Journal of Neurochemistry

JNC

JOURNAL OF NEUROCHEMISTRY | 2015 | 133 | 739–749

doi: 10.1111/jnc.13037

ORIGINAL ARTICLE

Alteration of mTOR signaling occurs early in the progression of Alzheimer disease (AD): analysis of brain from subjects with pre-clinical AD, amnestic mild cognitive impairment and late-stage AD

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Abstract

The clinical symptoms of Alzheimer disease (AD) include a gradual memory loss and subsequent dementia, and neuropathological deposition of senile plaques and neurofibrillary tangles. At the molecular level, AD subjects present overt amyloid β (Aβ) production and tau hyperphosphorylation. Aβ species have been proposed to overactivate the phosphoinositide3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) axis, which plays a central role in proteostasis. The current study investigated the status of the PI3K/Akt/mTOR pathway in post-mortem tissue from the inferior parietal lobule (IPL) at three different stages of AD: late AD, amnestic mild cognitive impairment (MCI) and pre-clinical AD (PCAD). Our findings suggest that the alteration of mTOR signaling and autophagy occurs at early stages of AD. We found a significant increase in Aβ (1–42) levels, associated with reduction in autophagy (Beclin-1 and LC-3) observed in PCAD, MCI, and AD subjects. Related to the autophagy impairment, we found a hyperactivation of PI3K/Akt/mTOR pathway in IPL of MCI and AD subjects, but not in PCAD, along with a significant decrease in phosphatase and tensin homolog. An increase in two mTOR downstream targets, p70S6K and 4EBP1, occurred in AD and MCI subjects. Both AD and MCI subjects showed increased, insulin receptor substrate 1, a candidate biomarker of brain insulin resistance, and GSK-3β, a kinase targeting tau phosphorylation. Nevertheless, tau phosphorylation was increased in the clinical groups. The results hint at a link between Aβ and the PI3K/Akt/mTOR axis and provide further insights into the relationship between AD pathology and insulin resistance. In addition, we speculate that the alteration of mTOR signaling in the IPL of AD and MCI subjects, but not in PCAD, is due to the lack of substantial increase in oxidative stress.

Keywords: Alzheimer disease progression, amnestic Mild Cognitive Impairment (MCI), mTOR signaling, autophagy, Preclinical Alzheimer disease.

J. Neurochem. (2015) **133**, 739–749.

This article is related to a companion paper: doi: 10.1111/jnc.13039.

The increasing age of the world's population leads to a high number of people diagnosed with dementia. Alzheimer's disease (AD) is a progressive disease, in which dementia symptoms gradually worsen over a number of years. Patients with AD often progress from stages of amnestic mild cognitive impairment (MCI) to complete dementia or late stage of AD. MCI patients have memory loss but normal activities of daily living and demonstrate AD like pathology, with amyloid plaques in the neocortex and frequent neurofibrillary tangles (NTFs) in the temporal lobe (Querfurth and

Received October 20, 2014; revised manuscript received December 19, 2014; accepted January 12, 2015.

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Abbreviations used: AD, Alzheimer disease; Aβ, amyloid β; DS, Down syndrome; IPL, inferior parietal lobule; IRS1, insulin receptor substrate 1; MCI, mild cognitive impairment; mTOR, mammalian target of rapamycin; NTFs, neurofibrillary tangles; OS, oxidative stress; PCAD, pre-clinical AD; PI3K, phosphoinositide3-kinase; PMI, post-mortem interval; SPs, senile plaques.

LaFerla 2010). Both senile plaques (SPs) and NTFs are characteristic hallmarks of AD diagnosis.

In some cases, the autopsy of brains from apparently cognitively intact individuals reveals an extensive SP load. Those individuals with normal cognitive function as shown by antemortem psychometric test scores but who have evidence of AD neuropathology at autopsy, were included in a new stage of AD called pre-clinical AD (PCAD). Literature on these individuals is limited, most likely because of the rarity of sample availability (Aluise *et al.* 2010, 2011). Researchers have begun to characterize the biochemical profile of PCAD, which includes neuronal hypertrophy, no hippocampal cell loss (West *et al.* 2004), increased synaptic plasticity (O'Brien *et al.* 2009), and alterations in zinc transporters (Lyubartseva *et al.* 2009). The etiology of AD remains obscure and the molecular pathways by which the various pathological alterations selectively impair cognitive domains related to learning and memory are unclear. Examination of brain of AD subjects during the different stages of disease may yield a better understanding of modification of different molecular pathways, especially in the early stage.

