental animals are kept and used for the proposed procedures are designed for the least possible disruption of natural behaviour and quality of life. We constantly work to improve husbandry and procedures to minimize actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable. For the protocol, there is provision for the appropriate anaesthetic and analgesic regimes as well as humane culling methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Relevant published literature will be used as template for experimental design and decision making (Workman et al., 2010. Guidelines for the welfare and use of animals in cancer research. BJC, 102, 1555-1577).

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)] administration of substances will be undertaken using a combination of volumes, routes and frequencies that themselves will result in no more than transient discomfort and no lasting harm.

Guidelines for Body condition score. [Ullman-Cullere, Lab Anim Sci. 1999 Jun;49(3):319-23]

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading 3Rs literature and participating in 3Rs workshops locally and nationally. Through discussing refinements with our NACWO, NVS and potentially ASRU. I will attend the Retrospective Review meeting and the 3Rs Poster sessions, which take place annually at our Institute.

42. Improving Cancer Therapy by Enhancing the Anti-Tumour Function of Immune Cells by Membrane Modification

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Immunotherapy, NK Cells, T Cells, Membrane binding proteins

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to evaluate the potential of cell therapy, utilising immune cells that have been modulated to enhance their function, as a treatment for cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One person in every two in the UK can expect to be diagnosed with cancer during their lifetime. Despite significant improvements in diagnosis and treatment, one person in every four will die prematurely as a result of cancer. The current main stay treatments for cancer, which include surgery, chemotherapy and radiotherapy can be traumatic, often have serious side effects and are limited in their effectiveness, especially against cancers that

readily metastasise. As a consequence, there is an urgent need to develop new and more effective treatments for cancer that avoid the drawbacks of existing therapies. It has been long known that immune cells, such as natural killer (NK) and T cells, play an important role in preventing and limiting the growth and spread of cancer. In recent years, this fact has been exploited in the form of cell therapies for specific cancers, such the YESCARTA and KYMRIAH and T cell therapies that have been approved by the FDA and EMA for the treatment of childhood leukaemia.

As yet, no immune cell therapies have been approved for the treatment of solid tumours, although a number have entered clinical trials. A major problem in treating solid cancers with cell therapy is the suppressive microenvironment within the tumour mass which prevent the immune cells from effectively recognising and killing their targets. As a consequence the transferred cells tend to localise at sites other than tumours and die off. A number of approaches have been used to enhance cell therapy with some success, including cytokine therapy with interleukin 2 or 15, and the use of checkpoint blockade antibodies, such as pembrolizumab and atezolizumab, which block the immunosuppressive Programmed Death Ligand (PD-L1) pathways and thereby reduce immune cell mediated suppression by the tumour.

The purpose of the work outlined below is to evaluate the potential of immune cells, either modified directly in the animal or manipulated within the laboratory to enhance their potential to target and kill tumour cells, for the treatment of solid tumours. The work will be focused on tumour types for which treatments are either ineffective or unavailable.

What outputs do you think you will see at the end of this project?

At the end of this project we expect to have gained new information on the expansion, survival and therapeutic efficacy of modulated immune cells targeting a range of cancers. This would lead towards the identification of candidate compounds, which can be patent protected, for progression towards first in human clinical trials.

New understandings on the cellular mechanisms that influence tumour growth will be published in peer reviewed scientific journals, conference posters and patent applications. New data acquired from using unique murine models will be shared with the community to inform future, similar experiments.

Who or what will benefit from these outputs, and how?

In the short term, the work is expected to benefit scientists working to develop more effective treatments for cancer by advancing the understanding of the mechanisms by which tumours evade the immune system and the effectiveness of manipulations aimed at overcoming these. Additionally, good animal practice and new data from murine models will help refine murine experimentation in these models.

In the medium term the work is expected to lead to the identification of more effective cell therapies for translation into first in human trials.

In the long term cancer patients and health care providers are expected to benefit through the development of more effective treatments.

How will you look to maximise the outputs of this work?

In order to maximise the outputs of this work we will consult with scientists in academia and industry to optimise the experimental design to ensure the work produces robust and

reproducible outputs. We will disseminate new knowledge gained via presentations at conferences and by publication in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The purpose of the outlined work is to develop novel cell therapies for the treatment of cancer in humans. To this end, it is necessary to use a species that shares a similar anatomy and physiology to

humans. Consequently, mice have been selected as they are the species with the lowest neurophysiological sensitivity that meet the criteria for the studies. In addition, the immune system of mice has been extensively studied and shown to correlate well with that of humans.

The necessary reagents to undertake the study are readily available for mice. Additionally, human forms of the proteins that we want to study, such as cytokines, a protein that boosts the function and proliferation of immune cells, are functional in mice with a similar degree of potency to that in human cells.

The availability of numerous conventional inbred and genetically altered strains of mice provides a repertoire of relevant immunological research tools that is not available in other species. We are especially interested in mouse strains with minimal immune systems that will accept donor human grafts of immune cells and tumour cell lines.

Mature mice, above 6 weeks of age, are essential to these studies as their immune system has fully developed and they are large enough to be handled, treated and bear a tumour for the duration of the study with minimal cancer related morbidity.

Typically, what will be done to an animal used in your project?

Most mice used in these studies will be purchased from a commercial supplier, however, some genetically altered strains may be bred in-house under Protocol 1. Genetically altered mice bred under Protocol 1 are not expected to experience any adverse effects because of the modification. Mice arriving from an external supplier will be acclimatised for a minimum of 5 days before use. All mice will be habituated to human contact, by regular handling, prior to the start of experiments.

Immunocompromised mice will be barrier housed to ensure conditions are optimum for maintaining good health.

In protocol 2 mice will be injected with immunotherapy in the form of immune cells or immune cell modulating lipid nanoparticles or virus in the absence of tumour. In some experiments an immune modulating reagent will be injected by a standard injection route to support the engraftment of the immunotherapy or to act as a control. The mice will be monitored for the onset of Graft Versus Host Disease (GvHD), Cytokine Release

Syndrome, Host versus Graft (HvG) responses and other potential treatment related symptoms by being weighed regularly and a clinical score chart will be completed regularly to monitor the health of the mice. Small blood samples may be collected at specific timepoints throughout the study to monitor the persistence of the immunotherapy.

Under Protocol 3 , mice may be briefly exposed to radiation or given a chemotherapeutic agent to improve the engraftment of human tissue including tumours and immune cells. Mice in Protocol 3 will be injected with a tumour cell line, of either mouse or human origin. In most cases, the tumour cells will be injected either below the skin or into the abdominal cavity. In some cases, the cells may be given by intravenous injection. Once the tumour is established the mice will be given immunotherapy in the form of immune cells or immune cell modulating lipid nanoparticles or virus. In some experiments an immune modulating reagent will be injected by a standard injection route to support the engraftment of the immunotherapy or to act as a control. Small blood samples may be collected at specific timepoints throughout the study to monitor the success of engraftment of the tumour and immune cells. For subcutaneous tumours, growth will be assessed by measuring the size of the tumour mass using a

calliper. For tumours grown at other sites, the tumour burden will be assessed using a non-invasive imaging system. In all cases the tumour burden will be limited to one that does not normally result in the animals developing overt signs of suffering and any animals that show overt signs of suffering will be killed.

Mice may remain on study for up to 90 days (maximum 40 days for those given tumour cell by intravenous injection). Throughout this period, tumour growth will be monitored carefully and animals will be killed if the tumour reaches the limits set out in the guidelines on the welfare and use of animals in cancer research (British Journal of Cancer. 2010 May 25; 102(11)) or if the animal shows any overt signs of suffering.

Typically, mice will experience mild, transient pain and no lasting harm from the injection of immunotherapy or tumour.

Mice may experience moderate suffering for 1-2 days after irradiation or the injection of chemotherapeutic reagents and they will be closely monitored.

At the end of the experiment, mice will be killed humanely to enable lymphoid tissues and other organs to be harvested for the analysis of immune cell infiltrates in the tumours and the distribution of immunotherapy agents to organs.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection and sampling: These procedures are not expected to cause more than mild, transient pain, similar to that of a vaccine jab, and all animals are expected to resume normal behaviour within a few minutes.

Anaesthesia: The induction, maintenance and recovery from anaesthesia is not expected to cause more than mild distress and all animals are expected to recover uneventfully and to resume normal behaviour shortly thereafter.

Irradiation or chemotherapy: These procedures may result in overt signs of suffering in some mice including, inappetence, weight loss, piloerection and reduced activity however, all mice are expected to recover fully within 2 days.

Tumour development: Mice with subcutaneous tumours are not expected to show signs of pain or suffering and tumours are not expected to spread from this site. In up to 10% of mice when the tumour becomes large, the skin overlying the tumour may become reddened and/or ulcerated. In the event of ulceration, the mouse will be killed humanely.

Mice with abdominal tumours are not expected to show signs of pain or suffering. In a small proportion of mice, tumours may spread to local lymph nodes and the liver. Tumours developing at these other sites may result in signs of suffering such as weight loss, a hunched posture, piloerection and impaired mobility. In the event that any such signs develop the mouse will be killed humanely.

Mice given tumour cells by intravenous injection may develop tumours at a number of sites consequently, the duration of these studies will be limited to a maximum of 40 days during which, overt signs of suffering are not expected to develop. A small proportion may develop overt signs of suffering

such as weight loss, a hunched posture, piloerection and impaired mobility. In the event that any such signs develop the mouse will be killed humanely.

Immunomodulation: There is a chance that mice may experience minor suffering as a result of treatment with immunomodulating agents, cell therapy or new biological compounds especially as some of these have not been previously tested in vivo. Mice will be carefully monitored for any adverse signs following administration and if any display more than transient signs of discomfort they will be killed humanely.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- In Protocol 2 :
- Mild 100%.
- In Protocol 3 :
- Mild 20%: Subcutaneous and abdominal tumour models.
- Moderate 80%: Metastatic tumour model, chemotherapy or irradiation treatment.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to undertake the outlined studies, it is necessary to have a tumour model that is representative of what happens in human cancers. We can then evaluate the tumour

killing potential of the modified immune cells and the processes involved in targeting and killing the tumour, which happen in lymphoid tissue, blood and at the tumour site and involve complex interactions between many different cell types that are at best only partially understood. Consequently, whilst non-animal assays will be used for primary screening, the efficacy of the outlined treatment can only be fully assessed using live animal models.

Which non-animal alternatives did you consider for use in this project?

Migration assays, using Transwells, can be used to study the migration of cells across a cell layer that can represent the movement of cells from the blood or the lymph into lymph nodes and tumours. Each transwell has a plastic membrane that is perforated by small holes and can be coated with a layer of cells. Chemicals that induce the migration of immune cells across the transwell layer are added and the migration of immune cells can be evaluated.

3 dimensional tumour spheroids can be made from tumour cell lines or patient derived tumour biopsies by growing them in a specially made gel. Spheroids represent a more physiologically relevant in vitro model than 2D cell layers because they create complex 3D structures similar to those seen in patients. These 3D structures are more resistant to immune cell killing than 2D cell layers.

In vitro killing assays, where immune cells are mixed with tumour cells and the immune cells' ability to kill tumour cells is evaluated, are being used by us to design membrane modifications that improve the killing of tumour cells.

Why were they not suitable?

Transwell migration assays do not provide an accurate model for studying the migration of immune cells through the endothelial layer that separates the blood stream from other tissues such as lymphoid organs or tumours because they are static and do not represent the forces and biological interactions present in the actively moving blood stream.

Tumour spheroids provide a 3D environment in which to test immune cells but they do not contain the blood vasculature or the complex mix of other cells that are present in tumours.

Mixed lymphocyte and tumour cell studies provide a more complex mix of cells but no blood vasculature or 3D tissue architecture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals required over a 5 year period based on three main factors:

1) The group size for each experiment (10-12 mice) is based on our previous experience, that of the contract research organisations (CRO) that we have worked with and a

literature search of similar studies, and the knowledge that up to 10% of mice given tumours will spontaneously reject them. We have also used the NC3Rs Experimental Design Assistant (EDA) to design our experiments and this has also suggested 10-12 mice per group for some models.

2) The number of groups (4-5) per experiment is based on :

a) the number of compounds we want to test

b) Time required to accurately record measurements and administer treatments

c) Splitting our team to allow group randomisation and blinding

d) Commitment to the 3R's to reduce the numbers of control groups required. Each experiment will include a tumour only control group and an immuno-modulating vector control group as well as 2-3 experimental groups. We will endeavour to increase the number of experimental groups in each experiment to reduce the number of experiments undertaken and thus reduce the number of control groups used.

3) The number of experiments (6-8) per year is based on our capacity to produce the cells and compounds that are needed for the studies as well as experiments in different tumour models that will be required to demonstrate the efficacy of our compounds in multiple tumour types.

4) An estimated 500 mice will be required for pilot experiments and untreated murine tissues. Pilot tumour growth studies using 5-10 mice will be used to establish patterns of tumour growth and enable humane endpoints to be identified. For pilot studies to establish an effective dose of immunotherapy to reduce the growth of the tumour 10-12 mice per group will be used. The variability of tumour growth will also be calculated to establish appropriate monitoring timepoints. Some dose response experiments may be required to establish a minimum effective dose for some new compounds.

Data and experience from each experiment will be reviewed at the end of each study to ensure that future experiments are run as effectively as possible as with as few mice as possible to reach statistically meaningful results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the NC3R's Experimental design assistant (EDA) when planning experiments. Data from our previous studies and where relevant, pilot studies, will be used for power calculations to determine the group size needed to generate statistically meaningful data.

We reviewed our previous in vivo experiments and the literature to identify successful randomisation and blinding strategies.

We employed an external consultant to review statistical analysis, power calculations and group sizes.

We reviewed our in vivo techniques and competencies to ensure that we can minimise treatment related variability in our models.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, we will purchase mice from an external supplier to ensure the correct number are available for studies and minimise the potential risk of overbreeding that can occur when maintaining small colonies.

Pilot studies will be used to establish the growth kinetics of tumours, the effective dose of immune modulating therapies and the variability in tumour growth to optimise subsequent experiments.

At the end of the experiment, we will harvest as many tissues as possible at post-mortem. Tissues not required for immediate analysis will be preserved by fixation or freezing for potential used at a later date.

We are active users of the internal tissue sharing network that makes any excess tissue available to other scientists.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The outlined studies will use established tumour models to investigate the potential of novel manipulations to enhance the immune systems' ability to kill cancer cells. The methods used for tumour induction have been refined to minimise any associated suffering. In most cases tumour growth is not expected to result in any overt suffering during the time course of the experiment. A monitoring regime will be used that ensures any suffering is detected at an early stage and clear endpoints defined so that prompt action is taken to minimise suffering. For some models, immunocompromised mice will be required. These will be maintained under barrier conditions that ensure the environment is optimum for their health.

The studies will evaluate the effectiveness of the treatment against several tumour types. The risks associated with these differs according to the strain of mice used, the tumour cell lines and the site of growth. To minimise these risks, wherever possible, tumours will be grown at a subcutaneous site as this provides the least invasive option for induction, and monitoring tumour development is easiest at this location. However, some of the tumours, for which we are developing treatments, can only be grown in association with specific tissues, such as the ovaries or blood/bone marrow, therefore other sites will be used when it is necessary to do so. In all cases, the duration of tumour growth will be limited to one that minimises the likelihood of adverse effects developing as a result of tumour growth.

To increase their susceptibility to tumour cell and immune cell engraftment mice may be briefly exposed to radiation or given a chemotherapeutic agent prior to the administration of immuno- modulating reagents and/or the immune cell modulations being evaluated. The influence of these treatments on tumour growth and development will be determined by comparing treated and untreated groups. Non-invasive methods will be used to measure tumour growth and all studies will be terminated at the earliest possible time point and not

later than the endpoint outlined in the guidelines for the welfare and use of animals in cancer research (British Journal of Cancer. 2010 May 25; 102(11) 1555).

Why can’t you use animals that are less sentient?

The outlined studies will investigate novel methods of modulating the immune system to enhance its ability to kill cancer cells with the ultimate aim of progressing promising interventions into first in human clinical trials. Non-mammalian animals are unsuitable for these studies as their physiology differs markedly from that of humans and a mammalian model is a prerequisite for translational studies.

Furthermore, the reagents needed to undertake this work are only available for rodents.

It is not possible to use embryos or very young mice for these studies as their immune system is immature and they would not be able to sustain the tumour growth required.

It is also not possible to undertake this work on anaesthetised animals as the time period required for tumour growth is too protracted.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pilot studies will be undertaken for any new tumour model to determine the required challenge dose, the tumour growth rate and early monitoring time points. The data generated will be used to develop a scoring sheet for clinical signs and to determine humane endpoint criteria that will be used to ensure that subsequent studies are designed such that the required data is obtained with the minimum level of suffering. All animals will be closely monitored for signs of ill health throughout the study, including regular weight checks and the frequency of monitoring will be increased in line with the development of the tumour and its associated risks.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE guidelines and use the NC3Rs 'Experimental Design' tool to plan our experiments so that they are safe, scientifically sound and fully address any animal welfare concerns. All surgical procedures will be conducted in accordance with the LASA guidelines on aseptic techniques and the Guidelines for the welfare and use of animals in cancer research as published by the NCRI (Br J Can, 2010)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through the attendance at conferences discussing the advances and applications of the 3Rs.

Through annual meetings organised by the host institute, focused on promoting the uptake of advances in the 3Rs.

Through information cascaded by the NIO and the NC3Rs regional programme manager.

43. Improving Disease Resistance in Farmed Fish

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Fish, Aquaculture, Disease resistance, Genome editing, Precision breeding

Animal types	Life stages
Rainbow Trout (Oncorhynchus mykiss)	embryo, neonate, juvenile
Salmon (Salmo salar)	embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Infectious disease outbreaks within aquaculture present an enormous threat to global food security and production. Genetic editing can help us understand disease resistance in fish, and has potential to produce fish with enhanced resistance to benefit global food security and aquaculture biosecurity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Aquatic animal disease is one of the most serious constraints to the expansion and development of sustainable aquaculture. Genetic editing therefore can contribute and help us understand disease resistance in fish, and has potential to produce fish with enhanced resistance. Subject to an appropriate regulatory authority and environment, this technology may enhance animal welfare significantly, sustainable aquaculture production and global food security in the future.

What outputs do you think you will see at the end of this project?

Aquatic animal disease is one of the most serious constraints to the expansion and development of sustainable aquaculture. Therefore the main long-term benefit of this work is a reduction in the incidence and severity of disease outbreaks in salmonid species aquaculture. Strengthening disease prevention at the farm level through responsible fish farming (including reducing antimicrobial resistance in aquaculture and application of suitable alternatives to antimicrobials) and other science- based and technology-proven measures.

Despite the phenomenal growth in aquaculture production, disease remains one of the largest hurdles to sustainable production, with estimated losses at ca. 40% (FAO 2012). Such concerns confirm disease as the major constricting factor for expansion of the aquaculture industry to 2050, and potentially cost the sector $6 billion in yield loss each year. As such, infectious disease presents the primary risk to sustainable development of this industry, which is critical to global food security. In addition, the frequency and severity of infectious disease outbreaks present a major animal welfare issue, and can also have impacts on the environment and wild fish, particularly in open marine water environments.

The development of genetically-edited farmed aquaculture finned fish species will reduce the mortality rate of farmed fish, plus reduce the environmental impact of pharmacological treatments on the environment, thus reducing the overall significant negative impact on global aquaculture. Improving disease resistance in farmed fish to such highly pathogenic diseases as IPNV (Infectious Pancreatic Necrosis Virus) or ISA (Infectious Salmon Anaemia), which can have cumulative mortality rates up to 90% and greater.

Genetic improvement by selective breeding can be enhanced by genetic marker-assisted selection, which can play a crucial role in helping aquaculture meet future demands for production; research into the genetics of disease resistance has played a major role in ensuring the health and security of aquaculture stocks. Where genetic marker-assisted selection has had a demonstrable large benefit in aquatic species is in resistance to infectious pancreatic necrosis in Atlantic salmon, where a single gene accounts for a large proportion of the overall genetic variation.

Another potential application of these genome editing technologies is to study surrogate broodstock technology.

The long-term objective overall is the selection for increased pathogen resistance in the development of a host where the pathogen does not infect the host so that the pathogen will not be able to reproduce and spread in farmed and potentially wild host populations. This application would improve global food security and biosecurity.

Who or what will benefit from these outputs, and how?

The two main categories of benefits of this research are (i) improved knowledge of disease resistance and host-pathogen interaction in fish via knowledge of the functional mechanisms underlying genetic resistance, and (ii) a new breeding tool to help tackle the problem of infectious disease in aquaculture. The potential long term benefit has positive implications for both fish welfare and for global food security and human health.

The research proposed under this license will use genome editing technology to understand the functional genetic basis of resistance to viral disease in farmed salmonid species. Specifically, the research will target resistance to Infectious Pancreatic Necrosis virus (IPNV) in Atlantic salmon and rainbow trout. IPNV is a disease of great concern in aquaculture, mainly among salmonid farmers, since losses in salmonid fish - mostly very

young rainbow trout (Salmo gairdnery (now Oncorhynchus mykiss)) fry and Atlantic salmon (Salmo salar) post-smolt frequently reach 80-90% loss of stocks.

IPNV, as such a serious pathogen, not only causes the significant mortality and morbidity to farmed Atlantic salmon, but has an associated negative animal welfare and economic cost.

In addition, the research will examine genetic resistance to another serious pathogen of farmed Atlantic salmon, specifically Infectious Salmon Anaemia Virus (ISAV) using similar techniques.

The success of developing disease-resistant genome-edited salmonid species will improve the welfare of these farmed fish species and reduce the burden on disease-control measures, i.e. pharmacological intervention, environmental impact, plus ensure a sustainable business.

The projects and work involved in our aquaculture unit has a key component of forming projects and collaborations with aquaculture breeding companies, which provide a clear route to downstream application of the results generated from the research.

How will you look to maximise the outputs of this work?The specific outputs of the project include:

(i) short-term: improved knowledge of the biology underpinning host response and resistance to IPNV and ISAV in salmon, and IPNV in rainbow trout. This improved knowledge will lead to benefits for the academic community, and downstream for aquaculture via development of vaccines, drugs, immunostimulants, etc. It will be disseminated by regular peer-reviewed publications and conference presentations.

(ii) short-term: New opportunities and techniques to enable breeding companies to use disease resistance markers in selective breeding, initially by using marker-assisted selection (or targeted genomic selection) to improve breeding values to disease resistance. This output may take the form of a patent or license, and will be communicated directly to the collaborating companies who are contributing to the funding of this research under research agreements with the establishment.

(iii) medium-term. When the regulatory landscape is suitable, genome-editing may be used directly in aquaculture breeding, and this has transformative potential for preventing and controlling disease outbreaks. The economic and animal welfare benefits to this are clear. Furthermore, this long term benefit will contribute to food security and therefore human health. A successful case study, such as IPNV or ISAV resistance will contribute to highlighting the benefits of this technology, and communication with government and public stakeholders will be critical to facilitating this output. The establishment has an active and well-managed public outreach programme, and exhibits on this and similar technology will be showcased at our open doors days and the local agricultural show, in addition to other events as the opportunities arise.

Species and numbers of animals expected to be used

- Rainbow Trout (Oncorhynchus mykiss): 8,000
- Salmon (Salmo salar): 8,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Infectious diseases present a major constraint on aquaculture production, causing high mortality levels and impaired growth due to infection. Atlantic salmon and rainbow trout are two important aquaculture species and therefore the improvement through genome-editing (GE) for viral disease resistance will overall improve animal welfare and reduce production losses, ultimately benefiting global food security.

Eggs will be fertilised and the genomic construct will be injected into the single cell of the developing embryo to generate the viral disease resistant genome-edited fish; these fish will be raised, with control fish, possibly until the free-feeding fry stage of development. GE fish fry will have the immediate advantage of viral disease protection; such molecular biotechnologies could also be extended bacterial pathogens.

The ultimate goal, although outwith this current licence, will be the disease challenge of GE fish; presently GE fish may be transferred to another licence with authority to conduct disease challenge studies

Typically, what will be done to an animal used in your project?

This project currently only seeks to develop genome-edited viral disease resistant fish and examine the genetic make-up and phenotype of these fish. Nonetheless, the present experience of an animal, under this current licence, will be the genome editing and observation of the outcome of that genome editing, i.e. the physical appearance or the success of the genetic integration. The expectation is to mirror the fish hatchery experience, from egg to free-feeding fish but with GE fish, which have been modiied to be resistant to certain viral infections. Fish will be raised in aquaria through the egg stages, through the alevin stage to the free-feeding fry stage; within this project the fish will only be maintained in a freshwater environment as juvenile fish (i.e. fry and parr) will not be taken through smoltification.

What are the expected impacts and/or adverse effects for the animals during your project?

Any impact of the genome editing will likely be observed before the free-feeding stage of development,i.e. non-protected stages.

The production of GE lines, initiated at the one-cell stage, will only carry forward animals beyond the free-feeding stage that have as a maximum of a mild phenotype. As a novel technology it is anticipated that these animals will live out their life through natural behaviour and therefore should have no adverse effects. Nonetheless, to err on the side of caution, close and careful welfare observation and checks will be continuously made during development. Any animals with an abnormal phenotype, whether GE-related or not, will be euthanised at the earliest possible time, which is anticipated to be before the free-feeding stage.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is anticipated, for the production of the GE fish lines, based on previous experience, the vast majority may experience mild severity through fin clipping for genotyping, with up to 5% of protected stages with a possible moderate phenotype, due to the GE, which would be euthanised before first-feeding.

This will be measured against non-injected control animals, which will help to understand rates of background genetic abnormalities. The highest probability will be that any genetic abnormality will occur at the pre-alevin stage, i.e. well before first feeding.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objectives of the project cannot be achieved without using animals because the traits we are interested in studying, and those of relevance to aquaculture production, are most effectively measured on the animals. Cell culture models will be applied to test the gene edits and investigate their effect on, for example, viral replication in vitro, and this will inform the choice of edits to be used for the animal experiments. Furthermore, extensive computer analyses will be performed to guide the choice of gene edit to perform. It should be noted that the expected outcome is a reduction in the need for animal disease challenges in commercial aquaculture production, via the use of genetic tests and genome editing for disease resistance.

Which non-animal alternatives did you consider for use in this project?

To fully understand the contribution of genetics and tissue response of disease-resistance an in-vivo model is required; here GE fish species, predominantly at pre-free feeding stages (i.e. non-protected stages), will have a significant and important role to determine the success of the genome-editing.

Why were they not suitable?

Genetic disorders with genetic and/or pharmacological intervention are dependent on complex interactions between genes, cells, tissues and organs in living animals. This can only be achieved to a very limited degree with monoculture cell- or organoid-cultures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous experience and record keeping has ensured that animals held under this production licence are closely monitored, with encouragement to those holding lines under licence to hold fewer and heathier lines. Furthermore, our databases of animal use, whether stock management or annual Return of Procedures, and forecast research projects, provide an insight to the animal numbers required.

The edited animals will be compared to unedited controls from the same family, which will control for background genetic effects. Our policy in these experiments is to use the minimum number of fish to provide robust scientific results and statistical power, in consultation with specialist statisticians. The experimental design proposed is based on the principle of observing differences in mortality level between edited and unedited control fry in the disease challenge experiment. Previous studies suggest that the difference in mortality between resistant and susceptible fry is 35 % under the challenge conditions we propose. It is critical to have sufficient sample size to have the statistical power to differentiate this 35 % difference from stochastic differences in mortality rate between groups of individuals. A statistical power calculation based on a chi-squared test indicates that n = 50 is required to detect this effect (with power of 0.95). We also plan to run replicate samples of each group (2 x 50 individuals) corresponding to two replicate challenges – this accounts for potential tank effects.

Mean mortality level will be compared to that in 100 control (unedited) full siblings. To achieve this number of individuals for challenge, we must account for mortality due to the microinjection process and a proportion of unsuccessfully edited embryos. Therefore we will microinject twice as many embryos as we plan to use in the disease challenge experiments in protected stages. We also plan to use several families (up to two resistant and two susceptible) to partially control for polygenic genetic background, and to increase chances of successful editing and subsequent embryo survival.

We have highly skilled and dedicated staff that are continuously focused on improving their skill set to refine welfare, husbandry and experimental procedures and handling. Dedicated staff and a coordinated central management of the aquatic facilities will ensure the appropriate support for any new users of these model systems.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Research groups are aware of our establishment's animal research design course through AWERB notification as well as the NC3Rs, Experimental Design Assistant tool. The facility manager and NACWOs will be aware of proposed projects, which will use GE fish and therefore can provide appropriate advice.

Researchers, holding animals under this production licence, are closely monitored, with encouragement to hold fewer and heathier animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The applicant’s experience with experimental design is extensive, however we have professional statisticians who can also be consulted to ensure that the animal experiment has adequate statistical power to detect impacts of the edit on the target trait (e.g. disease resistance).

The edited animals will be compared to unedited controls from the same family, which will control for background genetic effects. These measures will ensure that the project

maximises the chances of a successful result while only using the minimum number of animals necessary to achieve that result.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The research will focus initially on Atlantic salmon, the primary species used in UK aquaculture. This is because this species is most severely impacted by IPN virus (IPNV) and ISA virus, and it is the species that the impact of the research are ultimately targeted. It is plausible that other species will be used for similar experiments in the future, particularly if the experiments covered in this license are fully successful and act as a good case study. This will include work on IPNV in rainbow trout as it is an importance aquaculture species for which IPNV is a major commercial problem in hatcheries. Since it is possible to work with large numbers of young fish directly of the species of interest, and there are adequate genomic and immunological tools for those species, there is no need for model organisms – this is a key advantage of working with aquaculture species.

CRISPR/Cas9 biotechnology has already been tested to generate gene-edited salmon embryos, which were then reared to pre-first-feeding alevins to assess the efficacy of the technology. Therefore, the major goal of the research proposed in this project, is to target specific disease resistance loci, via CRISPR/Cas9 genome editing, of Atlantic salmon and rainbow trout embryos.

Suffering will be minimised by daily checking and monitoring of both the water and the health of the fish/ embryos. Fish or embryos showing signs of disease or distress or developmental abnormalities will be killed, taking advice from the named veterinary surgeon.

Why can’t you use animals that are less sentient?

Unfortunately, there are no alternative in-vitro model systems that can be provided and used for the studies that have use under this license. However, amongst all the currently used model organisms in scientific aquaculture research, we have chosen a lower vertebrate model species compared with mammals, i.e. pre-feeding, non-protected, salmonid developmental stages, which for the production of GE fish, are considered less sentient than mammalian model species.

The CRISPR/Cas9 technology, which has already been tested to generate gene-edited salmon embryos, will predominantly examine those juvenile fish that are reared to the pre-first-feeding, i.e. non-protected alevin stage, to assess the efficacy of the technology. Any animals taken forward to the free-feeding fry stage will only be healthy individuals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The facility manager and the facility NACWOs regularly monitor all fish stocks, i.e. non-protected stages and protected stages held under this licence, reporting issues to the responsible person and the NVS. Furthermore, with the NVS and a second NACWO for the facility there will be additional oversight of protocols. With this relationship there is continued shared information relating to welfare so that best practice is maintained and to ensure no conflict of interest.

Best practice will also include pain management, which will utilise anaesthesia during genotyping by fin-clipping and appropriate analgesia. Users will be encouraged to use, which will include training, alternative methods, i.e. skin surface swab and or fin-clip of non-protected stages.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

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How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Close liaison and the continued close working relationship is maintained between the NVS and facility NACWOs, which helps collate and disseminate welfare and best practice. Furthermore, with the NVS and a second NACWO for the facility there will be additional oversight of protocols. With this relationship with the NVS and second NACWO there is continued shared information relating to welfare so that best practice is maintained and to ensure no conflict of interest. A Home Office conflict of interest form has been supplied previously in relation to our establishment licence.

I have contributed to several fish welfare meetings, again disseminating the information and best practice following the meeting. Our NTCO and HOLC also keep us updated with 3Rs, Understanding Animal Research and Home Office related information, which can be

disseminated. Together we are registered with the fish Husbandry Association and attend meetings, following which we disseminate the information and best practice.

I attend and have run regional NACWO meetings and Zebrafish Facility managers meetings (UK) to share and communicate best practice. Over the years I have attend numerous workshops relating to best welfare practice. I maintain a close-working relationship with the a nearby aquaculture institue, with whom information is shared.

The aquatic facilities staff, and researchers, are of a high calibre and their skills are relevant and up-to- date, ensuring minimal number of animals are required to generate new GM or GE lines, to which the most efficient husbandry practices are adhered to.

Best practice is communicated to facility researchers through a series of forum meetings.

44. Investigating the Spatial Ecology of Wild Fish to Inform Fisheries Management

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Fish, Conservation, Ecosystem, Telemetry, Movement

Animal types	Life stages
Seabream	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to significantly advance the knowledge base on the movements and habitat use of a broad range of marine species of commercial, recreational and ecological importance.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Substantially depleted wild fish populations across the North Atlantic have resulted in an urgent need to manage human exploitation of marine species, to ensure that 1) exploitation occurs at a sustainable rate; and 2) the essential habitats required by fish (e.g. nursery, spawning and feeding areas) are identified and then protected from damaging human activities, and thus ensure population sustainability. However, the movement, habitat use and migration characteristics of many wild fishes within UK waters and across Europe remain very poorly understood. Therefore, this project will collect vital evidence on the movement and habitat use of several wild fish species. Working directly with various

regional fisheries authorities and national statutory bodies across the UK, the data from the project will directly feed into fisheries management plans and support sustainable fishing activity.

What outputs do you think you will see at the end of this project?

The outputs from this project will include highly novel data on fish movement and habitat preferences across several districts across the UK, which are managed by the Inshore Fisheries and Conservation Authorities (IFCAs), and Natural England (NE). The species have been selected because: 1) they represent critical components of marine ecosystems, 2) they are highly valued in both commercial and recreational fisheries and, 3) very little information exists on how they use marine and/or estuarine habitats. This data will be freely shared between the research team, the IFCAs and NE to help improve fisheries management policies. By collecting data at a wide spatial scale the project will also contribute to UK and EU level fisheries management policies. From this work a suite of peer reviewed publications will also be produced which will contribute to the wider understanding of how marine life exploit coastal seas across Northern Europe.

Who or what will benefit from these outputs, and how?

Data produced from the project will be freely and immediately shared with the relevant IFCAs and NE, and can therefore be rapidly incorporated into fisheries management plans within the lifetime of the project. The project will therefore provide short term benefits to the research establishment, IFCAs and NE via continued research participation in this field, as well as long term benefits to the marine environment. The primary goal of the project is to collect data to help improve sustainable fishing practices by identifying important habitats for a range of marine species, known as Essential Fish

Habitat, including breeding areas and migration routes. Protection and/or appropriate management of human activities within these critical areas will provide increased quality of life for the animals that depend upon them, but also economic and social benefits by increasing the sustainability of commercial and recreational fishing businesses, and coastal communities that they support.

How will you look to maximise the outputs of this work?

As part of project funding, social media videos will be produced to disseminate the project activities and key outputs to the general public. Videos detailing the outcomes of the work will be disseminated on Twitter, Facebook and on project webpages. Project results will also be presented at both academic and lay conferences. Where possible results from this work will be published in open-access journals. All fish tracking data will be uploaded to an online portal known as the European Tracking Network (ETN), if research opportunities arise data will be shared (via the ETN) to other research organisations globally.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project aims to track movement and habitat use of the following wild species (adult and juvenile): Bream (2 species: Sparus aurata & Spondyliosoma cantharus), European Seabass (Dicentrarchus labrax), Flatfish (2 species: Pleuronectes platessa, Solea solea), Mullet (3 species: Chelon ramada, Chelon aurata, Chelon labrosus), Pollack (Pollachius pollachius), Sharks (4 species: Galeorhinus galeus, Mustelus asterias, Mustelus mustelus, Scyliorhinus canicula), Skates and Rays (3 species: Raja clavata, Raja microocellata, Raja undulata) and Wrasse (5 species: Labrus bergylta; Labrus mixtus; Centrolabrus exoletus; Ctenolabrus rupestris; Symphodus melops). These animals have been selected because they are highly valued for commercial fishing or are extremely rare, with little to no information available on their habitat preferences.

Typically, what will be done to an animal used in your project?

We propose to use acoustic telemetry to track the movements and habitat use of the aforementioned fish species, across the south coast of the UK. Acoustic telemetry uses sound emitted from tags to record fish movements across the sea through a series of listening devices placed on the seabed (receivers). This will involve capturing wild fish from multiple sites across the UK. Fish may be held in aerated seawater tanks for up to 60 minutes. Fish will then be provided with general or local anaesthetic (depending on the species or capture method), and an acoustic/sound transmitter tag will be implanted either within the fish belly or externally attached. Implantation or attachment of transmitters will take approximately 1-5 minutes. Pain relief will be applied to the surgical or attachment site. Fish will be allowed to recover from anaesthesia in a holding tank and then released back into the wild. No attempt will be made to re-capture tagged individuals. Movement and habitat use of the tagged fish will then be monitored remotely via a network of specialist equipment (referred to as a receiver array) which will be deployed on the seabed.

What are the expected impacts and/or adverse effects for the animals during your project?

Fish will have moderate surgery to implant or attach an electronic device within the body cavity or the external surface of the animal so that their movements can be tracked when set free. As part of the procedure the fish will be exposed to either a general or local anaesthetic (depending on the species and capture context). There is a risk that fish may not recover from the anaesthesia (<5%) in which case they will be humanely killed using a schedule 1 method. However, prior experience of tagging fish with the same methods has shown a >95% survival rate. Mortality risk as a direct result of the tagging procedure is therefore extremely low, however when it does occur fish are humanely euthanized using Home Office approved methods. Fish may feel pain from the tagging procedure, therefore painkillers/analgesics will be applied to the surgical site. Animals are expected to recover quickly (5 minutes) and be released back to the wild. Subsequent monitoring of these fish's movements (via acoustic telemetry) has shown behaviours that are comparable to similar studies globally with some individuals making migrations in excess of 500km from the capture location.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Fish: 100% moderate

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Live, wild fish are required for the project so that their movement patterns can be monitored in their natural habitat, and therefore the results can be considered a reliable representation of wild fish movements in order to inform conservation and management. The use of wild fish is preferable because the swimming capabilities and behaviour of hatchery-origin fish may be biased by the condition, and learned behaviour of captive fish, and there are ethical concerns associated with releasing captive-reared fish into the wild.

Which non-animal alternatives did you consider for use in this project?

Interviewing local anglers and commercial fishermen; Interviewing local anglers and commercial fishermen would potentially indicate which habitats are required for each target fish species.
Computer models of fish movements; Modelling techniques could potentially be employed to forecast/predict fish movement and habitat use.

Why were they not suitable?

Interviewing local anglers and commercial fishermen: This method may be biased to size classes which anglers or commercial fishermen are targeting, and may therefore not provide robust data on fish habitat requirements. Angler interview data may also be biased to areas and seasons which are logistically easier to fish. While angler-derived knowledge is valuable, it provides little on the movements of individual fish outside of times and places that they are captured, and thus leads to a biased picture of how fish use the marine ecosystem.

Computer models of fish movements: Models require real-world data in order to validate them and ensure outputs are realistic. In the absence of real world movement and habitat use data for the model species in this project, computer models would not be of sufficient reliability to be considered useful for informing ecosystem-based fisheries management.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Following ARRIVE guidelines, the minimum statistically viable sample size will be used within this project. Due to the nature of telemetry studies, which do not allow for a formal power analysis to identify appropriate sample sizes, the research team's prior experience with acoustic telemetry and external expert judgment has been used to identify the minimum sample size required per POLE ("place other than a licensed establishment" i.e. in the wild). Doyle et al. (2017) suggested that to account for inherent variability in individual fish behaviour, 30 individuals of each species is the minimum sample size to monitor movement and habitat use patterns within a POLE. Unlike Doyle et al., (2017) and laboratory studies where the number of study animals can be controlled, the nominated POLES cover extensive areas of open coastline where tagged animals are targeted by commercial and recreational fishers, and can freely move outside of the POLE boundaries. International Council for Exploration of the Seas (ICES) are responsible for allocating fishing quota across the EU and UK. Statistics produced by ICES estimate that; natural mortality (e.g. predation) and human fishing (e.g.commercial and recreational fishers) can remove 9-39% of the adult fish population per year. Prior experience from the PPL suggests that approximately 25-50% of tagged animals may move outside of the study site(s), at which point their movement can't be easily monitored using acoustic telemetry. Due to these limitations, a sample size of 100 individuals per species per POLE is required, to ensure a minimum of 30 animals are monitored for the duration of study.
Tagged animals = 100, 9-39% removed due to natural mortality = minimum of 61 individual fish tagged, 25-50% move outside of the study area
= minimum of 30 individual animals tracked.

By replicating this sampling effort at each of the 6 POLEs extremely novel data can be collected on fish movement and habitat use characteristics, across a range of sites which vary in their habitat extent, hydrological conditions and human activity in the coastline. Fish movements are highly variable across each POLE therefore we need to tag fish across all POLEs to understand local and regional movements. Only with this data can fisheries management plans be developed which include the protection of essential habitats for each of the target species. For sharks, skates and rays, we anticipate lower sampling effort limited to fewer POLES, due to the greater time and effort required to capture these species relative to round bodied fish as well as their data-poor status. Lower numbers of sharks, skates and rays are thus estimated to be used.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

By purposefully selected study species with very little existing data on which to base fisheries management measures, we ensured that we were not conducting studies on species for which adequate data already exists.

By selecting transmitters with multi-year battery life over transmitters with short (<1 year) battery life, we have ensured that the maximum amount of data can be collected from individual tagged animals, reducing the need to capture and tag further individuals.

By taking significant steps to ensure cross-compatibility of acoustic telemetry equipment with colleagues in Europe (France, Belgium, the Netherlands, Spain, Ireland), we will be able to follow the movements of tagged individuals across the English Channel. Similarly, colleagues will be able to obtain data from our receiver equipment deployed in UK waters.

This will ensure that the maximum possible amount of movement data will be extracted for each tagged individual and thus reduce the need for unnecessary repetition of work.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The poles within this project are the districts of the following Inshore Fisheries and Conservation Authorities (IFCAS): 1) Cornwall 2) Isles of Scilly 3) Devon & Severn 4) Southern 5) Sussex 6) Kent & Essex. Each IFCA is responsible for managing commercial and recreational fishing within their district. Each IFCA is a devolved administration, which means fisheries management is enacted at a local/IFCA district level and therefore varies by IFCA district. The IFCA districts also encompass large portions of the UK coastline, which vary in seabed habitats, environmental conditions and the level of commercial fishing activity.

The project license holder may tag all species listed in each POLE, depending on the specific/local fisheries management policies enacted by the relevant IFCA. It is however anticipated that the exact number of individual fish and species tagged will vary depending on the research requirements within the IFCA district. The project license holder will therefore optimize tagging effort on a POLE by POLE basis to minimize the number of fish tagged to the lowest possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will tag a variety of fish species with either internal or external transmitter tags under local or general anaesthesia.

Internal implantation provides better long-term welfare outcomes for tagged fish as there is lower risk reducing fish swimming capability through drag. However, since internal implantation requires a surgical procedure, and the holding time required for internally-tagging animals is generally longer than for external tagging, it is considered more invasive at the time of tagging.

The primary advantage of external tag attachment is the increased speed of tag application, which last around 1 minute. In situations where deep water species are being tagged the application speed of the transmitter tag may be critical to increase post-release survival and reduce lasting harm. A disadvantage is that external attachment of tags creating drag at the attachment site, which potentially increases the risk of post-release tissue damage and infection.

External attachment methods will only be selected when internal tagging would result in lower welfare outcomes than externally tagging the animal. The anaesthesia and tagging

method selected will therefore be dependent on expert opinion at the time of tagging and the outcome of pilot studies conducted on each species and tagging method combination. The physiological condition and welfare of the animal will always be maintained at the highest possible level, by minimizing the time the animals are held during the tagging procedure and conducting physiological assessments of each individual at multiple stages throughout the procedure.

Why can’t you use animals that are less sentient?

This project is species-specific rather than using the animals as models, therefore using less sentient animals would not provide the required data.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Within our action plan, we have highlighted a “pilot study” step. This requires us to tag a sub-sample of 10 individuals of each species using the relevant tagging method(s) prior to tagging our sample size (100 individuals per POLE). This step will allow us to minimize the welfare costs for the species If mortality as a result of the procedure is higher than natural mortality (39% or 4/10) during the pilot study a full methodological review will be conducted with the NACWO, NVS and NTCO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Where relevant PREPARE guidelines, NC3Rs and Laboratory Animal Science Association will be consulted for best practice. The PPL holder will also review the latest published peer reviewed literature to refine tagging methods.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check information on NC3Rs website, we have signed up to the NC3Rs newsletter, we will meet the NC3Rs regional programme manager, and attend Regional 3Rs symposia.

NORECOPA will be used as a resource.

45. Macrophage Biology and Development of Anti-Inflammatory Drugs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Macrophage biology, Drug development, Diabetes and obesity, Cardiovascular disease

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is two-fold

1) To better understand the role of cells called macrophages in the process of inflammation

2) To develop new classes of anti-inflammatory drugs that both reduce the production of inflammatory mediators and enhance tissue repair processes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation is the physiological response to noxious stimuli such as infection and tissue injury. We have made considerable progress in understanding the rapid response to injury

and infection, which we call acute inflammation. However, there are many unanswered questions relating to persistent or chronic inflammation which characterises many important human diseases including but not confined to diabetes, cardiovascular disease, inflammatory bowel disease and arthritis.

We know that white blood cells called macrophages, which are found in nearly all the tissues of the body, play essential roles in all stages of the inflammatory response ranging from the initiation of acute inflammation by acting as sentinel cells to driving chronic inflammation through secretion of inflammatory mediators and causing tissue damage. One important unanswered question is what role macrophages play in terminating an inflammatory response, a process often referred to as resolution of inflammation and what role tissue-resident macrophages play in initiating tissue repair.

Currently available anti-inflammatory drugs target a limited number of molecular targets and many widely used anti-inflammatory drugs have significant side effects that limit their use in clinical medicine. By understanding what role macrophages play in resolving inflammation and initiating tissue repair in living animals we aim to identify new molecular targets for generating new classes of anti-inflammatory drugs that will enhance inflammation resolution and initiate tissue repair.

In recent work we have shown that a macrophage receptor called GPR84 is up-regulated at sites of inflammation and enhances ongoing inflammation. These properties make GPR84 an excellent target for the generation of novel anti-inflammatory drugs and we have developed a series of small molecules which alter macrophage biology via the GPR84 receptor. We want to turn these 'GPR84 tool compounds' into new anti-inflammatory drugs.

What outputs do you think you will see at the end of this project?

- new insights into macrophage cell biology and inflammation
- research papers published in peer-reviewed scientific journals
- review articles about drug re-purposing.
- consultation and collaborations with industry companies
- patent applications for new drugs and new indications for existing drugs
- public outreach talks on disease mechanisms and drug discovery
- novel anti-inflammatory drugs

Who or what will benefit from these outputs, and how?

In the medium term (2-5 years) academic collaborators will benefit from new scientific insights, which we will share through talks at scientific conferences and publication of research papers and review articles.

In the longer term (5-10 years) patients, clinicians and pharmaceutical companies will benefit through drug development and drug re-purposing.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this 5-year programme of work through academic collaborations within the establishment, through collaboration with other academic organisations in the UK and internationally. We will maximise the dissemination of new knowledge by presenting our research at local, national and international conferences and writing primary research papers and review articles with open access.

Species and numbers of animals expected to be used

- Mice: 21,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

One of the goals of our research is to understand the role of cells called macrophages in the physiological response to tissue damage called inflammation. Only by studying inflammation in adult mice can we see if the behaviour of macrophages treated with inflammatory mediators in tissue culture studies matches the behaviour of tissue-resident macrophages in living animals.

A longer term aim of our research is to develop new types of anti-inflammatory drugs to treat serious human diseases such as arthritis and diabetes so we need to use mice as a model in which we can replicate the human conditions that we want to treat with our new drugs.

We will use adult mice for our studies because they have fully developed and functional innate and adaptive immune systems. Mobilisation of immune cells is an important feature of both acute and chronic inflammation.

Typically, what will be done to an animal used in your project?

Genetically altered animals may be bred on this licence. The typical experience of an animal used in this project would be pre-treatment with a drug or control substance by injection or oral delivery followed by delivery of an inflammatory stimulus with or without continued drug treatment. All animals will be monitored for any adverse effects before humane killing and harvesting tissues for analysis.

Most experiments (75%) will be short term and will last less than 7 days as we are studying the role of macrophages in acute inflammation. In fewer experiments (25%) we will be studying the role of macrophages in chronic disease processes such as obesity, diabetes or atherosclerosis. This will involve feeding an altered diet for up to three months with a drug or control substance administration daily for periods of up to 6 weeks. The number of procedures used will be the lowest number needed to generate robust datasets to address specific hypotheses.

In some experiments (up to 5%) mice may be irradiated to facilitate bone marrow transplantation where host white blood cells will be replaced by blood cells from another strain of mice.

What are the expected impacts and/or adverse effects for the animals during your project?

Irradiation prior to bone marrow transplantation will render the animals more susceptible to opportunistic infections. These effects will be of short duration (less than 2 weeks) and may be ameliorated by treatment with antibiotics if needed.

For some experiments animals will be fed a high fat diet which will induce obesity, pre-diabetes or atherosclerosis. These animals may develop some self-limiting skin inflammation but the altered diet will not cause abnormal behaviour, pain or increased susceptibility to opportunistic infections.

To test the role of macrophages and drug candidates in inflammatory bowel disease (IBD) we will need to add dextran sulphate to the animals' drinking water to mimic the breakdown of barrier function in the colonic mucosa that is seen in human IBD. Animals will be monitored daily for changes in weight, stool consistency and faecal blood. We do not expect the animals to experience pain or exhibit altered behaviour.

In multiple protocols we will deliver drugs and control substances to animals. This will cause no more than transient (less than 10 minutes) pain and discomfort. Delivery of inflammatory mediators may cause slighter longer lasting pain or discomfort (up to 1 hour) but the doses of inflammatory mediators used will be the minimum needed to initiate the disease process. Analgesics will be administered when required and in consultation with the NVS.

In all experiments weight loss will be used to monitor loss of appetite, changes in feeding and drinking behaviour, and excess fluid loss.

In all experiments we will regularly monitor animals for changes in coat condition and lethargy as general signs of ill health.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 50% Sub threshold
- 30% Mild
- 20% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To study a complex biological process such as inflammation we need to use the most physiologically relevant system that integrates multiple inputs from immune cells, hormones and metabolism. We often study parts of the inflammatory response in primary cells in tissue culture systems but to understand the role of macrophages in inflammation we need to study this process in tissues containing many different cell types. This requires the use of adult mice in which we can initiate the inflammatory response in a timed manner in response to specific inflammatory stimuli.

We cannot get sufficient numbers of tissue resident inflammatory cells from human volunteers. We can generate human macrophages from blood cells called monocytes using tissue culture techniques but these macrophages do not recapitulate all aspects of the cell biology of tissue resident macrophages found in for instance atherosclerotic lesions or joints spaces in rheumatoid arthritis.

Studying the time course of mammalian inflammatory responses from initiation to resolution will need to be performed in adult mice despite advances in computer modelling and 3D cell culture techniques. Indeed, we are keen to use computer modelling to identify key variables in our animal experiments which we will then test in vivo which will require the use of adult mice.

Which non-animal alternatives did you consider for use in this project?

We routinely use literature searches and analysis of chemical databases to identify compounds with potential anti-inflammatory properties. We are increasingly using datasets produced by other laboratories that has been made publicly available in order to identify changes in macrophage gene expression in different inflammatory diseases. Testing candidate pathways needs to be performed in adult animal models of human diseases before we can prioritise these pathways for developing new drugs.

Nearly all of our drug development work involves the use of transformed cell lines or primary cells prepared from human blood donations or mouse tissues.

Key experiments need to be performed in mice despite advances in computer modelling and 3D cell culture techniques. We cannot perform proof of concept experiments with newly developed anti- inflammatory drugs in human volunteers until these experiments have been performed in adult mice.

Why were they not suitable?

Database searches and experiments performed using cell lines and clinical tissue samples help to implicate certain genes and signalling pathways, but they cannot tell us if these genes and pathways play an essential or non-redundant role in inflammation and tissue repair in the context of an ongoing inflammatory response.

Cell based studies and computer modelling cannot reproduce the complex physiological interactions between different cell types in different tissues that work together to produce an integrated inflammatory response in adult mammals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample sizes for our experiments are estimated from past experiments. Calculations typically show that we need group sizes of n= 10-15 to achieve the quality of results we need to test hypotheses about re-purposed drugs and novel GPCR agonists.

We have used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The minimum number of mice needed for each experiment will be calculated after reading widely into the existing literature. We routinely consult resources such as the NC3Rs Experimental Design Assistant and PERPARE guidelines.

Before performing experiments for the first time we consult with colleagues performing similar studies using experimental animals and perform pilot experiments using small numbers of animals to identify key variables in the experimental design and data collection.

Wherever possible experiments will have a block design so that different treatments share the same control animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If using a new experimental protocol or testing a new drug we will perform pilot experiments.

Breeding colonies will be managed in line with best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on identifying animals for future breeding and to assist in maintaining a suitable colony size to ensure only those animals needed for key experiments are produced.

We will harvest multiple tissues from experimental animals and store these tissues for future analysis in other projects and we will share tissue samples with other groups. Experimental datasets will be analysed using multiple well validated statistical methods and we will employ mathematical modelling to identify sources of variability in our experimental data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our protocols for inflammatory cell recruitment have been refined in over 20 years of use to cause only momentary discomfort and minimum pain and suffering and are constantly reviewed.

To study the process of inflammation caused by bacterial infection we need to use mice so that we can measure a range of physiological processes that are initiated when a bacterial pathogen activates the host immune response.

We will use endotoxemia experiments as a more refined method to follow key aspects of host immune response to bacterial pathogens with the least harm. This protocol will allow us to specifically down-

regulate important elements of the host immune response with repurposed drugs and new GPR84 drugs.

We are able to use the air pouch model of inflammation to generate quantitative data on inflammatory mediator production and leukocyte recruitment hence this protocol is the most refined method to study cellular recruitment and inflammatory mediator expression in a model of sterile inflammation.

Anti-inflammatory drugs are a mainstay of current therapy for inflammatory bowel disease (IBD) and we will monitor animals in our IBD experiments regularly for weight loss, changes in faecal consistency and changes in behaviour.

Our protocols for arterial inflammation, diabetes and obesity have now been integrated into a single procedure. The new protocol involves feeding animals with an altered diet to induce metabolic disease. Our disease models have been optimised to cause minimum lasting harm to experimental animals. We have chosen not to use protocols that mimic Type 1 Diabetes through damaging the pancreas because we want to study the role of inflammation in Type 2 Diabetes and obesity. Our new diabetes protocol will cause less suffering and long term harm than protocols that ablate pancreatic islets.

Why can’t you use animals that are less sentient?

The process of inflammation in foetal and embryonic mice is significantly different to the process in adults due to the development of innate and adaptive responses and the development of a microbiome in adult animals.

Non mammalian species that are less sentient e.g. nematode worms do not have the complex immune systems and physiological interactions that we want to study in the context of inflammation and so are unsuitable to address the aims of this PPL.

Systemic inflammation is common in human adults and increases with age so we cannot study this disease process in terminally anaethetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The routes of administration used to deliver inflammatory stimuli and anti-inflammatory drugs will be chosen to be the least invasive for instance including drugs in food or drinking water or injection just below the skin.

Irradiation used to prepare animals for bone marrow transplantation will be reviewed in line with best practice to limit lasting harm caused by this procedure.

Animals will be housed in a controlled environment with optimal light, heat, food and appropriate companion animals. Our procedures will be constantly reviewed to ensure that animals do not experience any lasting pain or discomfort.

When using a drug for the first time we will carry out pilot experiments with 5-6 animals starting with the lowest dose to look for any unanticipated adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the latest scientific literature in peer-reviewed journals (PubMed) and by consulting online scientific guidelines (including LASA, NC3R, ARRIVE) to ensure our experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend regular departmental animal welfare meetings and 3Rs days organised by the institution. The 3Rs will be discussed regularly in lab meetings and always reviewed before setting our standard operating procedures.

All scientists working under this licence benefit from regular updates provided by our Named Information Officer, the NC3R's regional manager and regular seminars on aspects of the 3Rs.

We will regularly review relevant websites such as www.nc3rs.com and https: //science.rspca.org.uk

46. Mechanisms Underpinning Neuronal Excitability in Health And Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Pain, Inflammation, Neurodegeneration, Cancer, Therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged
Naked mole-rat	adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To extend knowledge of mechanisms governing how nerves are switched on and off in the context of pain, control of breathing and neurodegeneration, conditions where levels of oxygen and interactions between the immune and nervous systems are important. Anticipated benefits are understanding potential new drug targets for pain relief and the treatment of neurodegenerative conditions associated with ageing, such as Alzheimer’s and Parkinson’s diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Chronic pain is a hallmark of conditions like osteoarthritis, irritable bowel syndrome, cancer and endometriosis, affecting approximately 40% of the adult population in the United Kingdom.

Unfortunately, current medications are deemed inadequate at controlling pain by two thirds of chronic pain patients, either because the drugs do not work, or the side effects experienced are too unpleasant.

Neurodegenerative diseases like Alzheimer's disease which affects approximately 1 million individuals in the United Kingdom, and disordered breathing syndromes such as central sleep apnoea, are similarly poorly managed, a few treatments improving symptoms, but no therapies providing a cure.

A major risk factor for both chronic pain and neurodegenerative diseases is ageing and in the United Kingdom the proportion of the population aged over 65 years of age will increase from 16% to 30% by 2030, thus the prevalence of conditions for which age is a risk factor is set to increase.

In both chronic pain and neurodegenerative conditions, changes in neuronal excitability and function lead to the symptoms observed. In recent years, it has become clear that changes in how the immune system interacts with the nervous system play pivotal roles in disease progression.

This work will focus on increasing our understanding of how the properties of nerves change from health to disease in the context of chronic pain, disordered breathing and neurodegeneration, and the role of non-neuronal cells in these processes. Results will open avenues to developing new therapies to treat pain and neurodegeneration.

What outputs do you think you will see at the end of this project?

The main output of this project will be new information about the mechanisms by which chronic pain and neurodegeneration (neuronal death) are caused.

For example, the way our experiments are designed, by comparing health to disease, we will be able to identify key molecules involved in specific disease processes, e.g. what molecules are released into the knee joint during arthritis and how do they alter neuronal excitability to cause pain? Similarly, by comparing the neurobiology of the long-lived naked mole-rat, which is highly resistant to cancer and neurodegeneration, to the biology of the mouse, we aim to identify molecules the support the healthy ageing of the naked mole-rat, which could be targeted to prevent and / or treat ageing-related conditions, such as neurodegeneration in humans.

All new information generated will be published, firstly online through the open access pre-print bioRxiv server, a process that ensures rapid dissemination of our outputs, and secondly in peer reviewed journals. In addition, we will communicate our findings at scientific conferences through poster and oral presentations.

We will also make sure that our work is communicated to the public in an appropriate way, for example, by providing lay summaries of our work and through the production of short films and animations, all of which we have a strong track record of delivering.

In the long-term, we would hope that our work would lead to the generation of new therapies for the treatment of chronic pain, disordered breathing conditions, and neurodegeneration, but this is beyond the scope of the current project.

Who or what will benefit from these outputs, and how?

In the short-term, the main benefits from our work will be to other researchers, both in academia and the pharmaceutical industry, as well as to the general public through our public engagement work.

Communicating with the public to increase understanding of what scientists do is not only crucial to educate and inspire, but also as a point of accountability considering how most research within the United Kingdom is funded. We currently collaborate with multiple pharmaceutical companies making us well placed to provide immediate benefit to their research programmes through our findings.

In the long-term, beyond the time of this project, we anticipate that findings from our research could lead to the development of new therapies to prevent and / or treat chronic pain conditions and neurodegenerative diseases.

How will you look to maximise the outputs of this work?

To maximise our outputs, we will continue our strong track record of collaborative work. For example, I run an animal sharing platform, which provides an online presence through which researchers can contact me to arrange collaborations and maximise the sharing of tissue to gain the greatest amount of information from any individual animal.

To maximise dissemination, we post all our findings, both positive and negative, on the open access pre-print bioRxiv server, which enables results to be posted online prior to peer review. In addition, we

will continue to post "Tweetorials" of our published work through Twitter to reach the maximum possible audience; these are often written in an accessible way for non-experts to also understand.

Species and numbers of animals expected to be used

- Mice: 13960
- Other rodents: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Chronic pain is whole body experience, involving activation of a peripheral nerve that generates responses in the central nervous system, and the experience of pain is modulated by how the nervous system interacts with other parts of the body, for example the immune system. Similarly, in neurodegenerative diseases, such as Alzheimer's disease, the disease occurs in a complex organ, the brain, and involves multiple different

cell types. Consequently, to study how nerve function changes between health and disease it is necessary for us to study whole organisms.

This project will use mice at all life stages, which are the most appropriate species for studying pain. Firstly, for studying pain affecting joints (e.g. osteoarthritis) and internal organs (e.g. endometriosis), it is necessary to use a whole organism that has the appropriate body parts, i.e. a purely in vitro (i.e. cell- based) study or one involving invertebrates like Caenorhabditis elegans that lack joints would not provide the same degree of insight that an in vivo (i.e. whole animal) study in a mammal can. In addition, technologies developed for genetically altering mice enable greater mechanistic insight to be generated than in other rodents. Mainly adult mice will be used, but in some experiments young mice will be used as well to study the developmental aspects of both pain and neurodegeneration.

We will also use naked mole-rats because they are long-lived rodents that display a variety of exceptional biology. For example, naked mole-rats are highly resistant to cancer, do not develop neurodegenerative conditions throughout their approximately 40-year life-span, can cope with fluctuations in atmospheric oxygen and carbon dioxide that regulate breathing, and display highly unusual pain biology. As a mammal, the naked mole-rat provides a unique opportunity to study biology at its extreme, for example, if we understand what is special about nerves within the naked mole-rat brain that make it resistant to neurodegeneration, this could be translated to be of benefit to humans. We will use naked mole-rats of all ages, from soon after their birth through to the end of their naturally occurring lifespan because this will enable us to see what changes do, or do not occur, within their nervous system that enable them to age healthily.

Typically, what will be done to an animal used in your project?

Many of the procedures detailed in this proposal will be conducted using general anaesthesia to ensure that pain and/or distress are limited during a procedure before animals are allowed to recover with appropriate post-operative care (e.g. soft food, pain relief post-surgery and heated recovery cabinets, as well as use of heat mats during surgery).

Small groups of animals will develop different forms of inflammation, such as arthritis and colitis, skin cancer, or experience fluctuating oxygen and carbon dioxide levels, procedures which will involve a moderate level of pain and distress; an animal would only experience one such experimental condition.

In arthritis models, this is likely to involve swelling of the joints; one arthritis model uses the joint lubricating fluid from human individuals (or dogs) with arthritis to induce arthritis-like conditions in mice and use of this substance will aid translation of our findings back to humans; synovial fluid will also likely reduce the severity of joint inflammation compared to synthetic agents, although these latter agents are also very useful due to their reproducibility of effect and known mechanism of action, how the pain they produce is evoked is less understood. Most of these inflammatory arthritis models will last a maximum of 7-days, but one that mirrors aspects of osteoarthritis last 28-days due to the progressive degradation of cartilage and bone that thus better recapitulates the human condition. A further arthritis model involves making a small injury to the knee joint that leads to gradual onset of osteoarthritis over several weeks, involving similar processes to the human condition, thus making it a good model to aid translation from bench to bedside.

In colitis models, animals are likely to show low activity and experience diarrhoea as occurs in human inflammatory bowel disease (IBD), but periods of colitis will generally last days, rather than weeks.

Some animals may experience 2-3 bouts of colitis to simulate the relapse and remission that humans with IBD also experience, remission from inflammation often not meaning a remission from pain, and thus our approach enables us to study pain mechanisms at all stages of disease.

In cancer models, cancer is induced in the skin on the back of the animal over a period of weeks and is not expected to induce any significant symptoms beyond tumour growth, metastasis to another body location being exceptionally rare.

In bladder pain and endometriosis models, animals will likely urinate more often, but produce a smaller volume, as occurs in humans with similar conditions. Bladder pain models are generally short lasting (e.g. up to 7-days) using an anti-cancer agent that causes bladder pain in humans (i.e. we accurately modelling a human condition). Endometriosis models are longer lasting (up 2-months) due to the gradual onset of the condition, i.e. animals do not experience symptoms until long after induction of endometriosis.

In some experiments, animals will be injected with substances that enable the tracking and modulation of sensory neurones in the body so that we can determine, for example, how the properties of neurones supplying the knee change during arthritis – these substances are inert and no adverse effects are expected beyond that experienced by the procedure itself; injection of substances to track neurones in the gastrointestinal tract (gut) and other internal organs requires surgery, from which animals are expected to make a rapid recovery.

From such work, we aim to identify new drug targets and these will be investigated by administering substances by the most appropriate route to determine how they ameliorate disease/pain progression,

i.e. drugs or other substances administered to modulate pain pathways in disease are expected to reduce the adverse effects of the, for example, arthritis being experienced.

A small number of animals will undergo surgery to insert wires that will enable us to measure the responses of muscles to distension of the gastrointestinal tract, bladder or vagina. Animals are expected to make a rapid recovery from surgery and the distension process itself evokes a short- lasting pain response that may be heightened in animals experiencing colitis, bladder pain or endometriosis. The distension procedure is required to simulate the type of natural stimulus that evokes pain in humans with such conditions, e.g. through passage of matter through the gut, a filling bladder and uterine contractions during menstruation.

In all animal models of pain-related diseases, animals will undergo behavioural tests to measure how disease and disease intervention affects their behaviour. Many of these behavioural tests will monitor the impact of pain on spontaneous behaviours, e.g. how much less burrowing does an animal display when a joint is inflamed, whereas other tests will measure the time taken to respond to a stimulus, such as a fibre being pushed against the hind paw.

In some studies, female animals will undergo drug-induced superovulation – a procedure to induce multiple eggs that is used in humans for in vitro fertilisation – harvesting of eggs will enable us to establish cell lines that in the long-term will lead to reduced animal use.

In some experiments, animals will be transported between two facilities (a 15-minute journey by an appropriate vehicle) to enable access to equipment that measures in a non-invasive way how cells in the blood respond to changes in the amount of inhaled oxygen and carbon dioxide. Results from these experiments will tell us how much oxygen and carbon dioxide get to the brain, which will help us understand the mechanisms by which breathing is regulated and how nerve damage can occur because long-term changes in brain oxygen and carbon dioxide are associated with nerves dying.

We do not expect animals to die as a result of the procedures performed and the minimum number of procedures will be performed on each animal to minimise the overall impact on an animal's well-being. All animals will be humanely killed at the end point of each study using approved methods and tissues used for further analysis, including sharing with other research groups to maximise the use of available tissues and thus minimise further animal use as far as possible.

What are the expected impacts and/or adverse effects for the animals during your project?

In all of our pain models, we anticipate that the animals will experience some degree of discomfort and heightened sensitivity in behavioural tests, due to accurate simulation of the human condition being modelled; the discomfort is expected to be lessened in our studies that aim to interfere with the pain mechanisms that we identify. Most arthritis, bladder pain and colitis models only last a number of days, but one arthritis model lasts up to 16-weeks because it is a slowly progressive condition with animals not experiencing measurable pain for the first 12-weeks, and similarly, our endometriosis model develops slowly and so although it lasts approximately 2-months, symptoms do not occur in the initial weeks. Weight loss is not generally expected apart from in the bladder pain and colitis models.

Behavioural tests used will sometimes evoke a transient sensation of pain, but that is the method required to measure how the disease affects pain and set a standard against which we can investigate how interventions affect the pain experienced.

In our cancer pain models, whereas mice develop tumours, naked mole-rats do not, moreover, because we are investigating skin tumours on the back of the animals, these are generally deemed to be low impact to the animal's well-being and no particular distress or weight loss is observed.

All surgeries conducted will be under anaesthesia, thus minimising the pain and discomfort experienced, although transient weight loss in the few days following surgery is anticipated; pre- and post-surgery pain relief will be administered to lessen the discomfort experienced where it does not interfere with the scientific objectives.

Exposure to low oxygen or high carbon dioxide will be relatively brief (maximum of 1-hour) and will cause mice to show altered breathing rate during the period of altered oxygen / carbon dioxide exposure, but this is not the case for naked mole-rats who are resistant to such changes in oxygen and carbon dioxide.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

There are no severe protocols associated with this project.

Most animals will experience surgery and / or a form of pain syndrome (e.g. arthritis) or cancer, which would be of moderate severity.

Mice exposed to low oxygen or raised carbon dioxide would be considered as moderate severity, but this would be mild for naked mole-rats that are resistant to low oxygen or raised carbon dioxide and continue to breathe normally.

Breeding of genetically altered animals will be of a mild severity, as will animals acting as control or sham conditions in our arthritis, colitis and other pain models.

Overall severity proportions:

Mice: 9.2% mild,
12.7% moderate
Naked mole-rat: 100% mild

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This research will investigate complex, whole organism pathologies, such as inflammatory bowel disease. To study such pathologies, it is necessary to study whole, intact organisms because of the interactions that take place between different body systems to drive disease pathology, e.g. the nervous system and immune cells in the blood.

Moreover, to correlate our work with humans experiencing similar conditions and to thus aid translation, it is necessary for us to study how an underlying pathology affects an animal's behaviour, which can only be done if whole animals are used. For example, humans with joint pain are less active due to activity-induced pain and we use similar assays to measure how arthritis and other conditions in mice affect their natural digging behaviours. Similarly, to study how changes in atmospheric oxygen or carbon dioxide affect breathing and the function of nerves in the brain, it is necessary to study an animal that has similar physiology to a human.

Studying less complex organisms, such as worms, would be inappropriate because they do not have the necessary physiology that would enable us to simulate human

pathologies, i.e. arthritis is a disease of the joints and worms do not have joints, nor do they have brains which precludes studying neurodegenerative conditions of the brain, or the brain regions that control breathing.

Lastly, using purely cell-based or organoid models prevents would be scientifically inappropriate as they do not permit the study of interactions between different body systems that are critical in the conditions that we will be studying, nor do they enable us to study how neuronal activity, and modifying that activity (e.g. via novel painkillers developed through our work) impacts animal behaviour.

Which non-animal alternatives did you consider for use in this project?

We do not only conduct whole animal work, but rather structure our computational, cellular and whole animal work to form coherent research projects, with the additional benefit of using human tissue when appropriate.

To meet the objectives of the proposed research, the following hon-animal alternatives that we have considered are as follows:

- Computational approaches to identify drug targets for treating disease

- Human induced pluripotent stell cell derived sensory neurones (human cells, which can be transformed to be like neurones)

- Other cell lines / organoids

- Non-protected animal species (e.g. worms and leeches)

Why were they not suitable?

- Computational approaches are ideal for screening through 1000s of compounds to identify top "hits" that might interact with a target of interest and this a method that we have used and published.

However, such computational approaches themselves cannot alone provide information on mechanisms of disease pathology, be it how endometriosis-mediated pain progresses over time, or how a change in oxygen level changes brain activity to result in altered breathing.

- Human induced pluripotent stell cell derived sensory neurones (hiPSC-SN) are neuronal cells of human origin that are helping for screening purposes (e.g. does my compound of interest modulate activity of a certain molecule in a human cellular background), which is how we use them in our lab to help validate results from rodents. However, such hiPSC-SNs are homogenous in nature, unlike the true diversity of sensory neurones in a whole animal where we and others have shown that neurones supplying different targets have different properties. In addition, a purely cellular system does not permit us to examine the complex pathologies of conditions such as osteoarthritis or inflammatory bowel disease where interactions between the nervous system and cells of the immune system and other non-neuronal cells are important for disease pathogenesis.

- Other cell lines/organoids, as with hiPSC-SNs, provide a good background for studying the structure - function aspects of how specific molecules function, an approach we regularly use in the lab. However, such a cell-based or organoid-oriented approach

does not provide insight into the complex, whole organism physiology and pathology that we are studying in this project where multiple body systems interact with each other to modulate behaviour.

• Non-protected animal species (e.g. worms and leeches), every organism with a nervous system has nerves dedicated to detecting potentially harmful stimuli and enabling safe interactactions with its environment. However, the physiology and pathology that we need to study in this research are specific to what occurs in mammals, e.g. one cannot study a condition affecting joints in a species without joints (e.g. worms/leeches), nor can one study regions of the brain involved in sensing oxygen or that undergo neurodegeneration in an organism that lacks a brain/a brain similar to that of humans (e.g. worms and leeches / fruit flies).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical analyses will be employed ahead of starting experiments to ensure that the minimum number of animals will be used, as is necessary to produce statistical useful results. Such analyses will be driven by prior experience and data from the wider scientific literature.

In addition, where appropriate, pilot tests will be carried out in a small number of animals to assist with experimental design and refine group size estimates. For example, to determine the efficiency with which neurones can be labelled with a certain agent, this would be conducted in small number of animals and refinements made where necessary.

Overall numbers have been estimated based upon two key factors:

• The number of mice required to breed the correct number of genetically altered animals of the required genotype for the different types of genetically altered animals to be used, which is based on our lengthy experience of working with such animals.

• Consideration of the number of different test and control groups that will we analyse for each scientific protocol involving mice and naked mole-rats. The group size for each experiment being estimated using statistical analysis for each specific protocol to ensure that the minimum number of animals will be used to produce statistical useful results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We keep up with the latest literature to make sure that we are only conducting experiments that are scientifically appropriate and to prevent the meaningless repetition of studies conducted by others elsewhere.

Where possible, we conduct computational and cell line work before proceeding to using animals. For example, computational modelling can help to identify compounds most likely to provide the desired effect on a molecule that regulates one aspect of neuronal excitability, which can then often (but not always) be tested in a cell line to validate computational results before taking the lead compound into whole animal work.

We have also conducted pilot tests to assist with experimental design and refine group size estimates, alongside use of online reference tools, such as the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The animals used are inbred to produce more reliable results (inbreeding reduces the genetic variation and thus limits one possible source of variation in experimental measurement) resulting in a lower overall number being used.

We regularly design experiments so that multiple tissues can be taken from each animal after its death to decrease the overall number of animals used. For example, subsequent to behavioural studies looking at inflammatory bowel disease, we would isolate the colon, as well as sensory neurone tissue to perform experiments on both tissue types, and from behavioural studies analysing joint pain we would isolate sensory neurones and knee tissue to support different forms of analysis; we often also take the spleen for harvesting immune cells. Where possible, we always conduct studies where the animal acts as its own control, for example, in many inflammatory arthritis models, we only induce inflammation in one joint of an animal, which means that the other joint acts as a healthy joint (pathology in the untreated joint, due to, for example, offloading from the treated joint has not been observed), thus reducing overall animal use.

Specifically, regarding the naked mole-rat, it is a highly unusual animal and therefore whenever an animal used experimentally, we ensure that tissues are shared widely using an online sharing platform.

Use of this platform means that it is very common that tissue from one animal supports multiple projects, for example different brain regions can be taken for studies looking at neurodegeneration and the regulation of neuronal activity by oxygen, the skin is taken as a source for developing cell lines, heart, liver and skeletal muscle support studies examining energy regulation with ageing, and further tissues are often shipped internationally (e.g. blood to country X to examine properties of the naked mole-rat immune system and skin to the country Y to examine properties supporting the mole-rat's unusually strong and supple skin that shows limited scarring after injury).

All members of the research team also subscribe to an internal tissue sharing scheme, which supports sharing of animals, techniques and resources.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used because they represent good choice of animal model for the study of the specific pain related conditions in this project e.g. colitis and arthritis, being an organism in which disease models, pathology and behavioural paradigms are well developed and understood. Furthermore, we have generated significant preliminary data using mice that will be used as a basis upon which to build in the experiments proposed in this project, and such preliminary data has helped us refine the experimental approaches used, the number of animals required and the severity of the procedures conducted.

In addition, mice are the mammalian species best developed in terms of our ability to manipulate their underlying genetic make-up and produce so called 'genetically altered animals', which enables us to make mechanistic insights into disease pathologies that were previously impossible, i.e. we can use such genetically altered animals in our studies to analyse the roles of specific of genes of interest in disease pathology and behaviour.

Many of the models we use simulate very closely the disease in humans. For example, the bladder pain model results from administration of an anti-cancer drug that is frequently used in humans with cancer and causes bladder pain as a side effect, the main colitis model is induced through a change in diet and shows relapse/remission like inflammatory bowel disease in humans, one of the key arthritis models involves mechanically destabilising the knee joint leading to gradual onset of osteoarthritis and injury initiated / slow onset pathology regularly underpins osteoarthritis in humans, and the endometriosis model to be used reproduces the types of internal injury that occur in the human condition. Because we are studying pain mechanisms, providing standard pain relief throughout a study would compromise our scientific objective of understanding the key molecules that drive pain. To provide as much comfort for animals as possible and thus to minimuse suffering, we will enrich their environment as appropriate, e.g. providing food in the form of mash (available on the cage floor to

prevent the need for animals to rear up to obtain food) and placing heat pads under individual cages as an extra supply of warmth.

When tamoxifen is used as a gene inducing/deleting agent, animals can experience weight loss. To minimise any potential harm to an animal, no experimental procedure will be conducted until any weight lost has been regained.

We will regularly carry out behavioural tests, many of which are non-interventional behavioural tests to measure the impact of a condition on an animal's behaviour, for example, how does the condition an animal experiences affect their digging behaviour, a natural exploratory behaviour exhibited by mice?

Naked mole-rats are being used because they display an insensitivity to carbon dioxide/tissue acidity and low levels of oxygen, alongside extreme cancer resistance and absence of neurodegeneration over a long lifespan, combined features that are unique among mammals. By comparing mice to naked mole-rats we can identify the molecular basis for the differences in naked mole-rats and thus learn more about oxygen/carbon dioxide sensing, cancer and neurodegeneration in other mammals, including humans. Because naked mole-rats are resistant to the conditions that we will be exploring, they will experience minimal pain and distress under experimental conditions, e.g. agents that

induce skin cancer in mice do not in naked mole-rats and they continue to behave normally in conditions of lowered oxygen. Some naked mole-rats will receive hormonal injections to help us generate cell lines, which would lead to reduced animal use in the future, but the hormonal injections themselves do not causing any suffering of lasting harm.

Why can’t you use animals that are less sentient?

In this project we will study pain, how oxygen regulates breathing and how nerves in the brain die with ageing. For all of these conditions, an organism with a nervous system and brain is required, i.e. use of worms or flies would be inappropriate as they do not have the parts of the mammalian body that we need to study to stand the best chance of translating our findings to aid human health and that of companion animals. Moreover, many of the human conditions that we wish to study, e.g. osteoarthritis and endometriosis, require the use of adult mammals as they are conditions that impact humans in adulthood. In addition, to study mechanisms of joint pain, it is necessary to study animals that have the same musculoskeletal apparatus as humans, and thus worms and fish that lack joints would be inappropriate species to study.

Because we wish to study how disease progression affects an animal's behaviour over time, use of terminally anaesthetised animals is not possible. For example, the main model of osteoarthritis that we use lasts 16-weeks with behavioural symptoms only occurring from approximately week 12, and therefore to study how the progressive nature of the condition affects behaviour (and how our interventions modulate the behaviours), it is necessary to monitor animals over time, not under anaesthesia.

Naked mole-rats are the only mammal exhibiting their highly unusual biology and hence it is not possible to replace them with another species. For example, certain reptiles also exhibit low prevalence of cancer, but translating our findings from a mammal are going to be far easier than from a non-mammalian species like a gecko.

Lastly, for all our work using mice and naked mole-rats, it is important that we can compare our results to others in the field and benefit from insights that they make. In all areas we are working, pain, cancer, neurodegeneration and regulation of breathing, mammals (primarily mice) are the species of choice because disease models are well-established and transgenic tools are available.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In general terms, the welfare impact on the animals will be minimized through use of: sterile surgical technique, pain relief when required that does not impair the scientific objectives of the study, and humane killing at end of study, or at predetermined humane endpoints to prevent unnecessary suffering. We also use non-interventional behavioural tests to measure the impact of pain conditions on an animal’s behaviour.

For animals experiencing conditions lasting several days or weeks, they are regularly monitored (e.g. using a disease activity index scoring system for mice with colitis and measuring joint width for animals experiencing experimentally-induced inflammation) and weighed.

On days where animals experience surgery, they will be regularly checked with peri-operative pain relief and easily accessible energy rich food provided.

We have constantly refined the environmental conditions that our animals live in, for example by providing ramped/bendy tubing and enlarged tunnel/chamber habitats for our naked mole-rats to more closely mimic the living conditions and hierarchical colony structure that they would have in their natural wild setting. Moreover, for experiments where we wish to measure how changes in inhaled oxygen and carbon dioxide affect the amount of oxygen and carbon dioxide in the blood, we will use a non-invasive method where the animal is under anaesthesia and placed in a machine that scans the animal with light to produce a form of ultrasound imaging, rather than taking multiple blood samples and measuring in an external machine.

Where genetically altered mice are used, inducible constructs will be used wherever possible to limit any impact of gene expression/deletion on behaviour. Moreover, where possible we will limit gene alteration to the cells of interest, which in the case of this project will usually be peripheral sensory neurones.

Lastly, we will use non-aversive handling techniques where possible, and always allow acclimatisation of the animals when brought into the facility before starting a study, as well as acclimatisation to any behavioural apparatus before initiating the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will conduct our research according to the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines and make sure that we report our findings in line with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

We will also use online resources, such as the humane endpoints webinar (https://www.humane- endpoints.info/en), NC3R's resources for breeding and colony management in genetically altered mouse colonies (https://www.nc3rs.org.uk/3rs-resources/breeding-and-colony-management), and NC3R's experimental design assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design- assistant-eda).

In addition, for all aspects of our surgical work we will use the Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery and for our cancer research following the advice of Workman et al (2010) Guidelines for the welfare and use of animals in cancer research, British Journal of Cancer, 102, 1555-1577.

Lastly, we will keep up with the latest scientific literature to monitor advancements in the field about how to look after our animals and conduct experiments in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will make the following steps:

- Routinely check the NC3R's resource library and keep abreast with, and implement where appropriate, any new advances in refinement, reduction and replacement that are relevant to our project.

- Have regular discussions with the Named Persons and animal technicians within the facility to review current approaches and whether there are any new 3Rs opportunities.
- Continue our subscription to an internal tissue sharing scheme, which also provides an opportunity to keep up to date with 3Rs news and events, as well as take advantage of opportunities to share tissues and knowledge.

- Regularly check attend NC3R's workshops where appropriate and monitor publications in journals, such as Laboratory Animals and Lab Animal to stay abreast of the latest developments in all aspects of the care and use of animals in biomedical research.

47. Mechanisms Controlling Adult Myogenesis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

stem cells, skeletal muscle, aging, cell signalling

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at deciphering the molecular and cellular processes implicated in the activity of a population of adult stem cells that reside in skeletal muscles that are essential for muscle repair following injury or disease, and contribute to muscle aging.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite recent advances in our understanding of the processes that control the activity of skeletal muscle stem cells, additional research is needed to increase our knowledge. Studying the biology of skeletal muscle stem cells is important as it could unravel novel players with important role in the regenerative capability of muscle stem cells that could be harnessed to improve the treatment of muscle aging, muscle injuries and muscular degenerative diseases like muscular dystrophies.

What outputs do you think you will see at the end of this project?

By the end of the project, we will have generated novel findings that increase our understanding of how skeletal muscle stem cell activity is controlled in normal conditions,

as well as during aging and upon injury. The output of these findings will be communicated to the broader scientific community through publications in peer-reviewed journals, and to the specialised scientific community through presentations (poster and oral) at conferences.

Who or what will benefit from these outputs, and how?

On a short-term basis the main recipients of this research will be other researchers with interest in skeletal muscle stem cells, in muscle aging, and in muscle repair as our findings will add further understanding to the processes that pave the way to muscle repair. Scientists are likely to use this knowledge to help with their own research. Because stem cells that reside in adult tissues and organs share common mechanisms of control, it is also likely that our findings will be relevant to scientists studying other types of adult stem cells (for instance, neural stem cells or mesenchymal stem cells).

A long-term benefit may be seen in the development of novel therapeutic strategies or drug to treat muscular dystrophies and aging based on the findings of this study.

How will you look to maximise the outputs of this work?

Where possible, my laboratory has established and will continue to seek collaborations to either speed the production of data or to increase the depth and quality of data produced.

