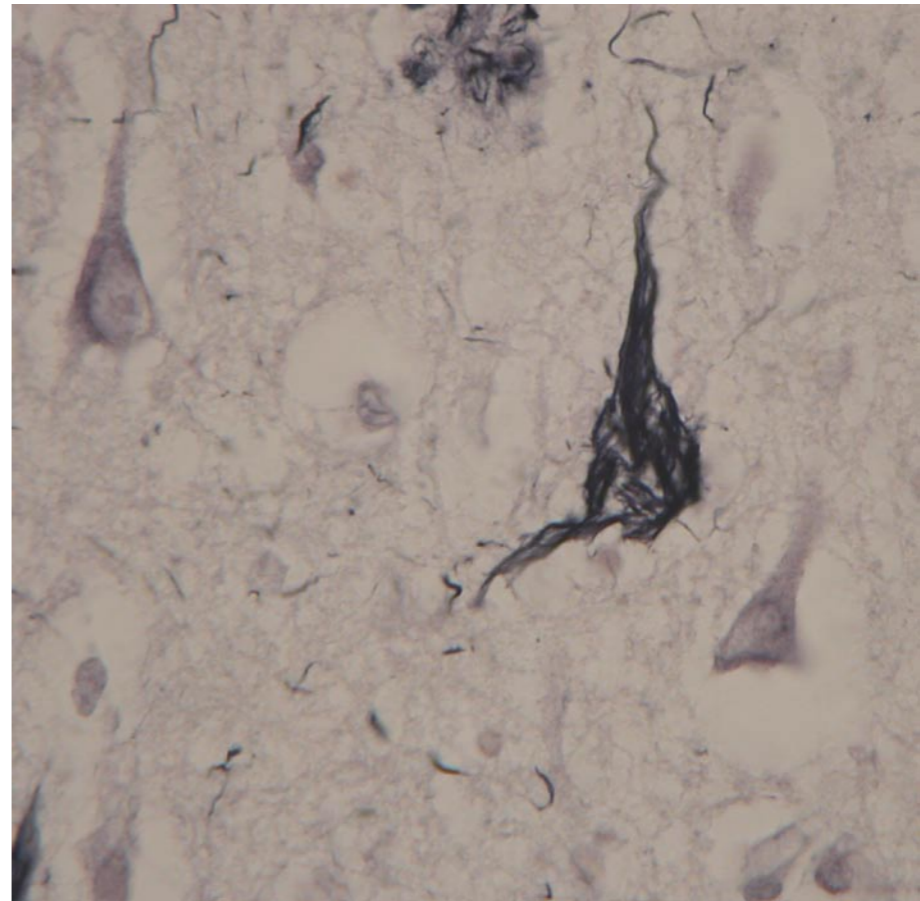


Thesis for doctoral degree (Ph.D.)
2007

Tau and neurofilament proteins in Alzheimer's disease and related cell models



Cecilia Björkdahl

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Institutet**

Stockholm 2007

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Cover picture: Gallyas silver impregnation of NFT (to the right in the middle) and healthy neurons (upper left and lower right). Courtesy of Nenad Bogdanovic.

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ISBN 978-91-7357-407-5

Have patience and endure.

William Shakespeare

ABSTRACT

Background and aims: Among those afflicted with dementia more than half suffer from Alzheimer's disease (AD). Pathological hallmarks of the disease include aggregates of A β , so called amyloid plaques, and neurofibrillary tangles (NFTs) made up of hyperphosphorylated cytoskeletal proteins. So far, no single pathological lesion has proven to be the sole cause of this slowly progressive disorder, with declining memory and cognitive deficits. No curative treatment exists for AD, even though some clinical symptoms can be alleviated. If this disease is to be defeated, probably the treatment must be directed against the disease-causing proteins, rather than downstream clinical manifestations. To find out more about the disease mechanisms, the studies included in this thesis focused on regulation of tau and neurofilament proteins (NFs), both part of the NFTs. Both tau and NFs are hyperphosphorylated in NFTs and the abnormal phosphorylation is dependent on dysregulated kinase and phosphatase activities found in AD. For example, the activity of the kinases GSK-3 β and p70S6K have been found to be increased in AD, and their effects on tau and NFs were investigated in the studies in this thesis. Kinase activity may be regulated by other kinases or by AD pathology in the form of A β , increased zinc concentrations or the reoccurrence of cell cycle markers to name a few, and this was also investigated in these studies.

Results and discussion: In **Study I**, sequential accumulation of A β variants and phosphorylated tau epitopes were demonstrated in AD brains. The levels of A β showed good correlation with phosphorylated tau; the strength of the correlation depending on the specific tau phosphorylation epitopes. Both A β and tau correlated well with different stages of the Braak or CERAD staging systems, which suggests that tau antibodies can be used selectively in AD diagnosis as a complement to morphological evaluations. In **Study II**, zinc treatment led to increased kinase activities, among them p70S6K and GSK-3 β , and a subsequent increase in tau phosphorylation. Tau translation was also increased through the activation of p70S6K, in accordance with increased tau levels in AD brains. These results indicate that p70S6K can regulate tau on both translational and post-translational levels, while the main effect of other kinases, such as GSK-3 β , is on tau phosphorylation. Many tau kinases are also capable of NF phosphorylation and when N2a cells were treated with zinc, in **Study III**, an increase in p70S6K activity was observed, together with a concomitant increase in NF phosphorylation. However, when p70S6K activity was blocked with rapamycin, the NF phosphorylation remained unchanged, despite the fact that the p70S6K activity was significantly decreased. Thus, zinc must induce NF phosphorylation in the N2a cells through other kinases. Other factors, such as small heat shock proteins, may influence tau and NF regulation. In **Study IV**, both Hsp27 and α B-crystallin were up-regulated in AD brains, and this correlated with increased tau and NF phosphorylation. Hsp27 overexpression in N2a cells led to increased pSer262-tau levels, probably regulated by p70S6K, while α B-crystallin overexpression actually resulted in decreased tau and NF phosphorylation. These differences reflect the complexity behind cellular regulation, and the picture gets even more complicated in the human brain, where the surrounding environment also has an effect. Since many pathways intertwine and affect each other, both directly and indirectly, sHSPs, for example, may be activated by tau hyperphosphorylation or the cell cycle. And they may in turn have reciprocal effects on tau regulation or the cell cycle, and so the circle continues. This complexity will affect the choice of possible future treatment strategies for AD since it is difficult to isolate one specific part of one pathway, without affecting any of the others detrimentally.

Keywords: AD, tau, neurofilament proteins, kinases, zinc, Hsp27, α B-crystallin

Tau and neurofilament proteins in Alzheimer's disease and related cell models.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text in bold by their roman numerals.

- I. Zhou X, Li X, **Bjorkdahl C**, Sjogren MJ, Alafuzoff I, Soininen H, Grundke-Iqbal I, Iqbal K, Winblad B & Pei J-J (2006), Assessments of the accumulation severities of amyloid β -protein and hyperphosphorylated tau in the medial temporal cortex of control and Alzheimer's brains, *Neurobiology of Disease* 22(3): 657-668
- II. An WL, **Bjorkdahl C**, Liu R, Cowburn RF, Winblad B & Pei JJ (2005), Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3 β in SH-SY5Y neuroblastoma cells, *J Neurochem.* 92(5): 1104-1115
- III. **Björkdahl C**, Sjögren MJ, Winblad B & Pei JJ (2005), Zinc induces neurofilament phosphorylation independent of p70 S6 kinase in N2a cells, *NeuroReport* 16(6): 591-595
- IV. **Björkdahl C**, Sjögren MJ, Zhou X, Concha H, Avila J, Winblad B & Pei J-J (2007), The small heat shock proteins Hsp27 or α B-crystallin and the protein components of neurofibrillary tangles: tau and neurofilaments, *Journal of Neuroscience Research*, *accepted*

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Abbreviations

aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
APP	amyloid precursor protein
CamKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cdc2	cell division cycle 2 kinase
cdk	cyclin-dependent kinase
Cdk5	cyclin-dependent protein kinase 5
CKI	Cdk inhibitor
CSF	cerebrospinal fluid
DLB	dementia with Lewy bodies
ELISA	enzyme-linked immunosorbent assay
Erk1/2	extracellular signal-regulated kinase 1/2
FACS	fluorescence-activated cell sorting
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GSK-3	glycogen synthase kinase 3
MAPK	mitogen-activated protein kinase
MAPs	microtubule-associated proteins
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MTs	microtubules
NPC	Niemann-Pick type C disease
NF	neurofilament protein
NFTs	neurofibrillary tangles
p70S6K	70-kDa ribosomal protein S6 kinase
PET	positron emission tomography
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP	protein phosphatase
pRb	retinoblastoma protein
PS	presenilin
ROS	reactive oxygen species
SCF	SKP1/CUL1/F-box
Ser	serine
sHSP	small heat-shock protein
Thr	threonine
TOR	target of rapamycin
Tyr	tyrosine
UBB	ubiquitin
UPS	ubiquitin proteasome system
ZnT	zinc transporter

BACKGROUND

Alzheimer's disease

Dementia is a very common disorder among elderly people and is becoming an extensive health problem with the ever-increasing ageing population. Among those afflicted, more than half suffer from Alzheimer's disease (AD; *Fratiglioni et al., 1999*). A century ago, Alois Alzheimer first described, in a deceased patient, the pathological hallmarks – plaques and tangles – of the neurodegenerative disease that later came to bear his name (*Alzheimer, 1907*). But it is mainly during the past two decades that the underlying mechanisms have begun to be understood, starting with the discovery of A β as part of the plaques and hyperphosphorylated tau as the main part of neurofibrillary tangles (*Goedert et al., 1991; Iqbal et al., 2005; Goedert & Spillantini, 2006*). Although much more is known today than two decades ago, the scientific community is still struggling to understand this devastating disease.

Alzheimer's disease epidemiology, genetics and risk factors

Most AD cases are sporadic, with millions affected worldwide (*Fratiglioni et al., 1999; Qiu et al., 2007*). The familial or inherited form of AD, representing only 1-10% of cases, is associated with several mutations affecting the proteins involved in the disease: presenilin (PS) 1 or 2, or amyloid precursor protein (APP) (*Blennow et al., 2006; Goedert & Spillantini, 2006; Turner, 2006*). Among these, the presenilin mutations, especially PS1 (~85%), are much more common in early onset familial AD than APP mutations (*Goedert & Spillantini, 2006; Turner, 2006*). No tau mutations are found in AD, but they occur in other tauopathies, such as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (*Williams, 2006*). The common denominator for the AD-associated APP and PS mutations is that they lead to increased production of the more aggregate-prone (spontaneously amyloidogenic) A β_{1-42} peptide, as opposed to the A β_{1-40} peptide (*Citron et al., 1992; Suh & Checler, 2002; Ling et al., 2003; Thomas & Fenech, 2007*). Although the A β in the amyloid plaques were considered to be the pathogenic, evidence found during the past couple of years increasingly points to soluble A β -oligomers as the disease-causing species.

Regardless of family history of the disease or other contributory factors, age is the most important and ever-present risk factor for AD. Other risk factors are: the ApoE ϵ 4 allele; factors associated with vascular disease, e.g. hypertension, atherosclerosis, coronary heart disease, smoking and obesity; and environmental factors such as low level of educational attainment or a history of head trauma

(severe enough to cause lack of consciousness) (Ling *et al.*, 2003; Qiu *et al.*, 2003; Karp *et al.*, 2004; Blennow *et al.*, 2006; Elbaz *et al.*, 2007; Thomas & Fenech, 2007; Qiu *et al.*, 2007). Polymorphisms of certain genes also increase the risk to develop AD, these genes include α 2-macroglobulin (a protease inhibitor), CYP46A1 (plays a role in cholesterol and phospholipid metabolism) and UBQLN1 (affects APP processing), to name a few (Bertram *et al.*, 2005; Hiltunen *et al.*, 2006; Thomas & Fenech, 2007). Those with Down's syndrome, who have three copies of chromosome 21, where the gene encoding APP is located, are also at risk of developing AD due to increased levels of this protein (Glennner & Wong, 1984; Goedert & Spillantini, 2006; Turner, 2006; Thomas & Fenech, 2007).

Alzheimer's disease symptoms, neuropathology, diagnosis, diagnostic tools, biomarkers and treatment

AD is a slowly progressive disorder with a decline in memory and cognitive deficits, such as problems with language, visuospatial skills, impaired judgement and decision-making (Gustafson, 1975; Gustafson *et al.*, 1977; Terry & Davies 1980; Cummings & Benson 1992). Only possible or probable AD can routinely be diagnosed clinically, and the only certain diagnosis of AD is made post-mortem, where AD is characterised by amyloid plaques, neurofibrillary tangles (NFTs) and neuronal cell loss (Duyckaerts & Hauw, 1997; Markesbery, 1997). Both plaques and NFTs can also be found in unaffected elderly people, but AD neurodegeneration usually precedes clinical symptoms by decades (Blennow *et al.*, 2006; Goedert & Spillantini, 2006).

An early clinical phase may be referred to as mild cognitive impairment (MCI), with clinical symptoms intermediate between normal ageing and AD, but this may also be the pre-stage of other neurodegenerative disorders (Winblad *et al.*, 2004; Albert & Blacker, 2006; Palmer *et al.*, 2007). In order to increase the efficacy of treatment, it should preferably be given during this early stage, but it is currently still very difficult to correctly diagnose cases corresponding to "MCI-leading-to-AD". Clinical experience has led to different international criteria for evaluating AD; for example, those of the National Institute of Neurological and Communicative Diseases and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). There are also various pathological criteria, some of which only consider one of the pathological hallmarks, such as those published by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD), which focus on the amyloid burden, while others, such as the Khachaturian, Regan criteria or Braak staging, consider both amyloid plaques and tau pathologies (Braak & Braak, 1991;

Turner, 2006). Due to advances made in diagnostic methods some researchers recently suggested a revision of the NINCDS-ADRDA criteria to take more modern methods into account (*Dubois et al., 2007*).

Diagnostic methods also involve neuroimaging and biomarkers. Neuroimaging can be used, for example, to investigate brain atrophy via MRI (magnetic resonance imaging) and glucose metabolism or A β plaques with the help of PET (positron emission tomography), while increased total and phosphorylated tau levels and decreased A β_{1-42} levels are used as biomarkers in cerebrospinal fluid (CSF) analysis (*Jelic & Nordberg, 2000; Sjögren et al, 2003; Blennow et al., 2006; Bailey et al., 2007; Ward, 2007*).

So far, no curative treatment exists for AD, but the clinical symptoms can be alleviated. Acetylcholinesterase inhibitors such as Donepezil, Rivastigmine and Galantamine are approved for clinical use and have been shown to decrease functional and behavioural symptoms, although they do not address the underlying pathologies (*Suh & Checler, 2002*). Another drug, Memantine (a non-competitive NMDA-receptor antagonist), alleviates cognitive and behavioural symptoms through neuronal protection against glutamate-mediated excitotoxicity (*Blennow et al., 2006; Turner, 2006*). Suggestions for other kinds of AD drug candidates, such as anti-inflammatory drugs, cholesterol-lowering drugs (e.g. statins), antioxidants (such as vitamin E) and oestrogens (*Suh & Checler, 2002; Ling et al., 2003*), have come out of epidemiological studies, but positive results obtained in experiments or indications from population-based studies are not always confirmed in clinical trials.

