ORIGINAL ARTICLE

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Disruption of leptin signalling in a mouse model of Alzheimer's disease

Anna King[1] · Anna Brain[1] · Kelsey Hanson[1] · Justin Dittmann[1] · James Vickers[1] · Carmen Fernandez-Martos[1]

Abstract

Disruption of leptin signalling has been implicated as playing a role in the development of Alzheimer's disease (AD). Leptin has previously been shown to be affected by amyloid-beta (Aβ)-related signalling; however, pathways that link leptin to the disease pathogenesis have not been determined. To characterize the association between increasing age-dependent Aβ levels with leptin signalling and the vulnerable brain regions in AD, we assessed the mRNA and protein expression profile of leptin and leptin receptor (Ob-Rb) at 9 and 18-month-age in APP/PS1 mice. Immunohistochemical labelling demonstrated that leptin and Ob-Rb proteins were localised to neocortical and hippocampal neurons in APP/PS1 and wildtype (WT) mice. Neuronal leptin and Ob-Rb immunolabelling was more prominent in the neocortex of both groups at 9 month of age, while, at 18 months, labelling was reduced in the hippocampus of APP/PS1 mice relative to WT. Immunoblotting analysis demonstrated decreased hippocampal leptin levels, concomitantly with an increased Ob-Rb levels, in APP/PS1 mice compared with WT controls at 18 month of age. While no *leptin* mRNA was found in either of the groups analysed, *Ob-Rb* mRNA was significantly decreased in the hippocampus of APP/PS1 mice at both ages analysed. In addition, a significant decreased protein kinase B (Akt) activity concomitantly with an upregulation of suppressor of cytokine signaling-3 (*SOCS3*) and protein-tyrosine phosphatase 1B (*PTP1B*) transcripts was present. Thus, these results collectively indicate alterations of leptin signalling in the hippocampus of APP/PS1 mice, providing novel insights about the pathways that could link aberrant leptin signaling to the pathological changes of AD.

Keywords Alzheimer's disease (AD) · Cognitive decline · Amyloid-beta (Aβ) · Leptin · Long leptin receptor (Ob-Rb)

Introduction

Alzheimer's disease (AD), the most common form of ageing-related dementia, is an irreversible neurodegenerative disorder that is characterized by the progressive loss of cognitive function associated with specific pathological changes in the brain (Hyman et al. 1989; Villemagne et al. 2013). With the increasing life span of the global population, AD is fast becoming a major health and socio-economic challenge for many countries, however, no current therapeutic disease-modifying intervention exists. Although the cellular basis for neurodegeneration in AD is

not yet fully understood, numerous studies have shown that the underlying disease process involves multiple complex factors, including metabolic alterations (Chakrabarti et al. 2015; Pedros et al. 2014; Petrov et al. 2015).

With respect to potential metabolic effects on neural systems, leptin is a polypeptide hormone primarily secreted by adipocytes that exerts its main biological function in the brain (Stephens and Basinski 1995; Zhang et al. 1994). Leptin acts by binding to receptors that are structurally related to the cytokine receptor class I family. Alternative splicing generates distinct isoforms of the leptin receptor, including long (Ob-Rb) and short isoforms (Ob-Ra and Ob-Rc-f), however, Ob-Rb is thought to transmit the majority of leptin's biological signals (Friedman and Halaas 1998). In addition to its classical role in the neuroendocrine regulation of food intake, the existence of leptin receptors in extra-hypothalamic brain regions such as the neocortex and hippocampus (Wilkinson et al. 2000) indicates that this hormone affects other biological processes. In the brain, the binding of leptin to the neuronal Ob-Rb receptor activates janus-tyrosine kinase 2 (JAK2), which in turn phosphorylates the insulin receptor substrate-1 and -2

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(IRS 1/2) that results in the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. JAK2 activation also leads to the phosphorylation of two tyrosine residues in the cytoplasmic tail of the Ob-Rb receptor, producing the activation of the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) and the signal transducer and activator of transcription 3 (STAT3) signalling pathways.

Clinical and epidemiological studies support the concept that disrupted leptin metabolism and/or signalling contributes to the pathogenesis of AD (Bonda et al. 2014; Lieb et al. 2009; Power et al. 2001; Rosenbaum et al. 1996; Tezapsidis et al. 2009). It is possible that alterations in leptin signalling in the brain lead to altered leptin levels in AD. However, while new studies indicate a state of insulin resistance in AD (Pedros et al. 2014; Petrov et al. 2015; Watson and Craft 2003), there remains uncertainty about the role of leptin under these disease conditions. In this regard, circulating levels of leptin have been reported to be significantly lower in patients with AD as compared to controls (Power et al. 2001). Recently, Lieb et al. (Lieb et al. 2009) reported that high plasma concentrations of leptin correlate with a significantly lower risk of AD. In contrast, Rajagopalan et al. (Rosenbaum et al. 1996) showed that high leptin levels in plasma correlate with volume loss in several brain regions, regardless of the body mass index. Another recent study reported significant elevation in leptin levels in both the cerebrospinal fluid and hippocampus in AD (Bonda et al. 2014). Notably, upregulation of leptin has been suggested as a potential approach to therapeutic intervention in AD (Tezapsidis et al. 2009).

Moreover, experimental studies demonstrate that alterations in leptin signalling could significantly contribute to the development of ageing-related cognitive decline and impairment of synaptic plasticity (Farr et al. 2006; Harvey 2007; Moult et al. 2009). Indeed, it has been shown that leptin is a potential cognitive enhancer, as genetically obese rodents with dysfunctional Ob-Rb receptors display impairments in hippocampal synaptic plasticity. Direct administration of leptin into the hippocampus can facilitate hippocampal long-term potentiation (LTP) in vivo via enhancing NMDA receptor function (Harvey et al. 2006). Importantly, a growing body of studies have demonstrated leptin's beneficial effect on improving learning and cognitive function in AD (Farr et al. 2006; Sato et al. 2011; Searcy et al. 2012). In fact, the mechanism through which leptin modulates cognitive decline involves mechanism of action closely linked to the regulation of Aβ levels, which highlights its neuroprotective capacities. With respect to this, leptin has a strong in vitro (Fewlass et al. 2004; Greco et al. 2009b, 2008) and in vivo (Fewlass et al. 2004) anti-amyloidogenic effect, attributable to its transcriptional regulation capacity (Niedowicz et al. 2013) and inhibition of amyloid precursor protein (APP) processing (Perez-Gonzalez et al. 2014) through the amyloidogenic pathway. Accordingly, leptin reduces extracellular Aβ protein

in vitro and this effect is dependent on activation of AMPK (Fewlass et al. 2004; Greco et al. 2009b, 2008). In addition, it has been reported that leptin can reduce total brain Aβ1–40 and Aβ1–42 in the Tg2576 mouse model of AD (Fewlass et al. 2004). Importantly, leptin reduces tau phosphorylation in neuronal cells through modulation of GSK-3β activity (Greco et al. 2009a), a protein kinase identified to be pathogenic in a range of neurodegenerative disorders (Koh et al. 2011). Therefore, these studies highlight leptin as an endocrine factor with a potentially important role in AD.

In the current study, biochemical, molecular biology and immunohistochemical techniques were used to examine the status of leptin signalling at 9 and 18 months of age in the APP/PS1 transgenic (Tg) line (Jankowsky et al. 2004). This Tg model of AD expresses mutant human presenilin 1 (PSEN1: deltaE9) and a chimeric mouse/human Aβ precursor protein harbouring the Swedish mutation (APP KM670/ 671NL). The APP/PS1 line demonstrates memory deficits as well as age-dependent Aβ plaque depositions in the brain (Arendash et al. 2001; Holcomb et al. 1998, 1999). Previous studies show that plaque deposition starts at between 4 and 6 months of age in these mice and steadily increasing with age (Jankowsky et al. 2004). Indeed, we detected very limited Aβ deposition around 6 months of age, whereas, from 9 months, plaque density and Aβ burden increased substantially up to 12-months of age (Fernandez-Martos 2017; Stuart et al. 2017). In this context, we examined leptin-related pathways through adulthood and aging to the background of increasing brain Aβ levels in these Tg mice. Our results indicated significant alterations in leptin and Ob-Rb levels in APPP/PS1 mice, corresponding with deregulated leptin-signalling at both 9 and 18 month of age, increasing with ageing. These findings suggest that AD may be associated with alterations in leptin signalling pathways that may result in a leptin resistant state, which could play a critical role to the irreversible cognitive decline and the characteristic pathological changes associated with this disease.