To maximise the dissemination of our findings, we post pre-print of manuscripts on repositories (for instance, biorxiv) and we present our work at local themed meetings, and at national and international meetings; this allows scientist to access our findings prior to their full publication.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our standard non-human model is the mouse, a mammal with a short gestation period and the ability to be relatively easily genetically altered, thus there are many mouse models available, both transgenic or with targeted genes/loci. For example, our project uses a genetically modified mouse line allowing the disruption of gene function specifically in adult skeletal muscle stem cells. Mouse is also a good model to study the regulation of muscle regeneration since there are many useful reagents available, particularly antibodies. In addition, there is a large literature of studies performed in this animal model, which allows us to compare our findings to previously published studies. Importantly, many of the key genes involved muscle repair in the mouse are conserved in human. We would therefore expect that our findings on mouse muscle stem cell regulation, muscle regeneration, disease progression and therapeutic intervention to be broadly applicable to human.

Typically, what will be done to an animal used in your project?

Typically, genetically altered animals will be bred to generate animals with a specific gene deficiency in skeletal muscle stem cells. The impact of this deficiency would be studied in ex vivo cultures of skeletal muscle fibres, and by analysis of muscle tissues following injury or aging.

What are the expected impacts and/or adverse effects for the animals during your project?

The breeding of our genetically altered mouse lines does not impact on the health of animals as we use inducible systems. This means that the genetic alteration is 'hidden' until we give animals a reagent to cause the alteration. This sort of approach is useful when a genetic alteration may have an effect on the normal development of the animal. Following induction of recombination, most genetic alterations described in this project will have no adverse effects on the animals. One particular mouse line may affect animals causing muscle weight loss or premature aging of muscles.

Our procedure to investigate the repair efficacy following muscle injury targets a single muscle of the back leg, which may generate a temporary discomfort but does not affect the mobility of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Breeding of genetically altered animals is a mild severity, and this is the largest proportion of our animals (around 70%). Some genetically altered animals are bred and maintained under a moderate severity as they are used to study aging (10% animals). Muscle injury is a moderate severity and this procedure is used in approximately 20% of our animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Muscle regeneration is a complex process involving the interaction of several cell types (mostly, fibroblasts, macrophages, blood vessels, nerves, in addition to stem cells). Tissue culture studies of muscle stem cells or their progeny provide limited information of the influence of metabolism or the complex cell-cell interactions taking place during muscle repair and aging. Therefore, in vitro studies may yield inaccurate information on the biology of muscle stem cells. The use of animals allows us to monitor the behaviour of

stem cells in their natural environment and ensure that our findings are physiologically relevant.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are immortalised cell lines, such as the mouse muscle C2C12 cell line. However, this cell line is notoriously distinct from skeletal muscle stem cells and numerous publications have reported differences between the mode of control of C2C12 cells and muscle stem cells. To date, no cell line has been reported to mimic precisely the behaviour of muscle stem cells in vivo.

Why were they not suitable?

There are no cell lines for skeletal muscle stem cells. Cell lines like the C2C12 cell line does not reproduce faithfully the behaviour of muscle stem cells; this makes any information derived from studies on C2C12 unreliable, unless the findings are subsequently checked in adult muscle stem cells grown in their natural environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are estimated from our usage in the previous period of our Project Licence, as well as from the various tools described below.

Typically, we will use 600 mice per year in protocol 1 to maintain the breeding colonies for the 5 genetically-altered mouse lines used on this project (Pax7CreERT2, Tg(Pax7-nGFP), Smoflox, Ptch1flox and Ift88flox) and their compound breeding lines (for instance, Pax7CreERT2;Smoflox/+). This figure includes also animals generated for experimental purpose.

We plan the use of 200 mice per year in protocol 2; this figure takes into account that for each genotype, groups of 3 animals will be injured totalling 6 animals for the mutant and control groups per time point and per experiment. As we typically analyse muscle repair by harvesting muscle at 4 different time points over a period of 2 weeks, this implies that 24 animals would be required per mouse line studied per experiment. 200 mice per year allow for 8 experiments to be carried out.

We plan the use of 200 mice per year in protocol 3. As for protocol 2, a minimum of 6 animals per time point and per experiment is required. This includes 3 control and 3 experimental mice. Here, animals will be analysed at various time to evaluate their aging (from 8 weeks to 8 months for the experimental animals, and from 8 weeks to 32 months for the control animals).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing our experiments, we make use of several resources to reduce the number of animals being used. These include: a) information from previous studies carried out in our own laboratory or reported by others in the literature; b) guidelines issued by the organisation TREAT-NMD, which aims at promoting best practices in the neuromuscular scientific community, in particular through the publication of standardized operating procedures (SOPs); and c) better design using the NC3R's Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our laboratory uses in-vitro methods, including the use of primary myoblast cultures and of single muscle fibre cultures, which have been shown to provide physiologically relevant information. Culture of isolated muscle fibres with their associated muscle stem cells already reduces the number of mice needed for this project, since multiple experiments can be performed on a single batch of fibres (e.g. hundreds can be obtained from a single muscle) from one mouse. So, these study models allow us to reduce the numbers of mice required for the in-vivo investigation stage. In order to reduce the number of breeding pairs, mice are kept as homozygous where possible. Where possible we initiate muscle regeneration in one leg and use the contralateral side as the control.

Pilot studies will be performed to estimate sample size. The aim is to use the minimum number of animals to obtain statistically significant results and thus be able to determine any difference between experimental and control group. The statistical principles involved entail first defining the experimental unit and controlling variation, which can be helped by pilot studies. If only two groups are compared (experimental and control), we will use Students T test; if the analysis performed involves the comparison of multiple groups or factors, one-way ANOVA will be employed. Finally, if we want to make no assumption about the scatter of the data we can use nonparametric tests such as Wilcoxon or Mann-Whitney.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use animal models with inducible conditional targeted mutations in specific genes with predicted roles in skeletal muscle regeneration and aging. The use of inducible recombinase mouse lines implies that mouse breeding does not generate animals with harmful defects; so there should be no phenotype until candidate gene expression or deletion is induced. Induction of gene knockout is tissue-specific and restricted to skeletal muscles; this minimises risks associated with the use of standard knockout strategies.

Our procedure to study muscle repair is designed to minimise pain and suffering, as animals undergoing muscle injury have a single minor hindleg muscle injured, which does

not impair their mobility, animals will be kept under anaesthesia throughout the procedure and analgesics will be provided after surgery.

Why can't you use animals that are less sentient?

The nature of our studies requires analyses in adult animals. Indeed, skeletal muscle stem cells form in later fetal development but adopt only their final role after the first 6 weeks of post-natal development; so the use of earlier developmental stages would not allow us to gain knowledge of their role in repairing adult muscles.

Other species used in muscle studies are zebrafish and Drosophila mainly. No stem cells with function related to that observed in human have been reported in Drosophila; in zebrafish, a population of cells with characteristics reminiscent of mouse and human skeletal stem cells has been reported. However, the scientific advances are currently not sufficient to consider these cells as a faithful model of stem cell-mediated muscle regeneration in human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures used in this project have been implemented for over 10 years in my laboratory and already use best practice, including close monitoring of animals undergoing a procedure and use of analgesics to manage pain. We have and continue to incorporate changes to our procedures and analysis methods as new information becomes available. For instance, we are in contact with TREAT- NMD which publishes SOPs related to the analysis of muscle function. Here are some common approaches we take to minimise harms to the animals: any techniques that may be painful or distressing to animals will be performed with anaesthesia and appropriate pain relief will be given to minimise pain (e.g. after surgery). All surgeries will be performed under aseptic conditions following best practice guidelines (e.g. LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery). Animals will be regularly monitored for possible adverse effects that may occur in response to the procedures or treatments. We will seek advice from the NVS and/or NACWO where appropriate (e.g. for administration of an antibiotic if an infection is suspected. Optimal treatment regimens (e.g. dose, frequency and route of administration) will be identified in small pilot studies before use in larger scale studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As already mentioned, we use guidance from the NC3R organisation in relation to breeding management and guidance from the Treat-NMD organisation in relation to the experimental design for neuromuscular studies. We also follow the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I maintain awareness of recent developments in animal studies through attendance at conferences, regular interactions with national and international colleagues, and through reading the peer-reviewed literature. I get regular news updates from https://www.nc3rs.org.uk/ to stay informed on advances about breeding management; for instance, I have been implementing intermittent breeding to reduce numbers.

48. Mechanisms of Neuromodulation in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

neuromodulation, dopamine function, brain circuits, basal ganglia, neurodegeneration

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary goal of this program of work is to identify the brain mechanisms that control the function of dopamine and related neuromodulators in the mammalian brain, and understand how these operate during the control of action selection. The secondary goal is to discover the abnormalities in these mechanisms that occur in neurogenerative and psychomotor disorders, including Parkinson's disease and addictions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The neuromodulator dopamine in the brain is critically involved in mediating movement, reward and motivation and is thought to be central to disorders such as Parkinson’s

disease and addiction disorders. Parkinson’s disease is the second most common neurodegenerative disease after Alzheimer’s, and leads to debilitating movement symptoms and problems with cognition, mood, and sleep. Addiction disorders give rise to one of the largest causes of morbidity worldwide. These disorders have devastating impact on the individuals affected, and pose significant socioeconomic burdens on society and healthcare systems.

Dopamine does not operate alone, but its function is regulated by other circuits and neuromodulators. Very little is currently understood about the range of related neuromodulators that might interact with dopamine, but new tools are making it possible to now study them. Dopamine neurons have been the focus of interest for efforts to develop new treatments for brain disorders. However, treatment options are very limited, and established treatments, such as direct dopamine replacement therapy for Parkinson's disease, have limited use. Many alternative treatment avenues have not yet been explored, in part because the underlying biology of the circuits and neuromodulators that could be involved are not yet fully understood. The outcome of this project will be a better understanding of the circuits and neuromodulators that impact on dopamine function and that might also go awry in disease. It is essential to better understand the functional significance of these mechanisms if we are to understand and improve options for treatment of dopamine-related diseases. Ultimately this could lead to the development of new drugs or treatment strategies.

What outputs do you think you will see at the end of this project?

The output of this programme of work will be:

Scientific data (e.g. in terms of recordings of neuromodulators, neural activity and behavioural performance).

New scientific knowledge (e.g. regarding the mechanisms that shape dopamine transmission and function).

Publications in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The following groups may benefit from our programme of work:

Short-term: our colleagues and collaborators working on related projects will benefit from the scientific knowledge that we will generate which will help them interpret their own data.

Medium-term: other researchers in both academia and industry will benefit from this knowledge in any peer-reviewed publications. We have a track record of collaborating with drug companies.

Long-term: ultimately patients will benefit, as improved understanding of the neural mechanisms that govern dopamine function and dysfunction in the mammalian brain will help us, clinicians and/or industry to develop new or improved treatments.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through several strategies:

Collaborators: We have an excellent track record of collaboration, internally within our institution, as well externally at national and international levels, including through a major current international collaborative research network supporting Open Science. Our results and the scientific knowledge generated will have a strong bearing on these collaborative research projects as well as the independent research of our collaborators. We also have a record of collaborating with industrial partners through industrial-academic consortia that allows us to influence their research programs.

Publishing and Presentations: In addition to publishing our work in respected, peer-reviewed journals, we will present the work orally and through poster presentations at local, national and international conferences.

Publishing null results: We will endeavour to design our studies in such a way that null results will be publishable (e.g. including positive control groups, validation measures of experimental manipulations, triangulation to determine generality of findings).

Data sharing: Wherever possible we will happily share our data (behavioural measures and recordings of neural activity) with other researchers, and have signed up to adopting Open Science pledges, publication of data and protocols.

Teaching: We will also include our research in our undergraduate and graduate teaching (lectures, seminars, tutorials), thus influencing the next generation of researchers, and will bring students into our research programs for their projects, thus building capacity and expertise for the next generation.

Outreach: We will share our findings with local patient groups, to develop the potential utility of our work, and in turn, their thoughts and experiences will inform our future work.

Species and numbers of animals expected to be used

- Mice: 15350

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We work on mice because they are the lowest vertebrate group whose brains sufficiently resemble human brains. In many experiments we will use genetically modified mice. These mice allow us to selectively manipulate the function of key proteins, specific cell types and circuits in the brain in order to assess their functions. They also allow us to examine the impact of genetic mutations that are found in patients so that we can then study how genetic mutations affect brain function. Most of our experiments will be performed in young adult animals as circuits are fully formed by this age. However, in some experiments we will study mice maintained in to old age to try and model the kinds of neurodegenerative changes that might contribute to certain disorders related to age.

Typically, what will be done to an animal used in your project?

Several of our experiments will involve animals receiving brain surgery e.g. to inject a virus to alter the genetics of neurons or to express a genetic sensor, or to make a brain lesion or to insert a microelectrode. In some experiments, animals will receive systemic injections or dosing with drugs that interfere with brain function. In some animals we will record the activity profiles of brain neurotransmitters, related molecules and cells, sometimes while the animals in anaesthetised, and sometimes while the animal is performing behavioural tasks, typically after a period of restricted access to food or water to motivate animal behaviour. Some animals might be singly housed. We might also examine the effects of brain lesions, drug treatments, various genetic manipulations. Some animals will be models of disease, and might be maintained in to old age as a model for age onset degeneration, or might be used to test whether potential treatments restore brain signalling and behaviours.

What are the expected impacts and/or adverse effects for the animals during your project?

There may be transient pain and discomfort after brain surgeries but animals are given analgesic drugs to cover the period when any pain would be expected. Injection of drugs might cause minor discomfort at the time of injection and some of the drugs that we use might result in transient abnormal behaviours (e.g. increased or decreased levels of movement). Some animals that receive a brain lesion might also experience changes in locomotion (e.g. turning behaviours) or risk of malaise for up to a week post-surgery. Any unexpected changes in motor activity that do not resolve by 24 hours would result in the animal being humanely killed.

Animals maintained into old age might experience some effects similar to those we would expect in aging humans, such as declines in muscle mass and activity.

Animals will be humanely killed at the end of the experiments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

2% of mice will experience non-recovery procedures 57% mice will experience sub-threshold procedures 2% of mice will experience mild procedures

39% of mice will experience moderate procedures

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to understand how the brain uses chemical modulators to change how neurons signal to each other in the context of a normal working brain and how this changes in disease. The brain is a complex structure with many different cells and circuits interacting and communicating with each other and no in vitro system can currently replicate this. Furthermore, we sometimes need to remove, silence, activate or measure particular brain chemicals and cells to understand their function, and this is not ethical or practical in humans. Computer simulations of the brain rely on the information that we will provide and while very useful as a companion to our work cannot replace the work that we do.

Which non-animal alternatives did you consider for use in this project?

We make use of in silico techniques and computational models for data analysis and modelling when we can, which helps us to generate hypotheses which can be used to assist in designing our animal experiments. We also collaborate with scientists using neurons made from reprogrammed human stem cells which allow study of several aspects of cell biology. In addition, we are collaborating with a fly-based research lab to understand which aspects of the biology we study is mirrored, and could be explored productively, in invertebrates.

Why were they not suitable?

Despite the utility of these alternatives for some aspects of our work, they are limited in their use as they do not allow us to test key aspects of how neural signalling operates in the context of normal physiological circuits that have developed in the intact brain. Computational models for brain function are generally constructed from information provided by data collected in animals and need to be developed further before they can fully recapitulate the brain environment. It is not possible to replicate in ex vivo or in vitro experiments the complex circuitry formed by the diverse range of cell types and neurochemicals that we will explore.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample sizes for our experiments are estimated from past experiments and closely related experiments (> 25 years). Estimates and calculations typically show that we need group sizes of 5-10 to achieve the quality of results we need. We've also used our annual return of procedures data to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will generate multiple brain tissue samples from one animal which can be used for multiple ex vivo experiments allowing the number of animals required to be reduced. Over

time we have also increased the number of recordings we can take from each tissue sample giving rise to a high yield of data further reducing animal numbers.

We will also minimize the numbers of animals used by making all the experimental manipulations (e.g. intracranial injections of viral vectors, lesions, genetic modifications) as accurate and appropriate as possible. We will also use genetically altered mice that allow us to combine anatomical targeting with genetic factors to make the manipulations and measurements in each animal as specific as possible reducing the numbers of additional mice that might be needed to further interpret the data.

We exploit newly developed tools from our collaborators as soon as they are developed to ensure that we are using the most powerful methodologies.

The design of our behavioural tasks has been informed by close collaboration with local colleagues who have over many years developed and modified our behavioural tasks such that they are better able to detect subtle changes in brain function.

We will also use what are called "within-subjects designs" in which we might use different halves of the same brain to take measures for control data versus experimental data, or the same animal might receive both the treatment condition and the control condition (e.g. on one day an animal might be observed in control conditions as well after manipulation of a key circuit). This reduces the number of animals required and also reduces the amount of variability in the experiment overall which then, in turn, further reduces the number of animals that we need.

In many of our experiments we will collect more than one set of data e.g. behavioural data and recordings of brain activity. We are therefore able to gather lots of information from the same animal, reducing the numbers of animals that we need overall.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

We will conduct pilot studies to ascertain whether experiments are feasible and to optimise the way the experiment is conducted.

We will share brain tissue generated for ex vivo work amongst team members to conduct several different experiments in this tissue to maximise data yield from each animal.

We will use computer modelling to help us get the maximum information from the analyses of our data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In order to study how the brain works we need to be able to record the activity of its neurons and molecules, and interfere with its normal function. This requires using techniques which disrupt brain function in different ways (e.g. selective brain lesions which remove a particular brain region, drugs which block specific receptors in the brain, or genetic mutations which alter brain function), or implanting electrodes or sensors which measure brain activity. We will use genetically altered mice that have mutations which allow us to manipulate brain function in very specific ways or which mimic human neurodegenerative and other psychomotor disorders. We will also use animals that have undergone manipulations of the brain or mimic brain disorders by either surgery or drug administration. The methods we use cause only minimal and short-lasting pain or discomfort. Behavioural testing is likely to cause only transient and mild discomfort as we aim to study behaviour under normal conditions. Some animals will be maintained into old age to understand age onset diseases, but the disease models we will use will represent mostly the earliest stages of dysfunction in disease meaning that the animals suffer only mild disturbances to behaviour to allow us to address our scientific questions.

Why can’t you use animals that are less sentient?

We can't use less sentient animals such as worms, flies or fish for these questions because their brains are not similar enough to human brains. They have different types of brain circuits, even using some neurochemicals and receptors not found in mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Operations on the brain are undertaken by qualified and experienced surgeons in surgical theatres, and the animals are given pain killers after the operations until they have fully recovered. They are also given highly palatable food to help them recover. The animals are monitored for at least 7 days after any surgery, but very quickly recover to seem the same as control animals. Similarly, the genetically modified mice are virtually indistinguishable from normal mice when viewed in their home cages. It is only with the sophisticated tests of movement that they can be distinguished. Animals undergoing behavioural tests are continuously monitored during the task. The tasks the animals are required to undergo are the least invasive to be able to answer our scientific questions and, in most cases, require the animals to exhibit natural behaviours or work for rewards. The animals will have extensive handling and training to perform the tests.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow best practice guidelines issued from the NC3Rs, and Laboratory Animal Science Association (LASA). We will follow ARRIVE and PREPARE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about advances in the 3Rs through attendance at local 3Rs meetings, through interactions with our NC3Rs regional manager and named information officer, by monitoring latest information on NC3Rs website, through 3Rs initiatives organised by our scientific societies (e.g. British Neuroscience Association) and through local project licence holders' meetings.

49. Studies to Assess and Improve the Humaneness of Stunning Methods for Poultry

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Animal welfare, Poultry, Waterfowl, Stunning, Slaughter

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	adult
Domestic goose (Anser spp)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To examine the effectiveness and humaneness of existing and new stunning and slaughter methods for poultry.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is an urgent need for more humane stunning and slaughter methods for poultry (including waterfowl) designed for human consumption. Stunning prior to slaughter is commonly used to render the animal unconscious prior to and during bleeding, with the aim of reducing pain and distress. The majority of poultry slaughtered in the UK are stunned using controlled atmospheric stunning (CAS) with CO2, or by electrical waterbath. However, it is recognised that exposure to CO2 can cause pain, anxiety, breathlessness

and nausea. Meanwhile electrical waterbath stunning is widely recognised as having significant welfare issues, such as the risk of pre-stun shocks, sub-optimum stuns, suspension of conscious birds upside down (which is stressful and, in some cases, painful) and variations in electrical current delivered to birds in multi-bird waterbath stunners. In addition, head-only electrical stunning (non-waterbath) that is often employed on small scale producer/processor waterfowl units, is widely recognized as ineffective. Nevertheless, this method is still used by a number of producer/processors (often with illegal DIY high voltage stunners). There is an urgent need for up-to- date research on the head-only electrical stunning of waterfowl, as there are no minimum required currents under UK (WATOK 2015) or EU (EC1099/2009) legislation for ducks or geese and little published peer-reviewed information on the effectiveness or otherwise of this method in these species.

What outputs do you think you will see at the end of this project?

The expected outputs of this project will be the potential development of new stunning methods and parameters for poultry that improve welfare at the time of slaughter. The results of this project will be disseminated by peer-reviewed scientific publications, presentation at scientific and industry conferences, the development and distribution of fact sheets based on the key findings of the project to the poultry industry, government bodies, animal welfare NGOs and stunner manufacturers. Potentially, part of this work will lead to new stunning and slaughter systems for poultry (including waterfowl).

Who or what will benefit from these outputs, and how?

The expected welfare benefits of the proposed studies are the refinement of existing methods and development and testing of new stunning systems which will reduce distress and improve the quality and duration of unconsciousness following stunning. The project will also develop stun parameter guidelines (e.g. minimum currents for waterfowl), which can be used for existing stunning systems to improve welfare at slaughter. The results from the proposed project would provide scientific evidence that could directly underpin legislation (UK, EU and worldwide). These findings would also have direct relevance to animal welfare NGOs (development of guidelines and training), other animal charities, poultry producers and stunner manufacturers.

How will you look to maximise the outputs of this work?

If appropriate and with permission of the funding bodies, key findings of the project will be used to help promote the welfare of poultry during stunning/slaughter to the general public, this will be in the form of press releases and invited presentations. Unsuccessful results of developed approaches will be submitted for publication.

Species and numbers of animals expected to be used

- Other birds: No answer provided
- Domestic fowl (Gallus gallus domesticus): 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Chickens, geese and ducks are being used, as they are the species in which these stunning methods are currently or will be used commercially for slaughter for human consumption. Birds used in this project will be of commercial slaughter age and weight to mirror industry practice.

Typically, what will be done to an animal used in your project?

The effectiveness and relative humaneness of stunning methods and parameters (magnetic induction heating, captive bolt, electrical stunning) will be assessed in this project. This will involve the evaluation of consciousness/sensibility with behavioural/brainstem indices and assessment of the electrical activity of the brain. This will be conducted in anaesthetised (non-recovery) and non-anaesthetised birds. The use of a non-recovery anaesthesia model for the first part of the magnetic induction heating study allows the assessment of brain function and temperature in response to this novel stunning method while reducing the potential for suffering. In this model stunning parameters will be revised and will be used in subsequent non-anaesthetised studies.

Anaesthesia and non-anaesthesia studies

Assessment of the electrical activity will involve the implanting prior to stunning of sub-dermal needle electrodes in the skin over the head. The recording electrodes will allow the assessment of the following electrophysiological indices: Global electroencephalographic (EEG), visual evoked potentials (VEPs) and/or somatosensory sensory evoked potentials (SEPs). These will be recorded from birds prior to and after electrical stunning and allow the assessment of brain function.

Anaesthesia magnetic induction heating only

In addition to the above, fibre optic temperature sensors will be implanted within the brain of anaesthetised chickens. This will allow assessment of brain temperature in response to the stunning method. Anaesthesia will be non-recovery with all birds Schedule 1 killed with an overdose of anaesthetic.

Non-anaesthesia studies only

The duration of behavioural and brain activity assessment after stunning will be until recovery of recorded electrophysiological and behavioural indices sufficient to meet the studies scientific requirements, this is envisioned to be a maximum of 1 minute of uncontaminated data, based on prior experience. With an absolute maximum total recording time of 5 minutes. This allows for assessment of stun-induced unconsciousness, recovery and the effects of the stun on the immediate health status of the bird. The determination of the period of induced unconsciousness is essential in determining if the period of unconsciousness is sufficient to prevent birds from experiencing pain and distress either prior to, during or after the bleeding process in commercial slaughter. A secondary benefit of this approach is the demonstration of recoverability which is an important requirement for acceptability for halal slaughter. The lack of demonstration of recoverability with the proposed stunning methods, could lead to the non-adoption of the proposed methods, which could result in birds being slaughtered without stunning, which would cause serious welfare harm. Immediately after recovery all the birds will be euthanised. The period of assessment will be refined during the experiment and if deemed appropriate will be reduced.

What are the expected impacts and/or adverse effects for the animals during your project?

Potential impacts could include:

- Stress from handling, this is unavoidable, but will be minimised when possible. Birds will be acclimatised to people and handling prior to use.
- Stress/discomfort during induction of anaesthesia, this is unavoidable, but will be minimised when possible with good animal handling and veterinary care.
- Pain/discomfort from positioning of EEG electrodes. This is unavoidable, small diameter EEG electrodes (27G) will be used to minimise discomfort.
- Pre-stun shocks with electrical stunning, will be continuously examined for in all birds. Any shock will be transient before the induction of unconsciousness.
- Spinal seizures with electrical stunning when the animal is conscious will be continuously monitored for with assessment of the raw EEG trace and animal behaviour/brainstem reflexes. If
- an animal is identified as having a spinal cord seizure it will immediately be euthanized. Spinal seizures in two birds in a treatment batch would serve as a stop point in experimentation and require re-evaluation and alteration of electrical stunning parameters.
- Pain/discomfort with heating of tissues prior to induction of unconsciousness with magnetic induction heating study. This will be continuously monitored for, any discomfort is expected to be transient before the induction of unconsciousness.
- Distress associated with recovery from the stun. This is unavoidable in recovery experiments. The assessment period will only be until recovery of recorded electrophysiological and behavioural indices for sufficient to meet the studies scientific requirements, this is envisioned to be a maximum of 1 minute of uncontaminated data. The total recording period (included periods of unconsciousness) will be a maximum of 5 minutes and the birds will be immediately euthanised after this period.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severities for each protocol/species type:

- Protocol I Magnetic induction heating of anaesthetised chickens, 100% non-recovery, chickens.
- Protocol II Magnetic induction heating, of chickens: mild 50% (non-recoverable stun to kill), moderate 50% (recovery).

- Protocol III Captive bolt stunning of ducks and geese: mild (90%), moderate (10%). Protocol IV Electrical stunning of ducks and geese: moderate (100%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Chickens, geese and ducks are being used, as they are the species in which these stunning methods are currently or will be used commercially for slaughter for human consumption.

Which non-animal alternatives did you consider for use in this project?

Prior to live animal experimentation, mathematical modelling (magnetic induction heating) and cadavers will be used to refine the developed methods and parameters prior to live animal testing.

Why were they not suitable?

There is no meaningful way to assess the time to induction of unconsciousness, time to return of consciousness and minimum time to induction of brain dysfunction in models, birds that are already dead or with other non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of experimental animals proposed are based on publications, which assessed the head- only electrical and captive bolt stunning effectiveness in chickens and turkeys. Where possible sample sizes have been reduced to the minimum required for generating meaningful results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The ARRIVE 2.0 guidelines were followed when designing the different aspects of this project. Study design in each protocol was designed to maximise data collection while reducing the number of birds required. Each animal is to act as its own control. Sample sizes were determined with standard power analysis based on recently published

research. In addition to this a step up/down approach is to be used in protocol 4. This has further reduced the number of birds which would otherwise be used for determining electrical stunning parameters.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A combination of computer models and cadaver testing have been (and will be) used to refine the stunning parameters and for testing theoretical performance. This has resulted in the number of birds required to assess stunning performance has reduce significantly from the previous project licence.

Where possible, information will be gathered for different protocols from the same procedure, ultimately minimising animal numbers. In addition, when it has been determined that sufficient data has been collected, sample sizes will be reduced and remaining animals instead used in later protocols.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The use of and constant monitoring of the terminal anaesthesia in the magnetic induction heating experiment will prevent pain, suffering and distress to birds. This model allows the testing and refinement of parameters without compromising welfare, this information will then inform subsequent experiments. In recovery experiments, birds will be carefully and constantly monitored for signs of excessive pain and distress (distress calls, rapid ventilation, rapid head shacking etc). After recovery experiments birds will be immediately euthanised to prevent lasting harm. Of the treatment groups electrical stunning has also been shown to produce a period electroanalgesia in poultry, when allowed to recover. This will reduce the potential for pain associated with recovery. Meanwhile captive bolt has been demonstrated to produce instantaneous irrecoverable stun in turkeys, which will prevent pain and suffering.

In a previous licence a local anaesthetic cream was applied to desensitise the skin prior to placing electrodes for assessment of the electrical activity of the brain. This was put into that licence as a refinement. However, it was found in experiments with turkeys that the additional capture and handling of the birds for placement of the LA cream caused moderate stress compared to the relatively mild pain associated with subdermal (under the skin) electrode placement. This requirement was removed in the amendment to that licence. In the subsequent experiments using these electrodes there were no obvious signs of pain and distress with the placement of electrodes without the local anaesthetic cream, birds were less stressed and easier to handle. This example demonstrates that in some cases the refinements made to improve animal welfare can have unforeseen

consequences and cause harms. This highlights the need for constant monitoring of welfare even after introduction of refinements.

Why can’t you use animals that are less sentient?

Chickens, geese and ducks are being used, as they are the species in which the stunning methods are currently or will be used commercially for slaughter for human consumption, there are no less sentient species that can be used for this research. The use of a terminal anaesthesia-based model removes the potential for pain and distress when evaluating the potential of magnetic induction heating as a stunning method. By using this model, parameters can be altered to test and improve potential effectiveness without causing further welfare compromise. This approach also reduces the number of non-anaesthetised birds needed in the following experiment. However, for time to induction of unconsciousness and time to return of consciousness, this can only be assessed in conscious birds.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Birds will be habituated to experimental staff prior to experimentation. This will include routine pen walks and low intensity handling. When birds are handled for experimentation care will be taken to avoid excessive or prolonged handling. All birds will be euthanised either during or immediately after experimentation.

The use of sub-dermal needle electrodes has been found in the previous two licences to provide sufficient signal quality without the need for anaesthesia and surgery, which is commonly used in welfare at slaughter experiments. In these licences this was a significant refinement that removed the need for induction of anaesthesia, surgery and recovery, which would have caused stress and pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ARRIVE 2.0 principles will be followed for experimental reporting in study design to ensure the most relevant and transparent experimental design. The methods to be used in this project have been standardised and published in peer-reviewed journals and a PhD thesis.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The institution, is active in dissemination of NC3Rs and3Rs relevant news, providing training and updates via a newsletter, emails and online notifications.

50. Supporting the Development of Medical / Surgical Devices

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

medical devices, ISO10993, safety, efficacy, surgical devices

Animal types	Life stages
Pigs	adult
Sheep	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide a service to biotechnology companies or research groups for the pre-clinical development of medical / surgical devices.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are many unmet medical needs within human medicine that may be solved or patients outcomes improved by the development of new medical devices.
For example: Improvements in visualization systems for conducting minimally invasive procedures will lead to reduced procedure time and improved patient outcomes.

Development of an off-the-shelf nerve regeneration device that will allow functional repair of nerves without the morbidity associated with nerve grafting.

What outputs do you think you will see at the end of this project?

The primary data outputs will be clinical, clinicopathological, histopathological, diagnostic imaging data to support or refute development and use of new medical or surgical devices for use in humans and / or animals. Data will relate primarily to clinical efficacy, safety and underlying biological mechanisms and will:

- enable an objective decision to be made regarding whether or not to progress a novel device or technique through further stages of product development
- provide an understanding of any potential adverse effects and allow for appropriate contraindications and precautions in any subsequent clinical trials
- increase the number of novel safe and effective medical devices available for a range of conditions affecting humans and/ or animals.

Who or what will benefit from these outputs, and how?

The benefit of this Project Licence is to support the development of novel medical devices in the management of disease in humans. There are a wide range of unmet medical needs, requiring new devices to be developed as approved medical devices for therapeutic use or supporting surgical or diagnostic techniques. This licence seeks to improve the treatment of spontaneous disease in humans.

Short term benefits:

- The acquisition of data to support development and improved design of medical devices.
- The acquisition of data to inform the regulator on the safety and or efficacy of novel medical devices.

Long term benefits :

Patients : Improved patient outcomes: advancing surgical devices should offer new treatment options to improve patient outcomes either by offering a solution to an unmet clinical need or by improving on current medical devices to reduce surgery time or patient trauma, thus enabling lower morbidity and/or quicker recovery.

Economy:

- advancing surgical procedures will reduce the cost burden on health care institutions by reducing surgery time and reducing hospital stay;
- UK plc through successful commercialisation of product for unmet health needs
- Environmental: advancing surgical procedures will reduce patient recovery times, thus reducing hospital stay and consumption of medical consumables and resources;

- Political: more effective surgical procedures align with government goals of reducing waiting lists while advancing the standard of care.

- Social: patient benefits by having a more effective, timely treatment reducing the time to return to society or to work;

How will you look to maximise the outputs of this work?

This work will provide data on safety and potential efficacy with respect to a novel or improved medical devices. If successful, it will support progression to further, substantive studies of a later prototype.

Data will be used by the Client to progress the medical device to clinical trials and market authorisation.

Studies conducted as a service or collaboration with academic research groups will likely be published by the relevant research group.

The Establishment has a cadaver sharing policy; methods of euthanasia include those that may allow unused tissues from this project to supplement other research projects.

Species and numbers of animals expected to be used

- Sheep: 70
- Pigs: 40

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Young or adult pigs and sheep will be typically be used for all experiments because they are the most appropriate species to determine efficacy and safety with respect to the devices tested. Their size, anatomy and physiology have similarities to the human that make them the most appropriate models for testing of medical devices, especially those devices of a larger size.

Non-rodent species are typically required by the regulatory authorities as part of the safety testing package to be submitted with the application for medical device marketing authorisation.

Typically, what will be done to an animal used in your project?

Typically animals will undergo a surgical procedure under general anaesthesia for implantation of the medical device (eg orthopaedic implant) or to use the device (eg surgical laparoscopy system) under sham surgery conditions. This may be a terminal procedure with no recovery or a recovery procedure for short or long term testing of the medical device. Some testing of the device or imaging of the device may be applied under general anaesthesia. For recovery animals, appropriate animal monitoring and analgesia plans are established with NVS prior to surgery.

Animals may undergo repeated general anaesthesia to allow non invasive (eg CT/ MRI / Xray) or invasive assessment of devices (eg contrast angiography) at scheduled timepoints during a long term study.

Blood samples may be collected throughout the study to monitor for known substances that can be released from the surface of components being tested (leachables), to provide pharmacokinetic data for any substance incorporated into the device or to provide clinical pathology data for the implantation period.

All animals will be humanely euthanased at the end of the study and appropriate tissue harvested for post mortem assessment - eg histology or biomechanical testing.

What are the expected impacts and/or adverse effects for the animals during your project?

The impacts of any conscious regulated procedures on the animal are typically expected to be mild and transient eg when performing blood / fluid sampling procedures only or conscious imaging procedures or data collection from implanted devices.

Those protocols involving recovery surgical procedures or imaging under anaesthesia are classified as moderate. Surgical procedures for implantation of medical devices will typically incur some pain. An expected recovery plan with appropriate monitoring and analgesia will be clearly defined in each protocol. Pain would be expected to improve during throughout the expected recovery period and scheduled post operative monitoring and endpoints will be clearly defined.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

100% of sheep and pigs undergoing recovery general anaesthesia in this PPL would be expected to be of Moderate severity.

Where appropriate, non recovery models will be used.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

ISO10993 guidance stipulates the testing requirements for regulatory testing of medical devices - these outline the in-vivo and in-vitro pre-clinical requirements prior to application for clinical trials. The Medical Device Regulations 2020 outline the approval process to achieve a Marketing Approval.

Which non-animal alternatives did you consider for use in this project?

Clients using this service would be expected to have completed any relevant in-vitro bench testing prior to moving into animal models to ensure the device has been optimised before proceeding to live animal use. The regulatory authorities approving medical devices stipulate the requirement for in-vivo testing to satisfy the Medical Device Regulations

Why were they not suitable?

Non-animal alternatives are not acceptable to the regulators that approve medical devices for use in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each study is individually assessed and the predicted animal numbers indicated on the relevant project protocol. For Pilot studies this will include a plan for the number of animals required to set -up or validate the model. For efficacy studies and regulatory studies, animal numbers will be designed to satisfy the study design and/or regulatory requirements.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Within each study animal numbers typically reflect the regulatory requirements for the device.

All clients are requested to provide information on previous in-vivo testing they have conducted to provide assurance that no unnecessary duplication of work is being commissioned across different sites.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Typically any new surgical model is first tested in a cadaver and/or terminal animals to optimise surgery techniques. For new models, a small-scale study of 1-2 animals is then planned to optimise recovery protocols before progressing to larger groups.

The Establishment has a cadaver sharing policy; methods of euthanasia include those that may allow unused tissues from this project to supplement other research projects.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and sheep will be typically be used for all experiments because they are the most appropriate species to determine efficacy and safety with respect to the devices tested. Their neuroanatomy and physiology are very similar to that in humans.

We will work with manufacturers and academic experts to ensure a continued refinement approach is adopted for all medical devices.

Recovery studies will only be performed once the surgical technique has been defined as much as possible using cadavers and/or terminally anaesthetised animals.

All methods used for recovery animals will be refined to minimise any pain; these include appropriate provision of analgesia, use of local anaesthetics where possible for conscious biofluid sampling and close monitoring of animals by large animal veterinarians and advanced trained animal technicians to recognise any adverse effects. All animals will be trained and habituated to the environment, staff and handling techniques prior to study to minimise stress.

Why can’t you use animals that are less sentient?

This PPL is aimed at large animal models to allow appropriate testing of devices at their intended scale for use in humans. Less sentient animals will not be appropriate for the models proposed under this PPL. Typically sheep or pigs will be used for all experiments as their size, anatomy and physiology, is similar to that in humans and allows appropriate testing of the device at its intended scale. For example, testing of laparoscopic equipment requires a pig around 70kg to reflect the size of an adult human abdomen.

Devices that are designed for implantation in humans are required to have safety and efficacy data for a time period designed to reflect use in humans or indicate longer time effects. These require implantation, recovery and long term observations. Where appropriate, acute studies in terminally anaesthetised animal may be used in pilot studies or for those medical devices that do not require long term contact.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To minimise discomfort/harm to the animals where possible studies are non-recovery in terminally anaesthetised animals with defined humane endpoints.
All animals will receive appropriate peri-operative care in terms of anaesthesia and pain management both during and after a surgical intervention.

Our in-house large animal vets’ expertise further enhances animal welfare by providing close collaboration with dedicated animal care staff and ready access to highly skilled

advice. Specific recovery plans have been designed to ensure the best recovery of any animal post-procedure and involve high levels of monitoring.

All animals are habituated to the environment and all recovery animals are trained prior to use for all handling procedures, such as use of a restraining crate.

Least invasive route of substance administration, appropriate needle gauge and local anaesthesia will be used where possible. Negative control groups (baseline groups) will be minimised whenever statistically feasible.

All individual study plans are reviewed by the Client, Study Manager, PPL Holder and key study staff including consideration of justification and implementation of refinement and reduction as part of the local protocol review process.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein, e.g. NC3Rs Blood sampling resource.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Monitoring publications and the NC3Rs website for new and alternative models that could be implemented as part of this project, or for review purposes prior to starting new models. In addition, articles on advances in the 3Rs are regularly published on the Internal Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.

51. Targeting Parasitic Helminths with Drugs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

parasites, drug discovery, therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify and develop novel chemical substances which get rid of parasitic worm infections effectively, and which have properties which would allow them to be developed into medicines. The project will also assess the ability of existing drugs, developed for other purposes, to act against parasites. To aid these aims we will also try to increase our understanding of how drugs are taken up into the parasite and how they work.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gut dwelling parasitic worms are extraordinarily common, impacting on the health and wellbeing of around one quarter of the world’s population. In addition, parasitic worms impact on the welfare of wildlife and domestic animal productivity. Drugs which are

currently used to treat these types of parasites are losing their effectiveness, therefore, new drugs are urgently needed.

Thus, this project aims to discover new drugs and assess the possibility of repurposing existing drugs as anti-parasite therapies.

What outputs do you think you will see at the end of this project?

The primary output of this project is to identify novel or repurposed compounds which target parasitic worms in vivo. To support this output we expect to also identify the route by which anti-parasitic compounds are taken up into parasite tissues, as well as an understanding of how they kill the parasite (their mechanism of action). This knowledge is important as it will help to strategically develop other drugs, that target these mechanisms and uptake routes.

We intend to share our findings with the scientific community and will publish at least three new papers describing novel compounds, mechanisms of action and/or of uptake. In addition, we expect to present our work, in the form of a poster or a talk, at both national and international conferences.

Who or what will benefit from these outputs, and how?

In the short-term, we will publish our work and discuss our data at scientific conferences. This will help the sharing of our compounds with other researchers, who will be able to test them against other types of infection. Moreover, we have established collaborations with other groups also interested in neglected tropical diseases, and have utilised these networks to test our compounds against other infections.

Our long-term goals is for new drugs which effectively kill parasites, to significantly help in controlling parasitic worm infections in developing countries. Within 5-10 years it is highly likely that we will have discovered and optimised, through chemical modification, new anti-parasitic drugs suitable for clinical trials in man and/or use in veterinary applications.

How will you look to maximise the outputs of this work?

Sharing our data sets, where appropriate, at conferences and invited talks will allow researchers to test our compounds against other types of infection. We have already embraced this approach and have published the efficacy of some of our existing novel compounds against worms we ourselves do not study.

In addition to publishing positive outcomes of our research we also strive to publish compounds which have failed to eliminate worms in vivo as this knowledge is useful when trying to understanding how drug structure relates to effectiveness in vivo.

We also strive to publish our methodologies and workflows allowing others to adopt our working patterns if appropriate.

Species and numbers of animals expected to be used

- Mice: 3750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our studies focus on the infection of adult laboratory mice with the gut dwelling parasitic whipworm Trichuris muris (T. muris). T. muris is the equivalent mouse parasite of Trichuris trichiura (T. trichiura), the whipworm which infects humans. T. trichiura and T. muris are virtually identical and therefore the mouse model enables us to develop new therapies to treat T. trichiura in humans.

Any chemical substances which we test in vivo (in mice) will already have been shown to successfully target the parasite in vitro. However, testing cannot be solely performed in vitro as the activity of any substance in vivo may differ to the activity seen in a petri dish in vitro.

Typically, what will be done to an animal used in your project?

Mice will be treated with chemical substances orally (no more than twice a day for five days) or by injection into the vein (no more once daily for 5 days) or by injection in to the abdominal cavity (no more than once a day for five days) or by injection under the skin (no more than twice a day for five days). Some mice will have received a parasitic infection up to 5 weeks before treatment with the chemical substances. Parasites will be given via the mouth. During the chemical substance treatment some mice will have small volumes of blood withdrawn for their tail veins, with no more than two samples taken in any 24 hours period and never exceeding the published guidelines on blood sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

Administration of parasites via the mouth will causes short term discomfort and stress from the restraint. This will occur at each administration but will resolve once the procedure has been completed, which typically takes less than ten seconds to complete.

Oral dosing of substances may enter the airways or damage the tube leading down to the stomach. This will occur rarely (<0.1%) and can be avoided by correct holding of the animal and good technique. If the airways are damaged we would expect to see an immediate increase in breathing rate, fur standing on end and a hunched posture. Such animals would be humanely killed.

Parasite infections can cause dehydration due to diarrhoea. This will occur rarely (<0.1%) and only when the later and larger larval stages and adult worms have developed in the gut. It can be managed by providing wet food ("mash") or fluid therapy and usually animals respond within 24 hours. In the rare occasion that an animal doesn't improve after one day of receiving fluid it would be killed humanely.

Giving a mouse a novel chemical substance may make the animal poorly, typically losing body weight, being less active and adopting a hunched posture. We very carefully assess our chemical substances, for example by analysing their ability to damage or kill cells (cytotoxicity) in vitro, prior to treating any mice in vivo and thus these effects are rare (1-

5% for tolerability testing and <1% in efficacy assessments). Careful daily monitoring of the animals immediately following dosing ensures we do not go above unacceptable harm as defined in our licence. Weight loss should plateau and begin to recover one day after the cessation of drug treatment.

Taking blood from the tail vein may cause local bruising and blood collecting outside of the blood vessels. This is rare (<0.1%) and associated with moderate discomfort. Monitoring of any mice experiencing bruising daily following blood sampling is important with bruising usually resolving within 3 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The procedures employed under this licence are classified as moderate. There are two main possible harmful effects, the first relating to administration of the novel chemical substances and the second to removing of blood samples. Both are expected to be rare events (1- 5% and <0.1% respectively).

For tolerability testing mice will be treated with novel chemical substances orally, or by an injection into the abdominal cavity or into the bloodstream, or under the skin. As these are novel chemical substances it is possible that, occasionally, the mice will react badly to these chemical substances. We expect these to be infrequent events (experience to date suggests between 1-5 %) as any chemical

substances administered to mice will have gone through rigorous testing in vitro to show that they do not kill cells. Mice will be checked daily after treatment with the novel chemical substance. We will look for signs that the animal is beginning to suffer and any mouse affected will be humanely killed.

Efficacy assessments: Chemical substances which are well tolerated by mice in tolerability studies will then be assessed in infected animals. This will enable us to establish how long the chemical substances last in the bloodstream and the ability of the chemical substances to eliminate the parasite from the mouse. Having tested these chemical substances in uninfected mice, we expect adverse effects due to giving animals the chemical substances to be rare (<1%). In order to to test the effectiveness of our chemical substances against the parasites, mice will be infected with parasites using standard procedures, chemical substances administered and blood repeatedly withdrawn from a tail vein. The main risk of harm to the mouse in these studies is excessive blood loss. We expect loss of too much blood to be very rare (<0.1%) as we will carefully control the frequency and size of the blood sample taken. We will ensure bleeding has stopped after each sampling. If the bleeding cannot be controlled, resulting in a blood loss greater than defined limits, the mouse will be humanely killed.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our studies are interested in identifying novel chemical substances which act against the gut dwelling parasitic whipworm Trichuris, utilising the mouse version of the human parasite, T. muris. T. muris in the mouse is a good model of T. trichiura infection in humans. Importantly, the mouse species of Trichuris is remarkably similar to the human species of in terms of its genes, molecules and interactions with its host. Thus the mouse model of the human disease enables us to develop new therapies to treat T. trichiura in humans.

In order to investigate novel chemical substances, which work against Trichuris, we need to grow the parasite in vivo as, despite work attempting to generate Trichuris outside of animals, in vitro the adult stages do not develop. Moreover, to determine if chemical substances have the potential to work in humans, they need to be tested in vivo as there are many additional factors, for example, how long the chemical substances last in the host, how available they are and how quickly they degrade, which cannot be effectively modelled in vitro.

Which non-animal alternatives did you consider for use in this project?

In vitro screening against T. muris: The aim of the project is to discover novel ways to treat gut dwelling worm infections, through the discovery of new drugs and the use of existing drugs which haven't been used against worms before ("repurposing"). In vitro screening assays allow us to examine particular properties of the chemical substances, for example, checking how good they are at killing the worm. This in vitro screening provides essential data on the effectiveness of chemical substances directly on the parasitic worms and will always precede any use of substances in vivo. Thus, using our automated high throughput in vitro screening assay, hundreds of novel chemical substances or drugs for repurposing will be screened against the adult stage of the parasite for anti-parasitic activity. However, this still requires mice to generate the adult parasite.

In vitro screening against T. muris eggs: We will also screen any novel chemical substances, capable of killing adult Trichuris worms, in vitro against early larval stages of the parasite via our in vitro egg hatching assay.

In vitro cytotoxicity screening: Chemical substances will be screened for any toxic activity against mammalian cell lines prior to any in vivo treatment in mice, to eliminate chemical substances which have a directly toxic effect on mammalian cells.

Substances may also be screened utilising the free-living non-parasitic nematode Caenorhabditis elegans (C. elegans) which can be used as a model for parasitic nemtodes in some instances.

In vitro pharmacokinetics (PK) analyses: in order to inform the fate of a chemical substances in vivo (its pharmacokinetics properties), substances which show good anti-parasitic activity will be screened in vitro to determine PK parameters e.g. half life and absorption.

Why were they not suitable?

In vitro screening against T. muris: whilst an important step to identify substances with anti-parasitic activity we know that anti-parasitic activity in vitro does not always translate to anti-parasitic activity in vivo. Thus, following in vitro screening, drug candidates need testing in mammalian systems to explore the effectiveness of the drugs in eliminating the parasites from their host.

In vitro cytotoxicity screening: whilst a good indicator of possible toxicity, the ability of an animal to tolerate a novel substance can ultimately only be established in a fully intact organism.

Screening utilising the free living model nematode C. elegans: we did consider screening utilising C. elegans, however, previous work in which we have screened against both T. muris and C. elegans has highlighted that efficacy across the two different nematodes does not always correlate. Therefore, this could lead to false positive and negatives.

Screening utilising larval stages of T. muris: similarly to C. elegans, we considered screening against the L1 larval stage of the T. muris parasite, as these can be generated in vitro from parasite egg stocks, without the infection of mice. However, published data demonstrates that chemical entities which are efficacious against the L1 stage do not always correlate with those that are effective against the adult stage. In addition, although targeting larval stages of the parasite could reduce the number of worms which reach adulthood, this is unlikely to treat the morbidity associated with T. trichiura infections which is due to the presence of adult parasites.

In vitro pharmacokinetic (PK) analyses: Whilst the in vitro assays can aid in prioritising substances which are likely to have a good profile, determining the full potential of the novel chemical substances requires studies in the animal to understand the fate of a substance. This includes measuring concentrations of the substance in the blood over time in order to understand how it is metabolised in vivo. These data are important and cannot be obtained in any other way.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The mice numbers projected have come from the sum of:

In vitro worm generation: from historical data we know the number of worms we anticipate to generate per mouse, and the number of replicates we need for robust data from our ex vivo screening platform. We can utilise this data to determine the number of mice required for generating worms for ex vivo screening.

Tolerability studies: the numbers of mice used in these studies have been projected based on the number of concentrations of chemical substances we test, multiplied by the number of substances we estimate to progress to testing in vivo to see how well a mouse tolerates the substance.

In vivo efficacy studies: these estimations have been based on the known numbers of mice required for each treatment group from historical studies which have allowed us to detect significant differences, combined with an estimation of the number of treatment groups we will test.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In vivo efficacy experiments have been designed utilising the NC3Rs Experimental Design Assistant as well as with consultation with an external statistical consultant.

Egg doses for in vitro worm generation has been carefully monitored over several years to optimise the numbers of healthy worms generated whilst minimising any adverse effects from a high worm burden.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Repurposed drugs: where possible we will use existing data on in vivo tolerability available for our analyses of repurposed existing drugs thus avoiding unnecessary animal usage for tolerability studies.

- We have revised our experimental design such that we no longer always include our "gold standard" drug treatment, which routinely results in complete worm clearance, as a positive control.

- We have carefully considered group sizes and experimental design, where possible, to test several chemical substances in one experiment to minimise the number of vehicle-treated control animals required.

- We routinely take samples for pharmacokinetics analysis for all our treatments. These samples are archived at -80C and thus if any substance is of interest we do not need to repeat the initial experiments in order to gain data about how the substance behaves in vivo.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of animal model

Our studies focus on the use of the laboratory mouse. T. muris in the mouse is a validated model of T. trichiura infection in humans. Importantly the mouse species of Trichuris is

remarkably similar to the human species of Trichuris in terms of its genes, molecules that stimulate the immune response and the way it interacts with its host. Thus the mouse model of human trichuriasis enables us to develop new therapies to treat T. trichiura in humans.

We use both wild type and severe combined immunodeficiency (SCID) mice. SCID mice lack part of their defence system (their immune system) that would normally get rid of parasites. As such, they provide a robust way to generate Trichuris worms without the need to repeatedly inject mice with an immunosuppressant. Importantly, if kept in a very clean environment, SCID mice are healthy.

The protocols employed are well established in our lab and designed not to induce suffering in animals.

T. muris is a natural parasite of mice and is well tolerated with no adverse effects. In order to assess how effective a drug is against the parasite (drug efficacy) we will usually infect mice with low infection doses (eg 40-50 eggs) to establish the numbers of worms typically found in wild mice and humans.

Novel chemical substances will only be delivered in vivo after careful in vitro analyses to exclude cytotoxic effects. Initial in vivo studies (tolerability studies) will be in the absence of infection with each mouse treated one at a time and observed for any adverse effects greater than short term distress (e.g. change in breathing pattern or mobility, poor grooming) prior to treatment of further mice.

Why can’t you use animals that are less sentient?

T. muris takes 35 days to develop to the adult stage of the parasite and therefore infections cannot be performed under terminal anaesthesia or at an immature lifestage.

We did consider screening our substances for anti-worm activity using the free-living model worm C. elegans or the L1 larval form of T. muris (which can be hatched from pre-existing egg stocks), however, previous work in which we or others have screened against both T. muris adults, T. muris L1 and C. elegans has highlighted that efficacy across the two different nematodes and the two different lifecycle stages does not always agree. Therefore, this could lead to false positive and negatives.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- We routinely perform T. muris infections with no side effects other than minor discomfort following scruffing / oral gavage which is very transient.

- Throughout treatment with novel chemical substances we will ensure the mice are well monitored with bodyweight, grimace score, body condition and general observations recorded following treatment to ensure no adverse effects are experienced.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the ARRIVE guidelines for all our experimental work. In addition, prior to any animal studies we will prepare and submit a full experimental study plan to the animal unit to ensure all studies are carried out in line with best practices. We will conform to the

principles described in the Working Party report "Refining procedures for the administration of substances" in Laboratory Animals (2001) 35, 1-41.

Use of male and female mice: when growing adult stage parasites in vivo we prefer to use male mice as they tolerate the infection better than females; equally in our initial assessment of how well the mice tolerate the drug and clear the worms we prefer to use male mice as they are larger and less likely to experience any harm. However if substances show anti-worm activity in these pilot studies it is important that we then test our novel candidates in females where the way the substances behave inside the mouse may differ and thus alter our assessment of how translatable our work will be into humans. To minimise harm to females used we will only use mice above 22g weight.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed of 3Rs advances through

- NC3Rs newsletters
- animal unit newsletters
- discussions with other in vivo researchers
- seminars put on through the animal unit

Any changes to best practice will be discussed with the NACWO and implemented where appropriate.

52. UK Rabies Diagnostic and Characterization Activities

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Diagnostic, Control, Innocuity, Isolation, Viruses

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is 'to support the research and diagnostic activities of the National Reference Laboratory for rabies and the National Laboratory for arthropod-borne and wildlife viruses of veterinary and zoonotic importance'. The project has been split into three tasks:

1. Production of positive control material for diagnostic assays.

2. Isolation of viruses of high scientific value in mice and propagation of pathogenic viruses for which in vitro models do not exist.

3. Innocuity testing of inactivated biological products to enable removal from high containment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

1. **Production of positive control material for diagnostic assays**. Diagnostic assays are used to determine the health status of humans and animals in or entering the UK. Some of the OIE/WHO recommended diagnostic tests used require infected brain tissue as positive control material e.g. rabies detection using the Fluorescent Antibody Test (FAT). Infected mouse brain is the only source of such material. As a WHO/OIE reference laboratory, the discovery of new lyssavirus species requires us to ensure that reagents used in our diagnostic tests are capable of detecting these new viruses. To achieve this, mouse brain tissues infected with these viruses may be required.

2. **Isolation of virus of high scientific value in mice and propagation of pathogenic viruses for which in vitro models do not exist.** Under previous licences, we have received material from animals that have been positive for viral nucleic acid in tested material but have been unable to recover virus using in vitro techniques. Additionally, some viral pathogens worked with are very difficult/cannot be cultured using tissue culture. Where required, we wish to use mice, with relevant justification, to propagate these viruses. Established virus stocks can then be shared globally to enhance virus understanding. The use of mice for this requirement is rare, but has been used twice in recent years (West Caucasian Bat Virus and Lleida Bat Lyssavirus).

3. **Innocuity testing of inactivated biological products to enable removal from high containment.** In order to remove inactivated biological products from high containment, it is necessary to demonstrate that the inactivation process has been successful. Currently there are a number of pathogens worked with in high containment for which in vitro methods to demonstrate virus inactivation are not available. Therefore we may wish to use mice to demonstrate the inactivation of these viral pathogens. Inactivated virus is used for the generation of pathogen specific sera produced in animals and as an antigen in stimulation assays to assess in vitro responses to inactivated viral derived products.

What outputs do you think you will see at the end of this project?

1. The requirement for mouse derived positive control material is essential for viral diagnostics, including lyssaviruses. Without these materials, ISO17025 accredited testing in support of statutory diagnosis of human and animal cases of rabies would not be possible. Information from diagnostic activities on notifiable diseases are reported to the scientific and global communities and policy makers to help inform their decision making.

2. Propagation of viruses of high scientific importance by in vivo methods (e.g. First isolation of lyssavirus species; West Caucasian Bat Virus and Lleida Bat Virus), where no in vitro culturing techniques are available yet or where in vitro methods have failed, is important. Where initial in vitro attempts have failed, in vivo propagated virus may be further passaged using cell culture techniques, for example, Ikoma lyssavirus, Lleida bat lyssavirus and West Caucasian bat lyssavirus have all been grown this way. In vivo propagated viruses may be used to determine cross-reactivity with existing diagnostic tests or used to develop new tests, ensuring that we have the necessary tools to detect incursions of harmful viruses into the country and/or monitor the spread of new outbreaks of existing viruses. Further, these viruses can be shared with scientific communities through collaboration or international virus archives, for the purpose of characterisation

and research. Characterisation of pathogens is of great importance to both the scientific and global communities to inform policy on both human and animal health, especially where potentially fatal zoonoses are involved. Data and information derived from the use of these viruses will be communicated to the scientific community through project reports and peer reviewed publications.

3. Validation that virus inactivation has been successful will be performed using in vitro assays where possible. In the event there are no validated assays for this purpose, it may be necessary to demonstrate virus inactivation by in vivo methods. This will eliminate the risk of live pathogens being removed from containment and allows them to be used safely for future experimental procedures. Like live pathogens, inactivated pathogens can be used to develop new diagnostic assays, normally by producing stocks of anti-sera used in serological assays. Again, these viruses can be shared with the wider scientific community for the purpose of research, virus characterisation and diagnostic development, with data and information being communicated through project reports and peer reviewed publications.

Who or what will benefit from these outputs, and how?

Statutory diagnosis of human and animal rabies uses the OIE prescribed gold standard Fluorescent Antibody Test 'FAT'. This test requires the use of tissue samples infected with various lyssavirus species, these are Rabies virus (RABV) and European bat lyssaviruses type 1 (EBLV-1) and type 2 (EBLV-2). Outputs from the use of these in vivo derived tissues is immediate through diagnostic test reports, without these tissues the test and results are not valid. Longer term, diagnostic tests,especially those on suspect reportable disease cases will be reported to the wider scientific community and policy makers to inform decision making.

The propagation of high importance viral pathogens or the validation of virus inactivation will produce biological materials that can be used for the validation and/or development of new diagnostic assays. These assays will be used to detect early incursion of new pathogens into the country or new outbreaks of existing viruses. The local community, including general population, local businesses, environment and economy will benefit from these tests. These biological products can also be shared with the wider global scientific community as described above. These products are extremely important for scientific and diagnostic advancement but the benefits are likely to be realised longer term.

How will you look to maximise the outputs of this work?

Please see above.

Species and numbers of animals expected to be used

- Mice: 42

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the biological system of choice for infection with many zoonotic viruses. They are sufficiently susceptible to infection for us to have confidence in the results gained from studies performed under this licence. Rodents are more amenable to laboratory experimentation at the organisation than dogs, cats or non-human primates which could be used as an alternative for pathogens such as rabies virus.

Under this licence, the intracranial (IC) route of challenge will be used and for this reason mice aged 3- 4 weeks before full ossification of the skull are preferred as they are easier to inoculate with less risk of trauma.

Typically, what will be done to an animal used in your project?

Typically, mice 3-4 weeks of age will be allowed to acclimatise in their new housing for 7 days, 3-4 mice per individually ventilated cage (IVC). On day of challenge mice will be anaesthetised (e.g. isoflurane) and then given pain relief (e.g. 150uls Buprenorphine via subcutaneous (SC) injection). Immediately afterwards, mice will be challenged with <50uls virus via IC route. Mice will be frequently checked, post challenge, looking for any adverse effects of the procedure or the inocula. Prior to any study and during the application stage for that study, the use of an alternative analgesic, one that can be given by injection prior to procedure and then in the drinking water during recovery, will be discussed with NVS and NACWOs. The new alternative analgesic regime will be used so long as it does not affect the outcome of the study.

Mice are monitored a minimum twice daily to check on the health of the animal and look for clinical signs. Clinical signs are monitored and when they reach our predetermined humane end point they will be euthanised using a Schedule 1 technique, e.g. heavily anaesthetised (e.g. Isolflurane) followed by cervical dislocation. Typically, mice will succumb to rabies 7-10 days post IC challenge. If not reaching a clinical endpoint, mice are typically monitored up to 28 days post IC challenge (for lyssaviruses) but this may be extended to 90 days depending on the virus under study, after which they will be euthanised as above.

What are the expected impacts and/or adverse effects for the animals during your project?

A comprehensive list of clinical signs has been produced and is used to monitor mice.

Clinical signs include: Signs of pain (squinting, pinched nose, flattened ears, hunched posture & rapid breathing), appearance (ruffled coat & visual loss of weight), behavioural (social isolation, lethargy, hyperactive, repetitive behaviour & nervousness) and neurological (loss of balance, local and systemic spasm, limb paralysis/ataxia & hind quarter paralysis).

Clinical Score and actions taken. Not all signs will be observed for each score

Score Clinical Signs	**Action Taken**
(0) Healthy	Monitor as normal
(1) Minor signs of pain (ruffled fur, hunched, pinched face), lethargy, social isolation	Monitor

(2) Increased severity of score 1 clinical signs plus affected gait, local spasms, loss of balance, hyperactivity, visual loss in weight.	Euthanise
(3) Score 2 clinical signs plus hind quarter paralysis, systemic spasms	Euthanise

The cut off for a humane endpoint has been established at clinical score 3. However, mice reaching score 2 will be euthanised. The discrepancy between Score 2 and score 3 euthanasia is because clinical signs can progress rapidly and skip from Sc1 to Sc3 within the period between checks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The cut off for a humane endpoint has been established at clinical score 3 (see above) which has previously been agreed as moderate severity.

Production of positive control material (Protocol 1) - it is expected that 100% of the mice used will succumb to disease (moderate severity).

Propagating viruses of high scientific importance where in vitro methods have failed (Protocol 2) - from previous studies it is expected that approximately 50% of mice used will succumb to disease (moderate severity) and 50% rest remain healthy (mild severity).

Validation of virus inactivation where in vitro methods cannot be used (Protocol 3) - it is expected that less than 10% of mice will succumb to disease (moderate severity) and 90% remain healthy (mild severity). Where assessment of innocuity is required, if clinical disease develops following inoculation of an inactivated sample in any mice then the remaining mice will be terminated to stop any adverse effects to them.

Protocol 1 = 100% moderate, Protocol 2 = 50% moderate & 50% mild, Protocol 3 = 10% moderate & 90% mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have replaced the OIE recommended in vivo mouse inoculation tests with the in vitro rabies tissue culture isolation test (RTCIT).

In all situations, in vitro methods are extensively explored before considering the use of animal models. Occasionally, the in vitro test is not sensitive enough and if the virus is of high strategic scientific value or the inactivated virus forms an integral part of a project, then mice will be required.

Virus infected positive control brain material is an absolute requirement for the OIE gold standard test for rabies diagnostics, FAT. Wherever possible, brain tissue from other lyssavirus in vivo studies, e.g. vaccine trials, will be used to replenish stocks.

Which non-animal alternatives did you consider for use in this project?

Tissue culture is used to isolate viruses wherever possible, including the use of mouse neuroblastoma (N2A) or baby hamster kidney (BHK) cell lines to isolate lyssaviruses. Where new or less characterised viruses are under study, extensive literature searches are undertaken to identify permissive cell lines to grow and characterise the virus e.g. Dog-SLAM Vero cells to propagate canine distemper virus.

Why were they not suitable?

Occasionally, the concentration of virus in the sample is too low to be detected by in vitro methods.

1. Chemicals used to inactivate virus are very toxic to living cells, therefore a sample must be diluted considerably before it can be tested to show inactivation of virus has been achieved. At these high dilutions, tissue culture techniques are often not sensitive enough to cultivate the virus, therefore alternative in vivo techniques must be sought. Prior to the main in vivo study a small pilot study will be carried out to ensure the in vivo model is sensitive enough.

2. Sometimes the concentration of virus in a diagnostic sample is too low or the virus itself is non- cultivable using in vitro tissue culture techniques and the use of in vivo models are the only option to cultivate the virus.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice to be used has been estimated based on the number of mice used in previous similar project licences and is dependent on the number and type of research projects agreed throughout the duration of this project licence. Prior to any use of mice for research or for diagnostic purposes an in vivo study application will be written, reviewed and approved by our local AWERB committee which includes a trained biostatistician.

8 mice (8 mice per isolate)- Infection of mice with viruses of high scientific importance (Objective 1)

20 mice (10 mice per virus control)- Infection of mice with zoonotic viruses for the preparation of positive control material. (Objective 2)
14 mice (14 mice per isolate, 4 mice to show that virus at the dilution used will be detected (cause disease) and 10 mice per inactivated isolate) - Innocuity testing of inactivated biological products to enable removal from high containment. (Objective 3)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Full statistical evaluation of each study will be performed by our experienced internal biostatisticians to ensure the minimal number of statistically relevant animals is used to validate experimentation whilst achieving the highest possible power. Where possible without jeopardising the interpretation of results, in-bred animals will be used to minimise random variation and therefore reduce the sample size needed to detect the same signal.

Study designs for objectives 1 and 2 are very basic and do not require any special design tools. Objective 3 requires blinded randomisation of cages on the rack system. Prior to the start of any study experienced staff, including biostatisticians are consulted.

Advancements in reagents used to inactivate viruses has progressed in recent years, reducing the need to perform high dilutions to remove the effect of the agent on tissue culture systems. This allows in vitro methodologies, where available, to demonstrate virus inactivation rather than using in vivo methods.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The research group has experience using many reagents used to inactivate viruses and knows what concentrations are toxic to cell lines as well as mice. Should a new reagent be required, a small pilot study will be performed to determine toxicity levels in mice prior to the main study. Tissues not required for the present study will be collected and stored for future requirements.

All studies performed for diagnostic activities are carried out to ISO17025 quality standards with research studies meeting ISO9001 standards. Our institute is committed to complying with PREPARE and ARRIVE guidelines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are the biological system of choice for infection with many zoonotic viruses. They are sufficiently immunologically similar to humans to allow confident extrapolation of findings, yet they are more amenable to laboratory experimentation at the organisation

than dogs, cats or non-human primates which could be used as an alternative for pathogens such as rabies virus. It has become apparent that although rabies virus is considered to be 100% fatal following the development of clinical disease, exposure to virus will not always result in disease. Indeed, infection of mice with lyssaviruses via different routes can result in different clinical outcomes. From established mouse studies inoculation of a neat preparation of virus via an intracranial (IC) route generally causes 100% development of clinical disease although exceptions can occur, albeit rarely, where IC inoculated animals survive infection. The mechanism behind this is unclear. Furthermore, inoculation of the same virus via a peripheral route will often lead to a reduction in the number of mice that succumb to infection. For this reason the IC route is favoured for the objectives of this project and will reduce the numbers of animals required. Suffering as a result of the IC challenge will be minimised using analgesia (eg. Bupronorphine) immediately prior to inoculation. Frequent checks post inoculation will

be performed following inoculation to monitor any adverse effects of the inocula/procedure. Suffering as a result of infection with lyssavirus will be reduced by adherence to a clinical scoring system that monitors inoculated subjects from inoculation to the development of clinical signs. All animals will be terminated using a Schedule 1 method when they reach the humane endpoint.

Further, where assessment of innocuity is required, if clinical disease develops and is confirmed in any mice, following inoculation of an inactivated sample, then the remaining mice will also be terminated to stop any adverse effects to the remaining mice.

A new scoring system for mice challenged with lyssavirus is in development. The new system removes some of the subjective decisions made by those monitoring the animals. It uses a cumulative score to decide when the humane endpoint has been reached. The new system has been run in parallel with the old score system for two experiments and is currently being updated prior to review by the local AWERB.

Why can’t you use animals that are less sentient?

At the present time, no less-sentient animal model exists to study rabies infection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are settled in for seven days prior to start of the experiment and will be kept group housed with 3- 4 mice per cage. Any single mice, after euthanasia of cage mates, will where possible be re-housed with other mice. The cages will contain various enrichments, including plastic climbing apparatus and tubes, cardboard tubes (gnawing) and enrichment foods (sunflower seeds).

Under advisement from veterinarians, procedures will be performed under general anaesthesia where required to reduce stress. Immediately prior to IC challenge, mice will be given pain relief (e.g. Buprenorphine), followed by a period of increased checks to monitor for adverse effects of the inocula or procedure performed. Cages are warmed post IC challenge to aid recovery.

We will investigate and wherever possible use an analgesic that can be administered before the procedure , by injection, and after the procedure, in their water.

Where animals need to be individually identified we will use animal safe marker pens to identify them instead of microchipping or ear tagging.

Where assessment of innocuity is required, if clinical disease develops following inoculation of an inactivated sample in any mice then the remaining mice will also be terminated to stop any adverse effects to the remaining mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Observations, guidance and recommendations are provided by vets and experienced NACWO's at wash up meetings at the end of a study. Recommended changes to procedure are reviewed on-line through 'NC3Rs' and 'procedures with care' websites and training provided by experienced (Named vet/NACWO) staff.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Before, during and at the end of each experiment, formal and informal meetings are held with veterinarians, NACWO's and other PiL/PPL holders, to discuss the experiment and best practice. Wherever reasonably practicable suggestions and guidance will be put in place. New guidance and practices will be written into procedural documents and outcomes from these new practices reported in the final project report.

During the proposal stage of new experiments the NC3Rs website will be referred to to determine best practice for procedures to be performed during that experiment.

Our establishment holds regular 'Species user and 3Rs committee' workshops, open to everyone to join and discuss all aspects of the 3R's. Members of the committee use information from a wide range of sources (inc. Laboratory Animal Science Association (LASA), Institute of Animal Technology (IAT), Procedures with Care & RSPCA web pages) to keep informed of new ideas and best practices.

53. Understanding the Role of Microglia During Glioma Growth

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

glioblastoma, microglia, zebrafish, cancer

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Microglia are specific immune cells within our brain. These cells have been shown to promote the growth of aggressive brain tumours called glioblastoma. The aim of this project is to understand how this is happening and how this can be prevented.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Glioblastoma represents a major threat to human health. Standard therapies have not proven successful and the average survival for patients is only 14 months after diagnosis. This urges the need of a better understanding of these tumours and the development of new therapies. As standard therapies (surgery, chemotherapy and radiotherapy) have not proven to be successful for these tumours, targeting the immune cells offers new promising avenues. It is now appreciated that these tumours grow within a complex microenvironment, which is crucial for their progression. The tumour microenvironment

(TME) contains many different non-cancerous cell types in addition to cancer cells, including endothelial cells, pericytes, fibroblasts, and immune cells. Amongst immune cells, the resident macrophages of the brain (microglia) and infiltrating macrophages can account for up to 30% of the tumoural mass and research points towards a tumour promoting role of these cells within the TME. It is therefore important now to understand how microglia promote the growth of these aggressive brain tumours, which signals within the tumour microenvironment lead to this activity and how this can be prevented. Finally, the hope is to find signals that reverse the activity of microglia to use these cells in future therapies to slow down glioblastoma growth.

What outputs do you think you will see at the end of this project?

At the end of this project we will have a better understanding of how our immune cells in the brain, the microglia, contribute to the growth of aggressive brain tumours called glioma. This is very important as current therapies are not efficient to treat gliomas. Understanding how microglia promote the growth of these tumours is a first step to develop therapies to stop this detrimental behaviour. We will publish our findings that describe our new insights in scientific journals, all of which will be open access to the public. In addition, we have generated and will continue to generate genetically altered zebrafish to share with researchers around the world. We will prepare press releases and social media-based outputs to convey the findings of our work to the public in a digestible manner. Our long-term goal is to help find new treatments for glioma, which can be combined with existing treatments leading to enhance patient survival.

Who or what will benefit from these outputs, and how?

Numerous groups will benefit from the outputs of our studies. The scientific community directly involved in studying immune cells and tumours in the brain will benefit from new knowledge that can be integrated into our growing understanding of the complexity of brain tumours.

The transgenic animals developed will be valuable to other scientists interested in immune cells and brain tumours as well as in the development of anti-cancer therapies.

Finding ways to inhibit the brain tumour promoting functions of immune cells will be beneficial to a wide group, including pharmaceutical companies, charities, patients and clinicians. The interest of pharmaceutical companies goes in line with benefits for patients and clinicians. Early interference upon tumour diagnosis with the immune cells in the brain is a promising strategy to inhibit tumour growth and metastasis and might result in the development of new therapies.

The benefits for charities and the general public arise from a combination of the importance of the results and public engagement activities that are planned for the next years. Due to the focus on live imaging studies the research is extremely well suited to achieve public interest. Complex research can here be explained in easy words to the general public. Eventually this has two main effects; on the one hand it broadens the public knowledge about cell functions and tumour growth and on the other hand shows impressively the impact of research on a ‘little fish’ on human health.

How will you look to maximise the outputs of this work?

We have established numerous collaborations both externally and internally to maximise the output of our work. We are collaborating with groups who have diverse expertise in

technology or the use of different model systems. We are publishing our work in international peer-reviewed journals and are promoting Open access to scientific publications: all articles from my lab are freely available, and the corresponding data and newly generated transgenic zebrafish lines are freely available to the community. In addition, we present our work widely at local, national, and international meetings.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 64000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The diagnosis of ~12000 new brain tumour cases in the UK every year and a long-term survival of only 12% urge the need of a better understanding of these tumours and the development of new therapies. As standard therapies have not proven to be successful for these tumours, targeting the immune cells offers new promising avenues. In the present project we are investigating the interactions of immune cells (microglia) and brain tumours aiming to understand why these immune cells promote the growth of brain tumours and how this can be changed. These are very complex 3-dimensional processes that cannot be modelled in vitro. Furthermore, the cells of interest are, as parts of the immune system, very sensitive to any alteration in their environment and cannot be studied without artefacts in vitro. Hence, we use larval zebrafish to understand how these immune cells interact with brain tumours. Zebrafish are vertebrates that exhibit remarkable conservation in terms of their molecular and cellular makeup with humans meaning that they often have shared mechanisms related to diseases and respond to gene and drug-based manipulations in similar ways. Importantly, the function of microglia in zebrafish is highly conserved when compared with their roles in mammals, which allows us to use this model to understand microglial regulation in health and disease. We primarily use larval zebrafish as these are small and optically translucent so that one can send light easily throughout the animal without need to any surgical intervention. These features, together with our ability to create genetically altered animals, and to treat young zebrafish with drug like compounds, means that we can directly see into the brain and observe microglia and brain tumours and their interactions as they occur over time. This then allows us to investigate how experimental manipulations, for example drug treatments, genetic and optical manipulations affect tumour growth in real time. Thus, the use of zebrafish allows us to gain insights into the role of microglia in brain tumours and to identify mechanisms underlying the disease at an unprecedented scale.

Typically, what will be done to an animal used in your project?

Most animals that will be used in our project will be genetically altered zebrafish that have fluorescent proteins in cells or tissues of interest that allow us to track biological events in real time using a range of cutting-edge microscope and camera systems. Some of the animals that we use in our project will have further genetic alterations that change the function of specific genes of interest, e.g. a gene that can change the physiology of a cell using, for example, optogenetics to activate specific cells using light, or a gene associated with a human disease. We will mainly use embryonic and larval zebrafish for experiments.

To induce brain tumours in these larval zebrafish we will employ different methods. We will for example activate human oncogenes in the larval fish brain. These oncogenes change the fate of cells which will result in tumour growth. Alternatively, we will transplant human brain tumour cells directly into the larval zebrafish brain. These human cells engraft in the larval brain and generate tumours. In some cases, animals will be treated with small molecule compounds applied to the water to activate specific genes, to alter the state of a cell, or to induce the selective death of specific cells.
Sometimes animals will need to be single housed to follow treatment outcomes on individual tumours. Some animals will undergo an irradiation regime. This is non invasive and will be done under anaesthesia. Imaging experiments under different microscopes are always non-invasive and will typically be carried out under anaesthesia. To do imaging, animals are typically embedded in a small drop of agarose to hold the animal in position while under anaesthesia. In some cases when we need to directly assess CNS activity, animals may be embedded in agarose in the presence of neuromuscular junction blockers.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the small size and rapid development of young zebrafish, we can carry out most of our experiments in largely non-invasive manners, and typically with no signs of adverse effects on the animal. To induce tumour growth in the larval zebrafish brain we will overexpress human oncogenes. The overexpression of these oncogenes will lead to cellular transformation, which results in increased

proliferation of these cells mimicking early stages of glioma growth. This has typically no adverse effect on the animal during early larval stages as the developing tumours are small and do not impact on the surrounding tissue.

The vast majority of experiments will be non-invasive imaging carried out under anaesthesia. These procedures may be repeated. However, we did in the past not observe accumulated impacts from repeated imaging as the animals recover fully from this non-invasive procedure.

In some cases, non-invasive imaging will have to be carried out on immobilised animals without anaesthesia to assess signalling activity in glioma networks. These procedures may be slightly uncomfortable to the animal, similar to a human experiencing an MRI scan, but do not have any lasting adverse effects from on the animal. Sometimes, animals are restrained in the presence of neuromuscular junction blockers to prevent them from moving whilst under high-resolution microscopes. Again, these are acute non-invasive procedures where the animal will not experience any pain, but we cannot exclude a discomfort from being restrained.

We are, however, also using xenograft models to induce tumour growth in larval zebrafish. Here we transplant human glioma cells into the brains of larval zebrafish. Transplantation protocols have been optimised under the previous project licence in a way that only the minimal number of tumour cells required for the experiments is transplanted. Thus, adverse effects due to the injection could be mitigated. In case the injections cause adverse effects (e.g. damage to membranes or vessels), these will become obvious within the first hours post transplantation and larvae will be humanely euthanized to prevent them from experiencing further harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our experience to date indicates that the vast majority of animals will not exhibit evidence of experiencing an adverse effect that is measurable. Most animals that we use are for breeding and maintenance reasons, and carry genetic alterations to visualise different cells of the nervous system with fluorescent markers. These alterations do typically not show any adverse effects due to their genetic alteration and are indistinguishable from wildtypes. Hence approximately 70% of animals will be considered sub-threshold.

We also study animals that exhibit mild adverse effects. Such effects could be caused by the impact of proliferating tumour cells, the transplantation of tumour cells, administration of anaesthesia, or mild stress due to being restrained during microscopy. We expect that up to 20% of the animals that we will use might experience this level.

We occasionally study animals that exhibit moderate adverse effects, due to their genetic alterations, and because very few of our experimental protocols elicit adverse effects of their own. As our genetic alterations and experimental manipulations principally affect cells of the nervous system, moderate effects may manifest in altered neural circuit formation, such as impaired motor outputs leading to altered swimming behaviour. We would only study animals experiencing moderate adverse effects for short periods of time and only when scientifically necessary. We expect that less than 10% of all the animals that we will use during our project would experience this level of effect.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the present project we are investigating the interactions of immune cells and brain tumours. These are very complex 3-dimensional processes that cannot be modelled in vitro. Furthermore, the cells of interest are, as parts of the immune system, very sensitive to any alteration in their environment and cannot be studied without artefacts in vitro. However, research on the tumour microenvironment of brain tumours is mainly performed in mice. Zebrafish represent an obvious refinement here as they are the vertebrate model of the lowest perceived sentience.

Which non-animal alternatives did you consider for use in this project?

Manipulation of signalling pathways of tumour cells will be performed and tested in vitro before implantation into the larval zebrafish brain. To test if manipulated signalling pathways might play a role for the interaction with macrophages/microglia glioblastoma cells will be co-cultured with macrophages/microglia. Only if clear alterations of interactions between macrophages/microglia and tumour cells are observed in vitro these cells will be injected into zebrafish larvae.

We also consider using cell culture techniques that now allow mini-brain-like "organoids" in the dish and are hopeful that in years to come technologies will become so refined that they can be used to study microglia - glioma interactions in vitro. Such cell culture systems may even allow us to model certain aspects of disease in the dish, and we look forward to incorporating such models into our work.

Why were they not suitable?

Standard in vitro system do not allow to model the complexity of the interactions between microglia and brain tumour cells. Microglia do not show their full repertoire of functions in vitro, hence only basic analysis can be done in vitro. Brain organoids are beginning to show great promise as an experimental tool for neuroscience, but have not yet been established to the point where they have all of the cell types that would be required to be able to probe the mechanisms of microglia interactions in the tumour microenvironment. Furthermore, brain organoids have not yet been developed where they can function under physiological conditions with a vascular system, the full complement of immune cells, that are known to influence tumour growth.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have calculated the number of animals that we are likely to use based on our use of zebrafish as a model system over the past 9 years.The present research relies on the ready availability of transgenic and mutant zebrafish lines for which there is no commercial supplier. Lines are therefore generated in house and have to be maintained by us in the fish facility to be used for experiments. Most adult fish will only be bred for line generation/maintenance and will not undergo experimental procedures.
Hence, the vast majority of fish will not experience harm. Due to the variety of cells and signals that will be studied in the context of tumour growth we require a collection of fish that allows us to monitor specific immune cells on their own but also in combination with other cells. Thus, we foresee the need of approximately 70 different transgenic and mutant zebrafish lines. For each fish line kept, approximately 60 individuals/year need to be bred and raised. We refresh stocks once per year, meaning that we will use 5 separate generations of each stock over the course of the project, giving a total of 21,000 animals.

In very few cases, animals will need to be maintained for breeding purposes where they may exhibit moderate adverse effects, which will be kept under protocol 2. We anticipate this to affect around 5% of all animals used for breeding under protocol 1 and therefore project a maximum number of 500 animals here.

The third protocol projects the use of up to 6,000 zebrafish for the generation of new genetically altered animals. This number largely reflects the new ability to target gene function at scale using new tools, including "CRISPR-cas9" gene editing. We can now assess the effect of changing gene function in animals very soon after injecting reagents

that can edit the genome. In the past, if we were interested in a gene's function, we would have to edit the genome and grow animals up to sexual maturity, maintain them through subsequent generations, and test if they affected a biological process of interest. Now we can look for the effects of disrupting gene function within days of such "editing". Although the number of animals we are likely to use may increase, the length of time that animals need to be maintained will be greatly reduced, representing an experimental refinement. Testing the effects of 30 genes over the course of this project in up to 5 assays using up to 20 animals per assay will require 3,000 animals. We will also generate stable mutant lines from genes that exhibit particularly important functions when assessed by acute gene editing, and expect to generate up to 10 such lines, with current estimations that we need to grow up 50 animals to successfully do so (500 in total). In addition, we are currently generating animals in which gene function is disrupted in a cell-type specific manner. At present we need to screen through many animals to find suitable ones for in depth study, thus meaning we require about 200 animals per new line we establish. We anticipate establishing another 10 such lines over the course of the project, meaning that we will need 2000 animals for generation of cell-type specific mutants. In addition, we will continue to establish further new transgenic reporter and effector lines for which we need up to 15 animals per line. Thus, we estimate that we may use a total of up to 6,000 animals for the generation of new genetically altered animals.

We predict using a further 30,000 animals in our experimental analyses upon oncogene overexpression to understand the role of microglia during glioma initiating stages. This is driven in large part due to fact that we want to perform studies that allow us to understand the changes in gene expression in the microglia and glioma initiating cells. For this purpose, we need to isolate these cells from the larval zebrafish. As individual larvae contain relatively low cell numbers, we need a large number of larval zebrafish to obtain sufficient material for our analysis. However, most of these larval fish will not undergo any additional procedures apart from oncogene overexpression and will be humanely killed using schedule 1 procedure. Furthermore, we will carry out in vivo imaging using transgenic reporter and effector animals. This enables us to have all experimental procedures being non-invasive, meaning that we directly observe and measure the intact living animal. Although we anticipate that many of our studies will be carried out at embryonic stages (5 days after egg fertilisation and under) before zebrafish are considered sentient enough to require legislative protection, we have established assays that do require analysis at protected stages after 5 days post fertilisation. For example, to understand the connectivity and maturation of networks between glioma initiating cells, larval zebrafish have to be analysed post day 5 of fertilisation. Similarly, studying network signalling and function requires analysis over 5 dpf as these are the stages when networks mature. Analyses of network signalling can we highly variable, so that we may need up to 50 animals per condition to obtain scientifically robust measurements. Therefore, we predict using up to a further 30,000 for experiments related to our oncogene overexpression model. We carry out very careful calculations to define how many animals are needed to find statistically meaningful effects in our experiments and will continue to do so for new studies.