If this disease is to be defeated, pharmacological treatment must be directed against the disease-causing proteins, rather than downstream clinical manifestations. APP is cleaved in two ways; one so-called non-amyloidogenic pathway and one amyloidogenic, pathological pathway. Attempts have been made to influence the cleavage enzymes (secretases) involved in these pathways. In the non-amyloidogenic pathway the cleavage enzyme is α -secretase, and the aim of treatment is to shift APP processing towards this non-pathological pathway. The first cleavage enzyme in the amyloidogenic pathway is β -secretase (BACE1), which shows promise for future therapy since BACE1 knockout mice show no pathological phenotype. Animal experiments in transgenic mouse lines have shown promising results for A β immunotherapy with regard to A β removal but, unfortunately, it caused dangerous side effects (aseptic meningoencephalitis) in a human phase II trial (*Ling et al., 2003*). However, work is continuing to make it safer for humans. Drugs against tau have focused

on abnormal hyperphosphorylation, and although several known kinase inhibitors can affect tau phosphorylation, the complexity of the signalling pathways in the cells causes problems associated with their multiple actions, as well as adverse side effects.

Neurofibrillary pathology and protein components in Alzheimer's disease

Tau

Tau belongs to the family of microtubule-associated proteins (MAPs) and normally binds to and stabilises microtubules (MTs) in neurons (*Weingarten et al., 1975*). Tau protein has four different domains: the N-terminal, the proline-rich, the microtubule-binding and the C-terminal domain. In the adult human brain six tau isoforms are expressed by different splicing of the same tau mRNA; they vary in the number of microtubule-binding domains (having either three or four) and in the number and size of N-terminal inserts (*Billingsley & Kincaid, 1997; Friedhoff et al., 2000; Shahani & Brandt, 2002; Avila et al., 2004; Goedert et al., 2006*). Tau can be post-translationally modified in several ways (e.g. glycosylation, ubiquitination and oxidation), but phosphorylation is by far the most extensively studied and is paramount to AD pathology (*Grundke-Iqbal et al., 1986; Mandelkow & Mandelkow, 1998; Gong et al., 2005; Goedert et al., 2006*).

The degree of tau phosphorylation varies with age and context, from ~ 7 Pi/mol in foetal brain (*Kenessey & Yen, 1993*), to ~ 2 Pi/mol in normal healthy adult brain and ~ 8 Pi/mol in PHF-tau (paired helical filament) in AD brains (*Kopke et al., 1993*). The list of serine (Ser) and threonine (Thr) phosphorylation sites (> 30) on tau continues to grow, as well as the list of kinases that phosphorylate tau (*see Figure 1 for examples*). When tau is abnormally hyperphosphorylated, especially on Ser214 and Ser262, it loses its ability to bind to MTs and may also sequester normal tau, preventing binding to MTs, resulting in disruption of the MTs (*Biernat et al., 1993; Xie et al., 1998; Alonso et al., 2001; Zhou L-X et al., 2006*). Hyperphosphorylated tau is prone to form PHFs in NFTs. Several protein kinases, such as glycogen synthase kinase 3 (GSK-3), cyclin-dependent protein kinase 5 (Cdk5), mitogen-activated protein kinase (MAPK), Ca^{2+} /calmodulin-dependent protein kinase II (CamKII), and cell division cycle 2 kinase (cdc2), are putative tau kinases in AD (*Schneider et al., 1999; Grimes & Jope, 2001; Pei et al., 2002; 2003a; Shahani & Brandt, 2002; Yamamoto et al., 2002; Hamdane et al., 2003; Gong et al., 2005; Wang et al., 2007*). The various

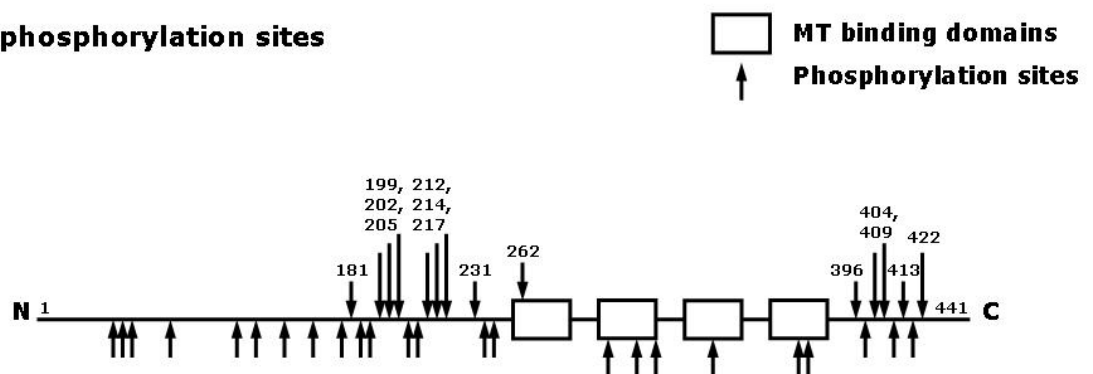
kinases may be activated by elements of AD pathology such as inflammation (Arnaud *et al.*, 2006), oxidative stress, A β and cell cycle re-entry.

Although no increase has been seen in tau mRNA levels, several studies have revealed increased levels of total and phosphorylated tau in AD – one reason for this may be increased eukaryotic translation factor 4E eIF4E activity (Li *et al.*, 2004). The eIF4E activity is increased particularly in cases of AD with late Braak diagnosis, and shows a significant positive correlation with both total tau and phosphorylated tau, suggesting that the increase in total tau levels seen in AD may be due to eIF4E-activated translation (Li *et al.*, 2004).

No tau mutations have been reported to cause AD, but differences in tau haplotype and mutations may be the cause of other neurodegenerative disorders such as progressive supranuclear palsy, corticobasal degeneration and FTDP-17 (Goedert & Spillantini, 2006; Williams, 2006).

Figure 1.

Tau phosphorylation sites



Some of the phosphorylation sites for the kinases GSK-3 β and p70S6K:

GSK-3 β : Thr181, Ser199, Ser202, Thr205, Thr212, Ser214, Thr217, Thr231, Ser262, Ser396, Ser404, Ser409, Ser413, Ser422

p70S6K: Thr212, Ser214, Ser262

Adapted from: Friedhoff *et al.*, 2000; Shahani & Brandt, 2002; Gong *et al.*, 2005; Gong *et al.*, 2006; Pei *et al.*, 2006; Wang *et al.*, 2007

Tau pathology

The NFTs are made up of abnormally hyperphosphorylated proteins, with tau proteins forming the principal component (Grundke-Iqbal, 1986a; 1986b; Goedert *et al.*, 2006). Tau pathology begins intracellularly with tau hyperphosphorylation and sequestration of normal tau and other microtubule-

associated proteins, causing microtubule disassembly, which impairs axonal transport, compromising neuronal and synaptic functions (*Iqbal et al., 2005; Gong et al., 2006*). “Geographically” tau pathology in the form of NFTs and neuropil threads starts in the transentorhinal region, and then spreads first to the hippocampus and amygdala before reaching the neocortex – this spread is consistent with the clinical presentation, starting with amnesia followed by progressive decline in multiple cognitive domains, leading to a vegetative state and finally death (*Braak & Braak, 1991*).

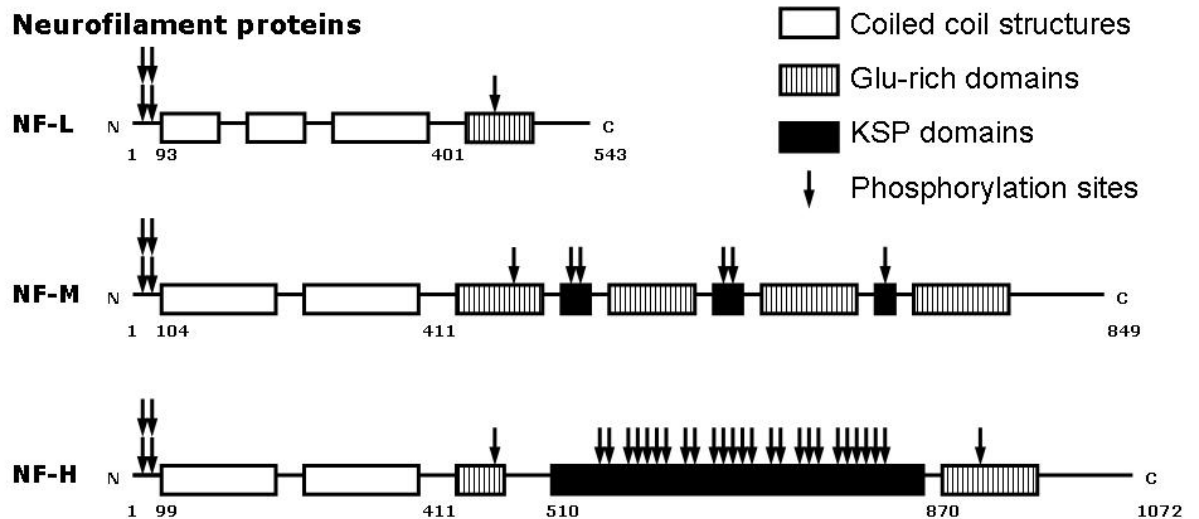
Other microtubule-associated proteins involved in Alzheimer’s disease

Not only tau, but other MAPs are also involved in AD. Abnormal hyperphosphorylation of MAP1b has been found in AD and is associated with NFTs (*Ulloa et al., 1994; Hu et al., 2002*) and other neurodegenerative diseases such as Niemann-Pick type C disease (NPC) and dementia with Lewy bodies (DLB; *Bu et al., 2002; Shepherd et al., 2002*), but the mechanism underlying the changes has not been fully elucidated. Protein phosphatases (PPs) known to dephosphorylate tau have also been shown to regulate MAP phosphorylation in rat brains, and the results suggest that PP2A is the major PP in MAP1b and MAP2 dephosphorylation (*Gong et al., 2000*). PP2B has also been found to regulate MAP1b dephosphorylation, but to a lesser extent than PP2A (*Gong et al., 2000*).

Neurofilament proteins

Neurofilament (NF) proteins are members of the intermediate filament family with a characteristic diameter of 8-10 nm and are important components of the cytoskeleton in neurons. The three NF proteins, NF-L, NF-M and NF-H, have molecular weights of 61-66 kDa, 90-100 kDa and 110-115 kDa, respectively, and they heteropolymerise to form filaments (*Lee & Cleveland, 1996; Julien & Mushynski, 1998*). The NF proteins can be post-translationally regulated in similar ways to tau through, for example, phosphorylation (*see Figure 2*) and glycosylation (*Julien & Mushynski, 1982; Pant & Veeranna, 1995; Lee & Cleveland, 1996*). During both murine and human brain development NF-L and NF-M are first detected during embryogenesis, and their levels progressively increase, while NF-H is barely detectable during embryogenesis but accumulates in the postnatal brain (*Julien et al., 1986*).

Figure 2.



The N-terminal head domain sites can be phosphorylated by kinases like PKA and PKC, while the KSP domains contain numerous sites ideal for proline directed kinases, such as Cdk5 and GSK-3 β . In contrast to tau antibodies, the NF antibodies seldom recognise specific phosphorylation sites, rather they recognise phosphorylation motifs covering several sites. The phosphorylation may also be checked with gel-shift assays.

Adapted from: Pant & Veeranna, 1995; Lee & Cleveland, 1996; Julien & Mushynski, 1998; Grant & Pant, 2000; Grant et al., 2006; Shihag et al., 2007

Neurofilament pathology

As for tau and MAP1b, the NF proteins are components of the NFTs and are abnormally hyperphosphorylated in AD (Perry et al., 1985; Sternberger et al., 1985; Lee et al., 1988; Ulloa et al., 1994; Hashimoto et al., 1999; Hu et al., 2002), and in other related neurodegenerative disorders such as NPC (Bu et al., 2002) and DLB (Shepherd et al., 2002). Abnormal distribution of NF-L has been found in brains with early onset types of AD, sometimes accompanied by tau, but not always (Nakamura et al., 1997), and the levels of all three NF subunits (NF-L, NF-M and NF-H) are increased in AD brains and in CSF from AD patients, especially in late-onset AD (Sjögren et al., 2000; 2001; Wang et al., 2001; Hu et al., 2002, Norgren et al., 2003). Both total NF and phosphorylated NF levels are increased in AD brains, and since no increase could be seen at the mRNA level this suggests that the degradation of NF is impaired in AD (Wang et al., 2003).

Many of the kinases known to phosphorylate tau are also capable of phosphorylating NF (Bajaj & Miller, 1997; Julien and Mushynski, 1998; Hashimoto et al., 2000; Sasaki et al., 2002), for example, Cdk5, induced by oxidative stress, phosphorylates NF, inhibiting NF axonal transport (Shea et al., 2004), and NF-H can be phosphorylated by Cdk5 and GSK-3 α in COS cells

(Bajaj & Miller, 1997). In metabolically active rat brain slices inhibition of PP2A induces phosphorylation and accumulation of NF (Wang *et al.*, 2001; Gong *et al.*, 2003). There is some connection between tau and NF pathology because tau-overexpressing transgenic mice, with NF knockouts, in particular NF-L, show decreased tau pathology compared with mice with normal levels of NF (Ishihara *et al.*, 2001).

Phosphorylation – kinases and phosphatases

An imbalance of protein kinase and PP activity is believed to be the main reason behind tau, NF and MAP1b hyperphosphorylation. Several protein kinases, such as GSK-3, Cdk5, MAPK, CamKII and cdc2, many of these components of the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, are considered to be tau kinases in AD (Grimes & Jope, 2001; Pei *et al.*, 1997; 1998; 1999; 2002; 2003a; Nordlinder *et al.*, 2002; Shahani & Brandt, 2002; Yamamoto *et al.*, 2002; Hamdane *et al.*, 2003b; Wang *et al.*, 2007). Previous studies have suggested that these kinases also are capable of phosphorylating NF (Pant & Veeranna, 1995; Bajaj & Miller, 1997; Julien and Mushynski, 1998; Hashimoto *et al.*, 2000; Sasaki *et al.*, 2002). Among the phosphatases, PP2A is believed to be the major phosphatase for PHF-tau dephosphorylation in AD (Tian & Wang, 2002; Wang *et al.*, 2007), and it has also been shown to dephosphorylate NF (Veeranna *et al.*, 1995; Gong *et al.*, 2003) and MAPs (Ulloa *et al.*, 1993; Gong *et al.*, 2000; 2001). Furthermore, PP2A activity is reduced in AD brain (Gong *et al.*, 1993). A putative link between A β and tau pathology is the fact that A β has been shown to activate several tau candidate kinases, such as GSK-3, MAPK and Cdk5 (Lee *et al.*, 2000, Zheng *et al.*, 2002; Takashima, 2006). The reoccurrence of cell cycle markers found in AD may aggravate tau phosphorylation since there are many similarities between AD and the cell cycle phosphorylation – the same kinases are involved and also the same type of phosphorylation (proline-directed Ser/Thr phosphorylation) (Lu KP *et al.*, 2003).

