CAFETERIA DIET INDUCES NEUROPLASTIC MODIFICATIONS IN THE NUCLEUS ACCUMBENS MEDIATED BY MICROGLIA ACTIVATION.

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Abstract

High-palatable and caloric foods are widely overconsumed due to hedonic mechanisms that prevail over caloric necessities leading to overeating and overweight. The nucleus accumbens (NAc) is a key brain area modulating the reinforcing effects of palatable foods and is crucially involved in the development of eating disorders. We describe that prolonged exposure to high-caloric chocolate cafeteria diet leads to overeating and overweight in mice. NAc functionality was altered in these mice, presenting structural plasticity modifications in medium spiny neurons, increased expression of neuroinflammatory factors and activated microglia, and abnormal responses after amphetamine-induced hyperlocomotion. Chronic inactivation of microglia normalised these neurobiological and behavioural alterations exclusively in mice exposed to cafeteria diet. Our data suggest that prolonged exposure to cafeteria diet produces neuroplastic and functional changes in the NAc that can modify feeding behaviour. Microglia activation and neuroinflammation play an important role in the development of these neurobiological alterations.

Key words

Neuroinflammation, Neuronal plasticity, Nucleus Accumbens.

1. Introduction

The incidence of overweight and obesity is reaching epidemic proportions worldwide with more than 1,9 billion overweight adults in 2013 (James, 2013). Obesity increases the risk for a wide spectrum of diseases including type-2 diabetes, hypertension, stroke and many types of cancer (Haslam and James, 2005), and has also been recently related to cognitive dysfunctions, such as Alzheimer's disease (Volkow *et al.*, 2011). Different brain areas are involved in food intake control, including the *dorsal striatum* (DS) and *nucleus accumbens* (NAc). The DS plays an important role in decision-making behaviours, particularly in goal-directed actions and selection of actions based on their currently expected reward value (Balleine *et al.*, 2007). The NAc is an important component of the mesolimbic reward system that participates in motivational and reward learning and the development of addictive behaviours (Smith *et al.*, 2016). This brain area is divided into two distinct neuroanatomical and functional sub-regions called the NAc core and the NAc shell with different inputs and outputs (Gangarossa *et al.*, 2013). In the present study, Nac core and shell have been studied separately due to their distinct roles regulating feeding behaviours (Floresco *et al.*, 2008; Castro *et al.*, 2015; Myal *et al.*, 2015; O'Connor *et al.*, 2015).

In addition, evidences suggest that alterations in dopamine and opioid signalling in the DS and NAc may contribute to the development of obesity (Berridge *et al.*, 2010). These alterations share similarities to those observed in drug addicts (Volkow *et al.*, 2008), and are suggested to enable the progress towards an addictive-like state that leads to maladaptive behaviours, such as excessive or compulsive eating. In agreement, recent studies in our laboratory have also demonstrated that operant food seeking behaviour for a highly palatable food modifies dendritic spine density in NAc neurons (Guegan *et*

al., 2013). Neuroadaptive changes in these reward circuits could also occur during the development of obesity and could participate in the associated maladaptive behaviours.

Overweight and obesity have also been associated with neuroinflammation mainly at the level of the hypothalamus (Purkayastha and Cai, 2013). Thus, changes in microglia functionality and proinflammatory molecules have been reported in this brain area upon exposure to high caloric food. This microglia activation may contribute to synaptic and neurocircuitry remodelling, affecting the functionality of hypothalamic neurons, which in turn may facilitate overeating (Argente-Arizón, 2015; Kälin *et al.*, 2015). Microglia activation and neuroinflammatory processes associated with obesity have also been recently reported in another brain area, the hippocampus, which could account for cognitive alterations (Erion *et al.*, 2014).

The present study investigated the consequences of obesity development by exposure to cafeteria diet (CD) on neuronal plasticity and neuroinflammatory processes in the reward circuit. Mice were divided in 3 experimental groups; (1) prolonged *ad libitum* access to standard food alone; (2) prolonged *ad libitum* access to standard food and intermittent access to CD, (3) prolonged *ad libitum* access to standard food and CD producing abnormal weight gain. These experimental conditions recapitulate different human feeding behaviour currently associated to the development of obesity. We evaluated changes in dendritic spine in the NAc and DS. Microglia activation and neuroinflammatory markers in the NAc and DS as well as amphetamine-induced locomotor responses to reveal functional alterations in this circuit were also studied. Finally, the consequences of chronic treatment with minocycline, an inhibitor of microglia activation, were evaluated on these behavioural and neuroadaptive alterations.

2. Material and methods

2.1 Animals

Male C57BL6/J mice from 8 to 12 weeks old at the beginning of the experiments were used. Animals were single housed and maintained in a controlled temperature (21±1°C) and humidity (55±10%) room with a 12:12-h reversed light/dark cycle (off at 8 a.m. and on at 8 p.m.). Animals had *ad libitum* access to water and standard food (CONTROL group of mice, n=54), to standard and CD food [FREE-CHOICE (FC) group of mice, n=43], or to standard food and intermittent access to CD (BINGE group of mice, n=26) during the whole experiment. Mice were habituated to the experimental room and handled for one week before starting the experiments. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC, Animal Welfare Assurance #A5388-01, approved as 06/08/2009, guidelines of the French Agriculture and Forestry Ministry for handling animals D34-172-13) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Institut Municipal d'Assistència Sanitària-Universitat Pompeu Fabra). All the experiments were performed under blind conditions.

2.2 Drugs

Minocycline hydrochloride (Sigma-Aldrich, Sant Louis, Missouri, USA) was diluted in PBS (0.1 M; pH=7.4) and administered intraperitoneally (i.p.) at a dose of 30 mg/kg once per day. (+)-α-Methylphenethylamine sulfate salt (D-amphetamine) (R&D Systems Europe Ltd, UK) was dissolved in saline and administered at a dose of 2 mg/kg (i.p.).

2.3 Experimental procedure

Mice were divided in 3 experimental groups with similar body weight at the beginning of the experiment. One group (CONTROL) was exposed to *ad libitum* access to standard food (2.85 kcal/g) for the entire experimental period of 43 days. The free-choice (FC) group was exposed to *ad libitum* access to standard food and to CD composed of an equitable mixed of 4 popular brand chocolate bars highly consumed by humans (MILKA®, SNICKERS®, BOUNTY® and MARS®, total 4.85 kcal/g) for the entire 43 days experimental period. The third group of mice (BINGE) was exposed to *ad libitum* access to standard food and to intermittent access to CD (24 h every 7 days) for the entire experimental period of 43 days, as previously reported (Czyzyk *et al.*, 2010). Mice from the FC and BINGE groups were pre-exposed before the beginning of the experimental period to CD for 3 consecutive days to avoid neophobia associated to the new food and taste. Body weight and food intake were evaluated regularly every week.

On day 29, half of the animals of each experimental group were treated with vehicle and the rest with minocycline (30 mg/kg, once daily, i.p.) for 14 days, until day 42. Eighteen h after the last minocycline injection, animals were sacrificed and their brain processed (see Suppl. Exp diagram for more details). In the BINGE group, the sacrifice coincided with the first 1:30 h period after the beginning of the last exposure to CD, a specific time frame period where the animals showed the maximum binge-like eating behaviour (kcal ingested per period of time).

Different groups of CONTROL, FC and BINGE mice were used to evaluate amphetamine-induced locomotor responses. These mice were kept under the same experimental conditions as described above. Half of the mice of each experimental

group were treated with vehicle and the rest with minocycline (30 mg/kg/day, i.p.) for 14 days, from day 29 to day 42. The protocol used to evaluate amphetamine-induced locomotor responses started on day 40 (3 days before the end of minocycline treatment) and lasted until day 54. Food exposure remained under the same experimental conditions during this entire 54 days period.

2.4 Amphetamine induced locomotor responses

All mice were habituated to the test apparatus, handling, and procedure for three consecutive days before the experiment. During this habituation period (day 40 to 42), mice were placed for 30 min in the activity box, received a first injection of saline, and were placed back in the box for 90 additional min. On day 4, the handling was identical, except that saline injection was replaced by d-amphetamine administration (2 mg/kg, i.p.) and locomotion was evaluated for 120 min after injection. Mice were treated daily with d-amphetamine (2 mg/kg, i.p.) for 5 consecutive days. This repeated exposure was followed by 6 days of withdrawal and by a challenge injection of d-amphetamine (2 mg/kg, i.p.) the following day. Horizontal locomotor activity was measured in a circular corridor (Imetronic, Pessac, France) as described previously (Brami-Cherrier *et al*, 2005). Counts for horizontal activity were incremented by consecutive interruption of two adjacent beams placed at a height of 1 cm per 90° sector of the corridor (mice moving through one-quarter of the circular corridor).

2.5 Ballistic labeling with the fluorescent dye DiI

See supplementary experimental procedures.

2.6 Dendritic spine analysis

Individual medium spiny neurons (MSN) in the NAc shell and core and DS and pyramidal neurons from the mPFC in vehicle treated animals were chosen for spine analysis based on the following criteria, as described previously (Lee *et al.,* 2006): (i) there was minimal or no overlap with other labeled cells to ensure that processes from different cells would not be confused, (ii) at least three primary dendrites needed to be visible for cells to be used for analysis and (iii) distal dendrites (from secondary dendrites to terminal dendrites) were examined.

To calculate spine density, a length of dendrite (at least 20 μm long) was traced by using the confocal microscope. All images of dendrites were taken at different z levels (0.13 μm depth intervals) to examine the morphology of dendritic spines. Measurements of dendrites and dendritic spine length and classification were made using trained automatic NeuronStudio analysis software (Rodriguez *et al.,* 2008) after previous deconvolution of the images with Huygens software (Scientific Volume Imaging, Hilversum, Nederland). All spine density measurements were performed in 3D reconstructions from the z-stack. The spine density was calculated by dividing the total number of spines automatically counted by the dendritic length. A spine was classified as thin spine if its head was <0.35 μm in diameter and presented a visible neck. A spine was classified as mushroom if its head diameter exceeded 0.35 μm and had a visible neck. A spine was considered stubby if short and no visual neck was present. Filopodia were separated from thin spines if the size of their head was insignificant or almost equal to its neck (Grutzendler *et al.,* 2003).

2.7 RNA extraction and reverse transcription

See supplementary experimental procedures.

2.8 Quantitative Real-Time PCR analyses

See supplementary experimental procedures.

2.9 Iba-1 Immunofluorescence

See supplementary experimental procedure.

2.10 Microglia morphological analysis

Confocal microscopic images of whole microglial cells stained with IBA1 were acquired with a glicerol immersion lens to evaluate the changes in microglial morphology (×20 objective; 3.0 zoom). Images were taken capturing 30 μm *z* levels (in 0.29-μm depth intervals) to evaluate the morphology of whole cells. Afterwards, the length of the microglial ramifications and the perimeter of the microglial soma were analysed with ImageJ software (NIH) in maximum *z* projections. The total number of microglial cells in a focal plane were analysed consisting in 11-28 cells per area per animal (n = 5–7 mice per experimental group).

2.11 Statistics

Three-way ANOVA with repeated measures was used to analyse intake measurements, weight increase and d-amphetamine locomotor responses. Diet and treatment were considered as between subject factors and day or week as within subject factor. Two-way ANOVA with diet and treatment as between subject factors was applied to compare data from the plasticity assays, microglia morphological analysis and neuroinflammatory factors quantification. A posterior *post-hoc* Fisher's F test was used when appropriate. The STATISTICA software was used and p values of less than 0.05 were considered significant. Data are presented as the mean + SEM.

3. Results

3.1 The increased in food consumption and body weight after prolonged exposure to CD were reverted by chronic minocycline treatment.

An increased caloric intake was observed in the FC group when compared with CONTROL animals during the entire experimental procedure (Fig. 1A). Around 60% of the kcal ingested by these animals came from the CD (Fig. 1C), demonstrating that this type of food was highly palatable and rewarding to the mice due to its high sucrose and fat content. The increased caloric intake facilitated body weight gain in FC mice, which reached statistical significance at the 4^{th} week of CD access when compared with CONTROL animals (Fig. 1B). Chronic treatment with minocycline (30 mg/kg, i.p., daily for 14 consecutive days) significantly decreased caloric intake only in FC animals (FC vehicle vs FC minocycline, $p<0.001$ on week 5 and week 6) (Fig. 1A) without modifying food preference (Fig. 1C). Accordingly, a progressive decrease in body weight was observed during minocycline treatment in FC mice, and a not significant trend was also revealed in CONTROL mice (FC vehicle vs FC minocycline, $p<0.001$ at the end of the treatment) (Fig. 1B). See Table 1 for detailed statistics values.