Finally, we predict using a further 6,000 animals for our xenograft model (protocol 5). Here we transplant human tumour cells into larval zebrafish between 3 and 5 days post fertilisation before they become protected animals. Analysis of these larval zebrafish will be similar as described for the oncogene overexpressing fish, however using lower numbers of animals in total due to the complexity of the model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To strongly reduce the number of animals we will do a large number of experiments between day 3 and day 5 post fertilization before the larva is protected under the act. Nevertheless, a certain number of larvae will have to be analysed post day 5. To investigate the role of macrophages/microglia most of these experiments can be performed before day 10 of development. Only a limited number of zebrafish need to be analysed at juvenile stages. Based on studies under the previous PPLs on macrophages/microglia, we have clear expectations of the variability enabling us to reduce the number of animals for our experiments as much as possible. Another important way in which we can reduce animal number is through live imaging of individual animals over time. Through time-course or time- lapse imaging, we can gain a wealth of information about the dynamic nature of biological events from single animals that would otherwise require multiple animals being assessed at many different time- points.

For breeding and maintenance protocols and the generation of new stocks of genetically altered lines adult fish will be kept. However, we can assess the efficiency of transgenesis and gene editing at unprotected stages, which reduces the number of animals taken on to protocols.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will work closely with our aquarium staff who are implementing trials that aim to adapt husbandry procedures to ensure optimal maintenance of our breeding stocks. Together with other groups we will continue to work to optimise the efficiency of transgenesis and gene editing, particularly cell-type specific gene editing, which we hope will reduce the number of animals that we use in our work.

We will continue our efforts on in vitro assays to test candidate signalling pathways in tissue culture. Only if clear alterations of interactions between macrophages/microglia and tumour cells are observed in vitro these cells will be injected into zebrafish larvae.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The optically transparent zebrafish larva is an excellent model for studying immune cells (in this case macrophages and microglia) in the microenvironment of brain tumours in vivo. In particular the opportunity to image cellular interactions at high temporal and spatial resolution in vivo, combined with the possibility of intervening genetically and pharmacologically, make the zebrafish the model of choice to study cellular interactions in brain tumours. This is not possible in any mammalian model.
Furthermore, the zebrafish has been established as a productive and informative cancer model. Overexpression of proto-oncogenes and transplantation of mammalian tumour

cells into zebrafish embryos have led to zebrafish models for different types of human cancer, ranging from B-Cell/T-Cell leukaemia and melanoma to glioma.

Our principal experimental methods involve live imaging zebrafish, and we can do so at different scales. We can carry out high-resolution screens of many animals to quickly assess how gene or compound function affect biological processes of interest, and in a manner that shows no signs of causing distress to the animal. In contrast we can also carry out extensive in-depth imaging over time of individual animals to understand specific types of interactions between immune cells and brain tumour cells. Time-lapse, time-course, of individual animals is a refinement, because we can gather enormous amounts of information from single animals. Such analyses provide insight into dynamic processes impossible to gain in other systems that would require the use of multiple animals if using other models.

We continue to pursue any innovations in husbandry practices, and when trialled and deemed successful, will be applied to our protected stocks, and may significantly reduce numbers of animals used for breeding and maintenance throughout the project.

Why can’t you use animals that are less sentient?

Zebrafish are the simplest standard model in which the complex interactions between microglia and brain tumours can be studied. Zebrafish also have an early onset of microglia development and hence are arguably less sentient than mammals by the time they have developed similar properties. We make every effort to study animals at the earliest stages at which we can address the questions that underpin the aim of our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Very few of our experimental protocols cause significant harm to animals and are already well refined. The main source of potential adverse effects to animals comes from the induction of tumour growth.
However, we mainly analyse tumour initiation stages during which a limited number of tumour cells is present in the larval zebrafish brain and hence the harm for the animal minimal.

Upon transplantation of mammalian cells zebrafish larvae will be kept at 33-35 C to ensure survival and growth of the mammalian cells. These temperatures do not affect welfare of the larvae and have also been found in natural habitats of zebrafish. Furthermore, our work under the previous PPL has confirmed that zebrafish tolerate these temperatures well. Transplantation protocols have been optimised under the previous PPL in a way that only the minimal number of tumour cells required for the experiments is transplanted. Thus, the total volume that is injected into the larval brains is strongly reduced and adverse effects due to the injection could be mitigated. In case the injections cause adverse effects (e.g. rupture of membranes or vessels), these will become obvious within the first hours post transplantation and larvae will be killed before they become protected.

For specific experimental aims larvae will be single housed upon tumour induction, as we will need to identify single fish for the experimental evaluations and single housing has been shown to be less stressful for zebrafish . These larvae will be closely monitored and will be immediately killed using a schedule 1 method in case adverse effects are detected. We will minimise animal suffering by performing a large number of experiments in larval zebrafish between day 3 and day 5-post fertilization and only a limited number of juvenile

fish will be used. All experimental animals will be closely monitored and killed by Schedule 1 if any adverse effects are observed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines issued by the National Centre for the 3Rs, and will follow the recently published guidelines put together by a group of zebrafish researchers in collaboration with animal welfare experts at the Federation of European

Laboratory Animal Science Associations (FELASA). In addition, we continue to refine practice across all experimental approaches as innovations and advances are published in the literature.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My group and I follow NC3Rs on social media, and stay informed about relevant innovations through our proactive bioresearch and veterinary services at the establishment. We will implement appropriate advances through discussions with our local vets and named animal care and welfare officer. In addition, we have proposed research proposals to NC3R in the past and plan to do so in coming years to actively contribute to the goals of NC3R in biomedical research.

54. Reproductive and Developmental Toxicology Safety Studies with Chemicals

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Reproductive/developmental toxicity, Regulatory, Safety, Rodent, Rabbit

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant
Rabbits	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence authorises the conduct of studies in laboratory animal species (rats, mice and rabbits) with the aim of evaluating the Reproductive and Developmental toxicity of non pharmaceuticals (agrochemicals, biocides, food additives/foodstuffs, ingredients of household chemicals (where legislation allows) and industrial chemicals). This is to aid in the development of new chemicals, and to provide mandatory information to regulatory authorities to allow marketing approval (i.e. to show they are safe when they come into contact with humans).

No cosmetic products or chemicals that are exclusively intended to be used as ingredients in cosmetics will be tested.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that their potential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain marketing authorisation or product registration.

This safety assessment is of immense importance along with other rodent, non-rodent and non-animal studies in demonstrating to governments and the public the safety of these substances.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safer more effective agrochemicals or food additives/foodstuffs, safer usage of industrial chemicals and biocides and the economic benefits from production/use of these substances, by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations.

Achievement of the objectives of this licence will enable safer agrochemicals, food additives/foodstuffs, industrial chemicals and biocides to be approved for marketing/registration and for the authorities to prescribe conditions for safe use of these substances and will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources.

Who or what will benefit from these outputs, and how?

Our customers will benefit, as the data we generate will allow them to progress their products under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain registration or marketing authorisation.

Humans and animals will benefit from these studies as this work will contribute to the development of safer or more effective agrochemicals or biocides, safer food additives/foodstuffs, safer usage of industrial chemicals and the economic benefits from production/use of these new substances, or identification of substances that are deemed unsafe for further development. We may, by our work, also contribute to better knowledge and understanding of these types of chemicals, and that knowledge may be used to develop further new chemicals.

How will you look to maximise the outputs of this work?

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for regulatory purposes or to support regulatory purposes (e.g. to

support marketing approval or registration). Previously however, we have collaborated with customers and shared data we have produced in the form of scientific publications that are in the public domain.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 162000
- Rabbits: 5150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most of our experiments will be carried out on conventional adult rats and rabbits as rodents and rabbits are mandated by regulatory authorities for the regulatory studies in this project. The life stages

evaluated on the various regulatory studies are also mandated by regulatory authorities. The rat is the standard animal for reproductive performance and developmental neurotoxicity tests and large amounts of background control data on all aspects of rat reproductive processes are available to help put study results into perspective. The rabbit has been selected as the second species for embryo-fetal development studies because it was found to be sensitive to Thalidomide, a notorious teratogen, whilst the rat was insensitive to this chemical.

In some specialist cases we may use the mouse for example due to unusual metabolism patterns or sensitivity in the rat and/or rabbit.

Typically, what will be done to an animal used in your project?

Typically on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken after they have been humanely killed for extensive toxicology analysis. Studies would range from a single dose, to those which last a matter of days (much less than a month) although some can last for 1, 3 or 6 months, and sometimes up to 1 year (to specifically examine whether a test substance can induce reproductive and developmental effects in two successive generations). Study durations are dependent on the specific regulatory test being performed. Some animals are left dose free for a few weeks after dosing is complete to see if any effects of the test substances can be reversed for example on OECD 422 studies.

Dosing of animals is commonly done orally using a flexible tube, or by admixture in the diet or drinking water. Other common routes are used such as inhalation (when animals are dosed in specially designed tubes) or dermal (via the skin).

Blood samples are usually taken from easily accessible veins in the neck or the tail of rats or mice, or from the ear arteries/veins of rabbits. We are limited to how much blood we can

take at once or, cumulatively, over a month. If we need a large blood sample, we would do this when the animal is anaesthetised and we would not let them recover consciousness. Where possible, we try and take as many of the tissues and samples we need after the animals have been humanely killed after all dosing had been completed.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a vet If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. If we can take blood samples when an animal is deeply unconscious then we do. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures are trained to a high standard.

Rarely we need to take a urine sample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around. Virtually every animal will get used to their new cage within about 15 minutes and are fine.

Dosing with chemicals may cause adverse effects in some studies. Experience shows that the majority (~65%) of animals are not expected to show any clinical signs of suffering (either no clinical signs or normal background signs expected of the rodent strain). A small percentage (~15%) may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals (~20%), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, but for preliminary studies that may be the first animal studies with limited data available, a very small percentage of animals may inadvertently show severe findings before they are immediately and humanely killed.

We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved in its care for advice, including vets.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

On the last project, about 85% of animals displayed mild severity, and around 10% of animals were classified as having displayed moderate severity. This is because these studies can last between a few days and weeks to up to a year, and although the individual procedures are usually mild in nature on their own, the cumulative effects make them moderate overall.

It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform.

What will happen to animals at the end of this project?

- Killed
- Kept alive

- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is currently no regulatory and scientifically acceptable alternative to the use of rodents and rabbits on the required regulatory studies.

These studies are run to satisfy the regulatory requirements of governments around the world to ensure agrochemicals, biocides, food additives/food stuffs and industrial chemicals are safe for humans and animals. These tests are very specific as to what they require in terms of testing in animals to ensure this.

We maintain a constant awareness of regulatory guidance and ensure that where non-invasive methods exist which fulfil the regulatory requirement they are used in preference to animal studies.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives for the work being undertaken on this project, but there are some exceptions. The regulations we are following do not allow safety decisions to be made on non- animal systems alone.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies .

Why were they not suitable?

We need to use animals because non-protected animal alternatives cannot replace the extremely complex series of events involved in reproduction and /or subsequent development of the young and the reproductive organs; these processes cannot in most cases be effectively modelled in the laboratory in test tubes/dishes or by the use of sub-mammalian animals.

That is why we need to test new chemicals in animals, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they were ever exposed to humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The regulatory guidelines we follow for each study usually indicate the number of animals in a study; otherwise, the number used is the minimum to achieve the aims of the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk having to repeat the study. For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist.

In vivo micronucleus tests may be bolted onto OECD 421 or 422 screening tests which reduces the overall numbers of animals used, and the OECD 422 study may be performed as an alternative to separate OECD 421 and 407 studies and again this reduces the overall numbers of animals used.

Otherwise reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will try and get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to get several different samples, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to use for the main studies. These studies are important as it gives us confidence that the doses we are using are correct prior to testing them in bigger groups of animals required by global regulators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our models involve dosing animals with test substances, and sampling them, with many outputs taken after the animals have been humanely killed. This is generally the least invasive set of procedures that can be done to give meaningful outputs to make scientific decisions about further tests, or to determine the safety of a test substance/device.

Throughout our studies, our animals are checked at least twice a day. This allows us to see over a period of time, whether dosing each individual animal is causing any adverse clinical signs. if this is the case, we can take action: get veterinary advice, add food supplements and extra bedding if needing, and even reduce dose levels or stop dosing completely.

Why can’t you use animals that are less sentient?

Rodents (rats and mice) and Rabbits will be used in all of the studies conducted under this licence. Rodents are considered to be of the lowest neurophysiological sensitivity (their brain function and physiology) that will allow us to achieve the study aims and are considered suitable for predicting what's likely to happen in humans (or animals). The rabbit is mandated to be used as the first choice non rodent species for embryo fetal studies because it was found to be sensitive to Thalidomide, a notorious teratogen.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Many of the procedures performed on our rodents like blood and urine sampling, cause only transient distress to the animals. Blood sampling procedures are similar to and about as painful as having a blood sample taken by a doctor or a nurse. Blood volumes are kept to a minimum within rigid volume guidelines. Confining animals in special cages to allow us to take urine samples is similarly of little distress to the animals.

For inhalation dosing, where animals are restrained in tubes, training of the animals occurs for increasing periods prior to treatment commencing to accustom the animals. Dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm and will be the minimum consistent with the scientific objectives of our studies. In addition, suffering will be further minimised by implementing clearly defined humane endpoints.

In addition, care is taken to provide as much environmental enrichment as possible. This includes plastic shelters in their cages, wood blocks and balls to gnaw on and push around; mice are given swings, and rats and mice on littering studies are given paper shavings from late gestation to use as nesting material. Rabbits are given key rings on their cages and wood blocks and hay and fresh vegetables and paper balls as appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For blood sampling and dosing then the following guidelines/literature will be followed:

Diehl et al. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Journal of Applied Toxicology: 21, 15-23 (2001).

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016).

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals.

Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. UK Home Office 2014

Regulatory guidelines.

This is not an exhaustive list and principally focuses on The Organisation for Economic Co-operation and Development (OECD) safety guidelines:

For low tonnage industrial chemicals and biocides guidelines include

OECD Test Guideline 421 Reproduction/Developmental Toxicity Screening Test, 29 July 2016

OECD Test Guideline 422 Combined Repeated Dose Toxicity Study With The Reproduction/Developmental Toxicity Screening Test, 29 July 2016

For human milk oligosaccharides guidelines include

OECD Test Guideline 408 Repeated Dose 90 Day Oral Toxicity Study in Rodents -dosing starts in juvenile rodents

For agrochemicals and high tonnage industrial chemicals and biocides guidelines include OECD Test Guideline 414 Guideline Prenatal Developmental Toxicity Study, 27 June 2018. For agrochemicals and high tonnage biocides and food additives/foodstuffs OECD Test Guideline 416 Guideline Two-Generation Reproduction Toxicity Study, 22 January 2001.

For agrochemicals and high tonnage industrial chemicals and biocides and food additives/foodstuffs guidelines include

OECD 443 Test Guideline Extended One-Generation Reproduction Toxicity Study, 27 June 2018 – there is always a specific regulatory request for the study detailing the exact study design required.

For agrochemicals and high tonnage industrial chemicals and biocides and food additives/foodstuffs – there is usually a specific regulatory request for the study

OECD Test Guideline 426 Developmental Neurotoxicity Study 15 October 2007.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?
This will be achieved by regular discussions with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

55. Characterizing the Tumor Ecosystem

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Initiation, Metastasis, Microenvironment, Ecosystem

Animal types	Life stages
Zebra fish (Danio rerio)	adult, embryo, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to understand how different cells in the body work together to promote cancer initiation and metastasis. This coordination of various cells is referred to as the tumor ecosystem.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite some progress, the majority of advanced cases of cancer result in patient death. We lack a fundamental understanding of how cells transition from benign to malignant states, and how this eventually leads to metastasis (spread). Almost all metastatic cancers are incurable, indicating that we need to understand this process to develop better treatments. A major insight over the past few decades is that cancer cells have a complex relationship with other cells in the body. These cells are sometimes referred to as the

tumor microenvironment. Together, the cancer cells and the tumor microenvironment create a tumor ecosystem. Therapeutic targeting of this ecosystem will lead to better outcomes for patients.

What outputs do you think you will see at the end of this project?

The major output of this project will be basic discoveries about the origins of cancer and how it spreads to new places. These will result in scientific publications and communication to the public through lectures and seminars.

Who or what will benefit from these outputs, and how?

The short term impact will be on other scientists who are interested in understanding cancer as an ecosystem composed of tumor cells and other cells in the body. We expect that our discoveries in this space will emerge within the 5 year proposed project and result in publications. In the longer term outside of the scope of this project, some of our discoveries may have therapeutic implications and could lead to medications used to treat cancer.

How will you look to maximise the outputs of this work?

In each step of the project, we will always publish our results in scientific journals, whether they are positive or negative. We will also attend scientific conferences and give lectures about our ongoing and completed work. This will lead to dissemination of new scientific knowledge.

Species and numbers of animals expected to be used

Zebra fish (Danio rerio): 46,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our cancer studies utilize the zebrafish (Danio rerio). The major advantages of the zebrafish compared to other models like mice is: 1) ease of genetic manipulation to test genes and pathways that we hypothesize are relevant, and 2) superior imaging of each step of cancer progression, from the very first cancer cell all the way through to metastasis. Because cancer is a disease of adulthood in humans, we utilize adult zebrafish to match this physiology as much as possible.

Typically, what will be done to an animal used in your project?

We will start our studies using genetically altered fish bred by natural methods. These genetic alterations are not expected to have any harmful phenotypes. Adult fish will have tumours induced either via microinjection of DNA constructs containing genes of interest, or via transplantation of cancer cells. For the microinjection studies, the DNA is suspended in water, placed into a glass microcapillary, and injected into either single-cell embryos or into adult tissues such as skin. The typical volume used for these injections is under 1

microliter. For the transplant studies, the cells are suspended in a phosphate buffered saline solution, placed into a glass microcapillary, and injected into adult tissues such as skin. The typical volume used for these studies is under 5 microliters. Once the tumours have developed the fish will be anesthetized, taken out of the water and then undergo rapid imaging within several minutes. They will then be returned to fresh water to recover, and then returned to the main aquatic system. They can be imaged up to 52 times (maximum once per week) over a period of up to a year. After this the fish will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

The major impact upon the zebrafish is the development of tumors. These tumors progress slowly in the zebrafish but eventually can metastasize to distant organs over a period of a year, which is one of our major biological questions. These fish with metastatic cancer can become ill (i.e. weight loss, lack of eating) similar to what happens in patients with metastatic cancer. At the earliest signs of this, the animals would be killed. The injection procedure itself is not expected to cause excess harms, as the volumes used and locations where we do the injections (i.e. single-cell embryo or adult skin) do not cause toxicity. For the anesthesia and imaging procedure, we use an approved anesthetic agent (MS222) which the fish typically fully recover from after theyare placed back in fresh water. We perform imaging as quickly as is feasible to minimize the time the fish is out of water. Many years of experience with this procedure (20+ years) has demonstrated that this is generally not toxic to the fish and they will fully recover from the anesthesia/imaging. In some cases, if the fish is burdened with a lot of cancer, they could fail to recover from anesthesia but would be asleep if death should occur.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold: 5%
Mild: 20%
Moderate: 75%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a disease not only of tumor cells, but also all of the other cells of the body. For example, while a melanoma skin tumor may start in a skin melanocyte, it interacts with many other cells types in the skin such as keratinocytes and fibroblasts. Beyond those early stages, the tumors may eventually metastasize to other organs such as the brain,

where the tumor cells then interact with completely different cells such as neurons. It is this interaction with the other cells in the body that make patients sick or die. Animal models such as the zebrafish are required because they are the only practical way to study these cell-cell interactions that are central to the nature of cancer. While cell-line based approaches (i.e. 2D or 3D organoids) have continued to improve, those technologies are not able to recapitulate the enormous complexity of all of the possible cell-cell interactions that can occur in cancer. Similarly, while bio-informatic approached can describe the nature of cell-cell interactions, these provide only correlative data, and do not definitively tell us the function of these various cell-cell interactions.

Which non-animal alternatives did you consider for use in this project?

The two major approaches that we use prior to animal studies are: 1) cell culture models, and 2) bio- informatic approaches. While each of these are important preliminary approaches, they are not adequate to tell us the actual function of the genes and pathways we believe mediate cell-cell interactions in cancer.

Why were they not suitable?

Cell culture models can test only 1-2 interactions at a time, which limits the range of potential interactions. Bio-informatic and computational approaches provide powerful correlative information, but do not tell us whether these correlations actually function in cancer.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The goal of our project is to study both tumor cell and microenvironmental influences on cancer progression. This is a challenging problem because we must take into account genes not only in the tumor cell, but also in a wide variety of microenvironmental cells. Thus to fully capture this complexity requires us to test many different combinations of genes that are altered in both cellular compartments. To achieve this, we first use extensive bioinformatic and cell culture approaches to come up with candidate genes or pathways that are dysregulated in either cellular compartment. Such informatic analysis usually results in a list of about 3,500 dysregulated genes. We then create transgenic zebrafish to test these. For each individual gene, we study approximately n=10 replicate animals. This then allows us to study approximately all genes that we identify from our bio-informatic analysis (10 x 3,500 = 35,000 fish).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have implemented multiple steps along the way to reduce the actual number of animals used:

1) We first perform extensive bioinformatic analysis of publicly available datasets that were generated from human tumor samples. This includes databases such as the TCGA, cBIO or Broad single cell portals. This in silico approach allows us to comprehensively identify candidates genes that we think may be important in cancer initiation, progression or metastasis.

2) We then test subsets of these genes in vitro using cell culture models. While this approach cannot comprehensively tell us about cell-cell interactions, it can sometimes tell us if a given gene acts solely in the cancer cell (i.e. independent of its surrounding microenvironment cells). If that is the case, then we will not need to test that particular gene in the animal, an important means of reduction.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A major advance from my lab has been the development of "somatic transgenesis", in which we directly alter the DNA of the animals without the need for germline transmission. This is a major

advance in reduction efforts, because it eliminates the need to breed animals which will ultimately not have the genetic background that is useful to us (a major bottleneck in traditional germline alleles in the mouse). Thus every single animal we use is directly informative, with no animals of the incorrect background that are uninformative. In the typical workflow, we will inject or electroporate transgenes into the animals, and then wait until they are a few months older when tumors will spontaneously form.

A second major reduction advance has been our ability to multiple genes within the same animal. In this scenario, we label one gene with a green fluorescent color, a second gene with a red fluorescent protein and a third with blue fluorescent protein. These fluorescent proteins are harmless and do not affect the animal. These are then co-injected or co-electroporated into the tissue of interest. We can then use imaging to figure out which of those genes directly contributes to the tumor. This allows us to study many more combinations of genes then would ever be possible by studying them one at a time in a cohort of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our studies use the zebrafish (Danio rerio), which has a long standing history of use in genetics and more recently disease modeling. We use this model to visualize certain cells using fluorescence, which causes no distress to the animal. We also use this animal to induce tumors, our major biological question. We can induce tumors either through introduction of DNA into the animal (which is called transgenesis) or through

transplantation of cancer cells into the animal. In some cases, these can be done in the embryonic stage, an important refinement method. In all of these methods, early stage tumors do not cause any distress to the animals, since they are small and very superficial. If the tumors become larger and/or metastasize, they can make the animals sick, just as they do in humans. We thus monitor the fish carefully for any signs of distress or ill health and kill them at any sign of such distress.

Why can't you use animals that are less sentient?

Cancer is largely a disease of vertebrates. Although some cases of cancer-like growths have been observed in plants or flies, these do not develop the full spectrum of the disease found in humans (i.e. metastasis) since they do not have a fully developed blood vessel network. Fish are the lowest vertebrate that is know to spontaneously develop cancers that are analogous to the human disease. While studying cancer at immature life stages in zebrafish is possible, this does not reflect the vast majority of the human disease. Pediatric cancers (i.e. earlier life stages) are relatively uncommon and not the focus of my laboratory. Instead we study cancers that almost exclusively develop in adults (i.e. melanoma, pancreatic cancer) and so we need to model this in adult stage zebrafish. It is not possible to induce cancer in terminally anesthetized animals since tumor development is a gradual process that requires genetic and epigenetic evolution over time, and does not occur in a single instance.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We monitor all fish on a weekly basis for the appearance of tumors or distress in the animal. If we find that a particular gene we discover causes the tumors to be more aggressive, then we will increase monitoring to more frequent intervals, such that we would kill any distressed animals quickly after this occurs. We will use a refined technique for genotyping (swabbing) instead of fin clipping if that is appropriate. We will use the improved anesthesia guidelines for zebrafish as they become available, which can include the incorporation of lidocaine hydrochloride buffered with sodium bicarbonate and ethanol for euthanasia of adult zebrafish.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the published NC3R guidelines for the care and use of zebrafish: https://nc3rs.org.uk/3rs- resources/zebrafish-welfare. These are encapsulated in The Directive 2010/63/EU. We will also use the LASA guidelines, found here: https://www.lasa.co.uk.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly attend the animal welfare lecture courses offered by the establishment, and I will work with the NC3R Regional Manager and Named Information Officer. I am also a subscriber to the ARRIVE guidelines web server, which provides updates to the NC3R publications. We will implement any suggested recommendations from either the internal establishment courses or from the ARRIVE publications as they arise. We will follow the ARRIVE guidelines for publications as they evolve.

56. Investigating Micro-Circuit Homeostasis in Ageing and Neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Synapse, Plasticity, Ageing, Neurodegeneration, Imaging

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to determine the synaptic, cellular and molecular mechanisms of neuronal homeostasis and test potential intervention strategies that may act to promote homeostatic control of neuronal firing rate in the aged and diseased brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We live in an increasingly ageing population, with this comes a greater prevalence of age-related neurodegenerative diseases including Alzheimer's Disease (AD). Such diseases carry great burden for patients, carers and the wider socio-economic structure. Currently, there are no successful treatments for AD or well-developed strategies to improve healthy neurological ageing. Our work aims to address these important issues, first by improving basic understanding and then by testing potential intervention strategies.

What outputs do you think you will see at the end of this project?

Our work will result in standard scientific outputs. These include, publications relating to the individual aims of the work set out. Invited talks and organised conferences to share relevant findings with colleagues and collaborators. Patents, relating to developed interventions and the leveraging of additional funding to support ongoing work. In addition, the programme will generate a rich data-set, consisting of functional and structural longitudinal measurements from thousands of neurons in ageing animals and AD models. The activity and plasticity of these neurons will be mapped to behavioural measures collected from the same animals. These outputs will be shared freely making them available to other researchers, computational neuroscientists and clinicians interested in neurodegeneration.

Who or what will benefit from these outputs, and how?

The global number of people living with dementia will increase from 50 million in 2018 to 152 million in 2050 (204% increase). https://www.dementiastatistics.org/statistics/global-prevalence/. There are currently no known cures for dementia. The cost to carers, healthcare systems and social services is enormous. However, most importantly, this myriad of neurodegenerative conditions is incredibly distressing and traumatic for sufferers. The work set out in this application aims to translate basic research into an experimental setting that will enhance our knowledge of how to slow the onset of neurodegeneration in old age. The aim is to determine critical plasticity processes and molecular targets that will form the building blocks of potential therapeutic strategies in the future. The benefits of the work cover a range of areas and stakeholders.

Short-term benefits: In the short-term, improved understanding of the early phases of neurodegeneration will be achieved by studying the efficacy of a key neural plasticity processes (homeostatic plasticity) thought to be critical for healthy network function. The benefit will be in the elucidation of factors that make the aged brain susceptible to dementia. As such, the most immediate short-term benefit will be to other scientists working in the same or related fields.

Medium-term benefits: In the medium-term, we will test potential strategies that may rejuvenate/enhance aged brain function/or slow progression of neurodegeneration in pre-clinical models. This benefit will specifically be achieved using the introduction of pharmacological agents, bioelectronic stimulation of neural circuits and/or manipulations of sensory factors, such as enriched environments, that can modulate neural plasticity. As such, the medium-term benefits will be to industry and those working in the translational space to take proof-of-concept preclinical work to an experimental medicine phase.

Long-term benefits: In the long-term, the work will lead to the identification of potential molecular targets for future therapeutic intervention. As such, the long-term benefits will be to patients and those working to develop clinical interventions.

How will you look to maximise the outputs of this work?

We work closely with clinicians and those conducting human experimental medicine trials to try and facilitate rapid translation of major findings from bench to clinic. For basic work, we will share all data generated from this project freely with both long-term collaborators and the wider scientific community as in previous work.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents are among the simplest mammals to study functions that depend on the cortex. Mice are the species of choice in biomedical research because production of transgenic animals is already well refined. The use of mice is justified as many neural-circuit features relating to investigated areas of research (ageing/pathology/plasticity and brain state oscillations) are comparable between mice and humans, making the investigation of preclinical models a useful testbed for developing understanding, related to human disease progression. In addition, mice carrying familial mutations linked to neurodegenerative disease can be used in conjunction with other technologies for reporting or

manipulating neuronal activity that impacts behavioural phenotypes such as sleep cycles and cognitive performance. Currently, animal experimentation is the only approach available to probe the impact of neural-circuit interventions on behavioural phenotypes.

Our proposed approaches very rarely show morbidity and so preclinical models of disease progression can be reliably tracked at the cellular and sub-cellular level over many months in vivo, which is not possible in either reduced preparations or human experiments. Our work focuses on adult and aged animals these life stages are selected as ageing is a key contributing factor to many age-related neurodegenerative disorders.

Typically, what will be done to an animal used in your project?

We use adult (2 - 14m) and aged (15-24m) wild-type mice as well as genetically modified mice that capture key features of neurodegenerative diseases such as Alzheimer's Disease (AD). Mice may be deeply anesthetised and then undergo transcardial perfusion with an oxygenated solution to facilitate brain slice preparation for electrophysiological recordings, this is a terminal procedure.

Alternatively, animals may undergo one of a series of brain imaging techniques that measure the activity of brain cells. In this work, a small glass window is implanted in the skull under general anaesthesia, during this step we may also inject a fluorescent reporter of neuronal structure/activity, electrodes (either on the skull or in the brain) or a small light-weight head mounting to help with later microscopy. For example, during our 2 to 4 hour surgeries, we will typically make very small windows in the skull to gain access to the brain, implant a glass coverslip and tiny screws/head fixings and probes no more than, then secure everything with dental cement before closing the wound.

Animals are then allowed to recover for ~1 month and given injections to help with any pain management (analgesic) which we would expect to be minimal. After recovery animals may undergo periods of sensory enrichment and/or deprivation. Enrichment involves stimulating with flashing lights/sounds in the home cage or adding toys or objects

for the mice to play with. Deprivation can involve surgery to reduce sensory input either through removal of whiskers, the eye, or ear-plugs, maintenance in a dark environment and/or social isolation. Again, after deprivation inducing surgery all animals are allowed to recover after surgery with analgesic.

Animals may also undergo a period of learning using standard behavioural approaches including automated touchscreen testing platforms, this can involve small amounts of food restriction to motivate the animals to press their nose against a touchscreen to receive a liquid reward.

Across periods of sensory manipulation or learning we image animals. Imaging always involves a period where mice can get used to the microscope (habituation). This may be conducted in anaesthetised animals, head-fixed awake animals or freely moving awake animals using small head- mounted mini-scopes.

Imaging, sensory manipulations and behavioural assessments can be accompanied by delivery of certain drugs that are thought to act as interventions in the ageing or disease process and/or may be combined with electrical brain stimulation or recording using electrodes.

Our experiments are longitudinal and so may span months to capture the ageing or disease process. However, the number of procedures is closely regulated so that a typical animal will undergo: one implantation surgery, one sensory manipulation, one behavioural assessment and limited periods of imaging or brain stimulation.

Animals may be given some pharmacological agents to promote or inhibit brain processes such as plasticity, label structures of interest, manipulate gene expression and/or modify AD-related pathology for example anti-amyloid drugs. Such agents are standard and well tolerated and may be given in the drinking water, food, by intraperitoneal and/or intravenous injection. A typical experiment may involve dosing a drug once a day for 7 -14 days.

Typically (80%), animals involved in imaging experiments will be imaged once a day over 5 days. However, some experiments may involve a lower frequency of imaging over a longer time period, for example 1-3 sessions over three weeks (10%). in addition, it may sometimes be necessary to conduct more high frequency periods of imaging, for example 4-6x per day for 2 days (10 %).

What are the expected impacts and/or adverse effects for the animals during your project?

Old age can be associated with increased incidence of cataracts, arthritis, obesity, spontaneous tumours, ulcerative dermatitis. We anticipate no more than 15% of mice will exhibit such clinical moderate severity. If two or more signs are seen the animal will be monitored more closely and NVS/NACWO advice immediately sought with attempts made to ameliorate the signs. The animals will be humanely killed within 2 days if no improvement is seen. Aged animals may die unexpectedly without showing prior signs of ill health, we estimate this may occur up to a maximum of 5 % in aged animals. Surgical steps, behavioural testing and imaging/electrical recording sessions are highly refined procedures but may still lead to bleeding, intermittent tremors, rare convulsions, enhanced aggression or subdued behaviour with decreased responsiveness. We anticipate no more than ~5% of mice in this protocol will exhibit such clinical signs of moderate severity. If two or more such signs are seen as a result of testing, the animal will be monitored more

closely. NVS and NACWO advice will be immediately sought and attempts made to ameliorate the signs. The animals will be killed within one day (end of working day) if no improvement is seen. Animals that exhibit convulsions will be killed immediately.

Cranial window implantation under general anaesthesia will be accompanied by appropriate level of anti-inflammatory/multi-modal peri-operative analgesia. Following recovery from anaesthesia mice will be returned to their home cage.. During the first few days of the recovery process animals are provided with soft food. Typically, 30 days will be allowed for recovery between surgery and further imaging/intervention. Repeated imaging sessions in awake animals will typically last 90 minutes, and very rarely more than two hours. If the experimental question does require imaging to exceed two hours, the time under the microscope would be progressively increased to gradually allow the animal to get used to (habituate) to the setup. For anaesthetised animals imaging/electrophysiological recording sessions will rarely exceed two hours, in the instances when they do (<5%) we will continue to closely monitor anaesthetic levels and physiological parameters such as temperature and heart-rate. In instances where the animals become unstable the experiment will be terminated and the animal humanely killed, we anticipate that this will be extremely rare in our experimental programme (<1 %).

Food restriction may be required to maintain motivation to perform automated behaviour tests in touch screen chambers and should not normally lead to adverse effects. However, animals will be closely monitored and will be weighed daily during experiments. If at any time, any animal drops below 90% of

its free-feeding body weight, it will immediately be given food ad libitum and monitored daily until its weight is above 90%. We will closely monitor the behaviour of the animals for signs of stress, including abnormally increased or reduced activity, stereotypic behaviour or reduced feeding and, if such signs appear, advice will be sought from the NVS/NACWO.

For monocular enucleation, eyelid suture, whisker trimming and auditory occlusion aseptic techniques will be employed to minimise post-operative infections. Multi-modal peri-operative analgesics will be given as required. Postoperative infection is the greatest risk and animals will be monitored regularly for signs of wound sepsis (such as redness and swelling around the relevant area) and appropriate veterinary consultation sought. Prophylactic antibiotics may be used. Likelihood of infection is low (<1%) and can be minimized with good surgical techniques under aseptic conditions. Animals will be monitored for signs of pain or discomfort (hunched posture, facial grimace, reduced appetite, decreased movement, increased freezing, ungroomed appearance) and will be treated with the consultation of the veterinary staff and if they do not respond to treatment/supportive measures within 1 day, will be humanely killed. There is a minimal risk of deaths resulting from anaesthetic or surgical complications (1 - 2%), and all deaths will occur under general anaesthesia or in the immediate post operative recovery period while still recovering from perioperative pain relief. This will be minimised by ensuring correct dosing of anaesthetics (inhalation anaesthetics are always preferred if possible) by accurate weighing and by good maintenance of body temperature. Whisker trimming is unlikely to induce adverse effects aside from the effect of anaesthesia as discussed above. Sham deprivation involves anaesthesia and/or handling matched controls and therefore has few adverse effects. Multiple sensory deprivation techniques are necessary as they induce different forms of neuronal plasticity that are differentially impacted by AD-related pathology. For example, whisker deprivation can be used to induce a competitive boundary between trimmed and spared whiskers that creates competition between brain areas. In contrast, monocular enucleation causes a global dampening of visually mediated

activity which triggers a homeostatic adaption of neuronal activity. Comparing the different types of plasticity impacted (or spared) in mouse models of AD and in the aged brain will allow us to determine which molecules could be targeted by pharmacological interventions.

Social isolation can induce stress in social animals such as mice. During social isolation the behaviour of the animals will be monitored for signs of stress, including abnormally increased or reduced activity, stereotypic behaviour or reduced feeding and, if such signs appear, advice will be sought from the NVS/NACWO. We do not anticipate any adverse effects due to environmental enrichment.

Administration of agents affecting brain functions and/or potential control substances are mild and will have minimal adverse effects, however animals will be closely monitored during the period of substance delivery. Insertion of a minipump may be associated with wound breakdown and infection. Other potential adverse effects may include hunched posture, abnormally increased or reduced activity, peritonitis or local irritation around the route of administration. If any of these becomes evident the NVS/NACWO will be consulted and the animal will be treated with the consultation of the veterinary staff and if they do not respond to treatment/supportive measures within 1-2 days, will be humanely killed. Maximum treatment frequency will be daily for a period of three months.

Animals given substances to induce transgene expression (e.g., tamoxifen or doxycyclin) will be carefully monitored, but no adverse effects are anticipated at the doses to be administered. In rare cases adverse effects may occur due to the method of dosing (including administration of the vehicle substance) resulting in local inflammation around injection sites, peritonitis, aspiration pneumonia or

reduced food or water consumption leading to weight loss or dehydration. Other effects include gastric ulceration, abdominal hernia, alopecia and enlarged testes. Incidence of adverse effects is usually rare. Mortality rates are expected to be below 1%. To screen for adverse effects animals will be checked and monitored, at least daily and weighed regularly. Animals displaying signs of peritonitis (hunched posture, reduced activity) or aspiration pneumonia (respiratory distress) will be immediately culled. Other signs of pain and distress include separation from cage mates, facial grimace, ocular or nasal discharge, piloerection and diarrhoea, mice will be monitored and humanely killed (Schedule 1) if these do not resolve within one working day.

Temporal interference brain stimulation uses low amounts of electrical current to stimulate the brain and is very unlikely to produce adverse effects. Electrical and sensory stimulation are considered safe modalities and have been used for decades in animal and human studies. The amplitude of stimulating currents will be similar to conventional low intensity non-invasive transcranial current stimulation (TCS). The adverse effects have been described in humans and cover mild tingling or light itching.

However, throughout stimulation animals will be closely monitored and should any abnormal behaviour or changes in physiological parameters (i.e., heart-rate, temperature or breathing rate) be observed the experiment will be terminated and the advice of the NVS will be sought. For sensory stimulation light will be delivered at low levels. No adverse effects are expected from light stimulation, as it is given at similar levels to that normally experienced by animals, however we will closely monitor aged animals during stimulation and after because they are more prone to seizures. Should we observe any seizures the animal will be humanely killed (Schedule 1) and the advice of the NVS sought.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 80 % of the animals will undergo surgery of moderate severity, the other protocols are mild 20 %.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Investigating the susceptibility of the aged brain to neurodegeneration by studying neural plasticity is a reasonably new idea. Therefore, there is not enough information to examine these relationships with computational models alone. We work with multiple computational modelling groups, which helps us to extract the most information from our experiments and make predictions for the best experiments to do, thus limiting the number of experiments in animals that will need to be performed. However, animal work is required to understand the interplay between neuronal circuitry and behaviourally assessed rates or learning or the expression of sensory induced plasticity. Animals also represent one of the only feasible strategies to study the process of neuronal ageing, as this is not well captured by in vitro assays, computational modelling, stem cell related work or even post-mortem human brain experiments.

Which non-animal alternatives did you consider for use in this project?

We will take every possible step to embrace feasible replacement options. This includes the following. First, the use of human induced pluripotent stem cell models for studying aspects of human disease. For example, neuronal culture systems derived from human AD patient stem cells and converted to neurons. Second, the use of in silico modelling of micro-circuit dynamics, this is a computational approach where previously collected experimental data is used to build simulations of disease. Finally, we are also collaborating to use manipulations of brain activity with non-invasive stimulation technology in humans.

Why were they not suitable?

The proposed future work uses experiments involving aging or complex disease/pathology progression and/or toxin clearance which is not feasible in preparations without vasculature such as cell cultures, reduced preparations or in silico given the complex multifactorial nature of senescence/disease related processes. In addition, mice carrying familial disease mutations can be used in conjunction with other technologies for reporting or manipulating micro-circuit activity that impacts behavioural phenotypes such as

cognitive performance. Currently, animal experimentation is the only approach available to probe the impact of neural-circuit interventions on behavioural phenotypes. These approaches very rarely show morbidity and so preclinical models of disease progression can be reliably tracked at the cellular and sub-cellular level over many months in vivo, which is not possible in either reduced preparations or human experiments. Furthermore, it can be challenging to model some features of AD related pathogenesis, such as changes in synaptic connectivity which are specific to certain parts of the brain, in less sentient non-protected animals, as such species do not have cortical architecture which parallels that in the human.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The projected number of animals reflects the number necessary to achieve the scientific objectives outlined in the programme of work described in this application. This number is based on previous workflows utilising the models described in this proposal.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use a centralised breeding facility to reduce the number of mice that are bred and not used by sharing animals with collaborators. We have designed all experiments to optimise power and minimise usage by considering the expected effect size whilst maintaining statistical power and the advice of statisticians (and the NC3R's Experimental Design Assistant) has been sought where necessary. In addition to careful calculation of predicted animal usage, additional experimental and technical approaches will be employed to meet the needs of reduction. For example, imaging approaches will be used to monitor volumes (rather than optical sections) of tissue so that data from more cells/synapses per animal can be collected thus reducing animal numbers.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most in vivo experiments will be longitudinal, so the probability of sharp deviations will be decreased by routine within-subject comparisons. Litter- or cage/age-matched animals will be used wherever possible to minimise experimental variability and thus reduce the need for additional experiments.

Where appropriate, data will be made freely available to other researchers, thus supporting reduction and group sizes will be based on small pilot studies estimating effect sizes to minimise underpowered studies or unnecessary use of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models we propose to use cause changes in cognitive rather than physical responses of animals and as such do not cause pain. We are also interested in the very early-stages of neurodegenerative disorders and so animals are often imaged/humanely killed at earlier adult time-points ahead of pronounced neurodegeneration. Few, if any, of the models we propose to use will be prone to seizure related events, as has been the case with some older preclinical Alzheimer's models.

Our protocol for repeated measurements from neurons has been developed because it only requires one surgical procedure, after which the animal can undergo measurements for weeks to months without any pain. After the initial surgery, the animal will be given pain relief and allowed to recover from the surgery for at least four weeks before any further procedures are carried out. These precautions will minimize any suffering the animals might have otherwise experienced. The most common adverse effects from either the surgery or the sensory deprivation would be infection and are expected to occur in a low percentage of animals (< 1%). The animals will be closely monitored throughout and treated humanely in consultation with veterinary staff in the event of pain or infection. Otherwise, the animals may be quiet and less active for a day or two following surgery, after which they return to normal behaviour.

Why can’t you use animals that are less sentient?

Our experiments investigate the interplay between neuronal plasticity, ageing, neurodegeneration and cognition. As such, animals are require to be sufficiently sentient to perform behavioural tasks and respond/adapt to sensory manipulations. In addition, we are interested in developing a better understanding of age-related neurodegenerative diseases and so the work needs to be completed in adult - aged animals as well as relevant preclinical rodent models of neurogenerative disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are the species of choice in biomedical research because production of transgenic animals, relating to rederivation, breeding and colony management is already well refined. All experimental procedures meet the requirements of the currently held PPL, specifically focusing on cumulative severity, experimentally defined end points and limiting the number of repeated interventions. Many of the proposed future work plans aim to refine experimental approaches further by reducing the impact of repeated anaesthesia. Specifically, by conducting imaging or electrophysiological approaches in awake, behaving mice. Similarly, a number of experiments investigate naturalistic behaviour in the animal home-cage, reducing stress associated with handling, novel environments and anaesthetic.

Furthermore, we have used state-of-the-art engineering and 3D printing facilities to support development of the lightest possible head mountings and wearable recording/stimulation devices. To ensure the behavioural needs of the animals are met, where compatible with experimental protocols, mice will be group housed where possible and institutional best practice will be followed with regards provision of environmental enrichment and other husbandry.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow LASA guidance ARRIVE and PREPARE guidelines as well as regularly consulting our local AWERB. We will also utilise available resources available through the NC3Rs, for example the online Experimental Design Assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I work closely with the NVS and veterinary support team, I also have close contacts with my local AWERB and receive regular updates on 3Rs conferences and opportunities. Other resources include

NIOs, other Named Persons, NC3Rs and NORECOPA. We will also utilise available resources available through the NC3Rs, for example the online Experimental Design Assistant

57. Investigating Multicellular Ecosystems in Cancer and Interstitial Lung Diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cell Biology, Cancer, Inflammation, Microenvironment, Immunotherapy

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how aberrant intercellular interactions contribute to inflammation, fibrosis and cancer. These findings will be used to identify novel therapeutic targets and develop these candidates for clinical translation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Solid cancers (e.g. lung, breast and oral) and interstitial lung diseases (ILDs) account for approximately 25% of avoidable deaths in the UK. A consistent feature of these distinct pathologies is sustained inflammation leading to alterations in tissue structure, which is a process involving many different types of cell and their interactions are yet to be fully understood. The development of immune checkpoint blockade as a cancer therapy has demonstrated that targeting intercellular interactions within these complex multicellular ecosystems can significantly extend patient’s lives, representing an unprecedented

advance in the clinical management of this disease. However, currently available treatments remain ineffective in the majority of patients.

In order to develop more effective treatment strategies a deeper understanding of the molecular mechanisms that regulate these multicellular ecosystems is required. The work carried out in this project will use state-of-the-art techniques to identify key pathogenic drivers and carry out pre-clinical testing of novel therapeutics to accelerate the translation of these findings into clinical benefit for patients.

What outputs do you think you will see at the end of this project?

We will develop a deeper understanding of how different cells interact in normal tissue compared to in cancer and interstitial lung disease. These findings will be communicated to the scientific community and general public through publications/conference presentations and public engagement activities (e.g. lay perspective press releases, social media posts and presentations at fundraising events) respectively.

In collaboration with industrial partners, we will test the efficacy of novel therapeutics. This will bring these drugs to the point at which they can be trialled in human patients.

Who or what will benefit from these outputs, and how?

In the short term (during the duration of the project) direct beneficiaries will include academics working in cancer or ILD research, who will benefit from the information generated regarding fundamental

biological mechanisms of disease progression through publications and conference presentations. Pharmaceutical companies will also benefit from this information as the findings are expected to identify novel therapeutic targets.

In the longer term (between the end of the project and the following 5-10 years), we expect patients to directly benefit from this study if the novel therapeutics that will be investigated are progressed into the clinic. If successful, the findings from this study may significantly improve overall survival rates and quality of life. These advances would also lead to indirect benefits for the UK economy, mitigating the extensive economic burden associated with solid cancers and ILDs, by increasing the efficacy of expensive immunotherapy drugs and reducing the loss in productivity caused by morbidity or premature mortality.

How will you look to maximise the outputs of this work?

During this project we will be working with existing industry partners and will establish new collaborations to maximise the output from any clinically relevant findings. As described above we will be using international journals and conferences as the major platforms for disseminating the novel information generated. These discoveries will be made widely available by ensuring that they are published in open access formats. In addition to this, where our work is not suitable for such platforms (for example describing unsuccessful approaches) we will make use of preprint repositories (e.g.
BioRxiv) to ensure that our findings are widely available and prevent unnecessary repetition in the future.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse model systems have been used for this type of research for many years. As a result, these animals represent the best understood model systems available to address our research questions and provide unique opportunities for precise experimental perturbations to be performed. For example, the tumour models we will be using harbour the same oncogenic driver mutations as those found in human patients; and the use of genetically altered animals will enable cell-type specific manipulation of key molecules under investigation. Unless required to address a specific experimental question, mature adult mice will be used as animals at this life stage are the only relevant option for the human disease processes under investigation.

Typically, what will be done to an animal used in your project?

Typically, the mice will be used in three types of experiment: 1) spontaneous tumour initiation due to oncogene activation; 2) tumour initiation through allograft injection; and 3) exposure to ILD/cancer risk factor mimetics.

1) Mice will be administered a drug (delivered to the lungs via injection, intubation or aerosol inspiration). Then over the course of 3-6 months the animals will be closely monitored to track the development and progression of tumours. Mice displaying signs of moderate discomfort/distress will be euthanised and the tumours will be analysed in the laboratory.

2) Tumour cells (and in some cases stromal cells) will be injected into mice. Then over the course of 1- 3 months the animals will be closely monitored to track tumour growth. Novel therapeutics may be administered to determine their efficacy in reducing tumour progression. Typically, these experiments will be terminated prior to the onset of adverse effects associated with tumour growth. In the event that any mice display signs of discomfort/distress they will be euthanised immediately and the tumours will be analysed in the laboratory.

3) Mice will be administered compounds that recapitulate known risk factors for cancer or ILD development, either alone or in combination with the tumour initiation methods describe above. As above, any mice displaying signs of discomfort/distress due to this exposure will be euthanised immediately and the affected tissues will be analysed in the laboratory.

What are the expected impacts and/or adverse effects for the animals during your project?

As a consequence of our experimental procedures the mice may develop a number of symptoms that will be closely monitored to ensure the animals are not subjected to undue pain, distress or suffering. These could include weight loss; hunched posture; pilo-erection; altered behaviour/activity; failure to eat/drink normally; dehydration; altered respiratory rate; pallor; and tremors. Mice will only be subjected to these symptoms where it is

necessary to address the scientific question. The majority of animals will not experience such symptoms. Where they are required, to uncover greater insight into the underlying biology, this project will not exceed moderate severity limits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild - 75%
- Moderate - 25%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to achieve the aim of this project and ultimately improve the clinical management of cancer and ILDs we must accurately recapitulate the genetic and cellular complexity found in human tissues and disease. The complex multicellular processes that we are investigating cannot be faithfully recapitulated in vitro, necessitating that this work is carried out using animal models. For example, to identify strategies for improving immunotherapy efficacy the trafficking and activation of immune cells needs to be accurately modelled, which is not currently possible using in vitro assays alone. Furthermore, the pre-clinical testing of novel therapeutics that we will carry out in this project is a fundamental requirement for these treatments to gain regulatory approval and progress into the clinic

Which non-animal alternatives did you consider for use in this project?

We have considered the use of in vitro assays using cell lines or primary cells isolated from surgical resection specimens. Indeed, these experimental methods will remain our principal mode of experimental testing. However, these systems are not appropriate for addressing all our research questions in a way that would enable the improvement of patient care. In addition, during this project we will be working to develop novel approaches that could reduce the reliance on animal models in future research.

Why were they not suitable?

The biological processes under investigation are regulated by complex multi-cellular ecosystems, where each cell-type/phenotype is sensitive to environmental and structural signals. In previous and ongoing work, we have developed 3D in vitro co-culture systems that attempt to recapitulate in vivo tissues. However, it is not yet possible to accurately maintain the phenotypes found in vivo for all the different cell types under investigation using these systems. Therefore, we initially test and optimise our hypotheses using

reductionist in vitro approaches that enable specific features of individual cell- types to be assessed. Once these experiments have yielded strong evidence for how a particular cell-type may impact disease progression or identify a promising novel therapeutic strategy, we need to ensure that these findings are validated in vivo. Without this in vivo validation it is not possible for these research findings to achieve clinical application.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For each experiment, we will use the minimum number of mice required to give us statistically robust and biologically meaningful data. Vehicle control groups will be used to ensure that statistical comparisons are made between relevant conditions, typically this will involve the administration of the relevant solvent without the active agent administered via the same method used in the experimental group. To minimise variation between biological replicates novel therapeutics will be tested using cell injection models (as opposed to inducible tumour models, which are known to exhibit high levels of variability in tumour progression rates).

The estimated number of animals to be used was calculated based on previous work using the experimental approaches proposed in this project, either from in house experience or published literature. Where this is not possible pilot studies will be performed prior to experimental testing, to ensure that reliable data can be generated before applying novel conditions to multiple animals.
Experiments involving in vivo readouts will be randomised using a random number generator to assign mice to different treatment arms. Where possible the operators responsible for administering therapeutics and taking measurements will be blinded to the experimental groups under investigation during data collection. To ensure the maximum efficiency of animal use, each experiment will have multiple readouts (e.g. in vivo measurement of tumour growth rates, followed by ex vivo analysis of cellular function and molecular phenotypes); and where possible tissue samples harvested from these animals will be archived (e.g. cryostorage or formalin fixed and paraffin embedded) to minimise the need for experiments to be repeated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We've followed guidelines detailed in the 'The Designing of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing, Overend, Borja and Berdoy; 2nd Edition) and used the NC3Rs Experimental Design Assistant online tool

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will collect tissues from euthanised mice for use in in vitro assays, to learn as much information as possible per mouse. We will also derive and culture cells from mice and will use those for analysis and further experiments, rather than performing procedures on live mice wherever possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will exclusively use mice for this project.

We will use non-invasive imaging to monitor lung tumour development. This enables quantitative and sensitive measurement of tumour burden, so that the majority of our experimental mice can be euthanized prior to the onset of any clinical signs of harm.

We will use cell-type specific promoters to regulate tumour initiation in the lungs, which improves the precision of this technique. This facilitates efficient monitoring and reduces off target effects, thereby reducing the pain, suffering and distress caused while enabling the relevant phenotype to be comprehensively investigated.

Why can’t you use animals that are less sentient?

The biological processes under investigation act in humans over many years to cause cancer or ILD in adults. These processes are not relevant to paediatric cancers, so we need to study adult mice.

In this project we will be using animals to model inflammation and cancer development in lung tissues, making mice the least sentient option with the relevant anatomy (e.g. less sentient animals such as zebrafish or drosophila are devoid of these organs).

We are also investigating novel therapeutics it is desirable/essential to conduct such studies in an organism that best approximates the action of a potential drug in patients, while avoiding the use of (for example) non-human primates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All work will be conducted by trained personnel, who will be given further training in specific techniques where necessary. This licence contains only protocols categorised as mild or moderate and anaesthesia and/or analgesia are used wherever appropriate. Animals will be closely monitored during all procedures and where possible we will consider refining existing techniques or incorporating new methods to minimise any suffering to the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Ullman-Cullere et al 1999 'A Rapid and Accurate Method for Assessing Health Status in Mice' Lab Animal Science; 49(3):319-323

Wilkinson et al 2019 'Progressing the care, husbandry and management of ageing mice used in scientific studies' Laboratory Animals 0(0):1-14.

Turner et al 2011 'Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider' J Am Assoc for Laboratory Animal Sci 50;600-613.

Workman et al 2010 'Guidance for the welfare and use of animals in cancer research' Br J Cancer 102;1555-157.

Diehl et al, 2001 'A good practice guide to the administration of substances and removal of blood, including routes and volumes' J. Appl. Toxicol. 21, 15–23.

'The Design of Animal Experiments: Reducing the use of animals in research through better experimental design' (Festing et al 2nd Ed).

We will also utilise online resources such as the PREPARE guidelines (https://norecopa.no/PREPARE), publications from the NC3Rs and the experimental design assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly attend relevant local meetings; liaise with our vet; read the latest relevant scientific literature; attend conferences and consult policies on animal research from our funders and other funding bodies. We will also use the NC3Rs website, newsletter and symposia to track advances in the 3Rs.

58. Investigating the Biology of Musculoskeletal Tissues in Health and Disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cartilage, short stature, osteoarthritis, osteoporosis, therapy

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to improve our understanding of the development, ageing and diseases of cartilage, bone, tendons and ligaments, and to find biomarkers (signals and proteins that can help to detect, diagnose and monitor disease) and test new therapeutic approaches for connective tissue disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Osteoarthritis (OA) is an age-related disease that causes joints to become painful and stiff. There are approximately 8.5 million people in the UK currently suffering from osteoarthritis which is becoming a heavy burden in the modern ageing society. OA can be exacerbated

by genetic influences, diet, injury and lifestyle, and as such, is a very complex disease to study. OA leads to progressive damage in the affected tissues that triggers repair responses similar to those seen in the developing embryo, however, these are not enough to counteract the disease progression. Osteoporosis (OP), an age- related condition leading to bone loss and fractures, affects 30-50% of women and 15-30% of men. Moreover, there are 450 individual genetic skeletal diseases (GSDs) that affect development of cartilage and bone. These range in severity from embryonically lethal to relatively mild, but even in the mild forms they dramatically impact on the patient's quality of life, leading to shorter bones and spines, bone malformation, joint pain and degeneration, mobility issues and disability. The GSDs often coincide with joint destruction through early onset osteoarthritis, and may present with other complications such as joint laxity and/or OP. Studying GSDs can yield important information about OA and OP susceptibility and progression and help tease out disease mechanisms important for skeletal maintenance and repair. Through this project, we propose to study healthy connective tissue (bone, cartilage, tendon, ligament, skeletal muscle) development to improve our understanding of development and ageing, and to use models of OA, OP and GSDs to find overarching disease mechanisms that could be the future therapeutic targets.

What outputs do you think you will see at the end of this project?

We are interested in development and aging of connective tissues, looking at the factors that are important for production of healthy functioning tissues and the ways to modulate them. We study changes in the components of the tissue that surround the cells, and their influence on health and disease. We also study changes that take place in connective tissues during development and how these tissues interact, forming a functional motor unit (for example and arm or a leg). Understanding the dynamic between various tissues is crucial for understanding how the human body works in health and disease and impacts on the physiotherapy regimes for the patients as well as on our understanding of other tissues. Several GSDs are associated with joint laxity, which is also an important complication in sports injuries and in healthy individuals, and it may lead to an aggravated OA and/or affect the overall bone mass and structure. We were the first group to show the limb weakness phenotype (an observable trait) in a GSD and successfully link it to the underlying tendon abnormality and the resulting early onset OA. Our models are ideal to study the disease progression and cause of GSDs, and understanding the biomechanical connections between the connective tissues may in future lead to better physiotherapies and management of GSD, OA and OP patients.

OA and OP are age-related diseases that are impacted by age, lifestyle and environmental factors as well as genetic susceptibility. This complexity makes them difficult diseases to study, however, therapeutic interventions for OA and OP are urgently needed in the modern ageing society. GSDs are individually rare diseases but collectively their frequency approaches that of the most common heritable disorders such as cystic fibrosis. GSDs are often associated with other disease complications, which are also found in healthy ageing population, for example osteoarthritis and osteoporosis. The frequency of osteoarthritis in the UK is extremely high and it represents a significant economic burden on the health service. Numbers of people suffering from OA are predicted to rise with increased longevity in the population and the disease is a major cause of decreased quality of life amongst the ageing population, whilst osteoporosis-related fractures will affect up to 50% of all women. There are currently no effective treatments for GSDs, OA or OP, and joint replacement and pain relief are the only treatment options. Our studies, continued from the previous licence and based on our expertise in OA, OP and GSDS, will highlight the function of specific molecules such as non-coding nucleic acids (microRNAs), components of the tissue that surround the cells (known as the extracellular matrix, or ECM),

mechanisms by which the cells sense their environment (mechanosensors) and other cartilage and bone proteins in tissue development. We will also identify biomarkers and sensitive read out assays to accurately monitor disease progression, these will ultimately enable better diagnosis and tailored treatment of the patients in the future. We will further characterise how mutations in proteins and microRNAs induce disease, define the genetic pathways and cellular mechanisms involved in disease progression and delineate whether pathway-targeted therapeutic strategies are capable of reducing disease severity. We have expertise in translating this type of preclinical data into successful clinical trials and we believe that work conducted under this licence will result in such outcomes as well.

Short term outcomes: publications, conference presentations, assay design, medical textbooks

Medium-long term outcomes: patent applications, diagnostic pipelines, clinical trials, new physiotherapy regimes, drug repurposing, musculoskeletal disease treatment

Who or what will benefit from these outputs, and how?

The results of this project will impact on the understanding of connective tissue biology and healthy ageing amongst the research and clinical community. They will also impact on patients' physiotherapy management, a better and shorter diagnostic journey thanks to the reliable biomarkers of specific disease, and novel therapeutic avenues that in the long term will lead to treatment of these currently untreatable conditions.

How will you look to maximise the outputs of this work?

The results of this study will be disseminated to the larger scientific community through presentations at national and international conferences, and publication in high impact factor open access journals. The investigators on this project are part of extensive national and international collaborative networks, and these links will also be used to disseminate the knowledge gained from this project, as well as for knowledge and skills transfer between many research centres in Europe. We will use our social media accounts and laboratory websites to publish soundbites about the project and we will engage our local press office in publishing any breakthroughs to the wider audiences. We have extensive links with patient organisations, ethical review bodies (AWERB, NC3Rs, UAR), and with clinical trials community, and we will use these links to engage with the patient and lay community, and to disseminate the results of our project.

Species and numbers of animals expected to be used

- Mice: 12,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the ideal model for our studies. Their skeletal system develops in a similar fashion to humans; they also develop osteoarthritis similar to that seen in humans with age, and in response to joint destabilisation in a relatively short (8-12 weeks) time frame;

and they are genetically tractable (we know the genetic code of the laboratory mouse strains and can engineer and track mutations that lead to specific conditions). Moreover, mice allow us to study the importance of individual cellular pathways and mechanisms in disease by crossing the disease models with mouse strains in which specific components of the cellular pathways have been deleted, and determining the effects of such deletion on the disease onset and severity. We also have all the relevant expertise, techniques and readouts in place to test treatment efficacy using mouse models in an efficient and time and resource-saving manner.

As we are studying the mechanisms implicated in the development and ageing of the skeletal tissues, we propose to use embryonic, juvenile and adult animals in our studies. Specifically, we will use mice from embryonic day 10.5 (onset of cartilage tissue) until 9 weeks of age (equivalent to human puberty) to study the role of proteins and the effect of mutations on long bone growth and development, and adult mice at 3 months to 18 months of age to study osteoarthritis and osteoporosis progression, both in injury-induced and age-induced manner.

Typically, what will be done to an animal used in your project?

Typically, an animal on this project will experience only mild to moderate severity. Specifically:

1) The disease-causing mutations and gene changes that we are investigating lead to short limbs but have no impact on the animal's activity or quality of life. In a bone development study, the mice will undergo three independent Xray imaging sessions under general anaesthesia with recovery, at 3, 6 and 9 weeks of age. The grip strength of some animals may be tested by allowing them to grip onto a bar and measuring the strength of the pull. Animals used to study the bone and cartilage dynamics will be labelled with a harmless labelling agent via intraperitoneal or subcutaneous injection, then humanely killed.

2) Tamoxifen injections may be used to delete genes specifically in adult tissues. All injection routes will cause momentary needle-stick pain. Intraperitoneal injection route has been chosen based on our previous results showing efficient delivery of injected substances to the avascular cartilage tissue within 2 hours of administration. Tamoxifen can have adverse effects on pregnant females and developing foetuses, therefore, only male and virgin female mice will be used in the experiments. Throughout the course of tamoxifen injections and any post-injection wait period, mice will be checked for any adverse reactions to the treatment.

3) The mice used in the study of therapeutic interventions will experience mild to moderate severity. Therapeutic agents will be delivered orally (via gavage, food or drink,; mild) or via subcutaneous/intraperitoneal (mild) or intraarticular/intravenous (moderate) injections, or subcutaneous implantation of drug pellets or mini pumps (moderate).

4) Osteoarthritis will be induced surgically via destabilisation of the medial meniscus (DMM), which is a crescent-shaped, cartilaginous band found between the shin bone (tibia) and a thigh bone (femur) that acts as a shock absorber and stabilizes the knee. This is a well-established and 100% reproducible procedure resulting in mild to moderate pain and joint similar to what is observed in humans. The DMM surgery will be performed under general anaesthesia with recovery on one of the animal’s hind-limbs and will result in mild post operative joint swelling and transient limping. Sham

procedure may be necessary for some of the surgical experiments as the progression of osteoarthritis may be affected by animal stress, use of anaesthetics, and the mechanisms involved in wound healing, especially inflammation. Unoperated control animals will be used, when possible, to minimize unnecessary procedures

5) Osteoarthritis will also be induced through ageing the animals up to 18 months (1.5 years) of age, equivalent to human 80 years of age, and will lead to mild to moderate pain and joint swelling, as well as some adverse effects of ageing (muscle weakness, obesity, fragility),

6) Ovariectomy (OVX, surgical removal of one or both ovaries) is the most commonly used and well- established method to induce osteoporosis, performed under general anaesthesia by a small incision on the side of the animal's body. After the wound has healed, the OVX procedure does not lead to any overt complications and does not affect the animal's quality of life; bone mass declines in the 4 weeks post operation. Sham procedure may be necessary for some of the surgical experiments as bone turnover may be affected by animal stress, use of anaesthetics, and the mechanisms involved in wound healing. Surgery also affects serum level hormones that may impact on bone metabolism. Unoperated control animals will be used, when possible, to minimize unnecessary procedures. The animals will be housed in a clean unit in individually ventilated cages with ample access to food and water and checked every day

What are the expected impacts and/or adverse effects for the animals during your project?

Areas of this project which may have adverse effects include:

1) Breeding of genetically modified lines: The mouse strains used in this licence have no overt skeletal phenotype or present with mildly shorter bones and in our experience exhibit no obvious pain or discomfort. Ear marking for the purpose of colony identification will be performed using the least invasive method (ear notching) causing only a momentary discomfort.

2) Behavioural testing: Grip strength testing should cause no discomfort. The animals will be habitualised to the grip tester; however, if the animal is experiencing stress, it will be returned to the cage and not used in the experiment.

3) Imaging: For the non-invasive imaging under general anaesthesia, animals may be imaged at 3 timepoints by Xray, once for a maximum of 5 minutes. Animals will never be re-anaesthetised until they have fully recovered from the previous anaesthetic, and the general anaesthesia sessions will be at least 48h apart. Sessions will be performed in a quiet procedure room, fresh bedding and cage enrichment will be provided after the procedure and animal's recovery will be closely monitored. Imaging may include the administration of well established tracer substances for which adverse reactions are not anticipated.

4) Injection of labelling substances and treatment: All injection routes will cause momentary needle- stick pain if they need to be given prior to the anaesthetic. In cases where multiple injections are given, to reduce stress animals will only be injected once per day. Alternative injection sites will be used to prevent repeated pain in one site. Discomfort and pain will be monitored by the monitoring the ability to carry out normal behaviours such as eating and drinking and managed by pain killers and cage

enrichment. Body weight and water consumption will be measured while undergoing daily injections. If the animal showed signs of distress, it will be excluded from the study and allowed to rest and recover in an enriched comfortable environment. Therapeutic compounds added to drinking water may change the water flavour and the animal's drinking habits - the volume of water consumed will be monitored over a three-day period to monitor hydration, sugar may be added to the water to make it more palatable. A small osmotic minipump or a drug pellet will be implanted subcutaneously where feasible in preference to multiple needle-stick administration. Minipumps or drug pellets will be implanted under general anaesthesia using an aseptic technique and analgesia will be used during the operation and post-surgery. Animals will be closely monitored after the surgery. In case of any adverse effects or discomfort the treatment will be discontinued, and the animals will be humanely killed. In the event a minipump or the drug pellet becomes re-exposed, it will be re-sutured .

5) Surgical induction of connective tissue disease: Most animals undergoing osteoarthritis induction will experience some inflammation (and possibly pain), and eventually arthritis, in their joint . Such effects may be mild or moderate depending on the duration of the study. After the procedure, the animal will develop a visibly swollen joint and minor impairment of movement (limping). The animals will be closely monitored for signs of pain and distress, andif required, pain caused by the onset of osteoarthritis will be controlled by the administration of appropriate analgesia administered as advised by the NVS. Age-related osteoarthritis may lead to joint stiffness, swelling and inflammation. These will be mild to moderate in severity and will be managed with appropriate analgesics .Ovariectomy would not result in any abnormal symptoms other than potential adverse effects associated with post operative wound healing. However, any animal showing adverse effects that cannot be relieved by appropriate treatment will be humanely killed. During observations of all animals, particular attention will be paid to respiratory rate, colour of mucous membranes, levels of normal cage movement and grooming and feeding behaviour. Post-operative pain will be controlled by the use of appropriate analgesics and monitoring of the animals immediately following the surgical procedure. Post operative score sheets will be generated for the purpose of close monitoring of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Sub-threshold 9,200

Mild 1,925

Moderate 875

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying the complex biological processes of long bone growth, bone remodelling, and articular cartilage degradation in osteoarthritis. Although we make extensive use of cell culture models and tissue engineering to study individual aspects of the process, the systemic processes of bone growth and homeostasis (a state of balance among all the body systems needed for the body to survive and function correctly) involve an integration of cell division, differentiation, cell death, vascular invasion, cartilage degradation, and bone degradation and deposition, and as such cannot be modelled in cells. Moreover, the pathobiology of GSDs and specifically the disease progression cannot be successfully studied using human samples due to the rarity of individual conditions, difficulty in obtaining relevant age-matched controls (healthy cartilage, bone and tendon/ligament biopsies) and the inability to successfully study the whole organism or create a replicable model of disease. Cartilage, bone and tendons are biomechanically challenged tissues, which are influenced by each other and other tissues in the system, such as the meniscus and the cruciate ligament. Therefore, studies of animal models are of importance in order to determine crucial factors in the biology of musculoskeletal conditions and to find potential therapies. Our previous cell and animal studies have already established that pathways that govern various types of cell stress are associated with defects in bone growth and

osteoarthritis, and we are now uniquely placed to conduct the animal studies necessary to further delineate the link between cell stress and these disease phenotypes. Furthermore, elucidating the specific disease mechanisms using transgenic animal models facilitates the discovery of translatable biomarkers circulating in the blood. These can then be applied in the clinic to stratify the patients, personalise the treatments and facilitate correct diagnosis.

Mice are the ideal model for our studies. Their skeletal system develops in a similar fashion to humans; they also develop osteoarthritis similar to that seen in humans in response to joint destabilisation in a relatively short (8 week) time frame, develop osteoporosis 4 weeks post-operation; and they are genetically tractable. The STR/ort mouse develops a naturally occurring osteoarthritis but because the disease is dependent upon the specific genetic background of this strain, it is not amenable to crossing with other lines to introduce new genetic alleles and was therefore rejected as a model. We have considered various models for inducing OA by sectioning various ligaments but the DMM model is the most reliable in terms of consistency of disease induction and is now the accepted model in the field.

We have previously generated genetic models of specific human GSDs through gene targeting and transgenesis. Moreover, we can genetically dissect the importance of various pathways in disease pathogenesis by crossing with other genetic strains harbouring knockouts of specified stress-related genes and determining the effects on disease onset and severity. We also have all the relevant techniques and readouts in place to test treatment efficacy using mouse models in an efficient and time and resource-saving manner.

Which non-animal alternatives did you consider for use in this project?

We make extensive use of cell culture models to study individual aspects of the effect the mutations are having on cell biology. We also use them to verify novel genetic variants of

disease and test the efficacy of potential treatments. We are involved in several national tissue engineering groups and centres of excellence and we have an NC3Rs funded project looking at harnessing the novel discoveries in tissue engineering to replace the use of animals in skeletal research. We constantly use the advances of these projects to reduce the number of animals used in our studies and to replace animal use where possible.

Why were they not suitable?

Whilst deleting genes in cells is relatively easy, engineering specific mutations is not, and often leads to changes in gene expression levels that are different from what is seen in the relevant patient tissues (i.e. overexpression). Many cells in the adult connective tissues have a low regenerative potential which means obtaining samples from patients and healthy controls through biopsies is near impossible and tissues can only be obtained at end stages of disease during total joint replacement. GSDs are relatively rare and access to patients is often limited. Finding healthy age matched controls is also difficult. Moreover, bone growth is a systemic process that requires input and interaction from many surrounding tissues, something that is extremely difficult to model in a cell culture dish. Connective tissue cells are highly responsive to mechanical stress and require gravity, compression and stretching, as well as a three-dimensional tissue environment to develop properly. At the moment, mouse is the closest organisms that allows us to model bone and cartilage development with accuracy maximising the results that we can obtain and accelerating the development of treatments and interventions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In order to obtain reliable, reproducible and statistically relevant data we have revised our previous studies and consulted a statistician. We have twenty years of expertise of generating and phenotyping animal models of musculoskeletal disease. In that time, we have developed efficient and standardised methods that allow us to obtain maximum information and reduce the number of animals used. We are therefore confident that the number of animals in this application is correct and the minimum for achieving the relevant goals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have made sure that our experimental design means that we are obtaining relevant and reproducible information from all samples. All of our studies are performed using inbred genetically modified mouse strains to reduce variation. For quantitative aspects of the programme, age and sex- matched (both male and female, apart from the OVX models) animals are used. Where feasible, the animals are littermates to reduce variation. Moreover, repeated imaging of the same animal under anaesthesia is used to collect bone growth data. This reduces the numbers of animals needed to quantitate bone growth. For

qualitative aspects, defined breeding pairs (where all progeny have the desired genotype) are employed to reduce numbers of animals with unwanted genotypes. All experimental outcomes pertaining to individual animals (e.g. genotype, sex, pedigree, body weights, bone lengths etc) are deposited in our electronic database (AniBio) devised to archive and analyse such data, and backed up in separate independent spreadsheets. Good data management practice leads to further reduction in animal numbers. Lines that are not required in next 6 months are frozen down (sperm freezing preferred where possible to reduce numbers of animals used) rather than maintained on as minimal colony. The most reliable and reproducible mechanically-induced model of OA (DMM) and the most reproducible model of OP (OVX) have been selected to minimise experimental variation in onset and severity of disease.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We expect to analyse up to 12 mouse lines during the lifetime of this licence. The design of individual experiments will generally involve factorial designs, which allow researchers to look at how multiple factors affect a dependent variable, both independently and together, and maximise the information obtained from the minimum resource. In planning our experiments, we have applied the results of our twenty years of experience of deep phentyping mouse models of skeletal conditions, including efficient

breeding of GA colonies, and ensuring that maximum data will be obtained from the collected tissues/animals. Some of the measures and read-outs are essentially qualitative. For qualitative experiments, the number of observations will be the minimum necessary to provide an adequate description (normally single or multiple samples from 3 animals per group). Other measures are quantitative and can be evaluated statistically. To generate statistically robust data, mathematical modelling (power calculations) have been performed to decide the optimal number of samples per experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this licence we will use mouse models harbouring mutations and/or deletions in extracellular matrix proteins, in proteins in primary cilia, which is a structure that the cells use to detect the matrix proteins and in microRNAs that regulate the production of matrix proteins. We will also use models in which components of the cell stress pathways have been deleted in order to understand the contribution of the pathway to the disease phenotype. We will use recombinase systems that are molecular tools that allow us to orchestrate tissue specific deletion of genes, reporter lines to verify the gene expression, and inducible systems to be able to orchestrate gene deletion in adult tissues only. All of our animal lines have a sub-threshold to mild severity, and that the mutation does not impact on the quality of life of the animal.

GSDs and osteoarthritis (naturally occurring or surgically induced) can also be studied in dogs. We rejected this model due to the lack of knowledge concerning genetic causes of canine GSDs and the extended timescale necessary for these types of study. Hartley guinea pigs develop spontaneous osteoarthritis and are a frequently used model of OA, especially for histological characterisation of disease progression and to test different treatments. However, not much is known about the genetic causes of spontaneous OA in guinea pigs and genetic manipulation of these animals is not possible at present. Ovariectomy has also been performed in sheep, rats and monkeys order to study osteoporosis. However, these studies require extended timescales, and genetic manipulation of these animals is impossible, or costly and unethical.

We have chosen DMM as the method to induce OA as it is the most established and consistent surgical method to model injury-related OA. It results in mostly mild discomfort to the animal and a mild to moderate disease develops in 8-12 weeks post-surgery with 100% incidence, thus reducing variability. OA can also be induced by anterior cruciate ligament transection (ACLT), or chemically induced by injecting monosodium iodoacetate (MIA) or collagenase into the knee joint. However, since MIA is a metabolic poison (which prevents enzymes from performing their necessary functions), cell death of the chondrocytes responsible for the production of collagen and the extracellular matrix that aid the maintenance of tissues within joints in this model is extensive, unlike in human OA, whilst

collagenase injections result in pronounced and accelerated joint degeneration (within 3 weeks post injection) that does not accurately mimic human disease progression. ACLT surgery requires more precision than the DMM and thus results in higher variability. We have over 5 years of expertise in the DMM surgery in our mouse models, therefore we have chosen DMM as the most reproducible and least painful method to induce OA.

OP can be induced by OVX surgery, unloading, or glucocorticoid administration. Among the rodents, rat is frequently used to examine the effects of glucocorticoid in bone. However, the severity of OP may vary depending on the age and dosage and the duration of glucocorticoid treatment. Several groups have shown the effectiveness of the OVX surgery to induce OP, making it the most established and reproducible OP model. Ovariectomy does not result in any abnormal symptoms, and has been reported in numerous studies to have minimal impact on the animal well-being. Osteoporosis can also be induced by joint unloading via tail suspension or microgravity simulation, however, these are likely to cause more stress and discomfort to the animal and have a significant impact on the general health and well-being, and were therefore excluded.

Methods to be used in this project include:

In vivo

- Generation of growth curves (by taking weight measurements; sub-threshold)
- Bone measurement using X-ray imaging under anaesthesia with recovery (multiple occasions per animal, allowing for longitudinal bone growth study and reducing the number of animals used in the experiments, mild)
- Grip strength measurements (by allowing animals to grip a grid attached to a force meter, upon habituation, sub-threshold)

- Destabilisation of the medial meniscus (DMM) induction of osteoarthritis (mild (60%) to moderate (40%))
- Ovariectromy (OVX) surgery (moderate)
- Injection with labelling substances and/or therapeutic solutions (mild to moderate)
- Gavage administration of therapeutic treatments (mild)
- Delivery of drugs via subcutaneous minipumps and pellets (moderate) Ex vivo
- X-rays taken after sacrifice
- Histomorphometric analysis of bone deposition and uCT analysis of bone parameters to corroborate and validate the Xray data
- CTX measurement of bone metabolism rates
- Biomechanical studies of extracted tissues: tensile testing of tissues
- Histological analyses of integrity of tissues, protein expression and localisation, cell division and cell death rates
- Gene expression and localisation: in situ hybridisation, qPCR and microarray analyses and proteomic studies: protein extraction and mass spectrometry analysis (allowing delineation of specific disease mechanisms that can then be further validated in cell culture and tissue engineered constructs thus leading to reduction of animals use)
- Biomarker detection in blood: Western blotting, ELISA, metabolomics (from blood extracted by cardiac puncture under terminal anaesthesia)

Why can’t you use animals that are less sentient?

Our work investigates the development of cartilage, bone and tendons, and as such, is restricted to the study of vertebrates. Certain aspects of cartilage development can be studied in zebrafish. However, not all human genes have their equivalents in zebrafish. Moreover, some mammalian microRNAs are not present in the zebrafish genome, and the mode of locomotion of zebrafish differs dramatically from that of mice and humans, meaning that the impact of mechanic stimulus on joint degeneration cannot be studied reliably in this model. Adult zebrafish skeleton contains less canaliculi (small canals running through the bone) compared to mice and humans, making studies of bone development difficult.

Moreover, whilst gene silencing and deletion are possible in zebrafish, engineering specific disease- causing mutations to model human disease remains problematic.

We aim to study development of skeletal disease from immature cartilage to ossification all the way through to joint degeneration and disease. These stages cannot be studied in immature life forms and require the full life span of the animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The mice will be checked every day by the Animal facility staff and by one of our researchers. All the procedure rooms ensure aseptic technique and are quiet and individually booked ensuring that the animals are not unnecessarily stressed throughout the procedure. We will use post-operative care score sheets to monitor the animals that have undergone surgery, and the animals will be held in a quiet room with gently heated cages to recover. NVS will be consulted for the best post-operative care and we will use analgesics to minimise pain and antibiotics to minimise infection, if necessary. Mash and easily accessible food will be provided to the animals with growth issues and tooth malocclusions. For grip testing the animals will be habituated to the device before testing in order to minimise stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All administration volumes and methods have been decided upon following consultation with the NVS and according to the NC3RS guidelines. The AWERB and the Animal Faculty organise regular researcher meetings and seminar series to disseminate the latest developments in the 3Rs, these will be attended by the members of this project and the guidance provided will be used to further refine the methods on this projects. The 3R responsibilities of the licence holder (Standard condition 4) and the personal licence holders (Standard condition 1), as described in the Animals (Scientific Procedures) Act 1986, will be adhered to. All the competency records are kept to date and will be refreshed and updated when required.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The main applicant attends regular Animal Facility researcher meetings, Animal Welfare and Ethical Review Body meetings and 3R seminars, and follows the NC3Rs news via Twitter and email newsletter. We also hold regular group meetings with the Colony Manager, Named Animal Care and Welfare Officers (NACWO) and the veterinary team (NVS). Before starting an experiment we submit a detailed study plan to the NACWO and NVS and discuss animal wellbeing and refinement procedures that could be implemented to further improve the technique and the animal experience.

59. Investigation onto the Role of Glucocerebrosidase Mutations in Parkinson Disease Mechanism and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Parkinson disease (PD), glucocerebrosidase (GBA), brain, gut, alpha synuclein (SNCA)

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the disease mechanism and to assess novel therapies of parkinson disease (PD) caused by mutations of glucocerebrosidase (GBA) and associated factors, with focus on the influence of GBA mutations on the gut microbiome and the spread of pathogenic alpha synuclein (SNCA) through the gut-brain axis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parkinson disease (PD) is the most common movement disorder. Global estimates in 2019 showed over 8.5 million individuals with PD. The cause for PD is not known. Currently there is no cure and, medicine, surgical treatment and other therapies only treat the symptoms and do nothing to slow or halt progression of the disease.

Two major breakthroughs in PD investigation in the last decade are the discovery that glucocerebrosidase (GBA) mutations are present in 5-15% of PD patients, and approximately 30% of those with GBA mutations develop PD. Recently, the gut microbiome has been implicated in the generation and spread of alpha synuclein (SNCA) pathology and PD pathogenesis is thought to occur through the gut-brain axis.

It is important to undertake this work considering it is addressing key issues as described above, in an intact higher organism. We also want to translate our findings to potential therapy.

What outputs do you think you will see at the end of this project?

1) The outcome of our previous PPL was the completion of a phase 2 trial of a repurposed drug based on data from our animal works that has led to the launch of the upcoming phase 3 trial. Biochemical and cellular experiments are being carried out to identify new drugs that can modify GBA activity and these will need to be characterised in vivo using mouse models of PD and mice with GBA mutations.

2) The new project will provide new information about the role GBA mutations play in contributing to the development of PD especially with regard to the gut microbiome and will further understanding of the role played by the gut-brain axis in the etiology and treatment of PD, which will identify new therapeutic targets that can be translated into new treatments.

3) Post-mortem tissues have been collected since we started this ongoing project and more are required to enable a thorough investigation of the pathology observed in GBA mutant mice and the cellular response to novel treatments.

Who or what will benefit from these outputs, and how?

1) Ultimately patients with PD will benefit from this work. Understanding mechanisms of disease will lead to the discovery of new therapeutic compounds within the time scale of this new PPL ie 5 years. These can be translated into new clinical trials within 5-10 years.

2) The scientific community as a whole and hence other researchers will benefit throughout the project as we publish our findings.

3) The post-mortem tissue bank will benefit future projects and our collaborators.

How will you look to maximise the outputs of this work?

This project is partly funded by an international agent emphasising collaboration, therefore we are obliged to share our experience and findings through the collaborative network in the scheduled meetings. In those meetings many of us present pilot and unpublished data, some of which may be negative data.

Part of the grant requirements is to share materials within the collaborative network, in this case the post-mortem tissues collected and archived.

Species and numbers of animals expected to be used

- Mice: 17700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using GA mice, the majority of them are young stage ie up to 15 months of age. We are also using embryonic and new born mice to set up tissue cultures. Only when necessary and scientifically justifiable will we use mice older than 15 months but no older than 24 months.

The mouse is the best choice of higher organism for use to study GBA mutations because of the wealth of research tools and experience of using mice for scientific research and because all the GA mice used in this project exist. Consequently, we do not need to create new GA strains for use in this project other than intercrossing existing strains. As a higher organism, mice have the brain and gut structure close to that found in humans. It is necessary to conduct our study in an intact higher organism with the gut-brain axis similar to that of human.