GSK-3 β

Glycogen synthase kinase 3 β (GSK-3 β) is one of the most studied and important kinases that are up-regulated in AD. GSK-3 β plays a role in numerous signalling pathways in cells, and regulates transcription factors and other proteins, also other kinases, involved in intracellular signalling, including pro-apoptotic pathways, and it also regulates microtubule stability through tau phosphorylation (Pei *et al.*, 1997; 1999; Grimes & Jope, 2001; Bhat & Budd, 2002; An *et al.*, 2005a; Takashima, 2006; Wang *et al.*, 2007). Phosphorylation of specific Ser or

tyrosine (Tyr) residues on GSK-3 β affects the protein activity, with Ser9 phosphorylation inhibiting and Tyr216 phosphorylation stimulating the activity (Grimes & Jope, 2001). Ser9 phosphorylation can be mediated by protein kinase B (PKB or AKT), protein kinase C (PKC), protein kinase A (PKA) and 70-kDa ribosomal protein S6 kinase (p70S6K) among others, while dephosphorylation is mediated by PP2A (Grimes & Jope, 2001). GSK-3 β plays a pivotal role in AD and has been linked to NFT pathology, mainly via tau and NF phosphorylation, but the kinase also interacts with PS, can be activated by A β , and has been shown to facilitate cell death (Grimes & Jope, 2001; Takashima, 2006). Abnormal expression of cell cycle markers has been found in AD brains and this may also affect GSK-3 β because GSK-3 β is normally involved in cyclin D₁ nuclear export and phosphorylation at the G₁-S boundary (Alt et al., 2000; Lu F et al., 2003). GSK-3 β phosphorylation of tau on Thr231 regulates tau's ability to bind to microtubules (Cho & Johnson, 2004), and overall GSK-3 β phosphorylation of tau has also been implied in the regulation of organelle transport (Tatebayashi et al., 2003).

With regard to other components of AD pathology, both A β and ApoE ϵ 4 can activate GSK-3 β , and GSK-3 β has been found to phosphorylate APP, increasing APP production (Bhat & Budd, 2002; Cedazo-Mínguez et al., 2003). Lithium is known to work as a selective inhibitor of GSK-3 β (Grimes & Jope, 2001) and transgenic mice overexpressing mutant human tau show significantly lower levels of tau phosphorylation and aggregation after lithium treatment (Noble et al., 2005).

p70S6K

The activated form of 70-kDa ribosomal protein S6 kinase (p70S6K) has been found in both pre-tangle neurons, that probably later develops NFTs, and NFT-bearing neurons (An et al., 2003). This kinase regulates and phosphorylates the 40S ribosomal protein S6 and activates translation (Ferrari & Thomas, 1994; Jefferies et al., 1997). Besides its translational capabilities, p70S6K also affects cell cycle control, cell differentiation and cell motility (Petritsch et al., 2000; Saucedo & Edgar, 2002; Gao et al., 2004). In a *Drosophila* model, tau-induced neurodegeneration is enhanced by target of rapamycin (TOR) and the following p70S6K activation in a cell-cycle-dependent manner (Khurana et al., 2006). Other forms of p70S6K-related regulation of the cell cycle may be initiated by PI3K, which has been shown to transmit its mitogenic signals through AKT and mammalian target of rapamycin (mTOR) to p70S6K (Gao et al., 2004). Studies also suggest that p70S6K affects GSK-3 β activation, as rapamycin treatment

decreased the half-life of cyclin D₁ proteins, regulated through GSK-3 β activation, and this may result in arrest of the cell cycle in the G₁ phase (*Dong et al., 2005*).

p70S6K consists of four domains: an N-terminal acidic domain, a catalytic domain, a regulatory domain and an autoinhibitory domain, and the kinase is regulated by phosphorylation at key sites (*Dufner & Thomas, 1999*). The activating phosphorylation occurs in a specific sequence: first Thr421 and Ser424 are phosphorylated, leading to a conformational change that facilitates the phosphorylation of Thr389 and then, finally, Thr229 is exposed, enabling phosphorylation and complete activation of the kinase (*Dennis et al., 1998; Pullen & Thomas, 1997*).

The number of neurons positive for activated p70S6K was increased in accordance with the progressive sequence of neurofibrillary changes according to Braak's criteria (*An et al., 2003*). The levels of total and activated S6 are also significantly increased in AD. Increased levels of tau and phosphorylated tau were consistently found in SH-SY5Y cells and rat brain primary cultures following p70S6K activation with zinc sulphate (*An et al., 2003*). This effect could be blocked by the inhibitor rapamycin which inhibits an upstream kinase, mammalian target of rapamycin (FRAP/mTOR) (*An et al., 2003; 2005b*). This suggests that when activated by zinc, p70S6K causes an increase in tau level through increased tau translation, as well as an increase in tau phosphorylation through the direct action of p70S6K. p70S6K has been shown to phosphorylate tau on Thr212, Ser214 and Ser262 (*Pei et al., 2006*). A relationship between PP2A and p70S6K has recently been demonstrated in a metabolically active brain slice system in which p70S6K was up-regulated while PP2A was selectively inhibited by okadaic acid (*Pei et al., 2003b*).

Protein phosphatases

Several protein phosphatases – PP1, PP2A, PP2B and PP5 – have been shown to dephosphorylate tau, but they vary in their specificity and efficiency (*Liu et al., 2005*). All these PPs could dephosphorylate the AD tau sites Ser199, Ser202, Thr205, Thr212, Ser 214, Ser235, Ser262, Ser396, Ser404 and Ser409 *in vitro*, while in human brain their total tau phosphatase activities varied from ~71% for PP2A to ~7% for PP2B, while PP1 and PP5 accounted for ~11% and ~10%, respectively (*Liu et al., 2005*). The different PPs favoured different sites, for example PP2A preferred to dephosphorylate tau on Thr205, Thr212, Ser262 and Ser409 (*Liu et al., 2005*).

Total phosphatase activity, and the activities of PP2A and PP5 were significantly decreased in AD brains, whereas PP2B activity was actually increased (*Liu et al., 2005*). PP2A activity was negatively correlated with the levels of tau phosphorylation at the various phosphorylation sites investigated and, together with the other results, this suggests that PP2A is the major tau phosphatase in the human brain (*Liu et al., 2005*). PP2A has also been shown to dephosphorylate MAP1B and MAP2 in the rat brain (*Gong et al., 2000*). Decreased PP2A activity may also exacerbate deleterious effects of inflammation in the brain by increasing cytokine synthesis (*Arnaud et al., 2006*).

Amyloid A β pathology and protein components in Alzheimer's disease

APP and A β

The amyloid precursor protein is a type 1 membrane protein (meaning that it spans the membrane once), and is ubiquitously expressed throughout the body (*Ling et al., 2003*). The APP695 transcript is most common in the brain, but other splice variants also occur such as APP751 and APP770, which contain an extra exon compared with APP695 (*Ling et al., 2003*). A β is derived from the region of the protein encoded by parts of exon 16 and 17 and contains between 40 and 43 amino acid residues (*Suh & Checler, 2002; Ling et al., 2003*). Not only neurons, but also other cells such as astrocytes, and microglia express APP in the brain (*Ling et al., 2003*).

APP cleavage

APP cleavage can take two pathways: the first is the non-amyloidogenic pathway where α -secretase cleavage is followed by γ -secretase cleavage, resulting in soluble sAPP α and the non-pathogenic p3 peptide, and the second is the pathological amyloidogenic pathway, which begins with BACE1 cleavage followed by γ -secretase cleavage, resulting in soluble sAPP β and A β (*Annaert & De Strooper, 2002; Suh & Checler, 2002; Ling et al., 2003; Dillen & Annaert, 2006; Vetrivel & Thinakaran, 2006*).

As mentioned above, the non-amyloidogenic pathway starts with α -secretase cleavage, resulting in sAPP α that has been shown to have neuroprotective properties, and several proteins with α -secretase-like activity have been found to date (*Ling et al., 2003; Dillen & Annaert, 2006; Vetrivel & Thinakaran, 2006*). Those most likely to be of relevance in AD are the two metalloproteinases ADAM10 and TACE (ADAM17), as both can cleave APP in cell systems, and

in the adult human brain ADAM10 shows the highest co-localisation with APP (Suh & Checler, 2002; Ling et al., 2003; Dillen & Annaert, 2006).

The path towards A β starts with β -secretase cleavage of APP, and studies have shown that BACE1 is the major β -secretase in the brain (Annaert & De Strooper, 2002; Suh & Checler, 2002; Ling et al., 2003; Dillen & Annaert, 2006; Vetrivel & Thinakaran, 2006). Overexpression of BACE1 in cell cultures led to increased A β levels, and BACE1^{-/-} knockout mice abolished A β production, without any adverse phenotypic effects (Ling et al., 2003). BACE1 can bind to nicastrin (Aph-2), a component of the γ -secretase complex, suggesting that BACE1 targets APP to the γ -secretase complex (Ling et al., 2003).

The γ -secretase intramembraneous complex is responsible for the final cleavage of APP resulting in the P3-peptide or A β , and the complex is made up of four different proteins: nicastrin, presenilin (PS1 or PS2; catalytic components required for γ -secretase activity), Aph-1 and Pen-2 (Annaert & De Strooper, 2002; Suh & Checler, 2002; Ling et al., 2003; Dillen & Annaert, 2006). A β can be cleared from the brain via different pathways, for example through degradation mediated by neprilysin or insulin-degrading enzyme (Suh & Checler, 2002; Ling et al., 2003).

A β pathology

Amyloid plaques are extracellular aggregates of A β . Levels of A β are increased in the brains of AD patients, and all currently known inherited familial forms of AD show an increase in A β levels (Citron et al., 1992; Cappai & White, 1999; Lee et al., 2005; Naslund et al., 2000; Religa et al., 2003). A β plaques in AD are first found in the temporal neocortex, and then spread to adjoining neocortical areas and the hippocampus, and are found in most neocortical areas at the end stage of the disease (Lee et al., 2005; Thal et al., 2000).

Whether A β is the initial cause of AD, according to the “amyloid cascade hypothesis” (see below) (Hardy & Selkoe, 2002; Selkoe & Podlisny, 2002), or whether it is just a consequence of other mechanisms (Lee et al., 2005) is still the subject of debate. For example, oxidative stress in neurons leads to an increase in A β , followed by a decrease in oxidative stress. A β may simply be part of the cellular defence against oxidative stress (Lee et al., 2005). No one denies that A β is an integral part of AD pathogenesis, but the role it plays is still uncertain – is it disease causing, or is it protective? The oligomeric forms of A β

are now considered to be the neurotoxic species, and the formation of plaque may actually be a way for the cell to survive by clearing away toxic A β out of the cytoplasm (*Suh & Checler, 2002; Ling et al., 2003; Lee et al., 2005*).

Hypotheses about the causes of Alzheimer's disease

Many believe that AD is explained by the amyloid cascade hypothesis, stating that an imbalance in the production and clearance of A β is the initiating event, leading to neurodegeneration and dementia (*Hardy & Selkoe, 2002; Annaert & De Strooper, 2002; Blennow et al., 2006*). However, there are some problems associated with this simplified view – one being that APP transgenic mice do not show NFT pathology. The only real case of true AD pathology was found in triple transgenic mice, where an FTDP-17 tau mutation was used (*Oddo et al., 2003*). The interplay between APP or A β and tau appears to be complicated, and studies in other mice models have shown that a reduction of endogenous tau results in decreased A β -dependent cognitive impairment, although it had no effect on A β plaque deposition or neuritic dystrophy, and no adverse effects on health or cognition could be seen in these mice (*Roberson et al., 2007*). So far, no single pathological lesion, not even amyloid plaques or A β , has proven to be the sole cause of AD. This has led some researchers to propose a “two-hit hypothesis” (*Zhu et al., 2007*), in much the same way as in cancer research. The two-hit-hypothesis basically states that something happens to affect the system negatively, but the system still functions more or less normally, then a second insult occurs and the system collapses or degenerates, and the consequences may be fatal (*Zhu et al., 2007*).

As mentioned above, the greatest risk factor for AD is ageing, and with age the cells in our body may suffer many perturbations in normal cell systems, e.g. the production and reduced scavenging of free radicals, in particular reactive oxygen species (ROS), leading to increased oxidative stress. Several studies suggest that oxidative stress is an early event in AD pathogenesis (*Arendt et al., 2000; Zhu et al., 2007*), and this may be caused by several factors, such as excessive deposits of metals (such as iron and copper) (*Huang et al., 2004*), activation of microglia surrounding senile plaques (*Suh & Checler, 2002; Arnaud et al., 2006*), the A β peptide, and abnormalities in mitochondrial metabolism and proteasomal function and protein degradation (*Zhu et al., 2007*). If oxidative stress is one of the two “hits”, then re-activation of the cell cycle in postmitotic neurons may be the other “hit” – normally no cell cycle markers should be present in postmitotic neurons, but in many AD cases the opposite has been found (*Nagy et al., 1997a*;

1997b; Vincent et al., 1997; Busser et al., 1998; Nagy, 2000; Ding et al., 2000; Pei et al., 2002; Zhu et al., 2007). According to the two-hit hypothesis, oxidative stress and cell cycle re-entry can independently initiate AD pathogenesis, but both are needed for propagation/progression of the disease (Zhu et al., 2007). According to Zhu et al. the “hits” may not necessarily be oxidative stress and cell cycle abnormalities; as long as the first “hit” requires compensatory adaptation of different pathways, it will make neurons vulnerable, while the second “hit” will trigger the degenerative process (Zhu et al., 2007).

Neuronal loss and cell death in Alzheimer’s disease

During AD pathogenesis, the number of neurons decreases due to neuronal death, leading to brain atrophy. The underlying reasons for the observed cell death in AD are still being debated, and one of the issues is whether or not apoptosis occurs in AD. Neuronal loss without the presence of necrosis, but with caspase activation, pronounced oxidative stress and increased A β found in AD brains, indicate apoptosis as a likely death path for the cell to take, and it has been hypothesized that the emergence of cell-cycle-specific markers found in AD brain, and cell cycle re-entry of terminally differentiated neurons, is one of the reasons behind cell death in AD (Nuydens et al., 1998; Raina et al., 2003; 2004; Appert-Collin et al., 2006). On the other hand, Bcl-2 (an anti-apoptotic factor) immunoreactivity has been shown to be elevated in human post-mortem AD tissue, and a DNA repair enzyme (Ref-1) has also been found to be elevated in AD neurons (Cotman, 1998; Roth, 2001), while it remains uncertain whether there is direct involvement of caspase-dependent neuronal apoptosis in AD pathogenesis (Roth, 2001). Apoptosis is a relatively quick form of cell death, and this would mean that at a given time only a subset of neurons would appear apoptotic (Roth, 2001). The classical apoptotic phenotypes that define terminal events, such as chromatin condensation, apoptotic bodies and membrane blebbing, are not seen in AD, perhaps due to the limited amount of cells exhibiting them at any given time. Initiator phases of apoptosis seem to be involved, but they do not lead to activation of the terminal commitment phase necessary for apoptotic cell death. This novel phenomenon has been termed “abortosis” by some researchers, and represents the inhibition of apoptosis at the post-initiator stage in neurons that survive in AD (Raina et al., 2003; 2004).