3.2 Exposure to CD altered structural plasticity in the NAc, which was reverted after chronic minocycline treatment.

At the end of the experimental procedure, mice were sacrificed and their brains processed to evaluate dendritic spine changes in the DS and the NAc shell and core. No differences in the total dendritic spine density were observed in MSN from the DS (CONTROL vehicle vs FC vehicle, n.s.) and NAc core between CONTROL and FC mice treated with vehicle (CONTROL vehicle vs FC vehicle, n.s.) (Fig. 2A). Interestingly, a significant decrease in dendritic spine density in the NAc shell was

revealed in vehicle FC group when compared with CONTROL vehicle animals (CONTROL vehicle vs FC vehicle, $p<0.05$) (Fig. 2C). Chronic treatment with minocycline (30 mg/kg, i.p., daily for 14 consecutive days) reverted the alteration in total dendritic spine density in the NAc shell in FC animals (FC vehicle vs FC minocycline, $p<0.01$), and increased spine density in the NAc core in this experimental group (FC vehicle vs FC minocycline, $p<0.05$). In contrast, no alterations of dendritic spine density were observed in the DS in any of the experimental groups (Fig. 2A).

The density of the different subtypes of dendritic spines was also evaluated. Prolonged exposure to CD in vehicle-treated FC mice decreased the density of mature-like mushroom-subtype spines in the NAc shell (CONTROL vehicle vs FC vehicle, $p<0.05$) and core (CONTROL vehicle vs FC vehicle, $p<0.05$) (Fig. 3Bi and 3Ci). Interestingly, chronic minocycline administration significantly reverted the alterations in mature mushroom-spine density in the NAc shell (FC vehicle vs FC minocycline, $p<0.05$) and core (FC vehicle vs FC minocycline, $p<0.01$) in FC mice. Moreover, minocycline treatment significantly increased mature-like thin-spine densities in the NAc core and shell of FC animals (FC vehicle vs FC minocycline, $p<0.05$ and FC vehicle vs FC minocycline, $p<0.01$ respectively). On the other hand, no modifications in immature subtypes (stubby and filopodia) were revealed in these brain areas.

Dendritic spine densities were also evaluated in mPFC pyramidal neurons. No differences in structural plasticity were observed in between CONTROL and FC mice treated with vehicle in this brain area. (Suppl. Fig. 2).

These data show that prolonged exposure to CD develops excess weight gain associated with alterations in neuronal plasticity in the NAc, mostly in mature-like spine subtypes (thin and mushroom subtypes). The decrease in food consumption and body weight

produced by minocycline chronic exposure in FC mice was accompanied by the reversion of these neuronal plasticity changes. See Table 2 for detailed statistics values.

3.3 Intermittent exposure to CD did not modify body weight gain nor structural plasticity in the NAc and DS.

BINGE animals had restricted access to CD only during 24h a week. This CD exposure did not modify body weight gain when compared with CONTROL animals (Fig. 4A). Similarly, no significant alterations in body weight were observed after chronic treatment with minocycline (30 mg/kg, i.p., once a day for 14 days). These animals showed binge-like eating behaviour on the first 2:30 h of CD access (data not shown). Brains were processed 1 h and 30 min after the beginning of the last exposure to CD, when binge-like eating behaviour was more relevant. Interestingly, no alterations in structural plasticity were observed in these animals neither in the NAc core, shell nor the DS. Moreover, minocycline treatment did not modify dendritic spine density nor dendritic spine sub-types in any of the 3 areas evaluated (Fig. 4Ci, ii, iii and Suppl. Fig. 1).

Structural plasticity in mPFC pyramidal neurons was also evaluated in these mice. No differences were observed between CONTROL and BINGE animals treated with vehicle in this brain area (Suppl. Fig. 2).

These data suggest that continuous and not intermittent access to CD is required to produce significant alterations in body weight gain and associated neuroplastic modifications. See Table 3 for detailed statistics values.

3.4 Molecular and cellular signs of neuroinflammation in the NAc induced by CD exposure were reverted by minocycline treatment.

We evaluated the expression of proinflammatory cytokines and microglia morphology in the NAc in CONTROL and FC animals, and the effects of chronic minocycline treatment in these neuroinflammatory processes. An increase in the expression of proinflammatory cytokines IL-1β ($p<0.01$) and IFN-γ ($p<0.001$) and a tendency to increase IL-18 was observed in FC vehicle mice when compared to CONTROL vehicle animals (Fig. 5A). No changes were revealed in the expression of TNFα/IL-1-dependent cytokine IL-6 (Fig. 5Aii). Chronic treatment with minocycline significantly reverted the increase in IFN-γ observed in FC mice (FC vehicle vs FC minocycline, $p<0.05$) (Fig. 5Aiii) and attenuated the effects observed in IL-1β and IL-18 in FC mice (Fig. 5Ai and 5iv). See Table 4 for detailed statistics values.

Morphological analysis showed that microglia cells acquired an ameboid-like phenotype characteristic of a reactive activated state in animals fed with CD. This was revealed by the significant increase in the perimeter of the soma of NAc core ($p<0.01$) and NAc shell ($p<0.001$) microglial cells and a tendency to decrease their branch length in both NAc areas in FC vehicle mice when compared with CONTROL vehicle animals. These changes in the perimeter of microglia soma in the NAc core and NAc shell were reverted by chronic minocycline treatment (FC vehicle vs FC minocycline $p<0.01$ in NAc core; and $p<0.001$ in NAc shell) (Fig. 5B). See Table 4 for detailed statistics values.

We also evaluated microglial morphological alterations in the NAc of mice with intermittent exposure to CD (BINGE mice). Our results show no differences in the perimeter of the soma or in the branch length of microglial cells in the NAc shell or core between CONTROL and BINGE mice treated with vehicle and minocycline (Fig. 4D).

3.5 Amphetamine locomotor responses were modified in FC mice and normalized after chronic minocycline treatment.

Neuroplastic alterations associated with CD-induced overeating and subsequent development of overweight might affect NAc functionality. In order to ascertain this hypothesis, amphetamine-induced locomotor effects, a response that involves NAc functionality (Vanderschuren and Kalivas, 2000), was assessed in CONTROL and FC mice. Basal locomotion during the three-day habituation period was not modified in the different experimental groups. Acute amphetamine administration increased locomotion in all the groups, although this response was significantly greater in FC vehicle animals in comparison with the CONTROL group ($p<0.01$) and FC animals receiving minocycline ($p<0.05$) (Fig. 6). Repeated amphetamine administration for 5 days produced a sensitization to its hyperlocomotor response in CONTROL and FC animals injected with minocycline ($p<0.001$). This effect was not observed in FC vehicle animals since amphetamine hyperlocomotor effects were not modified in these mice thorough the chronic treatment with the drug. This could be due to a ceiling effect to the hyperlocomotor response induced by amphetamine after the first administration of this drug in FC mice. Interestingly, previous exposure to minocycline did not modify amphetamine locomotor effects in CONTROL animals, but reverted the abnormal amphetamine locomotor responses observed in FC mice (FC vehicle vs FC minocycline $p<0.01$). These data suggest that the functionality of the NAc is altered during body weight gain in FC vehicle mice, which was reverted by microglia inactivation with minocycline treatment. See Table 5 for detailed statistics values.

Possible modifications in NAc functionality as a consequence of intermittent exposure to CD were also evaluated in BINGE mice. No differences were revealed in basal

locomotor responses nor after amphetamine treatment between CONTROL and BINGE mice treated with vehicle or minocycline (Fig. 4B).

4. Discussion

We show that prolonged exposure to highly palatable and caloric food leads to overeating and facilitates the development of obesity. These alterations in feeding behaviour are accompanied by important changes in structural plasticity and in the functionality of the NAc, a key brain area of the reward system involved in addictive behaviour (Smith *et al.*, 2016). In addition, our study demonstrates that microglia activation and neuroinflammatory processes play an important role in the development of CD-induced overeating behaviour and associated neuroplastic and functional alterations in the NAc.

In agreement with previous reports, access to highly palatable food led to consumption beyond homeostatic needs (Finlayson *et al.*, 2008; Liu *et al.*, 2016) and facilitated the development of obesity in FC mice. Alterations in dopaminergic and opioid neurotransmission in the mesolimbic system have been observed during the progression towards overweight (Murray *et al.*, 2014; Volkow *et al.*, 2008). These modifications are suggested to produce a weakening in the functionality of this brain reward pathway that triggers aberrant eating behaviours to compensate such rewarding deficits (Davis *et al.*, 2009).

In this study, we show that changes in neuronal plasticity in the reward system, specifically in the NAc, may also account for the behavioural alterations produced by prolonged exposure to high palatable and caloric food. Interestingly, no changes in neuroplasticity were found in DS nor the mPFC, other brain areas involved in food intake and the development of obesity (Volkow *et al.*, 2008). Previous studies have reported neurophysiological alterations in the DS in extremely obese humans, but not in subjects with a moderate degree of obesity (Haltia *et al.*, 2007; Karlsson *et al.*, 2015;

Steele *et al.*, 2010; Wang *et al.*, 2002). In agreement, our study described no alterations in neuronal plasticity in the DS of FC mice which showed moderate weight gain promoted by CD (20.77% increase compared to CONTROL mice). Moreover, we also did not observe any difference in the spine density in the mPFC in between experimental groups treated with vehicle (Suppl. Fig. 2). In contrast, a recent study reported that exposure for 3 weeks to high fat diet decreased spine density in pyramidal neurons from the infralimbic mPFC in rats (Dingess *et al.*, 2017). Differences in between species (rat vs mouse), type of palatable food (high fat diet vs mix of different chocolate brands) and dietary conditions (exclusive access to high fat diet vs freedom access to standard and CD) and the period of exposure to the diet (3 weeks vs 43 days) may explain the differences in the present and the previous study (Dingess *et al.*, 2017).

On the other hand, structural plasticity was not modified in any brain area evaluated in BINGE mice. These mice also showed no differences in body weight gain when compared with CONTROL animals. These data suggest that prolonged, but not intermittent, exposure to CD is required to produce these structural plasticity alterations in the NAc.

Our study describes important neuroplastic alterations in the NAc during the development of obesity. These neuroplastic changes have been previously suggested to affect the functionality of the brain reward system (Russo *et al.*, 2010). We observed that the most important modifications in structural plasticity occurred in mature-like spines (thin and mushroom) in the NAc shell, leading to a decrease in total dendritic spine density in FC mice. These findings complement previous studies from our laboratory revealing that seeking-behaviour for isocaloric palatable food in operant chambers increased dendritic spine density in the NAc shell, without modifying body weight (Guegan *et al.*, 2013). A correlation between food seeking behaviour alterations

and structural plasticity changes has been demonstrated in the present study and previous study (Guegan *et al*., 2013). Thus, mice learning active food seeking operant responding increased total spine density in NAc shell MSN (Guegan *et al*., 2013, whereas mice with *ad libitum* effortless access to high palatable food show the opposite alterations in the NAc shell (present study). Therefore, an increase in total dendritic spine density in the NAc shell might correlate with compulsive seeking behaviour (Guegan *et al.,* 2013), whereas the decrease of these dendritic spine densities might correlate with a decrease in food reward processing in overweight mice leading to food overconsumption to compensate such reward deficits. Although the most important changes occurred in the NAc shell, some specific modifications in neuronal plasticity were also observed in the core sub-region that were reverted after minocycline treatment (such as the decrease in mushroom spines, see Fig. 3). These selective neuroplastic changes in the NAc core might also account for the aberrant feeding behaviour revealed in FC mice, as previously suggested for the development of drugs addictive behaviours or impulsive food seeking (Guegan *et al.,* 2013; Robinson and Kolb, 2004).

However, our results suggest a prominent role of NAc shell neuroplasticity in the development of abnormal eating behaviours. In agreement, previous studies have revealed that high-frequency stimulation of the NAc shell decreases high fat food consumption in obese mice (Halpern *et al.,* 2013) and rats (Zhang *et al.,* 2015), whereas similar stimulation in the NAc core did not modify food intake (van der Plasse *et al.*, 2012). In addition, the NAc shell, but not NAc core or DS, has important GABAergic connections to other brain areas critically involved in feeding behaviour, such as the lateral hypothalamus, which could account for the role of this NAc sub-region in the control of food intake (Groenewegen *et al.,* 1999; O'Connor *et al.,* 2015; Stratford and Kelley, 1999; Urstadt *et al.,* 2013).

Previous studies (Berridge *et al.*, 2010; Johnson and Kenny, 2010) have suggested that neurophysiological alterations in different areas of the mesocorticolimbic system may lead to maladaptive functionality of this brain circuit facilitating the development of abnormal eating behaviours associated with obesity. In order to study the effects of the alterations in neuroplasticity in the NAc in FC mice on the functionality of the brain reward system, amphetamine locomotor effects were evaluated in CONTROL, FC and BINGE mice. Thus, FC mice, but not CONTROL or BINGE animals, showed anomalous locomotor responses after amphetamine administration. These results suggest that the alterations in structural plasticity in the NAc associated with prolonged exposure to CD modify the functionality of the mesocorticolimbic system. In agreement, previous studies also showed aberrant functionality of the brain reward system in overweight or obese rodents, revealed by the development of abnormal conditioned place preference or behavioral sensitization after drug treatment (Morales *et al.*, 2012; Robinson *et al.*, 2015).