Typically, what will be done to an animal used in your project?

Typically, a GA animal carrying gene mutation(s) found in PD patients will be born and a small tissue biopsy will be collected for genotyping when the animal is weaned. Mice of unwanted genotypes and in excess of requirements shall be killed, only the mice required for breeding to maintain the strain and for experiments shall be maintained for up to 15 months of age. Unborn (and dam) or new born may be killed to collect post-mortem tissues to set up tissue cultures. Only a limited number of mice shall be maintained beyond 15 months old to model aging until they reach 22 months of age, rarely the max of 24 months and be killed.

Experiments shall be carried out in mice mostly from 3 months old and ended by their 15 months of age. Mild behavioural tests and blood sampling may be conducted on some of the mice in order to characterise disease phenotypes and biochemistry. The majority of mice will be killed and post-mortem tissue removed. One type of surgery shall be performed in a very small number of them (max 400) once in lifetime in order to introduce a pathogenic protein into the brain or the guts. One course of substances treatment may be administered by either the parental or the non-parental route either before or after the introduction of the pathogenic protein again once in lifetime to a limited number of animals. Finally, mice will be killed. Animals will not be re-used.

What are the expected impacts and/or adverse effects for the animals during your project?

Majority of the GA animals should not experience pain, discomfort or distress. Only a very small number of animals undergoing surgery and recovering shall experience pain, discomfort or distress during that period of no longer than 2 weeks and they shall be given anaesthesia and analgesia and put under post-op care.

Progressive PD symptoms are expected to develop including loss in general activity, coordination and gait and these shall be characterised by mild behavioural tests.

Aging animal represents a distress therefore we shall only maintain animal beyond 15 months of age and as short duration as possible as experimentally required. We shall avoid single caging and sufficient enrichment and nesting will be provided to make their lives as comfortable as possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The general expected severity is mild (representing 88.1%). Only a very small number of animals undergoing surgery (max 400), and when they are aged (max 1700), shall experience moderate severity (representing 11.9%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We aim at characterising the role of GBA mutations in developing PD by use of mice with GBA mutations that are found in patients. We will focus on how the mutations influence the gut microbiome and the spread of abnormal SNCA through the gut-brain axis. Relevant studies at biochemical and cellular levels have already been carried out; our progress is at the stage necessary to further our works in intact higher organism with a similar gut-brain structure as human.

Data generated from non-protected animal alternatives is useful but limited in translating into clinical studies especially for PD considering the efficacy and dosing of therapeutic substances.

Which non-animal alternatives did you consider for use in this project?

Cell culture and stem cell derived organoids that we have experience on and are using for biochemical and cellular analyses.

Why were they not suitable?

They are helpful in generating some data but not the type of data related to connectivity between the gut and brain, nor do they model the complex structure of the brain (ie the blood-brain barrier is known to prevent substances crossing into the brain) and the gut or the influence of the microbiome on the gut-brain axis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is based on the estimation against the actual use of the precious licences and taking into account the addition of 2 more strains in this application.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We shall seek support from professional statistical support services and EDA (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) on study design and sample size calculations (EDA results available upon requests) in order to reduce the number of animals being used in this project.

Where relevant, factorial experimental designs will be used, rather than the one-thing-at-a-time approach, to maximise the information obtained from the minimum resource. For most of the quantitative experiments, sample sizes may be set using power analysis, generally using a significance level of 5%, a power of 80%, and a least practicable difference between groups of 25%. Otherwise, we will use our previous experience, or from the literature, to select sample sizes. In terms of the numbers of animals required, we expect that 6 to 8 animals per treatment group should be sufficient to provide sufficient power to the experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Good husbandry practice will be followed to reduce breeding of GAA for maintenance.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Existing monogenic GA mice presenting with no pain, suffering and distress shall be bred and maintained, biopsy and samples collected, and killed before 15 months of age. Only if absolutely necessary, animals will only be aged beyond 15 months, and rarely to 24 months.

Digenic GA mice shall also be bred. They too are unlikely to suffer from pain and distress considering some cases of digenic mutations are found in humans and their clinical presentation is similar to those of monogenic.

Mild behavioural tests may be conducted in order to assess neurological abnormalities. Blood samples may be taken for biochemical analysis.

As needs, once in a life time surgery will be performed on a minimal, small number of animals to produce the more pronounced model of SNCA accumulation/spread and they will experience moderate pain, suffering and distress which will be minimised by the use of analgesics.

To test for therapy, carefully chosen known and non-toxic substances will be administered once in a life time through non-surgical methods to target the disease pathways. Inclusion in food /water represents our preferred choice as animals are handled less frequently therefore less restraint.

Why can’t you use animals that are less sentient?

Mice represents the type of experimental model that has a sufficiently close physiology resemble humans and they are scientifically well characterised with a wealth of supportive tools available. Less sentient animals such as zebra fish and C elegans are too far as models for assessing parkinson disease etiology and modification particularly when investigating the effects of the gut microbiome on brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are opened to further refinements of our procedures in order to minimise harm to the animals. Described in this application, most of the procedures are fairly refined e.g. animal experiencing moderate severity after surgery/during recovery will be under close observation, pain management and post-op care.

The administration/sampling volumes are checked and they comply with current good practice guidelines (see: Laboratory Animals (2001) 35 1-41).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The PREPARE Guidelines (https://norecopa.no/prepare)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I constantly receive news from N3Rs and have taken part in their workshops and I will continue to do so.

60. Linking Seabird Behaviour to their Environment via Diet and Health Status

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Protection of the natural environment in the interests of the health or welfare of man or animals
- Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

diet, renewable energy, environmental change, health, foraging

Animal types	Life stages
European shag	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We would like to find out more about what seabirds eat and their health status so that we can better understand how they interact with their local environment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The natural environment is changing as a result of human activities and we know that more change is coming. If we want to protect natural populations of animals from the results of these changes, then we need to know more about how animals use the environment around them and what the consequences are of the decisions they make.

Seabirds are the world's most threatened group of birds because they are affected by a range of different pressures from human activities. These include predators that eat them, disease, competition with fishing activities and climate change. Ironically, they are also threatened by the presence of offshore windfarms, one of the strategies being used to slow down climate change.

Seabirds are also protected by law, which is helping to avert these population declines, but there is real pressure to increase the number of offshore windfarms to not only slow down climate change, but to improve energy security. Therefore, a major research goal at the moment is to try and work out how exactly future installations will affect where seabirds can go and what they might eat. We also need to do this considering what we know about the future climate and how changed weather patterns will also affect where seabirds can go, what they might eat and how this affects their health. Yet at the moment, we still know very little about these things as seabirds and the environments that they live in can be very difficult to study.

Thankfully simple techniques now exist to help us work out what seabirds eat and their health status. By understanding more about where birds go, what they eat and how this links to their individual health and to healthy populations, we can work out how and if they will be able to deal with a changed environment and provide advice to the organisations who are involved with new windfarm installation.

What outputs do you think you will see at the end of this project?

We will generate new information on what seabirds eat, their health and how that is related to where they get their food. This will better help us understand how they use their current environment. This in turn can help us understand how seabirds will manage in future environments that might be different due to global climate change or the installation of offshore windfarms. We will share this information via peer-reviewed publications in the first place.

Who or what will benefit from these outputs, and how?

The impact of the benefits will come at the end of the project when we can advise audiences beyond just the readership of our research papers. These broader audiences involve government departments and statutory nature conservation bodies, interested charities and private sector organisations such as windfarm developers and their representative groups. These organisations can all use the information to better understand the future of seabird populations. This will help them to plan where and when to place offshore windfarms and/or take other mitigation and compensation actions to help these birds.

How will you look to maximise the outputs of this work?

We have direct contact via our research funders to interested and relevant stakeholders such as those listed above. We are working with the funders to not only provide the information to these stakeholders, but in a way that is accessible to and usable by them. This process will ensure that our findings get to the people who need them the most and in way in which they can use them.

Species and numbers of animals expected to be used

Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Seabirds are the world's most threatened group of birds and we can only understand how we can help them by studying them in their natural environment. Our project focusses on adult breeding seabirds, during the breeding season, as this a period when they are highly vulnerable to change within the areas that are close to their nests and the chicks that they must provide food for.

Typically, what will be done to an animal used in your project?

Typically, we will capture breeding adult seabirds and equip them with a tracking device that records where they are feeding and other information about their behaviour such as how deep they can dive to. The device is attached to the lower back with tape and will fall off after a few weeks if the birds cannot be recaptured. However, we do recapture most birds at which point, the tracking device will be removed. We will then take a blood sample and/or an oral swab and/or a cloacal swab and/or a feather sample and/or have a brief search for ticks in the plumage. Some birds may be sampled in this way without first having a data logger attached. Any individual bird will only be sampled once in a year, but may be resampled in subsequent years in the same way. The birds are removed briefly from the nesting area to obtain these samples after which they are returned to close to their nest and released back in the wild, a process that typically takes less than ten minutes in total. In all these procedures, we expect that birds will experience short-term mild discomfort, but that there will be no long-term impacts of these procedures. Back in the lab we will use a variety of different techniques to find out what the birds have been eating and/or their health status in terms of stress, parasites, pollutants and infectious diseases like Avian Influenza (especially given the current outbreak), or other diseases that can be carried by ticks such as Great Island virus.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect no adverse effects. Occasionally a haematoma may form, but this is of little consequence. An extreme reaction to handling could lead to abnormal behaviour such as prolonged absence from the nest. This might have consequences for the current breeding attempt, but no long-term influence on the welfare or survival of the bird.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is mild in every case as the protocol is equal to or just exceeds the threshold for regulation.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand what seabirds eat, their health status and how those things depend on the environment they live in, the only option is to work on wild birds living in their natural environment.

Which non-animal alternatives did you consider for use in this project?

There is no non-animal alternative for studying wild animals.

Why were they not suitable?

See above. There is simply no suitable alternative when we want to understand more about natural populations and their interactions with the environment they live in.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We know from our earlier research how many seabirds we need to sample in order to capture where they go to feed. We also know that male and female birds can differ in where they go and what they eat and that sometimes these things can change between years. Our sample sizes are consistent with our studies in previous years under non-licenced procedures and take all of these factors into account so that we can understand what is happening with the whole population of each species.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We looked back at the data we have collected before about movement and checked how many seabirds we need to use so that we can describe the majority of feeding areas that they use around where they live.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each year we will see whether the areas used and food eaten by the birds are the same or different to previous years. If it becomes clear that there is not much variation between years then we will not need to keep sampling birds for following years. Each time we take a sample from a bird, we will ensure that the sample is stored and transported appropriately and where we can, use the sample for different objectives to make our sampling as efficient as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are studying wild populations of seabirds that are part of ongoing observational studies as they live in the natural environment around them. We regularly capture these birds to attach identification rings and/or tracking devices. In this project we will take samples from the birds to find out more about what they eat and their health status. The blood samples, mouth swabs and cloacal swabs are the least invasive techniques that exist to find out this information.

Why can’t you use animals that are less sentient?

We are studying how mature adult seabirds use the environment around them so cannot use less sentient species or life stages. The samples we propose are a very mild inconvenience to the birds. Terminally anaesthetising wild birds would be very hard to justify and we would not get permission from the relevant authorities in any case.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will refine the procedures that we undertake on the birds to ensure that any welfare harms are minimal, including minimising the length of time a bird is held, only undertaking procedures that are central to the research objectives, ensuring that all work is undertaken by experienced researchers, and returning the bird as soon as possible so that it can revert to its normal behaviour as a wild, free- living individual.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow advice from the NCRs on blood sampling, with modifications appropriate for birds (https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-general-principles). 'The Veterinary Nurse' includes detailed guidance on best practice for avian blood sampling.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project team are active members of the seabird research community, including via membership of The Seabird Group and various social media channels. The Seabird Group has a regular newsletter that would include relevant updates in regard to the 3Rs. The Project Leader receives the RSPCA's Animals in Science Newsletter which includes features on studies of wild animals similar to ours that would include updates on pertinent developments. Finally, our project team includes two academic veterinary surgeons who will be well-placed to advise on the 3Rs for our protocols.

61. Neural Activity for Learning and Executing Movement

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain, activity, learning, behavior, motor control

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The major goal of this project is to define a relationship between brain activity and behaviour. Specifically, it seeks to determine how brain structures work together to generate movement-relevant activity, and it will examine how this activity is shaped by learning new behaviours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our work is part of a global effort to decode brain activity. This research is central to understanding human behaviour, which is entirely dictated by the brain. Once we have discovered how brain activity translates into movement, we will have a more enlightened perspective on why and how people do the things that they do.

Determining why behaviour happens will have both personal and societal impacts. For example, the basal ganglia, one target region of our research, is thought to control the gradient from wilful to habitual movement. We have already transformed our interpretation of addiction as an extreme end of this system, which has replaced stigma with treatment.

Similarly, we can interpret and guide our own behaviour better once we know how the brain transitions between motivated actions and unconscious repetition.

Determining how behaviour happens will have every-day and interventional implications. For example, the same mechanisms likely underlie both normal skill learning and recovery from stroke. When we have identified the processes by which the brain learns and executes movements, we can hone the learning process both in a normal and abnormal context.

The findings from our research will also apply to future development of disease treatments. Movement disorders are prevalent and highly varied, ranging from an inability to move after stroke, to difficulty initiating movements with Parkinson's disease, to difficulty inhibiting movements with Huntington's disease. Since we do not have a firm understanding of how the healthy brain directs movements, we do not have biologically defined targets for treatment options. Our research works towards this goal by building fundamental knowledge of how brain activity leads to movement.

What outputs do you think you will see at the end of this project?

The primary output of this research will be contribution of knowledge through peer-reviewed journal publications. We hope to work towards three main discovery milestones.

First, we aim to generate causal descriptions of how activity in the brain leads to movement. This goal works towards a "translation" from activity to behaviour, which will allow us to interpret how neuronal activity is ultimately converted into movement. Despite being a fundamental aspect of the brain, this type of translation remains undiscovered.

Second, we aim to characterize brain activity as interactions across brain regions. Research on brain function has been largely piecemeal, with investigations focused studying on single regions at a time. We hope to draw a more holistic picture of brain activity by studying activity across multiple regions simultaneously.

Third, we aim to uncover how brain activity changes to support learning. By determining what structures are involved in learning, how they modify their activity, and how that activity can dynamically drive movements, we will strive towards a comprehensive understanding of the neural mechanisms of learning.

Who or what will benefit from these outputs, and how?

In the short term, we expect our research to primarily benefit the scientific community towards developing fundamental knowledge of the brain. However, we also believe that our work will be of interest to the general public. Neuroscience has become a popular subject of books, television, podcasts, and other media, and has emerged as an avenue for people to better understand themselves and others. As we make discoveries about the origins of behaviour, we hope to contribute to public engagement in neuroscience.

In the longer term, we envision our work benefiting patients by creating targets for novel treatments. Because we focus on general principles of the brain and the interaction between brain regions, we hope this aspect will be relatively wide-reaching. In other words, we hope to benefit future treatment of specific diseases (like Parkinson's disease, which originates in the basal ganglia), physical or emotional damage (like stroke or trauma), and normal processes like age-related motor decline.

How will you look to maximise the outputs of this work?

We are planning collaborations through shared resources, experiments, and publications. This collaborative approach is likely to produce more published science than our labs would otherwise generate individually.

In addition to research publications, we will also disseminate and contextualize our work with review articles and conference presentations.

We also publicly release and document all code and data upon publication, which is available for download without the need for requests or restrictions. We expect that our work will produce a large amount of data which could contribute to many different scientific questions, and therefore could be used indiscriminately by the larger neuroscience community.

Species and numbers of animals expected to be used

- Mice: 6200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most commonly studied animal in neuroscience due to their rapid breeding and maturation, well-established husbandry and infrastructure, genetic control, and standard mammalian brain plan. Prior work has demonstrated readily translatable properties between the brains of mice and all other mammals, so it is most efficient for resources and research impact to use mice.

We use adult mice since we are interested in studying behaviour and learning in the adult stage of life.

Typically, what will be done to an animal used in your project?

Mice will undergo surgery to implant a small head post to allow them to be restrained in a stable position for recording in future experiments.

Depending on the needs of the scientific question, we may create a transparent window on top of the skull or in the skull so that mice can undergo non-invasive imaging. This will be performed during the initial surgery to implant the head post.

Alternatively, we may create a small hole in the skull to allow direct access to the brain so that we can administer substances or insert very fine electrodes. This will be performed in the initial surgery to implant the head post if we aim to record neuronal activity at the start of the experiment. Otherwise, if mice require behavioural training before recording, this will be done during a second surgery.

After 5 or more days of recovery, mice will be habituated to consuming their daily water at a controlled time rather than from a water bottle in their home cage. They will also be habituated to handling and head-fixing in the rig for short durations.

From the point of surgery or water control, mice may need to be singly housed if co-housing is not possible. For example, some mice will have implants that can be chewed or damaged by a cage mate, or only one mouse in a given cage may be selected for water control experiments and cannot be co- housed with mice that have free access to water.

Mice will then be trained daily in simple head-fixed behavioural tasks, for example turning a wheel with their forepaws in response to a visual stimulus to receive a water reward. During this training, brain activity will be recorded or manipulated. Each training session typically lasts 1-2 hours and takes place across 2-6 weeks.

The daily water amount for each mouse will be calculated based on weight, which will either be attained in full during task performance or topped up after training. The weight of all mice will be monitored daily while they are on water control, with water amounts being adjusted as necessary to keep the weight within a target percentage of their starting weight.

At the end of a series of experiments typically lasting between 3 weeks and 2 months, the mouse will be killed.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will experience post-operative pain.

Mice on water control typically lose 10-15% of their baseline body weight, which will last for the duration of the water control period (typically 2-6 weeks). Some mice may lose 20% of their baseline body weight, and these will be closely monitored for signs of dehydration. Mice will be killed if weight loss reaches 25%.

Some drugs may cause temporary weight loss or minor abnormal motor function (e.g. increased propensity to turn in one direction).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 40% Subthreshold, 40% Mild, 20% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our research seeks to understand the link between brain activity and behaviour. To accomplish this goal, we need to study the brains of behaving animals. This work cannot be carried out on computer simulations or using in vitro preparations because they are too far removed from normal brains to provide insight into the mechanisms of behaviour. We also cannot carry out this work on humans because human neuronal activity cannot be measured with sufficient precision. Finally, to discover principles of brain function which are applicable to humans, our research uses mice as a mammalian model system.

Which non-animal alternatives did you consider for use in this project?

We will use computer models to help us interpret animal data and generate testable hypotheses for animal experiments, though these complement rather than replace animal use.

Why were they not suitable?

Computer models of the brain are limited by our current understanding of brain anatomy and physiology. Because there is so much still unknown about the brain, these models are not yet sophisticated enough to produce novel understanding about the brain.

For example, in one of the neural circuits of interest for this project, predictions from our current knowledge suggest that two particular areas should have opposite patterns of activity. However, these areas show similar activity patterns, indicating that we have not yet discovered a critical aspect of the biological system.

Instead, computer models are used to enhance our research by going back and forth from experiments to modelling. As an example, mouse behavioural data can be recapitulated with a computer model, and then we can test whether components of that model are present and necessary in the actual brain for the given behaviour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice is based on actual values from other laboratories performing similar experiments. It represents the estimated number of experimental mice necessary to generate statistically significant results, taking into account the expected fraction of transgenic-positive mice.

We have also conducted literature searches to confirm the typical number of mice necessary to achieve statistically significant results in these types of experiments.

We typically use about 10 mice per group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?
We have identified reliable genetically altered strains for our work, and we will perform routine literature searches to identify new strains that could further improve the data that can be collected from individual animals.

We have consulted the NC3R's Experimental Design Assistant as a tool for planning our experiments.

The use of cutting-edge techniques to perform large-scale recordings is also dramatically reducing animal numbers. For example, rather than recording from three areas across three mice, we can now record from three areas simultaneously in a single mouse.

Where possible, single mice are also used for multiple experiments. For example, where we seek to record and manipulate activity, the same mice can be used first for recording and then for manipulation.

We also can optimize multiple approaches in single mice (e.g. surgery strategies, task variants), which means fewer mice will be needed to reach an experimental standard to produce publication-quality data.

Finally, we have streamlined the tasks which we train mice to perform to be as simple as possible while retaining the components we need to answer our scientific questions. Up to 50% of mice do not learn more complex tasks, while nearly 100% of mice learn our simplified tasks.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use both genetically altered and wild-type mice in this project. The genetically altered lines are used to target modifications to specific cells and to express proteins to report or manipulate activity.

The mutations in the genetically altered mice are harmless and provides the same quality of life as wild-type mice. Importantly, these genetically altered lines reduce or eliminate the need for procedures previously used to conduct these types of experiments (e.g. virus injections).

We use water control as a method to motivate mice to perform tasks because it has proven to be both minimally distressful to the mice and effective for behavioural performance. Mice typically tolerate water control with no adverse effects, and body weight provides a robust measure of health before any more serious signs of dehydration are observed (e.g. hunched posture or piloerection). In this way, we can increase the amount of water for individual mice as and when necessary to prevent adverse effects.

Why can’t you use animals that are less sentient?

Our research is focused on understanding mechanisms behind learning and behaviour in the adult brain, which can only be achieved by studying behaving adult animals. Since we are specifically investigating the properties of the mammalian brain, our research requires mammals.

We are unable to use terminally anesthetised mice, because our scientific goal of understanding the link between brain activity and behaviour requires an actively behaving animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We closely monitor the health of our mice on a daily basis. For mice previously having undergone surgery, we provide additional measures for any mice with specific needs, including moist food to ensure weight maintenance, and medication to relieve pain or clean and heal wounds under NVS advice. For mice under water control, we weigh them daily and look for signs of dehydration, and increase their water or remove from water control as necessary. Mice are habituated to being handled and head-fixed in the rigs in increasing durations before experiments begin to minimize stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the Home Office minimum standards, ARRIVE, and PREPARE guidelines to ensure best practices for refinement. Our work stems from a laboratory included in the UK N3CRs working group on rodent head fixation and fluid/food control (Barkus et al. 2022 J Neuro Methods), and we will continue to follow this guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

There are continuous improvements and innovations in experimental protocols and technology to reduce and refine animal numbers. We have previously operated at the cutting-edge of these methods, and we will continue to adopt any new approaches that allows us to improve in the 3Rs.

We will also engage with ongoing institutional and national 3R’s efforts, including establishment welfare meetings and 3R’s days, interacting with the NC3R’s regional manager and the Named Information Officer, and signing up to the NC3R’s newsletter.

62. Repair and Resolution of Cutaneous Wounds and Inflammation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Wounds, Inflammation, Psoriasis, Healing, Therapy

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are

- the development of new treatments to improve the healing of wounds;
- the development of new treatments to resolve inflammatory skin conditions;
- to further the understanding of how normal skin function is maintained.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improved Wound Healing

Chronic wound care is an important area of unmet clinical need and was estimated to cost the NHS £8.3 billion a year in 2018 (Guest et al., 2020), an increase of 48% since 2013 .

Poor cardiovascular health and immunosuppression are the principal underlying reasons for impaired healing. Ageing, dietary insufficiency, infection, immobility, inflammation of blood vessels, diabetes and long-term steroid therapy are other contributory factors. Current therapies are inadequate, focussing on secondary symptoms (such as infection and the die-back of tissue around a wound) rather than the underlying cause, which can contribute to an increased risk of amputation, particularly with diabetic foot ulcers. Standard therapies provide a sterile, moist and anti-microbial environment to improve healing. Only one therapy that is not a dressing or debridement aid (removed dead/dying tissue in the wound) is currently approved by the US and European drug regulatory authorities for the treatment of wounds: Regranex®, which consists of a gel containing recombinant human Platelet-Derived Growth Factor.

A major part of this project will be to characterise novel therapeutic agents that will aid skin repair and combat inflammatory changes.

Treatment of Psoriasis

Around 3% of the UK population suffer from psoriasis, a chronic inflammatory disease of the skin, which bears similarity to and can be associated with other types of chronic inflammatory disease that affect the joints (Rheumatoid Arthritis) and the intestine (Crohn's disease). People who have psoriasis that affects more than 10 % of their skin show an increase in mortality rate. Both genetic and environmental factors contribute to the development of psoriasis. Current therapies, including drugs that suppress the immune system and ultraviolet light, all have some impact on symptoms; however, there is no therapy available that can cure the disease. Therapies can have adverse effects on the skin themselves, such as steroid-induced dermal atrophy (thinning of the skin), and may also be associated with side effects such as diabetes, osteoporosis (loss of bone density) and increased risk of infections and certain types of cancer.

As with cutaneous wound healing, this project seeks to facilitate the development of new therapies for the control and resolution of psoriasis.

What outputs do you think you will see at the end of this project?

The project will provide data that will help in the development of new treatments for skin injury, including trauma wounds and inflammatory skin conditions. In addition, studies may produce data to help with better understanding of skin regeneration.

These data may be used to support scientific submissions to regulatory bodies, in order to obtain the necessary approval to progress treatments into clinical trials. Data may also allow the identification of new therapeutic targets or strategies for the treatment of skin injury.

Whenever possible, data will be shared with the wider scientific community, thorough presentation at national/international meetings and publication in peer-reviewed journals, in accordance with ARRIVE guidelines.

Who or what will benefit from these outputs, and how?

Short-term, the benefits will be in the pre-clinical decision making process for progressing therapeutic candidates. Any new knowledge that is gained from this project may help and inform other research groups that are working in the same field. Medium-term, this should see more treatments progress to a clinical trial setting. Approval of new clinical therapies is a lengthy and rigorous procedure and so, it may be several years beyond the life of the project for those with clinical need to see any benefits from the outputs of this project.

How will you look to maximise the outputs of this work?

All studies undertaken in relation to this project are performed on a collaborative basis with other, independent researchers.

Whenever possible, data will be shared with the wider scientific community, thorough presentation at national/international meetings and publication in peer-reviewed journals, in accordance with ARRIVE guidelines.

Species and numbers of animals expected to be used

- Mice: 600
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult mice and rats in these studies. These represent the animals of lowest sentience in which we can perform these studies. Rats are more intelligent than mice but rat models give superior wound histology. Mouse models can have advantages over rats, particularly for diabetes-impaired wound healing, where use of the diabetic db/db mouse strain avoids the use of a chemical insult to induce diabetes in animals. The species and strain used in each study will be carefully considered, according to its aims.

Adult animals are of sufficient size that any wound sites are not too big relative to the size of the animal.

Use of animals allows us to study the multifactorial nature of the healing response which depends on a range of different types of cells.

Typically, what will be done to an animal used in your project?

First of all, animals used in this project will be anaesthetised and an area on the back of each animal will be shaved and any remaining hair removed using wax strips or cream.

Whilst still anaesthetised, animals may undergo procedures to the skin (the shaved area) that will result in injury. The injury may be an incisional wound (i.e. a cut made by a scalpel) or an excisional wound (i.e. a hole made by removing a plug of skin using a surgical biopsy punch). Animals will receive pain relief (usually by injection).

Various factors can affect the speed at which a wound heals, including infection, aging and diabetes. In order to study these, we will vary the procedure that we follow. For some studies, we may inject substances into the skin near the wound in order to mimic infection. In other studies, we may use aged animals or we may use female animals that have had the ovaries removed, in order to study the effects of aging. Finally, we may use animals that have been genetically-modified so that they develop diabetes.

For some studies, instead of a physical wound, a chemical may be applied to the skin that will result in inflammation. This is relevant for studying treatments for allergic dermatitis and psoriasis, for example, according to the type of chemical that is applied.

We may apply treatments to the animals in order to speed up the healing of the wound, or reduce the inflammation (if appropriate, this will be performed under anaesthesia); treatments may include dressings, gels, injections or substances given in the food or drinking water (or by a feeding tube directly into the stomach). Placebo treatments will be included in any study, together with any appropriate reference treatment (i.e. a treatment known to have a beneficial effect).

Studies will typically last one or two weeks. Animals will be weighed daily and welfare checks performed at least once per day. Experiment treatments will be administered/applied as appropriate for each study. Blood samples may be taken from animals during the study. Animals will be humanely killed at the end of each study and skin and other samples taken for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that there will be some discomfort from wounds and any chemically induced inflammation of the skin.

We expect there to be a temporary drop in body temperature (a couple of hours) and body weight associated with anaesthesia (one or more days).

During a study, the administration/application of substances may be associated with discomfort or localised irritation at a site of injection (a few seconds). The restraint of animals for the purpose of blood sampling may cause transient distress and minor pain/discomfort may be experienced when blood is sampled (a few minutes).

Diabetic animals may drink more and urinate more than healthy animals.

Application of a dressing to a wound may cause some discomfort and local irritation depending on its mechanical properties (i.e. how soft and flexible it might be) and how it is fastened to the animal (i.e. the adhesive used).

Unless stated, we expect to see some evidence of one or more of the effects for the duration of any study (up to two weeks).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the majority of animals to be classified as moderate (90 %), with some control animals being classified as mild or non-recovery (< 10 %).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The skin can be regarded as the largest organ of the body. It has many cellular and structural components and performs a range of roles including a barrier to infection and harmful chemicals, sensory perception (touch, pain, heat/cold) and temperature regulation. The skin also possesses a community of microorganisms on the surface (microbiome) which serves to modulate skin function in health and disease.

Animal models are necessary as we need to be able to study the integrated response of all the different cells in the skin, and those cells that migrate into the skin, following injury (whether caused by trauma or inflammation).

Which non-animal alternatives did you consider for use in this project?

Our skin research projects to make use of a range of animal alternatives. We can culture small pieces of skin (called explants) for short periods, which can allow us to study the effects of different treatments on specific processes that occur in the two major skin cell types, the epidermal cells (or keratinocytes), which form the top layer of skin (epidermis) and fibroblasts, which form the dermal layer, on which the epidermal cells sit. These models also allow us to study how molecules penetrate the skin.

We can make three-dimensional cell cultures, by making a synthetic dermal layer on top of which we can culture epidermal cells. This actually looks and grows a bit like skin, and the cultures are made from cells we can isolate from normal skin and keep frozen in the laboratory until we wish to use them. These cultures can be used in a similar way to the small pieces of skin, and can be used for looking at how substances protect skin cells from ultraviolet radiation (i.e. sun damage).

We have a cell culture system that allows us to monitor in real-time how epidermal cells or fibroblasts respond when a single layer of cells grown in culture is "damaged". This damage is caused by a metal pin which scratches through the layer of cells, making a gap. The cells migrate to fill the gap and we can look at how different chemicals can slow down or speed up this process. We can also perform experiments to study the formation and branching of blood vessels in culture dishes.

Why were they not suitable?

Currently, it is not possible to recapitulate all aspects of skin structure and function in cell culture models. All these culture systems lack a blood supply and immune cell populations,

and a microbiome (the population of resident bacteria and other microorganisms). Our three-dimensional cultures are quite fragile and so do not lend themselves to study the healing of wounds. All cell culture models depend on the availability of surgical skin samples from consenting individuals. This has particular relevance to wound healing, where the three-dimensional aspect of the healing process and the role of immune cells that migrate into the wound space need to be studied. A range of functional immune cell types are also required to study inflammatory conditions of the skin, such as allergic dermatitis and psoriasis; many of these cells are not usually resident in the skin and migrate, in response to inflammatory signals. Hopefully, as we become more able to add complexity to our cell culture models, we will see some replacement of animals in these types of study.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on running three mouse and one rat study each year for the project, combined with the use of current non-animal alternatives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have sought independent statistical advice based on data obtained from previous studies. Animal numbers used will be those required to meet the objectives of the project. To minimise animal numbers, we will set realistic limits for the size of effect that we wish to be able to detect in our studies (e.g. the fold improvement in the speed of wound closure). We will choose the most appropriate model for the project and utilise in-bred animals (i.e. animals that are genetically identical), in order to reduce variation in the results that we obtain.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We may perform small pilot studies in order to measure the concentration of drug that is achieved in the blood and in the skin following its administration; this will be done with small numbers of healthy animals per group (five or less), with one group per concentration of drug. Such studies can inform us of the most suitable concentrations to use in a bigger study to look at the effect of the drug on wound healing or inflammation. The range of concentrations tested will be within the toxicity limits of drug; this will have been determined by previous work by other laboratories, based on animal studies and/or cell culture experiments.

We always seek to maximise the amount of information that we can obtain from each study. This is achieved by collecting clinical observations during the study and collecting as many tissue samples as required, for analysis at the end of the study. We have available a wide range of analytical platforms to support our studies and give us access to

multiple readouts that can inform us about different aspects of a drug's ability to promote healing and/or to suppress inflammation. In collecting as much data as possible from each study, we minimise the risk of having to run another study in order to gain further knowledge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodent models of wound healing and skin inflammation. These models will include excisional and incisional wounding and chemically induced inflammation (psoriasis and dermatitis). These models are well-characterised in terms of their time-course and severity of harms. Because of this, a suitable frequency of monitoring animals for welfare purposes can be incorporated into study protocols and informed decisions about when it is appropriate to end a study can be made (according to the question we are trying to answer by running the study). Pain will be minimised by the use of pre- and post-operative analgesia for all surgical procedures and throughout the time-course of the study, where it does not frustrate the outcome (i.e. mask the effect of any treatments being tested). Also, humane endpoints can be set to maintain animals within moderate severity limits. All animals will be humanely killed at the end of each protocol.

Why can’t you use animals that are less sentient?

We require models to be robust and well-characterised in order to reproducibly evaluate new therapeutics of varying modalities, ranging from injectables to gels applied directly into wounds and material dressings. The chosen species and models best allow us to perform these tasks. Immature animals have immature immune systems; a fully functional immune system is fundamental to models of inflammation and the immune system is also important for cutaneous wound healing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A study will run only for the length of time required to meet the aims and objectives of that study. Monitoring animals for welfare purposes will be increased in response to any observed increased in signs of discomfort and informed decisions about when it is appropriate to end a study prior to any scheduled endpoint will be made. Pain will be minimised by the use of pre- and post-operative analgesia for all surgical procedures and throughout the time-course of the study, where it does not frustrate the outcome (i.e. mask the effect of any treatments being tested). Also, humane endpoints can be set to maintain animals within moderate severity limits.

A process of continuous review will operate, to ensure that the models we run are fit-for-purpose; this will involve looking at all the data that we collect and seeing how it may change with time and if required, making appropriate changes to models.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will perform procedures according to our own standard operating procedures. We will also follow guidelines issued by the Laboratory Animal Science Association (LASA) and guidance from the NC3Rs, as appropriate.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the 3Rs advances through attendance at lectures and seminars run locally, as part of a continuing professional development programme; through the NC3Rs website; by keeping up with the latest scientific publications in the field.

63. Studies of Experimental Ruminant Transmissible Spongiform Encephalopathies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Transmissible spongiform encephalopathy, ruminants, surveillance, experimental challenge, diagnosis

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to increase our understanding of the ruminant species spectrum that may be exposed to naturally occurring transmissible spongiform encephalopathies (TSEs) and its consequences: to learn whether the TSE agent can be transmitted to the particular ruminant species, whether the disease changes when transmitted to a different host and whether the resulting disease or exposure can be diagnosed by the diagnostic tests currently used for TSE surveillance.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The eradication of TSEs with the associated surveillance is currently a legislative requirement in cattle, sheep and goats whereby certain risk groups (healthy slaughtered

animals, emergency slaughter animals and/or dead animal on farm) are monitored for the presence of TSE [scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle] by examination of brain tissues. TSEs are notifiable disease, i.e. its suspicion has to be reported to the local authority, which also includes the TSE found in deer and other cervids (chronic wasting disease). BSE is zoonotic and can cause disease in humans (variant Creutzfeldt-Jakob Disease). There are multiple naturally occurring TSE strains in ruminants and some of these have already been shown to be transmissible to a different ruminant host. For example, it has been shown that BSE from cattle can be transmitted to sheep and goats, which is potentially more concerning in terms of possible transmission to humans. As ruminants may be exposed to various TSE strains naturally, it is important to know whether it would cause disease, how the disease looks like and whether it can be diagnosed in affected animals to remove them from the food chain and also protect other animals that may be exposed to these in future.
Achieving this objective requires experimental challenges in the species that may be potentially exposed to the TSE strains, which we seek in this project proposal.

What outputs do you think you will see at the end of this project?

The output is expected to provide new scientific information since these challenges are novel. The findings will be disseminated in the form of presentations and/ or peer-reviewed publications and may lead to policy/ legislative changes to prevent any occurrence naturally or surveillance requirements depending on the outcome.

Who or what will benefit from these outputs, and how?

Due to the novel nature of the work this will increase the scientific knowledge about TSE agents and their ability to change on passage. The scientific outputs from this also feeds into government policy (both UK and wider) to protect the human food chain and animal health. Depending on the results there may or may not be a change in surveillance requirements or modifications to current statutory diagnostic tests.

TSEs are slowly progressive diseases and results are usually not expected for several years, maybe not even within the lifetime of this licence, particularly if animals succumb to disease only several years after challenge. In fact, it is anticipated to continue the use of animals in this project, which are currently under a different licence that will expire soon. Thus, the impact is more likely to be long-term unless challenged animals succumb to disease very quickly and the disease can be diagnosed without difficulty.

How will you look to maximise the outputs of this work?

Scientific papers, information to government policy makers and material from the experiment will be archived and will be available to our network of collaborators and to other World Organisation for Animal Health reference laboratories for TSEs.

Although there is not currently a blood test available, blood samples will be taken at key points during the experiment and archived for potential blood test development to reduce the need to infect future animals.

Diagnosis of disease requires knowledge and skills in a range of diagnostic tests, which are not all available at the establishment. To describe any resulting disease, or confirm the absence of it, also often requires additional studies, for example transmission studies in rodents. Collaboration with other research facilities within and outside the UK is vital to characterise the disease in full and has happened in the past. Publication of research

findings often leads to interest from other researchers who request tissues from animals in these studies and thus maximise benefit. Even unsuccessful approaches (negative findings) will be published to make other researchers aware so that potential studies by others can be avoided or modified.

Species and numbers of animals expected to be used

- Cattle: 25
- Sheep: 10

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cattle and sheep are the natural hosts for TSEs. The project aims to investigate the consequences of exposure of ruminants to naturally occurring TSE agents. Diagnostic phenotype information will be obtained, including the dissemination of the TSE agent in the body and presence of the agent in the brain, which is in general used for the diagnosis of a TSE. This cannot be achieved in cell cultures, which have limited use in TSE research as they are usually only specific for a particular TSE strain and cannot address the question of agent dissemination. Using mice with a prion protein genotype of the host of interest is now common to investigate transmission of TSE agents and has already been done for some TSE agents but do not allow extrapolation to the actual host to answer questions about accumulation of the agent in peripheral tissues, which may suggest different routes of natural transmission, and do not address factors that may affect transmission other than the prion protein gene. In addition, it may be important to characterise the disease clinically in the actual host, which will differ between the different species.

Typically, what will be done to an animal used in your project?

Animals will be challenged by inoculation of brain material (or saline solution in control animals if applicable) into the brain under general anaesthesia. They will then be monitored clinically for signs of disease until clinical endpoint, development of an untreatable intercurrent disease or predetermined endpoint if no disease is evident. In sheep where peripheral distribution of the agent may be possible, a rectal biopsy may be taken, usually annually, to determine whether infection can be confirmed.

Samples will be collected during the life of the animals, which could include blood and faeces (usually annually and prior to death; faeces will be collected from sheep when rectal biopsies are taken) and saliva (usually prior to death; considered sub-threshold as it will only involve brief insertion of a swab into the mouth for the animal to chew on). Clinical monitoring may include recording of brain waves to external stimuli, which involves the insertion of small needle electrodes under the skin and is usually only done once prior to death.

Experiments are expected to last at least 15 months before any signs of disease are expected.

What are the expected impacts and/or adverse effects for the animals during your project?

Adverse reactions can be caused by the inoculation into the brain (e.g. bleeding, infection) or the anaesthesia (e.g. respiratory and cardiovascular problems) and at worst may result in death during or shortly after anaesthesia but could include dullness, incoordination, respiratory problems and inappetence. This will be treated but in case of lack of improvement within a week or deterioration will lead to euthanasia.

Blood sampling may result in swelling at the site of blood collection (neck) because of blood leakage (haematoma), which will resolve over a few days or weeks. Rectal biopsy will lead to minor bleeding, which generally stops after a few minutes.

Clinical signs of TSEs are variable but will generally include neurological signs, such as changes in behaviour, sensation and locomotion. Due to the slowly progressive nature of TSEs, early clinical signs can be subtle and may progress over weeks to months. The disease is fatal, and no suitable treatment currently exists.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Adverse reactions to inoculations have been rare [less than 1% of animals may die (severe severity]; none experienced over the last 15 years in both cattle and sheep). Similarly, swellings after blood were observed rarely (less than 1% in both cattle and sheep) without any evident pain or distress (mild severity). Rectal biopsies in sheep will lead to bleeding but in the past it has never required any treatment and has caused no obvious signs of distress in sheep (mild severity).

Clinical disease may be experienced in all animals inoculated with brain material. As there is no reliable diagnostic test, disease suspicion is based on clinical signs and proof of progression, which requires the animals to develop clinical disease (moderate severity). The ability of the animal to rise to its feet after laying down will be recorded and monitored by CCTV and if the animal is starting to struggle with this as part of the clinical signs it will euthanased. This is because in very rare cases (less than 1% in sheep and cattle), animals may be unable to get up and may die of cardiovascular failure if too long in lateral position and found too late to place them upright (severe severity).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is currently no alternative replacement technique to study how different TSE strains affect the body system of different species. Newly developed diagnostic techniques are able to amplify minute amounts of the infectious agent in a test tube but are only useful as additional test to confirm infection and would not be able to replace the study in animals itself, which is essential for surveillance and disease classification policies.

Which non-animal alternatives did you consider for use in this project?

Cell cultures, computer models

Why were they not suitable?

Development of cell cultures has been attempted but these are only suitable or available for certain TSE strains, none of which are under investigation here. Even if they were available, they would only address the question whether certain cells are susceptible to infection, but it would be impossible to conclude whether an animal becomes infected, what the clinical disease looks like, where the agent is distributed, and whether the current tests can diagnose it.

Every TSE agent behaves differently in different hosts. Whilst computer models can predict to some degree what may happen (infection or not), they would not be able to estimate the rate of infection and possible disease presentation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The sheep will be transferred from an already existing licence to continue being used under this licence. Cattle numbers are estimated based on previous studies where groups sizes of 5 are an established group size in farm animal TSE work and are considered the minimum number to give a robust answer. The limiting factor is animal housing, which restricts the number of cattle being used: due to the growth and size of the cattle, a pen is generally occupied by 5 cattle.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Sheep numbers already pre-determined because of their use in an existing licence.

As the proposed studies in cattle are novel it is usually unknown whether challenge would result in disease at all or only in a minority of animals, which would require the increase in animal numbers. Available accommodation limits the number of animals in a pen to a maximum of five per group. The input of a statistician will be sought to confirm that the animal numbers are adequate and at the minimum to achieve the objective.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Due to the novelty, it is usually uncertain what the optimum number will be, and all studies are essentially pilot studies. Serial passages in the same host, e.g. cattle inoculated with a sheep inoculum first and subsequently with an inoculum obtained from the cattle, usually leads to adaptation where the number of animals succumb to disease increases and the survival time reduces. This enables us to consider reducing the number of animals for a particular study, but past experience has also shown that the disease characteristics may not be uniform, which may be the effect of factors (e.g. genetic) not yet identified and cannot be controlled because the species under investigation is not in-bred, thus limiting the degree of animal reduction. A wide range of tissues in addition to the live animal samples

will be available for further use by us and any collaborators who have access to techniques we are not experienced in.

We use the intracerebral route of inoculation (injection in the brain) to increase the probability of infection compared to an oral route because it by-passes the gut as possible barrier. This ensures that we can reduce the number of animals (as oral challenge is likely to be unsuccessful in more animals).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals used will be the potential natural host (cattle, sheep) under natural conditions since the results will directly inform on the situation in those species susceptible to TSEs and subject to TSE surveillance.

Intracerebral inoculation will reduce the time to disease development compared to oral challenge so will reduce the time animals are in confinement in the establishment and may also shorten the clinical phase to reduce the time of potential suffering.

Why can’t you use animals that are less sentient?

TSEs are slowly progressive and even infection in more immature life stages (if this was possible) would result in disease manifestation in adulthood or close to adulthood based on natural disease where there is evidence that animals were infected in the uterus but do not develop disease until several months or years old. This is also the reason why terminally anaesthetised animals cannot be used. As this study may inform on disease surveillance in the natural host, sheep and cattle have to be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Inoculation via the intracerebral route will be done under general anaesthesia and appropriate pain relief will be provided to minimise pain and suffering. All inocula are screened for bacterial contamination prior to use and treated as necessary to minimise the risk of inflammatory brain disease.

Clinical monitoring of animals is very detailed and will include clinical examinations and passive observations from outside the pen and/ or camera observations from recordings. These will ensure that clinical signs suggestive of a TSE are captured as soon as possible and that the clinical progression is monitored until the animal reaches humane end-point.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use aseptic procedures for the inoculation according to good veterinary practice.

A scoring system based on expected clinical signs will be utilised to determine onset of disease, monitor clinical progression and determine clinical endpoint, which has been published. TSEs generally cause changes in behaviour and mental status, sensation and movement, some of which may not be specific enough to make a clinical diagnosis. TSEs are slowly progressive diseases and can be suspected if there is clinical progression over time (e.g. becoming more nervous) and there are changes in more than one category (e.g. more nervous and slightly unsteady gait).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

New refinements may include new diagnostic tests that would allow disease confirmation reliably in the host in accessible tissues or body fluids but would not necessarily allow earlier termination of the study as animals have to develop brain disease to determine whether the current diagnostic tests work. It would however help associating possible clinical signs with disease as early signs of TSEs are often unspecific and may be observed in a range of diseases. This may lead to refining clinical endpoints.

Similarly, we will utilise any results from studies in other countries using similar TSE agents if they are published during the duration of this licence and aid in refining the clinical endpoint.

64. Supply of Ruminant and Porcine Blood for Virus Infection Research and Diagnostics

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Blood, natural host cells, viruses, immunity, livestock

Animal types	Life stages
Cattle	aged, pregnant, adult, juvenile, neonate
Sheep	neonate, juvenile, adult, pregnant, aged
Pigs	neonate, juvenile, adult, pregnant, aged
Goats	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this licence is to “provide blood of cattle, sheep, goats and pigs to scientists in support of research and diagnosis of viral pathogens of livestock (including those also affecting humans termed zoonotic pathogens)”

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Critical research and diagnostic testing rely on the provision of blood from natural hosts of highly important exotic viruses of livestock such as Foot-and-mouth disease virus (FMDV), bluetongue virus (BTV) and African Swine fever virus (ASFV). The ability to use natural host blood and specifically blood-derived cells within our research as well as for diagnostic assays is essential to prevent the incursions of these viruses into the UK and to increase the scientific capability to control these viruses worldwide.

Blood from pigs and ruminants is required to isolate live cells derived from the natural host to be maintained as cell cultures in the laboratory.

Within the laboratory these cells will then be utilised to study the replication and immune response of highly important viral pathogens e.g. African Swine fever virus, Bluetongue virus, Lumpy Skin disease virus, Peste des Petits Ruminants Virus, Foot and Mouth Disease virus, Bovine Respiratory Syncytial virus as well as vaccine candidate antigens. Furthermore specific markers present on these immune cells and the immunogenetic background of individual animals and breeds will also be further characterised from this material to enhance our understanding of immune responses to viral infections.

By using these natural host cells in vitro the overall need of studying these viruses and their respective immune responses directly in animals is reduced, leading to an overall reduction in the use of animals.

What outputs do you think you will see at the end of this project?

Natural host cells obtained from blood of pigs and ruminant are a vital resource to carry out research and diagnoses for numerous important livestock and zoonotic pathogens. Additionally the immune response of blood immune cells derived from natural hosts both towards vaccine candidates against viral pathogens, as well as more fundamental understanding of how immune cells generate a response, will be analysed. Overall, the research conducted using these cells, as well as the use of these cells in diagnostic assays such as virus isolation, is vital to keep the UK free from important livestock and zoonotic viral pathogens.

Who or what will benefit from these outputs, and how?

The diseases studied cause major social and economic impacts in affected countries and if introduced to the UK will result in a ban on the movement of animals and loss of international trade in addition to causing animal suffering and welfare issues. Hence it is extremely important that appropriate diagnostic assays as well as new research aimed at preventing and controlling viral disease outbreaks are carried out.

How will you look to maximise the outputs of this work?

The materials collected as part of this PPL will be used to generate data which will, as appropriate, be published in open source peer reviewed papers specific to the objective of that study.

Species and numbers of animals expected to be used

- Cattle: 200
- Goats: 60
- Pigs: 60

- Sheep: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use cattle, pigs, sheep and goats raised on commercial farms and/or licenced establishments for which we have additional availability on this licence, which allow normal farm husbandry of these animals. None of the animals are genetically modified however some cattle and pigs are specifically inbred to reduce the variations in immune response determining genes The animals will be bled for the supply of blood from livestock allowing the isolation of cells or blood factors which need to be utilised fresh or from specified individuals. Some animals might be held long-term (years) at the named additional availability site in typical farm environments and husbandry. During this time these animals will donate blood in a tightly regulated frequency and under strict monitoring of adverse effects and stress responses

Typically, what will be done to an animal used in your project?

The overall severity is mild and adverse effects to blood sampling are extremely rare. The animals will be blood sampled by experienced animal technicians or veterinarians and will be placed under veterinary care should an unexpected event (such as inflammation of the vein) occur. Any animal showing adverse effects to blood sampling will not be sampled for an appropriate time defined by a veterinarian. Animals might also be vaccinated using appropriate routes, volumes and adjuvants for the respective species to allow the usage of their blood derived cells for specific immune assays.

Antigens which may be used are not expected to result in any adverse effects other than potential swelling/ inflammation at the injection sites. Following the procedures, animals might be released from the licence and returned to the national herd. Long term donors might also be euthanised humanely.

What are the expected impacts and/or adverse effects for the animals during your project?

Overall we are expecting 10-15 cattle bleeds each month, and 3-5 sheep, pig, and goat bleeds each month based on previous useage. Where animals are only bleed once for a specified purpose each bleed will count as single animal. Hence over the course of this licence we have asked for 200 cattle, 60 sheep, 60 pigs, and 60 goats. The majority of these animals will be released back to the national herd.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The overall severity is mild and adverse effects to blood sampling are extremely rare. The animals will be blood sampled by experienced animal technicians or veterinarians and will

be placed under veterinary care should an unexpected event (such as inflammation of the vein) occur. Any animal showing adverse effects to blood sampling will not be sampled for an appropriate time defined by a veterinarian. Animals might also be vaccinated using appropriate routes, volumes and adjuvants for the respective species to allow the usage of their blood derived cells for specific immune assays.
Vaccine preparations used are not expected to result in any adverse effects other than potential swelling/ inflammation at the injection sites. Following the procedures animals might be released from the licence and returned to the national herd. Long term donors might also be euthanised humanely.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the viral pathogens in question do not replicate within established cell lines or blood cells from model hosts, hence cells established from the blood of natural hosts are a requirement. Moreover, host cellular responses cannot be modelled or analysed using immortalised cell lines. The blood samples required under this licence will be used to immediately generate primary cell cultures of blood leukocytes by well-established protocols for use in fundamental and applied research into immune cell responses as well as use of these primary immune cells for use in diagnostic assays such as virus detection. These cells will have to be generated within 12 hours from taking the blood samples as cell death occurs progressively once the blood sample has been taken. The short window of opportunity to isolate viable cells makes the use of commercially available blood unrealistic as leukocytes would not be alive once the blood has been received.

We have arrangements in place with local abattoirs to obtain blood for some experimental studies were possible. However cellular viability in obtained blood varies and obtaining blood post mortem can affect the activation status of blood derived cells. Therefore, for validity and comparability reasons, blood samples need to be freshly harvested and cannot be taken in sufficient quantity or of adequate quality after death. Furthermore obtaining blood from abattoirs does not allow us to obtain blood from the same individual on several occasions which is important to reduce experimental variability and allow the investigation of seasonal and age effects.

Which non-animal alternatives did you consider for use in this project?

Creation of permanent cell lines to replace the need for primary cell cultures created from ongoing use of animals has been considered for both isolation and culture of viruses for both research and diagnostic purpsoes, but also for assessment of cellular immune responses.

Predecessors of this licence still covered the need for mice to maintain arthropod colonies. Over the years these Culicoides biting midge and mosquito colonies have been adapted to an artificial hemotek® membrane feeding system, allowing the reduction of mice needed. In the previous licence the provision of mice as emergency cover only was still included should valuable arthropod colonies suddenly cease to feed on these artificial membrane-blood systems. This requirement was removed 5 years ago, and is an example of how ongoing attempts for replacement are considered in this PPL.

Why were they not suitable?

The propagation of animal viruses often requires primary cells and/or cell lines from the target species for both use in research as well as a primary diagnostic assay. For example, African swine fever virus does not replicate in established cells lines or cells from species other than pigs. Many field strains of African swine fever virus will only replicate in primary porcine macrophages while failing to even infect porcine cell lines, meaning primary cells are required to produce virus for use in research platforms as well as to be used themselves for diagnosing ASFV by isolation.

Continuous attempts are in progress to establish cell lines to reduce the need for primary cells. The development and validation of a pig macrophage cell line for ASFV research and diagnosis is currently being carried out within the ASFV group with the long-term goal of replacing or reducing the requirement for primary cell cultures.

Furthermore primary host cells are essential to establish cellular immune responses to respective viral infections which are vital to inform appropriate vaccine design or to identify immune-pathogenic mechanisms. The usage of primary cells obtained from the same individual over time further allows addressing specific research questions such as variation of cellular immune responses to different viruses, influence of genetic backgrounds and difference in immune responses attributable to age and seasonal effects. These materials are not available from other sources such as commercial suppliers.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Reviewing the numbers of animals used during the course of the previous PPL, the number of animals used of each type varied from year to year based upon the individual projects however for cattle it was around 20 per year. The use of goats, sheep and pigs was substantially lower, and therefore estimates of animal use have been reduced substantially compared to the previous version of this PPL to reflect this. The numbers estimated for this PPL allow, on average, for each of 1 pig, sheep and goat to be bled per month for the duration of this PPL, and 3-4 cattle to be bled per month.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Specifically, when individual animals are bled regularly, they will have no more than 10% of the total blood volume on any one occasion removed and no more than 15% of the total blood volume taken in a 28 day period (estimated as 60 ml per Kg body weight). This is monitored by an internal process and rolling documentation of animal weights and volumes being taken.

For certain scientific studies it may be necessary to obtain blood on several occasions from the same animal to monitor responses over time and minimise variations within experimental repeats.

Specific animals might also be required as blood donors due to their specific genetic background.

Therefore an individual animal might be bled multiple times for a single scientific purpose. In addition this animal may also be bled for a different scientific purpose as a re-use.

Ruminants: Samples will not be taken more than once a week from the same animal

Pigs: Samples will not be taken more than twice a month from the same animals with a minimum interval of 1 week

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each request for blood is made visible to the PPL holder in advance to it being taken, and the PPL holder also ensures that each person requesting the blood is aware of the uses and objective permitted under the PPL as well as the requirement to minimize volumes taken.

The scientific acceptable justification for the provision of blood from healthy animals under this licence is the need for fresh blood which cannot be fulfilled by commercial blood supplies (for example isolation of primary blood derived cells or obtaining blood from specified individual animals). Cells, isolated from blood, which are excess to requirements may be frozen to enable them to be used later without the need for a further blood sample request. The volume of blood requested will, however, be the minimum required to achieve the objective of the request.

The capability to use genetically defined cattle and inbred pigs may enable fewer animals to be used to achieve the same experimental goals.

Most importantly the use of these primary cells obtained from the blood of livestock will reduce animals needed to be directly infected with the respective viral pathogens which both constitutes a reduction as well as a refinement since direct viral infections of the natural hosts with respective viruses would lead to clinical disease and higher severity categories of the respective procedure.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

It is of significant benefit to science that immune responses to viral infections of blood derived cells can be carried out using cells from the respective natural hosts. By using natural host target cells scientific results obtained are of most practical relevance and transferability to natural infection scenarios and subsequent vaccine development within the same host

The use of these primary cells obtained from the blood of livestock will reduce animals needed to be directly infected with viral pathogens. This not only leads to less animals being used but also constitutes a refinement as direct viral infections of the natural hosts with respective viruses would lead to clinical disease and higher severity compared to the mild procedure of obtaining bloods samples from superficial veins.

Why can’t you use animals that are less sentient?

The animals required to have blood collected under this licence are used as they are the natural hosts to the pathogens being studied / vaccines being developed and / or immune mechanisms being investigated.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As this PPL will be acquiring either blood samples or a simple vaccination only, which are very mild procedures, extended monitoring of animals after application of these procedures will not be required. Each animal will be monitored by the responsible person after blood samples and / or vaccination is undertaken until a time that the animals health has returned to normal. Where possible, animals will be trained to facilitate blood sampling, with positive reinforcement offered to encourage this behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The principals of "Diehl, K. H., et al. (2001). "A good practice guide to the administration of substances and removal of blood, including routes and volumes." J Appl Toxicol 21(1): 15-23" will be applied to blood sampling and volumes taken, and monitored by an internal system at the establishment for each individual animal.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By referencing online email groups, reviewing information circulated by the institute NIO, communicating with other establishments doing similar procedures, and referencing sites such as the NC3Rs, I will stay informed about advances in the 3Rs in this area.

65. The Development and Application of Pre-Clinical Cancer Models for Identifying and Screening Novel Drug and Imaging Agents for Cancer Diagnosis and Treatment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, therapy, imaging, refined models, tumour microenvironment

Animal types	Life stages
Mice	adult, juvenile
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to establish more clinically-relevant pre-clinical models of cancer, to apply imaging methodologies to assess their growth and spread, and to use them to validate new drug targets, drugs, drug delivery systems and diagnostic/prognostic (predictive) imaging agents.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Current pre-clinical (before clinical trials) cancer models do not provide a fully predictive setting for testing new treatments and imaging agents, meaning that they frequently fail in the clinic. As some aspects of drug and imaging agent development still require use of animal models to allow for e.g. full body exposure, dependence on the blood circulatory system for both delivery to specific organs or removal of the agents from the body, establishing more clinically-relevant models would reduce this failure rate and, importantly, ensure that, where animal models are required, knowledge gained is as informative as possible of how the agents are likely to perform in patients.

What outputs do you think you will see at the end of this project?

The project will result in new, more refined, cancer models that are more predictive of likely clinical outcomes. These new models will, be used to identify new drug targets, drugs, drug delivery and imaging systems. It will also result in an improved understanding of the role of the tumour microenvironment (the cellular environment in which tumour or cancer cells exist) in driving tumour progression and drug resistance, thus potentially providing insight into how to improve cancer treatment further.

Papers describing the models and 3Rs developments, as well as the scientific findings made using the models, will be published in peer-reviewed papers and disseminated at national and international meetings by members of the group. This will increase their availability to the wider scientific community and demonstrate their utility in achieving more relevant scientific outputs.

Who or what will benefit from these outputs, and how?

The main beneficiaries will be both the scientific community and cancer patients. We will contribute to improved understanding of the tumour microenvironment’s role in cancer and develop models which allow this to be better studied in vivo (in the body) and ex vivo (in the laboratory). In the short term we, and the wider scientific community, will be able to use these models to identify new cancer drug targets, to test potential new drugs, to identify improved methods for delivering drugs to the tumour, including secondary as well as primary sites, and new methods of imaging tumours within the body. In the longer term, this will also benefit cancer patients, as it will provide more effective drugs and reduced side effects. Furthermore, it should result in lower drug development costs because, by having more clinically relevant models, it will reduce the frequency of drugs that appear successful in pre-clinical trials and then fail in the clinic. Additionally, this will also reduce the numbers of animals used in such ineffective pre-clinical trials.

How will you look to maximise the outputs of this work?

We intend to report our findings at national and international cancer research conferences and at broader scientific and animal welfare meetings, and events aimed at improving public understanding of science e.g. via Patient Participation & Involvement groups. This will maximise the reach and impact of the outputs. We will publish our findings in high impact, open access peer-reviewed journals, but in addition, will also publish unsuccessful approaches, for example, as a Brief Report on the NC3Rs Gateway, or in peer-reviewed journals, to avoid unnecessary repetition by other groups.

We will make any excess tissue generated from the models we have developed available to other researchers e.g. for analysis of additional genes/molecules enabling the achievement of additional scientific outcomes from the studies.

Species and numbers of animals expected to be used

- Mice: 5500
- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The application of our experimental technologies requires a species with a body structure similar to a human, and it must also be able to generate a sufficient amount of tissue for our experimental needs. Rodents (rats and mice) are the lowest species in which a genetic mutation has produced an immunodeficiency (a full or partial impairment of the immune system) which allows the growth of human tumours without rejection. There are a number of different strains available with varying degrees of immunodeficiency so the specific strain choice is dependent on the level of immunodeficiency required to allow growth of the particular tumour type being investigated.

Experience has shown us which immunodeficient strain is most appropriate for particular tumour types, but we will always try to improve take-rates by trying new strains as they become available. Therefore, pilot studies may be carried out to identify the most suitable immunodeficient strain for a particular tumour type.

Adult animals are the most appropriate age as our models typically relate to adult humans.

However, we may use some younger juvenile animals (4-6wks) when investigating tumours which are paediatric in origin e.g. medulloblastoma.

Typically, what will be done to an animal used in your project?

All animals will be housed in enclosed, self-contained systems with appropriate “super clean” husbandry procedures, in order to prevent possible infections due to their lower immunity level.

All animals will undergo at least one procedure. For example, a mouse it may have tumour cells injected under the loose skin on its flank. The tumour cells may be mixed with other cell types and/or a biological support gel to stimulate or encourage growth. The tumour, which grows as a discrete lump under the skin, will be measured at least weekly with calipers and/or whole-body optical imaging (under general anaesthetic), depending on growth rate, until the maximum allowable size (1.2cm diameter) is reached, at which point the animal will be humanely killed and the tumour removed for laboratory analysis. This is a typical growth study.

For a simple therapy study, to investigate the effectiveness of a new drug, when measurements have shown that tumours, initiated as above, have become established, the mice will be treated with drugs according to the experimental protocol but, typically, they may be dosed twice weekly for 3 weeks with a drug given by mouth and, in some instances, another drug once a week injected into the abdomen. Tumours will be measured and the animal imaged as above. In this instance, where treatment is involved,

the tumours are allowed to grow to 1.5cm diameter but where a statistically significant effect on study objectives can be determined prior to reaching this size, the study will be terminated at that point.

For the more complex orthotopic (occurring at the normal place in the body) studies, the tumours will be initiated into the relevant organ by surgery under general anaesthetic prior to undergoing treatment as above, and growth and spread will be monitored by whole body optical imaging. Surgery typically last no longer than 20-30 minutes. These animals will experience some discomfort after surgery and some mild to moderate pain, which will be treated with analgesics under direction of the Named Veterinary Surgeon.

Hormone supplements may be required to stimulate certain tumours to grow e.g. oestrogen for breast tumours, and testosterone for prostate tumours. These supplements will be delivered via the feed or the drinking water for the duration of the experiment.

Growth rate of the tumour depends on both the type and location it is in, but most experiments end after 3 months, with around 20% lasting up to 6 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the variety of procedures potentially being carried out under this project, there are a number of potential adverse effects that need to be considered.

Tumour development

In a typical subcutaneous growth study, the mouse will experience minimal transient discomfort from the subcutaneous injection of tumour cells and physical restraint. The resulting tumour growth will be monitored by measuring with calipers, again while physically restrained by an experienced technician. The growth of the tumour should have no impact on the animal's health and welfare and the animal will be killed when the tumour reaches the maximum allowable size. Injected tumours grown subcutaneously may ulcerate or develop shiny patches that could lead on to ulceration, independent of tumour size, in around 5% of the animals. The animals will, therefore, be observed regularly as soon as growth is established, and any mouse will be humanely killed as soon as the skin covering the tumour exhibits pre-ulcerative signs such as skin stretching or reddening.

In an orthotopic model, the tumour may develop discretely in the organ of initiation and/or metastases (secondary tumours) may develop. Organs that tumours may spread to over time include the lungs, spine, brain, liver or the abdomen, increasing the tumour burden and potentially upsetting normal bodily functions.

Therefore, determining the tumour spread is vital to ensure the study will end before the animal begins to suffer beyond an acceptable level. This is done by the use of pilot growth experiments using small numbers of animals in order to allow characterisation of patterns of spread of different tumour types, to predict clinical signs and to define humane endpoints. Non-invasive optical imaging, where the whole body is scanned and any tumour deposits within the body identified, will be used to aid this process.
Reliance will also be placed on the general condition of the animal, together with assessment of tumours that can be felt by touch through the skin, and specific signs detailed in the current NCRI Guidelines on recognising tumour burden.

Surgery and imaging

If the animal is undergoing surgery or imaging any impact is mainly around anaesthesia. There is always a risk of death under general anaesthetic (<1%), but careful administration and monitoring by experienced technicians, and advice from the Veterinary Surgeon should prevent this.

It is likely that transient pain as a result of the surgery will occur. Therefore, appropriate pre-and post- procedure analgesia, as advised by the Veterinary Surgeon, will be employed.

Imaging is non-invasive and therefore not expected to affect the well-being of the animal.

Anaesthetic risk will be mitigated by ensuring there is a suitable period of time between repeated imaging events, to allow animals to recover fully between these events. Animals undergoing repeated anaesthetic events over a single day, for example when it is being imaged over a time course e.g. 0 hr and then 4hr post compound injection to establish a biodistribution over time profile, are at increased risk of dehydration and a drop in body temperature (<1%). Therefore, body temperature will be maintained by the use of appropriate warming, with monitoring to confirm that this is achieved.
Repeated anaesthetic events will only be carried out when animals have returned to normal eating, drinking and mobility, taking advice from the Named Persons or their nominees if deemed necessary,

including Veterinary advice to ensure that animals are able to be re-hydrated between anaesthetic events.

Dosing of substances

Any immediate post-dosing effects such as subdued behaviour, a hunched posture or disturbed normal behaviour will be monitored carefully for any developing clinical signs. Any animal displaying these adverse effects >2 hours after dosing will be killed by Schedule 1 method.

However, although unlikely, we may still see side effects common to most anti-cancer therapies including bone marrow depletion, diarrhoea, nausea and skin irritation. Animals will be closely monitored for loss of general body condition with particular emphasis on skin conditions, urine output, signs of infection or jaundice and will be weighed regularly for the duration of the study.

If adverse effects are minor e.g. slight weight loss (<10% of body weight) or mild diarrhoea (1-2 days), the animal will be given wet mash/diet gel, and may be placed on a dosing break. It will be monitored for signs of improvement, and may be returned to the study until termination day, if deemed appropriate. If there is no improvement within 2 days, it will be removed from the study and humanely killed . Animals with persistent body weight loss (e.g. 3 consecutive measurements of 10% body weight loss, on that day's weight) or those that lose bodyweight of ≥ 15%, jaundice, excessive or reduced urine output (as measured by observation of bedding, nesting material etc.), will be humanely killed.

There may be specific adverse effects to any hormone substances being delivered. Therefore, animals receiving such substances will be carefully monitored and any exhibiting mild symptoms, such as minor weight loss or signs of dehydration or urine scald, will revert back to normal diet or water until recovery is observed. Furthermore, testosterone, when given to the males, may cause increased aggression and fighting; animals will be separated and singly housed where appropriate.

Careful monitoring of general condition and, in particular, tumour growth and animal weights, alongside interpretation of data generated from pilot growth and tolerability studies, will enable us to identify scientific end points allowing termination prior to the onset of adverse effects and the need to introduce humane end points.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that around 60% of the mice will experience moderate severity, while 40% will experience no more than mild severity, while for rats the numbers will be 90% moderate and 10% mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Research and development of anti-cancer agents involves a multi-step process in which animal studies form an intrinsic part of the regulatory process linked to the approval of new drugs for e.g. by the European Medicines Agency. This approach has been adopted by the National Cancer Institute (US), the largest cancer research organisation in the world. This step is required to test drugs and imaging agents in a physiological setting where factors such as localization to organs and penetration into tissues can be assessed.

Additionally, cancer biology is now known to be affected by the microenvironment in which cancer cells grow. While our experimental programme is focused strongly on in vitro (laboratory-based) non-animal modelling, it is currently not possible to reproduce the tumour microenvironment or complex interplays between different body systems that affect both the growth of cancers and subsequent treatments.
For example, processes such as metastasis (tumour spread and invasion) which involve movement of cells between organ systems can still only be adequately modelled in a whole animal setting.

Therefore, some aspects of drug and imaging agent development still require the use of animal models to allow for e.g. metastatic spread, or the dependence on the blood circulatory system for both delivery to specific organs and/or removal of the agents from the body.

Which non-animal alternatives did you consider for use in this project?

We are pioneering the development of humanised (containing human cellular components) in vitro (laboratory grown) tumour models in our research unit. Thus, alternatives to

animals are being used wherever possible; for example, 3-Dimensional cell culture modelling of humanised tumours which can be used to identify candidate drugs suitable for in vivo testing, including effects on local invasion as well as tumour growth, are currently employed in our laboratory in preference to using animals wherever possible.

Why were they not suitable?

Although our humanised 3D models are superior to current 2D models, it is still not possible to mimic vascularization (vessel development) and thereby relevant drug access or the many homeostatic mechanisms (those that maintain the body's equilibrium) in play in a whole body environment that allows relevant tumour biology drug/imaging agent evaluation. Additionally, such models are not sufficiently developed to adequately mimic movement of cancer cells between organs. Therefore, animal models are still required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been determined based on past experience in model development and agent testing gained during work under previous PPLs, as well as the expected number of studies we hope to carry out to fulfil existing and anticipated new funding requirements.

Studies will be designed to use the minimum number of animals required for statistical significance, according to the specific questions being asked in the Study Protocol, which we will write for each study, as part of good laboratory practice.

The number of groups and, therefore, animals required for a specific experiment, is dependent on the particular study rationale, which will be detailed within its individual Study Protocol. For example, for a simple tumorigenicity study, there may be only 2 groups of 8 mice, each group being initiated with differing tumour cell concentrations, but for a novel therapy study, there may be 5 groups of 8-10 mice, namely

1) vehicle control (untreated control not required as growth rate already established through tumorigenicity studies)

2) drug A

3) drug B

4) drugs A+B combined

5) Standard of Care (already in clinical use) drug (positive control)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Laboratory based (in vitro) studies are used to identify lead compounds, evaluate dose ranges confirming target modulation/expression and relative off-target toxicity which can be used to inform on relevant doses for use in PK, PD and pilot tolerability studies. These methods include for example, 2D and 3D in vitro cell viability and death assays in response to compound insult including combination therapy assessment, and 3D assays for measuring drug/imaging agent penetration. Careful use of such assays reduces the numbers of in vivo experiments by rejecting candidate drugs, imaging systems and drug delivery systems that are unlikely to be effective.

All experiments will adhere to the PREPARE and ARRIVE Guidelines on design and reporting of animal experiments, and good principles of experimental design, including the use of the NC3R's Experimental Design Assistant, will be employed to ensure sufficient animal numbers and group sizes will be used to adequately test the hypothesis. We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tumour cell lines will be maintained in vitro and stored frozen to negate the need to use additional animals for maintenance in vivo.

Pilot studies will be performed on any new tumour model to facilitate the identification of experimental variation in tumour growth, and this will be used directly in powering studies to achieve statistical significance.

In addition, in vitro studies will be used to evaluate dose ranges confirming efficacy and lack of toxicity which will be translated into clinically relevant doses for use in the pilot tolerability studies, which will ensure the drug has no toxicity in a tumour bearing animal.

We will obtain advice from clinical colleagues regarding clinical treatment regimens (cycles, dosing and routes of administration) and pharmacy colleagues will be consulted when considering biodistribution / bioavailability of novel compounds.

The use of short-term tolerability assessments may be avoided if supporting data is already available in a relevant model (the correct animal strain, tumour model, compound batch and dosing route+regimen).

Conventional approaches to monitoring disease and the effects of therapeutic interventions in these models frequently rely on analysis of tissue obtained post-mortem, to look at the progression of the disease, which requires substantial numbers of animals to be killed at different time points to look at the development of disease over time and the effectiveness of any treatment. However, by using established whole animal imaging technology e.g. bioluminescence or ultrasound, we are able to look at the progression of disease and treatment within an individual animal in real-time. This approach removes inter-animal variation in the disease model and interactions, and reduces the number of animals required per study, as well as providing more quantitative measures of tumour burden and delivery of agents. For example, for an orthotopic model whose internal tumour dimensions cannot be measured, tumour growth would normally have to be characterised by timed terminations of multiple study groups; using imaging technologies, this could be assessed in a single group of animals with multiple imaging points. Likewise, for a biodistribution study looking at tumour and organ uptake of a fluorescent labelled

compound, sequential images at set time points can demonstrate both the compound targeting and clearance and would negate the need for multiple termination groups. Consequently, we can also combine both bioluminescent imaging and fluorescent imaging to investigate tumour growth and drug targeting in the same animal.

Definition of non-lethal end points is also a key aspect for refinement of existing models and imaging allows us to gain sufficient information of effectiveness of disease and treatment earlier in the disease progression, so where relevant and available, imaging will be used to reduce the numbers of animals needed.

However, while allowing an overall reduction in numbers, it should be borne in mind that the cost to an individual animal in terms of the number and severity of the procedures it may be subject to will increase. Therefore, each protocol has a maximum number of optional procedures any one animal will undergo, and the potential increase in side effects will also be carefully monitored.

We will make any excess tissue generated from the models we have developed available to other researchers e.g. for analysis of additional genes/molecules enabling the achievement of additional scientific outcomes from the studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For each study, as part of good laboratory practice, we will write a Study Protocol which includes: a statement of the objective(s)
a description of the experiment, covering such matters as surgical protocols, experimental treatments, imaging, the size of the experiment (number of groups, number of animals/group), duration, and the experimental material

tissues to collected and method of processing at termination

details of potential adverse effects and clearly defined humane end points

an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated)

All cells used for implantation are maintained in a sterile environment and regularly tested for the presence of bacteria. Prior to administration they are prepared using sterile techniques and initiation will use the lowest volume of cells required for tumour growth, determined from pilot studies.

Drugs are prepared and administered using sterile technique. The route of administration is via the least invasive method appropriate to the model.

Experience has shown us which immunodeficient strains are most appropriate for particular tumour types, but we will always try to improve establishment and growth rates. Therefore, small pilot studies may be carried out to identify the most suitable immunodeficient strains and optimal tumour cells concentrations for a specific tumour type, and to try new strains that may become available, in order to refine the methodology.

All animals will be housed in containment systems (isolators or IVCs), with appropriate barrier (ultra- clean) husbandry procedures, in order to protect them from possible infections due to their weakened immune status.

Non-tumour bearer studies will be used to assess drug tolerability, thus avoiding the need for tumour initiation. Use of pilot tolerability studies to ensure there are no unexpected adverse effects associated with new models or novel imaging agents and to ensure the drug levels used are not associated with any long-term cumulative effects. We will obtain advice from clinical colleagues regarding clinical treatment regimens (cycles, dosing and routes of administration) and pharmacy colleagues will be consulted when considering biodistribution / bioavailability of novel compounds.

Subcutaneous models are established through injection beneath the skin, avoiding the need for surgery requiring general anaesthesia.

Orthotopic models and models of metastasis require surgery to establish tumours. These procedures are carried out under general anaesthesia with appropriate pre-and post-operative care. Such models are only used where scientific objectives require growth of tumour in a more relevant setting and/or assessment of cancer cell movement out of the primary tumour or colonisation of different sites.

We have also developed and refined two tumour resection models for different purposes:
partial resection in a subcutaneous tumour
This model allows us to demonstrate proof of concept and efficacy of slow release drug delivery depots, before moving forward into the more complex orthotopic brain model for greater translational relevance.

full resection of mammary fat pad tumours

This allows the generation of metastatic tumours at secondary sites, which would otherwise not have time develop before the primary tumour has reached the maximum allowable size.

High Definition (HD) camera system will be employed to assist with the visualisation and introduction of cells in orthotopic surgeries, or in the brain tumour resection protocol, allowing us to accurately identify the correct area of the brain relating to specific tumour types (i.e. cerebellum for medulloblastoma, cerebral cortex for glioblastoma and ependymoma) thereby producing a more consistent and reproducible model while minimising adverse effects. Currently, rats are deemed the most appropriate species for this work rather than mice, for a variety of reasons, primarily relating to the smaller brain size of the mouse, where there is potentially a greater risk of brain damage during surgery, and the problems of growing a large enough tumour to allow resection and subsequent drug administration via the depot. However, as we develop and refine the procedure, we hope to be able to transfer the technologies into a mouse model during the course of this

licence, if deemed appropriate. Any drugs deemed suitable for this method of delivery will always be tested against a subcutaneous model first.

Real-time imaging modalities e.g. bioluminescence, facilitates the early termination of models in which the primary tumour is at an internal location, prior to the onset of any observable adverse effects, or in the early detection of metastasis, and will aid in the development of new models of clinically-relevant metastatic spread and of new cell lines in established models allowing us to choose the most appropriate scientific end point prior to the onset of any clinical signs.

Imaging will also allow us to remove from study at an early stage any animals found to have either nil tumour growth or the more unlikely event of tumours growing in the wrong site, which could confound results, and will prevent animals undergoing unnecessary procedures.

In the case of hormone-dependent tumours e.g. breast and prostate, we deliver the necessary hormones by incorporating them into the diet or water, rather than by pellets implanted under the skin. This refinement is less invasive, as it is not a surgical procedure, and will reduce the commonly seen effects of oestrogen pellets such as urine scald or bladder stones.

The maximum dose rates for hormones or other target inducers (substances which can activate genes within a cell), delivered via food or water, are based both on the existing literature and our own previous experience, and have been calculated to deliver the same continuous dose whether delivered in water or food. Pilot studies may demonstrate that a lower than maximum dose is sufficient for our experimental requirements, so this will be used in preference to the “standard” accepted maximum dose, wherever possible.

We select our end points on a scientific rather than an adverse effects basis, by our use of imaging modalities and by careful observation and monitoring by our experienced staff, thus allowing us to select and anticipate end points as soon as we have scientifically relevant data and prior to the onset of adverse effects. Clearly defined humane end points are written into both the Project Licence protocols and the individual Study Protocols, which will override any scientific end points should they become apparent. Where a statistically significant effect on study objectives can be determined prior to the end of the scheduled dosing phase, the study will be terminated at that point.

These refined methodologies ensure the procedures undertaken minimise any impact on the welfare of the animals.

Why can’t you use animals that are less sentient?

Adult mammals are required to replicate the body systems and life stages of the cancers under investigation, apart from medulloblastoma (a childhood brain cancer), when younger, but still post weaned, animals will be used, thereby ensuring appropriate comparison and relevance. Mice and rats are the lowest sentient species in which tumours will grow in this comparative manner, including the development of metastases and metastatic spread.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored by trained technical staff (the same ones who have carried out the procedures and, therefore, understand the models) daily; animals will be weighed and tumours measured at least weekly. The maximum allowable tumour size will be as detailed in the NCRI Guidelines i.e. a mean diameter of 1.2 cm for a growth study, 1.5cm for a therapy study. However, any loss of condition or mobility or e.g. reddening/thinning of skin which will lead to ulceration will lead to the animal being humanely killed, regardless of tumour size.

Clearly defined humane end points will allow early termination should these be breached prior to the establishment of a scientific end point.

Animals which have undergone surgery will be assessed on a daily basis, as a minimum, using the Unit Surgery Scoresheet, for at least a week post-operatively.

All procedures will be regularly reviewed to see if any further refinements can be introduced. Where practical, we will encourage and promote the use of novel husbandry techniques to improve animal welfare, such as handling mice by cupping or with tubes rather than by the tail.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The project will be carried out under the following guidelines- Norecopa PREPARE Guidelines

NCRI Guidelines for the Welfare and Use of Animals in Cancer Research
BVA/FRAME/RSPCA/UFAW Refining Procedures for the Administration of Substances Working Group report
NC3Rs Guidance for in vivo techniques

Findings will be reported according to the NC3Rs ARRIVE Guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will have regular liaison with the Named Persons, the Institutional AWERB and the NC3Rs.

I will keep up to date with publications and bulletins from bodies such as NC3Rs, RSPCA, Laboratory Animals etc.

My membership of Animal Science and Welfare bodies and attendance at their meetings and workshops and my network of colleagues working in similar fields will also keep me abreast of latest development, and I will take every opportunity to introduce any suitable refinements into the protocols, which I will then disseminate further via the above avenues.

66. The Impacts of Psychiatric Risk Genes on Brain Activity and Behaviour

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

neuroscience, mental health, neural networks, cognition, sleep

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate the mechanisms through which genes associated with risk of psychiatric disease in humans influence brain activity and behaviour. The findings will be integrated with clinical work in patients to inform new diagnosis and treatment approaches for schizophrenia and related disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Mental illness presents one of the greatest healthcare challenges of the 21st Century, afflicting at least 450 million people plus their families and carers. In the UK, mental illness affects 1 in 6 working age adults and 1 in 7 adolescents and is estimated to cost the economy over £100 Billion per year. The largest contributors to this burden are mood, anxiety and psychotic disorders. Current treatments for these conditions are reliant on

drugs identified by chance decades ago, which are prescribed largely on a trial-and-error basis and are associated with high rates of unpleasant and disabling side-effects. Quality of life therefore remains poor for many patients. For example, in schizophrenia, around 30% of patients do not improve with standard antipsychotic treatment, about 50% have intermittent long-term psychiatric problems, over 80% are unemployed and the average life expectancy of sufferers is reduced by between 10 and 20 years.

The outlined animal studies forms part of a collaborative endeavour, between scientists in academia and industry, that will combine data generated by brain imaging and clinical phenotyping in patients, molecular studies of patient stem cell-derived neurons and computational modelling to identifying novel therapeutic targets for the treatment and prevention of serious mental illness.

What outputs do you think you will see at the end of this project?

This project will investigate the mechanisms through which genes associated with risk of psychiatric disease in humans influence brain activity and behaviour, with the aim of informing new diagnosis and treatment approaches for schizophrenia and related disorders. Data generated during this project will be shared in the form of publications (open access preprints and peer reviewed journal articles), with non-proprietary behavioural and neurophysiology data advertised through a network of international collaborators and made open access for all interested parties.

Specific expected outputs include:

1. Back-translation of signatures of abnormal brain activity and cognition from people at genetically high risk of schizophrenia to genetically altered rat and mouse models.

2. Computational models of disordered neural circuit activity associated with increased schizophrenia risk.

3. Preliminary test data for novel drugs/interventions aimed at normalising abnormal brain activity in mouse models of psychiatric disorders.

Who or what will benefit from these outputs, and how?

Within the 5 years of this project, primary beneficiaries will be preclinical and clinical researchers working on the neurobiology of mental health in both academia and industry.

Ultimately, it is expected that the outputs of this work will benefit patients living with high genetic risk of psychiatric diseases through the development of more effective treatments. In so doing the work is expected to benefit all involved in the care of patients including their families, clinicians, health care providers and society.

How will you look to maximise the outputs of this work?

Sharing of experimental design through protocol pre-registration where appropriate.

Alignment of rodent work with analogous protocols using brain imaging and neurophysiology in patients.

Close liaison with Advisory Groups including patients and representatives.

Discussion of approach and results at public events and national and international academic conferences.

Timely publication of results as preprints and peer-reviewed articles. Collaboration with industrial partners on treatment development.
Liaison with clinical colleagues to optimise translation and patient benefit.

Species and numbers of animals expected to be used

- Mice: 500
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically altered mice and rats have been successfully established as models of genetic risk for psychiatric disorders. For example, mouse models of schizophrenia-associated genetic mutations show memory and attentional impairments, and abnormal brain waves comparable to those of human patients and thus provide a means of investigate the cellular mechanisms linking genetic risk to clinical symptoms.

Mental illness – particularly psychotic disorders like schizophrenia – most commonly first manifests during adolescence and early adulthood (around 10-25 years in humans). Both clinical and rodent work on this programme will therefore focus on the equivalent peri-pubertal time window, equating to approximately 30-50 days after birth in mice and rats.

Typically, what will be done to an animal used in your project?

Genetically altered mouse or rat lines carrying mutations simulating human psychiatric risk mutations will be bred to generate experimental animals of both sexes. The lines used are all well-established and are not associated with harmful phenotypes.

Following weaning, the animals will be habituated to the researchers by regular handling. The cognitive function and/or anxiety traits of the animals will be assessed using non-aversive behavioural tests.

Typically, cognition will be assessed using simple maze tasks and anxiety determined by studying their innate behaviour in an environment encompassing a choice between enclosed (“safe”) and exposed (“risky”) areas. Prior to assessment, the animals may have their access to food restricted to encourage them to explore the test arena to gain a food reward. Highly palatable food rewards will be used to minimise the need for food restriction. Typically, behavioural screening takes around 30 minutes and is conducted daily over a two-week period. Animals are not expected to show any adverse signs in response to screening.