In our previous work, we used Down syndrome (DS) brain with and without dementia to gain insight into the early molecular events that led to the alteration of mTOR (mammalian target of rapamycin) signaling and might contribute to the pathogenesis and progression of AD neuropathology (Butterfield *et al.* 2014a; Cenini *et al.* 2014; Perluigi *et al.* 2014). In DS brain, prior to development of significant AD pathology, the proteostasis network is dysregulated as a consequence of increased oxidative stress (OS) conditions and autophagy impairment (Di Domenico *et al.* 2013; Butterfield *et al.* 2014a). Several studies demonstrated that amyloid β-peptide (Aβ) is associated with increased OS in many brain regions (i.e., hippocampus and inferior parietal lobule, IPL) of AD and MCI subjects (Butterfield *et al.* 2001, 2006) while PCAD subjects demonstrate oxidative damage only in selected brain areas, not including the IPL (Aluise *et al.* 2010, 2011; Bradley *et al.* 2010). Moreover, growing evidence highlights the role of autophagy in several neurodegenerative disorders, including AD (Ling and Salvaterra 2009; Majumder *et al.* 2011; Nixon 2013). Therefore, the well-documented age-dependent decrease in autophagy function may contribute to the accumulation of proteins in the brain. Taken together, evidence suggests that increased production or decreased clearance of aberrant conformations of Aβ is the primary initiating trigger for the development of synaptic defects and cognitive complications of AD (Butterfield *et al.* 2001; Holtzman *et al.* 2011). Diverse Aβ species have been proposed to overactivate the phosphoinositide3-kinase (PI3K) Akt/mTOR axis, which plays a central role in controlling protein homeostasis (Caccamo *et al.* 2010; Gupta and Dey 2012). Moreover, levels of Aβ oligomers in AD brain have been correlated with increased phosphorylation of insulin receptor substrate 1 (IRS1) at its inhibitory residue, and with the increased activation of kinases including Akt and mTOR, that are able to target this residue (O'Neill *et al.* 2012).

An increasing number of studies demonstrated that memory alterations could be linked to abnormalities in circulating insulin levels and/or defects in insulin signaling pathways in AD (Griffin *et al.* 2005; Boura-Halfon and Zick, 2009; Craft 2012; O'Neill *et al.* 2012; Talbot *et al.* 2012; Butterfield *et al.* 2014b). Hence, a relationship may exist between insulin resistance and the development of AD in the typical elderly, adult population.

Based on these observations, the aim of the current study was to investigate the status of the PI3K/Akt/mTOR pathway in the IPL from three different stages of the disease: late AD, MCI, and PCAD. We tested the hypothesis that the autophagy pathway is modified as an early event in the progression of AD.

Materials and methods

Brain tissue

Brain tissue was provided by Sanders-Brown Center on Aging of the University of Kentucky. All the studies were performed on the IPL of non-disease control, PCAD, MCI and AD cases. Clinical diagnosis of stage of disease was made according as described previously (Aluise *et al.* 2010, 2011). Age and gender are listed in the Table 1. The post-mortem interval range is between 2 and 4 h and is comparable between the three groups. The degree of cognitive impairment was assessed using the Mini Mental State Examination (MMSE) (Table 1).

Aβ assay

Frozen IPL samples were extracted sequentially in ice cold isolation Buffer, (pH 7.4) with a complete protease inhibitor cocktail (4 μg/mL leupeptin, 4 μg/mL pepstatin A, 5 μg/mL aprotinin, 0.2 mM phenylmethylsulfonyl fluoride and phosphatase inhibitors), and centrifuged at 20 800 *g* for 30 min at 4°C. Following centrifugation, the supernatant was collected and the pellets were sonicated (10 × 0.5 s pulses at 100 W; Fisher Sonic Dismembrator, Fisher Scientific, Florence, KY, USA) in 2% sodium dodecyl sulfate (SDS) with PIC followed by centrifugation (as above, at 14°C). The supernatant was again collected, and the remaining pellets were sonicated in 70% formic acid (FA) followed by centrifugation at 20 800 *g* for 1 h at 4°C. Aβ was measured in tissue samples using a standard, well-characterized, two-site sandwich ELISA as described previously (Cenini *et al.* 2012). Briefly, an Immulon 4HBX plate was coated with 0.5 μg/well by antibody, incubated overnight at 4°C, then blocked with a solution of Synblock (Serotec, Minneapolis, MN, USA), as per the manufacturer's instructions. Antigen capture (AC) was performed using monoclonal antibody Ab9 (against Human Aβ 1–16). Antigen detection was performed using biotinylated and 12F4 (end specific for Aβ42; Covance, Princeton, NJ, USA).