Pioneer studies have reported microglia activation and neuroinflammatory processes in the hypothalamus during obesity, which seem to participate in the neurophysiological alterations leading to obesity (Thaler *et al.*, 2012). However, the role of neuroinflammation in the brain reward system in the development of excessive weight gain is still unknown. We evaluated changes in neuroinflammation measuring differential mRNA expression of cytokines and microglia morphological alterations in the NAc, the main brain structure showing neuroplasticity changes after CD exposure. We show that the NAc displays neuroinflammation and microglia activation in early stages of obesity. Based on previous reports (Ekdahl, 2012), we can suggest that these alterations in proinflammatory molecules and microglia functionality in the NAc can modify synaptic remodelling and dendritic spine pruning, a mechanism that may

account for the alterations in structural plasticity observed in FC mice. Different mechanisms have been suggested to explain the role of microglial activities regulating neuronal plasticity. Thus, recent studies have described evidences on the role of microglial cells in the removal of synapses (pruning) during adulthood (Siskova and Tremblay, 2013). In the healthy brain, microglia removes unwanted spines and synapses contributing to the maturation of neuronal circuits. However, inappropriate engulfment of synapses, a process that is suggested to be altered in pathological situations associated to neuroinflammatory processes (Wolf *et al.,* 2017), causes excessive loss of running or new-born synapses as reported for example in neurodegenerative diseases such as Alzheimer's disease (Hong *et al.,* 2016). A similar mechanism of action may also account for the alterations in neuronal plasticity in the NAc associated with the development of overweight reported in the present study. Moreover, recent studies also suggest that alterations in synaptic connectivity and related behaviours involve immunological pathways governed by microglial cells (Udeochu *et al.,* 2016). Interestingly, we have observed changes in mRNA levels of two major pro-inflammatory microglial cytokines, the IL-1β and IFNγ. Previous studies have shown that both cytokines can modulate neuronal plasticity in different brain areas (Maher *et al.,* 2006). These results suggest that microglia induced chronic inflammation can play an important effect in neuronal plasticity in the NAc. To evaluate this hypothesis, mice were exposed to chronic treatment with the inhibitor of microglia activation minocycline. Chronic minocycline treatment produced a relevant and significant decrease in food intake and body weight gain in mice exposed to CD when compared with those animals exposed to standard diet. However, minocycline also produced a weak non-significant decrease in body weight in CONTROL and BINGE mice. Low inflammatory processes may also be active in basal conditions in CONTROL or BINGE

mice. Minocycline by modulating other systems such as the 5-lipoxygenase and matrix metalloproteinase-9 activities in the central nervous system or regulating lymphocyte activities at the peripheral level (Song *et al.*, 2004; Koistinaho *et al.*, 2005) can also produce anti-inflammatory effects that can results in a weak decrease in food consumption and body weight in CONTROL and BINGE animals. By these alternative mechanisms, minocycline could be able to modulate inflammatory processes and subsequently affect feeding behaviour and body weight in CONTROL and BINGE animals. This possible effect was weaker than that mediated by its effects on microglia cells, which were clearly revealed in FC mice where microglia activation was relevant.

Chronic minocycline also reverted the decrease in dendritic spine density and the modifications of specific spine subtypes in the NAc shell and core after CD exposure, as well as, the functional behavioural alterations promoted by this food exposure. These data suggest that microglia activation and neuroinflammation contribute to the development of aberrant neuronal plasticity and functional changes in the NAc that correlate with abnormal eating behavioural and excessive weight gain. Interestingly, a recent study (Lewitus *et al.*, 2016) reveals the involvement of microglia activation and cytokine release in cocaine-induced locomotor sensitization in mice, supporting the role of these glial cells in the regulation of NAc functionality.

In conclusion, chronic exposure to highly palatable food leads to neuronal plasticity alterations in the NAc that affect the functionality of this brain area and favour overeating behaviour and excessive weight gain. These alterations are mediated by local microglia activation and neuroinflammation processes in the reward circuitry after highly palatable food exposure. The identification of this neuroinflammatory process underlines the relevance of the reward circuitry in the development of behavioural

alterations, leading to obesity and suggests the interest of targeting these neuroinflammatory responses for obesity treatment.

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Disclosure/Conflict of interest

None of the authors reported biomedical financial interests or potential conflicts of interest.

Authors contribution

RM, EV and MM elaborated the study design. M G-M, BG, S M-N collected the data. M G-M, MM and RM drafted the article. M G-M, EV, RM, JP and MM contributed to data analysis and interpretation. All authors critically reviewed the content and approved the final version for publication.

References

Argente-Arizón P (2015). Role of non-neuronal cells in body weight and appetite control. *Front Endocrinol (Lausanne)* **6**: 1–15.

Balleine BW, Delgado MR, Hikosaka O (2007). The role of the dorsal striatum in reward and decision-making. *J Neurosci* **27**: 8161–5.

Berridge KC, Ho C-Y, Richard JM, DiFeliceantonio AG (2010). The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. *Brain Res* **1350**: 43–64.

Brami-Cherrier K, Valjent E, Hervé D, Darragh J, Corvol J-C, Pages C, *et al* (2005). Parsing molecular and behavioral effects of cocaine in mitogen- and stress-activated protein kinase-1-deficient mice. *J Neurosci* **25**: 11444–54.

Castro DC, Cole SL, Berridge KC (2015). Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry. *Front Syst Neurosci.***9**: 90.

Czyzyk TA, Sahr AE, Statnick MA (2010). A model of binge-like eating behavior in mice that does not require food deprivation or stress. *Obesity (Silver Spring).***18**:1710-7.Davis CA, Levitan RD, Reid C, Carter JC, Kaplan AS, Patte KA, *et al* (2009). Dopamine for “wanting” and opioids for “liking”: a comparison of obese adults with and without binge eating. *Obesity (Silver Spring)* **17**: 1220–5.

Davis CA, Levitan RD, Reid C, Carter JC, Kaplan AS, Patte KA et al (2009). Dopamine for "wanting" and opioids for "liking": a comparison of obese adults with and without binge eating. *Obesity (Silver Spring).***17**: 1220-1225.

Dingess PM, Darling RA, Kurt Dolence E, Culver BW, Brown TE (2017). Exposure to a diet high in fat attenuates dendritic spine density in the medial prefrontal cortex.

Brain Struct Funct. **222**:1077-1085.

Ekdahl CT (2012). Microglial activation - tuning and pruning adult neurogenesis. *Front Pharmacol* **3**: 41.

Erion JR, Wosiski-Kuhn M, Dey A, Hao S, Davis CL, Pollock NK, *et al* (2014). Obesity elicits interleukin 1-mediated deficits in hippocampal synaptic plasticity. *J Neurosci* **34**: 2618–31.

Finlayson G, King N, Blundell J (2008). The role of implicit wanting in relation to explicit liking and wanting for food: implications for appetite control. *Appetite* **50**: 120–7.

Floresco SB, McLaughlin RJ, Haluk DM (2008). Opposing roles for the nucleus accumbens core and shell in cue-induced reinstatement of food-seeking behavior. *Neuroscience* **154**: 877–84.

Gangarossa G, Espallergues J, Kerchove d'Exaerde A de, Mestikawy S El, Gerfen CR, Hervé D, *et al* (2013). Distribution and compartmental organization of GABAergic medium-sized spiny neurons in the mouse nucleus accumbens. *Front Neural Circuits* **7**: 22.

Groenewegen HJ, Wright CI, Beijer A V, Voorn P (1999). Convergence and segregation of ventral striatal inputs and outputs. *Ann N Y Acad Sci* **877**: 49–63.

Grutzendler J, Tsai J, Gan W-B (2003). Rapid labeling of neuronal populations by ballistic delivery of fluorescent dyes. *Methods* **30**: 79–85.

Guegan T, Cutando L, Ayuso E, Santini E, Fisone G, Bosch F, *et al* (2013). Operant behavior to obtain palatable food modifies neuronal plasticity in the brain reward circuit. *Eur Neuropsychopharmacol* **23**: 146–59.

Halpern CH, Tekriwal A, Santollo J, Keating JG, Wolf JA, Daniels D, *et al* (2013). Amelioration of binge eating by nucleus accumbens shell deep brain stimulation in mice involves D2 receptor modulation. *J Neurosci* **33**: 7122–9.

Haltia LT, Rinne JO, Merisaari H, Maguire RP, Savontaus E, Helin S, *et al* (2007). Effects of intravenous glucose on dopaminergic function in the human brain in vivo. *Synapse* **61**: 748–56.

Haslam DW, James WPT (2005). Obesity. *Lancet* **366**: 1197–209.

Hong S, Dissing-Olesen L, Stevens B (2016). New insights on the role of microglia in synaptic pruning in health and disease. *Curr Opin Neurobiol.* **36**: 128-134.

James WPT (2008). WHO recognition of the global obesity epidemic. *Int J Obes (Lond)* **32 Suppl 7**: S120–S126.

Johnson PM, Kenny PJ (2010). rats : Role for dopamine D2 receptors. **13**: 635–641.

Kälin S, Heppner FL, Bechmann I, Prinz M, Tschöp MH, Yi C-X (2015). Hypothalamic innate immune reaction in obesity. *Nat Rev Endocrinol* **11**: 339–51.

Karlsson HK, Tuulari JJ, Tuominen L, Hirvonen J, Honka H, Parkkola R, *et al* (2015). Weight loss after bariatric surgery normalizes brain opioid receptors in morbid obesity. *Mol Psychiatry* doi:10.1038/mp.2015.153.

Koistinaho M, Malm TM, Kettunen MI, Goldsteins G, Starckx S, Kauppinen RA, *et al* (2005). Minocycline protects against permanent cerebral ischemia in wild type but not in matrix metalloprotease-9-deficient mice. *J Cereb Blood Flow Metab.* **25**: 460-7.

Lee K-W, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P (2006). Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing

medium spiny neurons in nucleus accumbens. *Proc Natl Acad Sci U S A* **103**: 3399–404.

Lewitus GM, Konefal SC, Greenhalgh AD, Pribiag H, Augereau K, Stellwagen D (2016). Microglial TNF-α Suppresses Cocaine-Induced Plasticity and Behavioral Sensitization. *Neuron* doi:10.1016/j.neuron.2016.03.030.

Liu S, Globa AK, Mills F, Naef L, Qiao M, Bamji SX, *et al* (2016). Consumption of palatable food primes food approach behavior by rapidly increasing synaptic density in the VTA. *Proc Natl Acad Sci U S A* doi:10.1073/pnas.1515724113.

Maher FO, Clarke RM, Kelly A, Nally RE, Lynch MA (2006). Interaction between interferon gamma and insulin-like growth factor-1 in hippocampus impacts on the ability of rats to sustain long-term potentiation. *J Neurochem.* **96**: 1560-1571.

Morales L, Olmo N Del, Valladolid-Acebes I, Fole A, Cano V, Merino B, *et al* (2012). Shift of circadian feeding pattern by high-fat diets is coincident with reward deficits in obese mice. *PLoS One* **7**: e36139.

Murray S, Tulloch A, Gold MS, Avena NM (2014). Hormonal and neural mechanisms of food reward, eating behaviour and obesity. *Nat Rev Endocrinol* **10**: 540–52.

Myal S, O'Donnell P, Counotte DS (2015). Nucleus accumbens injections of the mGluR2/3 agonist LY379268 increase cue-induced sucrose seeking following adult, but not adolescent sucrose self-administration. *Neuroscience.* **305**: 309-15.

O'Connor EC, Kremer Y, Lefort S, Harada M, Pascoli V, Rohner C, *et al* (2015). Accumbal D1R Neurons Projecting to Lateral Hypothalamus Authorize Feeding. *Neuron* **88**: 553–64.

Purkayastha S, Cai D (2013). Disruption of neurogenesis by hypothalamic inflammation

in obesity or aging. *Rev Endocr Metab Disord* **14**: 351–6.

Robinson MJF, Fischer AM, Ahuja A, Lesser EN, Maniates H (2015). Roles of "Wanting" and "Liking" in Motivating Behavior: Gambling, Food, and Drug Addictions. *Curr Top Behav Neurosci* doi:10.1007/7854_2015_387.

Robinson TE, Kolb B (2004). Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* **47 Suppl 1**: 33–46.

Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Wearne SL (2008). Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. *PLoS One* **3**: e1997.

Russo SJ, Dietz DM, Dumitriu D, Morrison JH, Malenka RC, Nestler EJ (2010). The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends Neurosci* **33**: 267–276.

Siskova Z, Tremblay ME (2013). Microglia and synapse: interactions in health and neurodegeneration.*Neural Plast.* 425845.