Protein aggregation and defective degradation

AD is a disease of protein aggregates and it is therefore logical to investigate what happens to protein quality control and degradation in AD. Ubiquitinated proteins accumulate in AD pathological hallmarks and an aberrant splice variant of ubiquitin (UBB), UBB⁺¹, is unable to bind to target proteins (Ciechanover &

Brundin, 2003; de Vrij et al., 2004; Song & Jung, 2004; van Leeuwen et al., 2006). UBB⁺ also accumulates in the neuritic plaques and tangles of AD and has been shown to block proteasome activity, which may result in neuronal death (*Ciechanover & Brundin, 2003; de Vrij et al., 2004; Song & Jung, 2004; van Leeuwen et al., 2006*). The involvement of the ubiquitin proteasome system (UPS) in AD is based on findings that ubiquitinated proteins accumulate in AD brains, proteasome subunits co-localise to disease-related areas, and proteasome activity is decreased (*Ciechanover & Brundin, 2003; de Vrij et al., 2004*). It has also been suggested that A β mediates proteasome inhibition (*de Vrij et al., 2004; Song & Jung, 2004*) but, at least in cell systems, A β may also be degraded by the proteasome (*Ciechanover & Brundin, 2003*). Normal ageing probably results in a cellular environment with decreased protein quality control capacity, laying the foundation for neurodegeneration caused by AD-related mechanisms (*Ciechanover & Brundin, 2003; de Vrij et al., 2004*). A link between oxidative stress and the proteasome has also been suggested, with a preceding decrease in proteasome activity, leading to an increase in oxidised proteins (*de Vrij et al., 2004*).

Proteins targeted for proteasomal degradation are normally polyubiquitinated, i.e. a chain of ubiquitin monomers (a minimum of four) is attached to the protein destined for degradation via a regulated process, where the enzyme E1 first activates free ubiquitin, followed by transfer to the E2 conjugating enzyme, before an E3 ligating enzyme transfers E2-conjugated ubiquitin to the substrate (*Glickman & Ciechanover, 2002; Ciechanover & Brundin, 2003; de Vrij et al., 2004*). The ubiquitinated substrate is now targeted for the proteasome, where it becomes degraded and the ubiquitin monomers are recycled (*Glickman & Ciechanover, 2002; Ciechanover & Brundin, 2003; de Vrij et al., 2004*). The activity of E1 is decreased in the AD brain (*Ciechanover & Brundin, 2003; de Vrij et al., 2004*) and a special E3 ligase has been found that recognises soluble hyperphosphorylated tau in AD and targets it for the proteasome (*de Vrij et al., 2004*). Most cytosolic and nuclear protein levels are regulated by the UPS, but secreted and then internalised proteins are handled by the lysosomal system (*de Vrij et al., 2004*). A protein quality control system is also present in the endoplasmic reticulum, degrading for example Pen-2, a component of the γ -secretase complex (*de Vrij et al., 2004*).

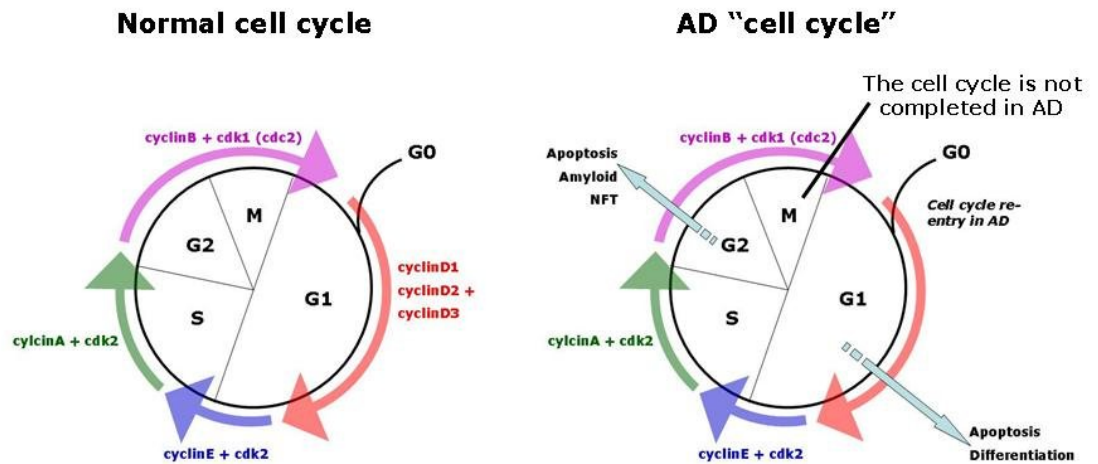
Cell cycle markers in Alzheimer's disease

Several regulated processes start when the sperm meets the egg, and many of these continue throughout life – one of these essential, and highly conserved, processes is that by which cells divide, i.e. the cell cycle. In fast-growing tissues

in mammals, the completion of a cell cycle usually takes between 12 and 24 hours. In a specific sequence, cell cycle regulators, mitogenic factors, cyclins, cyclin-dependent kinases, and inhibitors are expressed and degraded in a highly regulated manner – if regulation fails, cell death or cancer may result. The key component of the cell cycle is the complex between cyclin and Cdk that triggers downstream processes. A normal cell cycle (*see Figure 3*) is made up of four different phases: 1) the G₁ phase, the start of the cell cycle and the first “gap phase” in which the cells grow and carry out normal metabolism, and the organelles are duplicated, 2) the S phase, which derives its name from “synthesis”, in which the DNA is replicated in order to duplicate the chromosomes, 3) the G₂ phase, the second gap phase, in which the cell continues to grow and prepares for cell division, and finally, 4) the M phase, or mitosis plus cytokinesis, in which the cell divides into two daughter cells, completing the cell cycle. The cyclins were so named because their levels cycle up and down during a normal cell cycle, and they pair up with their specific Cdks during the different stages or phases of the cell cycle in an ordered manner. First the cyclin Ds (1, 2 or 3) pair up with Cdk4 or Cdk6 during the G₁ phase, and as the cell cycle progresses into the S phase, the cyclin D levels decrease and cyclin E levels increase. During the S phase the cyclin E levels decrease and are replaced by increasing cyclin A levels, but they both interact with Cdk2. During the M phase cyclin B levels predominate, and cyclin B forms a complex with Cdk1 (also known as Cdc2).

During a normal cell cycle in a dividing cell the process is checked several times – if any abnormality is detected, the cell will either regress to the resting (G₀) phase or will die by apoptosis. For the cycle to progress into the S phase, the retinoblastoma protein (pRb) must be phosphorylated and release its hold on E2F, a transcription factor that activates genes such as cyclin E and A. Another checkpoint protein is the transcription factor p53, which monitors DNA damage, and there is also a group of proteins that acts as specific Cdk inhibitors (CKIs) that are induced by events such as contact inhibition, mitogen withdrawal, DNA damage and differentiation/senescence.

Figure 3.



Adapted from: Nagy et al., 1998; Arendt et al., 2000; Nagy, 2000; Hamdane et al., 2003; McShea et al., 2007

Numerous studies have shown the up-regulation of cell cycle markers in AD (Nagy et al., 1997a; 1997b; Vincent et al., 1997; Busser et al., 1998; Nagy, 2000; Ding et al., 2000; Pei et al., 2002), and both G₁/S and G₂/M markers are found in neurons exhibiting neurofibrillary degeneration, suggesting that the cell cycle is aberrant and they do not follow classical apoptosis (Hamdane et al., 2003a; Zhu et al., 2007). Parallels have also been drawn between cancer cells and neurons in AD, where altered regulation of Cdk4, the inhibitors p16 and p21, and other cell cycle control elements effectively behave as oncoproteins in vulnerable neurons in AD (Raina et al., 2000). In AD both tau and A β are linked to the cell cycle (see Figure 3) – both affecting it and being affected by its progression (Copani et al., 2001; Frasca et al., 2004; Raina et al., 2004). Up-regulation of DNA replication also precedes neuronal cell death (Yang et al., 2001), but to date no evidence of a completed cell cycle has been found (Zhu et al., 2007). There is increasing evidence linking apoptosis in postmitotic neurons with a frustrated attempt to re-enter the cell cycle (Nuydens et al., 1998; Verdaguer et al., 2003), and neurons that have re-entered the cell cycle will either die or produce AD pathology (Nagy et al., 1998; Husseman et al., 2001; Zhu et al., 2007) as the neurons that show a cell cycle phenotype also exhibit features such as increased phosphorylation and kinase activity, found in degenerative neurons in AD (Vincent, 2000; McShea et al., 2007). Abnormal mitotic activation has also been found in many other neurodegenerative

disorders, suggesting a more general disease pathogenesis (*Nagy et al., 1997; Husseman et al., 2000*).

The transition from G₀ to G₁ is dependent on the activation of cyclin D1. Cyclin D1, together with its Cdk partners (Cdk4/Cdk6), phosphorylate and inactivate pRb, which promotes G₁ to S-phase progression. Cyclin D1 expression has also been found to be selectively induced in dying neurons, and cyclin D1 mRNA levels have been found to peak 15-20 hours after nerve growth factor withdrawal, concurrent with the time that neurons become committed to die (*Freeman et al., 1994*). In order to inactivate cyclin D1, the protein is removed from the nucleus into the cytoplasm during the S phase, and this is mediated by CRM1-dependent nuclear export through GSK-3 β -dependent phosphorylation of cyclin D1 at a conserved COOH-terminal residue, Thr286 (*Alt et al., 2000; Lu F et al., 2003*).

As mentioned above, age is the main risk factor for developing AD, and it may also affect cell cycle regulation. When the brain ages, it loses its ability to counteract or deal with age-related problems. For example, inflammation and/or oxidative stress may trigger mitogenic pathways, via PI3K and MAPK cascades, affecting not only cell-cycle-related events but also expression and post-translational modification of APP and tau (*Arendt et al., 2000*). However, APP and PS mutations may also cause the cells to enter a so-called “mitotic steady state”, in which cell cycle proteins are expressed but no true cell cycle is executed, which precedes amyloid deposits in transgenic mouse models, making them more vulnerable to continued AD pathogenesis (*Zhu et al., 2007*). Results from experiments in SH-SY5Y cells suggest that A β induction of the cell cycle is regulated via the MAPK cascade, and that A β treatment affects cell cycle progression and even apoptotic cell death (*Frasca et al., 2004*). A β -active fragments (β AP₂₅₋₃₅) can also activate the cell cycle in rat primary cortical neurons (*Copani et al., 2001*). As for tau, overexpression of either wild-type (wt) or mutated tau protein in a *Drosophila* AD model system (*Khurana et al., 2006*) has been linked to cell cycle re-entry; similar results have also been found in a mouse model (*Zhu et al., 2007*).

Small heat-shock proteins

α B-crystallin

α B-crystallin belongs to the family of small heat-shock proteins (sHSPs), which share the same molecular weight (about 22-23kDa) (*Iwaki et al., 1992; Klemenz*

et al. 1991) and have strong sequence similarity. They have the same nuclear localisation at high temperatures and cytoplasmic localisation under normal conditions (Klemenz *et al.*, 1991; Arrigo *et al.*, 2007). α B-crystallin can be phosphorylated on Ser residues in response to various types of stress, such as heat, arsenite, okadaic acid, H₂O₂ (oxidative stress) and high concentrations of NaCl or sorbitol (hypertonic stress) in human glioma cells and rat tissues (Ito *et al.*, 1997). The Ser phosphorylation sites have been mapped by mass spectrometry and found to be Ser19, Ser45 and Ser59, and each phosphorylated site can be recognised by specific antibodies (Ito *et al.*, 1997). Furthermore, kinases such as extracellular signal-regulated kinase 1/2 (Erk1/2) and MAPKAP kinase-2 have been found to phosphorylate α B-crystallin (Kato K *et al.*, 1998).

α B-crystallin has also been shown to interact with MTs by binding to MAPs (Fujita *et al.*, 2003; 2004), and is up-regulated in cells exposed to agents that promote MT disassembly (Kato *et al.*, 1996; Launay *et al.*, 2006). When MT depolymerisation is enhanced, α B-crystallin mRNA and protein levels are increased (Kato K *et al.*, 1996; Liang & MacRae, 1997), and α B-crystallin may also prevent microtubule aggregation (Xi *et al.*, 2006).

Experiments indicate that α B-crystallin is involved in the ubiquitin/proteasome pathway in a phosphorylation- and cell-cycle-dependent manner, since overexpression of α B-crystallin (with aspartate mutations mimicking phosphorylation on Ser19 and Ser45) together with FBX4, an F-box-containing protein that is a component of the ubiquitin-protein isopeptide ligase SCF (SKP1/CUL1/F-box), induced ubiquitination of one or more proteins in non-stressed cells (den Engelsman *et al.*, 2003). No FBX4 has been found in the human brain (den Engelsman *et al.*, 2003), but a similar factor contributing to protein aggregation may exist and may aggravate AD pathology. α B-crystallin, as part of the SCF^{Fbx4/ α B-crystallin} cyclin D₁ ubiquitin ligase, facilitates cyclin D₁ degradation via a GSK-3 β -dependent pathway (Alt *et al.*, 2000; den Engelsman *et al.*, 2003; Lu *et al.*, 2003; Lin *et al.*, 2006; Barbash *et al.*, 2007). In α B-crystallin knockout mice, tau expression is increased in lens tissues compared to wt mice, suggesting that α B-crystallin plays a role in tau degradation (Bai *et al.*, 2007).

Hsp27

Another small heat-shock protein is Hsp27, first discovered as an inhibitor of actin polymerisation (Miron *et al.*, 1991), but has later been found to be part of different intermediate filament inclusions (Head & Goldman, 2000) and to

associate with MTs in cells (*Hino et al., 2000*). Hsp27 has been found to be up-regulated in AD and can be localised to NFTs (*Renkawek et al., 1994a*); furthermore, it preferentially binds directly to hyperphosphorylated tau (*Shimura et al., 2004*).

Hsp27 shares high sequence homology with α B-crystallin (*Hickey et al., 1986*) and, overall, the sHSPs show similar chaperone activities (*Head & Goldman, 2000; Arrigo et al., 2007*). These two sHSPs are also regulated in a similar way by phosphorylation (*Richter-Landsberg & Goldbaum, 2003*), and can be phosphorylated by some of the same kinases, such as MAPKAP kinase-2 (*Rouse et al., 1994*). Ser, Thr and Tyr residues make up almost 20% of the Hsp27 amino acid sequence, and they are all possible phosphorylation sites (*Hickey et al., 1986*). After heat shock or mitogen activation, Hsp27 was phosphorylated on the key sites Ser78 and Ser82 (*Landry et al., 1992*). Ser15 phosphorylation also affects Hsp27 activity (*Lavoie et al., 1993*). Hsp27 can affect cell cycle progression through promotion of cell cycle re-entry into the S phase by facilitating ubiquitination and degradation of the cell cycle inhibitor p27^{Kip1} (*Parcellier et al., 2006*).