Methods

Animals

Cohorts of young (3 and 6 months age; $n = 5$ per group) and older (9 and 18 months; $n = 10$ per group) male APP/PS1 mice (Jankowsky et al. 2004) and age-matched WT littermates were used in this study. The maintenance and use of mice, and all experimental procedures, were approved by the Animal Ethics Committee of the University of Tasmania (Approval No A12780), in accordance with the Australian Guidelines for the Care and Use of Animals for Scientific Purposes. All analyses were conducted by personnel blinded to the genotype.

Tissue preparation

Animals were terminally anesthetized with sodium pentobarbitone (140 mg/kg) and transcardially perfused with 0.01 M phosphate buffered saline (PBS; pH 7.4) or 4% paraformaldehyde (PFA), in the middle of the light cicle (between 11 AM and 1 PM). Brains were immediately dissected, post-fixed overnight in the same fixative solution and then transferred to 18% then 30% sucrose solutions overnight (Liu et al. 2013). Serial coronal cryosections (40 μm thick) were cut on a cryostat (Leica CM 1850). For molecular biology experiments, the neocortex and hippocampus for each animal was split into two fractions and processed independently, for real time PCR or Western-blot analysis. Cortex and hippocampal samples were stored at −80 °C for later analysis.

RNA isolation and RT-qPCR

Total RNA was isolated from neocortex and hippocampus of APP/PS1 mice and WT mice tissue at 9 and 18-months of age ($n = 5$/age and genotype), respectively, using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as described previously (Fernandez et al. 2009). Relative quantitation of *leptin* (assay ID: Mm00434759_m1) and *Ob-Rb* (assay ID: Mm00440181_m1) was performed using 10 ng of reverse transcribed total RNA (cDNA) in TaqMan One-Step real time PCR Master Mix (PE Applied Biosystem). *β-Actin* (assay ID: Mm00607939_s1) with VIC as real time reporter was used as a control to normalize gene expression. Relative quantitation of *SOCS3* (Forward 5′- ATGGTCACCCACAGCAAGTTT -3′ and Reverse 5′- TCCAGTAGAATCCGCTCTCCT-3′; NM_007707.3) and *PTP1B* (Forward 5'-AGGAAGAG CTGTCCTCCACT-3′ and Reverse 5'-CACATTGA CCAGGAAGGGCT-3′; NM_011201.3) was performed using 25 ng of reverse-transcribed total RNA, 20 pmol/ml of both sense and antisense primers and the SYBR Green PCR Master Mix (Applied Biosystems) in a final reaction volume of 10 μl (Fernandez-Martos et al. 2011). The reactions were run on a Light Cycler® 480 System instrument and software (Roche). Relative quantification for each gene was performed by the ΔΔCt method (Livak and Schmittgen 2001). Primers were designed using NCBI/ Primer-BLAST software, and β-Actin (primer sequence obtained from (Gonzalez-Fernandez et al. 2014)) were used as an endogenous control.

Protein extraction and western blot analysis

Proteins from neocortex and hippocampus tissues of APP/ PS1 mice and WT mice at 3, 6, 9 and 18-months of age ($n = 5$/age and genotype), were extracted with RIPA buffer (Sigma Aldrich) containing a cocktail of protease inhibitors (Roche). Denatured protein samples (20 μg) from each group were electrophoresed into Bolt® Bis-Tris Plus gels (Invitrogen), transferred to PVDF membranes (BioRad) and incubated with primary antibodies [rabbit anti-leptin (1:1000; Thermo Fisher), rabbit anti-Ob-Rb (1:500; Abcam), rabbit anti-Akt (1:1000; Cell signalling), rabbit anti-Akt (Ser473) (1:1000; Cell signalling), rabbit anti-STAT3(1:500; Santa Cruz), and rabbit anti-STAT3 (Tyr705) (1:1000; Cell signalling)] overnight. Subsequently, a corresponding anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:7000; Dako) was used, as described previously (Liu et al. 2015). GAPDH (1:5000, Millipore) was used as a loading control and band intensity was measured as the integrated intensity using ImageJ software (v1.4; NIH).

Histology

All immunohistochemical procedures were performed, as previously described (Fernandez-Martos et al. 2015), in one coronal 40 μm sections (bregma position −2.54 mm) of APP/PS1 mice and WT mice tissue at 9 and 18-months of age ($n = 5$/age and genotype). Antigen retrieval was carried out prior to immunohistochemistry using 10 mM citric acid, pH 6.0, in a pressure cooker for 14 mins. Endogenous peroxidase activity was inactivated in the sections by incubation with 2% H_2O_2 in a 70% methanol solution at RT for 10 min, and sections were then blocked for 1 h at RT in blocking buffer containing 10% horse serum, 0.3% bovine serum albumin and 0.3% Triton X-100 in TBS. The sections were then incubated overnight at 4 °C with the primary rabbit anti-leptin (1:250, Abcam) or rabbit anti-Ob-Rb (1:250; Abcam) antibodies in blocking buffer, plus 1 h at RT. Antibody binding was visualized by sequential incubations with a biotinylated anti-rabbit secondary antibody (1:500; VectorLabs) and HRP-linked streptavidin (1:500; Perkin Elmer), and with the "Nova Red Kit" (VectorLabs) according to the manufacturer's instructions. The sections were finally dehydrated in graded ethanol, cleared with xylene and coverslipped with DPX (Panreac).Images were collected using the same acquisition parameters on an Axio lab A.1 microscope and AxioCam ICc5 camera (both Zeiss, Germany), with ×20 and ×40 objectives and Zen 2012 blue edition software (Zeiss, Germany). In all cases, the specificity of immunoreactivity was confirmed by processing sections lacking primary antibody (Suppl. fig. 1).

Statistical analysis

Values were reported as means ± standard error of the mean (SEM), and differences are considered significant at $p < 0.05$ (CI 95%). Differences between groups were evaluated using student's t-test, and two-way anova using
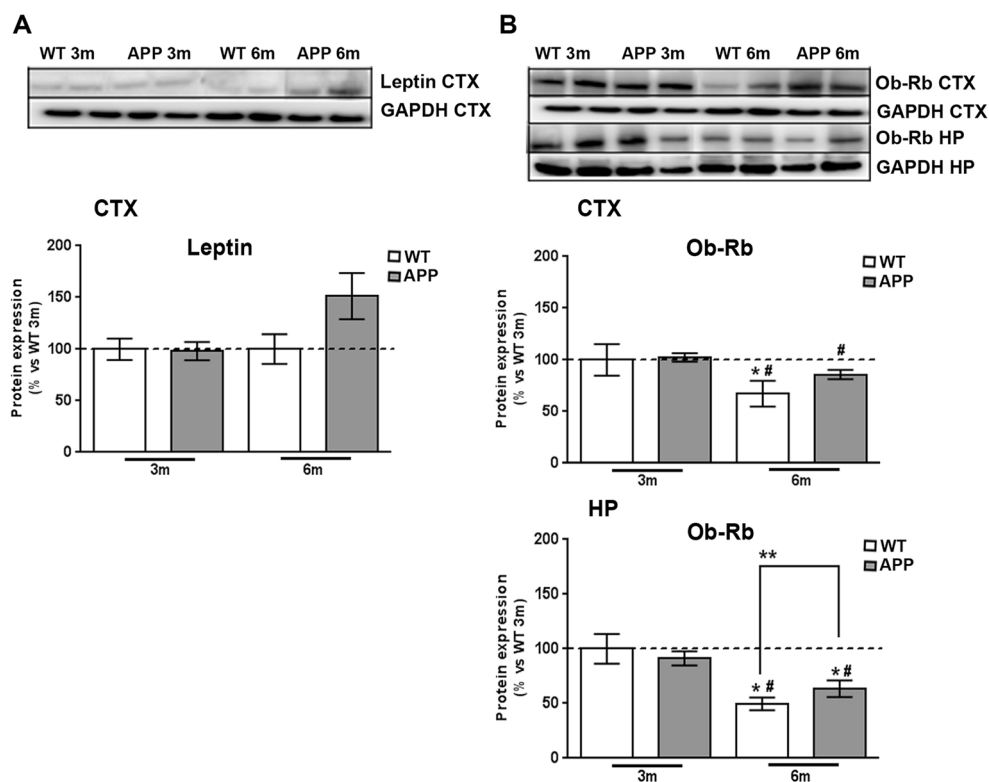
Dunett's post-hoc test, to compare to WT 9 month of age group, and Sidak's post-hoc test for multiple comparisons between groups. Statistical analysis was performed using GraphPad Prism software (version 6.0).