Smith D V, Rigney AE, Delgado MR (2016). Distinct Reward Properties are Encoded via Corticostriatal Interactions. *Sci Rep* **6**: 20093.

Song Y, Wei EQ, Zhang WP, Zhang L, Liu JR, Chen Z (2004). Minocycline protects PC12 cells from ischemic-like injury and inhibits 5-lipoxygenase activation. *Neuroreport* **15**: 2181-4.

Steele KE, Prokopowicz GP, Schweitzer MA, Magunsuon TH, Lidor AO, Kuwabawa H, *et al* (2010). Alterations of central dopamine receptors before and after gastric bypass surgery. *Obes Surg* **20**: 369–74.

Stratford TR, Kelley AE (1999). Evidence of a functional relationship between the

nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. *J Neurosci* **19**: 11040–8.

Thaler JP, Yi C-X, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, *et al* (2012). Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest* **122**: 153–62.

Udeochu JC, Shea JM, Villeda SA (2016). Microglia communication: Parallels between aging and Alzheimer's disease. *Clin Exp Neuroimmunol.* **7**: 114-125.

Urstadt KR, Kally P, Zaidi SF, Stanley BG (2013). Ipsilateral feeding-specific circuits between the nucleus accumbens shell and the lateral hypothalamus: regulation by glutamate and GABA receptor subtypes. *Neuropharmacology* **67**: 176–82.

van der Plasse G, Schrama R, van Seters SP, Vanderschuren LJ, Westenberg HG (2012) Deep brain stimulation reveals a dissociation of consummatory and motivated behaviour in the medial and lateral nucleus accumbens shell of the rat. *PLoS One.* **7** :e33455.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berl*). **151**: 99-120.

Volkow ND, Wang G-J, Baler RD (2011). Reward, dopamine and the control of food intake: implications for obesity. *Trends Cogn Sci* **15**: 37–46.

Volkow ND, Wang G-J, Fowler JS, Telang F (2008). Overlapping neuronal circuits in addiction and obesity: evidence of systems pathology. *Philos Trans R Soc Lond B Biol Sci* **363**: 3191–200.

Wang G-J, Volkow ND, Felder C, Fowler JS, Levy A V, Pappas NR, *et al* (2002).

Enhanced resting activity of the oral somatosensory cortex in obese subjects. *Neuroreport* **13**: 1151–5.

Wolf SA, Boddeke HW, Kettenmann H (2017). Microglia in Physiology and Disease. *Annu Rev Physiol.* **79**: 619-643.

Zhang C, Wei N-L, Wang Y, Wang X, Zhang J-G, Zhang K (2015). Deep brain stimulation of the nucleus accumbens shell induces anti-obesity effects in obese rats with alteration of dopamine neurotransmission. *Neurosci Lett* **589**: 1–6.

Figure legends

FIGURE 1: Prolonged exposure to CD led to overeating and the development of excessive weight gain in mice with free choice access, which was reverted by chronic minocycline treatment. (A) Daily average intake per week. Data represents the total kcal ingested per g of body weight on each experimental group. Doted bar determines the beginning of the treatment with vehicle or minocycline for 14 consecutive days (30 mg/kg, i.p. once daily). **(B)** Percentage of body weight increase per week in all experimental groups. Doted bar determines the beginning of the treatment with vehicle or minocycline. **(C)** Percentage of standard (SC) and CD intake in mice with free choice access treated with vehicle or minocycline. Data represents the total kcal ingested per g of body weight of each type of food. Doted bar determines the beginning of the treatment with vehicle or minocycline All data are expressed as mean + SEM (n= 19-35 per experimental group) ★$p < 0.05$, ★★$p < 0.01$, ★★★$p < 0.001$ (CONTROL versus FC diet, Fisher's F-test); ☆$p < 0.05$, ☆☆$p < 0.01$, ☆☆☆$p < 0.001$ (vehicle versus minocycline, Fisher's F-test).

FIGURE 2: Prolonged exposure to free-choice CD access led to alteration in structural plasticity in the mesocorticolimbic system, which was reverted by minocycline chronic treatment. Quantification of total dendritic spine densities (number/10 μm) in medium spiny neurons (MSN) from the dorsal striatum (DS) **(A)**, nucleus accumbens (NAc) core **(B)** and shell **(C)** of mice fed with free-choice cafeteria diet access (FC) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day). Data represent the average of 4-7 dendrites per animal (n = 5–9 mice per experimental group) and brain area. No more than 3 dendrites were evaluated from the same neuron. Data are expressed as mean + SEM. ★$p < 0.05$, ★★$p < 0.01$ (CONTROL versus FC diet,

Fisher's F-test); ☆p < 0.05, ☆☆p < 0.01 (vehicle versus minocycline, Fisher's F-test). Scale bar 2μm.

<u>FIGURE 3:</u> Prolonged exposure to free-choice CD access led to some alterations in spine subtype densities in the mesocorticolimbic system, which was reverted by minocycline chronic treatment. Quantification of the density (number/10 μm) of mushroom **i)**, thin **ii)**, stubby **iii)** and filopodia **iv)** dendritic spines in medium spiny neurons (MSN) from the dorsal striatum **(A)**, nucleus accumbens (NAc) core **(B)** and shell **(C)** of mice fed with free-choice cafeteria diet access (FC) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day). Data represent the average of 4-7 dendrites per animal; (n = 5–9 per experimental group) and brain area. No more than 3 dendrites were evaluated from the same neuron. Data are expressed as mean + SEM. ★p < 0.05, ★★p < 0.01 (CONTROL versus FC diet, Fisher's F-test); ☆p < 0.05, ☆☆p < 0.01 (vehicle versus minocycline, Fisher's F-test).

FIGURE 4: Intermittent exposure to CD led to binge eating maintaining normoweight and does not modify structural plasticity nor reactive microglia in the mesocorticolimbic system. (A) Percentage of body weight increase per week in all experimental groups. Doted bar determines the beginning of the treatment with vehicle or minocycline for 14 consecutive days (30 mg/kg, i.p., once daily) (n= 8-35 per experimental group). (B) D-amphetamine-induced locomotor effects of mice with access to CD only 24h a week (BINGE) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day; n = 6-13 per group). Mice were habituated for 3 days receiving vehicle injections and then 5 consecutive days with d-amphetamine (2 mg/kg; i.p.). After 6 days

of withdrawal mice were challenged with a last amphetamine injection (2 mg/kg; i.p.). Data are expressed as mean + SEM (C) Quantification of total dendritic spine densities (number/10 μm) in medium spiny neurons (MSN) from the dorsal striatum (DS) i), nucleus accumbens (NAc) core ii) and shell iii) of mice fed with standard food (CONTROL) and with access to CD only 24h a week (BINGE) and treated with vehicle or minocycline for 14 days (30 mg/kg, i.p., once a day) after week 4. Data represent the average of 4-7 dendrites per animal (n = 4–8 mice per experimental group) and brain area. No more than 3 dendrites were evaluated from the same neuron. Data are expressed as mean + SEM. (D) Morphological analysis of IBA1+ cells, quantification of the perimeter of the soma i) and total length of the branches ii) in NAc core, and quantification of the perimeter of the soma iii) and total lenght of the branches iv) in NAc shell of mice fed with access to CD only 24h a week (BINGE) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day). Data represent the average of 5-28 cells per animal; n = 5-8 mice per experimental group and brain area analyzed. Data are expressed as mean + SEM.

FIGURE 5: Prolonged exposure to free-choice CD access increases the expression of neuroinflammatory factors and activates microglia in the NAc, which was reverted by chronic minocycline treatment. (A) Quantitative RT-PCR analysis of the proinflammatory genes **i)** IL1β, **ii)** IL6, **iii)** IFNγ and **iv)** IL18 in the Nucleus Accumbens (NAc) of mice fed with free-choice cafeteria diet access (FC) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day) (n = 6-10 mice per group). **(B)** Morphological analysis of $IBA1^+$ cells, quantification of the perimeter of the soma **i)** and total lenght of the branches **ii)** in NAc core, and quantification of the perimeter of

the soma **iii)** and total lenght of the branches **iv)** in NAc shell of mice fed with free-choice cafeteria diet access (FC) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day). Data represent the average of 11-28 cells per animal; n = 5-7 mice per experimental group and brain area analyzed. Data are expressed as mean + SEM. ★★p < 0.01, ★★★p < 0.001 (CONTROL versus FC diet, Fisher's F-test); ☆☆p < 0.01, ☆☆☆p < 0.001 (vehicle versus minocycline, Fisher's F-test). Scale bar 40μm.

<u>FIGURE 6:</u> Prolonged exposure to free-choice CD access led to altered response to d-amphetamine hyperlocomotor effects, which was reverted by minocycline chronic treatment. D-amphetamine-induced locomotor effects of mice fed with free-choice cafeteria diet access (FC) or standard food diet (CONTROL) and treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day; n = 8-13 per group). Mice were habituated for 3 days receiving vehicle injections and then 5 consecutive days with d-amphetamine (2 mg/kg; i.p.). After 6 days of withdrawal mice were challenged with a last amphetamine injection (2 mg/kg; i.p.). Data are expressed as mean + SEM. ★★p < 0.01 (comparison CONTROL versus FC diet, Fisher's F-test); ☆p < 0.05 (vehicle versus minocycline, Fisher's F-test); ###p < 0.001 (1st amphetamine injection versus challenge, Fisher's F-test.

Supplementary experimental procedures

Ballistic labeling with the fluorescent dye DiI

On day 43, mice received an i.p. injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and rapidly perfused intra-cardiacally using a peristaltic pump delivering at 20 ml/min. Ten ml of $Na_2HPO_4/NaH_2PO_4/NaCl$ buffer (PBS) 0.1 M (pH 7.5) were initially perfused followed by 40 ml of 4 % paraformaldehyde (PFA) in PBS 0.1 M (pH 7.5). Brains were quickly removed from the skull and postfixed in 4 % PFA for 10 min. Brain coronal sections of 100 µm depth containing the NAc and DS (George Paxinos, 2001) were obtained by using a vibratome (Leica VT 1000 S, Nussloch, Germany) and kept in crioprotector solution containing glycerol at -20ºC until processed for fluorescent labeling.

Brain slices were labeled by ballistic delivery of fluorescent dye DiI (Molecular Probes, Eugene, OR, USA) using a gene gun apparatus (Helios Gene Gun System, Bio-Rad, Deutschland), as described previously (Grutzendler *et al*, 2003), and postfixed with PFA for 24 h at room temperature to further preserve structures and to allow the diffusion of the dye DiI. Sections were placed on microscope gelatine-coated slides and coverslipped with Mowiol mounting medium (Sigma-Aldrich, Sant Luis, Misuri, USA). Then, images were acquired with a confocal microscope (Leica SP5) using glicerol immersion lens (63x) and a 3.0 zoom to analyze dendritic spine density and structure.

RNA extraction and reverse transcription

Tissues corresponding to the different areas were collected and stored at –80°C. Isolation of total RNA was performed using an RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The quality of the total RNA

was assessed by the spectrophotometric ratio of A260/A280 (1.7:2.3). Total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) with 10 ng of total RNA from each animal to produce cDNA in a 20 µl reaction volume according to the manufacturer's instructions. Reverse transcriptase reactions were carried out at 25°C for 10 min, then 120 min at 37°C, and 5 min at 85°C. The resulting cDNAs tissues were diluted 1:2 and stored at –20°C until use.

Quantitative Real-Time PCR analysis

Real-time PCR was carried out with a LightCycler 480 (Roche, Basilea, Switzerland) using the SYBR Green PCR Master Mix (Roche, Basilea, Switzerland) according to the standard manufacturer's protocol. All the samples were tested in triplicate, and the relative expression values were normalized to the expression value of GAPDH. The following primers specific for mouse were used as endogenous housekeeping controls to standardize the amount of target cDNA:

IL-1β (sense, 5′-GAAGAGCCCATCCTCTGTGACT-3′;

antisense, 5′-GTTGTTCATCTCGGAGCCTGTAG-3′);

IL-6 (sense, 5′- ACCCCGGAAGGGAGACTTAC -3′;

antisense, 5′- GTGGGCATGTAGCTGACCATAC -3′);

IFN-γ (sense, 5′-GCTGATGGGAGGAGATGTCTACA -3′;

antisense, 5′-TTTCTTTCAGGGACAGCCTGTT -3′);

IL-18 (sense, 5′-CAGACAACTTTGGCCGACTTC -3′;

antisense, 5′-ATATCCTCGAACACAGGCTGTCTT -3′); and, finally,

Gapdh (sense, 5′-ATGACTCCACTCACGGCAAAT-3′;

antisense, 5′-GGGTCTCGCTCCTGGAAGAT-3′).

Samples were analyzed by the ΔΔCt method. ΔΔCt values were calculated as the ΔCt of each test sample (different pharmacological treatments and diets) minus the mean ΔCt of the calibrator samples (CONTROL vehicle group) for all the genes analyzed. The fold change was calculated using the equation $2^{(-\Delta\Delta Ct)}$.