Small heat-shock proteins in Alzheimer's disease

The small heat-shock/ α -crystallin proteins Hsp27 (*Renkawek et al., 1994a*) and α B-crystallin (*Iwaki et al., 1992; Renkawek et al., 1994b; Mao et al., 2001*) have both been shown to be up-regulated in AD. When protein levels in the frontal and temporal cortices of AD and control brains were investigated, a significant positive correlation between tau, Hsp27, α B-crystallin and other heat-shock proteins was found, but no correlation was seen with regard to senile plaques according to Braak staging (*Sahara et al., 2007*). The authors speculated that the sHSPs function as regulators of soluble tau protein levels and, after a while, the chaperone system is saturated and granular tau isoforms (intermediates of tau filaments) can form unhindered. These results suggest that the granular tau isoforms were formed before the NFTs, as early as in Braak stage I. α B-crystallin, and Hsp27 when Braak 0 samples were excluded, showed an inverse correlation to the granular tau isoforms. No NFTs were detected in the frontal cortices of Braak 0 or Braak I brains, but increased levels of granular tau and Hsp27 were found in Braak stage I samples. Hsp27 preferentially binds to hyperphosphorylated tau, so it may be possible that hyperphosphorylated prefilamentous tau was already induced in the frontal cortices of Braak I brains (*Sahara et al., 2007*).

sHSPs interact with microfilaments and intermediate filaments, affecting their polymerisation and protecting them from external insults by a phosphorylation-dependent mechanism (*Liang & MacRae, 1997*). Even in unstressed cells, both Hsp27 and α B-crystallin have been shown to affect intermediate filament interactions and may even protect the filaments from pathological aggregation (*Perng et al., 1999*).

Metals in the brain

Many cellular functions in the body are dependent on and/or regulated by various metals – the metal ions can act as co-factors for proteins and enzymes, affecting their activity, structure and function (*Burdette & Lippard, 2003*), and they are essential for the membrane potential in neurons. Increasing evidence suggests that metals such as aluminium (Al), iron (Fe), zinc (Zn) and copper (Cu) can promote A β aggregation and neurotoxicity in the AD brain (*Bush et al., 1994; Cuajungco & Lees, 1997; Lovell et al., 1998; Suh & Checler, 2002; Bush, 2003; Huang et al., 2004; Shcherbatykh & Carpenter, 2007*), and APP can transport both Cu²⁺ and Zn²⁺ ions (*Annaert & De Strooper, 2002*).

Zinc

Like other ions in the body, some metal ions such as zinc (Zn²⁺) can be transported across membranes via ion channels or special zinc transporters, e.g. ZnT3, which is present only in the brain and testes (*Frederickson et al., 2000; Burdette & Lippard, 2003; Mocchegiani et al., 2005*). The ZnT3 transporter ensures that a special group of glutamatergic neurons (gluzinergetic neurons) has zinc-filled vesicles near their synapses for signalling (*Frederickson et al., 2000; Burdette & Lippard, 2003; Mocchegiani et al., 2005*). Normally, 10-15% of the zinc in the brain, one of the most abundant brain metals, is localised in presynaptic vesicles and may be released upon neuronal activity and depolarisation – this process is normally highly regulated, but may be abrogated by pathology (*Assaf & Chung, 1984; Frederickson et al., 2000; Frederickson & Bush, 2001; Koh, 2001; Mocchegiani et al., 2005*). Throughout the past decade, several studies have been made on the zinc level in AD, with varying results. However, more and more studies indicate that zinc ions are unusually increased in brain regions such as the hippocampus, amygdala and cortex, that are heavily affected by AD pathology (*Deibel et al., 1996; Danscher et al., 1997; Lovell et al., 1998; Mocchegiani et al., 2005; Religa et al., 2006*).

Zinc neurotoxicity can lead to increased oxidative stress affecting apoptosis. Both pro- and anti-apoptotic properties have been seen depending on the

concentration and exposure time (*An et al., 2005b*), and zinc has also been shown to bind to A β and enhance A β aggregation (*Bush et al., 1994; Koh, 2001*). Altered zinc metabolism in the brain can accelerate plaque deposition in AD and exacerbate neuron injury (*Frederickson & Bush, 2001; Bush, 2003*), and is also associated with other neurodegenerative disorders, such as epilepsy, amyotrophic lateral sclerosis and Parkinson's disease (*Cuajungco & Lees, 1997*). Special metal chelators that bind Zn²⁺ have been suggested as a potential means of treating disorders with abnormally increased zinc levels (*Suh & Checler, 2002*). However, zinc has also been suggested to have a protective role in AD, since A β plaques may actually be better than soluble, monomeric forms (*Cuajungco et al., 2002*). Zinc deficiency has been proposed to lie behind NFT, due to deficient DNA-metabolising zinc enzymes, giving rise to abnormal neuronal DNA and the synthesis of pathological proteins (*Constantinidis, 1990*).

Zinc has also been shown to activate the PI3K and MAPK pathways, in a similar way to insulin (*Kim et al., 2000*), and can also activate other kinases such as PKC and ERK1/2 (*Koh, 2001*) implicated in tau hyperphosphorylation in AD. Concomitant elevated levels of zinc and NF phosphorylation, in particular in the most severely affected areas of AD brains, (*Perry et al., 1985; Cuajungco & Lees, 1997; Wang et al., 2001*) suggest that there is a link between zinc and NF phosphorylation in AD. In animal experiments, long-term zinc administration to Sprague-Dawley rats led to impaired cognitive functions in both their reference and working memory (*Flinn et al., 2005*), and in Tg2576 mice (with the APP^{SWE} mutation) it has been reported that zinc may contribute to gender differences in A β plaque formation (*Lee et al., 2002*). Female Tg2576 mice normally show more A β pathology than male mice of the same age, but in ZnT3 transporter knockouts the zinc levels were lowered, leading to decreased A β plaque burden and disappearance of the difference between the sexes (*Lee et al., 2002*).

AIMS OF THE STUDY

To better understand the pathogenic mechanisms of Alzheimer's disease it is important to understand the regulation and causes of the abnormal phosphorylation of tau and NF proteins throughout the course of the disease. Interactions among the sHSPs, A β and tau/neurofilament pathology, as well as cell cycle progression have been reported. A β has affects on cell cycle progression and sHSP activity, and activates kinases known to phosphorylate tau and neurofilaments, and this, in itself, may abrogate AD pathology affecting at least the sHSPs, completing the pathway or cycle.

Specific aims

The aims of the experiments in **Paper I** were to investigate the degree and accumulation of different antibodies recognising phosphorylated tau and different A β variants in AD and control brains, and to correlate tau hyperphosphorylation with both A β variants and AD pathological staging according to the Braak and CERAD staging systems.

Many kinases that are part of the PI3K- and MAPK-pathways are up-regulated in AD, but their effects on downstream kinases and their substrates are unknown. The aims for the experiments described in **Paper II** were to investigate how kinases within the PI3K- and MAPK-pathways are involved in the regulation of tau kinases, such as p70S6K and GSK-3 β , and the possible consequences on tau regulation. Zinc, known to be increased in brain areas affected in AD, was used to activate the signalling pathways in rat brain slice cultures and in SH-SY5Y human neuroblastoma cells, and the subsequent kinase activities and tau levels and phosphorylation were investigated.

Not only tau, but also NFs are hyperphosphorylated and form parts of NFTs in AD, and the NFs may be phosphorylated by the same kinases as tau. The aims of the experiments described in **Paper III** were to investigate whether zinc could induce NF phosphorylation in N2a mouse neuroblastoma cells, and whether this phosphorylation could be mediated by p70S6K.

The two sHSPs, Hsp27 and α B-crystallin, are up-regulated in AD, but to what extent or the consequences of their up-regulation is still largely unknown. The aims for the experiments described in **Paper IV** were to confirm the up-regulation of Hsp27 and α B-crystallin in AD brains compared to control brains, and to study possible correlations between the sHSPs and tau and NFs in AD

brains. To further study the direct effect of increased Hsp27 and α B-crystallin levels on tau and NF regulation, the sHSPs were overexpressed in N2a cells and the tau and NF levels and phosphorylation were investigated.

MATERIALS & METHODS

Brain samples (Papers I & IV): Medial temporal cortex tissue blocks from 22 AD and 10 control cases were obtained from the Kuopio Brain Bank. The AD-related lesions were examined in all samples using routine neuropathological diagnostic evaluations carried out according to a standard procedure (*Alafuzoff et al., 1999*). Degenerative changes related to AD were assessed on Bielschowsky silver-stained sections, as described by Mirra et al. (*1991*) and the cases were classified accordingly. Additional immunohistochemical staining with monoclonal anti-human PHF-tau, the anti-phosphorylated-tau clone AT8 (Innogenetics BR-03), and the monoclonal anti-human beta-amyloid clone 6F/3D (DAKO M872) was carried out to verify the accuracy of the assessment of lesions. Brain homogenates were prepared from grey matter homogenised in a buffer including 50 mM Tris, pH 7.0, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM ethylene glycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 2 mM benzamidine, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 0.1% β -mercaptoethanol, 20 mM β -glycerophosphate, 0.1% protease inhibitor cocktail, 2 mM Na_3VO_4 , 50 mM NaF, and 2% sodium dodecyl sulphate (SDS) at 4°C. Protein concentrations were measured using the BCA protein assay kit from Pierce (Boule, Stockholm, Sweden).

Cell culture experiments (Papers II, III & IV): SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 [1:1], supplemented with 5-10% foetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. N2a mouse neuroblastoma cells were grown in DMEM/Opti-minimum essential medium [1:1]; 5-10% FBS, with or without 1% PEST and 0.2% Fungizone[®], in appropriate cell culture plates, dishes or flasks depending on the experiments. Cells were harvested in ice-cold phosphate-buffered saline (PBS), centrifuged (5 min at 2500 rpm) and resuspended in cell lysis buffer containing: 2 mM EGTA, 25 mM NaF, 1% Triton-X 100, 200 μM Na_3VO_4 , 0.5 mM PMSF, 5 mM EDTA, protease inhibitor cocktail (1:200), 150 mM NaCl and 50 mM Tris. The cell lysates were sonicated on ice using a probe sonicator, followed by protein concentration measurements using the BCA protein assay kit. To reduce the basal level of active p70S6K before some of the experiments, the serum levels in the cell culture media were reduced in a step-wise manner. First the cells were grown in 0.5% FBS medium overnight, then, two hours before the experiments the medium was replaced by serum-free medium in order to decrease the endogenous kinase (p70S6K)

activity. After two hours the SH-SY5Y cells in the experiments described in **Paper II** were pre-treated with vehicle (0.1% dimethylsulphoxide; DMSO) or the inhibitors rapamycin, wortmannin, LY294002 or U0126 alone, or in different combinations, before 30 min or 4 hours of zinc treatment. In the studies described in **Paper II** cell viability was measured with an MTT assay after prolonged serum starvation or zinc treatment, with different zinc concentrations for different times (*Rodrigo et al., 2000*). For the serum starvation experiments described in **Paper III**, the N2a cells were deprived of serum in a similar way to the SH-SY5Y cells described in **Paper II**, before the cell viability was analysed using the WST-1 assay according to the manufacturer's instructions (similar to the MTT assay) (Roche, Stockholm, Sweden). The experiments described in **Paper III** also involved treatment with different zinc concentrations for different times, and the cell viability was again analysed with the WST-1 method. To determine zinc-induced responses of p70S6K and neurofilaments, the cells were grown in medium containing step-wise less serum, followed by treatment with either 100 or 200 μ M zinc at the times: 0 (control), 30 min, 3 hours and 8 hours. In some cases, N2a cells were pre-treated with rapamycin (20 ng/ml) for 1 h and then with both rapamycin and zinc. Similar experiments were carried out with kinase inhibitor where the cells were treated with either rapamycin [20 ng/ml] or lithium [20 mM] for 4 hours, before cell lysates were prepared as described above. These experiments are described in **Paper IV**.

Rat brain slice preparation & treatment (Paper II): Rat brain slices were prepared following the procedures previously described (*Pei et al., 2003b*). Wistar rats (male, 150-200 g, Grade II) supplied by Scanbur BK (Sollentuna, Sweden) were decapitated whilst deeply anaesthetised. The brain was rapidly removed and placed in oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH₂PO₄, 1.3 mM MgCl₂, 2.0 mM CaCl₂, 11 mM D (+)-glucose, 25 mM NaHCO₃, at pH 7.4, for 7-8 min at 4°C. The right and left hemispheres were then separated and 350- μ m-thick coronal slices were sectioned with a vibroslicer (Campden Instruments Ltd, London, UK). After 30 min equilibrium, the brain slices were treated with 1 mM zinc sulphate at different points in time, then homogenised in buffer containing 50 mM Tris-HCl, pH 7.0, 1.0 mM PMSF, 1.0 mM EDTA, 2.0 mg/l aprotinin, 10 mM β -mercaptoethanol, 20 mM β -glycerophosphate, 2.0 mM Na₃VO₄ and 100 mM NaF. The homogenates were sonicated and centrifuged at 16,000 g for 10 min. The resulting supernatant was used for Western blotting (see below).

Plasmid purification and pcDNA₃ transfection (Paper IV): The three pcDNA₃ constructs (pcDNA₃-empty [control], pcDNA₃- α B-crystallin [FLAG-tagged] and pcDNA₃-Hsp27 [FLAG-tagged]) were amplified in competent TOP10 *E. coli*, according to the protocol supplied by the manufacturer (*One Shot[®] TOP10 Competent Cells, Invitrogen*), and the plasmids were purified using the QIAGEN Plasmid Midi Prep kit before the DNA concentrations were measured. The purified constructs were cut with the appropriate restriction enzymes and were run on 1-2% agarose gels to check the construct sizes before transfection. Wt N2a cells were transfected in suspension with the respective plasmids (30 μ g DNA) using LipofectamineTM/PlusTM reagent from Invitrogen. Twenty-four to forty-eight hours after transfection the cells were passaged and selective antibiotics were added to complete cell culture medium before the cells were harvested in cell lysis buffer, as described above.

Immunocytochemistry (Papers II & III): SH-SY5Y cells were grown on FALCON[®] culture slides (8-well) for the experiments described in **Paper II**, and were treated with zinc for 4 hours before they were rinsed with HEM buffer (pH 6.85) containing 80 mM HEPES, 10 mM EGTA and 2 mM MgCl₂, and then fixed in 4% paraformaldehyde/HEM buffer [1:1] for 30 min. The cells were permeated with Tris-buffered saline (TBS) containing 0.1% Triton X-100 for 5 min, and washed with HEM buffer twice. Unspecific binding sites were blocked with buffer containing 5% bovine serum albumin (BSA, Sigma) and 0.1% Triton X-100 in TBS for 30 min, before incubation with primary antibodies overnight at 4°C. This was followed by incubation with biotinylated anti-rabbit IgG at a dilution of 1:200 for 2 hours, and visualisation with the avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA, USA) with 3-3'-diaminobenzidine-4 (DAB) HCl/H₂O₂ as a substrate. In the experiments described in **Paper III**, N2a cells were grown to \approx 60% confluence on coverslips in 24-well plates, and treated with 100 μ M zinc for 0 (control) and 3 h in the presence (20 ng/ml) or absence of rapamycin (3 coverslips/condition). Coverslips with cells were washed in 37°C HEM buffer, then fixed in 4% paraformaldehyde/HEM buffer [1:1] and permeabilised in TBS+0.1% Triton X-100. The coverslips were incubated with SMI34 [1:1000] in a moisture chamber at 4°C overnight. The coverslips were then incubated with biotinylated secondary anti-mouse antibody using the VECTASTAIN ABC-kit, and were visualised using a DAB substrate kit for peroxidase (Immunkemi, Järfälla, Sweden). Numbers of total cells and SMI34-positive cells in 8-12 continuous fields of vision per slide were counted with a 40 x objective lens under a microscope.