Results

Leptin and Ob-Rb levels in young APP/PS1 mice

We first examined the protein levels of leptin and Ob-Rb receptors at 3 and 6 months in APP/PS1 mice compared to age-matched WT mice, in order to determine both leptin and Ob-Rb expression profiles prior to significant cerebral Aβ deposition and before cognitive loss. Immunoblotting analysis demonstrated that leptin and Ob-Rb proteins were present in the neocortex of APP/PS1 mice and age-matched WT littermates at both these ages (Fig. 1a, b). While leptin levels were not significantly altered there was an age dependent reduction of Ob-Rb in the neocortex at 6 months of age in both APP/PS1 mice and WT. In the hippocampus, while leptin protein was not detectable at quatifiable levels in the APP/PS1 or WT mice at either ages (data not shown), there was also an age dependent reduction of Ob-Rb at 6 months of age in APP/PS1 and WT, with the level of the receptor being significantly enhanced in APP/PS1 relative to WT at this age (Fig. 1b).

Leptin levels in the brain of aged APP/PS1 mice

We next determined leptin protein levels in the brain of APP/PS1 mice in the context of amyloid pathology (9 months of age) and amyloid pathology in ageing (18 months of age), in order to examine its expression relative to age-matched WT littermates. At 9 months of age, leptin was detected, while there was no difference in leptin levels between genotypes in the neocortex (Fig. 2a) but significantly ($p = .048$) higher levels in the hippocampus of APP/PS1 mice relative to WT controls (Fig. 2b). At 18 months, leptin levels were significantly ($p = .003$) lower in the hippocampus of APP/PS1 mice compared to age-matched WT (Fig. 2b). Leptin levels significantly ($p = .02$) decreased with ageing in the neocortex of WT mice (18 months relative to 9 months), while no differences where determined in the hippocampus of WT mice (Fig. 2b).

To identify the source of leptin in the brain of aged mice, RT-qPCR analysis was performed on *leptin* mRNA purified from neocortex and hippocampus of APP/PS1 mice and WT mice at 9 and 18 months of age. No *leptin* mRNA was detected in either of the groups analysed, while white adipose tissue, as a positive control, showed high levels of *leptin* transcript (data not shown).

Immunohistochemical staining further demonstrated the presence of leptin protein in neocortical (Fig. 3a-f) and hippocampal (Fig. 3g-l) neurons in APP/PS1 mice and WT mice, respectively, at 9 and 18 month of age. In the neocortex, at 9 months of age, leptin was localized to the pyramidal cell cytoplasm and neurites, as reflected by strong immunolabelling
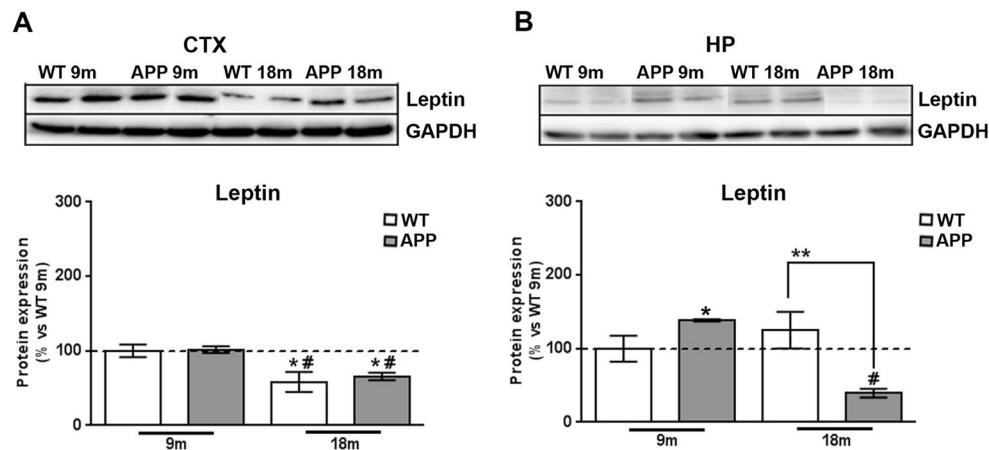

Fig. 1 Alterations in leptin levels in a pre-clinical state in APP/PS1 mice. Representative GAPDH-normalized immunoblot images and quantitation of leptin (**a**) and Ob-Rb proteins (**b**) in both neocortical and hippocampal extracts of APP/PS1 mice compared to WT controls at 3 and 6 month of age. Bar graphs represent the mean ± SEM. Statistical analyses were performed using two-way ANOVA where *$p < 0.05$ vs WT mice at 3 month of age; #$p < 0.05$ vs APP/PS1 mice at 3 month of age; **$p < 0.05$ vs WT mice at 6 month of age. The data are presented as a percentage (%) of the corresponding value in the WT control group at 3 month of age. Abbreviations: CTX, neocortex; HP, hippocampus; APP, APP/PS1 Tg mice

Fig. 2 **Alterations in leptin levels in APP/PS1 mice.** Representative GAPDH-normalized immunoblot images and quantitation of leptin protein in both neocortical (**a**) and hippocampal (**b**) extracts of APP/PS1 mice compared to WT controls at 9 and 18 month of age. Bar graphs represent the mean ± SEM. Statistical analyses were performed using two-way ANOVA where $^{*}p < 0.05$ vs WT mice at 9 month of age; $^{#}p$ < 0.05 vs APP/PS1 mice at 9 month of age; $^{**}p < 0.05$ vs WT mice at 18 month of age. The data are presented as a percentage (%) of the corresponding value in the WT control group at 9 month of age. Abbreviations: CTX, neocortex; HP, hippocampus; APP, APP/PS1 Tg mice

(Fig. 3a, b). At 18 month of age, qualitative analysis suggested lower neuronal leptin immunoreactivity in both genotypes, with the highest reduction in WT mice compared to APP/PS1 mice (Fig. 3g, h). Interestingly, while lepin immunoreactivity in WT animals showed a somal staining pattern within pyramidal neurons in the neocortex (Fig. 3g-g'), high immunolabelling was present in both cell bodies and neurites in APP/PS1 mice (Fig. 3h-h'). Furthermore, leptin showed a differential pattern of cellular immunolabelling in the hippocampal regions in APP/PS1 mice and WT mice, at 9 (Fig. 3c-f) and 18 month of age (Fig.3i-l), respectively. At 9 month of age, pyramidal cells in CA1 showed cytoplasmic immunostaining with dendrites labelled in the stratum radiatum of CA1 (Fig.3c, d), whereas, in areas CA2 and CA3, labelling of neurons was reduced (data not shown). In addition, granular cell bodies of the dentate gyrus and mossy cells in the hilus showed very strong immunolabelling for leptin (Fig. 3e, f). Nevertheless, at 18 months of age, leptin immunolabelling was lower in the hippocampus of APP/PS1 mice compared to age-matched WT mice (Fig. 3i-l). Taken together, the results of immunoblotting analysis and immunohistochemical staining suggest that leptin is present within neurons in the cortex and hippocampus, where it is localized to the cell soma and neurites and that there is a loss of leptin with ageing, which is most prominent in the hippocampus of APP/PS1 mice.