FA-extracted material was initially neutralized by a 1 : 20 dilution in TP buffer (1 M Tris base, 0.5 M Na_2HPO_4), followed by a further dilution as needed (1 : 100 to 1 : 400) in AC buffer [20 mM Na_3PO_4, 0.4% Block Ace (AbD Serotec; Raleigh, NC,

Table 1 Autopsy case demographics

	Age	SEM	PMI	SEM	MMSE	SEM
Control	85.1	2.5	3.9	1.0	28.7	0.2
AD	84.5	1.2	3.6	0.4	12.9	3.4
MCI	89.3	2.4	4.2	1.2	23.3	1.8
PCAD	86.3	0.8	2.3	0.8	29.3	0.3

USA), 0.05% NaN3, 2 mM EDTA, 0.4 M NaCl, 0.2% bovine serum albumin, 0.05% CHAPS, pH 7]. SDS-soluble fractions were diluted (1 : 20) in AC buffer alone. Phosphate-buffered saline (PBS) fractions were diluted 1 : 4 in AC buffer alone. A peptide standard curve of Aβ was run on the same plate for comparison, and standards and samples were run at least in duplicate; Aβ values were determined by interpolation relative to the standard curve. Plates were washed between steps with standard PBS containing 0.05% Tween-20 (2–4×) followed by PBS (2–4×). Plates were developed with TMB reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), stopped with 6% o-phosphoric acid, and read at 450 nm using a BioTek multiwell plate reader (Biotek Instruments, Winooski, VT, USA).

Sample preparation for western blot

IPL from Controls, PCAD, MCI, and AD subjects ($n = 8$ per groups) were thawed in Isolation Buffer (0.32 M Sucrose, 20 mM HEPES, 2 mM EGTA, 2 mM EDTA and 0.1 M GlcNAc, pH 7.4 with 4 μg/mL leupeptin, 4 μg/mL pepstatin A, 5 μg/mL aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors) using a Wheaton glass homogenizer. One aliquot was set on ice and sonicated 2 × 10 s on 20% power using a 550 Sonic Dismembrator (Fisher Scientific, Rockford, IL, USA) and centrifuged at 4°C for 10 min at 1000 *g* to obtain homogenates. Protein estimation was performed using Pierce bicinchoninic acid Protein Assay (Thermo Scientific, Rockford, IL, USA).

Western blot

For western blot, 30 μg of proteins from IPL homogenate (Control, PCAD, MCI, and AD groups) were separated by 12% SDS–polyacrylamide gel electrophoresis and blotted into a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% bovine serum albumin in Tween-20, TRIS-buffered saline (T-TBS) for 1 h and 30 min at 22°C and incubated individually over night at 4°C with primary anti-bodies: Akt and p-Akt (Ser473) (1 : 1000), mTOR and p-mTOR (Ser2448) (1 : 1000), p70S6K (Thr389) (1 : 1000), phosphatase and tensin homolog (PTEN) (1 : 1000), Beclin 1 (1 : 1000), IRS1 and p-IRS1 (Ser307) (1 : 1000) from Cell Signaling Technology (Beverly, MA, USA); p-4EBP1 (Thr36), Tau and p-Tau (Ser404) (1 : 1000), PI3K and p-PI3K (Tyr 508) (1 : 1000), GSK-3β, p-GSK-3β (Tyr 216) and p-GSK-3β (Ser9) (1 : 1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); MAP1LC-3 (1 : 1000) from Abcam (Cambridge, MA, USA). After three washes with T-TBS, the membranes were incubated for 1 h at 22°C with secondary antibody horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (1 : 5000; Sigma-Aldrich, St Louis, MO, USA). Membranes were developed with Clarity western-enhanced chemiluminescence substrate (Bio-Rad) acquired with Chemi-Doc MP (Bio-Rad) and analyzed using Image Lab software (Bio-Rad) that allows the normalization of specific protein signal with proteins total load obtained by fluorescent detection of monodimensional proteome on TGX stain free gels.

Statistical analysis

Data are expressed as the mean ± SD of eight independent samples per group. All statistical analyses were performed using a non-parametric one-way ANOVA with post hoc Bonferroni test $p < 0.05$ (*) was considered significantly different from control.

Moreover, we performed a correlation analysis between mTOR and Aβ levels. All statistical analysis was executed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA).

Results

Aβ amount

We analyzed Aβ 1–42 PBS-soluble, SDS-soluble and FA-extracted (fmol/mg) amounts in IPL of all the groups (PCAD, MCI and AD) compared to control subjects. Increased (Aβ 1–42) PBS-soluble and FA-extracted amounts were found in PCAD, MCI and AD compared to control (Table 2).