Animals selected on the basis of their performance in the behavioural tasks, (~50%) will undergo a surgical procedure, performed under general anaesthesia, to either attach electrodes to the scull for electroencephalography (EEG) recordings (~70%) or to implant

electrodes and/or a cannula into a selected brain region (20%) or (in mice only) to implant a head fixation post and create a cranial port (10%). The animals undergoing these procedures are expected to recover uneventfully from surgery and to resume normal behaviour within a few hours. Following surgery, all animals will be given pain killers, until they are no longer showing any signs of pain, and will be singly housed to prevent the implants from being damaged by a cage mates.

Recording of brain activity usually begins 1 week post-surgery. Prior to recording, the animals will be gently restrained to enable the recording wire to be connected to the implant. Recordings are then made under three scenarios 1) while the animal is resting quietly in its home cage, 2) while the animal listens to non-aversive auditory tones played at different pitches and patterns, (replicating those used on human patients in the clinical arm of the collaborative programme) or 3) whilst the animal is exploring a maze to obtain a food reward. Recording sessions typically last between one and three hours and may be repeated on up to five occasions over a two-week period.

Mice implanted with a head fixation post and port will be used (once only), in a terminal electrophysiological recording session during which non-aversive auditory tones will be played at different pitches and patterns. Prior to testing the mouse will be habituated to restraint by the head post.

At the end of the study the animals will be killed under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Some behavioural assays expose animals to conditions that may cause mild transient anxiety – for example placing the animal on a narrow linear track raised off the floor – but these exposures are brief, typically around 10 minutes and do not have any lasting effect on the animal’s wellbeing.

For some tests, mild food restriction may be required to motivate the animals to search for a food reward in a maze. Highly palatable foods rewards will be used for these test (e.g. condensed milk) to minimise the need for food restriction.

Animals undergoing surgery are likely to experience post-operative pain. In all cases, the animals will be given pain killers until they are showing no signs of pain

The surgical implants used in these studies are of a size and weight that does impact the wellbeing of the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% Mild (behavioural testing only) 50% Moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brain is the most complex organ in the body. Whilst clinical phenotyping and brain imaging of patients, and in-vitro analyses using cultured neurons, do provide some insight into it function, it is not possible to resolve the underlying cellular mechanisms responsible for psychiatric disease or to assess

the effectiveness of novel drug treatment using these methods alone. Consequently, animal studies are an essential component of the programme of work for (i) determining the cellular mechanisms through which risk mutations impact brain activity and behaviour; (ii) obtaining the deep-brain recordings critical to assessing brain activity and its influence on behaviour; (iii) Determining the influence of specific genetic mutations and; (iv) assessing the effectiveness of novel drug treatments.

Which non-animal alternatives did you consider for use in this project?

Studies using:

1. Human volunteers
2. Non-regulated species e.g. Drosophila
3. In silico computational modelling
4. Electrophysiology in terminally anaesthetised animals

Why were they not suitable?

Human Subjects: This work is part of a research programme integrating brain imaging, neurophysiology (magnetoencephalography, MEG and electroencephalography, EEG) and behavioural testing in patients diagnosed with psychiatric disease and/or carrying genetic mutations associated with increased psychiatric risk. However, neither MEG nor EEG allow access to signals generated by regions contributing to disease symptoms that are located deep within the brain. Also, MEG and EEG measure neural population activity, but cannot pinpoint mechanisms of disease at the cellular or molecular level. Finally, patients present at a wide range of ages, making the systematic interrogation of neurodevelopment very difficult. The in vivo components of this programme therefore require back- translation into animals. Patient and animal work will be complemented by neurophysiological and biochemical studies in patient-derived neuronal cultures.

Non-regulated species e.g. Drosophila: The nervous systems of non-regulated species differ markedly anatomy and physiology from that of humans and therefore are unsuitable for studying complex neurodevelopmental diseases like schizophrenia.

In silico computational modelling: The use of modelling requires simplified approximations and cannot be used to generate the initial data for this project. However, the overall programme of work will use computational models to integrate data generated in animal

studies with that of patient studies. The resultant in silico models may form the basis for provisional assessment of novel drugs.

Electrophysiology in terminally anaesthetised animals: This can offer important insights into neural network activity however, general anaesthetics significantly alter the brain state thus obscuring or confounding the effects of genetic variation. Anaesthesia also precludes the assessment of behavioural measures. Where possible, work in freely behaving animals will be combined with in vitro studies of neural activity (e.g. in brain slices) and with computational models of brain activity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on a 15 year track record of related experimental work describing changes in brain network activity across different experimental states and taking into account related literature and advice from collaborators.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals used is based on analysis of previous data collected by the team, cross- checked with the NC3Rs Experimental Design tool and using power calculations and a statistical framework recommended by the institute's Statistics Clinic. Where possible, the design has been developed to collect multimodal data from a single set of animals, rather than running separate behavioural and electrophysiological studies in multiple groups. Capturing both waking and sleep data from individual animals also maximises efficiency and enables more powerful, intra-individual comparisons.

The experimental design has been peer-reviewed by statisticians on a UK Research and Innovation funding panel.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The methods used are highly efficient at gathering large volumes of data from individual animals using a within-subject design, meaning the number of individual animals used is kept to the minimum required to allow robust statistical analysis. Each experiment is mapped out from the outset, working with facility staff to assign each animal ‘Study Plan’ to optimise its contributions to the dataset, typically addressing multiple objectives during a single procedure (for example by recording during both behaviour and sleep).

Our breeding scheme is designed using a standardised colony management tool, such that every pup generated can be used as an experimental animal, either carrying a genetic

mutation or as a littermate control. We expect to require approximately 15 litters per genetically altered line over the course of the PPL.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mouse or rat lines carrying mutations simulating human psychiatric risk mutations (for example, Copy Number Variants involving deletion or duplication of 1-40 genes) will be bred to generate experimental animals of both sexes. The lines used are all well-established and are not associated with harmful phenotypes.

Following weaning, the animals will be habituated to the researchers by regular handling. The cognitive function and/or anxiety traits of the animals will be assessed using non-aversive behavioural tests.

Typically, cognition will be assessed using simple maze tasks and anxiety determined by studying their innate behaviour in an environment encompassing a choice between enclosed (“safe”) and exposed (“risky”) areas. Prior to assessment, the animals may have their access to food restricted to encourage them to explore the test arena to gain a food reward. Highly palatable food rewards will be used to minimise the need for food restriction. Typically, behavioural screening takes around 30 minutes and is conducted daily over a two-week period. Animals are not expected to show any adverse signs in response to screening.

Animals selected on the basis of their performance in the behavioural tasks, (~50%) will undergo a surgical procedure, performed under general anaesthesia, to either attach electrodes to the scull for electroencephalography (EEG) recordings (~70%) or to implant electrodes and/or a cannula into a selected brain region (20%) or (in mice only) to implant a head fixation post and create a cranial port (10%). The animals undergoing these procedures are expected to recover uneventfully from surgery and to resume normal behaviour within a few hours. Following surgery, all animals will be given pain killers, until they are no longer showing any signs of pain, and will be singly housed to prevent the implants from being damaged by a cage mates.

Recording of brain activity usually begins 1 week post-surgery. Prior to recording, the animals will be gently restrained to enable the recording wire to be plugged into the implant. Recordings are then made under three scenarios 1) while the animal is resting quietly in its home cage, 2) while the animal listens to non-aversive auditory tones played at different pitches and patterns, (replicating those used on human patients in the clinical arm of the collaborative programme) or 3) whilst the animal is exploring a maze to obtain a food reward. Recording sessions typically last between one and three hours and may be repeated on up to five occasions over a two-week period.

Mice implanted with a head fixation post and port will be used (once only), in a terminal electrophysiological recording session during which non-aversive auditory tones will be played at different pitches and patterns. Prior to testing the mouse will be habituated to restraint by the head post.

At the end of the study the animals will be killed under terminal anaesthesia.

Why can’t you use animals that are less sentient?

These studies require the use of an animal with a central nervous system that is anatomically and physiologically similar to that of humans and a behavioural repertoire sufficiently diverse to model that of humans. Consequently, it is only possible to conduct the outlined work using a mammalian species. Rodents are ideally suited because established genetic lines are readily available that provide translational validity and compatibility with high-density neurophysiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have extensively refined the procedures conducted under this licence over the past decade, to minimise the welfare consequences on the animals. Prior to the start of the study, the animals will be habituated to human contact by regular handling. The behavioural tests used are non-aversive and do not cause any harm. When food restriction is required, the animals are monitored by regular weighing and weight loss is restricted to a maximum of 10% of body weight compared to age match controls. In addition, highly palatable food rewards are used for these studies which further reduces the need for food restriction. The surgical procedures are all performed by experience surgeons and are undertaken in-line with LASA guidelines. Post-surgery, all animals are given pain killers until they are no longer showing any overt signs of suffering. The electrophysiology studies conducted post-surgery have no impact on the wellbeing of the animals. At the end of the study all animals are killed humanely under terminal anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs Experimental Design Assistant, ARRIVE 2.0 guidelines for design and reporting, institutional SOPs for handling and injections, LASA guidelines for aseptic surgery, comprehensive and contemporary review of published papers in the field.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive regular 3Rs newsletters from our local and regional NC3Rs champions and participate to my institutional 3Rs events. In addition, I monitor advances in best practice by regularly attending conferences in the field, liaising with national and international collaborators, and through weekly journal clubs appraising recent publications. I also maintain live collaborations with engineers and technology developers in the field, optimising opportunities to pilot and/or implement the most efficient and minimally invasive methods of neurophysiological data collection.

67. Underpinning Mechanisms of Energy Balance, Obesity and Comorbidities

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

obesity, neurobiology, type 2 diabetes, food intake

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to define key components and circuits regulating food intake, energy expenditure, body fat influencing health over the lifespan. This work will advance our knowledge of how these biological processes work and how they can ”go wrong” in obesity and related comorbidities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity is a global pandemic and rates have tripled worldwide since 1975. In the UK (and Europe), more people are now overweight or obese than are a normal healthy weight. Prolonged obesity and altered body fat increases the risk for type 2 diabetes, heart disease, stroke, fatty liver and dementia. For this reason, obesity represents one of the biggest medical and economic challenges facing this century. However, effective obesity prevention strategies and treatments are limited. For the past 20 years, most obesity medications have produced unwanted side effects that have limited their use. Our strategy is to define the underpinning mechanisms of energy homeostasis and body fat so that we can identify new targets to be exploited to improve health worldwide.

What outputs do you think you will see at the end of this project?

The work performed will provide new information of the effect of food intake, energy expenditure and body weight and how they contribute to health. This will be important for advancement of our understanding in the obesity and related diseases fields and lead to future strategies to improve health and prevent/treat disease. The data generated will lead to publications and presentation to the scientific community at both the national and international level. There will also be public outreach to disseminate research findings to the general public.

Who or what will benefit from these outputs, and how?

A wide variety of benefits will be obtained through the completion and dissemination of the research. For example, through previous work, we identified a completely new way that type 2 diabetes may be treated, a discovery that led to benefit in patients. Other targets that we have worked on have led to

new obesity medications. We have also discovered how obesity medications work to produce their therapeutic benefit which led to the development of better drugs. Therefore, patients are expected to benefit through this research. The NHS may also benefit because people may live longer and healthier lives. The scientific community is also expected to benefit from the research by providing new knowledge about how energy balance and body weight is controlled. And the general public is expected to benefit through dissemination about the effect of nutrition on health.

How will you look to maximise the outputs of this work?

We will look to maximise outputs of the work through dissemination. Dissemination will include formal and informal presentations to colleagues, collaborators and other academics about successful and unsuccessful approaches. Additional forms of dissemination will include presentations of the work at other universities/institutes and national/international conferences, to the public in scheduled events or via social media, meetings with government officials and/or industry. In addition, findings related from this work will be hypothesis driven and published in peer reviewed journals.

Species and numbers of animals expected to be used

- Mice: 5800
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

At the core of this project is the ability to observe and manipulate specific cells in the brain or body in order to investigate the links between these cells and metabolism regulation and body weight/fat. It is not possible (or ethical) to do this in humans; thus, we need to use an experimental model, and rodents are ideal because their physiology, like that of other mammals, is very similar to human physiology, especially when it comes to food intake. Humans share over 85% of their genome with rodents. In addition, there is already a very large body of knowledge acquired using rodents, and that is an advantage because we can add to that body of knowledge instead of starting from zero (as it would be the case if we were to use alternative animal models). The diet can have life-sustaining and life-hindering properties beginning in pre-conception and extending through old age. Therefore, we propose to study the effect of diet on health throughout the lifespan.

Typically, what will be done to an animal used in your project?

Most animals will be genetically modified. Some animals will be given different diets (for example, but not limited to, varying in fat and/or sugar) and/or drinks (for example but not limited to varying in tastants) and we will measure their intake; or food will be withdrawn or animals put on a feeding schedule. Rarely, we might adjust the light and/or temperature in the room, for example to simulate

seasons. Some animals may also be treated with medications. For example, medications that may be expected to reduce appetite, improve blood sugar and improve learning/memory. On a subset of rodents, surgery will be carried out to a very high standard by fully trained and competent staff. For example, some mice/rats will undergo a 60 minute surgery to provide a way to deliver medications or other substances rapidly. Animals may also undergo a minor (typically 15 minute) surgery to implant a device under the skin that can release a medicine or substance slowly. Animals will be given painkillers and post- operative care just like people recovering in the hospital. Following these interventions, metabolic health will be assessed. This may include procedures where physiology or behaviour is precisely measured in a home cage environment with single housing (e.g. standard home cages or modified cages that are transparent allowing animals to see and smell one another and including environmental enrichment). Single housing will typically be for less than 2 weeks, but may be necessary for up to 4 weeks. Body weight and body fat may also be examined, glucose, insulin or pyruvate tolerance tests may be performed, blood may be sampled and blood pressure may be monitored (parameters associated with healthy ageing). In a small subset of rodents, we may record how rewarding rodents find high value foods such as those that people like and how food and brain circuits influence behaviour and cognition. Some animals will be genetically modified and will have no further intervention.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect that many of our interventions will improve obesity and health. As with people who have had surgery, we will manage possible post- operative pain with pain medications. We expect rodents to fully recover from anaesthesia and surgery within 24 hours and do not expect any changes in behaviour or physiology from the anaesthesia or surgery. In very rare situations animals might have hypersensitivity to anaesthesia.

Since the objective of this project is to clarify how food intake and body fat is regulated, we expect that our interventions (including but not limited to dietary, genetic, gene inducing/deleting, surgical, medicines (delivered by different routes), ageing/lifespan, photoperiod etc) will produce changes in feeding, body weight, body fat, metabolic health, cognitive health and/or general health. We also expect to see alterations in feeding patterns and energy expenditure. These changes may last for the full duration of the experimental tests or be transient. The precise measurement of energy homeostasis may require a period of single housing which we expect may cause stress. To minimise stress, animals will be provided with enrichment items, nesting and will have sensory access to other animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Up to 5800 mice and 1000 rats will be used. Specifically,

We expect 35% of mice and 70% of rats to undergo procedures classed as subthreshold.

We expect 65% of mice and 30% of rats to undergo procedures with a maximum severity limit of moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present, there is no alternative to animals for studying the complex processes of appetite, food choice, amount of food consumed, body weight and the regulation of metabolism. For example, cells can’t choose a biscuit over broccoli and can’t gain or lose body fat.

Which non-animal alternatives did you consider for use in this project?

Humans are not suitable for this work because the current technology is not sufficient to identify specific cells regulating energy balance. For example, fMRI can only identify gross brain regions that are activated in response to meals, but cannot provide any further information. We are already aware of the general brain regions and tissues regulating energy balance and body weight. What is now required is an identification of cells within these broad regions so that we can identify specific targets for the treatment of obesity and related diseases.

In principle, it should be possible to gain insight into complex mammalian behaviours using computer models specifically constructed for this purpose; however, no such model exists yet.

Why were they not suitable?

The technology is not yet advanced enough. For example, we do not yet have computer models sophisticated enough to be able to simulate complex mammalian behaviours and metabolism.

Moreover, to be plausible, computer models must be informed by the rules of biology: we first need to gather biological knowledge via experimentation (as outlined here) if we ever want to build useful computer models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals required for the experiments has been calculated based on past experience, scientific evidence and/or previous studies. Numbers have been based on work we have already done similar to that described in this project, discussion with colleagues and reading the peer-reviewed published literature. The number of animals needed for breeding purposes was calculated with the help of the staff at our animal breeding facility, and are also based on experience and best practices.

We anticipate that the majority of mice in Protocol 2-4 will come from breeding Protocol 1 and minority will be purchased. We anticipate that rats in Protocol 2 will come from either breeding Protocol 1 or purchase.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing the experiments, we perform statistical analysis to ensure that we use the minimum number of mice or rats per group that will be informative using power analysis and consultation with a statistician, where necessary. We also use online tools such as NC3R's Experimental Design Assistant when needed. We use the most stringent and rigorous techniques to make sure that our experiments are performed to give us clear answers to ensure against unjustified duplication of procedures. We also consult the literature and manufacturers of products to guide us to optimal experimental conditions before embarking on any experimentation. We also strive to reduce the numbers of rats and mice through breeding techniques to give us the experimental genotype every time, as long as this genotype isn't harmful. We also use in vitro and ex vivo techniques where possible to study specific cells in detail.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding practices will be as efficient as practically possible in order to minimise the number of experimental animals, including intermittent breeding, breeding for an experimental cohort and breeding for colony maintenance strategies as dictated by the strain and experimental need at any given time. Where needed, we will refresh the background strain in genetic lines to avoid genetic drift. We will also use tissue taken post-mortem for ex vivo/in vitro analysis as often as possible; this is to maximise the amount of useful experimental data obtained from each animal. In addition, pilot studies will be conducted if necessary to ensure that we can plan experiments in the best possible way.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rats and mice, both wild-type and transgenic. Our transgenic models do not cause suffering, distress, or harm. Most of our lines are phenotypically wild type. A small number may have genetic alterations in behaviour (e.g. energy balance) and/or body weight (e.g. lean or obese) and related behaviour and physiology. A small number require a gene induction protocol.

Some of our methods involve surgery. Such surgical intervention is the only method currently available to achieve the labelling and manipulation of cells in the brain required.

In addition to surgery, our methods also involve metabolic and behavioural analysis. These methods are based on observing natural behaviours (e.g. food/drink intake/choice, physical activity, energy expenditure, for example measured in the home cage or specialised TSE cages) and are thus unlikely to cause anything more than transient distress. The diet may be altered in both composition, amount and time of availability. Some rodents will have an implant in their head, used to manipulate or monitor brain cell activity. There are no known alternatives to using these implants, but they are not painful and do not cause any harm.

We assess body weight with balances and body fat/body composition with equipment such as DEXA and Echo-MRI. Body weight measurement can be performed with cupping and a balance. Body composition with Echo-MRI does requires brief restraint and DEXA requires anaesthesia. DEXA produces additional information to Echo-MRI, such as bone density, an image of body fat deposition, etc. and is used when this additional information is experimentally required and justified. It is usually experimentally necessary to collect body weight and body composition data at various experimental timepoints.

We will typically administer drugs by injection. This is the most effective way to give a drug to rodents; despite the needle, this method ensures the correct drug dosage. Occasionally, a constant dose of drug is needed and in this situation a minipump may be used. In some cases we will take blood samples (e.g. to measure glucose levels). This again is

unavoidable; no alternatives exist for measuring metabolites in blood or plasma. The expected distress is only transient.

Some interventions are expected to cause obesity in our experimental animals, but this is not expected to be severe: the degree of obesity will not cause pain or suffering. Some interventions are expected to improve obesity or reduce body fat and this will not be to a degree that will impact welfare.

Why can’t you use animals that are less sentient?

We want to study how the body and brain modulate behaviour and metabolism in mammals. This is only possible with freely-moving, awake mice/rats; terminally-anaesthetised rodents is not an option.

Non-mammalian species do not share the brain structures that are relevant for food intake regulation in mammals. These structures are an important part of the object of study in this project. This makes it impossible to use other, less sentient species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use tubes and cupping to handle rodents except where this isn't possible or desirable (e.g. handling to habituate rodents to the experimenter).

We have implemented a refinement in blood sampling with a tail prick as opposed to removing a small portion of the tail.

Animals that undergo surgical procedures are likely to experience a degree of post-operative pain or discomfort. This will be managed with appropriate perioperative care and pain relief. All animals are expected to fully recover from surgery within 24 hours. Because surgical interventions may be experimentally designed to influence energy homeostasis (for example, food intake) and/or body weight, this may be affected without being an indication of welfare concern/poor recovery. Following surgery, the animals will be checked on a daily basis for signs of ill health or distress to ascertain their recovery. Signs of ill health or distress (excluding changes in food intake or body weight) will be brought to the attention of the NACWO and/or NVS. Additional post-operative care may involve providing easily accessible soft food (wet food), to facilitate food consumption.

For our behavioural experiments, we will habituate the animals to their new, temporary environment (e.g. the cages (such as TSE) where food intake is measured). We will also handle these animals regularly to habituate them to the interaction with the experimenter, and we will use tube handling and cupping to minimise distress. Different types of animal housing (single, pair, group) will be considered in advance of each experiment, on a case by case basis, depending on the scientific outcome required. Most experiments involve group housing and only temporary single housing. Unless experimentally required, animals will be group housed in recommended husbandry and care conditions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For general guidance, we will consult the resources page on the N3CRs website (https://www.nc3rs.org.uk/3rs-resources) and the related videos available from https://researchanimaltraining.com/article-categories/procedures-with-care/.

For surgery, we will follow the Guiding Principles for Preparing for and Undertaking Aseptic Surgery, published by the Laboratory Animal Science Association (LASA), http://www.lasa.co.uk/publications/.

For planning and reporting experiments, we will follow the ARRIVE (https://arriveguidelines.org/) and the PREPARE (https://norecopa.no/prepare) Guidelines.

For substance administration, we will follow LASA guidelines and local guidelines (e.g. https://researchanimaltraining.com/wp-content/uploads/2021/05/lasa_administration.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will make sure that my team and I keep up-to-date by following the Latest News on the NC3Rs website (https://nc3rs.org.uk). Any advances relevant to the project will be discussed with the Named Persons at our University in order to implement these promptly and effectively.

68. Understanding Synaptic Dysfunction and Resilience in Neurodegenerative Diseases and Ageing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Alzheimer's, synapse, ageing, neuroscience, neurodegeneration

Animal types	Life stages
Mice	neonate, adult, juvenile, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Loss of synapses, the connections between neurons in the brain, is the strongest predictor of cognitive decline in Alzheimer’s disease and healthy synapses are important to avoid cognitive decline during ageing. In this project, we aim to better understand the reasons that synapses become dysfunctional and die in ageing and diseases like Alzheimer’s disease and related neurodegenerative disorders. The long-term goal is to develop therapies to prevent or reverse synapse loss to help people living with neurodegenerative disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Losing cognitive function is one of the most feared aspects of ageing, and age is the biggest risk factor for dementia. Dementia is one of the leading causes of death in the UK, and a growing problem for our society as we are living longer. Dementia is a devastating,

progressive decline in mental function that is caused when the brain is damaged by diseases such as Alzheimer's disease (AD) and related neurodegenerative disorders. In the UK, we spend over £30 billion per year caring for people with dementia, and currently, we do not have any treatments that can stop the devastating progression of the underlying diseases. The symptoms of dementia result when the cells in the brain are damaged and can no longer communicate effectively. Currently we do not fully understand these changes in the brain, which is why we do not have effective treatments. In this project, we aim to better understand the brain changes that cause cognitive decline during ageing, Alzheimer's and related diseases in order to develop effective ways to prevent or treat these devastating conditions. To help with reading this license, a glossary of terminology is included below.

Terminology

Neurons: Brain cells that form networks to allow mental functions such as memory and thinking. Neurons can be excitatory – which make other neurons more active, or inhibitory – which make other neurons less active.

Synapses: The contact points between neurons where they communicate. Changes in synapses allow learning and memory. The loss of synapses strongly predicts the loss of mental function in AD.

Glia: Support cells in the brain that maintain the blood brain barrier, provide nutrients and support for neurons, and make up the brain's immune system. Astrocytes are a type of glia that make up the

blood brain barrier, provide nutrients to neurons, and help maintain synapse function. Microglia are immune cells which clear toxic substances from the brain. During diseases like AD, glia work to maintain the health of neural networks, but eventually the disease processes make the glia harmful in a process referred to as inflammation.

Amyloid beta (Abeta): One of the proteins that aggregates abnormally in the brains of people with AD. Abeta forms large clumps called amyloid plaques in the brain. Genetic mutations that increase levels of Abeta cause rare forms of familial AD. These genes are used to create animal models of the disease.

Tau: The other protein that aggregates in AD and related diseases called "tauopathies". In tauopathies, tau becomes hyperphosphorylated and clumps into neurofibrillary tangles inside cells. These tangles spread through the brain jumping from neuron to neuron. As the tangles spread, disease symptoms worsen. Genetic mutations in tau cause a tauopathy called frontotemporal dementia and are used to make animal models of disease.

Apolipoprotein E (ApoE): ApoE is a protein important for cholesterol transport in the brain. It has 3 forms, ApoE2, 3, and 4. Inheritance of ApoE4 increases the risk for Alzheimer's and other neurodegenerative diseases.

Neurodegeneration: The loss of function and eventual death of synapses, neurons, and neuronal networks.

What outputs do you think you will see at the end of this project?

At the end of this project, we will have new knowledge about why synapses are lost in ageing and neurodegenerative diseases including Alzheimer's disease and other forms of dementia. This new knowledge will be published in scientific journals and the data will all be shared for free with scientists around the world so that they can build upon the new knowledge. It is possible that this project will result in new drugs to prevent or reverse synapse degeneration. We certainly work with industry and drug discovery agencies to try and translate our neuroscience work into treatments that will help people living with dementias.

As an indication of our ability to produce these benefits, during our preceding PPL from which this project develops, we have produced the following key outputs:

- Disseminated new data and knowledge through >30 peer-reviewed manuscripts (>10 using animal models and techniques described in this PPL), >20 presentations at scientific meetings and >20 invited seminars at academic institutions and companies
- Engaged pharmaceutical companies interested in using our models and methods to validate candidate therapeutics
- Influenced policy through work with professional associations.

Who or what will benefit from these outputs, and how?

We will use rodent models of dementias to study changes in the brain and to try and reverse damage.

The short term and highly likely benefits of this project include: The advancement of knowledge about dementias, knowledge shared with other scientists and drug companies that they can then use for further advances, scientific publications which are open access - raw data from experiments shared freely on web-based repositories. These short term benefits will mostly be used by other scientists.

Medium term benefits may include refinement of models of dementia useful to the entire field and identification of new molecular targets for therapeutics. This will mainly affect other scientists.

The longer-term potential benefits include: Development of disease-modifying therapeutics based at least in part on the work in this PPL that will help people with dementia; Influences on government policy about how to help people with dementia and how to fund the best types of research. These long term benefits will help clinicians, patients, and policy makers.

How will you look to maximise the outputs of this work?

We will maximise outputs by:

- Making all publications open access
- Sharing data freely from all papers
- Using preprint servers to share papers in advance of formal peer review
- Publishing negative data/unsuccessful approaches

Species and numbers of animals expected to be used

- Mice: 46,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice for these experiments as they are vertebrates that share many aspects of brain structure with humans. Mice are also used because of the availability of genetically modified strains that express genes that cause Alzheimer's and related diseases. We use adult/aged mice to model some aspects of these age-related diseases in an intact brain and some younger animals (pups) in experiments to culture brain slices and expose them to human Alzheimer's disease brain tissue to model the disease. We need to look for some things in ageing, intact brain to most accurately reflect the disease, but as a refinement and reduction approach, we have developed a brain slice model that is a stepping-stone before aged mice that accurately models some disease aspects. Many slices are generated from each mouse pup, reducing the number of animals needed for these experiments.

Slices grow best from young animals and are kept ex vivo in culture incubators for months to allow study of a limited amount of ageing.

Typically, what will be done to an animal used in your project?

Typically, mice carrying genes involved in dementias will be bred in our mouse unit. Most of the mice will be humanely killed as pups in the first or second week of life to make brain cultures. Some mice will be aged until they are adults then undergo behavioural testing to examine the effects of disease on brain function. There are potential harms with ageing mice with genetic alterations including some decreases in body weight and hunched posture, much like those experienced by ageing humans.

Ageing animals will be carefully monitored and will be humanely killed if the phenotypes exceed thresholds defined in the licence. Some animals will receive an injection into the brain to model or treat disease phenotypes or a surgery to implant a window to allow imaging of the brain as disease progresses. These surgical treatments have rare potential harms including infections or excess bleeding. During surgery, if there are major complications, animals will be humanely killed while still under anaesthesia. After surgery, animals will be carefully monitored and will be humanely killed if the phenotypes exceed thresholds defined in the licence. Some mice will receive treatments of experimental drugs to try and recover disease phenotypes. These treatments could have side effects, so mice will be carefully monitored and humanely killed if effects exceed thresholds defined in the licence. Each mouse will live for 3-6 months after the procedure. In some cases, mice will have both a surgical procedure and non-invasive cognitive testing.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of the mice on this licence will not undergo potentially harmful procedures as they will be used for breeding or post-mortem tissue collection after humane killing. We plan some mild procedures like cognitive testing. We also plan some moderate procedures including recovery surgeries to induce models of dementia and to observe brain changes and recovery with treatments over time. We plan some mild experiments to examine behavioural changes and treat mice with drugs that might help the dementia like symptoms and brain changes. We do not expect any common adverse effects from these procedures. Rarely, our procedures may have adverse effects such as infection after surgery or side effects from treatments. Any animals experiencing adverse effects will be examined by a veterinarian, and if the effects cannot be alleviated, the animals will be killed humanely. At the end of experiments, animals will be killed humanely. Some of our genetically modified mice may be provided to another Project Licence if appropriate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals in this PPL are expected to be within the sub-threshold category approximately 90% with the remainder of the animals experiencing a mild (approximately 5%)and moderate severity (approximately) 5%.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have consulted resources on the FRAME website and Altweb to identify any viable replacement possibilities, as outlined below.

The mouse work in this programme is part of a wider effort incorporating experiments in human post- mortem brain, human stem cell derived neurons, human resected brain tissue, and fly models of disease. Only by taking multiple approaches will we be able to come to a better fundamental understanding of disease that will lead to disease modifying therapeutics. The animal experiments are essential. Since the diseases and functions we study are neuronal, experiments cannot be done on cells which do not have neuronal functions. Wherever possible, we will carry out pilot work (e.g. Viral vector construct development) in non-animal cell lines.

The proposed project aims to study synaptic function and loss and how different brain cell types affect this during ageing and disease. The project thus requires tissue in which the neuronal networks and their glial support systems remain largely intact. Neither non-animal alternatives, such as human stem cells that have been differentiated into neurons,

nor cultured primary neurons, can provide information regarding intact network function in adult tissue and so cannot provide a viable alternative for these experiments. For some screening work, we are using fruit flies which do have an intact brain, however, these are invertebrates that do not have all of the same brain cell types and structures as humans, so the most promising fly work needs to be validated in mice before moving to humans.

The examination of synapse loss and dysfunction around plaques requires the formation of senile plaque pathology, which cannot currently be accurately recapitulated in vitro. These lesions form in an age-dependent fashion relevant to human AD, which is a disease of brain ageing. Thus, to test the important hypotheses about mechanisms of synaptic degeneration and to test potential therapeutics, we require intact, ageing brain with circuitry similar enough to humans to test memory function. Mice and humans share the basic brain structures involved in memory thus making them a meaningful model species. They are also amenable to genetic manipulation, which allows the introduction of genes that cause human disease into the mouse genome resulting in recapitulation of both pathology and memory impairments.

As a replacement, we undertake extensive work on post-mortem human disease and control brain tissue in a bank that we use in parallel with our mouse and fly studies. We design studies that will enable us to test whether the mechanisms we are investigating in mouse models are important to human disease. We have also recently developed and published a human stem cell derived neuronal model of synapse degeneration which is a promising new development that may eventually be able to completely replace the use of mice for dissociated cell cultures. However, these cultures cannot replace mice for in vivo behavioural and circuit work.

Computer modelling of network/synaptic dysfunction is not a viable replacement alternative as the necessary data to build these models is not yet available. However, data from these experiments will inform modelling work.

Which non-animal alternatives did you consider for use in this project?

As above we have considered and indeed use fruit flies, human stem cells, and human donated brain samples for complementary experiments.

Why were they not suitable?

The examination of synapse loss and dysfunction around plaques requires the formation of senile plaque pathology, which cannot currently be accurately recapitulated in vitro. These lesions form in an age-dependent fashion relevant to human AD, which is a disease of brain ageing. Thus, to test the important hypotheses about mechanisms of synaptic degeneration and to test potential therapeutics, we require intact, ageing brain with circuitry similar enough to humans to test memory function. Mice and humans share the basic brain structures involved in memory thus making them a meaningful model species. They are also amenable to genetic manipulation, which allows the introduction of genes that cause human disease into the mouse genome resulting in recapitulation of both pathology and memory impairments.

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Computer modelling of network/synaptic dysfunction is not a viable replacement alternative as the necessary data to build these models is not yet available. However, data from these experiments will inform modelling work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our scientific questions, we have designed experiments with animal numbers based on previous similar work and statistical calculations. All of our experimental designs incorporate looking at effects of sex since dementia is more common in women and there are likely important biological effects of sex.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We adhere to the ARRIVE guidelines designing and reporting animal research to ensure best practice in using enough animals to answer a scientific question without using too many and use online tools such as the NC3Rs experimental design assistant to inform numbers needed.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have taken several additional measures to reduce animal numbers: We routinely share tissues from individual animals that are killed for ex vivo work between experimenters in order to maximise the data collected from a single animal.

Slice culture experiments reduce numbers of animals as multiple slices are made from each individual mouse, allowing treatment and control conditions to be run in parallel from the same animal which reduces variability as well as reduces animals needed.

Behavioural and in vivo imaging experimental designs reduce animal numbers needed for each experiment as the brain is tested at baseline in each animal allowing it to serve as its own internal control. For example, we can image synapse density and assess memory function before and after drug treatments in the same animal allowing conclusive observation of whether these phenotypes recover. This avoids the need for large cohorts that would be needed to statistically show an improvement post-mortem by comparing drug and vehicle treated groups.

Wherever possible, an individual animal will be used for in vivo, ex vivo and post-mortem experiments. This practice will reduce numbers, and increase power of the data. This is a

particularly powerful approach with the collaborators within our centre as we will be able to correlate data about memory (from behavioural studies), synaptic function and plasticity (from in vivo imaging, electrophysiology and slice culture), synaptic structure (from in vivo imaging studies and post-mortem reconstructions of neurons), and synaptic volume and protein composition (from post-mortem array tomography studies). All of these data can be acquired within a single animal allowing for a complete picture of how synapses function in the brain, become dysfunctional in AD, and whether they can recover with treatments, from the molecular to behavioural level.

As mentioned in the replacement section, we will also use human brain tissue, human stem cell derived neurons, and fruit flies to refine our experiments and use mice only for the most promising questions. To the extent possible in post-mortem brain, we will confirm that the molecular pathways we plan to test in animal models are relevant to human disease. For example, if we do not see increased tau levels in human synapses in AD, we would not pursue this as a therapeutic target to manipulate in our mouse model, thus reducing numbers of mice used in experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of experiments will be carried out in models of dementias. The mice exhibit many of the pathological changes of these diseases, and so provide an appropriate model for the study of the consequences and causes of synapse loss.

The plaque bearing lines (APP/PS1, and APP/NLF/NLGF) have been specifically selected for several reasons. Firstly, these lines all develop amyloid pathology and synapse loss using different genetic approaches and with different timescales. This is important as some findings from a single mouse line have failed to be replicated by other groups in other lines, meaning they are more likely to be an artefact of the genetic manipulation than of the disease we are studying. As a field, we have come to the consensus that work needs to be replicated both by multiple scientists and in multiple transgenic lines. We also use mouse lines expressing genes that increase risk of dementias. For example, the APOE targeted replacement and knockout lines replace mouse Apoe with the humanized version of APOE in its three alleles, or knock out mouse APOE. These mice are essential for understanding the role that the strongest genetic risk factor for Alzheimer's plays in neuronal circuit dysfunction and are currently the only lines available for these studies. Tau transgenic lines recapitulate some of the tau pathology and cell death seen in Alzheimer's and other tauopathies, and will allow the investigation of mechanisms of tau induced circuit dysfunction, the spread of tau, and the role of glia in these processes. Viral constructs and bigenic promotor/responder lines will be used to introduce pathological proteins into specific brain regions to allow the study of the spread of proteins through the brain and the effects of reducing protein expression. Models of neurodegeneration will be combined with reporter lines allowing us to interrogate circuit function. We are very experienced in the

design and use of animal models to address specific questions about the disease and in relating them to human phenotypes and will use this experience to choose the most appropriate model for each scientific question.

Why can't you use animals that are less sentient?

For some questions, we can and do use fruit flies and mouse pups to make slices, However, aged living mouse models recapitulate AD age-related brain pathology that cannot be done without a living brain. Plaques and tangles similar to those in the human brain cannot be produced by cells in culture, and behavior cannot be tested in brain slices or cultured human cells.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be housed in groups wherever possible, and cages will be enriched with tubes and objects for exploration. These methods have been shown to reduce stress in mice. Up to 4 animals can be housed in a single cage even after surgical procedures such as electrode or cranial window implantation as long as mice are appropriately monitored.

For slice culture studies, animals are killed as pups and brain slices kept alive in an incubator for several months, which avoids the animals experiencing any procedures in life other than sacrifice. Slices can be made from any of the transgenic lines mentioned above and viruses can be introduced into them for further study.

For our in vivo drug treatment studies, we refined oral dosing. Instead of giving compounds by gavage (as requested by the pharmaceutical company based on the literature) or intraperitoneal injections, which is stressful, we worked with the veterinary staff to use drug dissolved in jelly. This is much less stressful and allows for accurate drug dosing based on weight. We are finding this an effective and less potentially harmful way to administer substances and plan to continue this wherever possible in this project.

We plan to use surgery to inject viruses, and implant electrodes and craniotomies and head posts for imaging. Surgical procedures will be conducted using appropriate aseptic techniques and general anaesthesia will be maintained throughout. Analgesia will be administered before starting surgery after the anaesthesia induction and post-operatively. Mice that recover from surgery for further recording or imaging will be monitored closely and sacrificed if adverse effects are found as detailed in the protocols.

We plan to have chronic experiments like behavioural studies and imaging of the brain through a cranial window for up to 12 months. The in vivo imaging will allow longitudinal studies of synapse degeneration and whether it is preventable and reversible with candidate treatments. This preparation has been used by our group for 10 years and is very well tolerated by mice. Potential problems include the glass coverslip and head post coming off or infection under the coverslip (which are both very rare occurrences). If these problems arise, the mouse will be immediately sacrificed as detailed in the experimental protocols. Despite these potential problems, we feel the refinement of using a longitudinal technique, which reduces the numbers of mice needed for each study, is worth the rare complications.

Animals not killed under schedule 1, will be killed under general anaesthesia to minimise distress. Non- schedule 1 methods such as exsanguination, decapitation and fixative perfusion will be used as required to optimise electrophysiological recordings or

immunohistochemistry. These will be under terminal anaesthesia excepting the decapitation of pups, which for scientific reasons cannot be done under anaesthesia (as it affects the quality of the cell cultures).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We adhere to ARRIVE guidelines and NC3Rs principles and use the NC3Rs experimental design tool to ensure refinement.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our PPL holder and PIL holders working on the project attend annual refresher courses on the 3Rs and discuss experimental design routinely at our weekly group meetings.

69. Brain Network Mechanisms of Memory

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Memory, Brain, Neuronal dynamics, Neural pathways, Behaviour

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to reveal how brain nerve cells cooperate to support memory-guided behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A growing body of research is now showing that memory is far more extensive than simply storing and retrieving information about life experience that has already happened, and thus picturing the past.

Indeed, the brain leverages our memories to also anticipate future situations, thereby enabling us to make choices most appropriate to ongoing circumstances and avoid repeating actions with negative consequences. However, the biological mechanisms allowing the brain to support such adaptive, memory-guided behaviour remains unknown. Notably, this gap in our knowledge about brain and behaviour has important implications for understanding what goes wrong in conditions where memories are not formed appropriately or, in contrast, expressed inappropriately. What prevents us from reaching a

comprehensive understanding of the brain mechanisms supporting memory-guided behaviour? Perhaps the two greatest challenges to this scientific endeavour are (1) it is not possible to record brain cell activity in behaving humans under physiological (non-pathological) conditions; and (2) the brain operations performed on mnemonic information rely on a complex division of labour amongst a large diversity of nerve cell types. Accordingly, the general aim of this project is to measure the activity of diverse brain cell populations and identify the neural pathways controlling their neuronal cooperation during memory processing. This work will be delivered using small animals (adult mice) to reveal the biological mechanisms allowing the mammalian brain to acquire, store, retrieve and utilise memories for adaptive behaviour.

What outputs do you think you will see at the end of this project?

Scientific publications of new knowledge

Who or what will benefit from these outputs, and how?

The scientific outputs of this project will primarily benefit the academic community:

Short-term: new discoveries that drive forward neuroscientific research to answer scientific questions at the nexus of brain and behaviour.

Long-term: new insights into the neural mechanisms that are targeted in conditions yielding defective memory.

This gain in knowledge will also benefit the general public since our improved understanding about how the brain supports memory and behaviour will be communicated and discussed during outreach events.

How will you look to maximise the outputs of this work?

By means of collaboration, sharing of datasets and codes, dissemination of new knowledge during conferences, seminars and public lectures

Species and numbers of animals expected to be used

- Mice: 10000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will involve using small adult rodents (mice) because: (a) they are the most widely used animals that have sophisticated spatial and other types of behaviours; (b) the anatomy and physiology of the adult mouse brain is well described; and (c) the proposed procedures have been specifically designed for and successfully used in this specie and life stage (adulthood). We will use adult mice that are 3-9 months old to allow investigation of memory mechanisms that can be related to those in the adult human brain.

Typically, what will be done to an animal used in your project?

Genetically altered mice without harmful phenotypes will be bred.

Animals may receive an intracranial injection under general anaesthesia and allowed to recover for up to 10 days. They will then undergo intracranial surgery to implant a micro device that will accommodate the analyses of neurons in the brain, or they may have a device implanted to assist in restraining the animal.

Animals will be trained to perform simple behavioural tasks such as exploration of a spatial arena or learning that a sensory stimulus signals the delivery of a food reward to collect. During these tasks, the animal may be freely moving or may be restrained by fixing of the head. When performing a behavioural task, freely behaving animals will typically also be able to rest and sleep for long sessions in a familiar home cage environment. Tasks will be performed for up to 4 hours per day and for up to a maximum of 6 weeks. Habituation to head fixing is typically 5 days and during experiments animals will be head fixed for no longer than 120 minutes per day for up to 3 weeks. Animals may be motivated to perform the tasks by controlled access to food and water but not allowing their bodyweight to drop more than 15% from its starting weight.

Craniotomies may be performed to insert recording electrodes in up to 6 regions of the brain or they may be given intracranial injections to induce a targeted manipulation of the neurons in the brain.

We will always allow full recovery of up to 10 days between each general anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Following cranial surgery, animals are expected to make a rapid recovery from the anaesthetic within two hours. This will be marked by their continued ability to eat, drink, rear, groom and perform any other fundamental behaviours. The animal can yet experience discomfort and pain from the wound for a few days. This will be managed by administration of analgesics, as it is the case for humane surgeries. Full recovery is expected within 7-10 days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild: 20%
- Moderate: 80%

What will happen to animals at the end of this project?

- **Killed**

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present there is no possible replacement for small rodent-related experimental work aiming at defining how neuronal networks operate within the brain to support memory-guided behaviour. In the proposed work we will use mice which show structural and functional equivalents to the human brain, despite differences in brain size. It is however not possible to perform brain microcircuit recordings of well-defined neuronal populations in behaving human beings. Importantly, there is also excellent availability of diverse mouse genetically altered strains for the proposed anatomical work and for use of causal manipulations.

Which non-animal alternatives did you consider for use in this project?

We have considered in silico simulations, recordings in humans, and in-vitro preparations.

Why were they not suitable?

In silico simulations are not suited for this project as they do not fully capture behaviour-related brain dynamics, and our experiments require actual quantification of behavioural performance in memory tasks. On occasions, we might however be able to use them to complement animal recording experiments by simulating the collective activity of larger cell populations in silico.

Recording brain activity in humans is currently restricted to the use of coarse non-invasive tools which measure the average response from thousands of cells. Therefore, activity at the level of well-defined cell types cannot be readily inferred from non-invasive measures in humans.

In vitro recordings provide limited insight as brain networks do not necessarily remain intact and animal behaviour cannot be preserved. On occasions, we might however be able to use them to complement animal recording experiments by assessing plastic changes occurring at the level of synapses.

To address the objectives of this project there is currently no alternative model for the recording of brain activity associated with cooperation between diverse well-defined neuronal populations, higher- order cognition and behaviour. It is therefore necessary to use animals to meet the aims of the project. This project will nevertheless generate valuable data sets that can in turn be used to inform future studies performed using the above alternative methods.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the numbers of mice required for our project primarily based on our last 15 years of extensive work in the field of behavioural neurophysiology. These numbers will ensure delivering our scientific objectives in a robust manner. This is notably achieved by the development of our multi- neuron recording technique, a high-throughput approach that leads to the collection of large datasets from each individual on each day.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The first step we have taken relates to the design of our rodent behavioural tasks. In these tasks, the experimenter will be able to take multiple independent recordings from each individual mouse, thereby reducing the total number of required animals. Moreover, we are using a within-subject design where each mouse is its own control. This will reduce statistical variance, further reducing the number of animals used.

Our experimental design also leverages techniques for blinding and randomisation, which will give better results and reduce the need to repeat experiments. Neural and behavioural data analyses will be conducted in an identical way regardless of the identity of the experimental condition from which the data are collected, with the investigators blind to group allocation during data analysis of experiments.

To avoid experimental bias, randomization will be implemented using computer-generated sequences. This includes randomizing allocation of animals to different experimental conditions; randomizing the assignment of stimuli to each mouse; randomizing the order in which stimuli are presented in the recording sessions.

To further reduce experimental biases and thus reduce mouse numbers, other variables will be held constant, including animal housing and handling protocols which will be logged across all experiments. Testing will be conducted around the same time each day. Variables that are more difficult to control, such as food intake relative to body weight, will be logged and accounted for in experimental analyses using appropriate statistical methods. Indeed, numbers will also be kept minimal because in addition to the multiple recordings made from each animal, we will collect multivariate data to allow application of rich, multifaceted data analysis techniques such as multiple regression and bootstrap-coupled estimation of effect sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

We also typically analyse data rapidly after each data collection. This approach effectively allows the initial experimental cohort to be used as pilot data for subsequent experiments. This reduces the number of animals used by ensuring that the project is fully optimised to address the aims of the study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project will use small rodents (adult mice) because: (a) they are the most world-wide used laboratory animals exhibiting sophisticated memory-guided behaviour; (b) the mouse brain is currently the best described mammalian brain with respect to neuronal anatomy and physiology; and (c) the proposed procedures have been specifically designed for and successfully validated in this specie.

These mice will behave in memory tasks while we will perform in vivo multichannel recording of brain activity. This recording technique involves a microdrive that stands as the product of a series of refinements by our laboratory since 2011. It represents state-of-the-art in this field of research and features our continued effort to refine material and design. Implanted mice typically express normal behaviour, being free of their movement as shown by their ability to explore an entire open field arena in a few minutes and voluntarily eat, drink, groom, nest, rear, and sleep/rest.

We will combine this behavioural neurophysiology approach to optogenetic interrogation in order to access causality between brain activity and behavioural response. Optogenetic allows identifying and manipulating well-defined types of neurons, thereby relating the activity of specific populations of neurons with behaviour and memory processing. The technique involves the injection of adeno- associated viral vectors to deliver a cell-specific promoter with the aim to transfect specific classes of neurons with light-activated ion channels and its fluorescent marker. This procedure has been widely used by the neuroscience community and there is no indication of specific adverse effects.

Why can’t you use animals that are less sentient?

We use adult animals rather than juvenile animals as this project focuses on the cellular activity that underlie adaptive behaviour in the mature mammalian brain. The hippocampus and related brain circuits, which are the focus of this project, are also not fully developed in more immature life stages.

We investigate higher-order neuronal operations performed by brain circuits centred on the hippocampus and therefore we cannot use non-mammalian animal models such as fish or invertebrates as these do not share the same brain circuits as adult mammals.

Brain recordings under terminal anaesthesia will not allow carrying our research project as behaviour- related neuronal activity will not be preserved.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will train animals in their behavioural tasks, and familiarise them with the recording environment to promote effective learning.

Our surgical procedures will continue to undergo refinement by liaising with our veterinary

surgeon. Analgesia is given prior to surgery and continues until the animal is fully recovered. Warmth and easily accessible food and fluids are also provided. Implanted animals will be housed alone as cage-mates can potentially damage the recording electrodes. However, animals will be placed in sight of other animals and, if possible, placed next to old cage-mates during rest periods. After animals have recovered from surgery and prior to recording, animals will be handled frequently. This will help minimise stress during behavioural tasks and recording, and help accustomize the animal to the experimenter. During a typical recording day we will include intermittent rest and sleep sessions, including in a familiar home environment. During controlled access to food or water, the body weight and condition of the animal will be monitored daily. Periods of fasting will occur during the day and controlled food/water delivered in the evening. Where possible, systemic administration of substances/drugs will occur via the animal's diet or drinking water.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA, NC3R’s, ARRIVE and PREPARE guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are monitoring progress in the 3Rs through our regular discussions with the Named Information Officer, the NC3R’s regional manager who sends out 3Rs News Letter and organises the annual 3Rs Research Day for the establishment. We also follow the communications posted on the NC3R’s website, the regular animal welfare departmental meetings and the ‘refresher courses’ organised locally by the establishment.

70. Models of Breast Cancer for Use in Breast Cancer Prevention and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Breast Cancer, Patient Derived Xenograft, Targeted treatment, Drug resistance

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to understand the molecular and cellular biology of breast cancer; with a particular focus on triple negative breast cancers (TNBCs) and patients with defects in DNA damage repair genes (BRCA1/2 and PALB2 mutations). We aim to better understand the drivers of breast cancer development, metastasis and mechanisms of resistance of tumour cells to anti-cancer agents. This project will aim to inform patient stratification for existing breast cancer treatments, to identify novel therapeutic targets and/or diagnostic biomarkers and to develop novel therapies for the treatment of this disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Breast cancer is the most common cancer in the UK, and the most common cancer in women. Around 55,000 women are diagnosed with breast cancer each year. Around 15% of breast cancer are triple negative breast cancers (TNBCs) and do not express oestrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2). TNBC is a heterogenous and highly aggressive subtype, known for its high drug resistance, poor prognosis and lack of targeted therapies. TNBC is the most common breast cancer subtype observed in patients with defects in Homologous Recombination (HR) DNA Repair deficiencies, such as pathogenic BRCA1/2 and PALB2 mutations.

Our work, by aiming to develop more effective treatments for breast cancer will have direct benefit for people with these diseases. PARP inhibitors (PARPi) and platinum chemotherapy drugs have been shown to be very effective treatments for breast cancer patients with BRCA-gene mutations. An international clinical trial testing the PARPi, olaparib, in early breast cancer demonstrated that adding olaparib to standard treatment for patients with high-risk, early-stage breast cancer with BRCA mutations cut their risk of dying by 32%. This resulted in more patients remaining cancer free and becoming breast cancer survivors. This has led to approval by global medicine regulators for olaparib in BRCA1/2 mutation associated breast cancer, and will hopefully soon lead to olaparib being offered through the NHS to this group of patients in the UK.

Unfortunately, despite these advances many women with breast cancer still die from metastatic recurrence. In studying resistance to these drugs, our aim is to design better ways of treating people with breast cancer including those with genetic forms (BRCA1/BRCA2 or PALB2 gene mutations) and potentially extending these treatment approaches to other patients with breast cancers with faults in other DNA repair genes. We also directly study samples and data from clinical trials of these agents to see if we can identify factors determining which patients will respond to PARPi. Our work also focuses on the preclinical investigation of drugs targeting a protein called DNA polymerase θ, which has the potential for use in BRCA1/2 and PALB2 mutant cancers that develop PARP inhibitor resistance.

Our intention is that our future work will also identify more effective treatments for specific subsets of breast cancer patient, such as patients whose tumours express specific genes or are resistant to chemotherapy or specific targeted therapies of interest. For some of these genes of interest, we have found preliminary evidence in the laboratory that these cancers have a particular weakness to drugs that are already in development for other types of cancer using large scale 'screening' studies

Ultimately our overarching aim is to impact treatment of breast diseases through innovative translation of basic science. This will be achieved by studying breast cancer subtype specific vulnerabilities using gold-standard models of the disease: including in vivo patient derived xenograft models (PDX) and in vitro 3D patient-derived organoid (PDO) cultures. Our research will assist the design of future clinical trials and the development of novel anti-cancer treatment combinations. We will identify new and more effective treatment combinations to treat breast cancers.

What outputs do you think you will see at the end of this project?

Work to be undertaken under this project licence aims to enhance our understanding of the drivers of breast cancer development, metastasis, progression and resistance to

treatment, with the hope of identifying a 'positive' diagnostic biomarker and developing novel therapies to improve patient outcomes.

We have previously identified target that drive the malignant phenotype of breast cancer for further pre- clinical in vivo investigation and have collaborated with other groups to establish biomarkers to improve diagnosis, prognosis and monitoring of breast cancers. Over the course of the last 10 years, my lab has developed a bank of patient derived xenograft (PDX) and patient derived organoid (PDO) models that encompass the range of phenotypes of human breast cancer. We aim to use these models, along with cell line xenografts, to validate biomarkers and druggable targets that could be used in the diagnosis and treatment of breast cancer.

We will publish studies carried out under the authority of this license in open-access high-impact journals and will present research at national and international conferences. Our group and other researchers (both academic and industry) will use these presented studies to inform drug development for breast cancer, with a particular focus on TNBC.

My group has a strong clinical focus. In addition to leading two academic research groups I am also a practicing consultant clinical oncologist and lead global clinical trials. Moreover, my group collaborates with academic and industry partners and numerous pharmaceutical companies. These collaborations will enable fast up-take of pre-clinical treatment strategies for treatment of TNBC that are developed under the authority of this license, allowing them to be translated into clinical practice with the ultimate benefit of impacting care and treatment of breast cancer patients.

Who or what will benefit from these outputs, and how?

Our research will assist the design of future clinical trials and the development of novel anti-cancer treatments and treatment combinations. We will identify new and more effective treatments to treat breast cancer, with a particular focus on TNBC.

The findings of this project will provide fundamental insights for drug development and clinical trials and will consequently impact the clinical field in the long term. Moreover, this work will benefit the basic research community by increasing our fundamental knowledge of breast cancer biology and mechanisms of resistance to therapy. Within the duration of this project, there is potential that findings from these experiments may influence how clinical trials are designed, thereby having an impact on patient care.

How will you look to maximise the outputs of this work?

We will publish our results in peer-reviewed open-access high-impact journal journals and will present our research at national and international conferences.

We will continue to collaborate with our colleagues and external collaborators including those in academia, industry partners and numerous pharmaceutical companies to share knowledge and techniques. These collaborations will enable fast up-take of pre-clinical treatment strategies for treatment of TNBC that are developed under the authority of this license, allowing them to be translated into clinical practice with the ultimate benefit of impacting care and treatment of breast cancer patients.

Species and numbers of animals expected to be used

- Mice: 28000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the only animal species to be used in this programme of work; they are widely used for preclinical models of cancer research. Mice have been selected as the experimental animal of choice as the availability of immunodeficient strains permits the growth of human-derived tumours and engraftment of human immune cells. Mice with functional immune systems would reject tumours of human origin.

Mice ranging from 3 to 16 weeks old will be used for tumour studies.

For the majority of our experiments, female immunodeficient mice which are capable of hosting human patient derived xenografts (PDXs) or cell line xenografts will be used. Immunodeficient strains (e.g.
SCID/beige, NSG mice) have been chosen for their tolerance to allogenic transplantation due to the lack of both functioning T and B lymphocytes, as well as having reduced macrophages and Natural Killer cells. This absence of these immune cells will permit growth of tumour cells of human origin.

Immunocompetent mice may be used for experiments in which we will investigate the role of the immune system, for example in experiments where we implant mouse tumour cells lines, that are analogous in many characteristics to tumours that would develop in humans.

We will also use immunocompetent genetically engineered mouse models (GEMMs) to study cancer biology and development. This will allow us to determine the roles of specific genes in the development of spontaneously occurring tumours, in a host with a functional immune system. This will closely replicate disease progression in the human body.

Together these animals will enable investigation of breast cancer biology, by allowing us to closely model the human disease. This will enable us to develop effective treatments which can potentially be translated for use in the clinic.

Typically, what will be done to an animal used in your project?

Breeding of Genetically altered Animals (Protocol 1): Genetically altered mouse strains will be bred, particularly those that are immunodeficient and immunocompromised and can therefore act as recipients of human tissues and cell lines in subsequent protocols, enabling studies of tumour biology and response to novel treatments. Immunocompetent genetically altered animals will be used to study tumour biology in the context of an intact functioning immune system. Mice will be kept on this protocol as maintenance to the age of 12 months. Mice on this protocol will undergo up to 4 procedures: breeding by conventional methods/generation of embryos or blastocysts; tissue biopsy to determine genetic status; maintenance to the age of 12 months; terminal procedures (exsanguination, perfusion fixation, schedule 1 killing)

Transgenic Spontaneous Tumour Models (Protocol 2): Some studies will use genetically altered mice with genetic mutations promoting the development of spontaneous

tumours. This will allow us to study spontaneous tumour development and metastasis. Tumour growth will be monitored by palpation, caliper measurement and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 24 months of age (expected that around 50% of mice will be culled before they reach 1 year of age). Mice on this protocol will undergo up to 15 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: identification/monitoring of spontaneous tumours (mandatory), imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, intraductal delivery of lentivirus, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, tumour biopsies, ageing to 24 months, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

We will use protocols 2-7 detailed below to create new breast cancer PDX models from patients of clinical interest (patients with TNBC, BRCA1/2 mutations, PALB2 mutations and/or patients with biological subtypes of breast cancer that have undergone specific treatment regimens). We will also use the model for cell line experiments using both human and mouse cells in immuncompromised and immunocompetent mice, respectively.

Subcutaneous or mammary fat pad tumour models (Protocol 3): Tumour fragments or cell suspensions will be implanted into the mammary fat pad of female mice. Tumour growth will be monitored by palpation, caliper measurement and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 24 months of age; animals without tumours will be culled a maximum of 12 months after tumour cell inoculation. Mice on this protocol will undergo up to 16 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: tumour establishment (subcutaneous/ mammary fat pad - mandatory), tumour monitoring and measurement, imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, tumour biopsies, surgical resection of tumours, ageing to 24 months, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Mammary intraductal injection (Protocol 4): Breast cancer cells will be transduced with lentiviral vectors and injected into the mammary ducts of female mice. Tumour growth will be monitored by palpation, caliper measurement and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 24 months of age; animals without tumours will be culled a maximum of 12 months after tumour cell inoculation. Mice on this protocol will undergo up to 16 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: tumour establishment (implantation into the mammary duct - mandatory), tumour monitoring and measurement, imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, tumour biopsies, surgical resection of tumours, ageing to 24 months, administration of

drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Intravenous inoculation with tumour cells (Protocol 5): Tumour burden will be established via intravenous injection of tumour cells. Tumour growth will be monitored by monitoring of body condition and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 12 months of age. Mice on this protocol will undergo up to 12 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: tumour establishment (intravenous inoculation with tumour cells - mandatory), imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Intracardiac inoculation with tumour cells (Protocol 6): Tumour burden will be established via intracardiac injection of tumour cells. Tumour growth will be monitored by monitoring of body condition and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 24 months of age. Mice on this protocol will undergo up to 13 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: tumour establishment (intracardiac inoculation with tumour cells - mandatory), imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, ageing to 24 months, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Intracranial or intracisternal inoculation with tumour cells (Protocol 7): Tumour burden will be established via intracranial or intracisternal injection of tumour cells. Tumour growth will be monitored by monitoring of body condition and/or non-invasive imaging. Animals will be kept on this protocol until a maximum of 24 months of age. Mice on this protocol will undergo up to 13 procedures, though we do not expect any mice to undergo all possible protocol steps. Protocol steps include: tumour establishment (intracranial or intracisternal inoculation with tumour cells - mandatory), imaging, inoculation with stromal cells/ECM components, endocrine modulation via hormone pellet implantation/administration of hormonal agents via the drinking water or surgical removal of the ovaries, suppression of immune response/administration of immunomodulatory agents or immune cells, administration of agents to activate/repress inducible gene expression, treatment with test agents, radiotherapy, blood/hair/urine/faecal sampling, ageing to 24 months, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Maximum Tolerated Dose (Protocol 8): Novel drugs or drug combinations will be tested in different mouse strains in order to determine safe and tolerable treatment regimens. Animals will be kept on this protocol until a maximum of 12 months of age, though we expect most mice to only spend a few months on this protocol. MTD studies will be performed in non-tumour bearing mice. Mice on this protocol will undergo up to 5

procedures, though we expect <5% of mice to undergo all possible protocol steps. Protocol steps include: treatment with novel test agents (mandatory), radiotherapy (<5% mice), blood/hair/urine/faecal sampling, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory) .

Tissue Collection from Non Tumour Bearing Mice (Protocol 9): Non tumour bearing mice may be used to allow for blood/fluid collection and tissue labelling and collection from new genetically engineered mouse models and mice from the same genetic backgrounds as those that will subsequently be used for tumour bearing experiments on Protocols 2-7. This protocol may also be performed on wild type mice. Animals will be kept on this protocol until a maximum of 12 month. Mice on this protocol will undergo up to 3 procedures, though only 50% of mice will undergo blood/fluid/tissue sampling. Protocol steps include: blood/hair/urine/faecal sampling, administration of drugs for labelling of tissues/specific cell types prior to culling, culling by schedule 1 or listed method (mandatory). 50% of animals are likely to experience mild severity as they will not undergo any of the procedure steps listed on this protocol expected to cause more than mild and transient adverse events. These mice will only undergo i tissue labelling (optional) and culling (mandatory).

Response of breast cancer cells to targeted therapeutics: Established breast cancer PDX models and breast cancer cell line models will be used for these experiments. Tumour cells may be modified in vitro prior to implantation, either to up or down regulate expression of genes of interest or to add tags such as bioluminescent and/or fluorescent genes or barcoded libraries. Tumours will be established either spontaneously, or by implantation of tumour cells via the following routes: subcutaneously, mammary fad pad, injection into mammary ducts following transduction with lentiviral vectors, intravenous injection, intracardiac injection or intracranial/intracisternal injection (via protocols 2-7). The majority of experiments will be performed in female NSG mice. Mice will be treated with therapeutic agents. Tumour cell growth will either be monitored by palpation and caliper measurement or by non- invasive imaging, such as In Vivo Imaging systems (IVIS). In brief, cells are tagged with a bioluminescent reporter, meaning that they are capable of emitting light and can be detected non- invasively by a machine capable of detecting these emissions within the body.

Please see table below for a summary of potential steps for each protocol:

	SUMMARY OF PROTOCOL STEPS									
	STEP	PROTOCOL								
		1	2	3	4	5	6	7	8	9
	Breeding	✓	✗	✗	✗	✗	✗	✗	✗	✗
	Tissue biopsy to determine genetic status	✓	✗	✗	✗	✗	✗	✗	✗	✗
Tumour Establishment/ Monitoring	Identification of Spontaneous Tumours/ tumour monitoring	✗	✓	✗	✗	✗	✗	✗	✗	✗
	Tumour Establishment (subcutaneous/mammary fat pad)	✗	✗	✓	✗	✗	✗	✗	✗	✗
	Tumour Establishment (intraductal)	✗	✗	✗	✓	✗	✗	✗	✗	✗
	Tumour Monitoring and Measurement (subcutaneous/mammary fat pad/ intraductal tumours)	✗	✗	✓	✓	✗	✗	✗	✗	✗
	Tumour Establishment (Intravenous)	✗	✗	✗	✗	✓	✗	✗	✗	✗
	Tumour Establishment (Intracardiac)	✗	✗	✗	✗	✗	✓	✗	✗	✗
	Tumour Establishment (Intracranial/Intracisternal)	✗	✗	✗	✗	✗	✗	✓	✗	✗
	Imaging	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Inoculation with Stromal Cells/ ECM components	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Hormone Pellet Implantation/ Administration of Hormonal Agents via drinking water	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Endocrine Modulation surgical (removal of ovaries)	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Intraductal Delivery of lentivirus	✗	✓	✗	✗	✗	✗	✗	✗	✗
	Suppression of Immune Response/ Administration of Immunomodulatory agents or immune cells	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Administration of Agents to Activate/ Repress inducible gene expression	✗	✓	✓	✓	✓	✓	✓	✗	✗
	MTD	✗	✗	✗	✗	✗	✗	✗	✓	✗
	Tumour Treatment	✗	✓	✓	✓	✓	✓	✓	✗	✗
	Radiotherapy	✗	✓	✓	✓	✓	✓	✓	✓	✗
	Blood/hair/urine/faecal sampling	✗	✓	✓	✓	✓	✓	✓	✓	✓
	Tumour Biopsies	✗	✓	✓	✓	✗	✗	✗	✗	✗
	Surgical Removal of Primary tumour	✗	✗	✓	✓	✗	✗	✗	✗	✗
	Maintenance to 12 months	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ageing to 24 months	✗	✓	✓	✓	✗	✓	✓	✗	✗
	Tissue Labelling	✗	✓	✓	✓	✓	✓	✓	✓	✓
	Terminal Procedures	✓	✓	✓	✓	✓	✓	✓	✓	✓

What are the expected impacts and/or adverse effects for the animals during your project?

The animals used in the experiment can be expected to experience the following harmful effects:

Pain may arise from surgical procedures including: implantation of tumour fragments or cells subcutaneously, into the mammary fat pad or injected through the nipple, hormone pellets insertion, clearing of the fat pad, ovariectomy, tumour biopsy or surgical resection. This pain will be mitigated using appropriate and refined analgesia. These effects are expected to be transient and to last for <1 week post surgery. In general tumour growth in our model does not affect animal behaviour, however, the location and size of the tumour can affect the animal's movements. This is not common; abnormal gait will be monitored daily and if gait impedes locomotion beyond 48 hours then mice will be culled within 24 hours.

Following ovariectomy sometimes mice gain more weight compared to the control.

Surgeries, IVIS measurements, dosing and blood sampling from the tail vein are stressful for the animals but this stress can be reduced by increased handling with gentle methods. Stress during and following these procedures is expected to be transient. If mice show signs of distress during a procedure, then procedures will be halted until they have recovered. Mice will be monitored for clinical signs of distress following procedures (such as hunched intermittently, isolation from peers or subdued behaviour) and will be killed by a Schedule 1 method is they show signs of distress for a period of >48 hours.

Immunodeficient mice are prone to infection but with our current methods, we have not seen infections.

Mice on the maximum tolerated dose protocol (Protocol 8) may experience drug induced toxicity. Since some test agents may not have been previously studied in either an in vivo setting or in a particular mouse strain, toxic effects will be unpredictable both in terms of nature and incidence. Possible acute drug-related effects may include: abnormal respiration, diarrhoea and lethargy. Over the course of the study mice may develop signs of toxicity (significant loss of body weight, lethargy, skin rash), and the development of abdominal bruising, irritation and/or tissue damage at the site of injection as a result of repeated test agent administration. Mice will be closely monitored for acute effects in the first hours after drug administration and for development of toxicity over the course of the study. If any signs of toxicity develop, dosing may be interrupted or terminated to allow recovery of the mouse.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities for each type of procedure are as follows:

mild: breeding. 50% of total mice are expected to be used on a protocol with mild severity (Protocol 1)

moderate: intraductal injection, implantation of tumour cells subcutaneously or into the mammary fat pad, ovariectomy, clearing of the fat pad, tumour biopsy, surgical resection, hormone pellet insertion, oral gavage, intravenous injection, intraperitoneal injection, subcutaneous injection, blood withdrawal, non-invasive imaging under anaesthesia, radiotherapy and administration of test agents as part of maximum tolerated dose protocol. 50% of total mice are expected to be used on a Protocol with moderate severity (Protocols 2-9). For protocol 9, 50% of mice are expected to experience a mild severity.

Based on the percentage of mice expected to experience each severity (mild/moderate) for each protocol (1-9), the total proportion of mice expected to experience each severity is: **Mild:** 50.89%;

Moderate: 49.11%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The human breast is the site of breast cancer. The breast is composed of a network of flexible tubes that contain cells that can become cancerous. The environment within and around these tubes containing cells that cannot yet be replicated so that the balance of hormones, growth factors, oxygen and mechanical forces are the same as that found in patients. The environment within the mouse mammary glands and the human breast are remarkably similar; mouse models are the best method we currently have to model and study breast cancer.

Many aspects of tumour biology can be studied *in vitro*; we have considerable expertise in these assays and are actively trying to refine them to more accurately reflect the tumour microenvironment. Extensive studies are first carried out *in vitro* to mimic as many as possible of the cellular processes and interactions as well as responses to drugs that we study. However, a large number of questions can only be addressed using immune competent animal models, as the tumour microenvironment and immune systems in mice and humans are too complex to be recapitulated in *in vitro* experiments.

Whenever possible we use in-house established 3D Patient Derived Organoid (PDO) models to both substitute and complement our *in vivo* work in an effort to establish *in vivo* replacements as often as possible. These artificial systems are used to test agents that can target the candidate genes and proteins that we suspect may affect tumour establishment and growth. Our methodology incorporates extensive control experiments to ensure that *in vitro* observations are “on target” and “off target” effects that might explain a phenotype are minimised before *in vivo* validation. If results in these multiple systems are negative, then we will not proceed to performing animal studies. Only if promising and robustly verified results are obtained will we proceed to using animal studies. We will use information gained from *in vitro* analysis to optimise the design of animal studies.

The reasons why the use of an animal model is necessary despite these *in vitro* models are outlined below:

There are many aspects of the tumour microenvironment which cannot be adequately modelled *in vitro*. These include the presence of a wide range of non-tumour cells which can influence the proliferation of tumour cells, the effect of blood flow and low levels of oxygen found in some areas of tumours. To discover the effect of these variables on the growth and movement of tumour cells, *in vivo* models are required.

Tumour cell proliferation has been extensively studied *in vitro* with often-contradictory results. These *in vitro* studies have been hampered by the highly variable and artificial nature of the assays used. In order to develop better *in vitro* models we need to understand how tumour cells interact with other cell types *in vivo*.

In order to understand the contribution of cell spread to metastasis and the potential benefits of inhibiting cell dissemination as anti-metastasis strategies it is necessary to evaluate the metastatic potential of tumour cell lines and patient derived human tumour explants. At present this can only be done *in vivo*.

It is vital for the therapeutic approaches to be discovered and further explored and validated in this project to be able to examine the effects of immune cells in the tumour stroma. This requires an *in vivo* model with competent or partially competent or immune xenotransplant complemented immune system as a function immune cell tumour cell interface cannot be achieved in an *in vitro* model.

Which non-animal alternatives did you consider for use in this project?

Numerous non-animal techniques are routinely utilised by our laboratory, including bioinformatic and computational approaches. This involves analysis of datasets generated in house (both from patient data, animal studies and in vitro experiments) and analysis of publicly available resources – such as TCGA datasets.

We also use multiple in vitro approaches – including 2D cell culture (which are used for large scale drug screening assays), culturing of primary cells and patient derived organoids. We closely collaborate and share funding with a team who focus on the establishment of patient derived organoids from both breast cancer patients and from normal breast tissue. In generation of these models, non- animal hydrogels are considered where appropriate. Whenever possible we use in-house established 3D Patient Derived Organoid (PDO) models to both substitute and complement our in vivo work in an effort to establish in vivo replacements as often as possible.

We also endeavour to use resources such as SEARCHbreast where applicable to avoid repetition of experiments where tissue is readily available from experiments conducted at other institutes.

Moreover, we constantly keep up to date with recent non-animal alternatives in the field such as organ- on-a-chip systems.

The experiments detailed in this project relate solely to experiments which we do not believe there to be a suitable non-animal alterative available that could be used to deliver comparable insights. All new therapies under investigation in this project will first undergo rigorous laboratory based testing using surrogate models (such as those described above) in order to ensure that only the strongest hypotheses are progressed to in vivo testing.

For the purpose of the experiments detailed in this PPL, in vitro culturing of primary tumour cells was considered as an alternative but it lacks the complexity of the tumour microenvironment, which interacts with the tumour notably through paracrine signalling.

Similarly, culture of Patient Derived Organoids (PDOs) was considered, and indeed are used where appropriate in our programme, but again these lack the complexity of the non-tumour cell tumour microenvironment. Organ-on-a-chip models also lack this complexity.

Why were they not suitable?

Replacement is not possible, as an examination of alternative methods indicated that in vitro culturing of primary tumour cells or Patient Derived Organoids lacks the complexity of the tumour microenvironment, which interacts with the tumour notably through paracrine signalling. A large number of questions can only be addressed using animal models, as the tumour microenvironment and immune systems in mice and humans are too complex to be recapitulated by *in vitro* experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on prior experiences with the model system in question, and best estimates of expected effect sizes. Where appropriate we carry out pilot studies to establish these parameters and allow us to accurately determine the appropriate animal number for larger experiments. Animal numbers are also calculated based on the resources we have available - both in terms of funding and personnel - and the lab's overarching experimental plans for the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All new therapies under investigation in this project will first undergo rigorous laboratory based testing using surrogate models (such as cell lines and patient derived organoid models) in order to ensure that only the strongest hypotheses are progressed to *in vivo* testing. We will only proceed to test novel therapies *in vivo* once *in vitro* studies have been completed which support the therapeutic potential of the approach under investigation so that ineffective approaches are not unnecessarily tested in animals.
Moreover, to minimise the number of mice used we will rely on our previous experience using established protocol design procedures that yield the highest rate of success using the least number of animals. This is also made possible due to our collaboration with experts in the same field. Where applicable, pilot experiments will be carried out to determine variability in tumour growth. Experiments will be designed and optimised with the aid of online resources such as the NC3R's Experimental Design Assistant. We will conduct careful power calculations using this and other resources to ensure that the minimum number of animals is assigned to a particular experiment, while maintaining power to detect meaningful differences between test and control groups.

We also apply principles of good experimental design such as randomisation and blinding (where feasible to do so) to avoid sources of bias and to ensure that experiments are robust and reproducible and truly add to the knowledge base. Moreover, we seek specialist statistical advice from external sources when required – for example during the preparation of grant proposals - in order to ensure experiments are appropriately designed and powered.

NSG-hprt (NOD.Cg-Prkdcscid Il2rgtm1Wjl Hprtb-m3/EshJ) mice will be used under this PPL. These mice are immunocompromised and harbour a frame shift and premature termination, resulting in functional knockout of the hypoxanthine phosphoribosyl transferase (Hprtb-m3) allele. This strain allows for implantation of human tumour xenografts. Once these tumours are excised and cultured ex vivo in the laboratory, Hprtb-m3 null cells can be eliminated by selection with hypoxanthine- aminopterin-thymidine (HAT). This means that mouse cells which are Hprt deficient can be removed from culture, whereas the Hprt proficient human cells will remain. We plan to use this system to generate PDX derived organoid cultures without the complication of mouse cell

contamination.

Generation of such organoids will increase the number of experiments we are able to do in vitro; and thereby reduce the number of animal experiments required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Numbers of breeding pairs will be subject to ongoing review to ensure that excessive numbers of mice are not produced for experimental work.

To minimise the number of mice, pilot studies will be used where appropriate to determine parameters such as tumour graft success rate for each model before proceeding to larger experiments. Power calculations will also be employed to determine the lowest number of mice required to give statistically valid results, thereby minimising the number of repeat experiments required. These will derive from online tools including the NC3Rs Experimental Design Assistant.

All tumour cell lines that are used in this project will be rigorously checked for their provenance, genetic identity and negative mycoplasma status, thereby preventing the wasted use of mice in animal experiments that are unnecessary.

The use of tumour biopsy will enable us to sample the same tumour pre-, during and post-treatment. This will reduce the need to setup large cohorts of animals to be sacrificed at different timepoints during treatment regimes. We also plan in some of our studies to use non-invasive imaging techniques which will allow us to detect primary tumours and metastasis at an earlier timepoint and to perform longitudinal analysis on these mice, therefore requiring less mice for these studies.

Moreover, tissues that are harvested from mice culled on experiments conducted within this PPL will be maximized for downstream ex-vivo analysis to reduce the number of subsequent *in vivo* experiments required. We will also discuss experimental plans with collaborators and make use of online tissue sharing resources such as SEARCHbreast, to avoid performing in vivo experiments where data could be sourced from those already conducted.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the only animal species to be used in this programme of work; they are widely used for preclinical models of cancer research. Mice have been selected as the experimental animal of choice as the availability of immunodeficient strains permits the growth of human-derived tumours and engraftment of human immune cells.

Immunodeficient strains (e.g. SCID/beige, NSG mice) have been chosen for their tolerance to allogenic transplantation due to the lack of both functioning T and B lymphocytes, as well as having reduced macrophages and Natural Killer cells. To model spontaneously occurring tumours in which an immunosuppressive microenvironment is present, genetically engineered mouse models (GEMMs) may be used.

Mice will be maintained in ventilated cages, provided with sterile food and bedding, and all procedures will be carried out in laminar flow cabinets to avoid infections. We will use cage enrichment to improve the living environment for the mice.

Mice under this PPL are expected to develop tumours; tumours may occur spontaneously in GEMMs (Protocol 2) or following implantation of tumour fragments/cells or tumours at the following sites: implantation subcutaneously or into the mammary fad pad (Protocol 3), by injection of tumour cells into mammary ducts (Protocol 4), intravenous injection (Protocol 5), intracardiac injection (Protocol 6) or intracranial or intracisternal injection (Protocol 7).

50% of animals on the project will be on a mild breeding protocol. the other 50% of animals under this PPL are expected to experience a moderate severity. We will use anaesthesia and analgesia as part of all surgical procedures, thus minimising suffering. Analgesia will also be delivered post-operatively where required. Mice will be monitored daily by BSU staff and a minimum of once per fortnight by PIL holders working under this PPL. Tumour burden will be assessed by either calipers (allowing for calculation of tumour volume) or by non-invasive imaging such as IVIS. Tumours will be grown to a suitable size to allow for routine passaging in subsequent cohorts of mice (PDX generation) or in the therapeutic setting to allow for analysis of tumour growth and metastasis compared to vehicle groups. Frequency of monitoring will be increased if animals exhibit clinical signs or if tumours are considered to be fast growing. Animal suffering will be minimised by keeping tumour burdens within acceptable limits. In studies where non-invasive imaging is used (particularly at sites where tumours are not easy to visualise and measure by calipers), this should facilitate earlier identification of tumours and monitoring of models in which metastasis is expected; and thereby allow for reduction of suffering by enabling us to cull animals before they exhibit clinical signs. Mice will be humanely culled using a schedule 1 method if mice develop signs of pain or distress, or if humane endpoints are met, as described in individual protocols.

Novel therapies will be assessed for toxicity and tolerability under our MTD protocol. Therefore, the majority of mice used under this PPL are expected to exhibit high tolerability to treatment regimes. We constantly refine our protocols to account for current best practices -including: modifying analgesia regimes on advice of the Named Veterinary Surgeon, and refining techniques and surgery skills through collaborations, continual ongoing training and review of recent advances in techniques being used in the research field.

Why can’t you use animals that are less sentient?

Mammary glands are a defining feature of mammals; mice are less sentient than higher mammals. We need to use a suitably aged mature female mammal in order to study how breast cancer cells progress and respond to therapies in a similar environment to that found in the human breast. Mice have teats (similar to human nipples) that are connected to flexible tubes (milk ducts) that mimic the architecture of the human breast to a remarkable degree. In order to study breast cancer, it is essential that studies are conducted in a model organism with mammary glands. Using less sentient species would

risk the research not being relevant to human disease.

Immune competent animals allow scientific interrogation of the interaction between cancer, therapy and the immune system. Whereas immunodeficient mice allow testing of the safety and anti-tumour activity of experimental anti-human therapies. This form of investigation is required by regulatory authorities prior to approval of first-in-man clinical trials.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are maintained in individually ventilated cages (IVCs) and are provided with an appropriate environment (e.g. nesting material, shelter, environmental enrichment, ad libitum access to food and water) to satisfy their normal behaviour. To minimise infection risk, animals are maintained under barrier conditions and cages are only opened within a laminar flow hood. Mice are only handled using clean, gloved hands which are disinfected in between cages to reduce transmission of infections.

To reduce stress, animals are not housed in isolation (if possible) and gentle handling techniques are implemented whenever possible, including picking up mice using a tunnel or cupped hands to avoid lifting mice by the tail.

Experimental design will constantly be re-evaluated based on current best practices in the field, and the mildest possible procedure will be used wherever possible with the aim of minimising animal pain and distress. This is based on investigation of the scientific literature and consultation with the NVS, NACWO, colleagues and collaborators to enable us to refine our techniques and minimise animal suffering through adoption of best practice. Moreover, competence of staff to perform procedures will be reviewed and documented on a regular basis, and additional training provided where necessary.

Individual protocols have also been refined to reduce suffering as much as possible. Spontaneous tumour models – mice will be moved onto this protocol as early as possible, post weaning, to ensure that tumour monitoring and monitoring of associated clinical signs is structured. For protocols where tumour cells will be implanted, this will be performed aseptically using sterile instruments to prevent infection. Cells injected via IV and intracardiac routes will be filtered prior to injection to ensure that clumping does not occur and to therefore reduce the risk of pulmonary embolism. For tumour implantation via intracardiac, intracranial and intracisternal routes, ultrasound guided imaging (may be used to ensure correct needle positioning. If surgery/anaesthesia are required animals will be allowed to recover on a heated pad and provided additional post operative monitoring including provision of peri and post procedural analgesia to reduce suffering as specified in individual protocols. The most suitable tumour establishment protocol will be selected for the research question under investigation – for example whether the aim is to study primary tumour development, or metastasis to specific organs such as the lungs, liver, CNS or bone.

Mice will be monitored daily for adverse effects. If adverse effects occur, animals will be subject to increased monitoring and interventions that aim to reduce suffering will be provided, in line with advice from the Named Veterinary Surgeon. This may include provision of a mashed diet, food supplementation (ie electrolyte gel, nutragel), extra enrichment, or increased analgesia.

Tumours will be checked regularly, at least once per fortnight. Animals will be monitored for tumour growth, weight loss, signs of metastasis, tumour ulceration and clinical signs

associated with pain and distress. Tumours will not be allowed to exceed the size limits described in Table 1 or tumour ulceration limits detailed in Table 2 – thus limiting the duration of suffering. For mice exhibiting signs of pain due to tumour formation, analgesia such as opioids, non-steroidal anti-inflammatory drugs (NSAIDs) or local analgesics may be provided. Majority of tumours will be tagged with luciferase. Non-invasive imaging may be used to monitor tumour burden, this allows us to track the growth of primary tumours and metastasis and ensures that mice are culled before humane endpoints are met. Non-invasive imaging may also be used immediately after tumour establishment via IV and intracardiac routes to confirm successful injections. Where tumour biopsies are taken, ultrasound guided imaging may be used to visualise tumours and placement of biopsy needle.

When receiving tumour treatments, treatments with known tolerability will typically be used to reduce potential adverse effects. Power calculations will be used to select appropriate group sizes, to ensure a suitable number of mice are used to detect a meaningful different in effect, therefore preventing repetition of experiments. Experiments will be suitably randomised and blinded to ensure that data outcomes are not biased.

Where treatment regimens (dose and administration route) have not previously been tested in comparable mouse strains, they will be determined in Protocol 8 (Maximum Tolerated Dose) prior to use to ensure low toxicity. Where tolerability of drug doses or therapeutic regimes will be tested, it will be refined to cause as little suffering and distress as possible. For full tolerability testing, a maximum of 3 dose level groups each with a maximum of 4 animals, plus a control group, may be used. Animals will be closely monitored in the immediate post-dosing period for 30-60 minutes and an acute toxicity observation chart will be used to enhance the standard health monitoring regimen over the course of the experiment. Mice will be closely monitored for acute effects in the first hours after drug administration, twice daily for the first 5 days and thereafter by daily inspection. If any signs of toxicity develop, dosing may be interrupted or terminated to allow recovery of the mouse. Mice which do not recover from severe symptoms (e.g. dyspnoea, convulsions) within 10 minutes, or moderate symptoms (e.g. agitation, loss of movement, moderate respiration) within 30 minutes will be culled.