Ob-Rb levels in the brain

We next determined whether altered leptin levels were associated with altered levels of its receptor Ob-Rb. RT-qPCR analysis demonstrated no significant differences in the expression levels of neocortical *Ob-Rb* mRNA between APP/PS1 and WT mice at 9 and 18 months of age (Fig. 4a). Interestingly, at both ages a significant ($p = .002$ and $p = .003$, respectively) downregulation of *Ob-Rb* transcript was detected in the hippocampus of APP/PS1 mice compared to age-matched WT controls. In addition, *Ob-Rb* mRNA levels were also significantly ($p = .043$) downregulated in APP/PS1 mice at 18-months compared to APP/PS1 mice at 9 month of age (Fig. 4b). These data suggest that *Ob-Rb* mRNA is downregulated with age and in the hippocampus of APP/PS1 mice.

Ob-Rb was also altered at the protein level in APP/PS1 mice. Immunoblotting analysis demonstrated significantly ($p = .0006$) lower neocortical Ob-Rb levels in APP/PS1 mice relative to WT controls at 9 month of age (Fig. 4c), while, in the hippocampus, Ob-Rb was signifcantly ($p = .0003$) increased in APP/PS1 mice relative to WT animals (Fig. 4d). Similarly, in 18 month mice, in the hippocampus levels of the receptor were significantly ($p = .0002$) higher in APP/PS1 mice relative to age-matched WT mice (Fig. 4d). Interestingly, there was also an age related effect of receptor expression in the neocortex with higher ($p = .005$ and $p = .01$, respectively) expression at 18 months in both WT and APP/PS1 mice relative to 9 months (Fig. 4c). This suggests that while at the mRNA level, *Ob-Rb* is downregulated with age, the protein expression levels are increased with age and in the hippocampus of APP/PS1 mice.

We performed immunohistochemical labelling to determine if the regional or cellular distribution of Ob-Rb was altered in APP/PS1 tissue and through ageing. Ob-Rb protein was detected in neocortical (Fig. 5a-f) and hippocampal (Fig. 5g-l) neurons in APP/PS1 mice and WT mice at both 9 and 18 month of ages. In the neocortex, Ob-Rb immunolabelling had a cytoplasmic expression in pyramidal neurons at 9 (Fig.5a, b) and 18 months of age

Fig. 3 Alterations in leptin localization in APP/PS1 mice.
(a-l) Leptin immunoreactivity in WT mice and APP/PS1 mice at 9 (A-F) and 18 month of age (G-L). Photomicrographs of immunoperoxidae-labelled sections with leptin immunoreactivityin the neocortex (CTX) and regions of the hippo-campus (CA1 and DG) from WT mice and APP/PS1 mice at 9 (A-E' and B-F') and 18 month of age (G-K' and H-L'), respectively. Images were collected using the same acquisition parameters on an Axio lab A.1 microscope and AxioCam ICc5 camera (both Zeiss, Germany), with ×20 and ×40 objectives. Scale bars = 20 µm. Black square indicates the region of interest magnified with the ×40 objective. Arrows indicate soma and dendrites. Abbreviations: CTX, neocortex; CA1, CornuAmmonis area 1; DG, Dentate gyrus
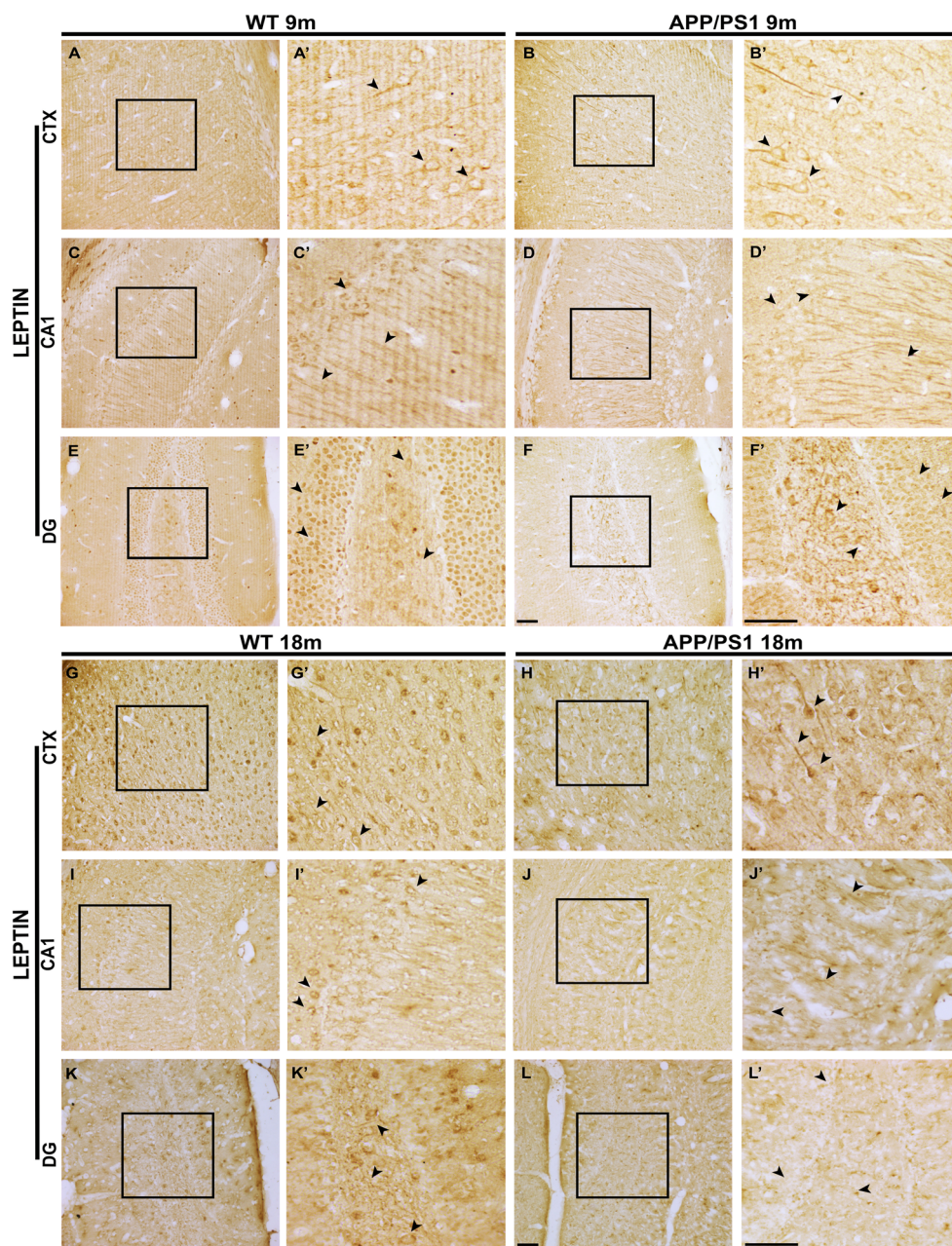
In the hippocampus,at 9 month of age, CA1 and the granular cell bodies of the dentate gyrus and mossy cells in the hilus showed strong Ob-Rb immunolabelling (Fig. 5c-f), whereas, in areas CA2 and CA3, relatively low labelling was present in APP/PS1 and WT mice (data not shown). At 18 month of age, although the cellular labelling pattern remained the same, compar-atively, there was a lower level of immunolabelling for Ob-Rb in WT mice as compared to APP/PS1 mice, espe-cially in the dentate gyrus (Fig. 5k, l). These data suggest that the pattern of Ob-Rb expression in the neocortex and hippocampus reflect the localization of leptin to specific regions.