Activation of PI3K/Akt/PTEN axis in PCAD, AD and MCI brains

Akt is one of the major targets of PI3K, and in this study we analyzed the phosphorylation of the PI3K p85α subunit on Tyr^{508} and Akt (Ser^{473}). p85α is a regulatory subunit most highly expressed and binds preferentially to phosphorylated tyrosine residues (Dhand *et al.* 1994). Moreover, we focused

Table 2 Aβ assay, Aβ (1–42) PBS-soluble, SDS-soluble, and FA-extracted amounts (fmol/mg) in IPL of all the groups (PCAD, MCI, and AD) compared to control subjects

			Control (fmol/mg)	AD (fmol/mg)	MCI (fmol/mg)	PCAD (fmol/mg)
Aβ1-42	HEPES	Mean	8.08	35.1**	29.7**	29.3*
		SEM	3.73	10	10.5	13.3
	FA	Mean	292.7	2271**	1027.82*	491.8**
		SEM	135.3	654.7	454.4	122

AD versus Ctr, $^{**}p < 0.01$; MCI versus Ctr, $^{*}p < 0.05$; PCAD versus Ctr, $^{**}p < 0.01$.

our attention on the PTEN, which specifically catalyzes the dephosphorylation of the 3′ phosphate of the inositol ring in PIP_3. PTEN is a key protein able to induce the inhibition of the Akt signaling pathway. Our results showed an increased phosphorylation of PI3K p85α subunit (Tyr^{508}) in both AD and MCI but not in PCAD IPL compared with control (Fig. 1a), no differences were revealed in PI3K expression. The increase in PI3K phosphorylation in AD was stronger than that in MCI, which increased by 70% with respect to controls instead of 40% (**AD vs. Ctr, $p < 0.01$; *MCI vs. Ctr, $p < 0.05$). Moreover, decreased PTEN expression was found statistically significant in AD compared to control (Fig. 1a) (about 20%; **AD vs. Ctr, $p < 0.01$); while the comparison MCI versus control resulted in a p-value close to significance ($p = 0.0597$). No alterations in PI3K and PTEN levels and/or phosphorylation were observed in PCAD brains with respect to control (Fig. 1a).

The phosphorylation of Akt (Ser^{473}) was significantly increased in AD and MCI compared to control (**AD vs. Ctr, $p < 0.01$; *MCI vs. Ctr, $p < 0.05$) but not in the PCAD group; in addition, no alteration in expression of Akt among the three pathological groups was detected (Fig. 1b).

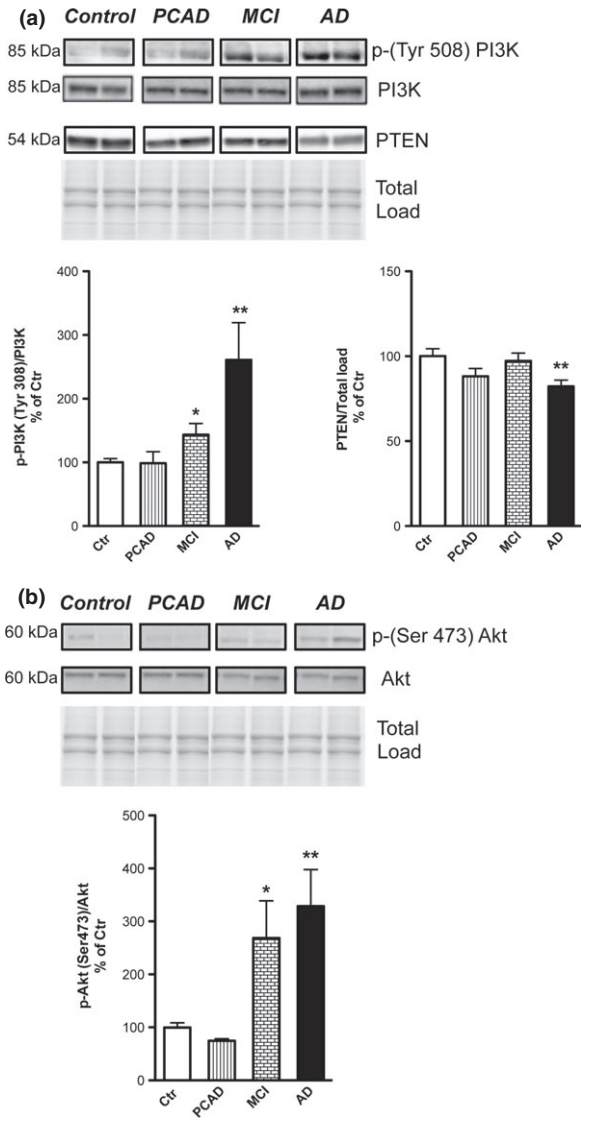

Fig. 1 PI3K (Tyr508) phosphorylation levels and PTEN expression levels (panel A), Akt (Ser473) phosphorylation levels (panel B) in IPL of Controls, PCAD, MCI and AD. Samples of IPL were assayed by western blot as described in the Materials and Methods section. Densitometric values shown in the bargraph are the mean of 8 individual cases per group normalized per total protein load and are given as percentage of control, set as 100%. On the top a representative blot image with protein bands is shown. **p < 0.05 and **p < 0.01.*