Immunoelectron microscopy (Paper II): For immunoelectronic microscopy after zinc treatment, cells were fixed in a mixture of 3% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.4, and ultra-thin sections of cell pellets were prepared following previously developed protocols (*Qinyang et al., 2002*). Non-specific binding was blocked by 10% BSA for 2 hours, and the sections were incubated with primary antibodies overnight in a humidified chamber at 21°C. The bound antibodies were detected with protein A coated with 10 nm gold. Gold particles with or without primary antibodies were counted under a Tecnai 10 electron microscope (Fei Co., Eindhoven, the Netherlands). Background staining of gold particles without primary antibodies was 0.2/μm².

Western blot (Papers II, III & IV): The cell lysates were diluted in buffer and boiled for 5 min at 95-100°C before loading onto 10% or 12% sodium dodecyl sulphate polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes and were blocked for 1 hour at room temperature with 5% fat-free milk in TBS + 0.1% Tween-20 (TBS-T). Incubation overnight at 4°C with the primary antibody was followed by repeated TBS-T washing before incubation with horseradish peroxidase-linked secondary antibodies (GE Health [Amersham], Uppsala, Sweden) for approximately 1 hour at room temperature on a shaker. The membranes were washed again after secondary antibody incubation, after which the bands were detected using an enhanced chemiluminescence (ECL) kit from GE Health (Uppsala, Sweden). The membranes were developed on X-ray hyperfilm (GE Health, Uppsala, Sweden) using a CEAPRO processing machine (CEA Group, SVK Röntgenteknik, Jeppsson Aktiebolag, Stockholm, Sweden) in a darkroom. Densitometric measurements were made on scanned films using QuantityOne™ 4.3.0 software from BioRad (Stockholm, Sweden) or NIH Image 1.63 software (<http://rsb.info.nih.gov/nih-image/download.html>).

Dot blots (Papers I & IV): The samples were diluted in cell lysis or brain homogenate buffer, depending on the sample origin, and were spotted onto nitrocellulose membranes. The membranes were then dried for 30 min at 37°C to allow the proteins to bind to the membrane, before blocking with 5% fat-free milk in TBS-T for 1 hour at room temperature. After blocking, the primary and secondary antibodies were applied as for the Western blot, and the membranes were analysed in the same way. The amount of protein was optimised by spotting dots with 0.625, 1.25, 2.5, 5, and 10 μg protein contents on

nitrocellulose membranes. The intensity of the dots was analysed and the final concentrations were based on the linear part of the concentration gradient curve.

FACS analysis (Paper IV): The cells were harvested and fixed with ethanol before the cell cycle phases were analysed using propidium iodide (PI) staining with fluorescence-activated cell sorting (FACS) using a FACSCalibur machine. The results were analysed with the CellQuestPro (Becton & Dickinson) and ModFIT™ software programs to give the number and percentage of cells in each cell cycle phase.

Statistical analysis (Papers I, II, III & IV): Statistical comparisons between different experimental groups were made by ANOVA, followed by a least significant difference (LSD) post-hoc test or non-paired Student's t-test where applicable. Relationships between the levels of AD-related proteins in the brain homogenates were analysed by bivariate Spearman correlations. For all the statistical analyses a p-value of 0.05 or less was considered significant.

RESULTS & DISCUSSION

Paper I – Assessments of the accumulation severities of amyloid β -protein and hyperphosphorylated tau in the medial temporal cortex of control and Alzheimer's brains

A β , in the form of amyloid (neuritic) plaques, and hyperphosphorylated tau, in NFT, are the key pathological features of AD. The degree of accumulation of the three A β variants (A β_{40} , A β_{42} and A β_{43}) and tau phosphorylation epitopes has not previously been investigated in any detail, and the possible correlations between them and/or with Braak or CERAD staging were not known. Brain samples from the medial temporal cortex of 22 AD and 10 control cases were analysed regarding AD pathology according to Braak (neurofibrillary pathology) and CERAD (neuritic plaques). The levels of A β and hyperphosphorylated tau were investigated in brain homogenates from the samples using dot blots or indirect enzyme-linked immunosorbent assay (ELISA).

The levels of all the phosphorylated tau epitopes were increased in the AD brains compared to the controls, except pSer422 (a stress-activated site), but the ratios of the epitopes (AD:control) differed from about 1.5 to above 10, with pThr217 showing the highest increase of 13.27 times. Among the A β variants, A β_{43} and A β_{40} showed an AD:control ratio of about 1.7, while the increase in A β_{42} was only a factor of 1.18.

Many researchers believe that APP and A β trigger tau pathology as a downstream event (the amyloid cascade hypothesis), but the relationship between tau phosphorylation and the different A β variants is still unclear. To investigate this in the brain samples, the phosphorylated tau levels were correlated with the A β levels. The highest correlations of phosphorylated tau epitopes were found to be with A β_{43} , while A β_{40} and A β_{42} were correlated to a lesser extent. The order of the specific epitopes was unique for all variants, for example the AT180 epitope showed a strong correlation with all A β variants and also had an AD:control ratio of 9.00. None of the A β variants showed a significant correlation with pSer422, and neither A β_{40} nor A β_{42} were correlated with the PHF-1 levels; and A β_{40} did not correlate with Alz-50. Both the A β variants and phosphorylated tau epitopes showed positive correlations to both Braak and CERAD staging, but in both cases the correlations were strongest for the phosphorylated tau epitopes.

The results of this study suggest that the different tau epitopes vary in their regulation and the progression of phosphorylation during the course of AD pathology. A β may play a role in the regulation of this progression.

Paper II – Mechanisms of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3 β in SH-SY5Y neuroblastoma cells

As mentioned above, one of the two pathological hallmarks of AD is NFTs, which are mainly made up of hyperphosphorylated tau protein. Several kinases are known to phosphorylate tau, and GSK-3 β is one of the most studied in AD. Some of these kinases not only phosphorylate tau but may also affect protein translation via p70S6K. Increased zinc concentrations have been found in AD brains (*Deibel et al., 1996; Danscher et al., 1997; Lovell et al., 1998; Mocchegiani et al., 2005; Religa et al., 2006*) and it is known that zinc can affect kinase activity. In this study zinc's effects on GSK-3 β and p70S6K and their signalling pathways were investigated. SH-SY5Y neuroblastoma cells were treated with zinc and various kinase inhibitors, and the levels and degree of phosphorylation of several kinases and their substrates (tau and ribosomal S6 protein) were investigated using Western blots, while immunocytochemistry was used for localisation studies.

From experiments using different amounts of zinc it was determined that 100 μ M zinc was the lowest concentration that could induce maximal p70S6K phosphorylation in the SH-SY5Y cells, so this zinc concentration was used for the subsequent experiments. To lower endogenous p70S6K levels and activity, serum was removed from the medium before zinc treatment. In order to minimise stress response induced by starvation, this was performed in two steps, where the serum content of the medium was first lowered and then removed. Zinc treatment caused a time-dependent increase in p70S6K and GSK-3 β phosphorylation and activation, followed by an increase in total tau levels (when p70S6K was active) and tau phosphorylation (when either p70S6K or GSK-3 β was active). These increases could be blocked by using the inhibitors rapamycin (inhibits mTOR, a kinase upstream of p70S6K, regulating p70S6K activity) or lithium (a GSK-3 β inhibitor). Other tau kinases such as PKB, ERK1/2, JNK and p38 were also time-dependently stimulated after zinc treatment, more dramatically in the second stage (4-8 hours), than in the first stage (0-2 hours). These findings were confirmed in metabolically active rat brain slices, suggesting that this cell system can be used to study the

mechanisms of aberrant activation of signalling pathways in AD involving, for example, p70S6K and GSK-3 β .

Paper III – Zinc induces neurofilament phosphorylation independent of p70 S6 kinase in N2a cells

NFTs also contain hyperphosphorylated NF proteins and many tau kinases can also phosphorylate NF, but the detailed mechanisms are still unknown. Increased concentrations of zinc have been found in AD brains, and zinc can activate p70S6K, a kinase found in NFT-bearing neurons that can affect tau levels and phosphorylation. The N2a cells were treated with zinc or rapamycin (which inhibits p70S6K activity) and the protein levels, degree of phosphorylation and localisation were analysed using Western blots and immunocytochemistry.

In comparison to the previous study on SH-SY5Y cells (**Paper II**), the N2a cells were more sensitive to the higher (200 μ M) zinc concentration, while the cell viability was less affected at the lower (100 μ M) concentration. This discrepancy is probably due to the different origins of the cells. In the current study, two different zinc concentrations (100 and 200 μ M) were used providing the opportunity to study the N2a cells during both favourable and relatively unfavourable conditions. Zinc treatment of the cells resulted in an increase in both p70S6K activity and NF phosphorylation. The p70S6K activity could be blocked by rapamycin treatment, while this had no effect on NF phosphorylation. The results indicate that zinc induces NF phosphorylation through a kinase different from p70S6K. In order to lower the endogenous levels and activity of p70S6K, the cells were grown in a medium with progressively decreasing amounts of serum, and for the final hours the cells were grown in serum-free medium – this serum starvation (stress) may affect other signalling pathways in the cells. The results after serum starvation suggest that not only tau (*Planel et al., 2001*), but also NF is sensitive to starvation, and this serum-starvation-induced phosphorylation of NF could be enhanced by zinc. Zinc treatment could also induce cell proliferation and increased cell size, but only the increased cell size and not cell proliferation appeared to be related to p70S6K activation, contradictory to other studies showing that p70S6K can activate cell cycle progression (*Gao et al., 2004; Dong et al., 2005; Khurana et al., 2006*).

Paper IV – The small heat shock proteins Hsp27 or α B-crystallin and the protein components of neurofibrillary tangles: tau and neurofilaments

Small heat-shock proteins, such as Hsp27 and α B-crystallin, are up-regulated in AD and interact with the cytoskeleton in stressed cells (*Iwaki et al., 1992; Renkawek et al., 1994a; 1994b; Mao et al., 2001*). Hsp27 has been shown to localise to NFTs and can bind directly to hyperphosphorylated tau (*Shimura et al., 2004*), while α B-crystallin binds to microtubuli-associated proteins and is up-regulated when MT is disrupted (*Fujita et al., 2004*). Both sHSPs can also affect cell cycle progression, which is interesting since numerous cell cycle makers have also been found in AD, suggesting an attempt to re-enter the cell cycle. Brain samples and N2a mouse neuroblastoma cells overexpressing Hsp27 or α B-crystallin were analysed using Western or dot blots, and changes in protein levels were investigated. Kinase inhibitors were employed to investigate tau and NF phosphorylation and regulation, while FACS analysis was used to detect cell cycle progression in the N2a cells.

The present study confirmed an increase in both Hsp27 and α B-crystallin in homogenates from the medial temporal cortex of AD brains. This increase showed good correlation with the expression levels of the majority of the phosphorylated tau epitopes used in this study. Phosphorylated NFs are recognised by different SMI antibodies, and Hsp27 only correlated with SMI34, whereas α B-crystallin showed significant correlations with both SMI 310 and SMI312. No correlations were found between the sHSPs and MAP1B or MAP2 in the brain homogenates.

To investigate the effects of the overexpression of sHSPs on tau and NF regulation, N2a cells were transfected with Hsp27 or α B-crystallin. The results obtained from the transfected cells differed from those obtained from the brain homogenates. When Hsp27 was overexpressed only tau phosphorylation at Ser262 showed a significant increase in the transfected cells, while α B-crystallin overexpression did not show any significant correlation with increased tau or NF levels. On the contrary, we found significantly reduced levels of total tau, pSer396 and SMI34. The results suggest that α B-crystallin overexpression increased tau degradation, possibly via its ubiquitin ligase functions (*den Engelsman et al., 2003; Lin et al., 2006; Barbash et al., 2007*), and protected tau and NF from phosphorylation by kinases or facilitated dephosphorylation. Rapamycin treatment (inactivating p70S6K) of the Hsp27 overexpressing cells led to a noticeable decrease in both total tau and tau phosphorylated at Ser262.

Although some of the tau and NF antibodies (pThr212, pSer262, pSer396, and SMI310) showed a significant decrease after rapamycin treatment in the α B-crystallin-overexpressing cells, no decrease in total tau level was observed. When the cells were treated with the GSK-3 β inhibitor lithium, no effect was seen for most of the Hsp27 or α B-crystallin transfected cells; the only exception being a decrease in tau phosphorylation at Ser396 in the α B-crystallin-overexpressing cells. However, control cells transfected with an empty vector showed increased GSK-3 β ^{Ser9} phosphorylation and hence lower activity. This suggests that the sHSP overexpression in the transfected N2a cells protects GSK-3 β from lithium inhibition, possibly through cell cycle regulation.

The sHSPs also had an effect on N2a cell cycle regulation, causing a shift in the total cell population from the S phase to the earlier G₁ phase. This suggests that the changes discussed above regarding tau and NF are linked to the regulation of N2a cell cycle progression from the G₁ to the S phase. The abnormal expression of cell cycle markers and the suggested cell cycle re-entry of differentiated cells in AD brains may be one of the major reasons behind tau and NF hyperphosphorylation and the neuronal degeneration seen in AD (*McShea et al., 1997; 1999; Nuydens et al., 1998; Hamdane et al., 2003*).

Model systems for Alzheimer's disease – why use cell lines? Pros and cons.

Cell lines are incapable of exhibiting complete AD pathology because they lack the temporal and spatial complexity that is found in whole organisms. However, they have advantages over other model systems allowing the study of specific AD-related processes such as phosphorylation. They are also very easy to handle, unlimited numbers are available, they represent a homogeneous population, and they can be manipulated in innumerable other ways.

SH-SY5Y cells are a commonly used human neuroblastoma cell line, and when comparing the results of different studies it is of great advantage to have a large amount of data to refer to. The cells are also human, which helps when extrapolating the findings to human brains and AD. N2a cells are also frequently used among AD researchers. They are easy to transfect and have low levels of endogenous tau (*Montejo de Garcini et al., 1992*), which is convenient when one wants to study NF and avoid, for example, cross-reactivity with tau. The disadvantage is that they are mouse neuroblastoma cells, and this may lead to differences in the results between different species.

CONCLUSIONS & FUTURE PERSPECTIVES

In the study described in **Paper I**, sequential accumulation of A β variants and phosphorylated tau epitopes were demonstrated in AD brains. The levels of A β showed good correlation with phosphorylated tau; the strength of the correlation depending on the specific tau phosphorylation epitopes. Both A β and tau correlated well with different stages of the Braak or CERAD staging systems, which suggests that tau antibodies can be used selectively in AD diagnosis as a complement to morphological evaluations. The results of this study also suggest that the difference seen in tau phosphorylation at the different sites indicates that the specific sites may have different roles in NFT formation, and here A β may affect the occurrence of the different phosphoepitopes. There are numerous tau antibodies and it is hardly convenient to test them all. The results presented in **Paper I** can serve as guidelines on suitable tau antibodies depending on the aim of, or the hypothesis to be tested in, different investigations.