Leptin signalling in the brain

Our immunoblotting analysis studies so far suggest that age-ing is associated with lower levels of leptin and higher levels of Ob-Rb expression which is particularly prominent in the hippocampus of APP/PS1 mice. We next investigated the sta-tus of STAT3 (pTyr^{705}-STAT3) and Akt (pSer^{473}-Akt) path-ways, which are downstream of the Ob-Rb receptor (Fig. 6), as well as, the expression levels of *SOCS3* gene and *PTP1B* genes (Fig. 7), the main inhibitors of leptin signalling in the brain (Fig. 8).

Immunoblotting analysis demonstrated an age dependent decrease ($p = .017$) in STAT3 activity in the neocortex, but not
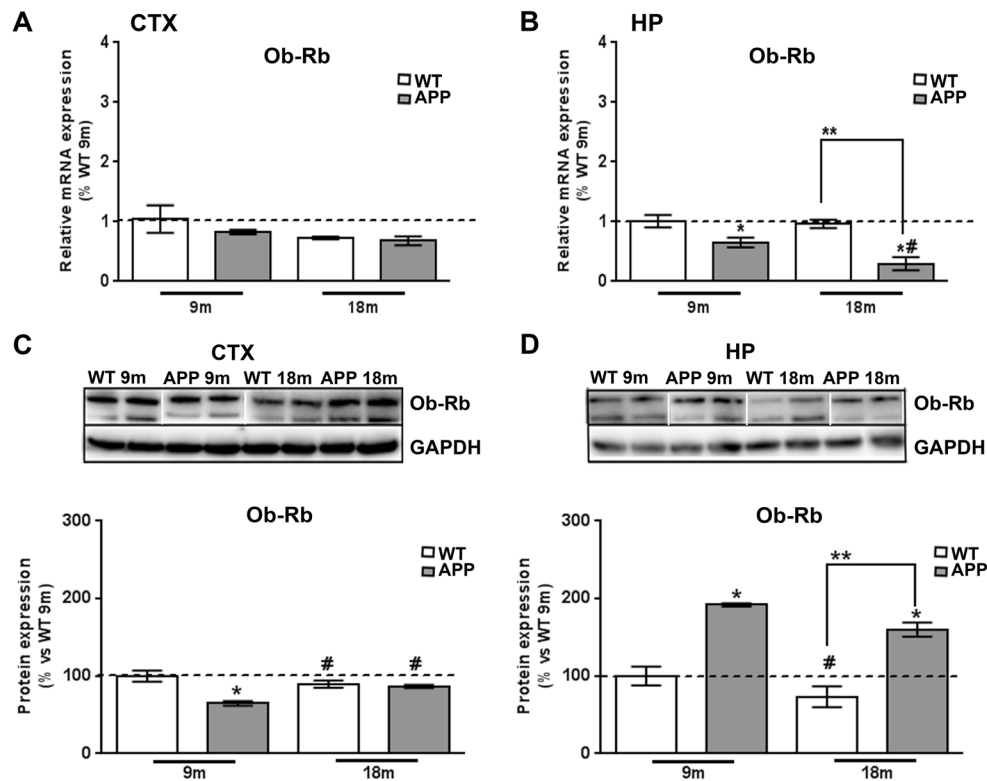
Fig. 4 Alterations in Ob-Rb levels in APP/PS1 mice. Neocortical (**a**) and hippocampal (**b**) *Ob-Rb* mRNA expression was assessed by qRT-PCR in APP/PS1 mice as compared to WT controls at 9 and 18 month of age. (**c-d**) Representative GAPDH-normalized immunoblot images and quantitation of Ob-Rb protein in both neocortical (C) and hippocampal (D) extracts of APP/PS1 mice compared to WT controls at 9 and 18 month of age. Bar graphs represent the mean ± SEM. The white lines in the western blot analysis (F) represent the lanes that were run on the same gel but were non-contiguous. Statistical analysis were performed using two-way anova where $^*p < 0.05$ vs WT mice at 9 month of age; $^\#p < 0.05$ vs APP/PS1 mice at 9 month of age; $^{**}p < 0.05$ vs WT mice at 18 month of age. The data are presented as a percentage (%) of the corresponding value in the WT control group at 9 month of age. Abbreviations: CTX, neocortex; HP, hippocampus; APP, APP/PS1 Tg mice

the hippocampus, of WT mice (Fig.6a). Conversely, at both ages, STAT3 significantly ($p = .0002$ and $p = .0009$, respectively) increase in the neocortex and hippocampus of APP/PS1 mice, and in both brain regions at 18 months relative to age-matched WT controls ($p = .0001$ and $p = .001$, respectively) (Fig. 6a, b). There was no difference in Akt activity between genotypes in either region (Fig.6c, d). Akt activity was significantly ($p = .0009$) decreased in the hippocampus, but not the neocortex, with age in APP/PS1 mice, and at 18 months relative to age-matched WT controls ($p = .029$). Interestingly, while there were no significant differences between genotypes at 9 months of age, ageing in both WT and APP/PS1 mice, was associated with higher Akt activity in the neocortex and lower activity in the hippocampus (Fig. 6c, d).

While no alterations in the *SOCS3* transcript were present with aging in WT mice, at 18 months RT-qPCR analysis demonstrated that the *SOCS3* transcript was significantly ($p = .001$ and $p = .031$, respectively) upregulated in the neocortex of APP/PS1 mice compared to WT controls and APP/PS1 mice at 9 month of age (Fig. 7a). Interestingly, in the hippocampus an age dependent increased ($p = .047$) *SOCS3* transcript was determined in APP/PS1 mice (Fig.7b). However, *PTP1B*

expression did not follow this pattern (Fig.7c, d). *PTP1B* mRNA was significantly ($p = .0001$ and $p = .004$, respectively) upregulated with ageing in the neocortex of WT controls and APP/PS1 mice, but significantly ($p = .001$) lower in APP/PS1 mice at 18 months than aged matched WT mice (Fig. 7c). In the hippocampus, however, there was a significant ($p = .0001$) upregulation only in the APP/PS1 mice with ageing ($p = .0001$), while *PTP1B* transcript was significantly ($p = .0001$ respectively) upregulated relative to WT mice at 9 and 18 month of age (Fig. 7d).