A downstream target of PI3K/Akt axis: mTOR and impairment of autophagy in PCAD, MCI and AD brain

mTOR is the major downstream target of the PI3K/Akt axis. mTOR is a direct substrate for Akt kinase and identified Ser-2448 was identified as the Akt target site on mTOR (Chiang and Abraham, 2005). We analyzed the expression levels and phosphorylation (Ser^{2448}) of mTOR (Fig. 2a). mTOR expression values did not show any significant difference among the groups, and also no difference was found in mTOR phosphorylation between PCAD and control IPL (Fig. 2a). The analysis of mTOR phosphorylation showed increased levels in both AD and MCI compared to control (*AD vs. Ctr, $p < 0.05$; *MCI vs. Ctr, $p < 0.05$). The p70S6 kinase is one of the major targets of mTOR phosphorylation at Ser^{2448} (Chiang and Abraham, 2005), and hyperphosphorylation of p70S6K at Thr^{389} is induced in both AD and MCI (***AD vs. Ctr, $p < 0.001$; *MCI vs. Ctr, $p < 0.05$) (Fig. 2b). Concurrently, we evaluated the phosphorylation of another mTOR target, 4EBP1, which is phosphorylated in four sites, including Thr^{36} and Thr^{45}, the two sites preferred by mTOR *in vitro* (McMahon *et al.*, 2002), causing 4EBP1 to dissociate from eIF4E-dependent translation. Our results showed a significant increased phosphorylation of 4EBP1 at Thr^{36} (Fig. 2c) in both AD and MCI (*AD vs. Ctr, $p < 0.05$; *MCI vs. Ctr, $p < 0.05$). This result supports the notion of hyperactivation of the PI3K/Akt/mTOR pathway not only in brain of AD subjects but also in MCI patients, while no modifications were found in PCAD brain. Moreover, to evaluate if mTOR hyperactivation-induced autophagy impairment, we analyzed LC-3, an index of autophagosome formation, showing significant decreased level of about 30% in AD patients and of about 25% in MCI (**AD vs. Ctr, $p < 0.01$; *MCI vs. Ctr, $p < 0.05$) (Fig. 3b). In addition, we analyzed the levels of Beclin 1, another autophagy marker, involved in stimulation and/or suppression of autophagy, demonstrating decreased levels of about 30% (Fig. 3b) in both AD and MCI groups compared to control, (*AD vs. Ctr, $p < 0.05$; *MCI vs. Ctr, $p < 0.05$). Further, decreased levels of Beclin 1 and LC-3 were found also in PCAD brain