Zinc is involved in numerous regulating and signalling pathways in cells and its levels are abnormally increased in AD brains. Studies were therefore carried out investigating the effects of zinc on tau and NF levels and phosphorylation in SH-SY5Y cells and rat brain slice cultures in the experiments described in **Paper II**, and in N2a cells in the experiments described in **Paper III**. The different cell systems differed not only in their species of origin (human for SH-SY5Y cells and mouse for N2a cells), but also in their responses to zinc treatment. In the experiments described in **Paper II** zinc treatment led to increased kinase activities, among them p70S6K and GSK-3 β , and a subsequent increase in tau phosphorylation. Tau translation was also increased through the activation of p70S6K, in accordance with increased tau levels in AD brains. These results indicate that the same protein (p70S6K) can regulate tau on both translational and post-translational (phosphorylation) levels, while the main effect of other kinases, such as GSK-3 β , is on tau phosphorylation. Many tau kinases are also capable of NF phosphorylation and when N2a cells were treated with zinc, in the experiments described in **Paper III**, an increase in p70S6K activity was observed (similar to that seen in the SH-SY5Y cell experiments described in **Paper II**), together with a concomitant increase in NF phosphorylation. However, when p70S6K activity was blocked with rapamycin, the NF phosphorylation remained unchanged, despite the fact that the p70S6K activity was significantly decreased. Thus, zinc must induce NF phosphorylation in the N2a cells through other kinases. Zinc can induce ERK1/2 activation, which can protect cells from apoptosis (*An et al., 2005b*). It is possible that after high-dose

zinc treatment for certain periods the surviving N2a cells undergo hyperphosphorylation of both tau and NF due to increased ERK1/2 activity (Harris *et al.*, 2004; An *et al.*, 2005a; 2005b). It should be noted that the cell lines differed in their sensitivity to different zinc concentrations; 100 μ M zinc treatment yielded similar activation of p70S6K, but the higher concentration (200 μ M) decreased SH-SY5Y cell viability to ~80%, and was even harsher on N2a cells, where only ~30% survived. These species differences between the cell lines must be considered when interpreting the data.

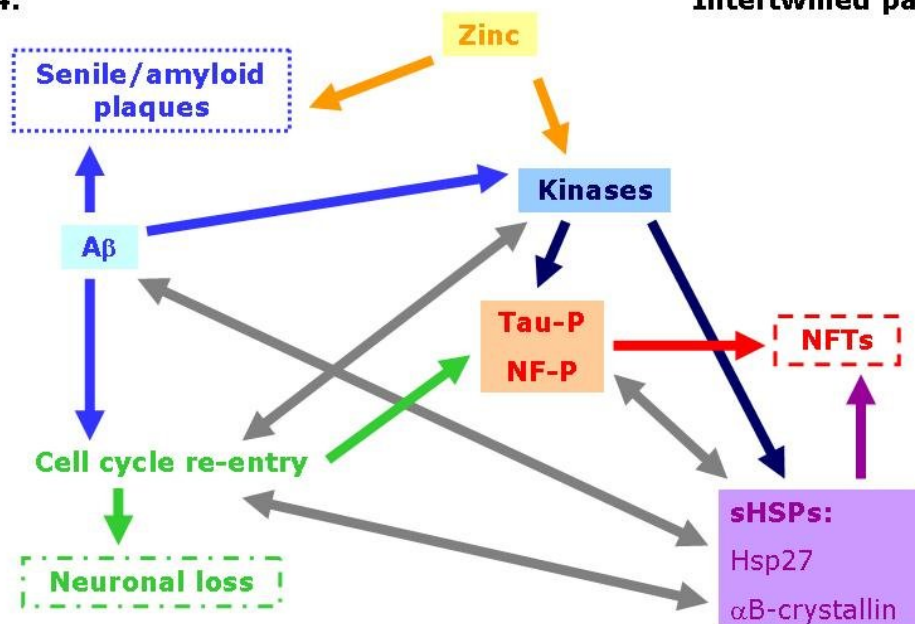
One of the greatest problems in AD research is that it is very difficult to exactly pinpoint what causes the disease, especially since most of the cellular mechanisms that are affected overlap and can affect each other in several ways, for example, through translational regulation, post-translational modification, degradation and their overall power to influence cell fate in the form of apoptosis etc. After the zinc experiments described in **Paper II** and in **Paper III** other factors that may influence tau and NF regulation were investigated. In these experiments, described in **Paper IV**, it was confirmed that both Hsp27 and α B-crystallin are up-regulated in AD brains compared to age-matched controls, and it was also demonstrated that this up-regulation correlates with increased tau and NF phosphorylation. The Hsp27 and α B-crystallin levels also correlate well with each other in the AD brains, and this may be due to similarities in activation by both external and internal stimuli (Head & Goldman, 2000). To investigate possible consequences of sHSP overexpression on tau and NF regulation, Hsp27 and α B-crystallin were overexpressed in N2a cells. The results showed that Hsp27 overexpression led to increased pSer262-tau levels, probably regulated by p70S6K, while α B-crystallin overexpression resulted in decreased phosphorylation of tau and NF. Tau phosphorylated on Ser262 detaches from the microtubules (Biernat *et al.*, 1993; Xie *et al.*, 1998; Zhou L-X, 2006; Pei *et al.*, 2006) and can be more easily hyperphosphorylated, making it more prone to aggregation, suggesting that Hsp27 overexpression actually aggravates tau pathology. However, α B-crystallin overexpression increased tau degradation, possibly via its ubiquitin ligase functions (den Engelsman *et al.*, 2003; Lin *et al.*, 2006; Barbash *et al.*, 2007), and protected tau and NF from phosphorylation by kinases or facilitated dephosphorylation. Although these two sHSPs have many similarities, they may actually play opposing roles in AD pathology.

From the results presented in **Paper IV** it also became apparent that signalling pathways in the same cell line, N2a cells in this case, may be differently regulated depending on “external stimuli”. Previous results, described in **Paper**

III, showed that zinc induced NF phosphorylation independently of p70S6K, but in the cells overexpressing Hsp27 the opposite seemed to be true, since rapamycin treatment decreased NF levels, as recognised by the SMI34 and SMI310 antibodies. The difference in results seems to be caused by the difference in conditions: Hsp27 overexpression in the experiments described in **Paper IV** compared to zinc treatment experiments described in **Paper III**. These differences reflect the complexity behind the regulation of, for example, protein phosphorylation evident in the cells through common signalling pathways, and even more so in the human brain, where the surrounding environment also has an effect. The reason behind the complexity of AD is that many pathways are intertwined and affect each other, both directly and indirectly. For example, sHSPs may be activated by kinases, tau hyperphosphorylation or the cell cycle. And they may in turn have reciprocal effects on tau, or the cell cycle, and so the circle continues. This makes it difficult to isolate one specific part of one pathway, without affecting any of the others detrimentally.

AD pathology involves both protein levels and their regulation via phosphorylation by increasing protein levels, kinase activities and substrate phosphorylation. Together, these result in protein accumulation that is detrimental to cellular function and viability. Since the different pathways are so intertwined (*see Figure 4*), effects in one of them will probably trigger effects in the others, making it very difficult to differentiate between the primary effects, the secondary effects and the consequences. It is tempting to try to halt this through the inhibition of different kinases and signalling pathways, but this is easier said than done since most signalling pathways play several roles in the cells, and very specific, targeted inhibition is essential.

Much more knowledge is required concerning these interactions and corresponding effects, and the overall aim of the studies presented in this thesis was thus to investigate probable causes and interactions at the protein level leading to AD neurofibrillary pathology. The results only explain a small part of the puzzle and the search must continue for increased knowledge on the signalling pathways and their mechanisms and interactions.

Figure 4.**Intertwined pathways**

The reason behind the complexity of AD is that many pathways are intertwined and affect each other, both directly and indirectly. For example, sHSPs may be activated by kinases, tau hyperphosphorylation, A β or the cell cycle. And they may in turn have reciprocal effects on tau, A β or the cell cycle, and so the circle continues (see *grey arrows for reciprocal pathways*). (The above figure is just an overview and do not describe the specific pathways in detail.)

It would be interesting to continue with the work focusing on the sHSPs. For example, investigating whether α B-crystallin overexpression actually may be used to help cells protect themselves from tau and NF hyperphosphorylation and aggregation, or if inhibition of Hsp27 can help ameliorate effects on tau hyperphosphorylation. Of importance would be to complement the experiments using cell systems with more animal or *in vivo* models. Several wt or mutated APP and/or PS transgenic mouse lines have been established, which develop varying degrees of AD amyloid pathology and express different degrees of A β ₁₋₄₂ compared to A β ₁₋₄₀, but they fail to develop any NFTs, and only some tau phosphorylation can be seen (*Suh & Checler, 2002*). The only transgenic mice that develop true AD pathology, meaning both amyloid plaques and NFTs, are the triple-transgenic APP^{SWE}/PS1^{M146V}/Tau^{P310L} mice (*Oddo et al., 2003*) – the only problem here being that the tau construct used is a mutation that has been found in FTDP-17, making this strictly speaking not an AD model. But the triple-transgenic mice may still be used for studying AD-related events. Experiments can be performed that either overexpress α B-crystallin or silence Hsp27 expression by injections of relevant viral constructs into the hippocampus or cortex in these triple-transgenic mice, and the effects on NFT-pathology could be investigated.

Continuing with the cell models is also possible, and then it would be of interest to see whether tau overexpression in SH-SY5Y or N2a cells could induce sHSPs expression or increase their activity. Here the pertinent signalling pathways should be checked as well, since both sHSPs also can be regulated through phosphorylation mediated by kinases involved in AD (*Hickey et al., 1986; Landry et al., 1992; Lavoie et al., 1993; Rouse et al., 1994; Ito et al., 1997; Kato K et al., 1998; Richter-Landsberg & Goldbaum, 2003*). Better knowledge concerning their phosphorylation state in AD could help give additional information about the signalling pathways involved in AD. Studies focusing on their protective effects on the cytoskeleton could also be carried out using the cell lines.

In AD there is not only problems with protein aggregation, but also with protein degradation and at least α B-crystallin has been shown to be part of an ubiquitin ligase complex that facilitates cyclin D1 degradation via GSK-3 β (*Alt et al., 2000; den Engelsman et al., 2003; Lu et al., 2003; Lin et al., 2006; Barbash et al., 2007*), and also Hsp27 is involved in degradation of cell cycle components (*Parcellier et al., 2006*). Experiments investigating in particular α B-crystallin's degrading properties, with regard to proteins such as tau and A β involved in AD pathology, could give additional information about the failure to keep up with the necessary protein degradation resulting in AD-pathology.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Introduktion

Den delen av världens befolkning som består av äldre människor ökar stadigt och det för med sig att allt fler drabbas av åldersrelaterade sjukdomar som demens. Av alla som drabbas av någon form av demens har mer än hälften Alzheimers sjukdom (*Fratiglioni et al., 1999*). Alzheimers sjukdom tar sig uttryck som en successiv försämring av kognitiva- och minnesfunktioner i hjärnan (*Gustafson, 1975; Gustafson et al., 1977; Terry & Davies 1980; Cummings & Benson 1992*). Kliniska tester som utförs kan inte säkert fastställa diagnosen Alzheimers sjukdom, utan det går bara att fastställa ”möjlig Alzheimers sjukdom”. Det är först efter dödens inträffande, vid en patologisk undersökning av hjärnan, som diagnosen Alzheimers sjukdom kan ställas genom att påvisa vissa karakteristika för sjukdomen. Vid Alzheimers sjukdom består patologier av olika ”proteinklumpar”, så kallade amyloida plack och neurofibrillära nystan, och celldöd i hjärnan (*Duyckaerts & Hauw, 1997; Markesbery, 1997*). Idag finns det ingen behandling som botar Alzheimers sjukdom, det enda läkare kan göra är att försöka minska några av de kliniska symptomen med hjälp av olika bromsmediciner (*Suh & Checler, 2002*).

Mycket av forskningen kring Alzheimers sjukdom har fokuserat på vad som gör att proteinerna klumpar ihop sig och skadar hjärnans funktioner. De amyloida placken återfinns mellan hjärncellerna och består av proteinklumpar av beta-amyloid. Beta-amyloid bildas när amyloid prekursor proteinet (APP) klyvs av olika enzymer, och när det sker kan beta-amyloid orsaka skador i hjärnan som medverkar till sjukdomsprocessen vid Alzheimers sjukdom (*Annaert & De Strooper, 2002; Suh & Checler, 2002; Ling et al., 2003; Dillen & Annaert, 2006; Vetrivel & Thinakaran, 2006*). De andra proteinklumparna återfinns inne i nervcellerna och består av olika proteiner som är kopplade till cytoskelettet i cellerna. Varje cell i kroppen har ett eget skelett, ett så kallat cytoskelett, precis på samma sätt som vi människor har ett skelett som håller oss uppe och som kan användas som stöd för kroppens funktioner. Cytoskelettet ger stadga åt cellerna och kan bland annat fungera som transportvägar för olika molekyler i cellerna. Vid Alzheimers sjukdom har många nervceller problem med sitt cytoskelett för att proteiner, som till exempel tau- och neurofilament-proteiner, inte längre kan fungera normalt. I vanliga fall så binder tau-proteiner till delar av cytoskelettet och hjälper till att stabilisera det, medan neurofilament-proteinerna själva hjälper till att bygga upp och koppla ihop olika delar av cytoskelettet. När tau- och neurofilament-proteiner inte kan interagera normalt med cytoskelettet, på grund av onormalt många fosfatgrupper som fästs på proteinerna, bryts delar av

cytoskelettet upp och tau- och neurofilament-proteinerna klumpar ihop sig och bildar de skadliga neurofibrillära nystanen (*Perry et al., 1985; Sternberger et al., 1985; Grundke-Iqbal, 1986a; 1986b; Lee et al., 1988; Ulloa et al., 1994; Hashimoto et al., 1999; Hu et al., 2002; Goedert et al., 2006*).

I cellerna finns det olika signalvägar som reglerar antalet fosfatgrupper på olika proteiner, vissa enzymer som kallas för kinaser sätter dit fosfatgrupper, medan andra enzymer som kallas fosfataser tar bort fosfatgrupperna. Vid Alzheimers sjukdom har man upptäckt en obalans mellan dessa kinaser och fosfataser, med en övervikt för kinaserna vad det gäller bland annat mängd och aktivitet – detta leder till att tau- och neurofilament-proteiner får för många fosfatgrupper på sig vilket bidrar till sjukdomsprocessen vid Alzheimers sjukdom (*Grimes & Jope, 2001; Pei et al., 1997; 1998; 1999; 2002; 2003a; Nordlinder et al., 2002; Shahani & Brandt, 2002; Yamamoto et al., 2002; Hamdane et al., 2003b; Wang et al., 2007*). Det finns väldigt många olika kinaser som verkar via olika signalvägar, men två av dem är extra viktiga för sjukdomsprocessen vid Alzheimers sjukdom: glykogensyntas kinas 3β (GSK- 3β) (*Pei et al., 1997; 1999; Grimes & Jope, 2001; Bhat & Budd, 2002; An et al., 2005a; Takashima, 2006; Wang et al., 2007*) och 70-kDa ribosomalt protein S6 kinas (p70S6K) (*An et al., 2003; Khurana et al., 2006; Pei et al., 2006*).