Discussion

A growing body of evidence supports a potential metabolic component in AD pathogenesis (Chakrabarti et al. 2015). Several studies have investigated insulin signalling in AD and suggest a state of insulin resistance (Pedros et al. 2014; Petrov et al. 2015; Watson and Craft 2003). While the importance of leptin in AD is suggested by several clinical and epidemiological studies (Bonda et al. 2014; Lieb et al. 2009; Power et al. 2001; Rosenbaum et al. 1996; Tezapsidis et al.
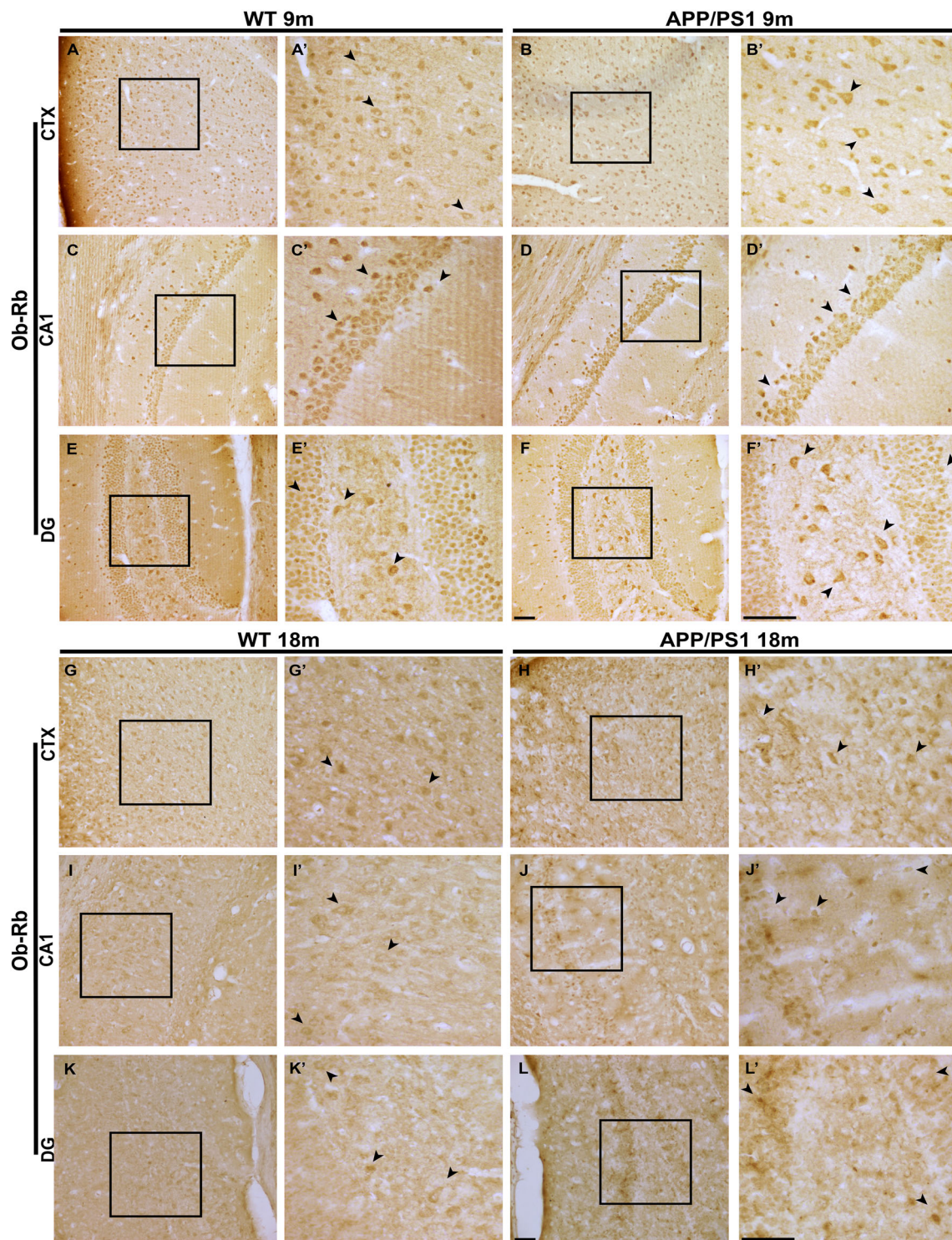
Fig. 5 Alterations in Ob-Rb localization in APP/PS1 mice. (a-l) Ob-Rb immunoreactivity in WT mice and APP/PS1 mice at 9 (A-F) and 18 month of age (G-L). Photomicrographs of immunoperoxidae-labelled sections with Ob-Rb immunoreactivity in the neocortex (CTX) and regions of the hippocampus (CA1 and DG) from WT mice and APP/PS1 mice at 9 (A-E' and B-F') and 18 month of age (G-K' and H-L'), respectively. Images were collected using the same acquisition parameters on an Axio lab A.1 microscope and AxioCam ICc5 camera (both Zeiss, Germany), with ×20 and ×40 objectives. Scale bars = 20 μm. Black square indicates the region of interest magnified with the ×40 objective. Arrows in black indicate soma. Abbreviations: CTX, neocortex; CA1, CornuAmmonis area 1; DG, Dentate gyrus

2009), the contributions of leptin to AD has not yet been fully characterized. Our study provides the first evidence of

alterations in leptin and leptin signalling in the brain of the APP/PS1 Tg model of early AD pathology, providing novel
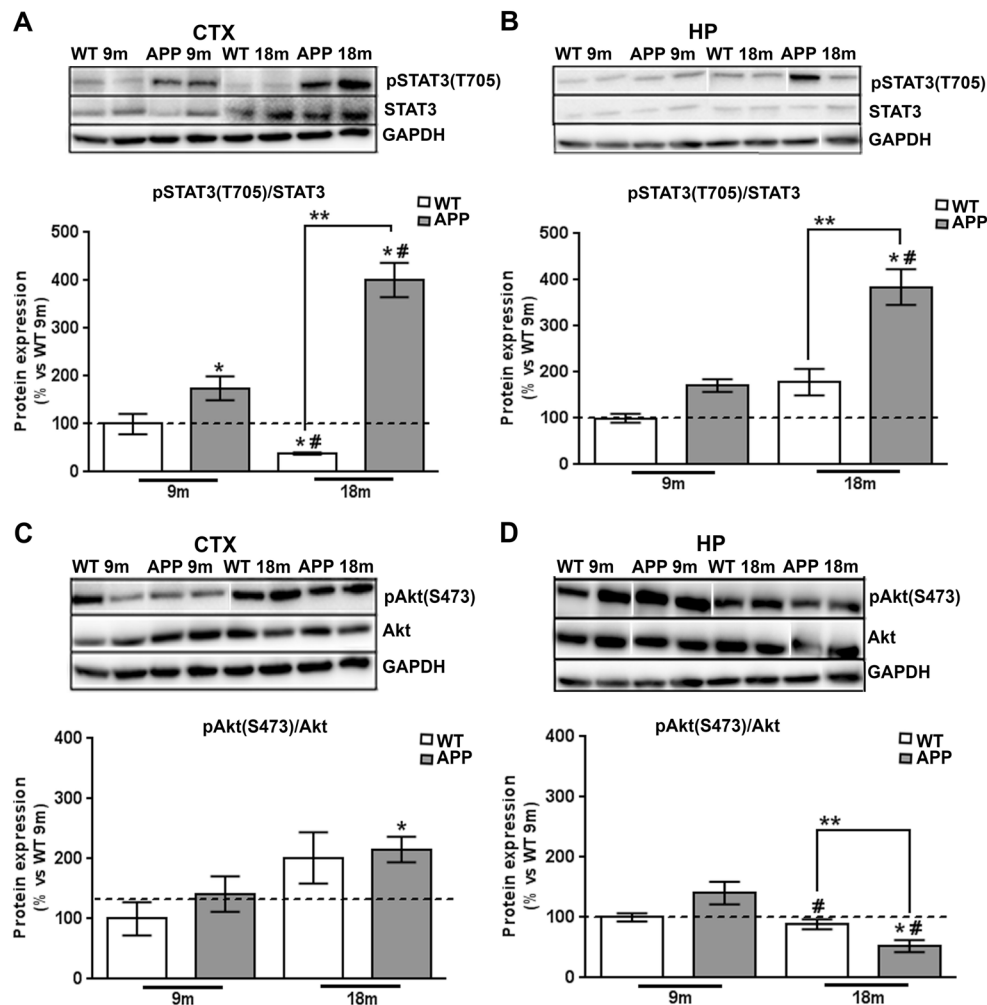
Fig. 6 Alterations in tyrosine phosphorylation of STAT3 in the neocortex and hippocampus of APP/PS1 mice. Representative GAPDH-normalized immunoblot images and quantitation of pSTAT3 (pTyr[705]-STAT3) protein in both neocortical (**a**) and hippocampal (**b**) extracts of APP/PS1 mice compared to WT controls at 9 and 18 month of age. (**c-d**) Representative GAPDH-normalized immunoblot images and quantitation of pAkt (pSer[473]-Akt) protein in both neocortical (C) and hippocampal (D) extracts of APP/PS1 mice compared to WT controls at 9 and 18 month of age. Bar graphs represent the mean ± SEM. The white lines in the western blot analysis (F) represent the lanes that were run on the same gel but were non-contiguous. Statistical analysis were performed using two-way anova where $^*p < 0.05$ vs WT mice at 9 month of age; $^#p < 0.05$ vs APP/PS1 mice at 9 month of age; $^{**}p < 0.05$ vs WT mice at 18 month of age. The data are presented as a percentage (%) of the corresponding value in the WT control group at 9 month of age. Abbreviations: CTX, neocortex; HP, hippocampus; STAT3, the signal transducer and activator of transcription 3; Protein Kinase B, Akt; APP, APP/PS1 Tg mice

insights about the pathways that could link alterations in leptin signalling with AD-like pathology.