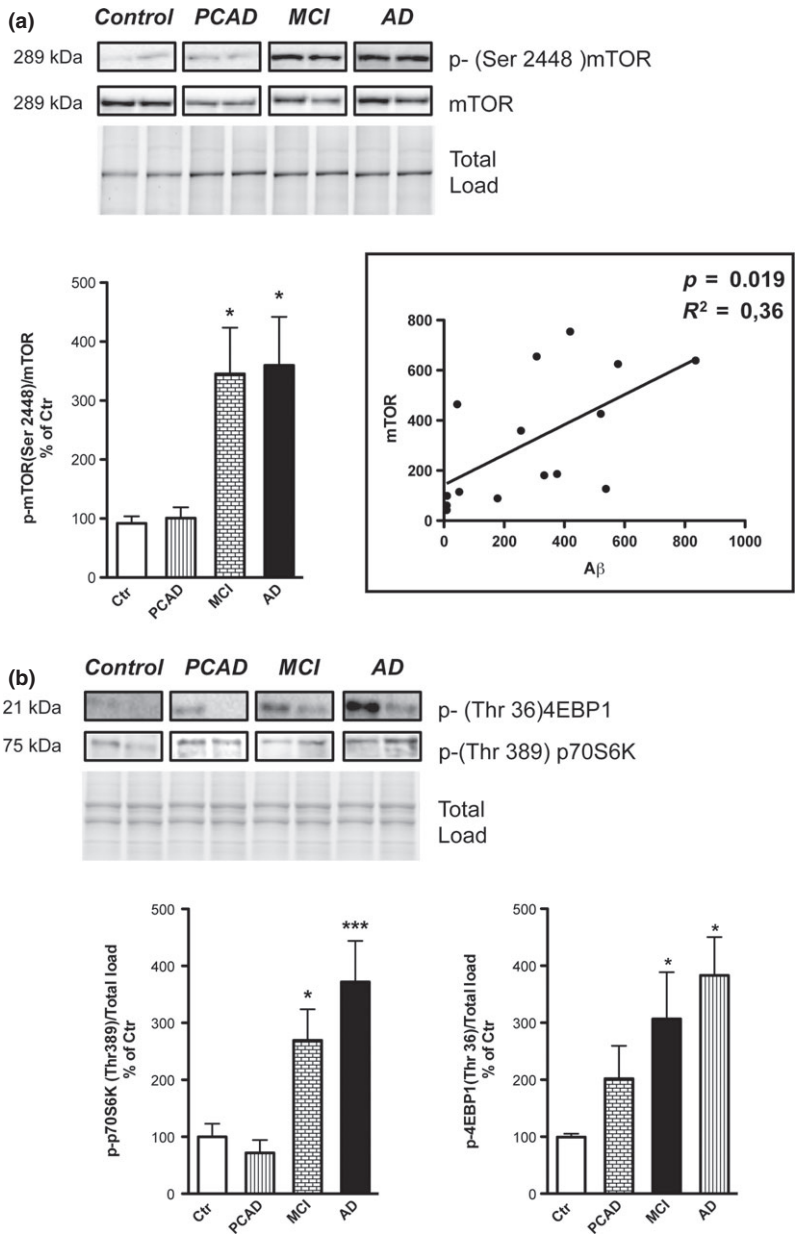

Fig. 2 mTOR (Ser2448)phosphorylation levels and associated Aβ amounts and p-mTOR (panel a), p70S6K (Thr389) phosphorylation levels and 4EBP1 (Thr36) phosphorylation (panel b) in IPL of Controls, PCAD, MCI and AD cases. Densitometric values shown in the bar graph are the mean of 8 individual cases per group normalized per total protein load and are given as percentage of Control, set as 100%. On the top a representative blot image with protein bands is shown. *(a)* also shows a positive and significant (p = 0.019) correlation between Aβ amount and p-mTOR. **p < 0.05 and ***p < 0.001.*

compared to control (**PCAD vs. Ctr, $p < 0.01$) (Fig. 3a and b).

An impairment of PI3K/Akt/mTOR pathway induce insulin resistance in PCAD, MCI and AD brain

Growing evidence supports the concept that neurons in AD become increasingly resistant to both insulin and insulin growth factor-1 (IGF-1). Insulin resistance is strongly sustained by the hyperactivation of the PI3K/Akt/mTOR axis throuogh the inhibition of IRS1 activity by a mechanism of negative feedback. Indeed, the prolonged activation of mTOR is able to phosphorylate IRS1 on its inhibitory domain (Ser^{307}). Our data show no difference in IRS1 expression of both AD and MCI IPL compared to control, while IRS1 phosphorylation was demonstrated to be increased by about two-fold in AD and MCI IPL with respect to control (*AD vs. Ctr, $p < 0.05$; *MCI vs. Ctr, $p < 0.05$). The hyperphosphorylation of p-(Ser^{307}) of IRS1 demonstrated that insulin resistance may be an early event in MCI and is maintained in AD subjects (Fig. 4). PCAD IPL did not demonstrate such changes (Fig. 4).

Phospho-Tau and GSK-3β in PCAD, MCI and AD

The increased activation of Akt in AD has critical downstream signaling consequences. We described above the mTOR activation in both clinical conditions (MCI and AD);

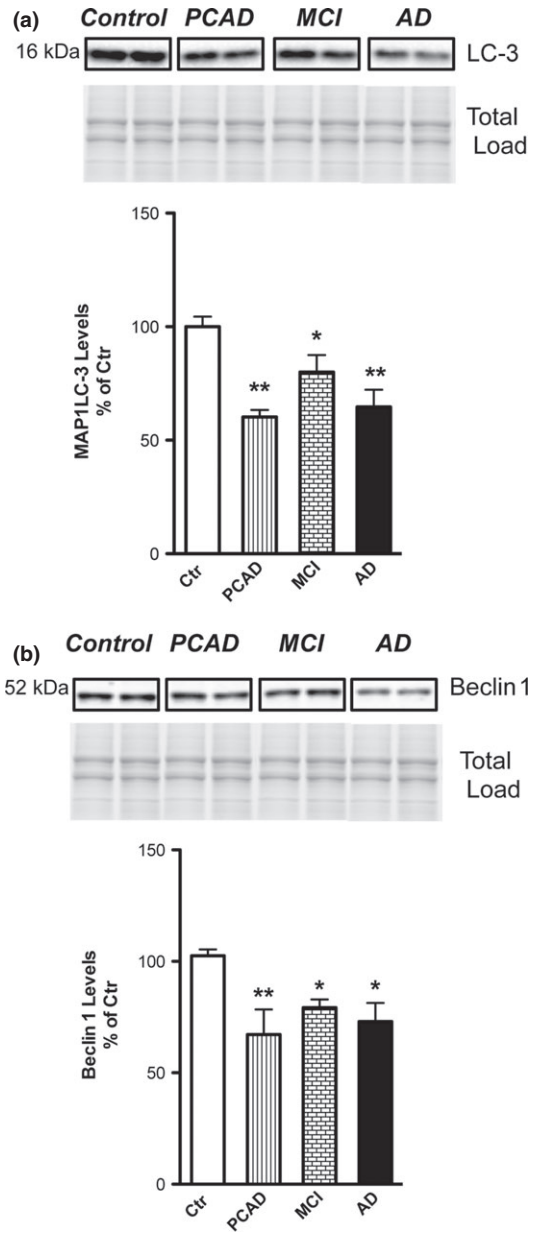

Fig. 3 Autophagy Markers, LC-3 (panel a) and Beclin1 (panel b) expression levels in IPL of Controls, PCAD, MCI and AD cases. Densitometric values shown in the bar graph are the mean of 8 individual cases per group normalized per total protein load and are given as percentage of Control, set as 100%. On the top a representative blot image with protein bands is shown. **p < 0.05 and **p < 0.01.*