Vissa forskare menar att det är beta-amyloid som är boven som startar hela sjukdomsprocessen vid Alzheimers sjukdom enligt den så kallade ”amyloid kaskad hypotesen” (*Hardy & Selkoe, 2002; Selkoe & Podlisny, 2002*), medan andra forskare anser att det är andra mekanismer som ligger bakom sjukdomsförloppet (*Lee et al., 2005*). Bland annat har paralleller gjorts med sjukdomsförloppet vid cancer när oftast flera olika saker måste gå fel för att sjukdom ska uppstå, det räcker alltså inte med bara en sak (*Zhu et al., 2007*). Åldrandet i sig självt ökar risken att drabbas av Alzheimers sjukdom, och det kan bero på att de normala försvarsmekanismerna i cellerna i kroppen sakta försämras med åldern. Till exempel så ökar produktionen av fria radikaler när vi åldras, som kan förstöra olika molekyler i kroppen, vilket leder till att cellerna utsätts för så kallade oxidativstress, samtidigt som kroppens skyddssystem försämras (*Arendt et al., 2000; Zhu et al., 2007*). Andra forskare har upptäckt att cellcykelmarkörer, som normalt inte ska vara aktiverade i nervceller som inte behöver dela sig längre, återfinns i nervcellerna vid Alzheimers sjukdom (*Nagy et al., 1997a; 1997b; Vincent et al., 1997; Busser et al., 1998; Nagy, 2000; Ding et al., 2000; Pei et al., 2002; Zhu et al., 2007*). Dessa cellcykelmarkörer kan påverka regleringen av kinaser som är inblandade i Alzheimers sjukdom, och

även tau- och neurofilament-proteinerna kan påverkas av detta återinträde in i cellcykeln (Copani et al., 2001; Frasca et al., 2004; Raina et al., 2004).

Nervcellerna gör sitt bästa för att skydda sig mot proteinerna som klumpar ihop sig, onormalt uttryck av cellcykelmarkörer och andra skador genom att aktivera så kallade små heat-shock proteiner. Två små heat-shock proteiner som är onormalt aktiverade vid Alzheimers sjukdom är α B-crystallin (Iwaki et al., 1992; Renkawek et al., 1994b; Mao et al., 2001) och Hsp27 (Renkawek et al., 1994a), de har bland annat som uppgift att skydda cytoskelettet och hindra proteiner från att ansamlas och klumpa ihop sig (Liang & MacRae, 1997; Perng et al., 1999; Alt et al., 2000; den Engelsman et al., 2003; Lu et al., 2003; Lin et al., 2006; Parcellier et al., 2006; Bai et al., 2007; Barbash et al., 2007).

Varför är det viktigt att studera hur många fosfatgrupper som sätts dit på tau- och neurofilament-proteiner vid Alzheimers sjukdom?

För att i framtiden få fram fungerande behandlingsmetoder för Alzheimers sjukdom måste de mekanismer som ligger bakom sjukdomsprocessen klarläggas tydligare. Det är viktigt att känna till orsakerna bakom bland annat det onormalt höga antalet fosfatgrupper på tau- och neurofilament-proteinerna som leder till proteinklumparna och de neurofibrillära nystanen. I studierna som ingår i denna avhandling ligger fokus på att just förstå orsakerna bakom och regleringen av bildandet av de neurofibrillära nystanen.

I **Studie I** så ingår experiment som undersöker ackumuleringen av tau-proteiner och beta-amyloid i hjärnvävnad från patienter som dött med diagnosen Alzheimers sjukdom, proverna jämförs med kontrollvävnad från icke drabbade. En jämförelse mellan olika tau-antikroppar som kan känna igen olika varianter av tau-proteinet, med eller utan fosfatgrupper, görs också och resultaten undersöks för samband relaterade till sjukdomsstadie (grad av Alzheimers sjukdom) och beta-amyloid nivåer i samma prover.

Olika metalljoner är av betydelse för olika cellulära funktioner i hjärnan, bland annat är zink normalt av betydelse för regleringen av vissa typer av signalering mellan nervcellerna (Frederickson et al., 2000; Burdette & Lippard, 2003; Mocchegiani et al., 2005). Men forskare har upptäckt att koncentrationen av zink är onormalt hög i hjärnregioner som drabbas vid Alzheimers sjukdom (Deibel et al., 1996; Danscher et al., 1997; Lovell et al., 1998; Mocchegiani et al., 2005; Religa et al., 2006) och zink kan aktivera kinaser som sätter dit fosfatgrupper på tau- och neurofilament-proteiner (Perry et al., 1985; Cuajungco & Lees, 1997; Kim et al., 2000; Wang et al., 2001; Koh, 2001).

Experimenten i **Studie II** fokuserar på effekten av zinkbehandling på regleringen av kinaserna p70S6K och GSK-3 β i hjärnvävnad från råttor eller SH-SY5Y humana neuroblastomaceller. De möjliga konsekvenserna för antalet fosfatgrupper på tau-proteinet undersöks också efter zinkbehandlingen. I vissa experiment så blockeras kinasernas aktivitet med hjälp av inhibitorer innan antalet fosfatgrupper på tau-proteinet undersöks.

Zink experimenten fortsätter sedan i **Studie III** i N2a mus neuroblastomaceller med fokus på huruvida kinaset p70S6K, som aktiverats med hjälp av zink, även kan sätta dit fosfatgrupper på neurofilament-proteiner. Även i denna studie så används en inhibitor (rapamycin) för att blockera p70S6K aktiviteten för att kunna utföra mer detaljerade studier kring regleringen av signalvägen och antalet fosfatgrupper på neurofilament-proteinerna.

I den sista studien, **Studie IV**, studeras de små heat-shock proteinerna Hsp27 och α B-crystallin, och sambandet mellan dem och tau- och neurofilament-proteinerna undersöks i hjärnvävnad från patienter som diagnostiserats med Alzheimers sjukdom. För att sedan även kunna studera möjliga effekter på tau- och neurofilament-proteinerna och antalet fosfatgrupper mer i detalj så fördes de små heat-shock proteinerna Hsp27 och α B-crystallin in i olika N2a celler. De olika N2a cellerna får en mätbart ökad mängd av antingen Hsp27 eller α B-crystallin i sig, vilket underlättar experimenten som utförs för att undersöka effekterna av detta på tau- och neurofilament-proteinerna.

Resultat och diskussion

I **Studie I** så undersöktes sambandet mellan beta-amyloid, tau-proteiner och mängden fosfatgrupper på tau-proteinerna i hjärnvävnad från avlidna patienter med Alzheimers sjukdom och kontrollpersoner. Det rädde ett signifikant positivt samband mellan beta-amyloid och fosfatgrupper på tau; de förekom även ett samband med graden av patologi i hjärnvävnaden. Fosfatgrupperna kan sättas dit på tau på olika så kallades "sites", och beroende på vilka sites som undersöktes varierade resultaten, från en knapp dubbling av antalet sites med fosfatgrupper när patienter med Alzheimers sjukdom jämfördes med kontrollpersoner, till mer än tio gånger så mycket vid vissa sites i hjärnvävnaden från patienterna med Alzheimers sjukdom i jämförelse med kontrollpersonerna. Resultaten visade att olika tau-antikroppar, som känner igen de olika "siten" med fosfatgrupper på tau, kan användas som ett komplement vid diagnostisering av Alzheimers sjukdom. Resultaten kan även underlätta val av antikroppar för framtida studier av sjukdomsprocessen vid Alzheimers sjukdom.

För att undersöka mekanismerna bakom regleringen av antalet fosfatgrupper på tau- och neurofilament-proteinerna utnyttjades det faktum att koncentrationen av zink är förhöjd vid Alzheimers sjukdom, och att detta bland annat kan aktivera olika kinaser. I **Studie II** och **III** så undersöktes zinks effekter på antalet fosfatgrupper som satts dit på tau- och neurofilament-proteiner i olika modellsystem. De olika modellsystemen reagerade olika på zinkbehandlingen. I experimenten med SH-SY5Y celler och hjärnvävnad från råtta i **Studie II** ledde zinkbehandlingen till ökad aktivitet av bland annat kinaserna p70S6K och GSK-3 β , med efterföljande ökning av antalet fosfatgrupper på tau-proteinet. Den ökade p70S6K aktiviteten ledde även till en ökning av den totala mängden tau-protein, helt enligt vad som även setts i hjärnvävnad från patienter med Alzheimers sjukdom. Resultaten tyder på att samma protein (p70S6K) kan påverka både mängden tau-protein och fosfatgrupperna på tau-proteinet, medan de flesta andra kinaser, däribland GSK-3 β , utövar sin effekt enbart på fosfatgrupperna som sätts dit på tau-proteinet.

Många kinaser som kan sätta dit fosfatgrupper på tau-proteinet kan även göra det på neurofilament-proteiner, så i **Studie III** behandlades N2a celler med zink för att aktivera p70S6K. Detta gjordes för att undersöka om p70S6K även kunde sätta dit fosfatgrupper på neurofilament-proteinerna. Efter zinkbehandlingen kunde en ökad p70S6K aktivitet registreras (liknande den som detekterades i **Studie II**), tillsammans med en ökning av antalet fosfatgrupper på neurofilament-proteinerna. Fast när sedan p70S6K aktiviteten blockerades med hjälp av inhibitorn (rapamycin) så registrerades ingen minskning av antalet fosfatgrupper, trots att p70S6K aktiviteten hade minskat signifikant. Det här tyder på att zink kan öka antalet fosfatgrupper som sätts dit på neurofilament-proteinerna via andra kinaser. Det är viktigt att ta hänsyn till skillnaderna mellan de olika cellinjerna (SH-SY5Y humana neuroblastomaceller, **Studie II**, och N2a mus neuroblastomaceller, **Studie III**) när resultaten ska tolkas, framförallt om man vill kunna dra några slutsatser för själva sjukdomsprocessen i människor.

De små heat-shock proteinerna, Hsp27 och α B-crystallin, kan tänkas påverka regleringen av antalet fosfatgrupper på tau- och neurofilament-proteiner då en ökad mängd av dem har påträffats vid Alzheimers sjukdom (*Iwaki et al., 1992; Renkawek et al., 1994a; 1994b; Mao et al., 2001*). Resultaten från **Studie IV** bekräftade att både Hsp27 och α B-crystallin finns i onormal mängd i hjärnvävnad från patienter med Alzheimers sjukdom i jämförelse med hjärnvävnad från kontrollpersoner. Denna ökning i mängd korrelerade positivt med en ökning av antalet fosfatgrupper som satt på tau- och neurofilament-proteinerna. För att undersöka möjliga konsekvenser som kan ha orsakats av den

ökade mängden små heat-shock proteiner så fördes Hsp27 eller α B-crystallin in i N2a celler. I de celler som hade en ökad mängd av Hsp27 i sig så ledde det till en ökad mängd fosfatgrupper på tau-proteinet (troligen genom p70S6K aktivitet), medan ökad mängd α B-crystallin i cellerna minskade antalet fosfatgrupper på både tau- och neurofilament-proteinerna. Resultaten från **Studie IV** tyder på att även om både Hsp27 och α B-crystallin mängden ökar vid Alzheimers sjukdom så kan konsekvenserna av den ökningen komma att variera. Medan Hsp27 verkar kunna bidra till att förvärra sjukdomsprocessen genom ökad mängd fosfatgrupper, försöker α B-crystallin skydda cellerna från den skadliga ökningen av fosfatgrupper på tau- och neurofilament-proteinerna.

Resultaten från **Studie I-IV** visar att det råder samband mellan antalet fosfatgrupper på tau-proteinerna, beta-amyloid nivån och var under sjukdomsprocessen som proverna tas (**Studie I**), att zinkbehandling av celler leder till aktivering av kinaser och en ökning av antalet fosfatgrupper på tau- och neurofilament-proteinerna (**Studie II-III**) och att små heat-shock proteiner kan påverka antalet fosfatgrupper som sätts på tau- och neurofilament-proteinerna (**Studie IV**).

Ett av de största problemen inom forskningen kring Alzheimers sjukdom är att det är väldigt svårt att exakt peka på vad som egentligen orsakar sjukdomen eller fungerar som startskott för sjukdomsprocessen, och det hela blir inte lättare av att många av de inblandade mekanismerna i cellerna ofta överlappar och påverkar varandra. Sjukdomsprocessen vid Alzheimers sjukdom påverkar både proteinnivåerna och regleringen av proteinerna via till exempel antalet fosfatgrupper. Detta resulterar i att proteinerna kan komma att klumpa ihop sig vilket skadar, och till och med kan döda, nervcellerna. Det är frestande att försöka förhindra detta genom att blockera de olika kinaserna och signalvägarna, men det är lättare sagt än gjort då de flesta signalvägarna i cellerna är delaktiga i många olika viktiga processer. Det här innebär att det krävs väldigt specifik blockering för att inte slå ut cellerna helt. Studierna som beskrivs i den här doktorsavhandlingen hjälper till att öka kunskapen om hur signalvägarna som reglerar antalet fosfatgrupperna styrs, vilket underlättar för att man i framtiden ska kunna ta fram möjliga behandlingar för Alzheimers sjukdom.

ACKNOWLEDGEMENTS

For help and support throughout my postgraduate studies, I would like to express my heartfelt thanks to the following people:

My main supervisor, **Jin-Jing Pei**, for the years in his group. We haven't always agreed on everything, but in the end we reached a consensus that I think we are both happy with, and we have definitely had some very interesting discussions over the years. You have helped me grow and improve as a scientist.

My co-supervisors **Bengt Winblad** – who gave me the opportunity and financial support to undertake this work; and **Magnus Sjögren**: although you spent most of your time away from Stockholm, you always had time for me and provided me with a voice of reason when I needed it most.

Former and present group members: **Wen-Lin, Lizzy, Xu Li, Xinwen** and **Haiyan**. Thank you for your willingness to help out in the lab and for interesting discussions. Special thanks to Wen-Lin – it was nice to know that I was not alone with my problems.

All my co-authors at KI and abroad who have generously shared their extensive knowledge with me, and for the great discussions and suggestions during the experiments and the writing process.

All the senior scientists and everyone working in the labs at Neurotec/NVS, for providing knowledge, skills and support. I am also grateful for all the administrative support I have received throughout the years.

All the other PhD students, former and present, who helped create a pleasant atmosphere at work; for always being helpful and willing to take part in discussions and sharing their thoughts and suggestions on the whole “PhD-experience”, as well as being great travelling companions to conferences.

My former and present office mates (**Alexandra, Annelie, Beata, Ewa, Louise** and **Tanja**) for always listening and helping out with both work-related issues and private problems. I could not have asked for better people to share an office with.

Special thanks to my “wailing wall” of old Södertörn friends – **Jenny, Karin** and **Tobbe** – without you, and our “Thursday lunches”, I would never have made it! I still feel a bit lost in the lab without you Jenny. 😊

My friends outside the lab world, who have put up with my strange working hours and even stranger complaints and problems – it felt great to have your support and enduring belief in me.

My family, who provided me with the foundation I needed to complete this work. I love you all to pieces!

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