According to our data, immunoblotting analysis showed that leptin and Ob-Rb proteins were present in the neocortex of young APP/PS1 mice and age-matched WT controls, while leptin protein was not detected at quatitable levels in the hippocampus of either groups analysed. In the APP/PS1 mice we did demonstrate a relatively enhanced hippocampal expression of Ob-Rb relative to WT controls, which could possibly contribute to leptin's reported beneficial effect on improving learning and cognitive function in AD (Farr et al. 2006; Sato et al. 2011; Searcy et al. 2012). Relatedly, high levels of Ob-Rb immunoreactity have been reported in this region (Harvey et al. 2006). Interestingly, in older animals of the current study, leptin was detected in the hippocampus, and our data indicates that there are higher leptin levels in the hippocampus of APP/PS1 mice relative to WT controls at 9 months of age, followed by an ageing-related reduction in expression across both genotypes and regions, which was more prominent in the hippocampus of APP/PS1 mice. Immunolabelling for leptin in the granular cell bodies of the dentate gyrus and mossy cells in the hilus was qualitatively lower in APP/PS1 mice compared to age-matched WT controls. The reduction in leptin levels can not be explained by a downregulation of *leptin* mRNA in the brain as we did not detect *leptin* transcript expression in cortex or hippocampus, as has been reported previously (Bonda et al. 2014; Maioli et al. 2015). However, the localization of the protein to neurons, suggests that leptin is internalized by these
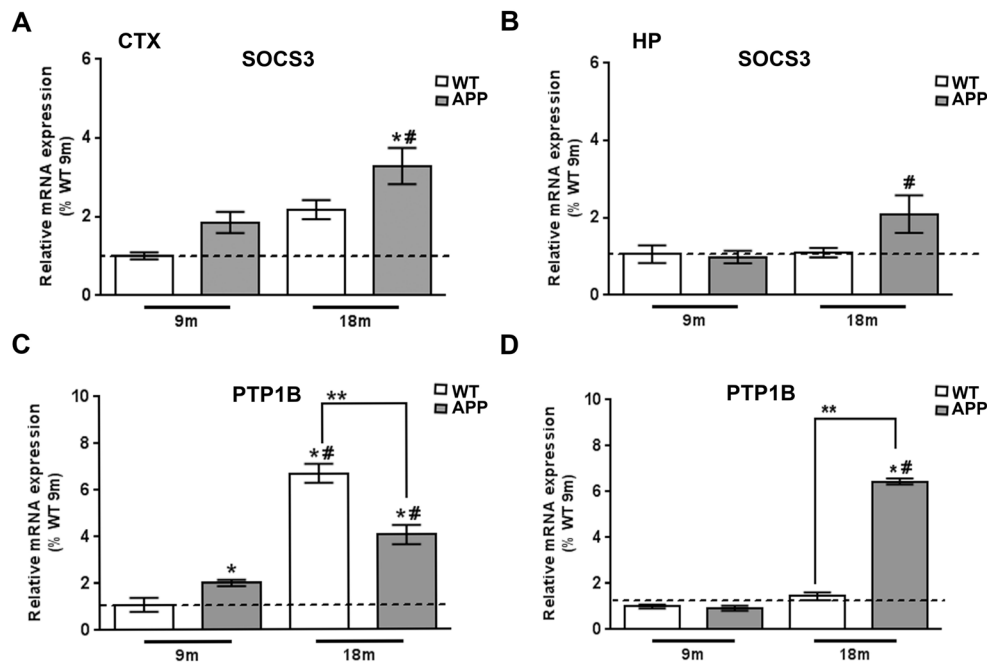
Fig. 7 Alterations in SOCS3 and PTP1B in the neocortex and hippocampus of APP/PS1 mice. Neocortical and hippocampal mRNA expression of *SOCS3* (a-b) and *PTP1B* (c-d) was assessed by qRT-PCR in APP/PS1 mice compared to WT controls at 9 and 18 month of age. Bar graphs represent the mean ± SEM. Statistical analysis were performed using two-way anova where *$p < 0.05$ vs WT mice at 9 month of age; #p

< 0.05 vs APP/PS1 mice at 9 month of age; **$p < 0.05$ vs WT mice at 18 month of age. The data are presented as a percentage (%) of the corresponding value in the WT control group at 9 month of age. Abbreviations: CTX, neocortex; HP, hippocampus; APP, APP/PS1 Tg mice; *SOCS3*, suppressor of cytokine signaling-3; *PTP1B*, protein-tyrosine phosphatase 1B
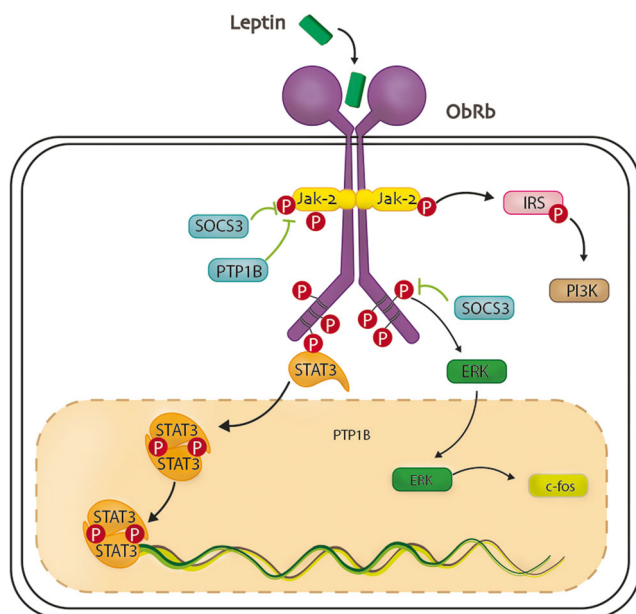

Fig. 8 Leptin signaling pathways In the brain, the binding of leptin to the ObRb receptor activates janus-tyrosine kinase 2 (JAK2), which in turn phosphorylates the insulin receptor substrate-1 and -2 (IRS 1/2) that results in the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. JAK2 activation also leads to the phosphorylation of two tyrosine residues in the cytoplasmic tail of the Ob-Rb receptor, producing the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the signal transducer and activator of transcription 3 (STAT3) signaling pathways. SOCS-3 and PTP1B interfear with leptin receptor signal transduction

cells (Fernandez-Galaz et al. 2010) and supports the concept that this hormone is synthesized in the periphery and actively transported across the BBB or blood–CSF barrier (Friedman and Halaas 1998). Hence, the alterations to leptin levels in the brain of the APP/PS1 mice may reflect altered blood–CSF concentrations, since leptin uptake by the target neuron or intracellular leptin-signalling resistance. In this regard, Holden et al. (Holden et al. 2009) reported that, in elderly individuals, higher serum leptin appears to protect against cognitive decline. Importantly, circulating levels of leptin were reported to be significantly lower in patients with AD as compared to controls (Power et al. 2001). In addition, it has been demonstrated that gliosis prevents circulating leptin from accessing neurons (Horvath et al. 2010) and interferes with neuronal leptin uptake in the brain (Jastroch et al. 2014). In this context, it will be interesting in future studies to determine the mechanism of leptin regulation in the brain of APP/PS1 mice through analysis of CSF samples and circulating leptin concentrations.