The minimal number of procedures possible will be performed on each individual mouse, in order to achieve the research question under investigation. Mice will be humanely culled using a schedule 1 method if mice develop signs of pain or distress, or if humane endpoints are met, as described in individual protocols. We expect that for the majority of mice, the experimental endpoint will occur earlier than the humane endpoint to minimise the duration and intensity of suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We continually consult the NVS, NACWO and colleagues to ensure best practice for all procedures undertaken, particularly with the aim to minimise animal pain and distress. The following resources will be used to ensure that experiments are conducted in the most refined way:

Workman et al (2010) Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102: 1555-77

NC3Rs website: https://www.nc3rs.org.uk/3rs-resources/search

https://www.ema.europa.eu/en/review-update-ema-guidelines-implement-best-practice-regard- 3rs-replacement-reduction-refinement,

https://www.lasa.co.uk

Refining procedures for the administration of substances:
https://doi.org/10.1258/0023677011911345

Guiding principles for aseptic surgery:
https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf

NC3Rs Grimace Scale

Mouse Body Condition Score: Ullman-Culler and Foltz (1999). Laboratory Animal Science 49(3): 319-332

FELASA Guidelines on Clinical Signs as defined in: Pain and distress in laboratory rodents and lagomorphs. Report of the Federation of European Animal Science Associations working group on pain and distress. Laboratory Animals (1994) 28; 97-112

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly review the literature and useful websites to identify new resources available to enable Reduction, Replacement and/ or Refinement in animal use. Examples of such websites include the NC3Rs and EURL ECVAM resources library. All team members are registered with the NC3Rs information list and hear about seminars and trainings that are available throughout the year.

Moreover, we liase with our NC3Rs Regional Programme Manager who provides a point of reference for advice on the 3Rs.

We also stay up to date and implement the 3Rs via discussion of experimental design and the 3Rs with colleagues and collaborators (both internally and externally) at laboratory meetings, departmental seminars, and at conferences.

71. Refined Breast Cancer Models for Use in Breast Cancer Prevention and Therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Breast cancer, Hormone resistance, Therapy, Breast development, Hormones

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Hormones: Keys to Breast Cancer Prevention and Therapy

The overarching aim of our research is to understand how hormones impinge on breast cancer in order to develop novel therapeutic and preventative approaches. This research will provide us with insights into how ovarian hormones oestrogen, progesterone, and testosterone act on the normal breast cells and breast cancer cells. This provides the basis for new preventative strategies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Seventy five percent of all breast cancers are hormone-sensitive, but progress in their

study has been hampered by lack of adequate models to study them. We have overcome this hurdle and shown that the environment that hormone-sensitive breast cancer cells sit in determines if they respond to hormones at all. We have found that placing hormone-sensitive breast cancer cells directly into milk ducts using a technique called "MIND", which is an abbreviation for Mammary Intraductal, is a much better way to study breast cancer cells. It lets breast cancer cells grow and first clog up the milk duct before growing out of the duct into the surrounding tissues as a newly formed breast cancer which is what is also seen in breast cancer patients. This technique allows us to grow and study most breast cancers, including notoriously difficult to study invasive lobular cancers (ILCs), which engraft with 90% success rates, without adding any hormones.

What outputs do you think you will see at the end of this project?

The output from this Project Licence is to obtain new information about how breast cancer and normal breast cells respond to treatment with hormones and therapeutic agents. The "MIND"/mammary intraductal model is much more effective at establishing cancer mouse models compared to conventional models when breast cancer cells are injected into other sites besides the breast duct.

This model also preserves important features seen in the original clinical sample such as the hormone levels, and expression of markers that the clinical team will look at to decide what therapy the breast cancer patient will receive. Furthermore, this xenograft model responds to therapies that the patient's tumours respond to (Sflomos et al., 2016).

Who or what will benefit from these outputs, and how?

The present research will provide us with insights into how ovarian hormones estrogen, progesterone, and testosterone act on the normal breast cells and breast cancer cells. This provides the basis for new preventative strategies.

Our work on one breast cancer target we study called LOXL1, may open new therapeutic options for lobular breast cancer, a difficult to treat subtype of breast cancer. Testing of the new drug candidates with the new lobular breast cancer cell lines we work with and patient-derived samples we work with will reveal the use of the new models for finding new ways to treat patients with lobular breast cancer.

Our searches based on multiple samples derived from breast cancer patients will help streamline efforts to translate into efficient and focused clinical trials and hence accelerate the transition from bench to bedside.

In the course of this project new breast cancer patient derived mouse models will be developed that will benefit the work of the wider breast cancer research community.

How will you look to maximise the outputs of this work?

We will continue to collaborate with our colleagues who are experts in the field of breast cancer research. We will publish our results in peer-reviewed journals and present at conferences.

Species and numbers of animals expected to be used

- Mice: 13,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mature, 8-12 week old immunodeficient female mice, are capable of hosting human breast cancer and normal breast cells therefore enabling studies of cancer biology and providing treatment efficacy data to allow rapid clinical translation. We sometimes use younger and older mice, which can also host human cancer cells.

Typically, what will be done to an animal used in your project?

New breast cancer models derived from breast cancer patient samples: We create new breast cancer mouse models derived from patient samples by labelling breast cancer cells from patients with visible markers and injecting them into the mammary ducts of female mice. Estrogen may be added to the water to promote tumour growth.

Response of normal and/or breast cancer cells to hormone stimulation: Normal mammary cells from patients and/or breast cancer cells from the sample patient can also be labelled with visible markers and injecting them into the mammary ducts of female mice. Mice will be implanted with hormone pellets. Blood samples may be taken. Biopsies may be taken. Both normal and tumour cell growth in mice is monitored by an imaging machine which can detect the markers we have labelled them with. Some mice may have their ovaries removed.

Response of breast cancer cells to targeted therapeutics: Established breast cancer models derived from patients and breast cancer cell lines models that have been transduced with lentiviral vectors are injected into the mammary ducts of female mice. Mice are treated with therapeutic agents. Blood samples may be taken. Biopsies may be taken. Tumour cell growth in mice is monitored by a variety of imaging techniques which use different machines to detect markers that we have labelled the cells with. Some mice may have their ovaries removed.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals used in the experiment can be expected to experience the following harmful effects:

pain from the surgeries in which cells are injected through trimmed nipples and pellets are inserted under the skin through small incisions or from removal of the mammary gland and removal of the ovary. This pain is mitigated by the use of appropriate and refined pain relief.

In general tumor growth in our model does not affect animal behaviour, however, the location and size of the tumor can affect the animal's movements removng the ovaries sometimes causes mice to gain more weight compared to the control.

Surgeries, tumour imaging measurements and blood sampling from the tail vein are stressful for the animals but this stress can be reduced by increased handling with gentle

methods.

The mice that are able to support breast cancer cell growth are prone to infection, but with our current methods we have not seen infections.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The harms caused to the animals in the experiment described here are to be rated as follows:

moderate (85%): injection of cells into the mammary duct, removal of ovaries, removal of mammary glands, removing part of mammary fat pad, administration of drugs through the mouth

mild (15%): hormone and control pellet implantation

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The human breast is the site of breast cancer. The breast is composed of a network of flexible tubes that contain cells that can become cancerous. The environment within and around these tubes containing cells cannot yet be replicated so that the balance of hormones, oxygen as well as mechanical force is the same as that found in patients. The environment within the mouse mammary duct and the human breast is remarkably similar and is the best method we currently have to model and study breast cancer.

Many aspects of tumour biology can be studied by growing cancer cells and cancer tissues in incubators; we have considerable expertise in these type of studies and are actively trying to refine them to more accurately reflect the tumour environment. These artificial systems will be used to test agents that can target the candidate genes and proteins that we suspect may affect tumour establishment and growth. Our methodology incorporates extensive control experiments to ensure that observations from growing cancer cells and cancer tissues in incubators reflect what happens in breast cancer patients. If results in these multiple systems are negative then we will not proceed to performing animal studies. Only if promising and robustly verified results are obtained will we proceed to using animal studies. We will use information gained from analysis of cell culture-based studies to optimise the design of animal studies.

The reasons why the use of an animal model is necessary despite availability of these

breast cancer cell culture models are outlined below:

There are many aspects of the tumour microenvironment, which cannot be adequately modelled using cultured cells. These include the presence of a wide range of non-tumour cells which can influence the growth of tumour cells, the effect of blood flow and low levels of oxygen found in some areas of tumours. To discover the effect of these variables on the movement of tumour cells, studies using mice are required.

Tumour cell growth has been extensively studied in cell based studies with often-contradictory results. These types of studies have been hampered by the highly variable and artificial nature of the tests used. In order to develop better cell-based models, we need to understand how tumour cells interact with other cell types studies using mice.

In order to understand the contribution of cell spread to organs outside of the original site of the cancer and the potential benefits of inhibiting cancer cell spreading to other parts of the body, it is necessary to evaluate the potential of tumour cell lines and patient derived human tumour tissues to spread to other organs. At present this can only be done with studies using mice.

It is vital for the therapeutic approaches to be discovered and further explored and validated in this project to be able to examine the effects of immune cells in the tissues surrounding the tumour. This requires studies using mice with active immune systems.

Which non-animal alternatives did you consider for use in this project?

Cell culturing of primary tumour cells was considered but it lacks the complexity of the tumour microenvironment, which interacts with the tumour notably through signalling between different types of tissues (Boone et al., 2015).

Why were they not suitable?

Replacement is not possible, as an examination of alternative methods indicated that cell culture based studies of primary tumour cells lacks the complexity of the tumour microenvironment, which interacts with the tumour notably signalling between different types of tissues (Boone et al., 2015).

In order to reduce the use of animals and reduce the strain on them in the future, we want to develop studies of cancer tissues from patients for high throughput analyses. Both primary tumours and tumours found to have spread to other parts of the body will be harvested and digested into microstructures. A natural material will be used to provide structural support for cancer tissue microstructures cultured after being grown in mice. Cancer tissue microstructures will be introduced on a test to identify personalised medicines for the individual patient.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We ensure that the maximum amount of information is obtained and analysed per experiment to reduce the need for repeats. statistical methods will be used to estimate the number of mice required to identify a statistically significant effect. We will consult as needed. In addition to this, all of the experimental hypotheses we test in animals are predicated by thorough cell-based studies and using studies where tissues from mice are grown in culture. This triaging of hypotheses to be tested in studies with mice by also using cell and tissue based studies reduces the number of studies we carry out in animals.

All outcomes will be presented using appropriate mathemathical analyses. For these studies, the primary analysis will compare the intervention group (drug treatment versus control) using rigourous mathemathical analyses. The difference in a measure signal from cancer cells from baseline to timepoints measured during the experiment will be the dependent variable. Mammary glands samples from mice will be considered as random effects while the treatment group (drug or control agent) is regarded as a fixed effect. Based on previous experience, given that treatment effects can be small, to reach a statistical power of 80% we would need at least 10-15 samples per group.

To assess treatment effects on growth of cancer cell spread, we will make measurements throughout treatment and analyse with appropriate mathematical tests. Based on past experience, to reach a statistical power of 80%, we would need at least 10-15 samples of organs that the cancer cells have spread to (e.g. brain, liver, lung) per group. When we work with the primary normal or tumour samples we need fewer mice because they will have plenty of material for other applications.

The approach we used in our mouse models is an improved method of cancer growth within the mouse breast We have documented 25%-95% growth rates depending on the type of the breast cancer cells being used in the studies. Because of this nature of the experiments, as a general rule, experiments will be performed using 3-5 mice per group or "condition". The same rule will be applied also for either treatments or oestrogen depletion studies where we will generate experimental groups of 3-5 mice/group. Henceforth, because of the nature of the treatments we have to generate experimental groups consisting of 10 mice/group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animal numbers will be minimised using three approaches. Firstly, we will rely on our previous experience in establishing procedures that yield the highest rate of success using the least number of animals. We have successfully competed studies under the authority of and are using the skills and knowledge informed in this work to yield results with minimum animal numbers. This is also made possible due to our collaboration with experts in the same field. We will also run, where possible, cell culture-based experiments to assess the validity of our hypothesis prior to applying the same principle to the setting using studies with mice. For instance, we are developing cancer tissue-based models to test hypotheses before proceeding to studies using mice.

A large reduction in the number of animals is not appropriate because a minimal number of animals is needed to allow relevant analysis. However, compared to other models using mice in which tumor pieces are grown in mice, our refined mouse breast cancer model is a refinement because we can inject up to 8 sites/glands per animal and use fewer mice to study a more individual tumours.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use non-invasive imaging where appropriate to minimise cohort size, so that we do not have to generate large cohorts of animals to be culled at different time points during the course of experiments. Additionally using tumour biopsy enables us to sample the same tumour pre- post- and during treatment. This reduces the need to setup large cohorts of animals to be sacrificed at different timepoints during treatment regimes.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been chosen as the experimental animal of choice as they are able to support the growth of human-derived tumours. The availability of special mouse strains permits the growth of tumours.

Mouse strains are chosen for their tolerance transplantation due to certain features of their immune systems. As a result of cancer cell injection or implantation, mice will develop tumours. Animal suffering will be minimised by keeping tumour burdens within acceptable limits. We use pain relieving drugs as part of all surgical procedures, thus minimising suffering. Animals will be monitored up to 3x times per week by staff, and research staff will assess the volume of the tumours. The tumours are allowed to grow to a suitable size for studies to begin or to be transplanted into other animals to provide animals for more studies.

The proposed experiments outlined here are appropriate for achieving the specified research goal because the mouse model we study offering an appropriate microenvironment similar to what is seen in breast cancer patients. The expected gain in knowledge lies in the achievement of a refined breast cancer mouse model for clinical and preclinical studies. It is important for the research field because cancer cell lines lack common genetic features of the diseases and current animal models require invasive surgery and lead to rapidly growing tumors that are painful for the animals. This is in accordance with legally recognized goals to decrease animal suffering, as the model is refinement to current models and decrease the number of animals per experiment, as each animal will be injected in 4-8 glands. If the mouse models we use in our studies can be translated to the cancer clinic, patient suffering and lives could be spared.

Why can’t you use animals that are less sentient?

Mammary glands are a defining feature of mammals. We need to use a suitably aged mature female mammal in order to study how breast cancer cells progress in a similar environment to that found in the human breast. Mice have teats (similar to human nipples)

that are connected to flexible tubes (milk ducts) that mimic the architecture of the human breast to a remarkable degree.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our preferred mouse model for growing human breast cancer cells in the mouse mammary gland is a refinement compared to current breast cancer models because it is less invasive than many current models. A further reduction of harms is not possible, because the needle that is used to inject the cells does not fit into intact teats. The width of the needle cannot be further reduced, because a smaller diameter would not allow cell injection. When appropriate for our experimental question, we use other methods to deliver tumour cells into mice. Pellet implantations are currently the most controlled ways to administer hormones to rodents and are less stressful for the mouse and the experimenter than oral delivery and injections. However, for some compounds, pellets do not exist and thus we still have to perform oral delivery. Animals are given wet diet after each oral delivery of drug to ensure if throat is sore they are able to eat food that is not dry/scratchy and do not have to stretch their neck if in discomfort. Appropriate pain relief and anesthesia have been discussed with the veterinarian to reduce the pain caused by our procedures. Animals are grouped housed and the cages contain appropriate enrichment material. Moreover, we are implementing gentle handling methods and avoid handling the tail, whenever possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We continually consult the Veternarian and colleagues in order to be using best practice for all procedures undertaken, particularly with the aim to minimise animal pain and distress. We have undertaken training and sharing of expertise by working with collaborating laboratories at our institute. This has allowed us to refine our surgical technique protocols and work together on shared knowledge of best practice.It has been highly beneficial to all teams and we will endeavour to continue similar interactions throughout the duration of this current project licence to share expertise and further refine techniques to minimise animal suffering through shared best practice adoption.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly consult the 3Rs resource library. We discuss 3R advances with BSU staff who we confer with prior to their implementation.

INVESTIGATION INTO THE PHYSIOLOGICAL DRIVERS OF POSTNATAL BRAIN DEVELOPMENT

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

neuroscience, brain development, psychiatric disorders, neural circuits, physiology

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To further understand how neuronal activity is co-ordinated by intrinsic and extrinsic signals to direct normal – and conversely dysfunctional, development in the mammalian forebrain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurodevelopmental psychiatric disorders place a significant life-long burden on an individual, their immediate family, and society as a whole. Understanding the fundamental rules for establishing the neural circuits that underpin higher order cognitive function is a pre-requisite to developing therapeutic interventions. Our recent work has identified transient circuits – physiological scaffolds – important for normal development in the mammalian cerebral cortex. Our proposed study will help us better understand the timeline of these circuits, how they are regulated and co-ordinated across brain areas to ensure the acquisition of normal function.

What outputs do you think you will see at the end of this project?

The project will identify molecular and physiological strategies used to co-ordinate cortical development. This will provide new information – in the form of peer review publications – to other researchers in the field of brain development, as well as inform clinical colleagues about the impact of environment and medication on the perinatal brain.

Who or what will benefit from these outputs, and how?

We anticipate that our findings will influence both short and long-term thinking, as well as lead to wider benefits to the UK and international biomedical research communities. Our immediate priority will be to inform the neuroscience academic community to help build consensus on future research objectives targeted at understanding normal and dysfunctional brain development. Ultimately it is envisaged that our outputs will have translational value and help address current debates in clinical practice, for example, as to whether or not various drugs such as selective serotonin reuptake inhibitor (SSRI) anti-depressants should be prescribed during pregnancy.

How will you look to maximise the outputs of this work?

The lab has extensive, world-leading experience in using optical approaches to investigate neural circuits off the developing mammalian forebrain. As such, we routinely collaborate with other groups, both in the UK and internationally, to assist them in their research endeavours. Dissemination of the results of the research to the immediate academic and clinical community is regarded as a top priority.

We are capable of generating significant amounts of data in a relatively short time frame; data that can be mined and interpreted from a variety of different angles. As such, we are intent on embracing online resources as a key way to communicate. Traditional means such as peer-reviewed journals, presentations at conferences and invited seminars are important benchmarks but one needs only look at other disciplines such as mathematics to see how we should maybe embrace more comprehensive and perhaps dynamic means of disseminating ideas, techniques and data – including those from unsuccessful experiments. To give an example of the current issues we face – the lab currently records and maps on the order of 4-5 cells per day in our in vitro experiments and obtained recordings from 50+ units in our in vivo electrophysiology analysis. These results speak directly to the question of diversity and function in the developing nervous system. Yet the current arrangement for disseminating research mean that some of the more unique and perhaps exciting findings will not make it into the public domain using traditional approaches. To address this, we continue to pursue online resources wherein researchers who have an interest in the field can browse all of our data with the capability to provide feedback on the analysis and direction of research in the lab. Such an endeavour is supported by the adoption of a research database in our Institute.

Species and numbers of animals expected to be used

- Mice: 18,000
- Rats: 2,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are by far the most common model system for studying the development of the mammalian nervous system. Traditionally, the rat has been preferred for the study of cortical physiology but advances in genetics now mean that mice represent the lowest and best vertebrate group in which to study the cellular and network properties of defined neuronal populations. The power of the genetic technologies is that we can target discrete populations of interneuron in the developing brain and understand their specific contribution to emergent network function. In addition, our studies in developmental psychiatric disorders benefit from an absence of maternal confound. Put simply, does a child develop a psychiatric disorder because of genetic or environmental reasons? Using rodent models enables us to control the former and test the latter, for example, the impact of anti-depressant SSRIs on brain development. These avenues of investigation have led us to also examine the role of behavioural state on development at early ages, when sleep predominates. To address this, we will include a number of neonatal rats as these are an established model for sleep analysis at early ages. Further, a number of genetically modified rat lines are available and relevant to our ongoing and future scientific objectives.

The majority of our experiments will be focused on early postnatal life, looking at early alterations in neural activity. However, there is significant utility in following some of these animals through to adulthood to examine if the pathophysiology is maintained or changes over the life time. Such investigations will enable us to better link adult phenotype with aetiology in neonatal cortical circuits, a key advantage of studies performed in rodents, and an aspect that is near impossible to track in human cohorts.

Typically, what will be done to an animal used in your project?

Our scientific methods include various physiological approaches from recording from acute brain slices to observing activity in live animals through imaging and implanted silicon probes. In parallel, we collect tissue for neuroanatomical studies that allow us to label and trace the location of cells, their pathways and molecular characteristics. This is often performed post hoc on the recorded tissue and provides further important information regarding the diversity of cells in a given brain region as well as confirming the targeting and therefore, validity of our physiology experiments.

The majority of experiments involve recordings from acute brain slices prepared from neonatal mice; an approach that allows us to record from and understand the contribution of individual neurons to the local circuitry of the developing cerebral cortex. For these experiments we breed genetically modified mice to generate neonates that mimic either normal or dysfunctional brain development and, in which, we can track and manipulate specific neuronal populations using optical and/or pharmacological means. We typically use 1-3 pups per day, anaesthetise them before decapitation and rapid dissection and slicing of the brain. These slices can then be maintained for up to 8 hours, during we time we can target and record neural activity.

More recently, we have come to appreciate that to truly understand the contribution of neurons to formative behaviour we need to record neuronal activity in live mice. To achieve this, we perform surgical techniques to implant either silicon recording probes (to directly record electrical activity) or a glass window over the surface of the brain, an intervention that allows us to image deep into the cortex over time without detriment to the

well-being of the animal. To date, we have performed these experiments in non-recovery anaesthetised animals but now we propose to use head-fixed animals that rapidly become habituated to the recording set-up and - similar to other animals at early ages - spend most of the time asleep. The animals will be imaged for up to 2 hours per day over early development to allow us to observe the impact of brain state (sleep versus wake) on early cortical activity. During this time, we observe neural activity associated with the animals fluctuating between sleep and wake. We also provide sensory stimulation, such as flicking the whiskers to see how normal sensory response are altered, providing further insight into the relationship between early sleep disturbance and psychiatric disorders. Imaging can be performed daily over the period of early, rapid brain development with the majority of experiments ending around the time the animals are weened. At this time point, we will humanely kill the animals, after which we may collect brain tissue for further anatomical studies.

What are the expected impacts and/or adverse effects for the animals during your project?

Pharmacological manipulation of early activity has the potential to lead to abnormal behaviours such as altered sleep-wake cycle, reduced interaction with littermates and social isolation; effects that are concordant with related pathophysiology in humans. Our investigations are largely focused on neuronal activity that occurs within the first three postnatal weeks. This, combined with clear humane end points in place, will minimize the impact on these animals.

Surgical intervention in postnatal mice can transiently disrupt the trajectory of normal development, most often observed as a failure to gain weight over the days immediately post-surgery. Our experience of performing such experiments to date suggest that we should not see prolonged adverse effects on the pups. The difference in weight gain observed in operated animals is transient, with pups starting to match the weight normally observed 2-3 days after recovery from the surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We estimate 50% of animals will be subthreshold and 20% mild. 18% will be non-recovery. 12% will be used under a moderate protocol.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed experiments will investigate not only when and how cortical interneuron circuits acquire functionality in the developing forebrain, but also how environmental factors such as pharmacological manipulation of neurotransmitters impacts on the normal developmental trajectory. As such, the objectives of our project require analysis of intact brains where we can observe and manipulate neuronal interactions in a way not possible in other models. This is only possible through the use of genetically modified mouse lines to identify circuit components and their contribution to emergent functionality under control and altered conditions.

Our adoption of a more systems-based approach, moving beyond single sensory systems to explore brain-wide co-ordination of development will require finer grain in vivo data to better model the contribution of the cells to activity in a state-dependent manner. This is of particular importance as ongoing projects point to the importance of sleep in brain-wide co-ordination of development. Sleep- wake cycles are controlled by a number of distributed brain centres with complex interactions that are challenging to recapitulate in vitro. As such our scientific questions necessitate the use of animals as opposed to alternative models.

Which non-animal alternatives did you consider for use in this project?

We have considered using in vitro organoids to provide key insights into possible hard-wired programming of early brain circuits. In addition, in silico modelling of brain development is an extremely useful tool. We collaborate with a couple of computational neuroscience research groups to further develop better in silico computer model of early interneuron circuits based on our data. To date, one of these models has been extremely useful in making predictions about circuit formation, enabling us to better target our experiments.

Why were they not suitable?

Our research is focused on the highly dynamic circuits of the early mammalian forebrain in the period immediately around birth. During this time, environmental factors start to influence and sculpt emergent function. Unfortunately, alternatives such as cell cultures are not a viable alternative as they poorly recapitulate these intricate, fluid interactions between the local environment and immature neurons.

More advanced in vitro systems, such as organoids, hold much promise and provide valuable information as to early genetic events such as general principles of fate determination and migration. However, again they have proven less useful as models of emergent neuronal synaptic interactions in that they fail to adequately capture the complexity of the highly dynamic early brain, nor the impact of the environment (nurture) beyond that of genetics (nature). We whole heartedly support endeavours to pioneer more effective in vitro organoid models and provide our expertise in developmental genetics and circuit analysis to collaborators to help refine their approaches.

In relation to computational, in silico approaches, our knowledge base is still lacking and therefore there is a real need to acquire more experimental data to build and validate the models. Over time these will likely improve further but perhaps never fully recapitulate the complexity and heterogeneity of brain development.

Further, neither in vitro or in silico approaches can, at present, provide useful insights into the impact of behavioural state - such as sleep-wake cycle - on emergent neural activity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers - the maximum scientifically justified by our proposal - are based on prior experience (running similar experiments in the lab for 15 years), our historical Home Office annual returns for animal use, and consultation with both veterinary and research colleagues.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the experimental design assistant (EDA) from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R) to plan our experiments. This has helped us identify that we have two arms of experimental design: (1) pharmacological, with 2 groups of animals (dosed vs. non-dosed); (2) genetic with three possible genotypes.

We further rely on our previous data to help define critical periods in early life and thereby better target our experiments to significant moments in circuit and behavioural development, such as the onset of sensory awareness around postnatal day 13.

We further rely on both formal (grant applications) and informal (joint lab and departmental meetings) peer-review of our proposed experiments that include animal number planning.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We engage in close consultation with animal care staff to promote effective animal husbandry and minimize the numbers of animals bred. Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

A number of further approaches will be employed:

- Genetically modified rodent lines no longer needed for immediate experimental aims are sperm or embryo cryo-preserved to avoid tick-over breeding.

- We co-ordinate with adjacent research groups. We share animals that are not required for our experiments with these colleagues who study neuronal activity in older animals.

- We also collaborate with computational neuroscience groups to develop models of brain development and thereby better target our experiments to predicted nodes – important cell types – that likely regulate key moments in development. By

understanding these, and the impact of perturbations on them, we can derive fundamental principle of brain development and extrapolate our findings to the wider nervous system.

- We have pioneered longitudinal imaging experiments that allow us to collect significantly more data from individual animals, reducing the number of animals needed for each line of investigation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare

costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use both normal and genetically modified rodent models to study brain development. Mice and rats represent the lowest species available with a brain structure and neuronal diversity similar to humans.

We use genetically modified rodent lines that exhibit no adverse signs when maintained on the appropriate background and used at the developmental ages that we study. They allow us to (1) identify specific neuronal subtypes that our data suggest are important for normal development, thereby avoid excess recordings; (2) manipulate neural activity so that we can directly test and assess the function of these specific cell types; (3) examine causative factors proposed to underlie neurodevelopmental psychiatric disorders.

The majority of experiments are performed in animal shortly after birth as this provides important insights into the origins of neural dysfunction thought to underpin neurodevelopmental psychiatric disorders. Experiments conducted under the previous project license have identified key time points in postnatal development allowing us to better focus our current experiments on these key junctures.

We will record neural activity in both brain slices and live animals, using surgical techniques in the latter to target our experiments to specific brain regions and thereby avoid systemic genetic modifications that can have the potential to lead to significant adverse effects to animals.

Why can’t you use animals that are less sentient?

Rodents are by far the most common model system for studying development of the mammalian nervous system.

The majority of our experiments to date have been in terminally anaesthetised animals. However, our shift to better understanding the impact of behavioural state - typically sleep in neonates, on brain development precludes this approach during in vivo experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all of our protocols we have defined our anaesthetic regime, possible outcomes and humane end points.

We continue to refine our surgical approaches in the light of our observations as well as new developments in anaesthesia, analgesia and surgery.

We have stopped using in utero surgery as postnatal surgery is less invasive and more targeted yet still gives us a unique ability to trace or incorporate cells, up- or downregulate gene function and study the long-term impact in the developing brain none of which is possible in vitro. This approach further represents a refinement over creating knock-out or knock-in animals in two aspects: (1) it enables us to

rapidly assess the impact of a genetic manipulation in a targeted, mosaic fashion. This is beneficial in terms of animal welfare as it circumvents adverse effects often observed following genetic manipulation that can arise from non-target tissue gene expression. (2) It enables us to avoid the protracted generation of genetically modified mouse lines reducing our use of animals.

Postnatal surgery will be done under general anaesthesia using aseptic conditions to prevent post- surgery infection. Analgesics suitable for the species will be applied peri-operatively as standard to minimise suffering, and monitored regularly. Animals will be humanely killed if any pain cannot be resolved by the administration of pain relief.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We use the followings sources of information:

The LASA (https://www.lasa.co.uk/current_publications), ARRIVE (https://arriveguidelines.org) and PREPARE guidelines (https://norecopa.no/prepare);

The 3Rs resource library on the NC3Rs webpage: https://nc3rs.org.uk/3rs-resources;

The journal LabAnimal (Nature journals, https://www.nature.com/laban/) for the latest research articles on implementation and refinement of in vivo techniques.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We participate in NC3Rs workshops on good practice in animal experimentation and attend termly meetings both at the Departmental and University level in which we share expertise and exchange examples of both good and bad practice. In addition, we received regular briefings from the NC3Rs regional manager and consult with the Named Information Officer.

72. Sea Lice and Gill Disease in Marine Aquaculture

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

sea lice, gill disease, salmon, trout, aquaculture

Animal types	Life stages
Salmon (Salmo salar)	juvenile, adult
Brown Trout (Salmo Trutta)	juvenile, adult
Rainbow Trout (Oncorhynchus mykiss)	juvenile, adult
Lumpsucker (Cyclopterus lumpus)	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to develop new approaches to disease in farmed salmon, trout and associated cleaner fish species.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Disease and parasites affect the welfare of farmed fish and the ability of aquaculture to contribute to the global food supply. Treatments are required to reduce the harm, but they must be shown to be safe and effective first by testing on infected fish. We will develop new methods to prevent and treat disease and to monitor the effectiveness of existing treatments, including sustainable alternatives to pesticides.

What outputs do you think you will see at the end of this project?

The most frequent short-term output of the drug studies will be data on the safety, efficacy and pharmacokinetics of candidate medicines, typically as commercially sensitive reports providing solid evidence to a client on whether their potential new approach to preventing or treating disease is safe and effective.

At later stages of development these reports may be submitted to regulatory authorities as evidence for licensing of the drug as a new medicine. This requires studies carried out and reported to the pharmaceutical industry's Good Laboratory Practice (GLP) standard.

Where the work is part of a collaboration with academic staff there will be publications in scientific journals. Even where data on pharmaceuticals is initially confidential, results will be included in peer reviewed publications following drug approval.

For non-medicinal approaches, the outputs will be reports and data showing the results of experiments e.g. how safe and effective a version of equipment or a device is, or information regarding the sensitivity of families within a broodstock.

Who or what will benefit from these outputs, and how?

The reports produced will identify promising candidate drugs for further study, development and use in fish farms. This is crucial to justification of the project because medicines used on fish farms benefit far more fish than are harmed during their development.

Drug resistance monitoring of sea lice will allow farmers to make informed treatment and management decisions by understanding when and where a pesticide has become less effective. Identifying the genetic basis of drug resistance will improve monitoring and inform new approaches to treatment.

Sustainable non-medicinal controls such as cleaner fish or improved cages will help farmers meet the Government calls for reduced environmental impact of marine cage farming including impacts on prawn and lobster fisheries.

In the long term, as new control methods come to market and the disease burden is reduced, improved fish health will facilitate increased production for public consumption, providing valuable protein and beneficial omega-3 oils that are good for heart health.

More effective treatments may have environmental or ecological benefits such as reduction in off-target effects of pesticide or sound pollution.

Farm companies and the farmed fish will benefit from improved resistance to disease following genetic selection.

How will you look to maximise the outputs of this work?

We will work with established commercial partners on drug safety, effectiveness and behaviour in fish to build evidence for approval of the right new products.

We will encourage clients and academic partners to collaborate on publishing papers to spread good practice and ensure unsuccessful approaches are not re-tested unnecessarily.

We will publish any refinements we make to our methods to improve fish welfare.

Species and numbers of animals expected to be used

Brown Trout (Salmo Trutta): 3500
Rainbow Trout (Oncorhynchus mykiss): 3500 Salmon (Salmo salar): 66000

Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have chosen fish species farmed at sea around the UK to find treatments for diseases that affect them on farms.

We will use post-smolt salmon and trout that are farmed on marine cage sites affected by sea lice or gill disease.

We will use juvenile ballan wrasse and lumpsuckers that are deployed from on shore hatcheries as cleaner fish on marine salmon and trout cage sites.

Typically, what will be done to an animal used in your project?

The new treatments we test are likely to be in feed, added to water or an injection under anaesthetic. For a trial looking at whether a new treatment works, a typical animal may be anaesthetised to allow measurement or marking and then allowed to recover, and will be infected, treated with either a potential or current medicine or placebo, and culled after a few weeks to allow disease progression to be compared between treatment groups.

For safety tests of new treatments there is no infection but fish are treated and may be anaesthetised. The order and timing of operations may vary; for example vaccines are given months to work before infection. Fish will usually only be infected and treated once but may require repeated anaesthesia over up to two weeks to follow parasite removal by cleaner fish in real time.

There will typically be some handling of fish using nets with associated minor mechanical damage such as scale loss. Non-medicinal treatments may result in increased physical contact, and fish will be monitored for resulting scale loss.

What are the expected impacts and/or adverse effects for the animals during your project?

Controlled numbers of sea lice typically cause some irritation and jumping behaviour on first exposure. Later stage infection will cause light damage to skin if adult lice congregate behind the dorsal fin, but any fish showing moderate damage will be culled.

Early stages of amoebic gill disease involve white patches of cell growth and scarring on the gills. Fish will not be allowed to progress to levels at which there are behavioural indicators of distress or suffering or if checked under anaesthesia to progress past 3 on the industry standard visual scale of 0- 5.

Potential treatments may have unpredicted side effects but fish will be monitored for signs of ill health.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Infected fish will be carefully monitored for signs of disease and we will aim to react as quickly as possible if any develop from mild to moderate disease. We anticipate that more than 90% of animals infected will suffer mild harm and fewer than 10% will suffer moderate harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Host-parasite interactions are complex and not completely understood. The infections we are testing treatments for depend on the health, physiology and immunity of the fish, and on the genetics of both fish and parasite. These interactions can not be recreated as computer models or in the laboratory.

Which non-animal alternatives did you consider for use in this project?

Sea lice that have been removed from fish will be used for early testing of products, but we cannot close the life-cycle and new drug treatments must be tested on infected animals to be approved. We have tried infecting fish using in vitro cultures of infectious isolates of N.perurans without success but will continue to try.

Why were they not suitable?

The sea lice life cycle cannot be maintained without a fish host. We can identify candidate medicines without using fish, but they must be proven effective and safe using infected fish before they can be approved for use on farms.

The amoeba that cause amoebic gill disease lose the ability to infect fish when grown in culture in a laboratory for several months, meaning that new isolates muse be sourced from naturally infected fish, which has proved both seasonally restricted and logistically difficult.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on previously published work and past experience of commercial studies we will need these numbers over 5 years if demand for trials proves consistently high, but the eventual total is likely to be lower in reality.

We anticipate using up to 16,000 fish per year. We anticipate safety and pharmacokinetic studies on 1- 4 candidate medicines per year, requiring up to 500 and 1,000 fish respectively. We expect to use 3,000 fish per year to maintain 6-10 strains of sea lice (40-100 fish per strain, partly replaced approximately every 2-6 months). We expect to use 1,000 fish per year to produce lice for in vitro drug sensitivity, a further 1,000 to produce lice to initiate in vivo experiments, and 2,000 for in vivo efficacy testing for candidate medicines. We anticipate infecting a further 1,000 per year with sea lice for testing non-medicinal treatments. We anticipate infecting up to 2,500 fish per year with AGD. We anticipate a further 4,000 entering from other project licences for continued use under Protocols 2 and 4.

It is likely that only a small proportion of trials will require rainbow or brown trout, or procedures on wrasse or lumpsuckers used as cleaner fish.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have analysed existing data to ensure that our experimental designs are based on robust and reliable information about the natural variation between fish and between tanks, thus reducing the need to repeat studies.

The US and European medicine regulatory authorities (FDA and EMA) set out requirements for the type, size and duration of studies required for approval of veterinary medicines for farm animals. This means following their advice avoids using too many animals by adding extra studies or testing at unnecessary doses.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We only keep strains of sea lice that are required for research, and keep colony size to a minimal viable level until demand increases just before a trial. The strains and numbers will be periodically reviewed and potentially reduced where possible and published literature will be reviewed as the project advances to stay informed of current best practices.

We aim to eliminate unsuccessful candidate drugs before testing in live fish. It takes fewer fish to grow enough lice to test drug efficacy once lice are removed from fish.

We supply excess sea lice to other researchers to reduce the need for dedicated infections.

We work within the Good Laboratory Practice (GLP) standard, which requires an experimental protocol that sets out the experimental objectives, plan, treatments, sample sizes, animals to be used, duration and data analysis. Following a plan ensures that there is the best chance of gaining useful data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use fish infected with either sea lice or amoebic gill disease. We restrict how many sea lice infect a tank of fish, and use fewer to infect smaller hosts. Progression of amoebic gill disease is temperature dependent. We aim to keep infection at levels that are easily supported by the fish without causing serious harm or distress.

Why can’t you use animals that are less sentient?

Sea lice and amoebic gill disease are both sea water pathogens that can only be grown on a few species of fish. Both infections develop over weeks, meaning that we must use fish that are conscious to maintain normal swimming and feeding, and we can only use fish that are old enough to go to sea.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use the NC3Rs online Experimental Design Assistant tool to ensure that we design valid experiments, use sensible numbers of animals, and analyse our data correctly.

We limit the parasite burden per animal to levels that are expected to cause mild harm based on the size of the hosts.

We use the minimum number of animals required to maintain a strain of sea lice or amoeba and only maintain strains that will be required for research.

All animals will be acclimatised to experimental set-ups prior to commencement of experiments. Where possible experimental tanks will be subjected to the same conditions (temperature, salinity, dissolved Oxygen, pH) as holding tanks to avoid inducing unnecessary stress.

Wherever possible samples, e.g. blood for drug testing, will be taken post-mortem to reduce handling under anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the following resource libraries of published best practice guidance to ensure experiments are conducted in the most refined way:

NC3Rs (https://www.nc3rs.org.uk/3rs-resources) Norecopa (https://norecopa.no/) European Union (https://etplas.eu/education/). In addition, we consult the following guidelines:

NC3Rs ARRIVE Guidelines 2.0: (https://arriveguidelines.org/) The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) are a checklist of recommendations to improve the reporting of research involving animals – maximising the quality and reliability of published research, and enabling others to better scrutinise, evaluate and reproduce it.

Norecopa PREPARE Guidelines: (https://norecopa.no/prepare) We also consult fish specific resources: Noble, C., Gismervik, K., Iversen, M. H., Kolarevic, J., Nilsson, J., Stien, L. H.& Turnbull, J. F. (Eds.) (2018). Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare 351pp.

RSPCA Welfare Standards for Farmed Atlantic Salmon

C. Sommerville, R. Endris, T. A. Bell, K. Ogawad, K. Buchmann, D. Sweeney. Veterinary Parasitology 219 (2016) 84–99. World association for the advancement of veterinary parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides for fish.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check updates to the regulatory guidance for testing veterinary medicines from the US and European Union to ensure that studies meet requirements.

In addition, we will regularly check for updates to the recommendations included in the ARRIVE 2.0 and PREPARE guidelines.

73. Gene Regulation in Neurological Development and Disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neuronal development, Gene regulation, Neurological disease, Vulnerability, Stress

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the molecular mechanisms underlying gene regulation during brain development and function, and to explore how these are altered in neurological disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We do not fully comprehend how the brain is formed during development. Understanding how genes are controlled during the complex processes of neuronal proliferation, migration, and connection is important for scientific research, and also to investigate and treat neurodevelopmental disorders.

Exploring the contribution of gene regulation to states of neurological vulnerability, such as after early life stress, is critical in understanding and intervening in a variety of neurological disorders.

What outputs do you think you will see at the end of this project?

This project will give information on how genes are turned on and off during development of the cortex. Mouse models will be used to address the functional significance of these in brain development and disease. Work in the laboratory will also identify the changes in gene regulation that occur after stressful events in early life, and how this increases the risk of later development of neurological disorders including depression, anxiety, and schizophrenia.

The main output will be publications relating to these data. The innovative nature of this work should result in high profile publications that are likely to gain the attention of a broad scientific and lay audience. The work will include innovative methodology and cutting-edge techniques that can be applied in other research areas.

The clinical impact on patients is long-term and beyond the scope of this proposal. However, the research will constitute an important step towards understanding, predicting, ameliorating, and preventing these significant neurological disorders.

Who or what will benefit from these outputs, and how?

The work is basic research, so we expect that benefit in the short term will be seen by increased understanding of biological processes during normal and altered human neurodevelopment. In the longer term, the identification of gene expression pathways altered in neurological disease may translate to new biomarkers of disease, and new therapies. We will also be investigating critical windows of adversity and of treatment, and this is critical for a nuanced discussion of vulnerability. The key cohorts who will benefit from the work outlined in this proposal are:

The scientific community: the results of the research will be published in high impact, open access, scientific journals that reach the wider academic community, as well as the lay public. Data and resources will be made available on publication. The research will be widely disseminated, for example through presentation at national and international conferences. The results will be discussed with world-renowned experts in the relevant fields throughout the project. The techniques developed in the laboratory will be shared with collaborators and other researchers. I will train and develop highly skilled researchers, including postgraduate students in the field of neuroscience.

Society: in addition to open access publication, we will increase public awareness and understanding of science through distribution of press releases when appropriate. We will also contribute to open days, school visits, and summer projects; these aim to introduce young students to state-of-the-art research and encourages them to consider a career in STEM (science, technology, engineering, maths).

Economic benefit: the commercialisation of scientific discoveries generated by the research described in this application will be a priority. Molecules and signalling pathways identified that may have relevance for neurological disorders will be tested in relevant disease models either in- house or via collaborations.

How will you look to maximise the outputs of this work?

We will collaborate with other neuroscientists in order to ensure that we obtain broad, as well as in- depth, knowledge of our animal models. We will collaborate with developmental psychopathologists to ensure our mouse model of early life adversity is relevant to human studies. Further, we will complement our animal work with work in human cells, samples, and patients, to feedback information gleaned from each system and ensure our work is of relevance in the clinic. We will disseminate our findings through open access publication

and make all datasets publicly accessible. Results will be discussed with world-renowned experts in the field and highlighted through presentation at national and international conferences. We will communicate with the public through public engagement events.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice throughout their lifespan. Mice share many physiological and genetic traits with humans and represent a useful model system to study brain development, maintenance, and regeneration in health and disease. Furthermore, mice are a well characterised model system for early life stress, with a reference genome and available high throughput sequencing data which will facilitate our study; there are also a variety of genetically modified strains available for disease modelling.

Understanding neuronal development requires the use of embryonic stages of mice, since this is when the cortex is formed. We are also researching the refinement of circuits after birth. A lot of our work focuses on early life stress which can occur in utero, through alterations to the mother, or during the first few weeks of life. We will then follow the mice through weaning and adulthood, to understand the neurological vulnerability engendered by the early life insult and its effect on the response of the animal to subsequent stresses received in adulthood. Investigating vulnerability across the lifespan is important to fully understand the impact. Critical windows across this period will be assessed for therapeutic intervention.

Typically, what will be done to an animal used in your project?

On the early life stress paradigm, pups will undergo adversity through either limited bedding and nesting for one week, driving the mother to frequently leave the litter to search for additional nesting material, or maternal separation, where the litter is removed from the mother for a short period daily during the early postnatal period. The deprivation is in relation to the quality of care provided by the mother, but does not result in an overt phenotype. After the period of early life stress, mother and pups are kept together until weaning, when the pups will go into single sex maintenance colonies until use. In adulthood, sub-threshold stress may be used to precipitate depressive-like behaviours in the early life stress mice, or in control mice for comparison. During setup of the paradigms, the pups will undergo behavioural testing after the early adverse events, after sub-threshold adult stress, or after the combination of the two together. We would anticipate seeing behavioural alterations only in the mice subject to both stresses.

We will endeavour to ameliorate the symptoms seen after the combination of early life and adult stress by environmental and gene targeting approaches. For environmental intervention, we will use enriched environment (giving the animals more space, new toys, more social interaction, changing the cage setup regularly to stimulate), or physical exercise (providing a running wheel), in different windows of time in the period between the stresses. For genetic intervention, we will inject genetic material into the brain of neonatal animals to deliver vectors that can activate or inhibit specific genes in the

postnatal period, either before or after the early life stress. To assess windows for genetic intervention, we will deliver vectors into the brains of genetically modified mice in which the vectors will only be activated in specific cells or at specific time periods (by using gene-inducing drugs in diet such as tamoxifen).

Genetically modified mice may be used in these paradigms to investigate the role of specific loci in the phenotypes. The breeding of genetically modified mice may also be used to develop techniques for mapping nascent transcription in neurons in vivo, and for investigation of neurodevelopmental disorders.

A small minority of pregnant animals will be subject to surgery that will be performed under anaesthesia and with the supervision of the named veterinarian. The surgery will be performed under general anaesthesia and will transfer genetic material and/or non-toxic substances into the brain of mice embryos. This is necessary to perform detailed analyses of the brain, and to understand how a lack of proper neuronal development may result in neurological and psychiatric diseases in the adult.

What are the expected impacts and/or adverse effects for the animals during your project?

Stressful events can induce depressive-like behaviours in mice including increased latency to eat, social avoidance/indifference, anhedonia (reduced ability to experience pleasure, such as not showing preference for a sucrose solution over water), and anxiety-like behaviours. These are relatively minor and are expected to only be experienced by the cohort of mice who undergo both early life stress and sub-threshold adult stress. Most mice in our studies will only undergo early life stress, as we are primarily assessing the interval between the two stresses, in order to study vulnerability. The early life stress alone is likely to cause transient and mild stress in the pups, and published studies have described no adverse behavioural phenotypes after weaning. Manipulations of pups can occasionally lead to rejection and cannibalism by the mother. This event will be minimised by using precautions, such as rubbing hands with cage sawdust before handling animals, avoiding overuse of surgical spirits, and by leaving some of the litter with the mother during the separation. Animals will be monitored closely after being reunited to the mothers and fostered if necessary.

Delivery of DNA vectors to brain cells in utero involves abdominal surgery of the pregnant mother. Good aseptic technique and appropriate use of anaesthesia and analgesia will be used to minimise pain and suffering during and after the surgery. Pregnant females will be monitored carefully after surgical procedures and further painkillers will be administered to reduce suffering if animals show signs of discomfort during recovery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect early life stress to be of mild severity, adult stress to be of mild severity, and the combination of both to be moderate severity. We anticipate interventions linked with the two-hit stress model (e.g. labelling injections, administration of genetic material into brain ventricles, environmental enrichment, behavioural techniques) to be of mild severity, and therefore, even in combination with the stressful events, the moderate severity would not be exceeded. We expect in utero electroporation surgeries to be moderate severity due to the potential of adverse effects due to the surgical procedure (infection of sutures, minor

pain after surgery). In my experience with these surgeries we find that animals recover well from the procedure and their behaviour returns to normal very quickly, suggesting that generally the surgeries are well tolerated.

Of the 4000 animals I have requested in total, 1125 (28.1%) would be expected to have a moderate severity (comprising 25% Protocol 1 animals, 50% Protocol 2 animals, and 100% Protocol 4 animals), with the remaining 2875 (71.9%) having a mild severity (75% Protocol 1 animals, 50% Protocol 2 animals, and 100% Protocol 3 animals).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This proposal uses mice as an animal model. The brain is a complex tissue and understanding its function fully requires studying it in the intact organism. My scientific focus will be on the changes occurring in neurons, but neuronal function is influenced by surrounding cells in vivo, and by the complex connections within and between brain regions. My research proposal centres on understanding the mechanisms through which early life stress affects neuropsychological function; this can be successfully modelled in whole animals but not mimicked in tissue culture systems. I also propose to rescue stress-induced phenotypes using environmental and gene-targeting approaches – this clinically relevant work requires insight that can only be gleaned by studying neurons in their natural tissue state. I will also use primary neurons derived from embryonic mice as an ex vivo model to confirm findings identified from neurons isolated from intact brain; these cultures ensure biological relevance and minimise culture artefacts.

Rodents represent an excellent mammalian model to study the brain during development, since their nervous system development shares many aspects with humans, as well as during adulthood in both normal and disease conditions. Mice can be easily genetically manipulated and for this reason, are widely used in the international research community to study the in vivo functions of mammalian genes. Mice are a powerful model for early life stress. Human studies of early life stress have focused on post-mortem brain, inappropriate tissue types (particularly blood samples), or epidemiological data – these studies provide valuable information but have confounding factors that do not allow us to fully probe the mechanisms through which environmental insults cause neurological dysfunction. The rodent 'limited bedding and nesting' and 'maternal separation' paradigms model parental neglect and induce anxiety behaviours in mice. They are well referenced, highly reproducible, and enable us to perform longitudinal assessment of the changes that induce and sustain neurological vulnerability.

Which non-animal alternatives did you consider for use in this project?

We work with human induced pluripotent stem cells (iPSCs) derived from adult blood or skin cells, in addition to animal models. Human iPSCs can model certain aspects of human neurodevelopment by differentiating the stem cells down neuronal lineages using established protocols. Human iPSCs can be used as neurological disease models through

the introduction of disease mutations, or through the use of patient-derived iPSCs (we are using this strategy to study Rett syndrome).

We also considered the use of human resected or post-mortem brain tissue, which we will use to complement the animal research but cannot replace it.

Another alternative to the use of animals is to use cell lines to attempt to model neurodevelopment in a dish. The laboratory will use mouse-derived cell lines (e.g. neuroblastoma lines) whenever possible, for example to optimise protocols.

Why were they not suitable?

Human iPSC-derived neurons have a human genetic background, are suitable to pharmacological human intervention, and are highly accessible for imaging, physiological recording, and biochemical analysis. However, the neurons differentiated from iPSCs have random network connectivity (not anatomically identified circuits) and are relatively immature (compared to adult human neurons).

Neuronal development cannot be reproduced completely in vitro; even the new organoid techniques recapitulate brain development only in part (Bhaduri et al., Nature 2020). Furthermore, neurons ‘in a dish’ cannot recapitulate environmental influences such as stress and ageing. Therefore, we feel that they can replace animal work in some but not all instances, and our use of both systems can enhance the translation of our findings into clinically relevant information.

We also plan to confirm appropriate findings from both systems in human post-mortem brain tissue. This has the advantage of being directly relevant to the human condition, with adult neurons in functional circuits. However, experimental interventions to understand cause-effect relationships are not possible. Moreover, available human post-mortem brain tissue is typically from older brains with advanced disease, has information for one time point only, and can have problems with tissue deterioration depending on the lapse between death and tissue harvesting.

The laboratory will also use mouse-derived cell lines, for example neuroblastoma cells, when appropriate. Transformed cell lines do not resemble brain cells and do not model the brain environment e.g., interaction with non-neuronal cells. Work using transformed cell lines will therefore be limited to protocol optimisation.

There is insufficient information available on brain function to generate accurate computer models which can predict the complex responses of neuronal tissues.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used for each experiment has been calculated using http://powerandsamplesize.com/ calculations that consider the statistical tests used to

analyse the results. All experiments will follow the guidelines indicated by UK Research and Innovation councils to ensure that the data are robust and reproducible.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical power will be assessed at the experimental planning stage, and throughout experiments. We will obtain behavioural, electrophysiological, and molecular data from animals of both genders.

Importantly, the experimenter will be blind to mouse genotype to avoid bias and will only be unblinded after data analysis. Whenever possible, experiments will be carried out in a sequential manner, to minimise the use of animals.

Our protocols will allow collection of tissue (in natural state or with perfusion fixation) and blood under terminal anaesthesia, maximising the material that we can use for immunohistochemical analyses or physiological/biochemical analyses per animal. Any surplus tissue will be stored immediately at -80oC for future use if not being used that day, generating a tissue bank that will be employed to obtain baseline values as required.

We will be conducting experiments to be able to publish results following the ARRIVE guidelines. By performing within-subject analyses which include cellular and cognitive information, data will be obtained from the same animal without compromising animal welfare, reducing animal numbers.

Statistical analysis will be performed so that all valuable information is extracted from the data (such as age/gender interactions). Statistical advice will be sought if required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before embarking upon in vivo experiments, relevant hypotheses will be tested using in vitro models, including neuron-derived cell lines and primary neurons. When in vivo experiments are appropriate, small pilot studies will be carried out to estimate the variability of the experimental data so appropriate statistical analysis can be used to minimise the numbers of animals required for a validated result. We will also conduct pilot studies to ascertain the most appropriate stressors (early life and adult) to model human early life adversity in mice.

I will carefully monitor animal breeding and adjust to provide the required animals for experimental need without excess. Breeding colonies will be kept to their minimum size so as not to over produce. Transgenic mouse lines that are not expected to be used in the next six months will be archived and embryos will be frozen for future use. We will conduct a full search before creating new transgenic lines to verify that there is no other worldwide availability. The Project licence holder will ensure that all people working on this project are appropriately trained and suitably competent to enable a high experimental success rate which will minimise the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This proposal uses mice as an animal model. My research proposal centres on understanding the mechanisms through which early life stress affects neuropsychological function, and I will therefore setup a model consisting of early life stress (maternal separation of postnatal pups for a short period daily, or limited bedding and nesting for the dam and pups) which can be combined with adult sub-threshold stress. Either early life or adult sub-threshold stress alone are not expected to induce adverse behavioural phenotypes in the mice, but the combination induces a depressive-like phenotype, modelling human early life adversity. Although the two-hit stress model does generate behavioural alterations in the animals to mimic the human condition, the majority of experiments will involve mice that have only experienced early life stress as our research questions focus on vulnerability. In other experiments with two stress hits, we will perform intervention measures with environmental enrichment or voluntary exercise, and gene-targeting approaches, which may reverse the behavioural impairments that our tests characterise.

To study neurodevelopment we will use in utero electroporation of timed pregnant mice to interfere with normal neurodevelopment in brain cells that can then be tracked throughout the lifespan. This procedure involves laparotomic surgery for the mother; animals will be closely monitored after the procedure for any signs of illness or discomfort. Appropriate anaesthesia and analgesia will be used during the procedure, and additional painkillers will be administered post-operatively as required; this will be done in consultation with the NVS. By targeting a subset of brain cells using this technique, we avoid the generation of genetic mouse lines with the mutation in a greater percent of cells which would likely induce more severe phenotypes.

The laboratory will also use mice carrying genetic modifications, which an effective way of investigating gene function in the brain. Although it is possible to inactivate genes and proteins during key developmental processes by administration of toxins or drugs, in many cases this approach suffers from lack of specificity. Moreover, unlike the use of transgenics, it does not allow the detailed dissection of the key physiological processes. All mice will be monitored daily.

Why can’t you use animals that are less sentient?

The mouse represents an appropriate species to study human-relevant biology with high significance, as this model system is used extensively world-wide. Importantly, rodent brain development shares many aspects with humans. Thus, work conducted with mouse model systems can provide insights into diseases of the human nervous system. More generally, mouse anatomy, physiology, and genetics are relatively similar to humans, and mice represent a powerful system to model human biology and disease.

Less sentient species such as the nematode worm Caenorhabditis elegans, the fruit fly Drosophila melanogaster, and the zebrafish Danio Rerio, are used to study some aspects of nervous system function. They are less sentient than mice, and have the advantage being easily genetically manipulated. However, the nervous systems of these species are much simpler than mammalian nervous systems. For example, Caenorhabditis elegans has only 302 neurons, Drosophila melanogaster has about 105 neurons, and Danio Rerio

has about 106. Therefore, the structure of the brain is very different. In contrast, the mouse has about 108 neurons, several orders of magnitude closer to humans (1012). Moreover, the developmental timescale and the behaviours controlled by the brain are very different in mammalian and non-mammalian species. Our research is based on the discovery of new gene functions that may play a fundamental role in regulating the mammalian brain in health and disease. In the past decade, extensive sequencing of most genomes has shown that many genes expressed in rodents and humans are not found in lower species. Thus, it would be impossible for us to investigate neurodevelopmental processes and neurological disorders using species that are so genetically distant from humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animal facility runs a comprehensive health-monitoring programme. Animal health and welfare records are maintained and include any adverse effects that may develop, particularly in genetically altered strains. The animals will be maintained under conditions where their health status can be protected as far as is reasonably practicable.

In advance of behavioural testing, animals will be handled daily to ensure that they do not get distressed by the handling required for the tests themselves. Modern methods of handling will be used to minimise distress (cup/tube handling).

When pups are separated from their mothers during the maternal separation paradigm, they will be maintained with littermates and will be carefully monitored. The mother will be left with some pups to minimise distress, and will also be monitored during separations. Manipulation of litters will be minimised, and we will implement strategies such as rubbing hands with cage sawdust before handling animals and avoiding overuse of surgical spirits, in order to prevent litter rejection when they are reunited. We will also use virgin dams to generate these litters, to minimise litter rejection and to maintain consistency and reproducibility. Animals will be monitored closely after being reunited to the mothers and fostered if possible if signs of cannibalism or rejection are evident.

During the limited nesting and bedding paradigm, pup body temperature and wellbeing will be monitored using an infrared thermometer. If pup body temperature drops, cages will be placed on a heated blanket to offset any potential impaired body temperature regulation.

In utero electroporation surgeries will be carefully managed to minimise harms. Appropriate anaesthesia and analgesia will be implemented with advice from the Named Veterinary Surgeon.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow published guidelines issued by Laboratory Animal Science Association (LASA), National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) to ensure that experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All associated staff in the lab will have extensive training in all the regulated procedures in this licence, and will use their experience to ensure suffering is kept to a minimum. Staff will attend all relevant and recommended courses including regular users meetings and PIL

refresher courses every 3 years, to maintain best practice and to keep animal welfare up-to-date with Home Office guidelines. The animal facility also disseminates a regular newsletter that highlights changes and improvements in animal research. I will conduct regular searches on the FRAME (Fund for the Replacement of Animals in Medical Experiments; frame.org.uk), NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research; nc3rs.org.uk), and norecopa (https://norecopa.no/) websites to ensure that we adapt our strategies in line with relevant advances. By staying up-to-date with the scientific literature in our field of research (e.g. using ncbi.nlm.nih.gov) we will also be made aware of developments that have repercussions on the 3Rs.

74. The Microenvironment in Organ Injury and Repair

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

liver regeneration, stem cells, t lymphocytes, inflammation

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the programme of work is to investigate how the immune cells affect the regenerative microenvironment and the liver progenitor cells after injury.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Liver disease in the UK is increasing in prevalence. Currently the only treatment for end stage liver disease is liver transplantation. As a result, the number of livers available for transplantation is much lower than required. The aim of this work is to find out 1. How liver disease occurs, 2. How the liver repairs itself following injury and 3. What happens when regeneration goes wrong: does it lead to liver cancer formation? By understanding these processes we can design drugs to specifically target these processes with the ultimate goal of preventing disease and enhancing repair in the liver; thus eliminating the requirement for whole organ transplantation.

What outputs do you think you will see at the end of this project?

Scientific outputs of this project will include research articles, presentation / posters at scientific conferences, public engagement activities at science fair. In addition, materials, protocols, and methods generated in this project will be shared with other researchers who are interested in studying liver regeneration and the immune system.

Who or what will benefit from these outputs, and how?

The benefits of this work primarily focus on liver disease and the treatment of such. These includes: 1) the understanding of the adaptive immune system during liver disease and regeneration. 2) Interventions that could promote regeneration. 3) Potential drug targets for chronic liver disease However, many of the processes which occur during liver disease also occur during disease in other tissues so our work in the liver will contribute to disease progression in a range of organs.

How will you look to maximise the outputs of this work?

Our research and knowledge regarding liver disease and regeneration will be communicated with the academic community, industry, patients and the public through several science communication activities. The findings of this project will be of interest to academics in the field, clinicians, patients with liver disease and the public. In terms of academic dissemination, data arising from our research will be available to the scientific community through early publication in peer-reviewed journals and presentations at relevant scientific meetings. We will publish in journals that will ensure the findings each the widest possible readership and maximize its impact. The importance of public engagement is recognised by our establishment. Our establishment has a dedicated Public Engagement team that actively liase the engagement between researchers and the public to disseminate new research knowledge and promote interest in science. To maximise our outputs, we will engage with other researchers through conferences with the aims to exchange ideas/ protocols and set up new collaborations.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mouse models of liver injury as our main models. These include genetic modified animals which enables the labelling of specific cell population or the specific ablation of T regulatory cells.

Regeneration in the liver is a complex, multi-staged process in which there are many different cell types interacting with one and other. It is impossible to model such complexity without using animal models. However, most experiments will be carried out in the laboratory at cell level before experimenting on live animals. Throughout the project, we will constantly, seek, review and incorporate alternatives to replace the need for animal studies. For example, in- co-culture experiments to confirm preliminary data, targets, and effect of small molecules/ recombinant protein before for the use in vivo enables the labelling of specific cell population or the specific ablation of T regulatory cells.

Typically, what will be done to an animal used in your project?

In our programme of work, we will use mainly dietary and chemical models of liver injury to model different aspects of human liver disease. Manipulation of immune cells can be achieved using intraperitoneal injections of antibodies or chemicals. Intravenous injections will be used of labelling cell for fate mapping experiments or induce liver injury. Cell transplantation experiments will be performed using surgical procedures such as intrasplenic injections or intravenous injections. For examples, adoptive transfer of T cells in combination with liver progenitor cells. We propose 3 protocols for this programme of work, with protocol 1 covers breeding of transgenic animals; Protocol 2 for induction of chronic liver injury (up to 20 weeks) , and protocol 3 for the induction of acute liver injury (up to 14 days). Mice will be humanely culled at the end of experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

As we are modelling liver disease, our animals will progressively demonstrate symptoms of the liver disease under the models that simulate chronic liver injury (with dietary or water). For example, weight loss and temporarily reduced mobility. These resembles the symptoms of human chronic liver disease such as liver fibrosis and fatty liver disease. The disease related symptoms will persist during the injury model regime, which range between 2- 6 weeks. In some situations, animals will experience interventions such as injections through intraperitoneal or intravenous, oral gavaging, blood sampling from peripheral vein. Animals might experience temporary pain due to the injections but will return to normal behaviour rapidly. The expected level of severity in these liver injury models are categorised as moderate severity. However, we will closely manage these symptoms such as monitoring animal body weight, behaviours, and condition of fur coating to ensure that the animals do not undergo any undue suffering. For animals that undergo intraperitoneal or intravenous injections, extra caution will be taken when performing injections to prevent injury to other organs of the occurrence of haemorrhage.

The animals that received liver injury models or ablation of immune cells may experience loss of weight, reduced mobility, and abnormal condition of coat such as piloerection. Animals weight and condition will be monitored frequently, such as: if weight loss exceed the threshold of the injury model, prolonged piloerection, hunched postures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The planned work will be conducted over five years and we will use a maximum of 8000 animals with approximately 5000 animals with moderate severities, 1500 animals with mild severity, 1500 with sub- threshold severity including breeders.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Regeneration in the liver is a complex, multi-staged process in which there are many different cell types interacting with one and other. It is impossible to model such complexity without using animal models. However, most experiments will be carried out in the laboratory at cell level before experimenting on live animals.

Which non-animal alternatives did you consider for use in this project?

Throughout the project, we will constantly, seek, review and incorporate alternatives to replace the need for animal studies. For example, in- co-culture experiments to confirm preliminary data, targets, and effect of small molecules/ recombinant protein before in vivo use.

Why were they not suitable?

Although relatively simple to conduct, in vitro co-culture experiments have the limitations of recapitulate the multicellular interactions in an complex organ. For example, the interaction of multiple cell types and the composition of extracellular matrix, recruitment and replenishment of new immune cells.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is possible to calculate the numbers of animals required for experimentation based on data from previous experiments and results. In all cases we ensure that we have calculated the minimum number of animals required for the experiment to give us useful data. This approach reduces the animal numbers required, and also reduces the likelihood that the animal experiment would have to be repeated. We also try to develop new models (genetic altered and non-genetic altered) to reduce the number of animal used. For example, we have developed Cas9 expressing cell lines to enable CRISPR-Cas9 genetic screening in vitro to reduce the number of animals used for the programme of work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use tools available online such as the NC3R's Experimental Design Assistant on the NC3R website (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda) to have an estimation of the number of animals required. We will also estimate the animal number required based on our previous experiments and experience in running similar type of experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will perform small pilot studies before new experiments to estimate the variability of individual type of injury models. Furthermore, we will share tissue with other research groups if multiple groups are working towards similar goals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our project aims to aim to investigate the effect of dysregulated immune system during liver disease using mouse models. The disruption of the immune system tends to occur during human chronic liver disease, and it is inevitable to use mouse models (both transgenic and non-transgenic) to simulate human chronic liver disease which takes several years to develop in human. We typically use mice for experiments as they model accurately human diseases and organ injury. Furthermore, genetic altered mouse models

are widely available between research communities enabling the reduction and refinement of animal models easier.

The animal models of chronic liver injury we used in this study (both dietary and transgenic) are well established and published across the field. However, we will perform small scale pilot studies for experiments such as altering the immune system. The optimal regime to alter the immune system without triggering systemic autoimmunity will be determined before using this protocol in conjunction with other liver injury model, as we do not intend to trigger systemic autoimmune response in our study

The behaviour of animals and signs of discomfort will be monitored throughout the injury regime, we will constant seek improvements on our current protocols through searching published protocols or exchanging knowledge with researchers within the field to reduce discomfort and establish models that are more relevant to human chronic liver disease. For example, the inclusion of a newly established diet in our project licence as a model of fatty liver which does not induce weight loss in mice, this has reduced the adverse effect on experimental mice.

If the animals undergo surgery pain relief will be provided prior to and after the surgery following veterinary advice.

Furthermore, when the toxicity or effect of a new agent is unknown a small dose finding study will be carried out to identify a safe and effective dose. This will aid the identification of potential therapeutic targets to aid healthy organ regeneration. Strict humane endpoints for these protocols, have been established through years of similar work and refinements of the techniques.

Why can’t you use animals that are less sentient?

Mice have one of the most developed immune system in mammalian experimental animals, and resembles human immune system and organ regeneration well. One of the key expects we aim to investigate is the recruitment and turnover of cells, and these experiments cannot be performed on animals that have been terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We typically use mice for experiments as they model accurately human diseases and organ injury. Genetic altered mouse model are widely available. We regularly refine the disease models we use to reduce animal symptoms and to improve the effectiveness of our models. For example, we are proposing pilot studies with a small cohort to identify the optimal dose for Diphtheria Toxin (DT) administration for our studies to reduce the suffering of animals whilst providing useful information simulating the disease phenotype. Experimental animals will be monitored with multiple criteria such as weight, sign of pain, piloerection, and standard operated procedure for pain management. For example, flexible tubing will be used for oral gavage to reduce complications and pain during administration

of drugs or tamoxifen. Furthermore, the administration of tamoxifen has been refined to the oral gavage route rather than intraperitoneal injections.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will perform our experiments with adherence to the ARRIVE guidelines and use the guideline to gain information about the minimal numbers of animals are utilised in order to gain valid experimental outputs. Careful breeding management will be conducted to ensure the animals with required genotypes are bred with the most efficient breeding strategy with minimal wastage.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will pay attention to latest published techniques and protocols to seek alternatives to refine our protocols.

75. Reproductive and Developmental Toxicology Safety Studies with Medicinal Products

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Reproductive/developmental toxicity, Regulatory, Saftey, Rodent, Rabbit

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult, pregnant
Rabbits	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This licence authorises the conduct of studies in laboratory animal species (rats, mice and rabbits) with the aim of evaluating the Reproductive and Developmental toxicity of medicinal products including, pharmaceuticals/biopharmaceuticals (human or veterinary/animal health), biological materials or entities (for human or veterinary/animal health), substances with potential medical utility other than therapy (eg diagnostic imaging agents, nanoparticle carriers), substances associated with drug candidates (eg metabolites, impurities, excipients, degradation products, placebos and/or novel vehicles for clinical trials), therapeutic/prophylactic vaccines, therapeutic cell preparations and Medical devices and/or their components or therapeutic agents.

This is to aid in the development of new medicines and devices, and to provide mandatory information to regulatory authorities to allow human/veterinary trials or marketing approval.

No cosmetic products or chemicals that are exclusively intended to be used as ingredients in cosmetics will be tested.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Governments require (and the public expects) that substances we are exposed to are safe or that their potential hazards are well understood and documented.

The data generated from the studies performed under this project will be used to inform decision- making processes on substances under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain clinical trial approval, marketing authorisation or product registration.

This safety assessment is of immense importance along with other rodent, non-rodent and non-animal studies in demonstrating to governments and the public the safety of these substances.

What outputs do you think you will see at the end of this project?

The overall benefit of this project is that it supports the development of safe, new medicinal products to improve the health and quality of life of human and veterinary patients by generating high quality data that is acceptable to regulatory authorities and enables internal decision making within our clients' organisations.

Achievement of the objectives of this licence will enable safe drug development candidates to progress and will also help to remove unsuitable candidates from the development pipeline at an early stage, thus saving animals and resources

Who or what will benefit from these outputs, and how?

Our customers will benefit, as the data we generate will allow them to progress their medicinal products under development and, where appropriate, to satisfy governmental regulatory requirements necessary to gain clinical trial approval or marketing authorisation.

Patients and animals will benefit from these studies as this work will contribute to the development of new drugs that help alleviate human and animal conditions. These new drugs may work better in the clinic, relieve or cure diseases and have better side effect profiles. We may, by our work, also contribute to better knowledge and understanding of these types of drugs, and that knowledge may be used to develop further new drugs.

Similarly for medical devices, they may be better than existing devices or a new device which can improve patient outcomes (examples of medical devices include dressings for wounds).

The toxicity information obtained is important when planning future trials in humans and animals, to make sure any starting dose in a clinical trial is safe for the participants taking it.

How will you look to maximise the outputs of this work?

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for regulatory purposes or to support regulatory purposes (e.g. to support drugs/devices progressing to clinical trials). Previously however, we have collaborated with customers and shared data we have produced in the form of scientific publications that are in the public domain

Species and numbers of animals expected to be used

- Mice: 3420
- Rats: 63000
- Rabbits: 4500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most of our experiments will be carried out on conventional adult rats and rabbits as rodents and rabbits are mandated by regulatory authorities for the regulatory studies in this project. The life stages evaluated on the various regulatory studies are also mandated by regulatory authorities. The rat is the standard animal for reproductive performance and developmental neurotoxicity tests and large amounts of background control data on all aspects of rat reproductive processes are available to help put study results into perspective. The rabbit has been selected as the second species for embryo-fetal development studies because it was found to be sensitive to Thalidomide, a notorious teratogen, whilst the rat was insensitive to this chemical.

In some specialist cases we may use the mouse for example due to unusual metabolism patterns or sensitivity in the rat and/or rabbit.

In some protocols we may use genetically altered animals. These animals may be immunosuppressed so that they do not reject the test substance (for example, biological

test substances such as human stem cells) or these alterations mean the animals produce specific medical conditions that we need to assess toxicity against. The use of genetically modified animals is very low compared to conventional animals.

Typically, what will be done to an animal used in your project?

Typically on this project, animals are dosed over a period of time with test substances, and usually sampled (e.g. blood or urine) before having tissues taken after they have been humanely killed for extensive toxicology analysis. Studies would range from a single dose, to those which last a matter of days (much less than a month) although some can last for 1, 3 or 6 months, and sometimes up to 1 year (to specifically examine whether a test substance can induce reproductive and developmental effects in two successive generations). Study durations are dependent on the specific regulatory test being performed. Some animals are left dose free for a few weeks after dosing is complete to see if any effects of the test substances can be reversed for example on juvenile toxicity studies, whilst offspring of treated dams on pre and post natal studies receive no direct treatment as mandated by the regulatory authorities .

Dosing of animals is commonly done orally using a flexible tube, or by injection using a syringe and needle, maybe directly into a vein, into a muscle in the leg, or under the skin. Other common routes are used such as inhalation (when animals are dosed in specially designed tubes) or dermal (via the skin).

Blood samples are usually taken from easily accessible veins in the neck or the tail of rats or mice, or from the ear arteries/veins of rabbits. We are limited to how much blood we can take at once or, cumulatively, over a month. If we need a large blood sample, we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try and take as many of the tissues and samples we need after the animals have been humanely killed after all dosing had been completed.

Some animals we use are genetically altered, so they better represent disease more applicable in humans, and make toxicity testing more relevant (and often shorter).

In some protocols we also have to surgically prepare animals for testing, when a normal animal would not be suitable. This maybe, for example, to implant a cannula into a vein for prolonged intravenous dosing, or for intravenous dosing over a period of hours. Surgery may also be required as part of the safety evaluation of a medical device that will be surgically implanted into a body cavity, or other therapeutic agent that requires injection into an internal tissue. Surgery is only performed when there is no other way forward.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, the amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to

repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. If we can take blood samples when an animal is deeply unconscious then we do. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures are trained to a high standard.

Animals undergoing surgery receive the same sort of care as a patient would in veterinary practice. We discuss their pain relief and use of antibiotics with a vet before we start and administer drugs as necessary.

The genetically modified animals we use are usually immunocompromised or modelling human disease.

Rarely we need to take a urine sample for analysis, so we would then put an animal into a special cage which is smaller than their normal cage. The animal can still move around. Virtually every animal will get used to their new cage within about 15 minutes and are fine.

Dosing with drugs may cause adverse effects in some studies. Experience shows that the majority (~65%) of animals are not expected to show any clinical signs of suffering (either no clinical signs or normal background signs expected of the rodent strain). A small percentage (~15%) may show transient subtle to mild clinical signs. Moderate signs of adverse effects may be seen in some animals (~20%), usually in the higher dose groups. Lethality and/or severe effects are not study objectives in any of the protocols within this licence, but for preliminary studies that may be the first animal studies with limited data available, a very small percentage of animals may inadvertently show severe findings before they are immediately and humanely killed.

We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved in its care for advice, including vets

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

On the last project, about 85% of animals displayed mild severity, and around 10% of animals were classified as having displayed moderate severity. This is because these studies can last between a few days and weeks to up to a year, and although the individual procedures are usually mild in nature on their own, the cumulative effects make them moderate overall.

It's impossible to predict the proportion of severities expected on a service licence like this, as this will be dependent on what study types we are asked to perform.

What will happen to animals at the end of this project?

- Kept alive
- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is currently no regulatory and scientifically acceptable alternative to the use of rodents and rabbits in fertility studies, pre and post natal studies and juvenile toxicity studies and The Organisation for Economic Co-Operation and Development (OECD) 416 and 443 studies and in most cases embryo-fetal toxicity studies.

However, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) S5 (R3) guidelines for safety testing of human medicinal products permit a number of options to use validated non animal or non mammalian assays to reduce or negate the use of animals and/or mammals for embryo-fetal development study testing in the following circumstances for pharmaceuticals expected to be embryo-fetal toxicants based on mechanisms of action, pharmacologic class or target biology; for pharmaceuticals intended to treat severely debilitating or life-threatening diseases; for pharmaceuticals intended to treat late-life onset diseases and, a qualified alternative assay which predicts the outcome in one species can be combined with preliminary embryo-fetal development study from a second species to enable the inclusion of women of child bearing potential (up to 150 women for up to 3 months treatment).

Labcorp scientists will advise customers of these options when relevant to the pharmaceutical being developed.

These studies are run to satisfy the regulatory requirements of governments around the world to ensure pharmaceuticals and medical devices are safe for humans and animals. These tests are very specific as to what they require in terms of testing in animals to ensure this.

We maintain a constant awareness of regulatory guidance and ensure that where non-invasive methods exist which fulfil the regulatory requirement they are used in preference to animal studies.

Which non-animal alternatives did you consider for use in this project?

We do not perform any validated non- animal alternative studies at Labcorp but study Sponsors may use such studies as permitted in the ICH S5 (R3) guidelines thus reducing or negating animal use in some circumstances and Labcorp scientists will advise customers of these options when relevant to the human medicinal products being

developed. There are no other non-animal alternatives for most of the work being undertaken on this project, but there are some exceptions (see previous section). The regulations we are following do not generally allow safety decisions to be made on non-animal systems alone, but there are some exceptions for human pharmaceuticals as detailed in the previous section.

In vitro and in silico methods (test tube or computer work not using animals) are used in combination with animal studies to inform study designs and assist in understanding of potential toxicity but cannot yet replace in vivo (animal) studies in most cases.

Why were they not suitable?

With a few exceptions listed in previous sections for human medicinal products, we need to use animals because non-protected animal alternatives cannot in most cases replace the extremely complex series of events involved in reproduction and /or subsequent development of the young and the reproductive organs; these processes cannot in most cases be effectively modelled in the laboratory in test tubes/dishes or by the use of sub-mammalian animals.

That is why we need to test new drugs and medical devices in animals in most cases, as they have similar physiology and processes as humans, and that testing gives us a good idea what may happen if they were ever tested in, or exposed to humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

The regulatory guidelines we follow for each study usually indicate the number of animals in a study; otherwise, the number used is the minimum to achieve the aims of the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Studies are designed to provide maximal data and statistical power (where appropriate) from the minimum number of animals considering that it is better to increase the number of animals used to achieve the objective than to use too few animals and risk having to

repeat the study. For regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist.

Conventionally reproductive assessments on human medicinal products are often performed as separate phases: fertility, embryo fetal and pre and post natal as adverse effects at an early stage may compromise ability to assess effects at later stages. However, if there is evidence from preliminary work or from similar materials, it my be possible to combine stages, particularly fertility and embryo fetal studies, to reduce the total numbers of animal used without compromising scientific integrity; the combined fertility/embryo fetal study is the preferred approach to separate studies by some customers.

Otherwise reference is made to standard study designs with input from the Department of Statistics, where appropriate, to identify the optimum number balancing the need to achieve study objectives while avoiding excessive animal use. These internal designs are reviewed and updated in line with changing external guidelines and internal refinements that either minimise numbers or reduce severity.

Whenever possible, common species of animals are used such that a large amount of control background data is available. This reduces the need for large control groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will try and get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to get several different samples, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Before our main studies, we use smaller groups of animals to get an idea of the doses we need to use for the main studies. These studies are important as it gives us confidence that the doses we are using are correct prior to testing them in bigger groups of animals required by global regulators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of our models involve dosing animals with test substances or devices, and sampling them, with many outputs taken after the animals have been humanely killed. This is generally the least invasive set of procedures that can be done to give meaningful outputs to make scientific decisions about further tests, or to determine the safety of a test substance/device.

Throughout our studies, our animals are checked at least twice a day. This allows us to see over a period of time, whether dosing each individual animal is causing any adverse clinical signs. if this is the case, we can take action: get veterinary advice, add food supplements and extra bedding if needing, and even reduce dose levels or stop dosing completely.

Why can’t you use animals that are less sentient?

Rodents (rats and mice) and Rabbits will be used in all of the studies conducted under this licence. Rodents are considered to be of the lowest neurophysiological sensitivity (their brain function and physiology) that will allow us to achieve the study aims and are considered suitable for predicting what's likely to happen in humans (or animals). The rabbit is mandated to be used as the first choice non rodent species for embryo fetal studies because it was found to be sensitive to Thalidomide, a notorious teratogen.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Many of the procedures performed on our rodents like blood and urine sampling, cause only transient distress to the animals. Blood sampling procedures are similar to and about as painful as having a blood sample taken by a doctor or a nurse. Blood volumes are kept to a minimum within rigid volume guidelines. Confining animals in special cages to allow us to take urine samples is similarly of little distress to the animals.

For inhalation dosing, where animals are restrained in tubes, training of the animals occurs for increasing periods prior to treatment commencing to accustom the animals. Dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm and will be the minimum consistent with the scientific objectives of our studies. In addition, suffering will be further minimised by implementing clearly defined humane endpoints.

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a

vet what pain killers or antibiotics the animals need both before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they start behaving normally again. We then check them at least twice daily before they go on study.

In addition, care is taken to provide as much environmental enrichment as possible. This includes plastic shelters in their cages, wood blocks and balls to gnaw on and push around; mice are given swings, and rats and mice on littering studies are given paper shavings from late gestation to use as nesting material. Rabbits are given key rings on their cages and wood blocks and hay and fresh vegetables and paper balls as appropriate.

In some tests we use animals that are genetically altered, to mimic conditions seen in humans or more commonly, transgenic mice because of their susceptibility to tumours. These animals are specially bred and don't display any harmful clinical signs due to their conditions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For blood sampling and dosing then the following guidelines/literature will be followed:

Diehl et al. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Journal of Applied Toxicology: 21, 15-23 (2001).

Gad et al. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. International Journal of Toxicology: 1-84 (2016).

LASA/NC3Rs: Guidance on dose selection for regulatory general toxicology studies for pharmaceuticals.

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery

Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. UK Home Office 2014

Regulatory guidelines.

This is not an exhaustive list and principally focuses on ICH safety guidelines for human pharmaceuticals:

ICH S5 (R3) guideline on reproductive toxicology: Detection of Toxicity to Reproduction for Human Pharmaceuticals, 30 July 2020.

ICH S11 Guideline on nonclinical safety testing in support of development of paediatric pharmaceuticals, 26 September 2020.

Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals ICH Guideline M3(R2)

Guideline on the evaluation of control samples in non-clinical safety studies: checking for contamination with a test substance. Committee for Medicinal Products for Human Use (CHMP), 2005.

CHMP/SWP/1094/04

Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies ICH Guideline S3A

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals ICH Guideline S6(R1) Nonclinical Evaluation for Anticancer Pharmaceuticals ICH Guideline S9

EMEA Committee for Medicinal Product for Human use (CHMP)
EMEA/CHMP/410869/2006: Guideline on human cell-based medicinal products.

For veterinary pharmaceuticals guidelines include:

OECD Test Guideline 414 Guideline Prenatal Developmental Toxicity Study, 27 June 2018.

OECD Test Guideline 416 Guideline Two-Generation Reproduction Toxicity Study, 22 January 2001. OECD 443 Test Guideline Extended One-Generation Reproduction Toxicity Study, 27 June 2018.

For medical devices guidelines include:

The International Organization for Standardisation (ISO) 10993-1 – Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process.

ISO 10993-3 – Biological evaluation of medical devices – Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicology.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

76. Defining immune mechanisms of intestinal inflammation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Colitis, inflammatory bowel disease, Immune mediated inflammatory diseases, Immunotherapy, IBD treatments

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand the underlying causes of intestinal inflammation, and how these responses differ between inflammation occurring in other target organs in other diseases, or protective responses occurring following infection and cancer, especially in the context of a new form of immune checkpoint colitis (CPI-C) induced by some new cancer drug therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We anticipate that the findings from this project will help us to discover targets methods for the treatment of inflammatory bowel disease, which may well be extendable to other

important immune- mediated inflammatory diseases (IMIDs), such as psoriasis, asthma, arthritis and hepatitis. Our data will inform the development of new mechanistic understanding and novel therapeutic approaches for help us understand and develop new treatments for these important diseases.

In the context of immune checkpoint colitis (CPI-C), there is currently not much known about this new type of immune checkpoint colitis (CPI-C) and there aren't any established mouse models of CPI-C, apart from the data from our own lab. Therefore, this novel animal model of CPI-C is incredibly important to the field of inflammatory bowel disease, in order to understand more about what causes this immune response and how we can help treat the CPI-C, which will have direct clinical relevance in the clinics.

What outputs do you think you will see at the end of this project?

The outputs of the project will include developing a new *in vivo* model of immune checkpoint induced colitis.

We will define how genetic pathways impact on physiological metabolic responses and how these effects can be reversed.

These above outputs mean that we will be able to identify key genes, cells or molecules, which can be targeted to help treat inflammatory bowel disease.

These data will then most likely be published by peer-reviewed open access publications.

Who or what will benefit from these outputs, and how?

Academic: The new *in vivo* models and genetic datasets will be made available to the academic community and will be of use to specialities that are investigating mucosal biology, cancer, immune homeostasis and autoimmunity (such as investigators in cardiovascular medicine, diabetes and metabolism). This will have both short-term benefits, allowing the group to research and perfect the colitis models, and long-term benefits with the eventual publications from this project.