Iba-1 Immunofluorescence

After pharmacological treatment and/or behavioural testing, mice received an i.p. injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and rapidly perfused intra-cardiacally using a peristaltic pump delivering at 20 ml/min. Ten ml of Na2HPO4/NaH2PO4/NaCl buffer (PBS) 0.1 M (pH 7.5) were initially perfused followed by 40 ml of 4 % paraformaldehyde (PFA) in PBS 0.1 M (pH 7.5). Brains were quickly removed from the skull and postfixed in 4 % PFA for 10 min. Brain sections (30-µm) were obtained with a microtome (Leica, Wetzlar, Germany) and kept in crioprotector solution containing glycerol at -20ºC until processed for immunofluorescence analysis.

Free-floating slices were rinsed in PB 0.1M, incubated for 2 h in blocking solution containing 0.3% Triton X-100, 3% Normal Goat Serum in PB 0.1M, and then incubated overnight at 4°C with the same solution and anti-IBA1 (1:500) primary antibody (Wako Pure Chemical Industries, Osaka, Japan). The next day (16 h later), after 3 rinses in PB 0.1M, sections were incubated for 2 h at room temperature with fluorescent anti–rabbit-Alexa455 1:300 (Invitrogen, Carlsbad, California, USA) in blocking solution. After three 10-min washes, tissue sections were mounted onto gelatine-coated slides with Mowiol (Sigma-Aldrich, Sant Luis, Missouri, USA).

References

George Paxinos KF (2001). *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates*. *London, Acad Press* .

Grutzendler J, Tsai J, Gan W-B (2003). Rapid labeling of neuronal populations by ballistic delivery of fluorescent dyes. *Methods* **30**: 79–85.

Supplementary Figure legends

Experimental scheme: Schematic diagram showing the experimental design followed in the study. The number of animals per experimental group on each session that were used for neuroplasticity and Iba1 immunofluorescence studies, for the evaluation of neuroinflammatory markers and for amphetamine-induced locomotor effects are also indicated.

SUPPL. FIGURE 1: Intermittent exposure to CD produced no alterations in spine subtype densities in the mesocorticolimbic system that were not affected after chronic minocycline treatment. Quantification of the density (number/10 μm) of mushroom i), thin ii), stubby iii) and filopodia iv) dendritic spines in medium spiny neurons (MSN) from the dorsal striatum (A), nucleus accumbens (NAc) core (B) and shell (C) of BINGE and CONTROL mice treated with vehicle or minocycline during the last 14 days of diet exposure (30 mg/kg, i.p., once a day). Data represent the average of 4-7 dendrites per animal (n = 4–8 mice per experimental group) and brain area. No more than 3 dendrites were evaluated from the same neuron. Data are expressed as

mean + SEM. In the DS two-way ANOVA revealed no significant differences in mushroom ($F_{(1,19)}$ = 0.0032, n.s.), thin ($F_{(1,19\ )}$ = 0.0073, n.s.), stubby ($F_{(1,19)}$ = 0.9426, n.s.) and filopodia ($F_{(1,19)}$ = 0.5675, n.s.) between experimental groups. In the NAc core two-way ANOVA revealed no significant differences in mushroom ($F_{(1,20)}$ = 1.8260, n.s.), thin ($F_{(1,20)}$ = 3.5504, n.s.), stubby ($F_{(1,20)}$ = 3.7052, n.s.) and filopodia ($F_{(1,20)}$ = 3.4789, n.s.) between experimental groups. Moreover, in the NAc shell two-way ANOVA revealed no significant differences in mushroom ($F_{(1,20)}$ = 1.1940, n.s.), thin ($F_{(1,20)}$ = 1.1519, n.s.), stubby ($F_{(1,20)}$ = 2.4638, n.s.) and filopodia ($F_{(1,20)}$ = 0.2338, n.s.) between experimental groups.

SUPPL. FIGURE 2: Mice chronically treated with saline from the CONTROL, FC and BINGE experimental groups presented no alterations in the total dendritic spine density in mPFC pyramidal neurons. Quantification of total dendritic spine densities (number/10 μm) in mPFC pyramidal neurons. Data represent the average of 4-7 dendrites per animal; (n = 4-5 mice per experimental group). No more than 3 dendrites were evaluated from the same neuron. Data are expressed as mean + SEM. One-way ANOVA revealed no difference in between experimental groups.

Table 1

Three-way ANOVA for intake measurements included in Figure 1A

	Intake					
	Diet	*p*-value	Treatment	*p*-value	Weeks	*p*-value
	F(1,93)= 169.446	0.000	F(1,93)= 9.902	0.002	F(5, 465)= 56.448	0.000
Interaction F X T	F(1,93)= 3.964	0.050				
Interaction W X F	F(5,465)= 6.769	0.000				
Interaction W X T	F(5,465)= 14.576	0.000				
Interaction W X F X T	F(5,465)=5.913	0.000				

F = diet; T= treatment, W= week; n.s. = not significant

Three-way ANOVA for weight increase measurements included in Figure 1B

	Weight increase					
	Diet	*p*-value	Treatment	*p*-value	Weeks	*p*-value
	F(1,93)= 35.378	0.000	F(1,93)= 0.175	n.s.	F(5, 465)= 182.331	0.000
Interaction F X T	F(1,93)= 0.469	n.s.				
Interaction W X F	F(5,465)= 52.428	0.000				
Interaction W X T	F(5,465)= 76.005	0.000				
Interaction W X F X T	F(5,465)=22.212	0.000				

F = diet; T= treatment, W= week; n.s. = not significant

Table 2

Two-way ANOVA for spine densities measurements included in Figure 2 and 3

		Spine density					
		Diet	*p*-value	Treatment	*p*-value	Interaction	*p*-value
dorsal striatum							
	Total	F(1,22)= 0.101	n.s.	F(1,22)= 0.789	n.s.	F(1,22)= 0.077	n.s.
	Mushroom	F(1,22)= 7.030	0.01	F(1,22)= 1.008	n.s.	F(1,22)= 1.186	n.s.
	Thin	F(1,22)= 0.017	n.s.	F(1,22)= 0.480	n.s.	F(1,22)= 0.345	n.s.
	Stubby	F(1,22)= 0.000	n.s.	F(1,22)= 0.014	n.s.	F(1,22)= 0.296	n.s.
	Filopodia	F(1,22)= 0.009	n.s.	F(1,22)= 0.914	n.s.	F(1,22)= 0.006	n.s.
NAc core							
	Total	F(1,21)= 3.245	0.09	F(1,21)= 0.756	n.s.	F(1,21)= 7.533	0.01
	Mushroom	F(1,21)= 0.485	n.s.	F(1,21)= 0.626	n.s.	F(1,21)= 11.940	0.00
	Thin	F(1,21)= 5.048	0.04	F(1,21)= 1.172	n.s.	F(1,21)= 6.012	0.02
	Stubby	F(1,21)= 0.208	n.s.	F(1,21)= 0.029	n.s.	F(1,21)= 1.969	n.s.
	Filopodia	F(1,21)= 0.533	n.s.	F(1,21)= 0.199	n.s.	F(1,21)= 1.766	n.s.
NAc shell							
	Total	F(1,20)= 0.189	n.s.	F(1,20)= 4.197	0.05	F(1,20)= 6.514	0.02
	Mushroom	F(1,20)= 0.218	n.s.	F(1,20)= 0.528	n.s.	F(1,20)= 5.674	0.03
	Thin	F(1,20)= 0.035	n.s.	F(1,20)= 3.602	0.07	F(1,20)= 5.347	0.03
	Stubby	F(1,20)= 0.009	n.s.	F(1,20)= 1.323	n.s.	F(1,20)= 0.312	n.s.
	Filopodia	F(1,20)= 0.618	n.s.	F(1,20)= 0.393	n.s.	F(1,20)= 1.257	n.s.

Table 3

Three-way ANOVA for weight increase measurements included in Figure 4A

	Weight increase					
	Diet	*p*-value	Treatment	*p*-value	Weeks	*p*-value
	F(1,76)= 0.455	n.s.	F(1,76)= 0.936	n.s.	F(5, 380)= 46.592	0.000
Interaction F X T	F(1,76)= 1.495	n.s.				
Interaction W X F	F(5,380)= 0.086	n.s				
Interaction W X T	F(5,380)= 24.196	0.000				
Interaction W X F X T	F(5,380)=0.477	n.s				

F = diet; T= treatment, W= week; n.s. = not significant

Two-way ANOVA for spine densities measurements included in Figure 4B

	Spine density					
	Diet	*p*-value	Treatment	*p*-value	Interaction	*p*-value
dorsal striatum						
Total	F(1,19)= 1.318	n.s.	F(1,19)= 3.149	n.s.	F(1,19)= 0.350	n.s.
NAc core						
Total	F(1,20)= 0.508	n.s.	F(1,20)= 0.024	n.s.	F(1,20)= 0.4.888	0.04
NAc Shell						
Total	F(1,20)= 0.067	n.s.	F(1,20)= 0.945	n.s.	F(1,20)= 2.060	n.s.

Table 4

Two-way ANOVA for neuroinflammatory factors expression measurements included in Figure 5A

	Expression of neuroinflammatory factors					
	Diet	*p*-value	Treatment	*p*-value	Interaction	*p*-value
IFNγ	F(1,26)= 7.306	0.012	F(1,26)= 11.128	0.003	F(1,26)= 6.718	0.015
IL-18	F(1,24)= 0.831	n.s.	F(1,24)= 4.785	0.039	F(1,24)= 1.892	n.s.
IL-1β	F(1,26)= 3.776	0.063	F(1,26)= 0.178	n.s.	F(1,26)= 4.209	0.050
IL-6	F(1,18)= 0.128	n.s.	F(1,18)= 4.321	0.052	F(1,18)= 0.180	n.s.

Two-way ANOVA for microglia morphology measurements included in Figure 5B

		Microglia morphology					
		Diet	*p*-value	Treatment	*p*-value	Interaction	*p*-value
NAc shell							
	Perimeter of the soma	F(1,20)= 16.228	0.001	F(1,20)= 7.029	0.015	F(1,20)= 7.420	0.013
	Lenght of branches	F(1,20)= 5.466	0.030	F(1,20)= 4.413	0.049	F(1,20)= 2.594	n.s.
NAc core							
	Perimeter of the soma	F(1,20)= 4.168	0.055	F(1,20)= 5.005	0.037	F(1,20)= 6.019	0.023
	Lenght of branches	F(1,20)= 5.492	0.030	F(1,20)= 6.311	0.021	F(1,20)= 2.311	n.s.

Table 5

Three-way ANOVA for d-amphetamine-induced locomotor effects included in Figure 6

	Locomotor effects of amphetamine					
	Diet	*p*-value	Treatment	*p*-value	Days	*p*-value
	F(1,30)= 6.565	0.016	F(1,30)= 0.845	n.s.	F(8,240)= 114.286	0.000
Interaction F X T	F(1,30)= 0.025	n.s.				
Interaction D X F	F(8,240)= 1.555	n.s.				
Interaction D X T	F(8,240)= 1.642	n.s.				
Interaction D X F X T	F(8,240)= 1.264	n.s.				