however, Akt phosphorylation also is able to target and phosphorylate GSK-3β on Ser^{9} and Tyr ^{216} residues, respectively, indicating the inhibitory and stimulatory phosphorylation sites (Griffin *et al.* 2005). Therefore, we investigated GSK-3β levels and phosphorylation at Ser^{9} and Tyr ^{216} in PCAD, MCI and AD IPL. Expression values of GSK-3β decreased significantly in AD cases (about 30%, *AD vs. Ctr, $p < 0.05$, quantitative data not shown) compared to control (Fig. 5a), while the inhibitory phosphorylation was increased in the AD group by 70% more than control. On the other hand, the stimulatory phosphorylation on Tyr ^{216} showed a significant decrease in AD of about 60% (**AD vs. Ctr, $p < 0.01$). Hence, the ratio of p-(Ser^{9}) GSK-3β and p-(Tyr ^{216})GSK-3β to total GSK-3β expression was respectively increased and decreased only in AD compared to control (***AD vs. Ctr, $p < 0.001$; **AD vs. Ctr, $p < 0.01$) (Fig. 5a). No differences were found in PCAD and MCI compared to control (Fig. 5a). These results supported the potential inhibition of GSK-3β, linked to a decreased activation on Tyr ^{216}, induced in AD IPL by Akt activation.

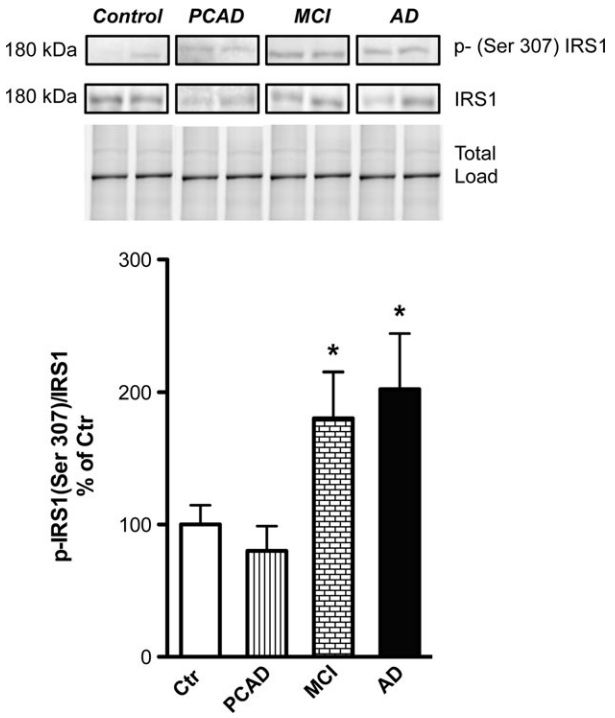

Fig. 4 IRS1 phosphorylation levels (Ser307), in IPL of Controls, PCAD, MCI and MCI AD cases. Densitometric values shown in the histograms are the mean of 8 individual cases per group normalized per total protein load and are given as percentage of Control, set as 100%. On the top a representative blot image with protein bands is shown. **p < 0.05.*

On the other hand, the hyperphosphorylation of tau in all cases was demonstrated (about 40%, **AD vs. Ctr, $p < 0.01$; 50%, *MCI vs. Ctr, $p < 0.05$ and 70%, **PCAD vs. Ctr, $p < 0.01$), suggesting that p-tau may be dependent on other kinases in addition GSK-3β activity (Fig. 5b). No differences were found in tau expression in PCAD and MCI IPL compared to control, but a slight increase was detected between the AD and control groups.