Public sector: Understanding the mechanisms that underpin the maintenance of a healthy immune system has potentially huge impacts across the NHS. If it is possible to develop a greater understanding of the causes and treatment of the diseases we are studying, this would be of great importance in the long term to future patients. With regular contact with gastroenterologists at different hospitals, we are well placed to contribute to the diagnosis and treatment of autoimmunity, cancer and infections. This will ensure that useful discoveries are rapidly developed for maximum patient benefit.

Industry: Manipulation of immune system has been shown to hold therapeutic promise, with new drugs now entering clinical trials and others already in routine clinical use worldwide. The potential for developing a novel clinical treatment to maintain healthy immunity and treat disease would be of significant interest to UK industry. This work has the potential for substantial economic benefits in the long term. We have frequent collaborations set up with different industry companies and we are able to test new treatment drugs in our colitis models in this project.

Staff training: This project will directly lead to the training of many staff members and students in these *in vivo* colitis models. Staff will gain expertise in a number of cutting-edge research areas that will expand the skills base of the UK and lead to further

discoveries and technological advances as this expertise is shared through the research community. Staff will also gain training in skills useful to the wider economy, including time management, communication, presentation, IT skills and university teaching.

How will you look to maximise the outputs of this work?

As a research group, we will continually be looking to work with other research groups and companies in order to further the understanding of the mechanisms that are involved in IBD. These data will also be presented at national and international conferences and published in open access journals.

Any datasets regarding gene expression will be made available to public downloadable datasets.

Species and numbers of animals expected to be used

- Mice: 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have been chosen as the experimental animal of choice as they have an immune system of similar complexity to the human. For these reasons, mice are the most frequently used animals in studies of the human immune system.

The mouse models are important, not only in providing new insights into the processes that cause disease, but also as models in which to study treatments for the disease. The models proposed in this program will help us develop therapies for treatment of cancer and ulcerative colitis by enabling us to study potential treatments.

This project expects to use wild type mice with the different colitis treatment models from 6-8 weeks old onwards and before the mice are considered 'aged', thereby being classed as adult mice. This lifestage is the most representative of the adult human lifestage that we are most interested in for this project.
This project will also breed specific gene knockout mice, which may over time develop adverse effects. Mice will be used/bought in in this project in order to identify key inflammatory causing cells within the disease models.

Typically, what will be done to an animal used in your project?

Procedures will typically involve either changing the diet of the mice, orally injecting a drug or solution to change the gut bacteria (as good or bad bacteria is known to impact gut health), or by injection into the belly in order to induce colitis depending on which model of colitis is used. In this project, we will be utilising a few different well established and published colitis models. Each colitis model used in this project will be described below:

Dextran Sodium Sulphate (DSS): This is an acute model whereby a chemical (DSS) is introduced into the drinking water for 5 days and then 5 days back on standard water to

allow mice to recover. Sometimes multiple cycles, typically around 3 more times maximum, to induce a chronic DSS, alternates several cycles of this 10 day (5 days DSS and 5 days normal drinking water) routine as described above.

Oxazolone, Trinitrobenzene Sulfonic acid (TNBS): Typically mice will be be injected once into the rectum with this chemical under anaesthesia. This model usually lasts 2 weeks before the animal is humanely killed.

T cell transfer model: T cell transfer colitis is induced by transferring T cells from a separate healthy donor mouse (humanely killed under schedule 1) that are subsequently administered into the recipient mouse. This is one injection given at the beginning of this colitis model and mice are monitored weekly. The experiment typically lasts around 4-6 weeks, as it takes around 4 weeks before mice develop colitis and then they are assessed and monitored for increasing signs of colitis development.

Faecal microbiota transplant (FMT) model with colitigenic bacteria: This model involves the modification of the gut microbiome by an oral injection of a "bad" inflammatory inducing known population of bacteria. This oral gavage injection happens at the beginning of the experiment and typically mice develop colitis within 3 weeks.

Anti-CD40: Model requires an injection of an activating drug on the surface of immune cells. Mice are given the injection once at the beginning of the experiment and the experiment lasts 1 week, where mice develop colitis within 2-3 days but recover after a week. Sometimes multiple 1-week cycles of anti-CD40 will be used following recovery after a week of treatment.

Immune checkpoint colitis (CPI-C) induction model: Mice will be treated with either a weekly injection of immune checkpoint drugs, or an oral gavage of proinflammatory microbiota only at the beginning of the experiment. Some mice will receive both of these at the beginning of the experiment. Mice will be treated with this for either 1 week, 3 weeks or 6 weeks and be humanely killed at the end of that timepoint.

What are the expected impacts and/or adverse effects for the animals during your project?

Some mice will be held and be given an injection into the peritoneum, some mice will be held and fed once through a tube into their stomach (before being returned to normal feeding) and some mice will be given both. All of these procedures may cause initial mild pain but mice should show no immediate adverse effects or signs of sickness afterwards.

Possible or expected adverse effects would include poor response to injections, although these would normally not occur very frequently.

After a couple weeks, some might develop symptoms of colitis which can include any of the following: mild diarrhoea, some weight loss, very rarely rectal prolapse, rarely some might get rectal bleeding, signs of piloerection and possible some hunched posture. The expected duration and initial appearance of adverse effects have been listed for each model below:

Dextran Sodium Sulphate (DSS): This model lasts 10 days. However, adverse effects, with some very early mild signs of colitis listed above like 1-5% weight loss, will start to be visible within day 2 of DSS administration. Typically by day 5, the signs of colitis will peak with typically around 10-15% weight loss, signs of diarrhoea, signs of piloerection and

other physical signs of illness. However, once the mice get put back on water after day 5, they should start to recover with visible signs of weight gain, reduction in physical signs of illness and reduction in diarrhoea.

Oxazolone, Trinitrobenzene Sulfonic acid (TNBS): This model lasts 2 weeks. However, adverse effects, with some very early mild signs of colitis listed above like 1-5% weight loss, will start to be visible within 2-3 days of administration of this disease. Typically by day 7, the signs of colitis will peak with typically around 10-15% weight loss, signs of diarrhoea, signs of piloerection and other physical signs of illness and will require more monitoring.

T cell transfer model: This model typically lasts 4-6 weeks. However, adverse effects, with some very early mild signs of colitis listed above like 1-5% weight loss, will start to be visible after 3 weeks. Before week 3, the mice will usually be completely asymptomatic before this time point and carry on gaining weight. Typically by day 21, the mice receiving T cells will begin to stop gaining weight and the weight change will plateau. By week 4, the weight loss will begin to start and other signs of illness (listed above) will start to become visible as the week progresses. From week 4, mice should be monitored more closely. By week 6, the adverse effects listed above should be more obviously observed and the experiment usually ends at this point.

Faecal microbiota transplant (FMT) model with colitogenic bacteria: This model usually lasts 3 weeks. The mice here will typically be picked up and given a metal needle put down their throats, whereby a solution of the FMT will be given directly into the mice. There should be no discomfort after this procedure has been performed. Depending on the mouse strain (i.e. healthy normal mice or an genetically knocked out line etc), differing severity of adverse effects will occur, but the most extreme would be moderate severity. In normal wildtype mice, even after 3 weeks, there are typically no visible adverse colitis (no weight loss, no signs diarrhoea etc) in the wild type mice. Other gene knockout strains may develop signs of colitis after a week in this model, which could typically be similar to the adverse effects listed above and therefore after a week, if signs are visible, they will need to be monitored more closely.

Anti-CD40: This model lasts 7 days. However, adverse effects, with some mice losing 5-10% weight loss, will start to be visible within day 2 of anti-CD40 injection. Typically by day 4, the signs of colitis will peak with typically around 10-15% weight loss, signs of diarrhoea, signs of piloerection and other physical signs of illness. However, mice should start to recover from day 6 onwards and begin to start regaining weight.

Immune checkpoint colitis (CPI-C) induction model: This model usually lasts 3 weeks. This involves an injection into the stomach with a drug that blocks the inhibitory molecules on the surface of immune cells, and possibly the FMT model too. In this model, the use of wild type mice is the only known used mice in this so far, the adverse effect signs of colitis listed above are not even seen after 3 weeks and therefore the disease severity is mild. In other gene knockout mice though, this may not be the case and could become moderate level of disease severity after a week or two. These could typically be similar to the adverse effects listed above and therefore after a week, if signs are visible, they will need to be monitored more closely.

All mice involved in this project will be humanely killed by approved methods at the end of experimental procedure or if it becomes necessary to alleviate suffering at any time.

Expected severity categories and the proportion of animals in each category, per

species.

What are the expected severities and the proportion of animals in each category (per animal type)?

sub-threshold - 0%
mild - 50%
moderate - 50%
severe - 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Complex complete immune system responses can only be accurately simulated in animal models and there is currently no available theoretical model that adequately describes the role of innate cells in the immune response. In particular, studies in which the location and migration of immune cells to other organs and peripheral tissues are addressed best in animal models.

Which non-animal alternatives did you consider for use in this project?

Our group will actively work to develop alternative non-animal assays to assess cell-specific individual aspects of immune functions. For example:

Cultured lymphoid cells will be derived from each of the gene-targeted mouse strains and these will be used, rather than the whole animal, for multiple functional studies in vitro.

We will work to optimise other alternative systems described in the literature that make use of commercially available cell lines instead.

Finally, we're working on optimising a mouse *in vitro* colon organoid system. These colon organoid systems involve extracting stem cells from the colon of one mouse and being able to grow an artificial colon *in vitro* and then being able to use that system to add other immune cells or bacteria populations. Once we can get these to work, this will help both at reducing and replacing the need for the use of as many mice in the future.

Why were they not suitable?

For inflammatory mediated models, using a full mammalian in vivo system is still required and useful. Many auto-immune diseases have the potential to have off-target effects on other organs. For example, this is shown in certain cancer patients who are on some drugs and can develop inflammation in the colon, but also in other organs. Therefore, in vivo animal work is needed so that we can view the entire systemic effect of other organs of this colitis model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

9000 in a 5 year period for immune system experiments and breeding is 3500 mice a year.

Each experiment currently has consisted of 12-16 mice to enable appropriate controls and the experimental test, with experiments staggered every week or two, this would equate to the number of mice estimated here.

The projected number of animals reflect the number necessary to achieve the scientific objectives outlined in the programme of work described this licence and that this has been informed by our previous experience and that of other published work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We can use the NC3Rs Experimental Design Assistant to aid in designing experiments with mice in order to get a better and more accurate idea of the number of mice required. We'll also access previously published data on what concentration of drugs, treatments and colitis inducing methods, so that we don't have to do unnecessary in vivo dose responses ourselves. A pilot study using only a small number of mice initially will also be planned for any new drugs or treatments used on mice for the first time.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Tissues from all mice will be shared across the group and with collaborators to maximise the data that can be extracted from a single animal. Furthermore, multiple organs will be analysed per experiment in order to reduce the number of experimental repeats that are necessary.

Pilot studies using only a small number of mice will also be planned for any new drugs or treatments used on mice for the first time.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm

to the animals.

The mouse models of colitis and methods used will be ones that are already well established over the past few decades. Therefore, they've been heavily refined and tested by multiple lab groups in the past. We have also had our own experience with all the models and methods used before, so therefore know the optimum dose and method of procedure to apply the model.

The models of colitis mentioned above are the most refined due to their extensive use and also being published by numerous different research groups. They've been well documented and produce good clinical data.

Why can’t you use animals that are less sentient?

Mice are the least sentient species available that gives the most sufficient comparison to human IBD. Less sentient animal models do not have complex immune system comparable to human to gain useful insights

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This project expects to generate new strains of mice, which may develop with time adverse effects like colitis. In order to prevent these signs, most studies with such mice will be conducted within 8 weeks of age, when no adverse effects are observed.

Animals on study/ bred for purpose are monitored frequently and more when necessary e.g. when expected to see adverse effects. Animals will be culled appropriately if adverse effects are noted. Mice will also be acclimatised to handling and procedures before any actual experiments on mice are performed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Following all guidelines in the Home Office regulations and by LASA guidelines for dosing and administration. We can also refer to NC3Rs guidelines and website for up to date refinements of techniques. As well as ensuring all members of the group on this project are sufficiently trained and supervised by me and my lead research associate before they're allowed to undertake any work on their own. Constantly, monitoring publications in case doses to the models used get refined or reduced. Monitoring and ensuring our own doses are refined to the best method and doses.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Checking the Home Office website often and keeping up to date with the laws and regulations. We will also aim to continue to monitor the NC3Rs webpage, resources, and the establishment's 3Rs named programme manager.

77. Establishing mechanisms in the resolution of inflammation and tissue repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Resolution of inflammation, Phagocytes, G-protein coupled receptors, Chronic inflammation, Lipid mediators

Animal types	Life stages
Mice	juvenile, adult, aged, pregnant, embryo, neonate
Rats	juvenile, adult, aged, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Studies underpinned by procedures described in this license will aim to elucidate fundamental mechanisms that regulate the host immune response to resolve inflammation and to repair damaged tissues. These studies will also establish the role that disruption(s) in these mechanisms have in the propagation of disease in experimental settings of chronic inflammatory conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic inflammatory conditions, such as rheumatoid arthritis and cardiovascular disease, and bacterial infections remain one of the key causes of morbidity and mortality in both humans and animals. Whilst recent years have seen a marked increase in the available treatments for these conditions, these are not effective in all patients. Furthermore, the unprecedented increase in antimicrobial resistant bacterial species has spurred a search for alternative treatments.

One key aspect that has limited the development of new, and potentially more effective, treatments is the lack of understanding of how the immune system is finetuned in health and what leads to its dysregulation in disease. Gaining insights into molecules that are produced during health to maintain a functional immune system and how dysregulation in the production and activity of these leads to disease onset and propagation will be essential in the development of a new generation of treatments that are likely to be more effective since they will leverage the body's own defence systems.

What outputs do you think you will see at the end of this project?

Procedures described within this license will allow us to dissect fundamental mechanisms that regulate the immune response. Therefore, we anticipate that the studies will uncover novel biological mechanisms that govern immune cells to terminate inflammation and promote tissue repair. Thus, we anticipate that the studies will result in several publications. We also anticipate that some of the work will lead to the development of new intellectual property that could form the basis of new treatments for these conditions.

Who or what will benefit from these outputs, and how?

Results from these studies are anticipated to advance our understanding into mechanisms that regulate immune cells and how their disruption lead to disease. Therefore, it is anticipated that in the short-term scientists interested in studying host immunity in distinct settings, including healthy, cardiovascular disease and bacterial infections, will be the main beneficiaries of results obtained from these studies.

In the medium to long term, we anticipate that our findings will have a wider impact given that chronic inflammatory conditions and bacterial infections are of relevance to both human medicine and veterinary medicine. Thus, we anticipate that results produced from procedures conducted under this project licence, in particular those identifying novel therapeutic targets, will benefit both humans with chronic inflammatory conditions and unresolved bacterial infections as well as veterinary animals with similar conditions.

How will you look to maximise the outputs of this work?

To maximize the output we will collaborate with experts both within and outside the institution to ensure that findings made are robust and that the best insights are obtained from these studies. We will also disseminate the findings via publications in peer reviewed journals and at both national and international conferences. Furthermore, we will submit large datasets, such as sequencing, proteomics and lipidomics, in open access repositories together with detailed documentation on the procedures employed to generate the findings to ensure that the datasets are available to other interested investigators. We will also publish the findings of experiments which yield negative results, to ensure that all data generated from these studies is available to the community.

Species and numbers of animals expected to be used

- Mice: 3000
- Rats: 200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The procedures described herein will focus on the use of mice and rats. The choice of these animals to study fundamental mechanisms in the regulation of host immunity is based on:

the extensive knowledge already available detailing aspects of the host immune response in these animals and the aspects that recapitulate host immune responses observed in other animal species including humans

the extensive tools available in these species, including transgenic animals where specific proteins of interest are fluorescently tagged or deleted, which will allow us to study their role in the modulation of host immune responses

the extensive expertise available in my group (>15 years) with studying host immune responses in these organisms

To allow us to 1) study the evolution of immune mechanisms and 2) how they change throughout the life course we aim to use pups (the use of animals in this life stage will be restricted to limited protocols and linked with the treatment of lactating mothers), young adults and old adults (the use of animals in this life stage will be limited to select protocols) in our studies. We will also use both males and females to evaluate sex specific differences in the regulation of host immunity.

Typically, what will be done to an animal used in your project?

In this procedure we aim to employ a wide range of experimental models of disease to study how host immune responses are regulated in response to a range of inflammatory insults. These will include models of rheumatoid arthritis where animals will be challenged with arthritogenic serum via injection; models of cardiovascular disease where transgenic animals will be fed a high fat diet and models of bacterial infections where animals will be challenged with different bacteria, such as for example Escherichia coli. We will also aim to study the role of various organ systems, such as the vagus nerve, in controlling aspects of the host immune response to the various inflammatory stimuli. For this purpose, we will use a surgical procedure to disrupt the vagus nerve prior to inflammatory challenge. Finally, to evaluate the dynamic mechanisms regulating host immune response, we will also employ different imaging modalities to study cellular trafficking to and from the site of inflammation.

What are the expected impacts and/or adverse effects for the animals during your project?

The proposed procedures will primarily only yield relatively short-term adverse effects on

the animals being studies. For example, animal models of bacterial infections my result in a transient malaise leading to reduced feeding and activity which are anticipated to last no more than 1-2 days. Models of arthritis are anticipated to lead to mild to moderate joint pain and swelling that may also lead to a transient, usually lasting 3-4 days, loss of weight. Models of intestinal inflammation are anticipated to lead to malaise, and diarrhoea, that are expected to last around 4-5 days and result in weight loss as well as reduced activity. In a small proportion of the experiments these studies may also result in bloody diarrhoea. These adverse effects are also anticipated in models of sepsis, where bacterial dissemination is anticipated to induce malaise, diarrhoea and enhanced sensitivity to pain. In certain instances, to study the impact of chronic inflammatory conditions, we will use models of arthritis and gut inflammation where joint swelling and related pain will be longer lasting (e.g. up to 50-60 days).

Models of bacterial endocarditis may lead to pain and weight loss. These are likely to be transient and only last a few days. In aged animals we anticipate to observe adverse effects associated with ageing that will include stiff muscle, poor coat, a higher incidence of tumours, obesity and reduced spontaneous activity. Furthermore, inflammation in these animals is anticipated to be more pronounced.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We anticipate that the large proportion of animals (both mice and rats) used for procedures described in the present license will fall under the mild category (~55%) with a smaller proportion falling in the moderate category (45%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst recent years have seen a significant advancement in the mechanisms that govern the immune system in both health and disease there are still fundamental aspects that remain unknown. This knowledge gap has limited our abilities to develop in vitro systems that fully replicate the environment that white blood cells are exposed to which in turn limits the applicability of these systems in studying the fundamental mechanisms regulating host immune responses. This is of particular relevance since there is an increased appreciation that interaction of immune cells with molecules produced by other cell types are central in regulating their biology in both health and disease. Therefore, the use of animal models is essential, since these allow us to study these complex interactions and the nature of factors that govern immune cell biology.

Which non-animal alternatives did you consider for use in this project?

As part of our efforts to minimize the use of animals we have explored the use of in vitro system, Organ-on-a-Chip models as well as human primary cells and tissues. We believe each of these systems can offer benefits in understanding mechanisms that be used to offset limitations with animal models and reduce mouse models.

Why were they not suitable?

Since our aim is to evaluate the molecular and cellular mechanisms that govern host immune responses in both health and disease single cells, in vitro models are not suitable since these models are devoid of the complex cellular and molecular cues that are essential for immune cell regulation.

Organ-on-a-Chip models provide some level of cellular complexity, however since our understanding of the environmental cues (both cellular and molecular) that are relevant to immune cell regulation is limited the utility of these models is limited since they do not fully replicate the tissue environment that immune cells are exposed to. Whereas primary human cells and tissues only provide a snapshot of the cues that regulate immune cells which limits our ability to study temporal changes that may be linked with disease onset and propagation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers were estimated based on >15 years' experience with these models and known variability for each of the experimental models and systems employed. We have also used data from studies published in our laboratory and by others to estimate group sizes that will allow us to achieve a significance threshold of 0.02 to refute the null hypothesis, with a power of 80%. Furthermore, we also ensure that the group sizes are sufficiently big to allow us to repeat the experiments on at least two separate occasions in order to ensure that any findings made are robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To minimise the number of mice the experimental design assumes that mice will be used to obtain different experimental outcomes in each of the models. It was also assumed that where possible alternative non-animal models, such as Organ-on-a-chip model will be employed to address relevant questions. We will also use the NC3R's experimental design assistant to further refine our experimental design are reduce the animal numbers. We will also information gathered from historical experiments where appropriate (e.g. for unchallenged controls).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Throughout the project we will ensure that breeding will be tightly reviewed and efficiently planned in order to produce the required animals and reduced the generation of surplus animals. We will also aim to use heterozygous breeding in order to obtain littermate controls which will increase the robustness of our findings and reduce variability thereby reducing the number of animals required for the desired experimental outcomes. We also aim to use findings made using the in vivo models to develop new Organ-on-a-Chip models that would help to further reduce animal use. As detailed in the previous section, we will also seek to measure multiple outcomes from each animal, this will help us reduce the number of animals used as well as enhance the robustness of our findings by allowing us to link the biological observations made. Were appropriate we will also employ OMIC approaches to gather as much information as possible from each animal which can then be mined by other investigators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The primary objective of my laboratory is to explore fundamental mechanisms of disease. Due to both ethical and technical limitations this it is not always possible to perform such studies in humans or with human tissues. Thus, as a surrogate we employ models, both in vivo and in vivo models of disease.

The majority of the experimental in vivo models that we employ for our studies are well established and extensively characterized in mice. Experiments in mice will be used to obtain further mechanistic insight, and therefore complement, findings made with human samples (from healthy volunteers and patients) and primary human cells. Of note, although the mediators and some of the processes they regulate (e.g. leukocyte trafficking and tissue repair) are conserved throughout evolution, the biosynthetic processes and enzymes involved in the formation differ between mammals and other vertebrates. Therefore, mice represent an ideal model organism to complement studies with humans and to identify mechanisms of disease, studies which are not generally possible with humans. We also have extensive experience with these models as well as with the use of transgenic animals, i.e. animals where the gene of interest has been modified to influence its function. In specific cases where we require a second species to investigate the biological actions of interest, we plan on using rat models given that the tools and models currently available, including an increased availability of transgenic tools, will allow us to better address the biological questions described in the previous sections. This will allow us to address our fundamental aim, which is to identify and develop novel Resolution Pharmacology-based therapeutics that accelerate the termination of ongoing inflammation and promote tissue repair and regeneration. Therefore, it is essential for this development process to be successful that we conduct thorough pre-clinical investigations.

Why can’t you use animals that are less sentient?

The choice of species is based on the wealth of knowledge available detailing key immune processes in mice that are of relevance to the mechanisms of relevance to our investigations (e.g. the contribution of different innate immune cells to the propagation and resolution of inflammation). In addition, there is a wealth of publicly available datasets that can be leveraged to minimize the use of animals and also to refine our experimental design. Whereas the life stage selection is based on the specific biological questions being investigated, given that immune responses change animals (and humans mature). For example, we will use pups to study mechanisms relevant to the establishment of the immune system whereas adult (both young and aged) animals to evaluate mechanisms that become altered with ageing. Due to the duration of the studies, we are also unable to terminally anaesthetized animals for most of the studies proposed since this would not be practical and would also likely interfere with normal, thereby having an impact on the results obtained.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The protocols employed for refinement will be dependent on the model being employed. For example, in models of arthritis we will provide analgesics where it does not interfere with outcomes being measured. We will also provide soft food to ensure that the animals can eat easily, and soft bedding to minimise pain, we will also monitor the animals daily to ensure their well-being. In surgical models we will provide both peri and post-surgical analgesics and rehydration to facilitate the animal recovery and minimise pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Here we aim to use both the ARRIVE guidelines as well as the NC3R experimental design assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To stay informed on advanced in 3Rs the licence holder will receive regulate updates from NC3Rs and other relevant organisations as well as will discuss aspect of model refinment with both the NVS and NACWO.

78. Genetic and non-genetic modulation of blood cells in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Blood, Stem cells, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to improve treatments for patients with blood cancers by characterising the impact of damaged genes on the development of blood cancers and to understand how blood cells carrying damaged genes interact with the microenvironment in which they reside.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Leukaemia and related blood cancers such as myelofibrosis are devastating diseases affecting all age groups with a major impact on morbidity and mortality. Current treatments for blood cancer are unsatisfactory and the large majority of blood cancers are currently

incurable. There is therefore a major unmet need to develop new approaches to treat patients with blood cancer as well as to discover new ways to test blood samples from patients to better diagnose and classify patients with cancer.

The haematopoietic (blood cell) system remains the only system in which cell replacement therapy has an established and widespread clinical role by way of bone marrow/stem cell transplantation, typically as a treatment for blood cancer patients. However, despite this, there is considerable morbidity and mortality associated with current clinical transplantation, often due to slow or incomplete recovery of specialised blood cells. Thus, a precise understanding of the composition and regulation of blood cell production is critical to help further refine and improve bone marrow transplantation approaches and treatment of patients with various forms of cytopenias (low numbers of one or more mature blood cell lineages), for example to specifically identify drug targets that can enhance endogenous regeneration of required cell types. Some growth factors in routine clinical use represent excellent examples of potential therapeutic targets that might be identified through this work.

Current therapies, mainly employing chemotherapeutic agents available for decades, fail in the majority of cases to eradicate cancer/leukaemia stem cells, which are the source of disease recurrence and poor survival in many blood (and other) cancers. A central question in haematology (and more widely in cancer biology) is to understand the identity of the blood cancer stem cells and to define how they survive and evade current treatments to cause cancer. Understanding the molecular mechanisms involved will help to identify novel therapeutic targets to allow selective eradication of blood cancer stem cells, whilst leaving normal blood cells intact, with broad applicability to cancer biology.

What outputs do you think you will see at the end of this project?

The research described in this project will result in new information relating to blood cancer development and other diseases involving blood cells that will be published in scientific journals and presented at scientific meetings, therefore furthering knowledge in the field of blood cell research.

Ultimately, the purpose of this research is to improve the management of patients with diseases involving abnormal blood cells, through the development of better diagnostic techniques and treatments. For candidate therapeutic targets that we identify, our ultimate aim is to develop a treatment that will be taken forward to early phase clinical trials in humans within a 5 year timeframe.

Who or what will benefit from these outputs, and how?

These outputs will benefit other scientists working in the field of blood cancer research and blood cell research more broadly.

Our ultimate aim is to identify candidate therapeutic targets and new ways of testing blood samples that might be directly implemented to improve the care of patients within a 3-5 year timescale. If any therapeutic candidates are taken forward for clinical development, there is also the possibility that industry partners will benefit from this research.

How will you look to maximise the outputs of this work?

Our research group has a strong track record of dissemination of the results from projects such as the current application with the all stakeholders in the relevant international

community as well as engaging with the public and the families of persons affected by the diseases we are studying. For example, we typically present our work at international meetings. Datasets will be made publicly accessible to scientific collaborators and ultimately the wider scientific community when the work is published. We will publish in open-access journals and will endeavour to make the work available as early as possible through the use of preprint servers.

Our research group has had a particular interest in public engagement and has fostered the development of leukaemia patient and carer groups to provide structured feedback to the research team for future development of scientific plans.

Species and numbers of animals expected to be used

- Mice: 40000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Blood formation and leukaemia development are precisely controlled processes that require the living environment, such as bone marrow. Blood forming stem cells are known to interact with other bone marrow cells and are affected by other factors in the bone marrow microenvironment such as inflammation. The mouse is the most widely used system to study the formation of normal blood and blood cancers. Mouse models have demonstrated to be highly relevant and essential for development of an understanding and clinical application of the blood forming system in man, not the least application of bone marrow transplantation and understanding of leukaemia since mouse and human stem cells share similar properties. Other advantages of the mouse model (apart from it being mammalian) include the availability of laboratory reagents to study blood functions. Furthermore, availability of various mouse strains allows the study how genes of interest function in the blood system, including in vivo study of human blood cells in mice.

It is also known that blood cell development changes dramatically through different life stages. Some blood cancers originate in utero and cause cancer in children. It has more recently been recognised that some blood cancers arising in adults also have an in utero origin. Other blood cancers are associated with ageing. To understand how the life stage affects blood cancer development, we need to study animals through all these life stages.

Typically, what will be done to an animal used in your project?

Genetically altered mice may be bred with mutations that are not expected to be harmful until the some of the genes of interest are activated by the administration of substances such as Tamoxifen. Mice may be exposed to gamma irradiation at a dose designed to deplete the bone marrow. In some cases, the dose of irradiation is such that it would be lethal to the animals due to ablation of blood cell production in the bone marrow and spleen, so called ‘lethal irradiation’, and in all such cases the animals’ blood cells will be ‘rescued’ by transfer of haematopoietic cells. Typically, the dose of irradiation is divided into two sessions so as to reduce the risk of irradiation sickness. Within 24 hours of irradiation the mice will be given blood cells from other animals, which may be genetically altered, by

intravenous injection to replenish the bone marrow. Sometime ‘sub-lethal irradiation’ is used, this involves a lower dose of radiation which is not lethal to the animals and does not require transfer of cells for haematopoietic rescue. This is typically used to induce DNA damage to the haematopoietic cells. This will allow us to study bone marrow transplantation and to understand how this is perturbed by genetic alterations of non-genetic factors.

Mice may undergo serial blood or bone marrow sampling in order to study the development of blood cells over time.

Mice may also undergo low dose irradiation to study the impact of damage to DNA on the way blood cells develop. To minimise the side effects of irradiation, it will be given as a split dose (minimum 4 hours apart), and a transplant of blood cells to 'rescue' blood cell production will be carried out in cases where higher doses of irradiation are administered.

Mice may also have their immune system or blood cells perturbed by the administration of substances such as chemotherapy agents or biologic therapies such as antibodies or small molecular inhibitors.
This is required in order to understand how and why such treatments might work (or fail to work) for the treatment of blood cancers.

Mice may also be aged up until 2 years of age to allow us to study how ageing might alter blood cell development and response to treatments.

Some mice will undergo surgery to implant tissue under the kidney capsule to study how local tissue environment alters blood cell development (as opposed to systemic factors circulating in the blood stream).

What are the expected impacts and/or adverse effects for the animals during your project?

The potential adverse events primarily relate to:
- Immunesuppression and resulting infection
- Irradiation
- Surgery; laparotomy to access the kidney
- Leukaemia development

Administration of substances

Welfare of animals at risk of adverse effects due to above procedures will be carefully and regularly checked as outlined in the proposal. If some animals are in pain or exhibit other adverse effects, pain- killers or other treatments may be given under veterinary direction and mice will receive wet food and will be more frequently monitored. Mouse strains showing any unexpected ill-health will be humanely killed. At the end of each protocol, animals will be killed by a schedule 1 method, in all cases ≤24 months of age. Some mice may be supplied to other projects with appropriate authority.

Mice will have increased susceptibility to infection when they undergo irradiation and immunosuppression however, we will mitigate this by housing them in cages that are designed to protect the animal from pathogens.

Exposure to gamma irradiation, followed by the administration of blood cells may lead to

transient weight loss of up to 15%. Typically this occurs 7-10 days post irradiation but is fully resolved by day 14. Mice will be given moist palatable food to help maintain their weight.

Some genetically altered mice will be at risk of the development of blood cancers which may cause problems with anaemia, increased risk of infection and bleeding.

Administration of substances may cause specific adverse effects which is largely dependent on the exact substance administered. For many, no adverse effects are anticipated. For other, such as chemotherapy agents, this may cause a transient reduction in blood counts with a temporary increase in the risk of infection, bleeding and anaemia. Some substances used to induce genetic modifications may cause transient weight loss, skin redness if given by subcutaneous injection. Rarely, substances administered by oral gavage may cause damage to the oesophagus or lungs.

Ageing mice can have a number of adverse effects including rectal prolapse, skin abnormalities, corneal opacities, dental disease, weight loss or seizures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

30% subthreshold, 50% mild, 20% moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Haematopoiesis, and particularly the regulation of haematopoietic stem cells (HSCs) and early haematopoietic progenitors, are precisely controlled processes that require regulation from complex environments that can only be found in animals, such as the bone marrow niche. Attempts to model or maintain haematopoiesis in cell culture have demonstrated that the properties of early haematopoietic cells change so that they prematurely lose their key functions: the abilities to self-renew, and to maintain their potential to differentiate into different blood cells. It is also important to study blood cell development in a mammalian system, with blood cell development and immune system functions which closely parallels that of humans. Therefore, the answers to our scientific questions can only be answered by using animals.

When exposed to culture (non-living conditions), blood cells change their properties dramatically and it is very difficult to maintain blood forming stem cells in the laboratory for prolonged periods. Therefore, to understand these processes there is no alternative but to study them using animals.

Which non-animal alternatives did you consider for use in this project?

We regularly (and whenever possible) perform a large number of analyses in vitro, such as colony assays and long-term colony forming initiating cell assays (LTC-IC) and only use animals where in vitro experiments are not possible. Such *in vitro* assays are used, for example, to identify candidate therapeutic agents suitable for further evaluation.

There are two important recent developments that we will make use of. First, a human bone marrow organoid model has been developed, and this will be used to study patient samples whenever possible to replace mouse (xeno)transplantation experiments.

Second, an in vitro system for the culture of mouse haematopoietic stem cells has been developed, a system that we are now using to carry out screening approaches that would otherwise require very large numbers of animals for experimentation.

Both of these new technical developments are major, groundbreaking advances in the field and are expected to have a major impact in terms of replacement strategies.

Why were they not suitable?

They are suitable for many experiments and we are using these model systems widely. However, they do not fully recapitulate the *in vivo* microenvironment and study of blood forming stem cells, and how these cells interact with the many other cell types in the mouse bone marrow and other organs, can therefore only reliably be conducted using animal experimentation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals on the basis of previous experience in our laboratory, our previous home office returns (to aid in the numers for the breeding protocol) and strategic planning of my research groups programme of work over the coming 5 years. The large majority of mice are required for breeding protocols in order to generate complex mouse models. In order to estimate the number of animals in each experiment we have carried out experimental planning and statistical considerations to maximise the amount of information obtained from each animal. For example, to perform a genetic knockdown screening approach in protocol 2 for functional validation of candidate genes, we carried out a power calculation that determined that 4 replicate experiments (3 mice per experiment) would provide a 90% power to detect a 4-fold impact of gene knockdown on engraftment (relative to control vectors and/or CD45.1 competitor cells) with a type I error of 0.05. Standard deviation was calculated using previous transplantation data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Genome Editing to introduce leukaemia associated mutations

One of the challenges of introducing multiple different mutations to accurately model haematopoietic malignancy is that this requires very complex and large breeding programmes. We have developed protocols which allow genome editing to introduce multiple mutations ex vivo prior to cell transplantation which will significantly reduce the numbers of mice required for breeding.

Use of appropriate number of animals in each experiment

Experiments are carefully planned in discussions between the PPL holder and PILs to ensure:

Experiments are planned to maximise the amount of information obtained from each animal e.g. serial blood sampling or BM sampling

That the experiment is set up in an optimal manner to most effectively achieve our objectives, including the use of appropriate controls.

Where possible mice will be randomised into different experimental groups (blinded to the investigator). In smaller, pilot experiments, randomisation may not be possible.

New experiments will initially be conducted in a small pilot to ensure that the experimental protocol is optimal before conducting larger experiments.

Maximising yields of haematopoietic cell populations

We have put extensive effort into maximising the number of the cells we can obtain from each animal by rigorously testing the optimal antibody staining concentrations, centrifugation speeds and enrichment methods. We have also optimised and introduced new methods for gene expression analysis on lower numbers of cells, including nanofluidic and next-generation sequencing approaches for gene expression analysis, leading to a major reduction in the numbers of cells required for each analysis, thus consequently dramatically reducing the number of mice required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colony management

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Extensive research over decades have established the mouse as the most suitable animal model for studies of normal and leukaemic haematopoiesis, and as such key procedures for irradiation, bone marrow transplantations and genetic modifications have been most optimal refined in the mouse when compared to other species. It is also the only animal model in which normal and leukaemic human hematopoietic cells can reconstitute well in immune-deficient strains, of key importance for our studies. Animal models such as fish, worms or fruit flies cannot be used to achieve the scientific aims of the project as these systems are far removed from blood cell development in mammalian systems. During the last few years we have further refined some of the key and potentially harmful procedures through the following measures:

We will study blood cell development in mouse model systems either by inducing genetic modification in transgenic models or by cell transfer (transplantation) in vivo which will allow us to study the properties of purified populations of blood cells.

These experimental protocols have been developed over many decades and are now very robust and reproducible and have undergone extensive refinement over many years to minimise pain, suffering, distress or lasting harm to the animals. We have selected methods that enable us to study the mechanisms of blood cancer development and to test treatment approaches while minimising the pain, suffering and distress caused to the animals. For example, we use models with established experimental endpoints that can be used to assess development of disease using laboratory techniques (advanced blood cell analyses) before the mice experience pain, suffering or distress relating to the development of the blood disease. Similarly we always use the most refined approaches in order to minimise harm to the animals as detailed in the ‘refinement’ section of the application.

Why can’t you use animals that are less sentient?

The mouse is the most widely used system to study the formation of normal blood and blood cancers. Mouse models have demonstrated to be highly relevant and essential for development of an understanding and clinical application of the blood forming system in man, not the least application of bone marrow transplantation and understanding of leukemia since mouse and human stem cells share similar properties. Other advantages of the mouse model (apart from it being mammalian) include the availability of laboratory reagents to study blood functions. Furthermore, availability of various mouse strains allows the study how genes of interest function in the blood system, including in vivo study of human blood cells in mice.

Non-mammalian animals are limited in their use because they either do not have the right type of haematopoietic cells to provide relevant results. We can’t use embryos or very young animals as blood cells types change dramatically during ontogeny and it would not be possible to study relevant cell types for the development of blood disease in adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice that undergo exposure to gamma irradiation are given a split dose to limit the risk of irradiation sickness. Where the mice have their immune system suppress, we maintain them in specialised individually ventilated cages that protect them from pathogens.

When it is necessary to give substances such as tamoxifen for gene activation, the most refined route will be used. Typically, we use oral gavage and subcutaneously injections for this purpose. The dosing regimen is designed to ensure the least harm to the animals with volumes being in line with published guidelines.

For surgical procedures we will follow aseptic procedures to minimum home office and LASA standards and will supply animals with an appropriate course of analgesics until they are fully recovered.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow NC3Rs (https://www.nc3rs.org.uk/) and associated resource library. We will keep up to date with LASA, ARRIVE and PREPARE guidelines. As a general note, we will employ the NC3Rs' experimental design guidance and experimental design assistant (EDA) to plan our experimental design, practical steps and statistical analysis utilising the advice and support for randomisation and blinding, sample size calculations and appropriate statistical analysis methods. We will use the EDA diagram and report outputs to support experimental planning with animal users.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep on top of the literature and regularly review state of the art in the field. I will regularly access the NC3R webpage and follow tutorials, webinars and symposia. I will attend both departmental and institutional 3R meetings. I will consult with the NC3R’s regional manager and the Named Information officer.

79. Identifying and characterising novel anti-schistosomals (2023 - 2028)

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Schistosoma mansoni, Helminth, Vaccine, Drug Development, Functional Genomics

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall goal of our research programme is to identify novel immunoprophylactic, chemotherapeutic and immunomodulatory agents useful in combating the neglected tropical disease schistosomiasis. By applying *in vitro*, *in vivo* and *ex vivo* models, we will characterise how selected chemicals/biomolecules affect parasite development, mammalian cell phenotypes and host/parasite interactions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Schistosomiasis affects millions of people around the world, killing approximately 300,000 every year, and there is currently no suitable anti-schistosome vaccine. Existing control strategies predominantly rely on the effectiveness of a single drug, which is incapable of preventing reinfection in endemic areas and could lead to the development of drug-

resistant schistosomes. The identification and characterisation of novel schistosome targets to be used in translational chemotherapeutic or immunoprophylactic investigations will provide information essential to understanding the mechanisms schistosomes use to orchestrate long-term survival in infected hosts. This information is needed to direct more effective host physiological/immune responses during our search for urgently-needed, novel anti-schistosomal drugs or vaccines.

What outputs do you think you will see at the end of this project?

Our project will identify several (10-20) promising schistosome biomolecules that may be taken forward by us or other research/development groups within the greater schistosomiasis/helminth community as promising immunoprophylactic or chemotherapeutic targets. It will also lead to the identification of novel molecules/mechanisms utilized by parasitic helminths to regulate their complex development or to modulate host immunological responses during infection. We finally will provide schistosome material to other academic/industrial laboratories enabling external studies of schistosome biology or diagnostics of infection to be sustained.

We, therefore, expect that our research will have the following beneficial outputs:

Output 1) New academic knowledge of high impact (i.e. basic research purpose) disseminated via the publication of peer-reviewed scientific manuscripts/articles as well as communicating research progress at scientific conferences, public engagement events, governmental consultations and funding body meetings.

Output 2) Information relevant to the translation of our basic research activities to applied anti- schistosomal vaccines, immunomodulatory products, biomarkers or drugs (i.e. translational or applied research). This information (i.e. intellectual property) could be embedded in patents.

Output 3) Provision of schistosome material for use by other academic and industrial organisations.

Who or what will benefit from these outputs, and how?

The primary beneficiaries of our research are those individuals who are at risk of infection with schistosomes and will eventually develop disease. While benefits to this target population may not happen until after the end of the project, it is the long-tem goal and supported by our three secondary outputs.

Output 1) New academic knowledge will be continuously produced (overlapping with existing PPL). Current grant activity supports immediate to long term benefits of other scientists within the academic community and include:

Understanding the function of schistosome biomolecules during schistosome development and host interactions. These basic science outputs can be achieved within the 5 year license.

Delineating the role of epigenetic processes and protein-coding RNAs/non-coding RNAs involved in instructing cellular trajectories during schistosome development and host interactions. These outputs will originate within the 5 year license and be continusouly advanced after the project has completed.

Improving existing novel high-throughput platforms for quantifying metazoan parasite phenotypes (contributing to 3Rs). These developments will be completed in the 5 year license.

Output 2) Translation of our basic research activities into new interventions will be continuously developed (overlapping with existing PPL). Current grant activity supports immediate to long term benefits including:

Identifying new compounds that have *ex vivo*/*in vivo* efficacy in killing schistosomes/other parasitic flatworms. These new compounds could enter a Pharma/academic drug-discovery pipeline for producing novel anthelmintics. We are currently working with key industrial partners within both Animal and Human health sectors to translate our basic science discoveries. Drug discovery outputs often take decades to achieve; therefore, a new drug to treat schistosomiasis (target beneficiary population are humans infected with schistosomes) will be realized after the 5 year license has ended.

Providing *in vivo* efficacy data for progressing new schistosome biomolecules as human/animal anti-schistosome vaccines. Good Manufacturing Conditions orientated companies/organisations are currently being engaged to achieve this benefit. Human vaccines for neglected tropical diseases such as schistosomiasis often require decades of research to achieve; therefore, a new vaccine to prevent schistosomiasis (target beneficiary population are humans at risk of infection with schistosomes) will be realized after the 5 year license has ended.

Output 3) Provision of schistosome material for use by other academic and industrial organisations will be continuous. Current activities will continue and include:

Supplying soluble egg antigen to healthcare affiliate organisations to allow them to diagnose schistosomiasis in return travellers, asylum seekers and immigrants.

Supplying parasite material to UK Higher Education Institutes for research and teaching purposes.

How will you look to maximise the outputs of this work?

We will disseminate outputs of this work via preprint publications, peer-reviewed publications, patents, sharing of best practices with others in the community, shaping new standard operating procedures with others engaged in similar activities and consultations with other stakeholders (e.g. government, funders). As part of our practice of publishing in Gold Open Access formats, all (positive and negative) results will be submitted to appropriate publishers (e.g. Wellcome Open Research).

Species and numbers of animals expected to be used

- Mice: 3470

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse model (adult) is the most easily adaptable and appropriate mammalian definitive host for studying schistosomiasis in the laboratory as it is fully permissive, supports the full sexual development of the parasite and produces fully viable, infective larval stages. In addition, the mouse model can easily be genetically manipulated with a wide range of knockout and transgenic lines available. This allows for specific dissection of particular host factors that may or may not be involved in the proposed experimental procedures discussed herein. The only way to currently obtain juvenile and adult worms in sufficient numbers for molecular and biochemical investigations as well as *ex vivo* assays is from infected animals. To reduce the number of mice needed to generate juvenile and adult worms in some experiments, schistosomula, which can be produced by mechanical transformation of infective-stage cercariae obtained from snails, will be used in a triaging step.

Finally, the mouse is the most highly tested animal model system for determining levels of vaccine-induced protection or effects of chemotherapeutic treatment in our field.

Typically, what will be done to an animal used in your project?

In all protocols outlined in this PPL application, adult mice are used to propagate the *Schistosoma* (e.g. *S. mansoni*, *S. japonicum* or *S. haematobium*) lifecycle or to assess the efficacy of an intervention (vaccine or drug) on the numbers of schistosome parasites residing in an infected group of mice.

Parasite eggs, harvested from livers/intestines of infected animals, are used to generate infective larvae (miracidia) for infection of susceptible species of molluscan intermediate hosts (e.g. *Biomphalaria glabrata* NMRI albino and pigmented hybrids), which we keep in our snail facility or can obtain through collaborators. Starting at 4-5 weeks post-snail infection, the infective larvae (cercariae) emerge and are then used to infect 6-7 week-old, female mice via the percutaneous route. Typically, to propogate the lifecycle for producing adult worms or to initiate efficacy experiments, 150-180 cercaria are used during percutaneous infection (lower numbers of cercaria are used if duration of experiment exceeds 49 days). If juvenille or lung stage worms are required, then approximately 4000 cercaria are used to initiate the percutaneous infection. At the end of a procedure, mice are humanely euthanised by an overdoes of general anaesthetic containing heparin (100 units/ml). For the production of adult worms, termination of the experiment typically occurs at 49 days post-infection. For the production of juvenile worms, termination of the experiment typically occurs at 21 days post-infection. For the production of lung stage worms, termination of the experiment typically occurs at 6 days post-infection. For adult and juvenille worms, parasites are recovered by reverse perfusion during exsanguination of the hepatic-portal venous system. For lung stage worms, worms are recovered from surgically- dissected lungs after confirmation of death (e.g. cervical dislocation after overdose of general anaesthetic containing heparin). When assessing the efficacy of a chemical intervention, previously infected (typically initiated with 150-180 cercaria for 5 weeks) female mice (typically 6-7 week-old) will be administered a formulation of chemical/excipient, typically via the oral route (i.e. gavage). The numbers of oral administrations will be at least once, but could be up to 5 times (delivered daily).

Parasite recovery by reverse perfusion during exsanguination of the hepatic-portal venous system typically occurs two weeks after the final oral administration of chemical/excipient. When assessing the immunoprophylactic efficacy of a vaccine formulation, 6-7 week old mice will typically be administered an immunogen via the intraperitoneal, subcutaneous or intramuscular route (at least 4 injections, separated by a 3-4 week interval). After a final rest of up to several weeks, the immunized mice will be challenged with approximately

150-180 cercariae via the percutaneous route. Parasite recovery by reverse perfusion during exsanguination of the hepatic-portal venous system typically occurs seven weeks after percutaneous infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Our protocols typically involves the percutaneous administration of *S. mansoni* larvae (obtained from snails) to mice. In order to generate schistosome parasites of different lifecycle stages for our studies, these organisms must develop and mature in a suitable definitive mammalian host. The expected adverse effect of this protocol will be short term stress due to handling (100%), short term irritation due to parasite infection (100%) and deterioration of condition (e.g. piloerection, lack of provoked movement, diarrhea) due to parasite development/natural disease progression (100%). Some experiments assess the immunoprophylactic potential of characterised schistosome biomolecules.

Here, we will administer protein or mRNA/DNA vaccines (intraperitoneal, subcutaneous or intramuscular injections) to mice before percutaneously infecting them with schistosomes. At predefined timepoints (before and after immunizations), we will withdrawal blood by superficial venepuncture or superficial venesection. At seven weeks after infection, we will assess the efficacy of vaccination when compared to control animals. The expected adverse effect of this protocol will involve stress due to handling (100%), mild discomfort of vaccination/muscle electroporation (100%), short-lived pain due to blood withdrawal (100%), irritation due to parasite infection (100%) and deterioration of condition due to parasite development/disease progression (100%). Other studies assess the function of schistosome biomolecules or anthelmintics. By using RNA interference (RNAi)/genome editing/transgenesis or targeted drug treatment, we will generate information critical to our understanding of proteins necessary for intra-mammalian parasite development. Here, *ex vivo* manipulated (using RNAi/CRISPR-Cas genome editing/transgenesis) parasites will be administered to mice (typically intraperitoneally/intramuscularly/percutaneously) or percutaneously infected mice will be treated (typically orally) with an anti-schistosomal chemotherapeutic agent. Both of these *in vivo* manipulations can synergistically be used to assess the importance of key schistosome biomolecules. At predefined timepoints, we will withdrawal blood by superficial venepuncture or superficial venesection. The expected adverse effects of this protocol include stress due to handling (100%), irritation due to chemotherapy administration or parasite infection (100%), short-lived pain due to blood withdrawal (100%) and deterioration of condition due to parasite development/disease progression (100%). In all protocols, mice will be subjected to terminal general anaesthesia (containing 100 Units/ml of heparin). This will involve mild discomfort due to terminal anaesthesia administration (100%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice (100%) subjected to protocols would be classified within the moderate severity category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Schistosomes are mammalian parasites and, thus, necessitate a suitable mammalian host for completing studies outlined in our research programme. While we can employ other non-mouse alternatives (e.g. *ex vivo* assessment of schistosome gene function) for some study objectives, this is not possible for *in vivo* determination of efficacies induced by novel chemotherapies or vaccines (i.e. disease progression or lack of disease progression requires studies in a whole animal). Mammals are also necessary to maintain the full *S. mansoni* lifecycle and to assess the function of schistosome biomolecules during administration of RNAi-treated/genome edited/transgenically-modified parasites.

Which non-animal alternatives did you consider for use in this project?

Non-mouse alternatives include *ex vivo* parasite culture, appropriate *ex vivo* cell model surrogates (e.g. 2D cell culture, 3D organoids, 'organ-on-a-chip' technology) to assess how a schistosome biomolecule or compound can affect a mammalian cell, molecular biology manipulation of schistosome genes, comparative genomics/bioinformatics to computationally interrogate schistosome biomolecules, functional genomics (e.g. RNAi and genome editing) to suppress schistosome gene expression, epigenetics to assess schistosome development, biochemistry to assess enzyme activity and proteomics/lipidomics/glycomics/transcriptomics to understand the biological nature of key schistosome molecules. All of these non-mouse alternatives will be synergistically used to prioritise schistosome biomolecules prior to performing *in vivo* experiments in the mouse model of schistosomiasis.

Why were they not suitable?

Assessment of *in vivo* efficacies induced by vaccination or chemotherapies, maintenance of the full *S. mansoni* lifecycle and characterization of schistosome biomolecules essential to intra-mammalian development (via administration of RNAi treated or genome edited parasites) all require a definitive, and permissive, mammalian host. There are no current alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Some 2,200 mice will be needed to maintain a continuous supply of parasite material for *ex vivo* studies, production of parasite biomolecules for molecular/biochemical/cellular

assays and lifecycle maintenance. The assessment of the anti-schistosomal efficacy of ~80 defined immunogens/adjuvants will require approximately 500 mice. Quantification of the anti-schistosome efficacy of ~80 defined compound/excipient formulations will require approximately 500 individuals. A further 350 mice will be needed to assess functional characterization of targets.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Greater than 10 years of historical data detailing worm burdens of experimentally infected mice (from the last two PPLs) will be used to calculate the number of mice/group needed to identify a significant difference between control and experimental groups as part of efficacy experiments. This information, as part of power calculations described later, will be used to reduce the number of animals used.

Triaging our *ex vivo* drug discovery experiments through the schistosomula (larval) lifecycle stage (derived from material obtained from snails and not mice) will also reduce the numbers of mice needed to generate juvenile and adult schistosomes for progressing drug discovery (i.e. only compounds active against schistosomula will be pursued against juvenile and adult worms).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have over 10 years of historical data (worm burdens) related to *S. mansoni* infection of mice. This worm burden data will be used to determine the optimal number of mice to use/group to assess efficacy of an immunogen or chemotherapeutic. We also will perform pilot studies with minimal numbers of animals (1-3/group) to assess immune responses (antibody and cellular) generated by different adjuvants or tolerability of compound/excipient formulation. Before progressing any *in vivo* assessment of anti-schistosome chemotherapeutics, additional metrics including (e.g.) overt mammalian cell cytotoxicity (using human HepG2 cells), compound solubility, membrane permeability, *in silico* compound docking into target models and Lipinski Ro5 violations will be quantified. These additional measures will be collectively used to progress only the most promising chemotherapeutics/vaccine candidates, which will optimise the number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse model is the most easily adaptable and appropriate mammalian definitive host for studying schistosomiasis in the laboratory as the mouse is fully permissive, supports the full sexual development of the parasite and produces fully viable, infective larval stages.

The methods employed in this license have been carefully developed over the last several decades in laboratories around the world and have been the subject of peer review and refinement. Our specific experiences utilizing mouse models for the study of schistosome/host relationships have come about from 29 years of practical experimentation. All procedures use animals obtained from a licensed supplier and all animals are housed in conditions that comply with the Home Office Code of Practice. While the methods employed require experimental infection and the development of immunopathology in mice, this is an unavoidable consequence of infection.

Why can't you use animals that are less sentient?

Adult mice (the lowest vetebrate/mammal group) are required to support the full sexual development of schistosome parasites and to assess the efficacy of immunogens or chemotherapeutics. There are no less-sentient animals that can be used to replace adult mice for our planned studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Increased monitoring of infected mice - We have leveraged a popular social media platform (Whatsapp) to enable secure and private communication with end-to-end encryption on all chats between license holders and laboratory members. This real-time communication ensures that the condition of mice on protocols do not exceed welfare metrics in breach of the moderate severity limit of our license. The welfare metric scoring system has also been refined by us, updated twice and used to score individual mice during later stages of percutaneous infection or during pilot studies exploring tolerability of chemotherapeutic formulations.

Improved restraining of mice - we are currently refining the way in which we restrain mice during percutaneous infection. Utilising 3D printer functionality and expertise within our in house engineering team, we will create restraints of variable diameters to accommodate mice of different body shapes (e.g. larger diameters for larger mice; small diameters for smaller mice). This refinement is intended to minimise the stress associated with percutaneous infection of schistosome parasites. The restraints will also be made of red perspex (instead of clear), which will also minimise stress due to the animals being maintained in a darker environment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Two seminal 'best practice' methodology chapters detailing the protocols to study schistosomiasis in experimental animal models have been consulted to refine the protocols used in initiating and maintaining the schistosome lifecycle in our laboratory.

These include:

Lewis FA and Tucker, MS. Schistosomiasis. Adv Exp Med Biol. 2014;766:47-75. doi: 10.1007/978-1- 4939-0915-5_3.

Tucker MS, Karunaratne LB, Lewis FA, Freitas TC, Liang YS. Curr Protoc Immunol. 2013 Nov 18;103:19.1.1-19.1.58. doi: 10.1002/0471142735.im1901s103.

As part of our activities, we continually review best practice guidelines for refinements in animal research by accessing both Laboratory Animal Science Association (LASA; https://www.lasa.co.uk) and NC3Rs (https://www.nc3rs.org.uk) publications, guidelines and resources.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are active readers of NC3Rs and have developed refinements in our own methodologies based on information obtained from this web-based resource. We also regularly converse with the NACWO and NVS at this establishment as well as other schistosome-based investigators at other HEIs to identify areas for improvements in our methodologies. Some examples are provided in the following section.

80. Immune and cardiovascular regulation during and after pregnancy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pregnancy, Maternal, Offspring, inflammation, Cardiovascular

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the how maternal immunological and vascular responses impacts pregnancy outcomes

Determine the consequences of maternal immunological and vascular responses during pregnancy on postpartum maternal health

Assess the consequences of maternal immunological and vascular responses during pregnancy on postnatal offspring health.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A direct consequences on the health of both mother and child during and after pregnancy. The maternal immune system undergoes major adaptations to protect both the mother and fetus from pathogenic insult. Thus, maintaining normal, quiescent maternal immune responses during pregnancy ensures that the health of mother and child is also maintained. Aberrations in maternal immune responses during pregnancies can have dire consequences on pregnancy outcomes, including recurrent pregnancy loss (miscarriages) and preterm birth (premature birth), which in turn has major negative impacts on the health of the child.

The blood vessel network is extremely important during pregnancy, with the maternal heart pumping

~50% more blood during pregnancy compared to non-pregnant women, indicating the maternal heart and her blood vessels must be in perfect working order to ensure the baby is developing properly. This is compounded by the necessity for normal blood vessel development of the placenta, a highly specialised organ specific to pregnancy, because of the importance of this organ in regulating the transfer of oxygen and nutrients to the developing fetus via specialised vessels.

A major pregnancy complication, preeclampsia, affects 2-5% of all pregnancies worldwide and is the leading cause of maternal and child mortality and morbidity worldwide. The condition is instigated by exaggerated maternal inflammatory response that prevents normal blood vessel developmental pathways, particularly in the placenta. The consequences of these immune and vascular alterations directly affect both mother and child. These include serious cardiovascular complications jeopardising maternal heart, kidney and liver function. With respect to the fetus, the poor vascular development in the placenta leads to sub-optimal transfer of vital oxygen and nutrients, leading to growth restriction. Aside from the antenatal and in utero complications arising from pro-inflammatory pregnancy complications, are the long-term implications such conditions have on both mother and child. For example, mothers who have had preeclampsia during their pregnancy have a significant increased risk of developing cardiovascular complications later in their lives; and children born as a result of a preeclamptic pregnancy have an increased risk of developing congenital heart defects. Together, these highlight the importance of the maternal immune system during pregnancy and its long-lasting impact on the health of mother and child.

This licence will focus on understanding how inflammation and vascular changes, during pregnancy impacts the health of mother and offspring during and after birth. By investigating these pathways, our work will help to better understand the mechanisms involved in development of cardiovascular complications in mother and child as a direct consequence of pregnancy complications. Furthermore, this work could pave the way for identifying novel therapeutic strategies for the treatment of pregnancy- induced cardiac pathologies in mother and child.

What outputs do you think you will see at the end of this project?

The data from this project will provide novel information of how maternal immune and vascular responses impacts the overall health of mother and child during and after pregnancy; will provide preclinical data that will simulate clinical complications that occur during pregnancy in both mother in child, thereby giving the licence a clear translational relevance. The licence will underpin key mechanisms in maternal immune and vascular responses that govern normal and pathogenic pregnancy outcomes and will be relevant to clinical manifestations, such as preeclampsia and congenital heart diseases.

Consequently, the project will lead to data that will increase our understanding of:

- How and why certain pregnancy complications occur
- How such complications affect maternal and fetal health during pregnancy How such complications impact the long-term health of mother and child
- Increase our knowledge of immune and vascular responses in normal, uncomplicated pregnancies.

Aside from enhancing our knowledge in these key areas of pregnancy research, the data from this project will provide a wealth of publications in international journals and conferences.

To achieve the objectives of this project we will generate novel tools and methodologies applicable in the lab, in animal models and potentially in the clinic. To this end we will use the most refined models that can best represent human pregnancy complications, specifically placental inflammation and find interventional agents that can reverse the adverse outcomes of pregnancy complications. Examples of these include the administration of anti-inflammatory agents, and placenta-targeted therapies using nanomedicines. By taking such approaches and developing new scientific tools we will enhance our studies application to both basic research and clinical science, forging collaborations and driving the spread of the research.

Who or what will benefit from these outputs, and how?

The overarching aim for this project licence is to understand why pregnancy complications arise due to perturbations in immune and vascular responses, and what impact these aberrations have on the health of both mother and offspring, highlighting an important unmet need in pregnancy research. By unpicking these mechanisms, the project will provide novel information and provide researchers in the field a greater understanding of how and why pregnancy complications arise.

Moreover, given the clear translational relevance of the objectives of this project, the outcomes of this project will also benefit clinicians, where findings from this project could ultimately guide patient care and treatment.

The latter outcome is compounded by plans within this licence to find effective treatments to restore, for example, normal cardiac function in the mother both antenatally and postpartum. This will highlight efficacy of commercially available drugs that are safe to use in pregnancy, for the treatment of maternal cardiovascular complications. Additionally, a targeted placental anti-inflammatory approach, described in the licence, to treat offspring congenital defects could offer a potential alternative to current invasive in utero interventions to treat such anomalies.

How will you look to maximise the outputs of this work?

This work will be undertaken with a broad range of expert collaborators from both basic and molecular biology to clinical practice. Each step will be validated to ensure that only targets that are applicable to both mouse and human pregnancy are investigated. New knowledge gained from these projects will be presented at national and international conferences while completed studies, irrespective of success, will be published in open-access journals. In accordance with the Concordat on Open Research Data, outputs from publicly funded research will be made freely accessible to maximise use of all data

acquired, broaden the research benefits and allow transparent scrutiny of research findings.

We are also keen to disseminate knowledge to lay audiences through public engagement including festivals, imaging competitions and talks to allow public debate about the use of animals in medical research.

Species and numbers of animals expected to be used

- Mice: 5310

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Complications during pregnancy affect all stages of life, from embryonic to neonatal, through to adulthood. Thus, the work to be carried out in this project will encompass these life stages.

Placentation varies between human and mouse. However, the mechanisms involved in the induction of placental aberrations and subsequent pregnancy-induced pathologies share common pathways between human and mouse. Therefore, using mouse pregnancy models will enable better characterisation of how and why such complications arise and their long-term impact on maternal and offspring life.

The project will be focussed on investigating impacts on:

Antenatal maternal health. Hence female mice will be used between 8 and 12 weeks, for optimal breeding ages for female mice.

Postpartum maternal health. The project will aim to investigate the long-term effects of pregnancy on maternal health.

Assessing the immunomodulation of the cardiovascular system throughout the life stages To maximise the specificity of studies proposed, in some cases we will apply inducible genetic manipulation immediately prior to induction of each protocol and enable us to make the most of the wide and valuable array of genetically altered (GA) strains available.

Typically, what will be done to an animal used in your project?

This project will generate genetically altered mice to target and visualise key aspects of immune and vascular biology and assess the dynamic changes that occur during and after pregnancy.

In this project we will use some animals in a breeding programme to develop new strains of mice that incorporate fluorescence proteins with specific immune and vascular genes associated with pregnancy. These mice will be used in study protocols designed to enhance our understanding of immune and vascular interactions that take place during pregnancy, provide robust, reproducible, and quantitative measurements of immune and

vascular changes during and after pregnancy, and allow tracking cells and vessels in real time with both non-invasive and invasive fluorescence imaging. Mice used for breeding will be housed in appropriate living environment that allows them to perform their natural behaviours and maintains their welfare. All genetic modifications will be previously established to minimise any impact on health and wellbeing. Any genetic changes that are known to impact development will be bred with additional 'conditional controls' whereby the gene/s can only be activated or deleted in the presence of additional dietary or drug supplements. Mice assigned to the breeding protocol will not be expected to experience more than transient or mild discomfort.

The three study protocols detailed in this project will rely on time-mated pregnancies to assess the immune and vascular responses that occur during pregnancy and their impact on maternal and offspring health. To address these impacts most animals under a study protocol will undergo injections or a series of injections during pregnancy that will induce immune and vascular changes. Additionally, some adult animals may undergo dietary changes (such as high-fat or Western diets) or exercise- induced stress to assess any metabolic shifts that are intimately linked to both immune and vascular changes during and after pregnancy. The vast majority (>90%) of mice in this project will be humanely culled prior to displaying any clinical signs of distress with quantitative data derived following the collection of tissues, blood and bodily fluids.

Approximately 40% of all adult animals and pregnant female animals under study protocols will undergo non-invasive imaging to increase the data acquired, refine the monitoring of processes being investigated and reduce the mice required for each procedure. To assess the long-term implications of pregnancy on immune and vascular health in maternal and offspring mice, adult animals are likely to undergo the optional steps of having blood taken and undergo 2-3 imaging sessions, during their lifespan, under 1 hour in length under recovery anaesthesia.

Grouping will be determined by ensuring experiments are designed with comparable control groups that account for genetic background and environmental factors. We will use matched mouse strains to minimise variation between experimental groups and maximise reproducibility.

Animals that are under procedure will be subject to enhanced supervision to ensure their well-being and ensure that experiments are conducted within the parameters detailed in the licence.

What are the expected impacts and/or adverse effects for the animals during your project?

(Protocol 1). Almost a third of the mice used within this project will be assigned to the breeding protocol and will not experience anything more than mild and transient distress. By breeding already established genetic altered strains with fluorescent reporter mice and using 'conditional' controls of gene deletion/activation we will mitigate any unforeseen impacts that genetic modification could have on normal development and behaviour.

The mice that are assigned to the subsequent three study protocols will most commonly experience moderate discomfort associated with single or repeat injections of the conditional gene regulators followed by immune and vascular-regulating substances at doses that are well tolerated. In the unlikely event that mice display localised inflammatory reactions that such as swelling or redness, acute weight loss or abnormal behaviour for more than 24 hours in response to these procedures will be euthanised.

(Protocol 2). 2000 time-mated pregnant mice will undergo up to two mating cycles (30% of the total project) and will be assigned to receive immune or vascular regulatory substances during pregnancy to induce placental inflammation only during their first mating cycle. While these models result in placental dysfunction, they are not associated with pain or abnormal behaviour and are only clinically apparent by the release of indicative proteins into the blood steam. Therefore, most mice will only experience mild and transient distress related to substance administration and blood sampling.

200 pregnant mice will also be put on modified diets during pregnancy (3 weeks) to induce pregnancy- associated metabolic inflammation. Mice receiving modified diets may experience some mild impact on their well-being and condition which will be closely monitor throughout. Mice will be assessed by combining defined parameters such as weight loss/gain, abnormal behaviour, clinical signs of liver dysfunction to ensure mice do not experience more than moderate and transient distress. Any mice that exceed our defined criteria will be euthanised.

(Protocol 3). To assess the immunomodulation of the cardiovascular system during all life stages, 200 pregnant mice will be euthanised using a schedule 1 method up to embryonic day 18.5. Embryos resulting from these pregnancies will be immediately removed from the euthanised mother and culled by cutting the umbilical cord and decapitated. Embryonic organs including the heart and liver will be harvested for further ex vivo analyses. 200 time-mated pregnancy mice will go to term of which 20% offspring will be culled at the neonatal stages – postnatal day (P)1-P7. Pups will be humanely euthanised and blood and organs, including the heart and liver will be assessed using ex vivo techniques. 20% of offspring from term pregnancies will be assessed at the juvenile stages (P12-P21).

(Protocol 3). 20% of all adult mice will undergo models of cardiovascular stress including modified (e.g high-fat or Westernised diets) up to but no longer than 8 weeks or exercise-induced stress.

During all study protocols approximately a quarter of all adult mice and 5% of juvenile offspring mice will be assessed by non-invasive imaging session/s conducted under short-term, inhaled anaesthesia. Short-term anaesthesia is well tolerated with mice experiencing mild and transient discomfort. To reduce the potential risk of transient respiratory distress, less than 10% of these animals will undergo longer-term anaesthesia (over 1 hour) and these longer sessions will be less frequent if repeat imaging is required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Of the total 5310 animals detailed in this project, 33% are assigned to breeding and maintenance, experiencing a mild severity. The remaining 67% of the total animals will be assigned to investigating the immune and vascular changes during and after pregnancy and will experience a severity no greater than no greater than moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The impact of pregnancy on the immunomodulation of the cardiovascular system in both mother and offspring is multi-factorial and complex involving both local and systemic interactions between blood vessels, surrounding tissue and circulating immune cells occurring at different times. To fully understand and assess how these different features of biology work together we need to employ approaches that allow assessing cells in the lab, producing models and determining how whole tissues, such as the placenta and heart, respond during the progression pregnancy and how such responses impact the health of both mother and offspring. While lab and approaches have made great strides in recent years, they are unable to accurately simulate the environmental and cellular changes that occur in living organisms, especially during pregnancy. Thus, in vivo approaches, for now, remain the most insightful method of assessing all aspects of cardiovascular modifications as a consequence of pregnancy.

Which non-animal alternatives did you consider for use in this project?

We have utilised laboratory-based methods such as culturing cells to reduce and replace our use of animal models. Specifically, we have investigated how organ-on-chip platforms, which can recreate some characteristics of living tissue, can be used to undertake pilot studies of cell-cell interactions, genetic manipulation. Additionally, we use human blood and placenta samples to look at ex vivo changes during pregnancy in circulating cells and the placental tissue.

Why were they not suitable?

While these approaches were extremely informative, they were unable to simulate the whole system linking pregnancy to immune and cardiovascular changes; and although we had access to maternal blood and placental tissue, we did not have access to tissues involved in the cardiovascular system, such cardiac tissue, which is one of the main tissue types we need to investigate in this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number estimated includes general breeding of genetically modified animals. These animals will be very useful to look at both immune and vascular changes that occur during and after pregnancy. The estimated numbers also include using embryonic, neonatal and adult tissues of offspring from time-mated pregnancies. Since numbers of animals to be

used should include embryos 2/3 of pregnancy duration (estimated duration of 21 days) and thus numbers include embryos from E14.5 onwards.

The number of mice stated above is based on mice used under the number of adult pregnant mice used in 2022 in the current PPL protocol I am using. The animal return for this protocol was 354 over 1 year. As my PPL will for 5 years and looking at both adult and offspring; as well as antenatal and postpartum time points, the estimated number has been based on: 354 X 5 (years) X 3 (adult and offspring and postpartum) = 5310

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This project will use genetically altered, fluorescently labelled, mice to allow us to readily visualise how different cells behave during fibrosis. This approach is a very well-established method that enables us to target selected cells, in this case those within the placenta and cardiovascular system, including the heart , and image them using non-invasive microscopy. The application of non-invasive imaging will greatly increase both the amount and quality of data obtained from each individual mouse thereby reducing the number of mice required for each protocol. In addition, tissues from the same animal will be used in as many analyses as possible to minimise the number of animals required. Where possible will minimise the number of control mice (those used to compare specific biological response in genetically altered or drug treated mice) by performing internal, comparative controls within the same mice (e.g before and after treatment) thereby reducing the number of mice required per group.

To ensure reproducibility of our experiments we will include randomisation of treatment or control groups, allocation concealment, and blinded assessment to prevent bias analysis of the data obtained. These approaches will reduce the number of experimental groups required and the impact of biological variation between animals. Furthermore, we ensure our statistical approaches are robust by utilising tools such as the NC3Rs online experimental design tool.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The breeding strategy will mainly focus on crossing existing genetically altered (GA) mice with fluorescently labelled, vascular reporter animals. If the existing GA mice are associated with any adverse phenotypes, such as developmental abnormalities, we will utilise inducible gene deletion or over-expression only induce genetic modification in adult animals thereby preventing adverse developmental effect. We will continue to utilise literature searches to confirm we are using the most up-to-date methodologies for the project. This will also ensure there is no duplication with previous reports. Ongoing statistically assessment of quantitative experiments will also enable us to re-assess animal numbers by improving our power analysis. With further regards to minimising waste due to breeding, we will employ the NC3R’s Best Practice approach. This will include keeping detailed records of individual strains to ensure optimal colony maintenance. Such record keeping will include information on the following:

- Litter size
- Litter interval
- Reproductive lifecycle
- Fertility

- Welfare concerns
- Known phenotypes

Regarding the breeding strategy, we will ensure the breeding colony is maintained according for its use: if the colony will not be used for more than 6 months, we will archive a strain through cryopreservation. This is something we have already done with one of our strains. We will also endeavour to carry out intermittent breeding, as per the NC3R's guidelines rather than continuous breeding to minimise waste, whilst also maintaining the minimum number of stock animals to maintain a colony. Both strategies will be dependent on the strain of mice being bred and/or maintained, which our record keeping will inform us on.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will exclusively use mouse models of pregnancy and its effects on cardiovascular events in mother and offspring, as well as the long-term implications of pregnancy on cardiovascular health in both mother and offspring in this project. These three study protocols have been chosen as they represent central roles for pregnancy and its impact on cardiovascular function. The dysregulation of both inflammation and pregnancy is a characteristic of placental dysfunction which can have major knock- on effects on the way the cardiovascular system functions in the mother during and after pregnancy.

Moreover, such placental dysfunction can have major effects on both the development and function of the cardiovascular system in the offspring. By assessing them individually, and in combination with clinically relevant metabolic changes such as high-fat diet, we aim to build a comprehensive picture of how the pregnancy status can have long-term consequences of cardiovascular outcomes in both mother and offspring. Most mice will experience moderate discomfort due to cumulative intervention such as gene and antibody depletion and inflammation for us to precisely investigate the role of pregnancy dysfunction. The generation of genetically altered fluorescently labelled mice through our breeding programme will refine our methodologies to allow non-invasive imaging and maximise the data obtained from each mouse.

Why can't you use animals that are less sentient?

This work will be carried out exclusively on mice due to the substantial existing data that describes the extensive similarity in inflammatory and fibrotic mechanisms with humans. There is also a wide and valuable array of genetically modified strains that are being constantly refined to improve the quality and breadth of data that can used to translate finding to clinical care. Less sentient animals can provide important developmental data, for example heart development, which this project will be investigating. However, such models do not have placentas, which we believe is a key determinant in cardiovascular

development. Furthermore, less sentient animals cannot inform us on pregnancy- induced cardiac changes in the mother, nor in postnatal vascular response as creatures such as zebrafish and xenopus lack complex immune and pregnancy response, which are restricted to mammals.

The use of mice also provided comparison with this large-scale basic and translational study and extensively studies tools (e.g drugs and antibodies) and pathologic mechanisms that have strong association with developing translational human studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures detail continued monitoring for all animals to ensure that animals requiring post- procedural care, pain management or improved environment stimulation will be addressed. The majority of work will routinely involve brief anaesthesia to minimise the distress associated with the initiation of each protocol. Clear criteria are set to assess animal welfare before and during all procedures for signs of discomfort, weight change and condition will be routinely monitored. Any appearance of these will lead to the animals being considered for early humane endpoints to ensure that no animals suffer unnecessarily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We describe protocols where we have been at the forefront of developing within our research field. We will continue to review our methodologies with other experts within the fields of pregnancy, inflammation, and cardiovascular biology while also seeking the advice and guidance of the animal care staff and veterinary support provided within the establishment. All procedures will follow best practice guidelines laid out by NC3R and LASA guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All methodologies will be continually reviewed through contemporary literature and by attending national and international conferences to ensure best practice. The applicant and all researchers who work within the protocols described will stay up to date with advances in 3Rs by engaging with expert staff within the establishment and also the wider community via the N3CR website.

81. Improving poultry production and welfare

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Welfare, Sustainability, Reproduction, Bone, Genetics

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	juvenile, adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Overall aim; to improve welfare and sustainability in meat and egg type poultry using a combination of genetic and genomic analysis and in vivo experiments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Poultry provide the largest amount of high quality animal protein in the form of meat and eggs in human diets. This is achieved with the smallest environmental footprint. A large

part of that success, including the very low environmental cost, has been achieved by traditional genetic selection. However, by selecting for production traits there has been some associated consequences for the welfare of poultry. There are also aspects of poultry biology which can lead to welfare issues that we can improve. Examples are the trade-off between growth and reproduction in meat type birds.

Breeding animals have carefully controlled diets to ensure health and reproductive success. The problems of keeping hens in aviaries or free range rather than in cages has led to the paradoxical increase in bone strength but more bone damage. It has also made injurious pecking more difficult to control. Our work tries to understand and reduce the unintended consequences of selection and the way be keep hens and improve the sustainability of the poultry industry. We do this by understanding the way the animal works and how the genetic make-up of the animal contribute to the characteristics that are important for production and welfare and by producing methods to improve or ameliorate them.

What outputs do you think you will see at the end of this project?

The outputs of the project can be divided into two main areas;

Knowledge and data that can help balance genetic progress and the way we keep poultry in production against the welfare costs to the birds. This will maintain the benefits in terms of food production efficiency for the human population. The outputs will take the form of papers, presentations and deposited data and direct contact with the industry.

Practical methods to improve genetic selection or improve the management methods for welfare and product safety traits. These will be in the form of new phenotyping tools (e.g. observing physical or biochemical characteristics) as well as diets or management changes to manipulate physiological traits such as the age when reproduction commences.

Who or what will benefit from these outputs, and how?

By understanding the mechanism of feed intake, we can devise strategies to control growth whilst maintaining reproductive output and reduce feed seeking behaviour of fast growing meat type broiler breeders. Feed restriction in broiler breeders is considered a welfare issue and therefore anything that can be done to ameliorate the issue will be seen as a benefit for the ethics of fast growing broiler meat production.

By measuring and understanding the genetics and physiology of bone weakness we can help select laying hens or derive nutritional or management strategies that will reduce the chances of bone breakage. Bone damage, in particular of the keel bone that is familiar to consumers as where the breast meat is attached, is considered a welfare issue. Therefore anything that can be done to ameliorate the issue will be seen as a benefit for the ethics of egg production.

By devising new measurement strategies and understanding egg formation we can improve the selection of hens to reduce waste, increase biosecurity and protect consumers.

How will you look to maximise the outputs of this work?

We have a good track record of publication and dissemination through giving

presentations at key stakeholder meetings that include all breeding companies. More directly in many cases we are working with breeding companies which gives an excellent route to implementation.

We collaborate across the world, particularly on the issue of Keel bone health and our methods are being adopted.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 3600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are investigating poultry in their own right as livestock species for agricultural production. Animals must be used for this work because the production and welfare traits are integrated products of whole animal function and rarely can this be investigated or measured entirely in vitro. As such, it is necessary to study the animals directly to improve production, reduce waste, prevent diseases that can be passed to humans, improve product quality and improve animal welfare.

The majority will be post hatch including hens in lay. Feed restriction in fast growing broiler breeders is greatest around 10 weeks of age and laying hen issues necessitate working on reproductively active animals.

Typically, what will be done to an animal used in your project?

By far the most likely experience for an animal under this project licence is to undergo restraint for less than a minute to facilitate an x-ray. Additionally a blood sample may be taken for the determination of genotype from red blood cell DNA or to measure circulating factors in the plasma or both simultaneously.

In a small number of cases in addition, a substance will be administered, for example to influence gut motility or feed intake. For some animals, they will be sex reversed during incubation in the egg and subsequently raised to study the effect on feed intake and growth.

What are the expected impacts and/or adverse effects for the animals during your project?

Short-term bruising due to blood sampling may be experienced in some situations.

For the short-term disruption to the endogenous clock by light patterns a reduction in weight may be experienced.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild	98%
Moderate	2%
Severe	0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are investigating poultry in their own right as livestock species for agricultural production. Animals must be used for this work because the production and welfare traits are in most part integrated products of whole animal function and rarely can this be investigated or measured entirely outside the animal. As such it is necessary to study the animals directly to improve production, reduce waste, improve product quality and improve animal welfare.

The work on bone health is largely to develop new phenotypes or to test management or nutritional interventions that cannot be replicated without using an animal.

The work on satiety or fullness relates to the overall activity of the structures, rather than what can be gathered from an isolated piece of tissue. The effect of feeding in animals is a complex interaction between gut and brain that cannot be modelled outside the animal, although small parts can. These are mentioned below.
For the production of egg shell there is currently no system that can replicate the deposition of the cuticle.

Which non-animal alternatives did you consider for use in this project?

Where it is appropriate, in vitro approaches will be used. In particular when the studies reach a point where very specific aspects of function can be elucidated using isolated tissues. Studies on gut motility in tissue samples and on the cells which form and remodel bone are suitable for this approach and we will use these where appropriate. We have previously studied the effects of enzymes and satiety factors in the test tube as well as using cell culture to study gene function.

Why were they not suitable?

As discussed, some methods are suitable for understanding the function of a gene for example. However, the work proposed for animals are cases were we are developing methods to phenotype animals or when there is no model or system available to study *ex-vivo*.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number is based upon the validation of novel methods to define new phenotypes for bone quality, particularly the keel. Alongside that are experiments to study nutritional supplements to reduce homocysteine and improve bone quality. These applications account for the majority of animals to be used. A smaller number (~200) will be used in the pursuit of the biology of food intake and (~150) for the understanding of cuticle deposition.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The most efficient designs are chosen to achieve the objectives of the experiment, and the number of animals used will allow differences of biological significance to be detected. The use of 2 or 3 way designs which maximises power for the number of animals will be used where appropriate.

The group size appropriate for each of the studies where effects on bone quality or changes in hormone or cuticle deposition is concerned is made after a power analysis of data from past experiments. Therefore, each experiment will be designed to minimise the number of animals used but at the same time ensuring that any results obtained will have statistical meaning. The Establishment retains the services of a statistician who is involved in study form review and who will be consulted in matters of data analysis or design. We utilise power analysis tools in Genstat and Minitab for this purpose.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We endeavour to control variation by ensuring housing is as good as possible and stocking density is controlled, this can include using cages to ensure the data quality is as good as can be achieved for egg traits or for food intake recording. Body weight is often an important variable and we usually rank and randomise on this factor when attributing birds to treatment groups.

We frequently use repetitions in time to give experimental replication. This also means if for some reason a method or technique does not work we reduce animal usage. The experiment can then be abandoned or modified without all animals scheduled for the experiment being affected. In cases where we do not know the size of effect or the variance we may carry out pilot studies to gather the information to provide an informed power analysis.

Where possible cadavers are used during the development of imaging techniques to ensure the approaches are appropriate for data collection when applied to live animals.

Commercially produced animals are used in the majority of instances; these are produced in their millions. Some birds bred from in house stock are also used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

No models will be studied in this proposal, chickens will be studied directly as a livestock species for the purposes of their welfare and production.

Blood sampling, injection and imaging will involve temporary restraint of birds but birds are handled gently at all times and the task is performed quickly. Options involving catheterisation we believe result in greater stress to the animal because the risks to the bird from loss of the catheter and the restraint required to prevent the bird removing the catheter.

Administration of dietary additives such as amino acid analogues, vitamins and changes to calcium and phosphorous or dietary restriction are known to be tolerated. The majority of the dietary manipulations proposed are used in some form already in industry. Manipulation of photoperiod to disrupt the biological clock is not known to have any harmful effect but can reduce growth in the long term. This will not be applied for more than a week.

Why can’t you use animals that are less sentient?

This PPL focuses on poultry production. We need to be able to correlate our finding with that of the industry and hence chickens are used. Chickens are studied for the purposes of their welfare and production.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For methods that are likely to pose risk, such as reducing skeletal health in laying hens or regimes to control growth and where there may be effects on body weight and/or skeletal health these parameters will be monitored closely in the studies. The proposed interventions are not known to involve any lasting harm. Where possible chickens will not be housed alone unless the experiment demands it and pens have perches and nest boxes if appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have guidelines developed for work on poultry in terms of blood sampling and other common procedures. However, guidelines for poultry are not generally available and we

are a large user of poultry in research in the UK, and our guidelines are probably relatively authoritative, although lessons can always be learned. We do extensive research in the literature for methods before undertaking new methods and all studies are reviewed by our AWERB.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The NC3R's newsletter and web site provides up-to-date guidance. Veterinary and NACWO provide input to study protocols which are required for every planned experiment. General poultry welfare conforming to the governments guidelines are practiced https://www.gov.uk/government/publications/poultry-on-farm-welfare/poultry-welfare-recommendations or if superior the home office guidelines. Additional experience from vets used to chicken systems is available from specialist poultry veterinary sources and from staff with longstanding experience within the facilities.

82. In vivo studies of pathways and cells involved in detecting damage and commensal microbes to elicit cancer immunity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer immunity, Cell death, microbes, Immune resistance, Immunotherapy

Animal types	Life stages
Mice	adult, juvenile, aged, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overall aim is to understand the general principles that enable the immune system to detect cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This research will help to build an understanding of cells and molecules that regulate immune responses to cancer. Longer term this could lead to improved and effective anti-cancer therapies.

What outputs do you think you will see at the end of this project?

An improved understanding of the mechanisms by which the immune system reacts to cancer e.g., its role in boosting the immune response to cancer or its role in switching off the immune reactions that would prevent a full response to the cancer.

This understanding might be used for the design of better vaccines and immunotherapies for cancer. The expected outputs of this project can be summarised as follows:
1) Knowledge of the extent to which the immune system represents a barrier to cancer development and progression.

2) Knowledge of the cellular and molecular factors that trigger/contribute to cancer immunity or suppress/subvert cancer-inhibitory immune responses in the context of defined genetic and non- genetic (e.g. diet composition) alterations in the tumours and/or the host.