F = diet; T= treatment, D= days; n.s. = not significant

Figure 1

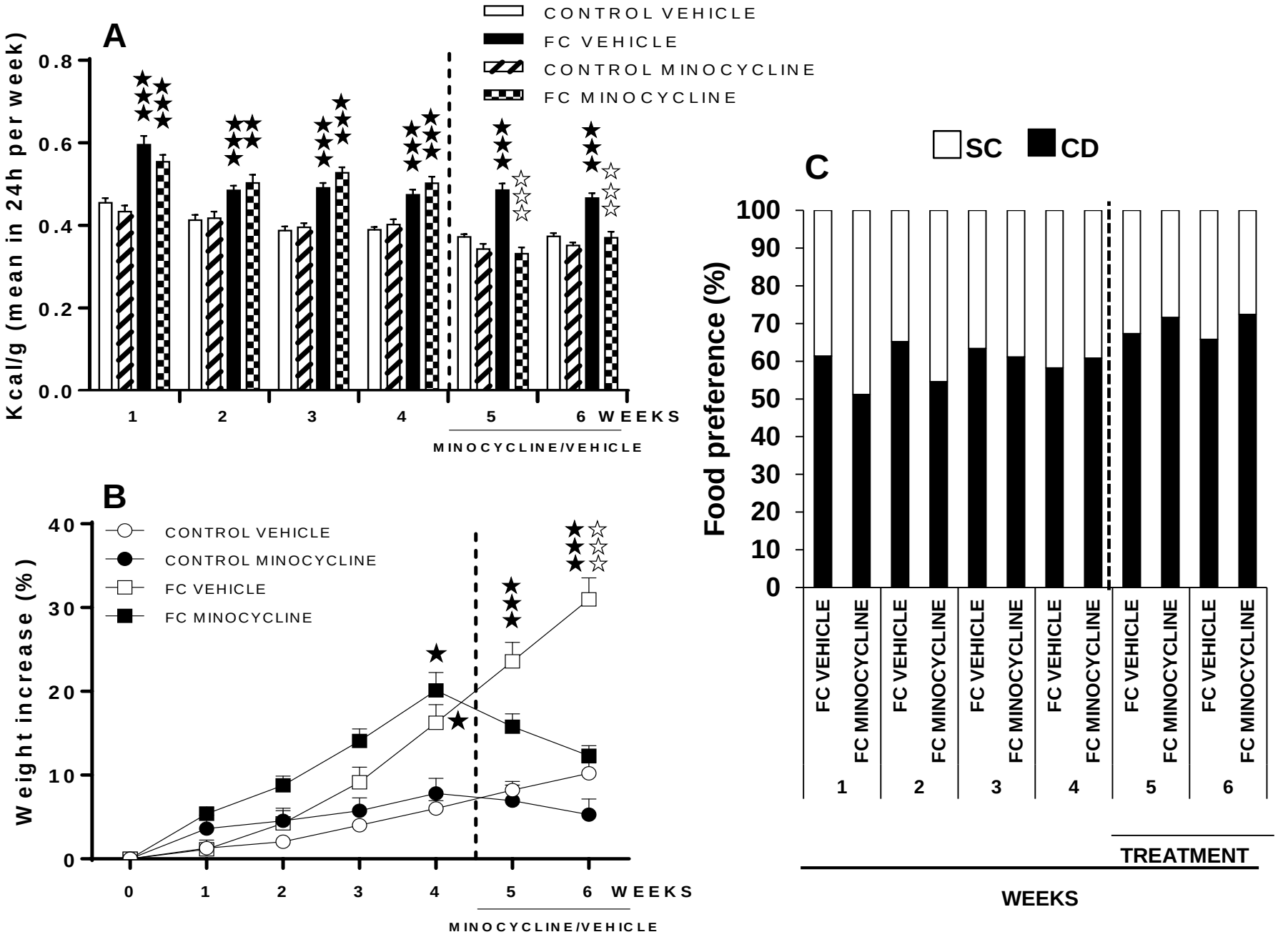

A
CONTROL VEHICLE
FC VEHICLE
CONTROL MINOCYCLINE
FC MINOCYCLINE
Kcal/g (mean in 24h per week)
0.0
0.2
0.4
0.6
0.8
1
2
3
4
5
6 WEEKS
MINOCYCLINE/VEHICLE
B
CONTROL VEHICLE
CONTROL MINOCYCLINE
FC VEHICLE
FC MINOCYCLINE
Weight increase (%)
0
10
20
30
40
0
1
2
3
4
5
6 WEEKS
MINOCYCLINE/VEHICLE
C
SC
CD
Food preference (%)
0
10
20
30
40
50
60
70
80
90
100
FC VEHICLE
FC MINOCYCLINE
1
2
3
4
5
6
TREATMENT
WEEKS

Figure 2

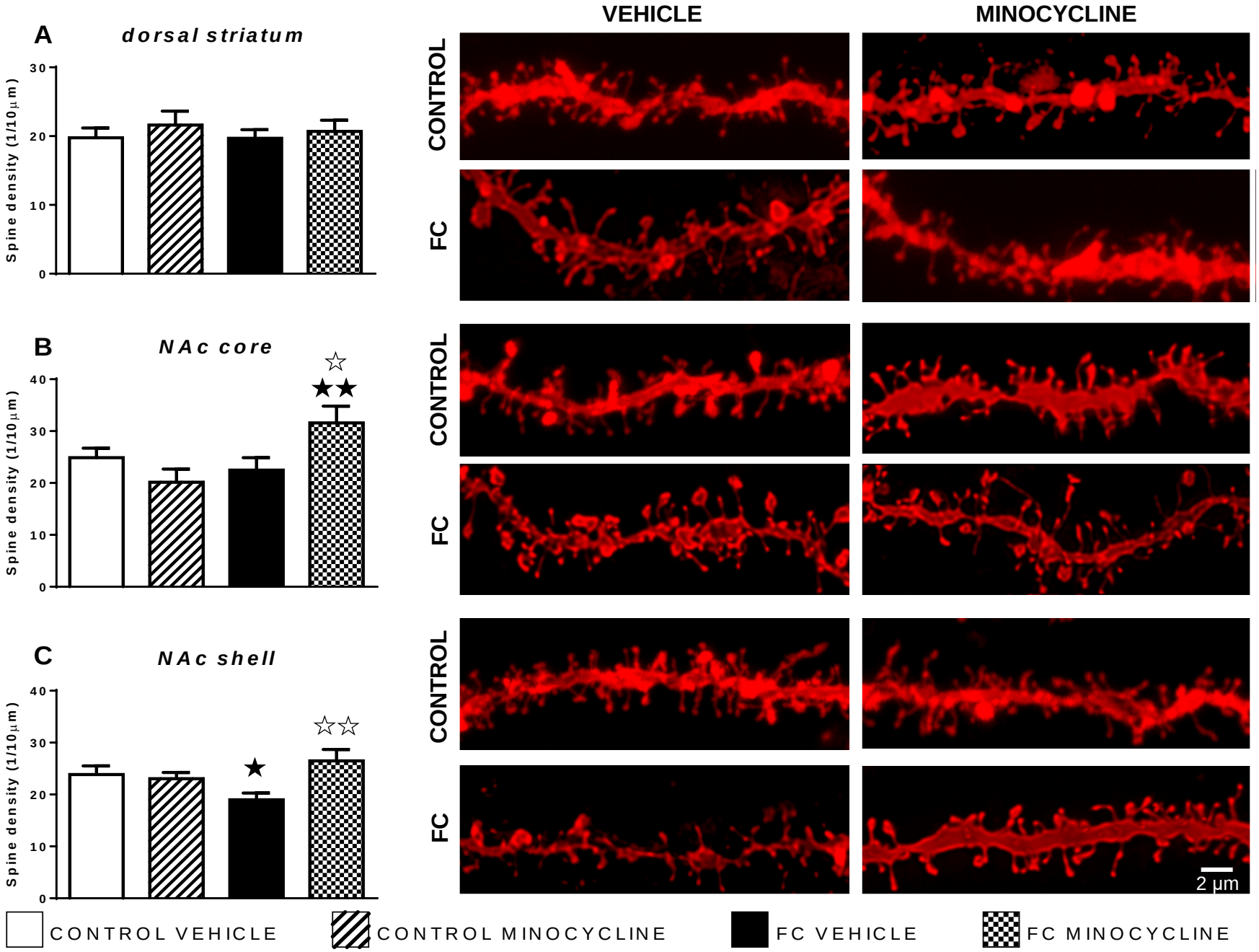

A
dorsal striatum
Spine density (1/10μm)
30
20
10
0
B
NAc core
40
30
20
10
0
C
NAc shell
VEHICLE
MINOCYCLINE
CONTROL
FC
2 μm
CONTROL VEHICLE
CONTROL MINOCYCLINE
FC VEHICLE
FC MINOCYCLINE

Figure 2

Gutiérrez-Martos M

CD induces neuroplastic modifications in the NAc mediated by microglia activation