Notably, in the APP/PS1 model, robust Aβ deposition is detected in both neocortex and hippocampus up to 12 months of age (Garcia-Alloza et al. 2006; Jankowsky et al. 2004). A severe gliosis commences around 6 months, especially in the vicinity of plaques, and the number of Iba-1 and GFAP-positive cells progressively increases with age (Kamphuis et al. 2012). Importantly, studies show links between Aβ and leptin but cause and effect still unclear (i.e. which cause which and/ or is possible that both affect each other?) (Fewlass et al. 2004; Ishii

2016), suggesting that decreased levels of neuronal leptin in the hippocampus of APP/PS1 mice at 18 month of age may be associated with disease-Aβ associated pathology.

Along with the marked decrease of leptin protein in the hippocampus of aged APP/PS1 mice, our immunoblot results showed significantly increased Ob-Rb levels in APP/PS1 mice compared to WT controls at both ages analysed. In addition, our results confirm previous studies conducted in both human AD and Tg2576 mice (Bonda et al. 2014; Maioli et al. 2015), where we demonstrate a significant decrease in *Ob-Rb* mRNA levels in the hippocampus of APP/PS1 mice compared to WT controls, at both ages analysed. Thus, although our immunohiostochemical analysis primarily identified ObRb receptor labelled cells with a neuronal profile, it is possible that other cell types (i.e. astrocytes) contribute to increasing ObRb levels in the hippocampus of APP/PS1 mice. Indeed, Maioli et al. (Maioli et al. 2015) have found increased Ob-Rb levels in astrocytes in the hippocampus of old Tg2576 AD mice (2-year-old), while leptin protein was reduced in neurons when compared to age-matched WT controls. Interestingly, a significant upregulation of Ob-Rb levels was determined in cultured astrocytes treated with Aβ$_{1-42}$ for 24 h (Maioli et al. 2015). Hence, these results may be indicative of age-dependent Aβ overproduction inducing alteration of Ob-Rb levels in the brain of APP/PS1 mice. However, the precise dynamics of Ob-Rb regulation in the brain are incompletely understood (Hikita et al. 2000; Mitchell et al. 2009), and the mechanism of Ob-Rb regulation in the APP/PS1 Tg line mice needs to be determined in future studies.

Alternatively, the alterations to leptin levels in the APP/PS1 mice may reflect intracellular leptin-signalling resistance. For that reason, we also investigated the association of leptin signalling pathways with increased Aβ pathology in aged APP/PS1 mice. The binding of leptin to the neuronal Ob-Rb receptor activates four major signal transduction pathways (Folch et al. 2015), JAK/STAT pathway, ERK pathway, PI3K/Akt/mTOR pathway and AMPK/SIRT1 pathway (Fig.8). However, we were particularly interested in STAT3 and Akt signalling downstream of Ob-Rb receptor, as the STAT3 pathway has been recently implicated in Aβ-mediated neurotoxicity in AD (Wan et al. 2010). Importantly, in aged rats, a decline in STAT3 activation is observed that is linked to a decrease in leptin responsiveness (Scarpace et al. 2000), which indicates that age-related changes in leptin receptor-dependent signalling cascades occur. In addition, new experimental studies conducted in mice have shown that high-fat diet consumption induced hippocampal leptin resistance produced by the desensitization of the Akt pathway downstream neuronal Ob-Rb receptors (Valladolid-Acebes et al. 2013). Importantly, leptin increases adult hippocampal neurogenesis in vivo and in vitro by a mechanism involving STAT3 and Akt signalling pathways (Garza et al. 2008). In the current study, an increased tyrosine phosphorylation of STAT3 was determined in the brains of aged APP/PS1 mice compared to WT controls, at both ages analysed. This is in agreement with

previous work showing increased pSTAT3 levels in APP/PS1 mice (Wan et al. 2010). Notably, increased pSTAT3 has been determined in post-mortem studies of AD (Wan et al. 2010). However, a significant decrease in hippocampal Akt activity was found in APP/PS1 mice compared toWT mice at 18 month of age. Concomitantly, we determined a robust upregulation of *SOCS3* and *PTP1B* transcripts, which could further contribute to desensitizing the Akt pathway by altering phosphorylation of Jak2, after ligand binding to an Ob-Rb homodimer, and may negatively regulate activaton of neuronal Ob-Rb in the hippocampus of AD mice. Indeed, it has been demonstrated that elevation of SOCS3 and PTP1B interferes with leptin receptor signal transduction in aged animals (Morrison et al. 2007; Peralta et al. 2002). Importantly, PTP1B regulates leptin signalling in vivo, by targeting Jak2 (Zabolotny et al. 2002). This was an interesting preliminary result of our study, because it may indicate the existence of a downregulation of signalling towards leptin resistance associated with age-related Aβ overproduction, which may support the link between age-related cognitive decline and impaired leptin responsiveness. Indeed, new preliminary data indicates that people with AD may benefit from leptin replacement therapy (Tezapsidis et al. 2009). In addition, a growing body of experimental studies have demonstrated leptin's beneficial effect on improving learning and cognitive function in AD (Farr et al. 2006; Sato et al. 2011; Searcy et al. 2012). Leptin has also been shown to enhance performance in memory tasks in SAMP8 mice, which display Aβ-induced neuronal toxicity (Farr et al. 2006). Leptin treatments leads to improvements in novel object recognition in APP/PS1 (Perez-Gonzalez et al. 2014) and TgCRND8 mice (Greco et al. 2010). In addition, improvements in contextual and cued fear conditioning tests have also been reported following 8 weeks of leptin treatment in TgCRND8 mice (Greco et al. 2010). Leptin has been shown to have strong in vitro (Fewlass et al. 2004; Greco et al. 2009b, 2008) and in vivo (Fewlass et al. 2004) anti-amyloidogenic effect, attributable to its transcriptional regulation capacity (Niedowicz et al. 2013) and inhibition of amyloid precursor protein (APP) processing (Perez-Gonzalez et al. 2014) that contributes to the amyloidogenic pathway. Accordingly, leptin reduces extracellular Aβ protein in vitro and this effect is dependent on activation of AMP-activated protein kinase (AMPK) (Fewlass et al. 2004; Greco et al. 2009b, 2008). It has also been reported that leptin can reduce total brain Aβ$_{1-40}$ and Aβ$_{1-42}$ in Tg2576 mice (Fewlass et al. 2004) and leptin reduces tau phosphorylation in neuronal cells through modulation of GSK3β activity (Greco et al. 2009a), a protein kinase identified to be pathogenic in a range of neurodegenerative disorders (Koh et al. 2011). This suggests a feedback loop where high concentrations of Aβ or metabolic disease could cause a state of leptin resistance and further contribute to AD pathology.

In terms of study limitations, our research provides the first experimental evidences of disruption of leptin signalling in the brain of the APP/PS1. Further mechanistic studies on the

consequences of leptin signalling's alterations may require larger sample sizes at defined timepoints. In addition, immunohistochemical staining was conducted to evaluate protein localization within brain between genotypes on sections relative to Bregma. Further quantitation of immunohistochemistry studies, investigating both expression profiles, may be warranted.

In summary, the data reported here provide the first experimental evidence for the pathways that could link aberrant leptin signalling to evolving pathology in the hippocampus of aged APP/PS1 mice. Altered leptin signalling may play a critical role in the pathophysiological changes of AD. Therefore, although the *primum movens* cause of AD is not yet fully understood,determining the role of leptin and related signalling in the brain may provide a new avenue for therapeutic development for this condition.

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