3) Identification of novel therapeutic agents to be investigated further for efficacy in disease models of cancer.

4) Publication in peer-reviewed journals and presentation at conferences and other appropriate communication platforms. This may include new approaches for other researchers to build on.

Who or what will benefit from these outputs, and how?

We expect that in building on this body of work and utilising the experience we have gained in the field of immune regulation of cancer we will contribute to the generation of new knowledge about how and when the immune system detects and attacks cancer. Ultimately this may benefit cancer patients by generating knowledge that can be translated into effective therapeutic strategies.

How will you look to maximise the outputs of this work?

The output of our work will be maximised by dissemination to the wider scientific community through publications in peer-reviewed journals, presentations in conferences and other appropriate platforms (pre-prints, online databases, social media etc).

Species and numbers of animals expected to be used

- Mice: 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The human and mouse genomes are approximately the same size and display a similar number of genes, many of which are involved in similar processes. Further, mice have genes that are involved in functions of the immune system that are not represented in invertebrate model organisms (e.g., nematode worm, and fruit fly).

Mice will be used at any life stage (juvenile and adult) to characterise the impact of genetic alterations, intestinal bacteria and diets on the development of the immune system. Adult mice with fully developed immune systems bearing spontaneously developed or transplantable tumours will be used to study anti-cancer immune responses.

Typically, what will be done to an animal used in your project?

The characterisation of existing and novel genetically modified strains of mice is the essential bedrock for the work to be undertaken in this project. The immune system of typical and genetically altered Germ mice will be studied at different time-points during their lives using lab-based analysis of blood samples or non-invasive imaging methods. These methods track specific cell-types following administration of substances (such as anti-cancer drugs) or after the use of different diets and which target a specific cell type or cellular function of interest. Imaging may sometimes require placing anaesthetised mice in apparatus to keep them still.

Typically, mice will experience up to 3 procedures for up to 12 months. The following mouse models of cancer may be used: i) injection of tumour cells via appropriate routes, ii) administration of cancer-causing chemicals (topical application or injection via appropriate route), iii) Ultra-Violet (UV) exposure,

iv) use of genetically altered mice that are prone to develop cancer due to their genetic composition.

Some of these models will be conducted in mice lacking the normal gut organisms in order to help understand these organisms can influence the immune system's actions on cancer cells. The effects of novel treatments aimed at harnessing the anti-tumour activity of the immune system will be studied, using the lowest dose that produces an effect. Tumour development and progression will be assessed by monitoring tumour growth. Where dosing is required, this may involve oral (by mouth) administration either once or twice daily for up to 3 months or injections daily for up to two cycles each of up to a maximum of 2 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be monitored closely, and veterinary advice will be sought when needed. Appropriate guidance will be used to define severity by assessing adverse effects observed.

In general, potential adverse effects may include:

- Wound breakdown or pain post-surgery following implantation of tumour fragments. Analgesia will be used.

- Impact of tumour growth: Most tumours are expected to be small, which will have no significant impact on the general well-being of the mice.

- Following administration of substances mice might experience transient reduction in activity and weight loss but this should last no more than two days.

- Following UV exposure: Areas of the skin may become dry and irritable. This will be treated with cream.

- Radiation toxicity may occur resulting in reduced mobility and reduced food and water consumption.

- Mice lacking the normal bacteria and other organisms that live in the gut produce semi-solid stools resulting in a chronic diarrhoea state. This is an expected observation for these mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected the majority of mice used will experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system is composed of many different cell types that work and interact in a dynamic three-dimensional environment. Our research aims to study the ability of the immune system to detect and attack cancer cells and thus it cannot be carried out in tissue culture systems in the laboratory where it is isolated from the other cells and tissues of the host and can only be addressed by the use of animals. The mouse is an ideal model organism to study immune responses to cancer in contrast to many others, including worm and fly, because it exhibits high similarity in components and function of the immune system to those present in humans.

Which non-animal alternatives did you consider for use in this project?

The use of cells, human tissues and analysis of the biological data generated using cells and human tissue (bioinformatic analysis) will minimise the use of mice wherever it is feasible, with the aim of reducing the number of mice required for future experiments.

Why were they not suitable?

Cells, human tissues and bioinformatic analysis will be used where possible. Their use is limited for this research as they aren’t suitable experimental systems to study the dynamic and complex interactions between the immune system and cancer.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will use normal and genetically altered mice. The genetically altered mice will have been bred under other projects.

The proposed number of mice have been estimated for the 5-year programme of work based on previous experience using similar protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use normal and genetically altered mice. The genetically altered mice will have been bred under other projects.

The proposed number of mice have been estimated for the 5-year programme of work based on previous experience using similar protocols.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design, mouse use will be minimised in several ways:

1. By minimising variability in results, for example by housing the mice under similar and consistent conditions.

2. Where no specific information is available in the literature, we will generate preliminary data in pilot studies. Effects worth investigating further may then use larger numbers of mice to determine if the observed difference is statistically significant. Pilot study numbers will be determined using power calculations.

3. By considering on-going statistical estimation of power requirements in each of the studies, using prior results in order to use the minimum number of mice.

4. By combining, where appropriate, different experimental groups with shared control groups.

5. By using, where possible, tissues from different sites on one mouse for both treatment and control samples.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Enrichment will be provided to the mice to support natural behaviour.

We will use optimised composition of diets and concentrations of substances. The route of administration of cancer cells and substances, the number of cancer cells, the concentration of carcinogens, the frequency, the dose of ultraviolet exposure and the duration of the model are refined through reviewing the literature and pilot studies to improve animal welfare, and harnessing the expertise of the vet and animal technicians.

Why can’t you use animals that are less sentient?

The choice of mice is based on the fact that mice are bred easily, and they have an immune system and physiology similar to humans, unlike other model organisms including fish, worm and fly.

Furthermore, a variety of validated and established genetically altered mice with altered immune components already exist and are used worldwide in studies aiming to characterise the functions of the immune system in physiology and disease. No other model organism provides this combination of characteristics.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All mice will be maintained in specialised cages to avoid infections. Written protocols will be provided to all staff involved in the experiment which will include details of possible adverse effects and clear humane endpoints. General or local anaesthesia, and/or analgesia will be given to mitigate potential pain and distress of procedures.

The choice of route to administer a substance or cells will where possible be the least invasive to minimize or avoid adverse effects, reduce the number of mice used, and maximize the quality and applicability of results. Standard routes of administration will typically be use e.g., orally or in the vein. However, the active concentration, volume, stability, and toxicity of a particular substance or cells may require administration through a non-standard route e.g., in the tumour.

Mice with superficial tumours will be monitored daily for and if local reactions are seen advice from the vet will be sought on appropriate treatments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgical procedures will be carried according to the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

We will follow guidelines of good practice [Morton et al., Lab Animals, 35(1): 1-41 (2001); Workman P, el al. British Journal of Cancer, 102:1555-77 (2010)] and for aged mice we will refer to Wilkinson (Laboratory Animals 2020, volume 5(34)).

We will follow the guidelines of Langford et al Nature Methods 2010 to assess pain by using the Mouse Grimace Scale.

We will review the NC3Rs resources for refinements.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date about the advances in the 3Rs by following the NC3R's guidelines, reading 3Rs literature and participating in 3Rs workshops locally and nationally. We will also take advice on refinements from the Animal Care and Welfare Officer and the vet.

83. Proteostasis in neurons and glia in neurodegenerative diseases

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Neurodegeneration, Alzheimer's disease, Glial cells, Proteostasis, Disease mechanisms

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the cellular and molecular mechanisms underlying neurodegenerative diseases in neuronal and non-neuronal cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Currently there is no treatment to stop the onset or progression of neurodegenerative diseases such as Alzheimer's disease and other related dementias. This lack of appropriate treatments is due to an incomplete understanding of the cellular and molecular changes that occur in the brain in these human diseases. These changes occur in nerve cells or neurons but also in other brain cells different neurons, named glial cells.

In order to find efficient therapeutic strategies, we need to better understand changes that occur in neurons and in glial cells. These changes are difficult to mimic in non animal models and therefore in this project we will be using mouse models of neurodegeneration. In this project we will use mice to generate primary cultures that mimic in vitro the functions of brain-derived cells and enable us to investigate their contribution to disease.

What outputs do you think you will see at the end of this project?

Outcomes of this project will result in publications in international peer reviewed journals. This will be made open access so they can be widely accessed. When large datasets are obtained, these will be deposited in open repositories where they can be accessed by any researchers.

Prior to publication, data will be shared by presentation at national and international conferences, generation of pre-prints, and discussed with other neuroscientists with an interest in neurodegenerative diseases.

Who or what will benefit from these outputs, and how?

This project will enhance our understanding of the changes that occur in the brain of mouse models of neurodegeneration. In the short term, findings will inform our future research projects but also the research of other scientists working in similar fields.

In the long term, the outputs will inform the development of potential therapeutic targets in neurodegenerative diseases.

How will you look to maximise the outputs of this work?

We regularly discuss our findings with other researchers in the field, either by attending conferences, discussing with colleagues, etc. Wherever relevant, we will combine our data with others to enhance the relevance of our findings. When necessary, we will also seek to establish new collaborations to complement our existing expertise.

Negative data or datasets generated in this project that are not directly relevant to our research will still be published in manuscripts as Supplementary Information, or when relevant these will be published in journals such as PlosONE or Scientific Reports that encourage the submission of data that is generated with good scientific standard, independently of the novelty or relevance.

Species and numbers of animals expected to be used

- Mice: 6,600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this licence, we are using mouse models of neurodegenerative disease, including Alzheimer's disease and other related disorders. These animals express human and mutant forms of disease- related proteins to recapitulate some of the disease features, to improve our understanding of the disease mechanisms. When possible, we will use mouse models that express genes in their wild type forms and/or are expressed at low levels, to avoid artefacts and/or damaging phenotypes. In this case, animals will not show any clinical signs.

Only when strictly necessary, we will use mouse models with mutant forms of disease related genes such as tau or amyloid precursor protein (APP) and/or a high level of gene expression, as these will provide models that will show a progression of phenotypes as they age that mimic those found in human patients. These models will experience moderate severity.

In the first instance, we will mostly use animals either before or a few days after birth, to prepare primary cultures that will be grown in vitro. Embryos (E15-E18) or few days old (P0-P10) mice will be used to prepare cultures of 1) neuronal or non-neuronal cells to investigate cell specific disease mechanisms, or 2) brain slice cultures that can be grown for long periods to recapitulate some of the disease features while maintaining the brain architecture. These cultures will provide an in vitro system in which to undertake treatments and repeats, while using a minimum number of animals and without the need to maintain the animals for long periods.

Only when data in these models show relevant results, we will consider the use of adult animals. After sacrificing the animals, the brain and other tissues will be collected and kept for further post-mortem analysis.

Typically, what will be done to an animal used in your project?

Animals will be bred to maintain colonies from genetically altered mice.

Regular breedings will be set up to obtain offspring that will be decapitated to prepare cultures from these animals.

Other animals may be culled to take the brain and other relevant tissue at different ages. Animals that are not used for breeding or for sample collection will be killed.
What are the expected impacts and/or adverse effects for the animals during your project?

In order to investigate neurodegenerative disease mechanisms, we will use mice that express human and mutant forms of proteins to recapitulate some of the disease features. Some mouse colonies will not develop any clinical sign derived from their genotype.

In some instances, we will need to model more aggressive disease forms and these mice may progressively display harmful phenotypes as they are modelling a progressive and harmful human disease. In most cases, these phenotypes are predominantly cognitive, thus not resulting in significant welfare issues in otherwise healthy animals. However, some mice can show evidence of motor impairment, including altered grip strength, hindlimb clasping or impaired rotarod performance, with the severity of these adverse effects increasing with age. Mice such as the Tau35 animals that will be used in this project, are also prone to eye infections.

Decapitation of either embryos or few days old mice is a procedure where animals do not experience any pain or harm as it is done swiftly, using sharp instruments for this specific purpose, in very young animals that have not yet developed any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Protocol 1. Breeding and maintenance of GA mice (mild). 10% mild, 90% sub-threshold
Protocol 2. Breeding and maintenance of GA mice (moderate). 10% moderate, 90% mild
Protocol 3. Decapitation of neonates. 100% mild

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the disease mechanisms underlying neurodegenerative disorders, we need models that mimic these diseases.

In the first instance, our research will be done in vitro using primary cultured cells, or ex vivo using organotypic brain slice cultures. While these models allow us to generate cells and slices that can be used for several weeks to undertake different treatments and time points, and therefore minimizing to a large extent the number of animals used, they still require the use of animals to prepare these cultures.

Primary cultures are the simplest model that we can employ to study neurons, astrocytes, and their interactions, in a relevant context. Mammalian neurons and astrocytes are specialised cell types and specific protein function cannot be modelled accurately in traditional cell lines.

Organotypic slice cultures will allow us to study glial and neuronal cells in a relevant context that preserves brain structure and cellular networks, while providing the advantage of working in vitro to perform drug treatments and to test multiple conditions. The slices provide a tool with which we can study disease development without invasive treatment and/or manipulation of adult mice.

Only when relevant and after generating sufficient evidence with experiments in cells or brain slices, we will use adult mice to obtain further information. Neurodegenerative diseases can only be partially modelled in culture. Animal models that recapitulate disease pathology and cognitive and motor symptoms are required to fully understand the mechanisms underlying neurodegenerative diseases.

Which non-animal alternatives did you consider for use in this project?

Immortalised human cell lines. These cell lines have been manipulated in such a way that they can proliferate indefinitely and therefore, can be cultured in vitro for prolonged periods.

Neurons and glial cells derived from human induced pluripotent stem cells (iPSCs). iPSC are derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of different types of human cells. iPSCs can be derived from patient cells that retain some disease properties.

In silico methods using computational approaches allow making predictions using databases and data analysis tools.

Why were they not suitable?

Immortalised cell lines have undergone manipulations that result in changes in their metabolism, function and genome, in most cases resembling cancerous cells. While different cell lines exist and some of them have a neuronal origin, these cannot fully recapitulate the specialised functions of neurons and glial cells.

iPSCs do not always fully recapitulate the disease conditions, particularly those of late-onset disorders, as is the case for neurodegenerative diseases. While this could be a useful model to study specific cell

types, they still cannot replace research in integrated systems such as in organotypic brain slice cultures or in mice.

When possible, we use in silico approaches to obtain information that could accelerate the project. For example, using software for scientific literature mining can avoid research duplication and can help identify mechanisms to test; or pathway and gene ontology analysis of existing databases can help in the identification of targets or mechanisms. While these are useful, these can only help during some stages of our project, but these substitute the research in experimental models where hypothesis need to be tested.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are calculated based on previous use and the objectives set in this project, and considering the typical variations obtained in our previous experiments. We are using about 300 animals per year per colony and we estimate that we will maintain one mouse colony in Protocol 1 and one mouse colony in Protocol 2. For Protocol 3, we routinely used 2 animals per month and each animal will have litters of an average of 12 pups which will be decapitated.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We first will determine the hypothesis to be tested and decide on the appropriate controls. When animals are used to generate in vitro cultures, positive and negative controls will be included within the culture prepared from the a given animal or a littermate. This will minimize variability and will reduce the number of additional animals needed. In this case, experimental units will be obtained from each culture, and experimental and control groups will be chosen from a pool of brains, reducing any bias during the selection.

To calculate sample size, we use G Power, an online softwares such as the NC3R's Power Calculation G Power, that allows the user to determine statistical power based on a wide variety of tests. The program estimates the sample size needed for our experiments, based on the effect size obtained in previous data, specifying the type of test and 90% of power at 0.05 significance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Mice on Protocols 1 and 2 will be bred to generate litters that will be used in Protocol 3, while females may still remain in Protocol 1 and 2, minimising the number of animals needed. From the same littermate, neonates can be used to generate different type of cultures, to make sure that each animal born is used and minimising the number of animals that need to be bred.

Brains and other tissues will be collected from animals that are no longer needed for breeding and culled in Protocols 1 and 2. This tissue will be used to generate data to be used in this project or to inform further projects. We routinely share available tissue with other researchers interested in these mouse models.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The disease models to be used in this study are well established. Wherever possible, we use mouse models that express genes in their wild type forms and/or are expressed at low levels, to avoid artefacts and/or damaging phenotypes. These mouse models do not result in any harm to the animals, and we do not expect suffering as these mice do not develop harmful phenotypes that affect their wellbeing.

Only when strictly necessary, we will use mouse models with mutant forms of disease related genes such as tau or amyloid precursor protein (APP) and/or a high level of gene expression, as these will provide models that will show a progression of phenotypes that mimic those found in human patients.

Embryos and neonates will be used to generate cultures to use in vitro. To obtain embryos, pregnant female mice will be sacrificed by schedule 1, and after confirmation of death, the abdominal cavity will be opened to remove the uterus containing the embryos. After removing the placenta, embryos will be decapitated following by brain dissection. Neonates will be separated from the mother and immediately sacrificed by decapitation before animals can develop any harmful phenotype.

Decapitation does not cause any pain or distress to the animals, as it is done swiftly with sharp instruments.

Why can't you use animals that are less sentient?

This project aims to study the role of glial cells in neurodegenerative diseases. The glial cells present in less sentient animals such as zebrafish do not fully recapitulate the type of glial cells present in the human brain and for this reason, we can only consider using rodent models for this project.

Our first choice in this project will be to use neonate mice to prepare cultures that can be used for different treatments and times and that can also be aged to mimic some of the disease phenotypes.

This not only minimises the number of animals required but also the need for adult animals.

A minimum number of adult animals is necessary to maintain the mouse colonies required to obtain neonates, or to obtain tissue. We will not undertake any harmful manipulation of adult animals in this project, tissue will be obtained from sacrificed mice or those that have been under terminal anaesthesia. The only harmful phenotypes observed are related to some motor and cognitive dysfunctions exhibited by mouse models of neurodegeneration as these are used to mimic human disease phenotypes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The clinical signs that may arise in mice used in the protocol with moderate severity are an unavoidable consequence of modelling human disease that progressively worsens with age/disease. We will carefully monitor these animals and those mice that are going through more suffering than expected in this project will immediately be humanely killed.

When possible, we will refine our models to use a lower number of genetically altered mice. For example, using viral methodologies to express the human tau protein in cultures from wild type animals, instead of using genetically altered mice that express tau protein to obtain these cultures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines in the Norecopa website (https://norecopa.no/prepare), and we will seek for new guidelines and publications from the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Periodically, we receive news by email regarding advances in the 3Rs through articles published in the NC3R newsletter. In addition, our Establishment regularly shares with licencees the news and training opportunities from the NC3Rs Regional Programme Manager. We will periodically attend conferences and events to stay up to date with 3Rs related topics. In addition, updates are shared regularly within the animal users' departmental meetings where these will be discussed with colleagues.

84. Improving therapies for diabetes

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

diabetes, islet, transplantation, beta cells

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to improve therapies for the treatment of diabetes through studies of insulin- producing cells. One focus will be on improving cell replacement therapies. Another focus will be to maintain or increase numbers and function of insulin-producing cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diabetes is a disease characterised by increased blood glucose levels (hyperglycaemia) due to a relative or absolute insulin deficiency. The incidence is increasing at an alarming rate and the current cost to the NHS is already around £14 billion (around 10% of the total budget).

What outputs do you think you will see at the end of this project?

The main outputs will be generation of new information, publication of scientific articles and presentations at conferences.

Who or what will benefit from these outputs, and how?

The outputs will benefit other scientists through increasing knowledge in our field which will help in the collective effort to develop new therapies. In the long-term it will benefit doctors and patients by leading to the development of new therapies.

How will you look to maximise the outputs of this work?

We often publish in "methods" books and will continue to do so. This allows us to discuss particular protocols in details, including specific measures we have taken to ensure success and/or implementation of the 3Rs. We collaborate with other scientists in a variety of projects and share tissues from diabetic animals. We also participate in outreach activities with schools and patient groups.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used as they have similar blood glucose homeostasis mechanisms to humans. We already have a wealth of information that we can build on and we know that drugs that work in humans to lower blood glucose concentrations also work on mice which validates the models as relevant. Diabetes can affect people at different ages so although we will primarily use adult mice, in some cases younger mice will be studied as will pregnant mice.

Typically, what will be done to an animal used in your project?

In our most common experiment a mouse (either wild-type or with a specific genetic alteration) would be given a high fat diet followed by administration of a drug (usually by injection). A glucose tolerance test and/or an insulin tolerance test would be used to test whether the treatment affects blood glucose control.

In experiments we conduct less frequently, diabetes would be induced by injecting streptozotocin or a mouse with spontaneous diabetes would be used. They may be given a treatment which may prevent or reverse diabetes such as injection of a specific drug or insulin producing cells transplanted under their kidney capsule. The blood glucose concentrations of the mice would be monitored.

What are the expected impacts and/or adverse effects for the animals during your project?

Injections and measurement of blood glucose have only transient effects on mice and are more associated with handling rather than the needle pain. We have previously measured blood glucose concentrations by implantation of continuous glucose probes that means we can measure blood glucose concentrations without holding the mice as the signal is sent through radiowaves (telemetry). Using this, we were able to estimate any interaction

increases blood glucose concentrations by about two hours (this also includes husbandry) and there is little to no additive effect of a needle prick or blood glucose measurement.

Most of the surgery we carry out is less that 20 minutes long and involves implanting cells or a device to deliver drugs. Animals typically under return to normal behaviour rapidly (within an hour) and the surgery does not tend to cause weight decrease. Rarely we carry out more invasive/longer surgery such as implantation of continuous glucose probes. This surgery is typically between 30-60 min and we have noted a transient weight decrease (a few days) and despite normal appearance and behaviour it can take around 5 days for mice to regain a normal blood glucose profile.

Mice which have diabetes can lose weight or fail to gain weight normally. With induced diabetes, the weight loss tends to be around 10-15% and in the no-treatment group may persist for the duration of the experiment (most often 4 weeks or less). For the models of spontaneous diabetes we use the weight is not significantly less than wild-type before 18 weeks of age (and most experiments are conducted before this).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Around half of the mice will be classified as subthreshold.

Around 25% will be classified as mild.
Around 25% will be classified as moderate.

What will happen to animals at the end of this project?

- Killed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Blood glucose metabolism requires the interaction of several different organs, including the pancreas, liver, fat, muscle and brain. It is therefore essential that any new therapies are tested in a whole animal to take into account these different systems that could be affected.

Which non-animal alternatives did you consider for use in this project?

The main non-animal alternative is cell lines, which we use in preliminary experiments. We also use human islets (clusters of insulin-producing cells) in vitro and are investigating the use of stem cell derived insulin-producing cells.

Why were they not suitable?

Cell lines can be used in very early stages of the research, but ultimately they do not act in the same way as cells do in the body. For example, cell lines grow indefinitely and one problem in diabetes is that the beta cells do not grow. Human islets are really useful but we have only access to limited numbers. Stem cell derived beta cells are not functionally equivalent to fully developed beta cells so are currently not suitable to study beta cell function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based our estimations on previous experiments using similar protocols. In some cases, animals can act as their own control (e.g. in beta cell transplantation experiments, which end in graft removal). This reduces the need for sham operations. Pilot studies may be used to determine the most relevant dose, which will prevent larger studies using several different doses. Tissues will be offered to researchers, for example kidney and eyes from diabetes models may be collected for researchers interested in secondary complications in these organs. We have previously documented what can increase variability in blood glucose concentrations (our main end-point) and plan experiments in accordance (for example, avoiding a full cage change immediately prior to a procedure). In addition we will, where necessary include both sexes which will maximise the relevant information gained from our experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the NC3Rs experimental design assistant and have considered in what circumstances animals can act as their own control (such as in cross over designs). We also have previously carried out telemetry experiments which has given us a good overview of ways we can reduce variability, which then allows fewer animals to be used. This includes group housing animals, avoiding overnight fasting and avoiding cage changes immediately prior to an experiment. Randomisation is used where possible but in some studies occurs after animals have been placed in groups to ensure similar starting glycaemia. Blinding is also used where possible but in some cases it may be evident which group is which (obese mice, different sexes) in which case blinding can be done at the analysis stage.

Exclusion criteria is set prior to starting the study to, for example, determine which animals will be considered diabetic.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies allows us to access which dosing strategies will be optimal. Efficient and well-planned breeding means that there is not an over-production of animals. We regularly

share tissue with different members of the group but also collaborators outside of our group.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered mice and mice with symptoms of obesity and/or diabetes. By using a genetic model of diabetes where possible, we can avoid potential toxic side effects of beta cell toxins such as streptozotocin. Our novel genetic models also seem to be less severe than similar models

such as the Akita mouse, with mice maintaining good condition despite hyperglycemia. The most common method we will use is measurement of blood glucose concentrations. This is done by using a very small pin prick and a tiny sample of blood. This can be done without restraining the animal and only takes a few seconds. We monitor animals with diabetes very carefully to ensure that they maintain good form.

Why can’t you use animals that are less sentient?

Blood glucose metabolism is well conserved throughout the animal kingdom but it is within mammals that is closely maps on to human blood glucose metabolism. Many of our experiments are measuring outcomes over a longer period of time so terminally anaesthetised animals are not appropriate. In addition, blood glucose metabolism is altered under anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are constantly reviewing our procedures. Some examples include using bedding retention when changing cages, peri-operative analgesia, using reduced fasting periods in glucose tolerance tests and training animals to voluntarily consume glucose in a glucose tolerance test.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152- 156, 2017.

Refining procedures for the administration of substances. Laboratory Animals. 2001;35(1):1-41.

LASA 2010 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section (

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have close contact to the NC3Rs and attend relevant seminars held by them. We discuss 3Rs advances at lab meetings and journal clubs and attend events covering 3Rs topics. In addition we will do literature searches and discuss procedures with national and international colleagues at scientific meetings. Advances are implemented through talking to researchers at lab meetings, listening to their feedback and offering training if necessary. We will make use of the 3Rs self assessment tool within our group to check how well researchers are engaged with 3Rs culture.

85. Interactions between the immune system and connective tissue in fibrosis and inflammation of the gut

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Intestine, Fibrosis, Inflammation, Fibroblast

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the mechanisms of fibrosis and inflammation of the gut, by assessing interactions between immune cells and connective tissue.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Almost every patient with Crohn's disease (CD), one of the two major clinical manifestations of inflammatory bowel disease (IBD), will require at least one surgery in

their lifetime to remove parts of the small intestine affected by fibrosis. Chronic intestinal inflammation in IBD has been identified as a key trigger for developing fibrosis, however it is now clear that currently available anti-inflammatory therapies in CD do not reverse or stop fibrosis progression.

Whilst this suggests a link between inflammation and fibrosis, it highlights that the mechanisms driving fibrosis, once established, are very distinct to the inflammatory pathways we can target. We therefore have to study models of established small intestinal fibrosis, in order to reveal patho-mechanisms and develop alternative therapies.

What outputs do you think you will see at the end of this project?

Pursuing this project, we will generate novel insights into mechanisms that drive fibrosis in the small intestine, in particular the ones mediated by fibroblasts. We will also probe the potential of targeting candidate pathways as anti-fibrotic therapies, which will establish a proof-of-concept before being taken forward into future drug development. These outcomes of these studies will be made available to researchers and clinicians through scientific publications.

Who or what will benefit from these outputs, and how?

Short term, we will generate unprecedented insights into the pathology of intestinal fibrosis, which will inform other researchers' own studies on the topic, accelerating research in the field. Medium term, the tools (models) developed and validated in this project may be useful to researchers in a variety of unrelated research areas. If we or others identify promising candidates to treat intestinal fibrosis, these can be developed into drugs in the future. Then, in the long term the project will benefit a wide research community and benefit patients and clinicians if future drugs are being developed by pharmaceutical industry and clinicians based on our work.

How will you look to maximise the outputs of this work?

We will work with national and international collaborators, who are experts in the field, to assure we minimise the time to reach meaningful research outcomes. We work with patients affected by the condition, as well as with clinicians and pharmaceutical industry, in order to ensure our research is optimally aligned with the needs faced in clinical practice. We collaborate with experts in the relevant in vivo models, and experts in the techniques required, to assure we use the most refined methods to test our hypotheses. Any successful or unsuccessful findings made in the project will be disseminated to the researchers, clinicians, patients and the public alike. This will involve scientific publications (open access), pre-prints, open access data repositories, and possibly by depositing new transgenic mouse strains.

Species and numbers of animals expected to be used

- Mice: 23000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of in vivo mouse models is pivotal to deliver the proof-of-concept studies that precede drug development and testing in large-scale clinical trials in patients. Only by this can we open avenues to develop effective anti-fibrotics that are desperately needed in the clinic.

A mammalian species is required to adequately reflect the complex interactions in intestinal pathophysiology. The mouse represents the most appropriate species for our studies, as research over the last decades has validated that mouse models recapitulate main features of intestinal disease. The mouse is lowest neurological sensitive species to show striking similarity of intestinal architecture, connective tissue cells and immune cells, to humans. Since we are focussing on the role of fibroblasts, we will require to target these cells specifically. The Cre-loxP system widely used and established in the mouse is an excellent tool available for this purpose, with a multitude of defined genetic mutant strains readily available.

Typically, what will be done to an animal used in your project?

Typically, disease models of fibrosis and inflammation will be induced in adult mice. As a possible refinement for fibrosis models, we will test the induction of fibrosis in juvenile mice, with the aim to reduce the total duration mice have to be under the fibrosis protocol whilst achieving equally informative results. All mice will be typically studied at experimental endpoints of 1 up to 6 months after fibrosis induction.

Fibrosis will be induced by either the short initial administration of a gene activating substance, or the administration of non-lethal, attenuated infectious agents, by standard injection routes, via oral gavage, or in diet/drinking water.

Additional intestinal inflammation - or intestinal inflammation only without fibrosis – may be either induced by the short administration of infectious agents (such as helicobacter), immuno-modulating substances (such as antibodies), or chemical substances (such as DSS), via standard injection routes, an oral method, or in drinking water. Some of these substances may be given repeatedly to model chronicity of inflammation. Additional inflammation will only be induced after a recovery period of 2 weeks from start of induction of fibrosis.

At later stages of the project, few mice may be given disease modulating substances such as antibodies, antibiotics or non-pathogenic infectious species, so that we can test potential therapeutic effects on fibrosis progression. To ensure adequate levels of the substance over the full duration of the model, these substances may need to be repeatedly given via standard injection or the oral route.

Wherever possible, we will administer substances through an implanted slow-release device to avoid repeated injections. Non-pathogenic infectious agents may be given via the oral route.

Mice may undergo non-invasive imaging, such as Micro CT to allow us to monitor a possible thickening of the intestinal wall and rule out possibel resulting adverse effects from pathological narrowing of the intestinal lumen. Alternatively, invasive imaging via a colonoscopy may be carried out to assess the extent of intestinal fibrosis and inflammation. During the colonoscopy we may take a small tissue biopsy for further investigation. Both imaging methods will be performed using general anaesthesia.

To test the impact of specific microbial components in the most refined way, mice may be bred under germ-free conditions and colonised with defined microbiota before fibrosis induction.

We have built in mutually exclusive steps in this project, to prevent that all or most of the above- mentioned steps could be carried out on the same mouse.

At the experimental endpoint the mice will be humanely killed and tissues collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The administration of any substances and agents will be carried out using routes and volumes that are not expected to have any impact on the animal, other than short discomfort. Some administered substances or agents result in progressive inflammatory and/or fibrotic disease. In theory, fibrosis and inflammation could eventually lead to a narrowing of the intestinal lumen, which we however do not observe in mice studied at the typical endpoints (up to 6 months), or even rare, longer endpoints (12 months).

Mice under fibrosis/inflammation protocols may experience transient weight loss and soft but formed stool, from which they recover within a short timeframe. Some mice may experience transient mild discomfort or abdominal pain they recover from quickly. If biopsies of the bowel are taken during invasive imaging, some mice may experience discomfort from the short healing process in 24 hours following the procedure. Any animals experiencing pain will be given painkillers and post-procedure care just like people recovering in hospital.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity	% of mice
severe	0%
moderate	24%
mild	4%
sub threshold	72%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you

have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Only animal models reflect pathophysiologic processes in its full complexity, and are therefore necessary to deliver the proof-of-concept studies that precede drug development and testing in large- scale clinical trials in patients.

Which non-animal alternatives did you consider for use in this project?

We profile patient tissues to identify the pathologic changes (cell types, molecular pathways) occurring in the fibrotic and inflamed intestine. Once a pathology-associated cell type and pathway is identified, we study it in great detail in an isolated setting in a dish. This typically involves one or several cell types, such as connective tissue cells co-cultured with immune cells.

The above approaches usually precede animal experiments, which we only consider when we have to establish causation, study a pathology in its whole complexity, and trial therapeutic candidate compounds.

Why were they not suitable?

By profiling patient tissues we can reveal the cell types and molecular pathways that are present in small intestinal fibrotic lesions. Similarly, we can study identified fibrosis-associated cell types in vitro in a dish, to manipulate specific intracellular and molecular mechanisms that are potentially promoting or counteracting fibrosis. Whilst we follow both of these approaches in our research program, they do not allow us to establish definitive causation and cannot model an organ (intestine) or organism in its full complexity. For example, only by studying the intestine when embedded in an organism can we tell whether a cell type or pathway is capable of driving/progressing intestinal fibrosis. Similarly, assessing the speed and extent of distribution of therapeutic candidate compounds, as well as their efficacy, requires the whole organism, and an accurate reflection of human organs and cell types.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Expert statistician advice was sought to help us with calculations using typical variations from our own earlier experimentation, or published experiments, to calculate minimum numbers of animals to be used whilst ensuring that the results are statistically significant. Sample sizes for our experiments are estimated from past and published experiments. Calculations typically show that we need group sizes of 6-8 to achieve the quality of results we need. We've used our previous experience in breeding, and expected ratios of the genotypes of transgenic mice required for experiments to estimate the number of animals that we will need to use for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible, we consulted the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/) for recommendations on the experimental design. The recommended suitable statistical analysis method was determined, in order to calculate required sample sizes using the tool or alternatives (e.g., G*power).

We have taken the following steps to reduce the number of animals to be used:

where possible, randomise (e.g., RandoMice https://github.com/Rve54/RandoMice/releases/) and blind experiments and analyses refresh cross by frequent back-crossing in order to reduce variation from genetic variation control for inter-group variation by using blocking in experimental design, i.e. age-, sex-, cage- and litter-match where possible conduct only adequately powered experiments that will likely yield a definitive answer

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding colonies will be managed in line with the best practice guidelines. Particular attention will be paid to genetic stability and good breeding performance. Data from breeding animals are readily available from the in-house database and will be used to make decisions on future breeding animals and to assist in maintaining a suitable colony size to ensure only those animals needed for experiments are produced.

In addition, we will:

- deploy pilot studies whereever possible with few mice to determine for example overall experimental variation, or the required frequency of substance administration to reach adequate drug levels. This will then inform the design of the eventual experiment monitor mice through non-invasive (e.g., microCT) or invasive imaging (Colonoscopy), in order to gain an insight of disease progression over time which allows to pick most informative timepoints and mice in a cohort.

- maximum tissue usage of killed animals, for example for deriving primary cell lines (e.g., fibroblasts or immune cells) to be studied in vitro, or profiling archived tissues (formalin preserved) in situ

- share animals with other researchers to harvest multiple tissues from the same experiment

- use in vitro models and profiling of patient tissues instead wherever possible

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Use of genetically altered (GA) mice

We will typically use genetically altered mice that have the mutations of interest switched off until they are activated using gene-activating substances, such as Tamoxifen. This method allows the animals to be normal until the point at which we need the gene to be active or inactive for our studies. It plays a significant role in reducing the time the animal is exposed to the harms from fibrosis, and potential effects of switching on/off genes. Only in exceptional circumstances where no inducible transgenic strains are available, we will use mice where genes are switched on or off constitutively.

It also allows us to induce genetic modification only in the cell type of interest, for instance a connective tissue cell type like the fibroblast. This prevents adverse effects resulting from unspecific targeting of either all cell types, or additional cell types that are not relevant to the disease process studied.

Models of Fibrosis

We will use two methods to induce fibrosis:

- the use of genetically altered mice where the genes responsible for the development of fibrosis can be activated;

- a model induced by chronic infection with adhesive enterobacteria. Either of these models allow us :

- to avoid alternative methods of inducing fibrosis through inducing inflammation, for instance by using harsh chemicals which produce acute disease. We do not need this level of suffering to achieve our aims.

- to limit clinically established fibrosis only to the intestine at the time points assessed, which is the site of disease we want to study. This avoids harm to the animals by established fibrosis manifesting in other organs, and reflects the relevant disease process observed in patients.

- for the genetically induced fibrosis model, we will test the potential benefit of inducing fibrosis in juvenile (as opposed to adult) mice, with the aim to reduce the total duration of the fibrosis model.

Use of models of intestinal inflammation in combination with models of fibrosis

We will assess how inducing intestinal inflammation impacts on the progression of fibrosis, as inflammation and fibrosis are commonly observed in patients. This may have the additional beneficial effect of reducing harm to animals, by accelerating fibrosis progression (inflammation is well- established risk factor for developing fibrosis), which will enable us to achieve our objectives earlier when studying animals for a shorter time, minimising exposure time of animals to potential cumulative harm.

Use of therapeutic candidate substances and non-pathogenic infectious agents

A major objective of this project is to identify substances and pathways that, when targeted, can slow down, stop or reverse intestinal fibrosis progression. Therefore, the genetic targeting and administration of substances or non-pathogenic infectious agents is expected to have beneficial effects on the animal, by ameliorating disease.

Use of germ-free models

The use of germ-free mice is the most refined way to specifically and conclusively study the impact of microbial components on fibrosis and inflammation progression. Use of germ-free mice avoids the use of antibiotics, which do not clear 100% of bacteria, and to which mice may develop resistance.

Use of monitoring techniques

We will monitor fibrosis and inflammation extent by both invasive or non-invasive imaging techniques. Whilst the harm caused to the animal by the monitoring is minimal (general anaesthesia), the benefits are that we will be able to determine the best timepoint to stop the model in each experiment, preventing unnecessary time under procedure for animals. For colonoscopy, we use a rigid endoscope to monitor only the first 2 cm of colon of mice, which is a very short procedure (few minutes). Since models of fibrosis and inflammation are expected to manifest in this part of the colon, we do not need to go further which would prolong colonoscopy. If endoscopic biopsies need to be taken, we will combine this with the monitoring step to avoid two separate procedures. Similarly, the intra-rectal administration of therapeutic substances will be combined with this monitoring step.

Options for severity and further reductions in adverse effects or duration

In order to study a disease process that is reflective of the disease observed in patients, it is important that the key clinical features of that disease are reproduced. If animals showed no clinical signs of fibrosis or inflammation it would indicate that they did not have disease, and this would therefore be a poor model of human disease. The key clinical signs of fibrosis and inflammation required to reflect human disease could not be modelled without seeing harms such as transient weight loss, soft but formed stool, and abdominal pain which are considered moderate severity.

Why can’t you use animals that are less sentient?

Non-mammalian animals are limited in their use because they either do not have the right type of connective tissue or immune cell, or the overall organ architecture or disease manifestation is too different from the human to provide relevant results. We can't use embryos or very young animals as their immune system is not fully developed yet, and the disease we are studying is slowly progressing and only established in adulthood.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have established monitoring schemes tailored to different procedures to assess the extent of clinical signs of intestinal pathophysiology and to detect any adverse effects in experimental animals. Animals will be visually monitored for general signs of pain and discomfort along with fibrosis/inflammation-specific clinical signs of weight loss, soft but formed stool, and anal inflammation (swelling). We also use non-invasive imaging

methods such as micro CT to monitor the thickness of the bowel wall and invasive methods such as colonoscopy to assess the redness of the intestinal wall. Established scoring systems incorporating these aspects will be used to regularly monitor animals under procedure, and monitoring frequency will be increased when signs of the disease become manifest. The main model to study intestinal fibrosis is the most refined with respect to achieving the aims of the project, as it uniquely manifests at a location (intestine) and as it is driven by a cell type (fibroblast, a connective tissue cell) that closely reflects human pathology.

The use of genetically altered mice (Cre-loxP system) allows for the temporally controlled and specific targeting of pathogenic pathways only in the relevant cell types (e.g. fibroblasts or immune cells). By the temporal control the duration of potential harms is kept to a minimum, and by targeting specific cell types the likelihood of harms arising from the off-targeting of irrelevant cell types is reduced. By inducing inflammation in addition to fibrosis, we anticipate to accelerate fibrosis pathogenesis, minimising the time animals could potentially experience cumulative harm.

Substances and infectious agents will be administered in line with published guidelines for volumes and routes and are not expected to cause any welfare issues. Where possible we will aim to add fibrosis and inflammation-inducing agents to either drinking water or feed to reduce the number of injections needed. We will consider combining substances into a single administration where they are compatible. We will consider the use of slow-release mini-pumps as an alternative to multiple administrations. Compounds required to control gene expression in genetically altered animals will be given for the shortest period possible which is typically 5 days. Where procedures require the use of general anaesthesia we will aim to combine these so that the anaesthesia burden is reduced.

All mice will be maintained under barrier conditions to reduce the chance of opportunistic infections. Aseptic techniques will be used for all recovery surgery. Analgesia, for example the use of opioids such as buprenorphine, will be given peri-operatively and post-surgery to minimise pain and suffering. Highly palatable food, jelly, or another fluid source will be placed at the bottom of the cage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will frequently consult the following online resources: www.nc3rs.org.uk; https://norecopa.no; https://www.lasa.co.uk.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Besides staying up-to-date using online portals such as www.nc3rs.org.uk or https://science.rspca.org.uk, we will use the ease of contacting our NC3R’s regional manager and our Named Information Officer. In addition, we will attend regular internal 3R’s meetings.

86. Pharmacokinetics studies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Absorption, distribution, metabolism, pharmacokinetics

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this work is to conduct relevant studies during the development of new medicines, to assess how much and how quickly the body absorbs the test medicine, and then how much and in what ways the medicine is changed within the body and then excreted.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The data produced from animal studies described in the protocols are intended to build on information already known to the clients, from animal studies and/or non-animal studies. Initial studies of absorption, distribution, metabolism and excretion (ADME, or pharmacokinetics), typically blood level assessment, with or without pharmacodynamic assessments, are commonly conducted to assist with selection of test items for regulatory development.

What outputs do you think you will see at the end of this project?

To produce data on absorption, distribution, metabolism and excretion of test items in animals, to enable judgements about selection for further development.

Who or what will benefit from these outputs, and how?

The data generated from the ADME studies will be used to plan further studies at other Establishments, within a regulatory programme for a test item, it may then be conducted, to provide information on ADME, commonly including routes and rates of excretion, and/or retention of test item within the body. Identification and assessment of specific metabolites may be conducted in this phase.

Before using new methodologies, the tests may be validated by use of relevant equipment and/or reference compounds with known effects. Successful completion of this work would then enable use of the new methodology in the production of data mentioned above.

How will you look to maximise the outputs of this work?

This work takes place in the context of an integrated drug discovery effort, where medicinal chemistry, in vitro biology and pharmacokinetic scientists have collaborated to produce novel compounds that fulfil appropriate properties (e.g. affinity, selectivity, brain exposure, target engagement) that deem them worthy of being tested in in vivo preclinical assays as detailed in the program of work. Through an iterative process, the program of work will provide evidence that novel molecules do or do not possess characteristics predictive of clinical efficacy, thus determining whether they are subjected to further toxicological assessment and clinical development.

Study Design will be key to ensure integrity of data collection is maintained at all time points, to produce data on absorption, distribution, metabolism and excretion of test items in animals, to enable judgements about selection for further development.

Species and numbers of animals expected to be used

- Mice: 10,000
- Rats: 5,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These studies are designed to identify metabolic change and excretion, using standard procedures seen to have been suitable for drug development programmes of clients over many years. Elements of this include ensuring administration of test items to a scientifically appropriate animal model (Rats and Mice) by the same route by which humans would be exposed, defining the extent of absorption through blood sampling over a time course, and identifying rates and routes of excretion though collection of excreta.

The animal models have been selected on the basis they can provide the data needed for the ADME studies and they are well established models to screen drug candidates before moving into other species. Animal numbers will be reduced where possible.

Typically, what will be done to an animal used in your project?

Dosing administration- test item/control material may be administered on one or more occasions via one or more of the following dose routes - no anaesthesia unless otherwise noted

- orally, including by gavage
- by injection: intramuscular, intravenous, subcutaneous, intraperitoneal.
- by discontinuous intravenous infusion (Local anaesthesia may be used) under manual restraint-within a restraint tube for up to 3 hours
- -while in the home cage (for up to 3 hours, with a temporary tether to protect the infusion line) - rats only
- by discontinuous or continuous intravenous infusion via a previously implanted vascular cannula. Typically requires restraint and single housing (rats only)
- Dermal application (with or without use of protective devices such as jackets, collars, or a rubber ring attached to the skin to delineate the dosing site). Animals will be single housed while wearing skin protective devices .

Transdermal by needle-free injection

- Intranasal by spray or instillation (brief recovery anaesthesia may be used) Oropharyngeal dosing (brief recovery anaesthesia may be used) - single occasion only
- Single Housing may be required on occasion (e.g. following surgery and exteriorised vascular catheter, due to aggression or to collect excreta)

Although there is no need or expectation that test items will cause adverse effects, it is possible that pharmacological or toxicological effects of test item may be seen, and expected only to be transient in nature, for a short period after dosing. Effects may include reduced activity or subdued behaviour for up to about 3 hours, changes in breathing rate, abnormally hunched posture, vocalisation, very occasionally mild neurological effects. Such effects would not normally be expected to result in evident or lasting distress.

What are the expected impacts and/or adverse effects for the animals during your project?

Oral gavage mis-dosing is rare but may be associated with tissue damage, dosing into the lungs or biting and swallowing the plastic gavage tube by rodents. Rarely, an animal may be found in significant discomfort shortly after dosing, or potentially dead. Such cases would typically be acute events; death is not an anticipated event in any individual case.

Discomfort associated with the various injection routes is anticipated to be transient in

nature. It is possible that local swellings may arise.

Intravenous dosing of rodents typically involves whole body warming to stimulate peripheral circulation of the tail vein. This has occasionally been associated with generalised seizures or death of an animal during or immediately after warming, as an acute event.

Restraint associated with some dosing routes may result in discomfort or distress. Rarely, an animal may be found dead in a restraint tube with no premonitory signs. Such cases would typically be acute events; death is not an anticipated event in any individual case.

Restraint required for protection of an implanted vascular cannula can cause a degree of discomfort, and potentially skin lesions.

Administration of substances directly into the lung may impair breathing in anesthetised rats. On occasion, this may result in non-recovery from anaesthesia.

Decannulation of restrained animals undergoing intravenous infusion via a temporary percutaneous device may occur (estimate of up to about 10% where free moving with a tether is enabled), requiring repeated venous cannulation, on up to three further occasions.

Single housing may result in a degree of distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We would expect moderate severity greater than 80% of mice and rats due to the potential surgical intervention, under another PPL, and continued use of this PPL, plus the cumulative effect of serial sampling throughout all of the studies.

Although there is no need or expectation that test items will cause adverse effects, it is possible that pharmacological or toxicological effects of test item may be seen, these are expected to be transient in nature, for a short period after dosing. Effects may include reduced activity or subdued behaviour for up to about 3 hours, changes in breathing rate, abnormally hunched posture, vocalisation, very occasionally mild neurological effects. Such effects would not normally be expected to result in evident or lasting distress.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have consulted the FRAME, NC3R and UAR websites for feasible alternatives to studying behavioral outcomes in other preparations, but generally a whole animal approach is necessary to produce reliable preclinical data that can be related to humans.

These studies are designed to identify metabolic change and excretion, using standard procedures seen to have been suitable for drug development programmes of clients over many years at the company. Elements of this include ensuring administration of a test item to a scientifically appropriate animal model by the same route by which humans would be exposed, defining the extent of absorption through blood sampling over a time course, and identifying rates and routes of excretion though collection of excreta.

The numbers of mice used sought is on the basis of two distinct areas of growth within the industry:

More discovery screening work due to availability of GA strains for comparison of targeted therapies.

Increase in development of biologics using mouse models for tissue distribution studies, these studies require larger groups of animals to categorise the distribution over time at multiple dose levels to reach receptor occupancy.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are typically used in preliminary testing, before clients seek conduct of in vivo studies in this project.
There are no other models that can be used at present to replicate the ADME protocols

Why were they not suitable?

Regulatory guidance still requires the assessment of pharmacokinetics in animals in advance of the conduct of large scale trials with human patients (ref).

Reference:
ICH M3 (R2) on the non-clinical safety studies for the conduct of human clinical trials

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based upon average number of rats /mice used per PK study and anticipated growth at our Establishment to supply this number of ADME studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experimental groups will be dependent upon the aims of the study. A typical study will

examine one or more of the following outcomes: blood/plasma kinetics, excretion balance, metabolite profiling and tissue distribution.

Where multiple endpoints are being addressed every effort will be made to combine these endpoints within the smallest number of animal groups possible – however this may be limited by experimental logistics and/or sample sizes that may be available. For example, combining blood/plasma kinetics with mass balance may not be possible as removing animals from the specially designed mass balance housing to collect blood samples may lead to a loss of excreta.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Group sizes will be determined to be the smallest number required to meet the study aims based on historical study experience and known scientific data of the compound.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and Mice will be used for this project.

These studies are designed to identify metabolic change and excretion, using standard procedures seen to have been suitable for drug development programmes of clients over many years within the company. As a result, the methods to be used will be administration of test item to a scientifically appropriate animal model by the same route by which humans would be exposed, defining the extent of absorption through blood sampling over a time course, and identifying rates and routes of excretion though collection of excreta.

Standard procedures within the organisation exist to ensure appropriate dose level selection for administration of materials by the most relevant route(s) and by the most refined method, to achieve the scientific aim, while expecting to remain within the severity limit established for the protocol. This is achieved by discussions with the client to confirm the existing information on tolerance or toxicity of the test item in the relevant species, the amount of dosing required, and the degree and type of sampling required. Dosing is commonly on a single occasion, but in cases of test items with long half-life, dosing can potentially be for up to 14 days in order to reach steady state of tissue/blood concentration. If data are lacking, a pilot study may be conducted in order to clarify these elements.

Why can’t you use animals that are less sentient?

Animals that would normally be considered less sentient that rat and mice would not be considered to have the complexity to deliver meaningful results for an ADME study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Standard procedures within the organisation exist to ensure appropriate dose level selection for administration of materials by the most relevant route(s) and by the most refined method, to achieve the scientific aim, while expecting to remain within the severity limit established for the protocol. This is achieved by discussions with the client to confirm the existing information on tolerance or toxicity of the test item in the relevant species, the amount of dosing required, and the degree and type of sampling required. Dosing is commonly on a single occasion, but in cases of test items with long half-life, dosing can potentially be for up to 14 days in order to reach steady state of tissue/blood concentration. If data are lacking, a pilot study may be conducted in order to clarify these elements.

Refinements include acclimation of animals within the facility prior to any procedure including regular handling, daily checks (including out-of-hours cover), habituation to restrain devices, and procedural work carried out by trained competent personnel.

Other measures that we undertake to minimise welfare issues include housing in socially compatible group sizes wherever possible, and by the provision of enrichment and supplements during the study work.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance from the client on their model requirements, however we will continue to regularly check information on the NC3R's website and are signed up to the NC3R's newsletter, also we will work to PREPARE guidelines. We are AAALAC accredited and review publications on the laboratory animals science association (LASA) website, as well as attending and contributing to seminars and meetings with LASA.

We will consult the NC3R's website for up-to-date information as well as regular documents that are circulated through our AWERB.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through our AWERB and 3Rs meeting s, we review any new information on advances in these type of studies. This may involve reducing number of animals to meet experimental design and group sizes will always be the smallest number required to meet the study aims based upon historical data from clients and known scientific data of the compound.

Every study design will be evaluated to ensure the 3Rs are applied and where possible the same animals are utilised for multiple endpoints.

87. Vaccine Development against Neisseria Gonorrhoeae

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccines, Infectious disease, Bacteria, Antimicrobial resistance, Immunology

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Development of a new vaccine candidate against Neisseria gonorrhoeae

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The primary aim of the proposed research is to develop a vaccine against Neisseria gonorrhoeae (gonococcus), the aetiological agent of gonorrhoea. Gonorrhoea is a sexually transmitted infection of major global health significance. It is responsible for considerable morbidity through pelvic inflammatory disease, ectopic pregnancy and infertility, and increased transmission of HIV infection. Importantly, it presents an enormous additional public health threat through the ability of gonococcus to acquire and transmit antimicrobial resistance (AMR), such that untreatable gonorrhoea is a real possibility. The major burden of disease lies in low and lower middle-income countries (LMICs), particularly among

women. In addition, there are highly susceptible groups in the UK, including men who have sex with men and commercial sex workers. A vaccine is urgently needed but presents a significant scientific challenge since natural infection does not necessarily lead to protection. No vaccine is currently available.

What outputs do you think you will see at the end of this project?

The potential benefits for the 5-year duration of this project are to:

- discover new vaccines or vaccine components against gonococcus.

-Establish the proof of concept and the mechanism of protection induced by these vaccine candidates

-Provide sufficient data to support the progression of the successful candidates to a clinical trial

-Compare and identify different mechanisms by which vaccines induce the desired immune responses

Who or what will benefit from these outputs, and how?

A vaccine against Neisseria Gonorrhoeae will benefit all individuals engaging in sexual contact globally by protecting them from gonococcal infection.

The vaccine will also be of particular benefit to individuals from low and middle-income countries and in key populations including men who have sex with men (MSMs) and commercial sex workers (CSWs) where the prevalence of Gonorrhoea is higher.

The new methods developed will also be shared with the scientific community who are studying vaccine development.

How will you look to maximise the outputs of this work?

To maximise the outputs of this work, we follow different strategies:

Publication: we will publish the successful as well as the unsuccessful approaches to gonococcal vaccine development

Conferences: we will present our data at relevant conferences (specific for the gonococcal and Neisseria infection which we are targeting and conferences focusing on vaccines and vaccine development generally)

We will engage with potential collaborators for all our programs. Our expertise is in vaccine development and thus we will collaborate with experts on the gonococcal pathogen and gonorrhoea disease, allowing fruitful collaborations as these skills and knowledge are complementary.

Protection of intellectual property, in order to attract a commercial company to take up further vaccine development and commercialisation.

Species and numbers of animals expected to be used

- Mice: 8000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice because they are the least sentient species that can be studied, with an immune system that is well characterized, and an understanding of how immune responses in mice may translate into what we would find in humans.

We use adult mice because they will have a fully functional and developed immune response.

Typically wild type animals are used for our studies, however genetically altered animals (GAA) may be used to investigate the role of specific genes in the immune response, and to identify which part of the immune system provides the immune responses and protection.

Typically, what will be done to an animal used in your project?

For the immunogenicity studies, mice receive the vaccines by injection 3 times and blood samples are taken 5 times to evaluate the immune response.

Very rarely, other explorations of the immune responses may be carried out, using genetically modified mice.

What are the expected impacts and/or adverse effects for the animals during your project?

For the immunogenicity studies, the adverse effects expected are mild, 25% of animals may possibly experience moderate severity and may include:

Transient and general malaise (2-3 days post vaccination).

Local reaction (transient swelling or redness) of the skin at the vaccination site that is unlikely to last more than 2-3 days post injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild – 75%
Moderate – 25%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The program of work involves research on live animals because there is no alternative: the evaluation of the immunogenicity and efficacy of our vaccine candidates is an essential part of the research, and there is currently no in vitro system available that can mimic the complexity of the entire immune system. In particular this work proposes to develop vaccine for human use, and thus requires use of a mammal in order to mimic as closely as possible the human immune system. Mice are the standard species used for immunogenicity testing of almost all vaccines. There is a unique body of research on vaccines in mice allowing comparisons with previous work and there are uniquely extensive sets of reagents available for analysing vaccine-induced responses in mice.

Which non-animal alternatives did you consider for use in this project?

There is to date no suitable non-animal alternative to evaluate the immunogenicity of a vaccine. However, several steps of the project benefit from non-animal work:

A correlate of protection is suggested for gonorrhoeae (serum bactericidal activity). We will thus seek to establish the in vitro assay in house or with collaborators, with the aim of routinely using this to improve the screening and selection of vaccine candidates and avoiding the need for challenge experiments in animal models.

Evaluation of antigen expression in vitro: We quality control and verify the antigen composition / appearance of the vaccines in vitro using appropriate techniques, prior to injection into animals: for example for our outer membrane vesicle vaccine candidates this is done by evaluating the size and appearance of the vesicles in the vaccine.

Why were they not suitable?

While non-animal alternatives are available to establish some vaccine characteristics (such as measuring the amount of antigen), these are not suitable to measure the immune response a vaccine can induce. The in vitro methods do not mimic the complexity of the entire immune system, from antigen presentation, detection and response to the danger signals, migration of immune cells to lymph nodes, generation of germinal centres and generation of antigen-specific B and T-cell responses.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is based on our experience of how many animals are used for vaccine candidate development up to clinical proof of concept.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We follow the ARRIVE guidelines, use statistical power calculations and the NC3R's Experimental Design Assistant to inform our experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Combining studies together to minimise the number of control mice required: Many experiments necessitate the inclusion of control groups such as unvaccinated animals or control vaccines. To minimise the repeated use of control groups during the project, several test conditions are included simultaneously in each experiment, so that one control group is used as a comparator for many vaccines, rather than assessing one vaccine candidate per experiment which would mean that a control group is required in each experiment.

Sequential sampling: Across a time course, we perform tail-bleeds rather than terminal bleeds, and have developed protocols allowing the evaluation of the immune responses in small blood volumes. This allows the reduction of the number of animals needed to conduct these studies, as it allows data from multiple time points to be generated from the same group of mice rather than requiring a separate group of animals for each time point

Collaborations: We have established collaboration with groups who have a pre-optimised mouse gonococcal challenge model to test vaccine efficacy. This allows us to perform challenge experiments without having to use extra mice for optimisation of the model. In addition, this challenge models is well characterized, reproducible and performed by experts, thus allowing a minimum number of animals per group and a minimum stress to the animals.

-Pilot studies: for a new vaccine composition, a short pilot study is performed to assess reactogenicity and immunogenicity, and if the results are acceptable, we then expand to more extensive protocols (including comparator groups and long-term responses).

Sharing tissues: we collect several organs for assessment of the immune response, and whenever possible provide tissues not relevant to our protocols to colleagues for their research studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of mouse model: Mice have been chosen as they are the standard model species used for initial immunogenicity testing of almost all vaccines.

The project mainly involves the use of wild-type mice which are proven in the field.

The project also involves genetically altered animals (GAA) in order to investigate the role of specific genes in the immune response (for example lymphocyte subsets, cytokines, TLR receptors, chemokines). We will use only established GAA strains with known phenotype, so the appropriate care and monitoring can be assigned prior to their arrival. For mice with immunocompromised phenotype (for example knock-out of a TLR receptor), the risk of infection should be absent due to the housing in IVCs.

Multiple injections will be used over a defined duration because it is important to determine how many injections are required to give the best immune response.

Why can’t you use animals that are less sentient?

We use mice because they are the least sentient species that can be studied, with an immune system that is well characterized, and an understanding of how immune responses in mice may translate into what we would find in humans.

We use adult mice because they will have a fully functional and developed immune response.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use the most refined route for the specific vaccine formulations, in keeping with the scientific end point. We will use short anaesthesia with isoflurane with most routes when anaesthesia is appropriate. The routes and maximum volumes follow the code of practice and guidelines set out in The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, Fourth Edition (2013).

We use anaesthesia for some parenteral injections, such as intramuscular injections, as these can be painful.

Increased monitoring is used upon detection of a side effect, and during challenge studies, and pilot studies allow us to understand predictable patterns of side effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, Fourth Edition (2013).

Published literature on mouse challenge models: regular checks ensure we consider the shortest and mildest possible challenge models.

LASA Best Practice Guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive the regular NC3Rs newsletter. One of our team members is also a member of an animal ethics committee, and thus regularly exposed to the improvements made by others working with animals on science. We also keep up with the literature specific to the vaccine platforms we are using, and to the diseases and pathogens we are targeting. We have the flexibility to implement these new advances within projects, or when initiating new projects. Most of the times, 3Rs advances also improve the workload of experiments, or their reproducibility, and ultimately results in use of less animals, thus benefiting not only the animals but the science too.

88. Modulation of Wound Healing and Wound Infection in Swine

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Wound Healing, Acute, Chronic, Wound infection, Therapy

Animal types	Life stages
Pigs	juvenile, adult
Minipigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to assist in the development of new therapies to minimise disfigurement after traumatic injury, promote or otherwise improve the healing of acute and chronic wounds, and control wound infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acute traumatic injury to the skin and underlying tissues (due to accident or elective surgery) is extremely common, affecting millions of people each year. The development of disabling and disfiguring scars is a key physical and psychological issue for patients following such acute trauma.

Chronic wounds (such as venous leg ulcers, pressure ulcers/bedsores and diabetic foot ulcers) are wounds that take a long time to heal. Some may require several years to heal whilst others remain unhealed for decades and some never heal. Those that do heal often recur within weeks or months.

Patients with these wounds often experience severe pain, significant emotional and physical distress, embarrassment (due to wound odour and leakage of fluids through dressings), reduced mobility and social isolation – which leads to loss of self-esteem, depression, anxiety, sleep disturbances, reduced quality of life - and further social isolation. It is also known that chronic wounds, as with most chronic diseases, not only severely impact the patient but also their families and friends.

These physically and emotionally debilitating, and frequently bacterially-infected, wounds particularly afflict and most severely impact the elderly - approximately 3% of people over 65 have a chronic wound of some form. Reflecting the progressively aging population, their prevalence is rapidly increasing, year on year.

Chronic wounds such as diabetic foot ulcers, frequently result in permanent disability due to amputation, or death. In the USA, 85% of lower limb amputations are preceded by the development of a diabetic foot ulcer, and worldwide, there is a lower limb amputation every 30 seconds due to diabetic foot ulceration. The 5-year mortality rate following amputation is reported to be approximately 30% which is comparable to that of cancer. Unfortunately, events such as infections, amputations and death as a consequence of a wound are all too common - though they may be avoidable with rapid and accurate diagnosis, and early use of effective treatments.

It has been estimated that the NHS spends £8.3 billion annually on treating wounds, approximately

£5.6 billion of which is spent on chronic wounds.

Wounds are a significant burden to patients, their families, the NHS, and the economy.

Existing wound therapies are largely ineffective; new, more effective therapies that can promote wound healing and clear wound infection are urgently required if the suffering of patients is to be minimised.

What outputs do you think you will see at the end of this project?

Based on past experience (gained from work undertaken under the authority of previous project licences), it is anticipated that work undertaken on this project will assist in the development and clinical uptake of new, more effective, therapies able to reduce scarring after acute traumatic injury, accelerate or otherwise improve the healing of acute and chronic wounds and clear bacterial infection – and in doing so reduce patient suffering

While our work is invariably commercially sensitive and undertaken under confidentiality agreements, we have and will publish our findings in respected peer-reviewed journals - wherever possible.

Who or what will benefit from these outputs, and how?

Primarily, it is hoped and expected that patients with problem wounds or individuals that display abnormal responses to injury will be the principal beneficiaries of our work under this licence.

The development of new more effective wound healing therapies that accelerate or otherwise improve wound healing and/or promote the clearance of infection would also be expected to reduce costs incurred by the NHS.

The process of new product development, testing and receipt of approval for clinical use is very time consuming - and as such, it is unlikely that any of the therapies tested in this project will achieve approval for clinical use within the lifetime of this licence.

That being said, three developmental therapies tested over the past 5 years (under our previous Project Licence) are now nearing approval for clinical use.

How will you look to maximise the outputs of this work?

We will maximise the value of our outputs by dissemination through a variety of means. Subject to approval from study sponsors, we will endeavour to present our findings at scientific wound healing meetings and publish as much information as possible from studies conducted under this Project Licence.

Species and numbers of animals expected to be used

- Pigs: 174
- Minipigs: 120

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Juvenile domestic (farm) pigs, weighing 35-50kg, and adult (sexually mature) minipigs (typically 15-30 kg) will be used in this project as they have repeatedly been shown to be effective platforms by which to assess the impact of a wide range of therapies on wound healing and wound infection.

As porcine and human skin are similar (biochemically, histologically and immunologically) and porcine studies are frequently predictive of clinical research, the pig is considered to be the species of choice for wound healing research. This acceptance extends to regulatory authorities responsible for the approval of new medical treatments (including the US Food and Drug Administration [FDA]).

Typically, what will be done to an animal used in your project?

Prior to any procedures, pigs will be acclimatised to their new environment for a period of between 7 and 10 days - during which they will be largely left undisturbed other than to familiarise them to husbandry staff, replenish their food and water provisions, and to refresh their bedding materials.

All pigs will be singularly housed during acclimatisation and for up to 20 days after wounding (or until all wounds are fully healed); but, will have continuous aural and daily visual contact (during pen cleaning) with animals in adjacent holding pens. Pigs will be group housed, after 20 days or once all wounds have fully healed, unless there are reasonable grounds (scientific, animal welfare or otherwise) not to do so. During group housing pigs have a tendency to groom/bite one another - which invariably results in

dressing and wound site damage. This can result in loss of test materials/devices and contamination of wounds with soiled bedding - which can in turn impact on the progression of wound healing and the microbial content of wounds, and thereby invalidate the study being undertaken.

After acclimatisation, pigs will then be given pre-medication sedatives (by intramuscular injection) to calm them and facilitate transportation to the operating theatre. Animals will then undergo gaseous general anaesthesia and will be given pre-emptive analgesia.

Under general anaesthesia, the hair at the planned wound sites will usually be removed by clipping and/or depilatory cream or wet-shaving.

The subsequent 'typical experience' of animals will depend on the protocol being followed.
Wound healing protocol

The skin at planned wound sites will usually be cleaned and disinfectant (e.g. iodine, chlorhexidine, or 70% alcohol) applied unless the purpose of the study is to document or investigate the impact of treatment on the normal microbial content (i.e. the microbiome) of skin and wounds.

Pigs will usually be given appropriate long-acting antibiotics to minimise the likelihood of wound infection (this may be omitted in microbiome studies).

Standardised (incisional or excisional) wounds will be created under general anaesthesia and (usually) under aseptic conditions.

Treatments (substances or non-invasive medical devices - such as dressings attached to Negative Pressure Wound Therapy pumps) will be applied to wound sites, surrounding normal skin or to the animal as a whole (systemic treatments).

Wounds will usually be individually dressed to prevent contamination with soiled bedding and uncontrolled cross-contamination of wounds.

Incisional wounds will usually be sutured closed (unless the purpose of the study is to investigate an alternative /developmental wound closure device).

Secondary dressings and outer jackets may be applied to protect wound sites from further damage. Pigs will then be allowed to recover from anaesthesia under warmed conditions.

Animals will be re-anaesthetised at various time points after injury (typically every 2 to 4 days) to permit: photography, wound site assessments, bacterial swabbing, re-application of treatments and re- application of dressings (if employed).

Supplemental pain-relieving drugs will be given whilst under anaesthesia prior to recovery on each occasion. Pain relief will be maintained for the first 14 days after injury by administration of analgesics by injection or using slow-release patches.

These studies will typically run for up to 14-20 days after injury - though longer-term studies, lasting up to 6 months, examining the effect of treatments on long-term parameters (such as scarring, microbiome re-establishment and wound implantation studies) may be performed.

Animals following these protocols will typically receive a marker of cellular proliferation (or other tracer) by injection into the abdomen whilst under anaesthesia - approximately one hour prior to killing.

Humane killing of animals will be performed during the final anaesthetic session

After confirmation of death, tissues will be harvested for histological investigation (or other analysis). Infected wound protocol

The skin at planned wound sites will usually be cleaned and disinfectant (e.g. iodine, chlorhexidine, or 70% alcohol) applied.

Standardised (incisional or excisional) wounds will be created under general anaesthesia and aseptic conditions.

Bacteria (one or more species/strains) will usually be applied to the surface of wounds.

Treatments (substances or devices) will be applied to wound sites, surrounding normal skin or to the animal as a whole (systemic treatments).

Wounds will usually be individually dressed to prevent contamination with soiled bedding and uncontrolled cross-contamination of wounds.

Incisional wounds will usually be sutured closed.

Secondary dressings and outer jackets may be applied to protect wound sites from further damage. Pigs will then be allowed to recover from anaesthesia under warmed conditions.

Animals will be re-anaesthetised at various time points after injury (typically every 2 to 4 days) to permit photography, wound site assessments, bacterial swabbing, re-application of bacteria or treatments and re-application of dressings.

Supplemental pain-relieving drugs will be given whilst under anaesthesia prior to recovery on each occasion. Pain relief will be maintained for the first 14 days after injury by administration of analgesics by injection or using slow-release patches.

These studies will typically run for up to 8-14 days after injury - though longer-term studies, lasting up to 3 months, examining the effect of treatments on long-term parameters (such as re-establishment of the normal microbial content [microbiome]) may be performed.

Animals following these protocols will typically receive a marker of cellular proliferation (or other tracer) by injection into the abdomen whilst under anaesthesia, approximately one hour prior to killing.

Humane killing of animals will be performed during the final anaesthetic session.

After confirmation of death, tissues will be harvested for histological investigation, microbial content analysis, or other analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

Based on over 20 years of conducting studies using similar protocols in pigs and minipigs, we believe the protocols to be followed in this project to be very well tolerated.

The adverse effects we expect to observe in animals following the protocols in this project are localised wound pain (associated with experimental wounding), and discomfort due to unintentional (unplanned) or planned wound infection.

Post-surgical pain

Pigs may experience transient discomfort after surgical wounding of the skin which is usually mild and self-limiting. Pigs will be wounded under general anaesthesia, and will be given appropriate levels of pain-relieving drugs prior to surgery (and thereafter on assessment days) in order to reduce post- surgical discomfort. Pigs will be monitored within 2 hours of the conclusion of general anaesthesia and at least daily thereafter for the development of adverse effects and any animal found to be displaying signs of distress or discomfort, that does not respond to remedial actions (as advised by the NVS) within 24-48 hours, or deteriorates during that time frame, will be killed by a Schedule 1 method.

Wound infection (unintentional)

The loss of the skin barrier as a result of experimental wounding in pigs (in the absence of intentional infection) can rarely result in localised wound infection (occurrence under previous Project Licences <0.5% approx.).

In order to minimise the likelihood of unintentional infection, surgical procedures will be carried out in accordance with Laboratory Science Animals Association Guiding Principles for Preparing for and Undertaking Aseptic Surgery, wherever possible. Animals will also be given appropriate antibiotics (as advised by the NVS) prophylactically (unless their use has the potential for interfere with scientific data- such as in microbiome studies).

Wound site infection is associated with increased (cloudy) exudation, discolouration of wound tissues and wound malodour together with elevated peri-wound inflammation (heat, pain, redness & oedema). Increased inflammation due to infection may result in delayed wound healing and erosion of the skin surrounding wounds - resulting in wound enlargement. These indicators will be monitored at each planned assessment point/dressing change (typically every 2-4 days), and if evident, topical or systemic treatments will be administered (as advised by the NVS) to control infection and minimise discomfort.

Where remedial treatment proves ineffective, and the infection is considered to be compromising well- being, animals will be killed by a Schedule 1 method. In our experience, localised infection of one or more wounds on a given pig has limited impact on their overall well-being.

With frequent monitoring for wound infection, the duration of this adverse effect will be limited to 2-4 days, which we believe is insufficient time for significant clinical manifestation of infection-associated adverse signs.

Wound infection (intentional)

The application of bacteria to surgically-created wounds will result in the localised development of clinical signs of infection - that in certain wounds (e.g. control-treated wounds) may last for the duration of the study (typically 8-14 days)

Wound site infection is associated with increased (cloudy) exudation, discolouration of wound tissues and wound malodour together with elevated peri-wound inflammation (heat, pain, redness & oedema). Increased inflammation due to infection may result in delayed

wound healing and erosion of the skin surrounding wounds - resulting in wound enlargement. These indicators will be monitored at each planned assessment point/dressing change (typically every 2-4 days), and wound infection-associated discomfort will be controlled by the routine use of pain-relieving drugs (as advised by the NVS).

Pigs with intentionally-infected wounds will be monitored regularly for the development of adverse effects - and any animal found to be displaying signs of distress or discomfort, or excessive wound enlargement - that does not respond to remedial actions (as advised by the NVS), will be killed by a Schedule 1 method.

Sepsis and Septicaemia (blood poisoning)

Although we have not experienced this in our previous studies, intentional or unintentional localised infection of wounds can rarely lead to sepsis (a whole-body response to a localised infection) and septicaemia (where bacteria travel from a localised infection via the blood stream and infect various organs).

Although the development of sepsis and septicaemia are very unlikely adverse events in healthy pigs with a normal immune system - such as in our work, the risk is present.

The clinical signs of sepsis and septicaemia are largely similar - and include:

1. Fever - elevated body temperature
2. Loss of appetite/reduced feed intake
3. Depression where pigs sit or lay in the pen and are unresponsive to disturbance.
4. Purple discolouration of the ears
5. Swollen joints
6. Red, blotchy skin
7. Lack of co-ordination
8. Unusual posture
9. Inability to stand
10. Increased respiration

Pigs will be monitored daily (twice daily for pigs with intentionally-infected wounds) for the development of the clinical signs associated with sepsis and septicaemia in terms of demeanour/appetite/excretions, temperature and above signs - at the pen side - and any animal found to be displaying such signs, that does not respond to remedial actions (as advised by the NVS), will be killed by a Schedule 1 method..

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Pigs - wound healing protocol - expected severity **MODERATE** - proportion of animals 100%

Pigs - infected wound protocol - expected severity **MODERATE** - proportion of animals 100%

Minipigs - wound healing protocol - expected severity **MODERATE** - proportion of animals 100%

Minipigs - infected wound protocol - expected severity **MODERATE** - proportion of animals 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mammalian responses to both injury and bacterial infection are multifaceted and complex - involving actions, interactions and responses of numerous cell types present at the site of injury and delivered to the site from other parts of the body via the blood stream.

Wound healing/infection research in animals is necessary as, while laboratory investigations on cells or pieces of skin (from animals and humans) can generate important preliminary data (such as toxicity data, and some indication of 'likely' efficacy); they are unable to fully replicate the multiplicity of physical and biochemical reactions, cell types and cell interactions, that occur in and around wounds (infected or otherwise) as they heal.

In vivo wound healing / wound infection studies in animals also offer the possibility of investigating mechanisms of action of, and/or, the development of unexpected adverse reactions to substances/therapies at the tissue/cell/molecular level, which, as this invariably requires wound tissue excision, would be largely ethically unacceptable in the clinical setting.

Which non-animal alternatives did you consider for use in this project?

We considered, and use (together with academic collaborators), a variety of non-animal approaches to achieve our research aims.

For our wound healing work, these include undertaking wounding assays (called scratch-assays) on sheets of cultured skin cells, and studies of the healing of wounds created on pieces of cultured (live) human skin (taken from patients with excess abdominal skin or following surgical limb amputation).

For our wound infection/antimicrobial work, these include Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) assays, membrane biofilm assays, and bacterially-infected human (and porcine) skin wound assays.

Why were they not suitable?

While these non-animal alternatives can and do provide us with useful information (for example in terms of effective dose), that can assist and guide the design of animal studies, such alternatives cannot replace them. The complexity of the wound healing response, and that in response to bacterial infection, particularly the involvement and interaction of numerous cell types from different parts of the body - means that wound healing and wound infection, and the impact of developmental therapies, can only be studied in intact animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use based on our previous experience of using these animal models and our anticipated future requirements.

This includes both our experience of the minimal number of replicate wounds / animals required, for each treatment group to provide valid and useful data, alongside our experience in, and understanding of, the wound healing and anti-infectives sectors, and the likely demand for our research models. We have made assumptions on future requirements based on our best assessment of the science, current research climate and our previous experience.

Because of their size, multiple wounds can be created on individual pigs without substantially elevating discomfort. In studies investigating local-acting treatments (i.e. where treatments only impact on the wound to which they are applied) each wound is considered a 'statistical unit' - this permits the testing of both experimental and control treatment regimens on the same animal - which reduces the number of animals used in a given study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals to be used is based on 20 years prior experience with these animals and the protocols to be followed, relevant scientific literature and power calculations. We utilised available online resources such as the NC3Rs experimental design assistant to plan experiments and performed power calculations to determine group size. These calculations were based on knowledge of the mean values and variability of the primary outputs for each protocol - taken from our prior experience and that of others.

For example, based on recent historical porcine wound healing data, the number of replicate wounds necessary to detect an increase in wound closure of 20% (i.e. a change in mean wound area from 372mm2 [SD=53.37] to 298mm2 [SD = 39.60]) at an alpha level (‘p’ value) of 0.05 and a power level of 80% is 6. This rises to 8 replicates at a power level of 90%.

Our group sizes are the lowest possible to allow for the very infrequent “in study” loss of animals/wounds (due to death or other complications - such as inadvertent infections); whilst maintaining a high probability that a study will be sufficiently powered for statistical analysis on completion. This reduces the likelihood that animals will undergo unnecessary procedures in statistically underpowered studies.

We design experiments so that multiple experimental readouts can be derived from a single animal. We use imaging (primarily digital photography) and other non-invasive/minimally-invasive methods of wound / wound infection assessment - such as measurement of colour, trans-epidermal water loss and bacteriology swabs (where possible and appropriate), so that wound healing and wound infection can be tracked non/minimally-invasively and confirmed by analysis of wound tissue taken after humane killing.

The creation of multiple wounds on individual animals allows for several formulations to be tested at the same time - and “within animal” controls to be used (where appropriate) - both of which reduce the total number of animals required for a given study.

We standardise our experimental variables in order to minimize variation, such as using animals that are closely matched in terms of age and genetic background. This also reduces the total number of animals required for a given study.

Over time, we have managed to reduce variability in outcomes to control treatments between studies. This means that it is not always necessary to repeat certain control groups and that historical control data can be used – thus reducing the number of wounds (and hence animals) required in a given study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where appropriate, non-animal alternatives such as microbiology assays, 'scratch' assays on sheets of cultured skin cells and wound healing and wound infection assays on cultured (live) human skin will be used to screen-out inappropriate/ineffective investigational agents and determine the most effective dosing regimens for subsequent animal studies - thereby reducing the numbers of animals required for effective in vivo investigation.

In instances where the pre-existing data (in relation to potential wound healing or antimicrobial efficacy) is considered insufficient to undertake a fully powered study, or where a large number of formulation variants exist, preliminary pilot studies, involving small numbers of animals/wounds, will be undertaken to determine the need for more extensive investigation or to screen-out less effective variants – and thereby reduce the numbers of animals used.

We regularly share animal tissue with other research groups and have a good communication network within the establishment to alert other groups to available tissues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use large white (farm) pig and minipig wound healing and wound infection models in this project. These strains of pigs are used in preference to other less common pig strains due to their availability and, in the case of minipigs, due to their size, genetic standardisation and production for biomedical research purposes.

Porcine skin, and the biological mechanisms and processes by which it heals after injury, is more similar to humans than that of rodents and other small animals. This together with the fact that porcine studies have been found to be more predictive of subsequent clinical research has made them the species of choice for wound healing research.

Our overriding rule, is to use the model and methods with the least likelihood of causing pain, suffering, distress or lasting harm that is necessary to address the scientific question being asked.

Dermal wound healing in mammals is known to be significantly influenced by gender - with females displaying an advantage in healing rates. Similarly, males (both human and animals) are more susceptible to bacterial infection than females. These gender differences would be expected to result in greater variation in studies involving both sexes than in single sex studies. This in turn would be expected to increase the number of replicate wounds (and animals) necessary to detect a clinically significant benefit. For these reasons, wound healing and wound infection studies are often undertaken on just one gender. That being said, as we are fully aware of the need to undertake studies using both male and female animals, it is our intention to make clients fully aware of the potential impact/misdirection of single gender studies on the subsequent 'usefulness' of developmental treatments in the clinical setting. We will also encourage our clients to undertake selected early and late (clinically-defining) pre-clinical work on animals of both genders.

The surgical methods used to create wounds, the routine use of pain killing drugs and warmed recovery, the size and number of wounds per animal, the smallest bacterial inoculums and the frequency, form and duration of follow-up assessments and dosing procedures/dosing regimens that we use, have been progressively refined to minimise harms - during the course of our previous licences (and by others).

For a given animal, we create the smallest and fewest wounds under general anaesthesia, provide appropriate levels of pain relief, and undertake the fewest and shortest follow-up assessments and substance administrations, by the most refined route, possible.

Group-housed pigs have a tendency to groom/bite one another, which for our protocols invariably results in damage to test materials/test devices and/or damage to overlying dressings - which in turn can lead to damage and/or contamination of wounds - and ultimately invalidation of the study. That being the case, all pigs are individually-housed in single pens for up to 20 days or until all wounds have healed. Whilst they cannot come into direct contact with one another, they are aware of the presence of other animals in adjoining pens.

Wherever possible, environmental enrichments (e.g., forage food, treats such as fruit and vegetables and toys) will be provided to animals throughout our protocols.

Why can’t you use animals that are less sentient?

The mammalian responses to injury and wound infection are multifaceted and complex – and as yet, these complexities haven't been fully recapitulated in less sentient species. Recent research undertaken on Zebrafish suggests that whilst their response to injury is similar to mammals in some ways – it is clearly different in others; and it is well known that not all data derived from Zebrafish studies and other less sentient models is relevant to humans.

Because wounds take days or weeks to heal, and wound infections can take a similar period of time to clear, it is not possible to study these processes (and the impact of treatment on them) under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As noted earlier, the surgical methods used to create wounds, the routine use of pain killing drugs and warmed recovery, the size and number of wounds per animal, the composition and form of bacterial inoculums applied, and the frequency, form and duration of follow-up assessments and dosing procedures/dosing regimens that we use, have been progressively refined to minimise harms - during the course of our previous 3 project licences.

Opportunities for further refinement are continually sought.

The following refinements were made during recent project licences:

1. Rather than physically trapping/restraining pigs in order to facilitate administration of premedication sedatives (which is very stressful) we now use a 1-metre-long spiral catheter (between the syringe and needle). This allows free movement (after insertion of the hypodermic needle) during the sedation process and is noticeably less stressful for pigs.

2. We routinely calm pigs prior to sedation (and at other time points) by rubbing/scratching them behind their forelimbs - this is effective for both 'farm' pigs and minipigs, and has an immediate calming effect in the majority of animals.

While we do not expect any significant physical, behavioural or physiological deviation from normality in animals following our protocols, we undertake regular monitoring of key well-being parameters which facilitate the early detection of unexpected adverse effects (that may impact on animal pain, discomfort or distress) and thereby allow the rapid deployment of remedial action.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the following resources in planning and conducting experiments:

ARRIVE Guidelines 2.0. https://arriveguidelines.org/arrive-guidelines PREPARE Guidelines. https://norecopa.no/prepare

NC3Rs Experimental Design Assistant. https://eda.nc3rs.org.uk/

NC3RS guidance on blood sampling in pigs. https://www.nc3rs.org.uk/3rs-resources/blood- sampling/blood-sampling-pig

The assessment of Facial expressions in Piglets Undergoing Tail Docking and castration: Toward the Development of the Piglet grimace scale. Di Giminiani et al 2016. Front. Vet. Sci., 3 (100).

Development of a Piglet grimace scale to evaluate Piglet Pain Using Facial Expressions Following castration and Tail Docking: a Pilot study Viscardi et al. 2017. Front. Vet. Sci. 4:51.

Is the Piglet Grimace Scale (PGS) a Useful Welfare Indicator to Assess Pain after Cryptorchidectomy in Growing Pigs? Vullo et al. 2020. Animals 10, 412.

Lou et al. 2022. The Application of 3D Landmark-Based Geometric Morphometrics towards Refinement of the Piglet Grimace Scale. Animals 12, 1994.

NC3Rs guidance on anaesthesia. https://www.nc3rs.org.uk/3rs-resources/anaesthesia
NC3Rs Guidance on analgesia. https://www.nc3rs.org.uk/3rs-resources/analgesia

NC3Rs Guidance on handling and restraint. https://nc3rs.org.uk/3rs-resources/handling-and-restraint

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery. https://www.lasa.co.uk/wpcontent/uploads/2018/05/Aseptic-Surgery.pdf

EFPIA/ECVAM good practice guide to the administration of substances and removal of blood, including routes and volumes. https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/abs/10.1002/jat.727

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our group will stay informed through the NC3Rs website. Relevant information, including the NC3Rs newsletter, is circulated within our establishment by email to all personal and project licence holders. We will attend local events organised by our Animal Welfare and Ethical Review Committee and information sessions on NC3Rs funding streams organised by our establishment's Research & Innovation Service. We will share best practice within our establishment and have well developed interdisciplinary networks to facilitate this. We attend regular local user-group meetings for project licence holders at which the group will receive updates on any changes to best practice or requirements.