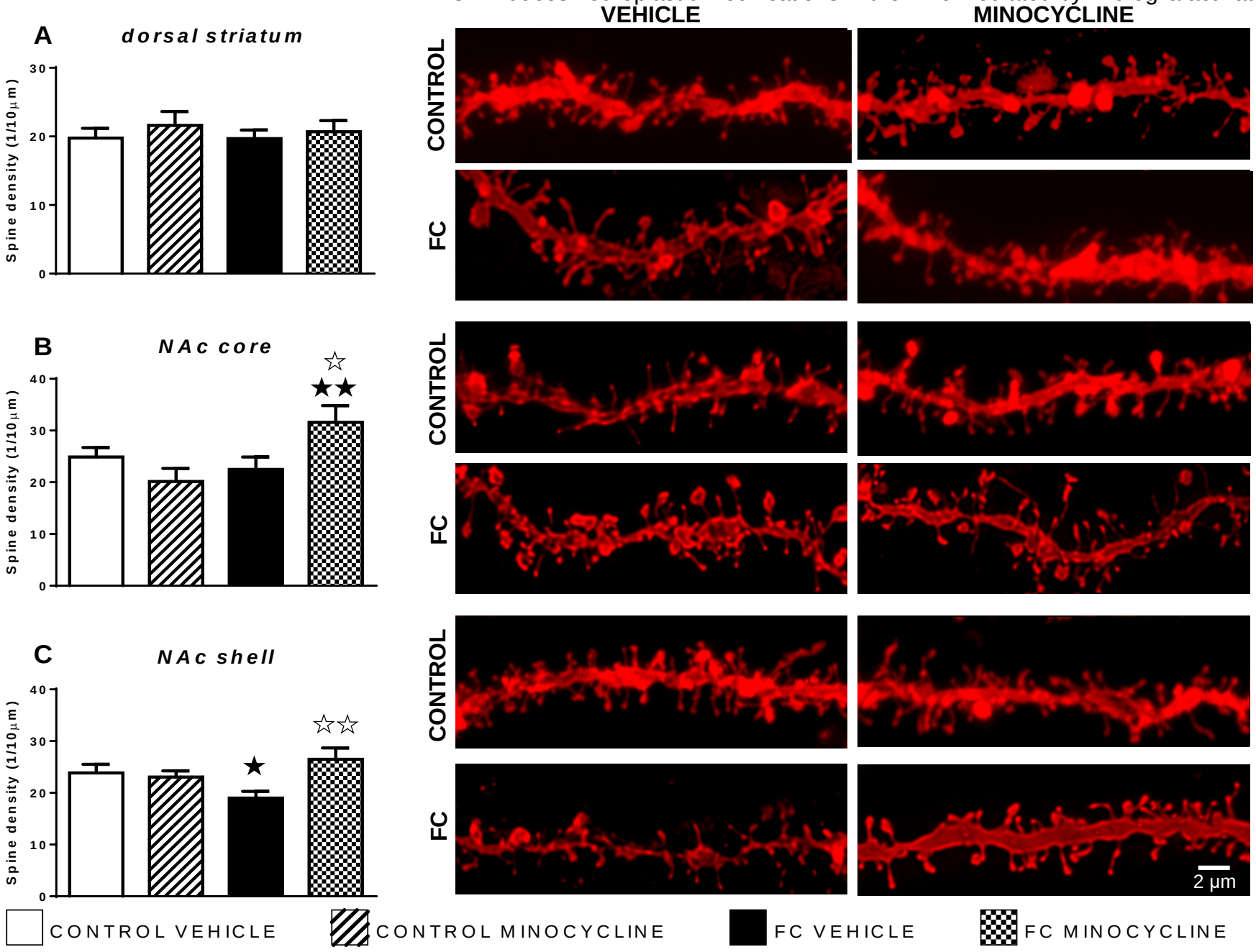


Figure 3

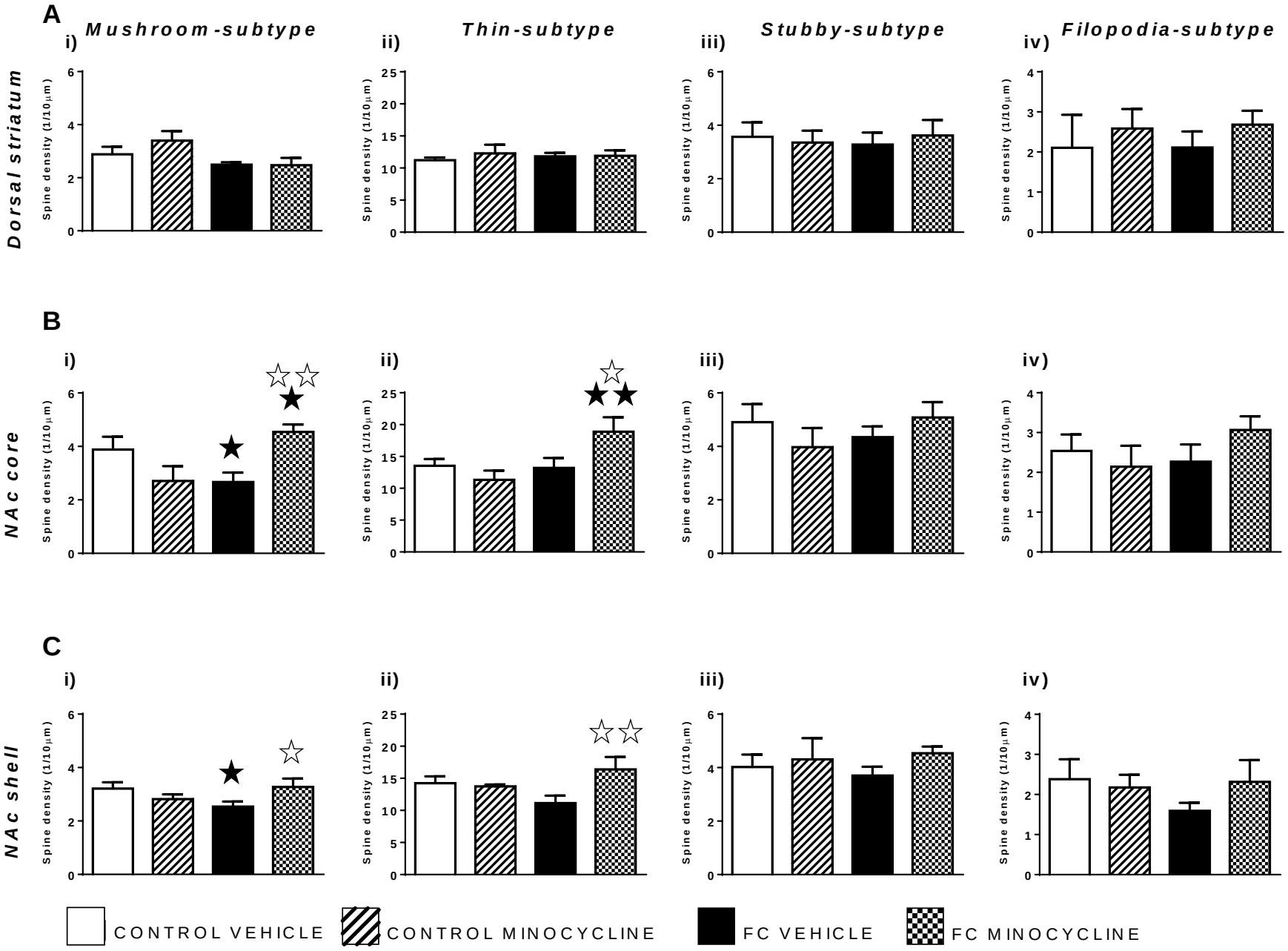

A
Mushroom-subtype
Thin-subtype
Stubby-subtype
Filopodia-subtype
i)
ii)
iii)
iv)
Dorsal striatum
B
NAc core
C
NAc shell
Spine density (1/10μm)
CONTROL VEHICLE
CONTROL MINOCYCLINE
FC VEHICLE
FC MINOCYCLINE

Figure 4

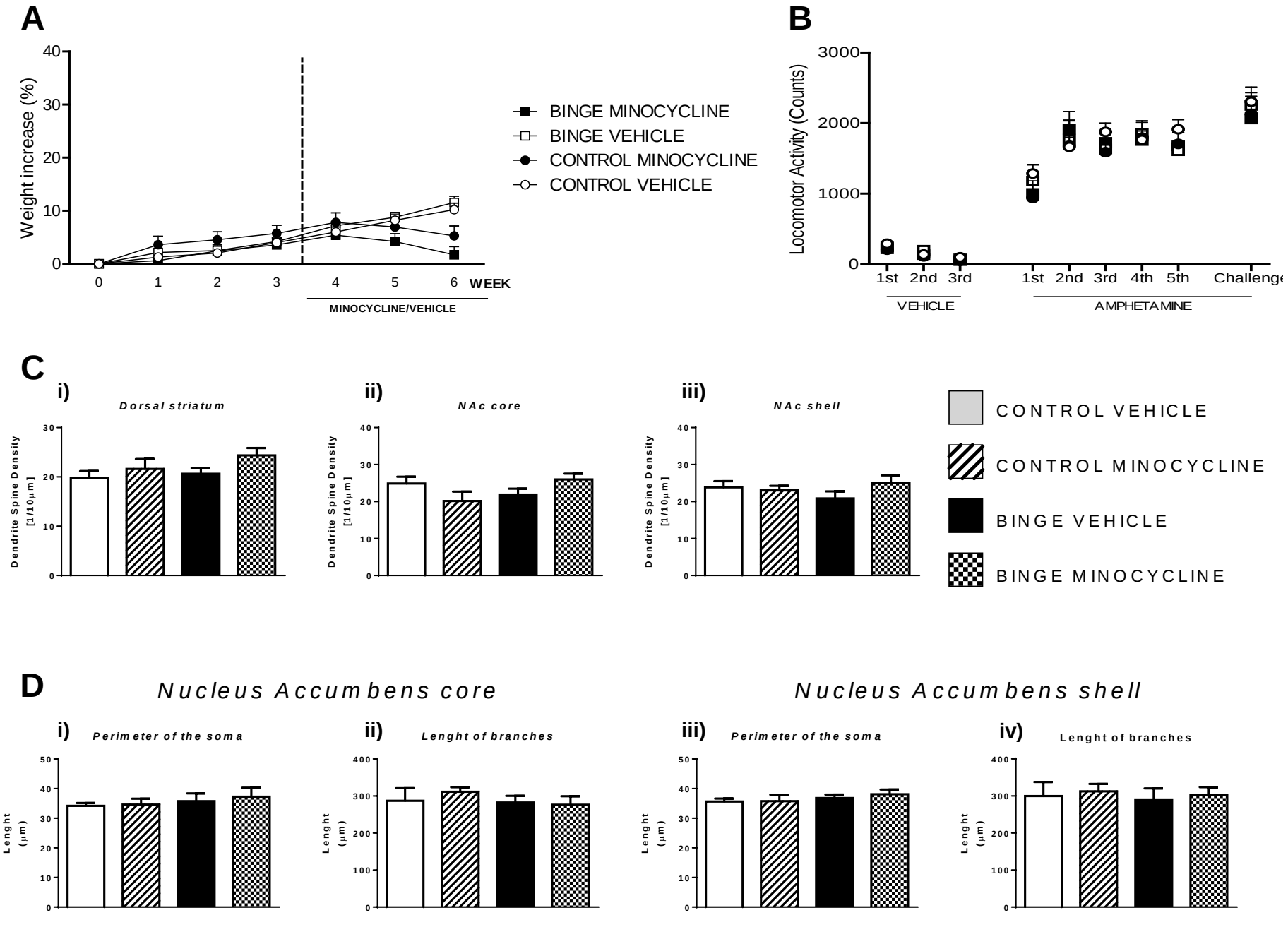

A
Weight increase (%)
40
30
20
10
0
0
1
2
3
4
5
6
WEEK
MINOCYCLINE/VEHICLE
BINGE MINOCYCLINE
BINGE VEHICLE
CONTROL MINOCYCLINE
CONTROL VEHICLE
B
Locomotor Activity (Counts)
3000
2000
1000
0
1st
2nd
3rd
1st
2nd
3rd
4th
5th
Challenge
VEHICLE
AMPHETAMINE
C
i)
Dorsal striatum
ii)
NAc core
iii)
NAc shell
Dendrite Spine Density [1/10µm]
CONTROL VEHICLE
CONTROL MINOCYCLINE
BINGE VEHICLE
BINGE MINOCYCLINE
D
Nucleus Accumbens core
Nucleus Accumbens shell
i)
Perimeter of the soma
ii)
Lenght of branches
iii)
Perimeter of the soma
iv)
Lenght of branches
Lenght (µm)

Figure 5

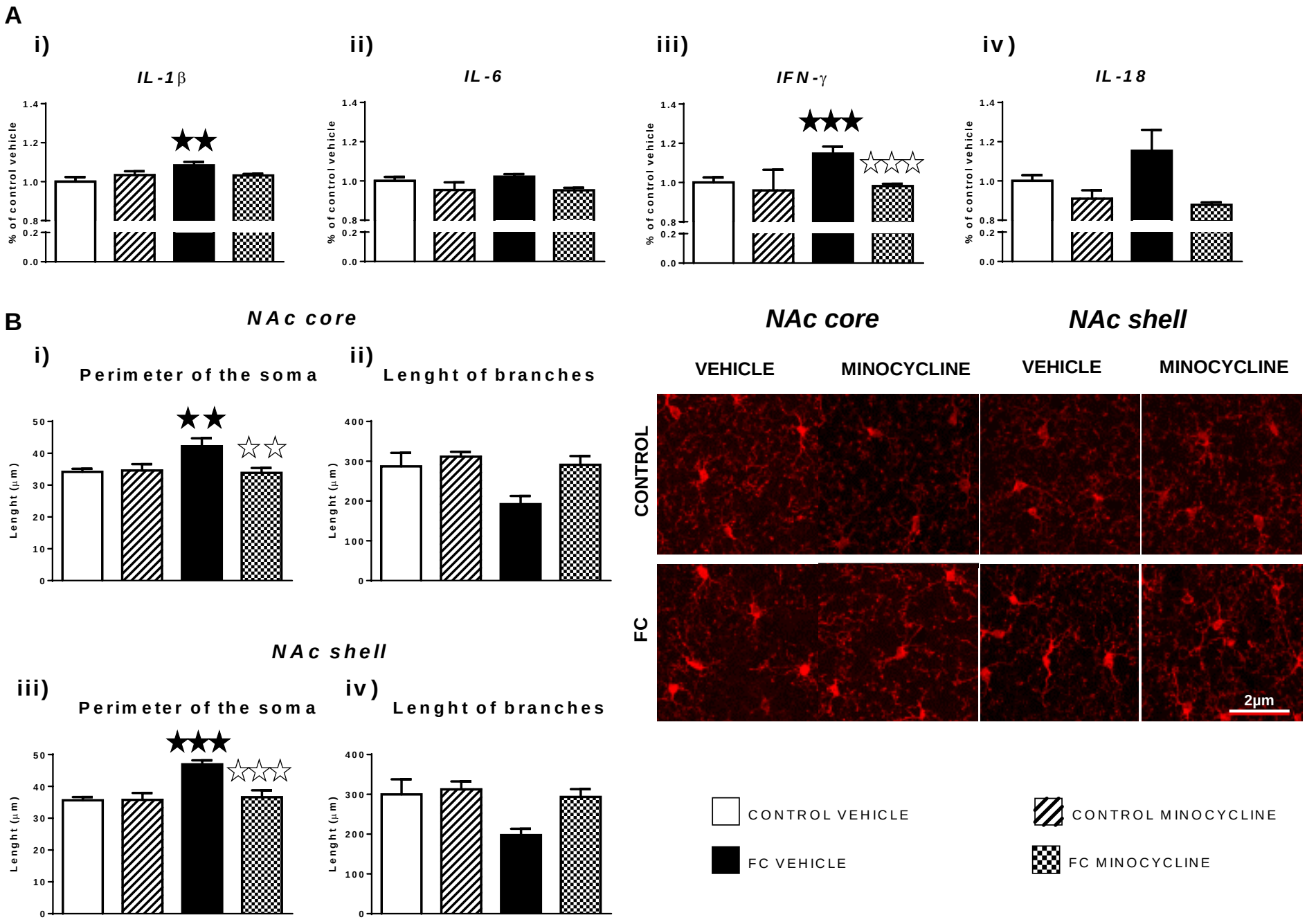
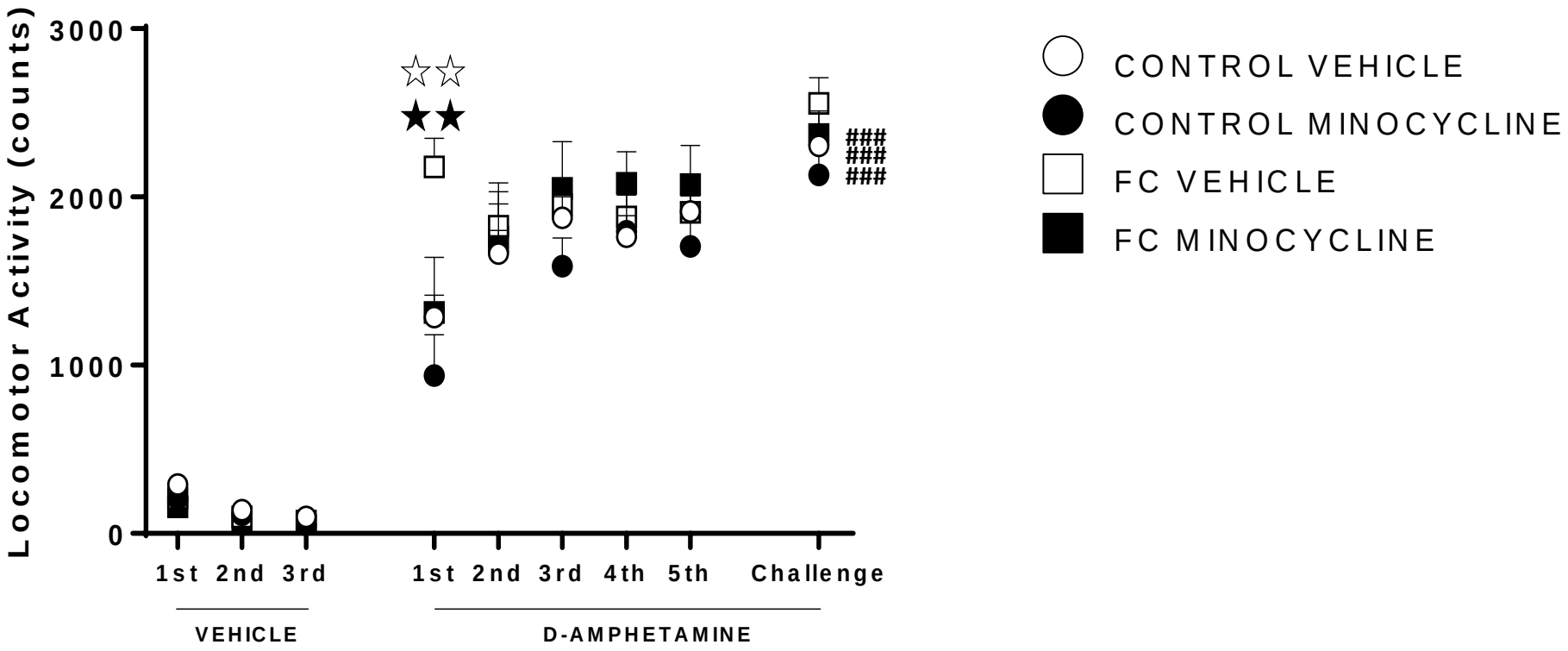
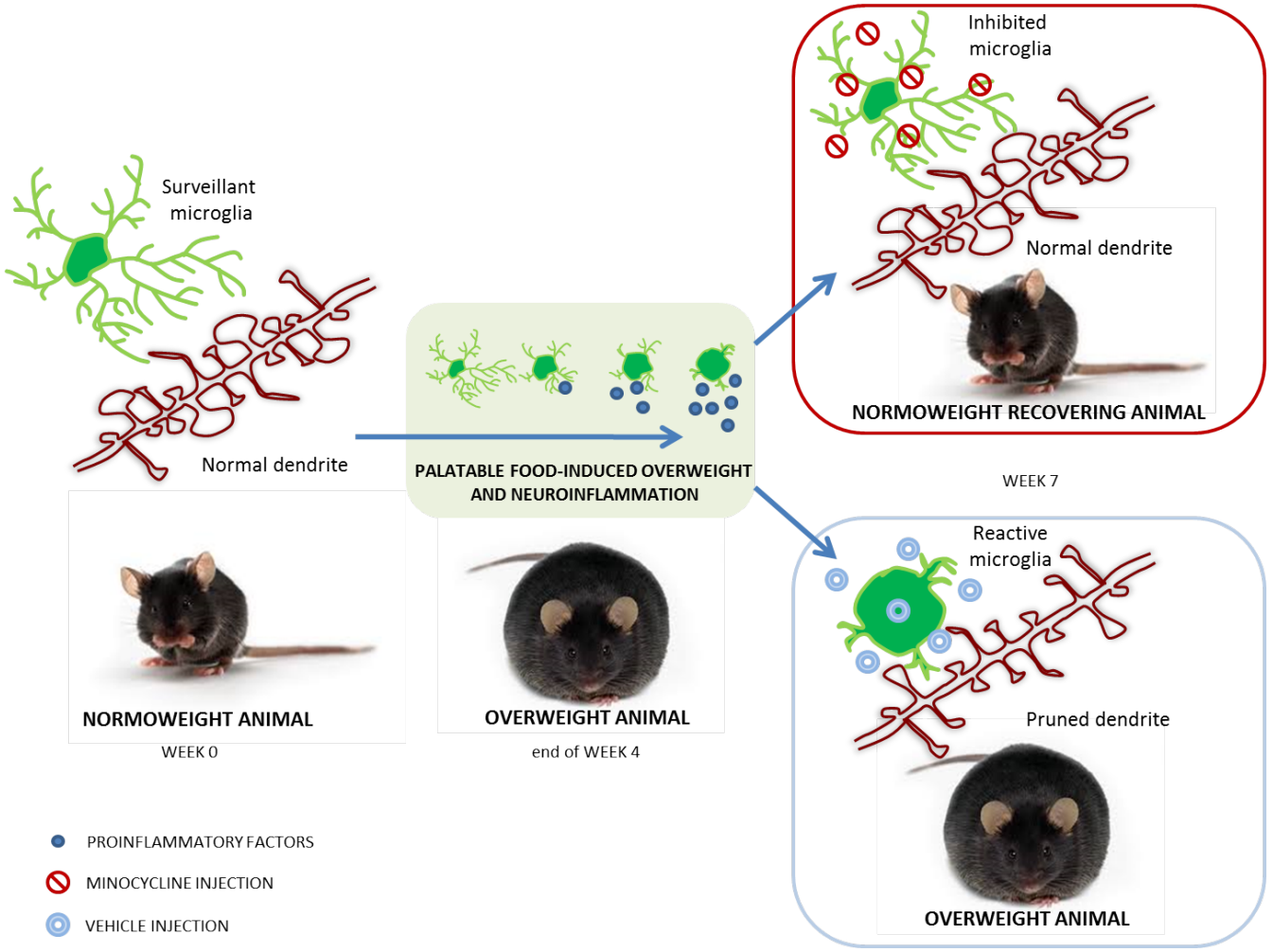

A
i)
IL-1β
ii)
IL-6
iii)
IFN-γ
iv)
IL-18
% of control vehicle
B
NAc core
i)
Perimeter of the soma
ii)
Lenght of branches
Lenght (μm)
NAc shell
iii)
Perimeter of the soma
iv)
Lenght of branches
NAc core
NAc shell
VEHICLE
MINOCYCLINE
VEHICLE
MINOCYCLINE
CONTROL
FC
2μm
CONTROL VEHICLE
CONTROL MINOCYCLINE
FC VEHICLE
FC MINOCYCLINE

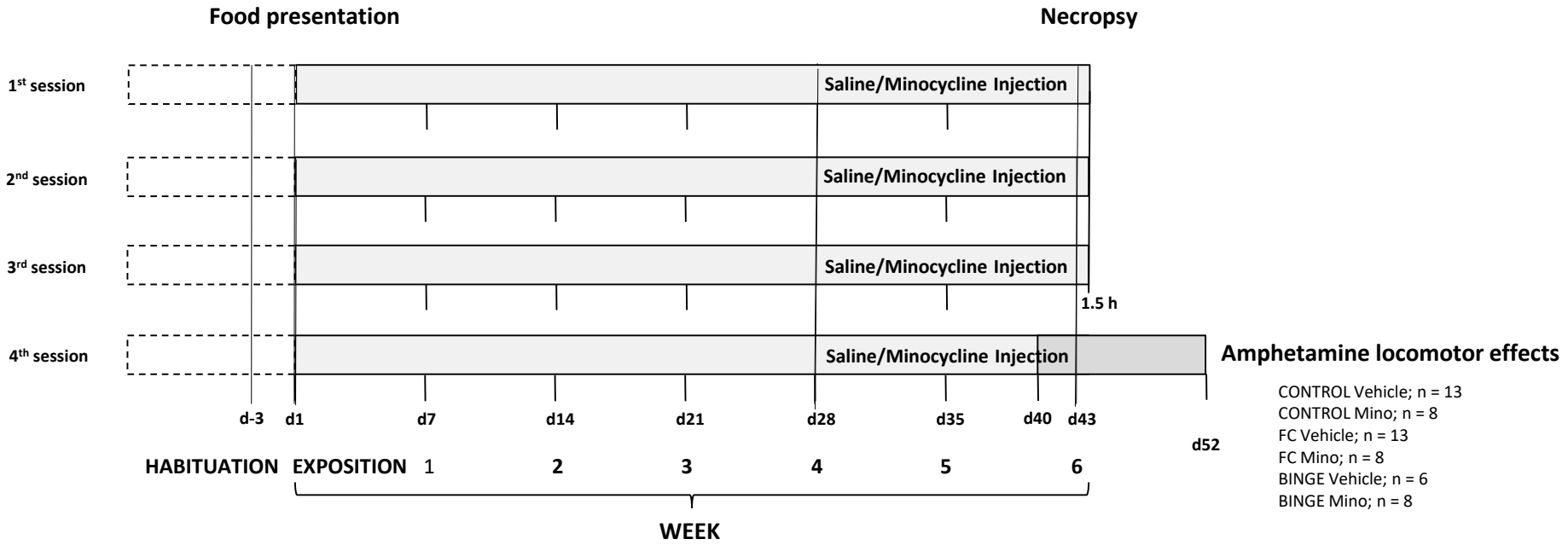
Figure 6


Locomotor Activity (counts)
3000
2000
1000
0
1st 2nd 3rd
1st 2nd 3rd 4th 5th
Challenge
VEHICLE
D-AMPHETAMINE
☆☆
★★
###
###
###
CONTROL VEHICLE
CONTROL MINOCYCLINE
FC VEHICLE
FC MINOCYCLINE



- Prolonged exposure to high caloric cafeteria diet leads to overweight and alterations in structural plasticity, neuroinflammation and functionality of the nucleus accumbens.
- Inhibition of microglial activities in overweight mice normalized these neurobiological alterations and weight gain.

Supplementary Experimental scheme.



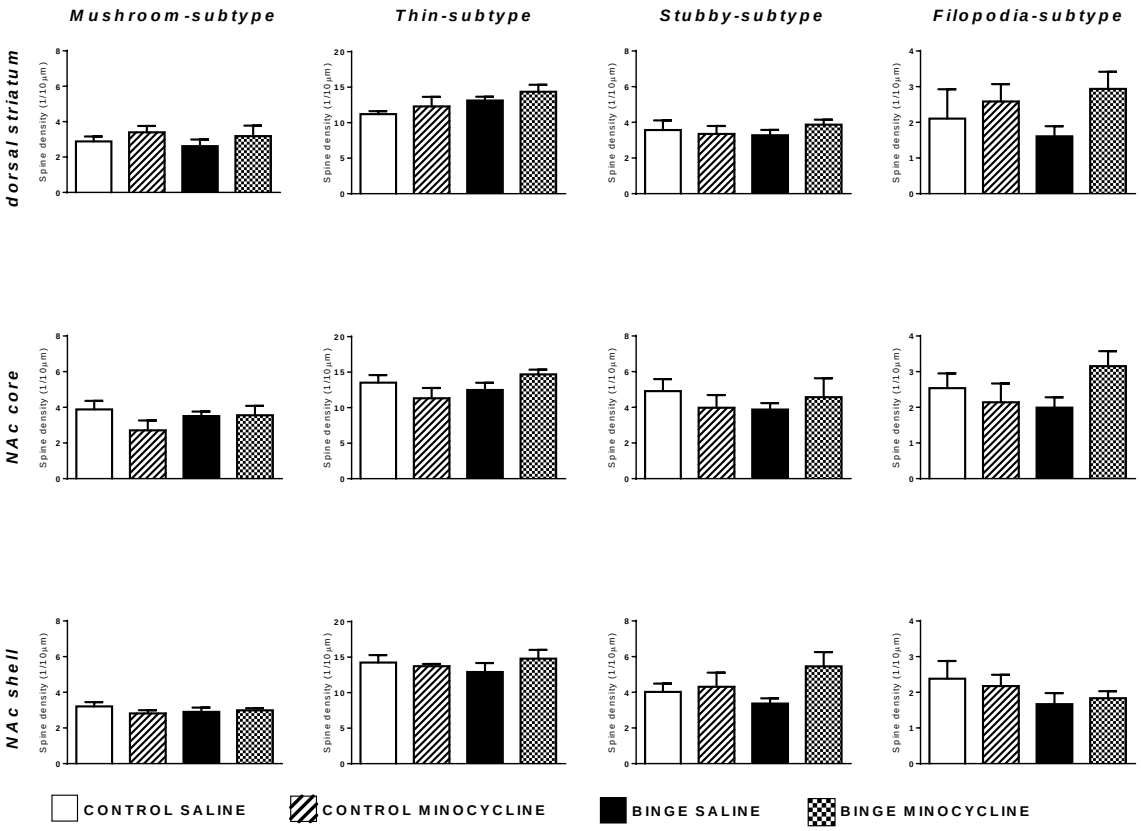
Plasticity and Iba1 quantification

	1st SESSION	2nd SESSION	3rd SESSION
Ctrol SALINE	3	2	2
Ctrol MINO	3	3	0
FC SALINE	5	2	2
FC MINO	2	3	0
BINGE SALINE	5	2	1
BINGE MINO	1	3	0

Neuroinflammatory markers quantification

	1st SESSION	2nd SESSION	3rd SESSION
Ctrol SALINE	3	3	4
Ctrol MINO	3	2	1
FC SALINE	3	2	3
FC MINO	2	2	2

Supplementary figure 1



Supplementary figure 2