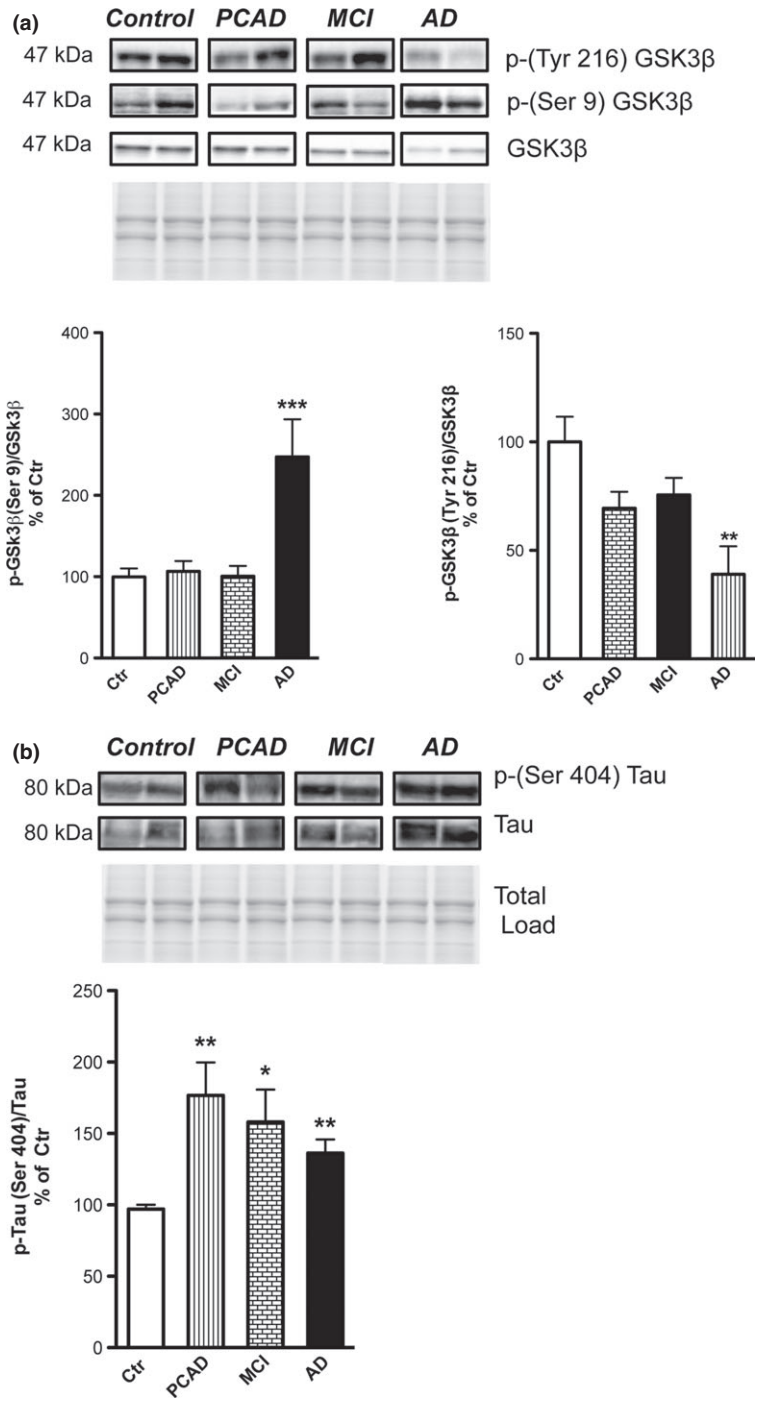

Fig. 5 GSK3β (Ser9 and Tyr216) phosphorylation levels (panel a) and tau (Ser404) phosphorylation levels (panel b) in IPL of Controls, PCAD, MCI and AD cases. Densitometric values shown in the bar graphs are the mean of 8 individual samples per group normalized per total protein load and are given as percentage of Control, set as 100%. On the top a representative blot image with protein bands is shown. ****p < 0.001; *p < 0.05; **p < 0.01.*

Discussion

The present study describes the alteration of the PI3K/Akt/mTOR pathway in the brain (IPL) of three different stages of AD (PCAD, MCI and late-stage AD). We observed a significant up-regulation of the PI3K/Akt/mTOR pathway in both MCI and AD but no differences between PCAD IPL compared to that of control. So far, the literature on PCAD is limited and recent studies showed that in the IPL of PCAD subjects no significant changes in global OS relative to controls was observed (Aluise *et al.* 2010, 2011). The results on PCAD subjects presented in the current study are in agreement with previous studies and could be explained by the fair amount of Aβ and hyperphosphorylation of tau in the brain of these subjects. Moreover, the PI3K/Akt/mTOR axis regulates cell survival, growth and metabolism, and the lack of modifications of this axis in PCAD IPL might be related to the absence of cell loss (West *et al.* 2004) as well as neuronal

hypertrophy (Iacono *et al.* 2008) and increased synaptic plasticity (O'Brien *et al.* 2009) that occurs in PCAD brain. Thus, our data support the notion we posed in 2010 (Aluise *et al.* 2010), that in PCAD there is a relative protective period against AD-related neurodegeneration. In contrast, we found significant decreased levels of Beclin 1 and LC-3, both markers of autophagy activity that can be translated as an onset of autophagy impairment. Deficiency of Beclin 1 in cultured neurons and transgenic mice is shown to be consistent with the deposition of Aβ peptides, whereas overexpression of Beclin-1 reduces the accumulation of Aβ (Pickford *et al.* 2008).

A different scenario is observed in MCI brain where the changes in cognition exceed those in normal controls and the spectrum of neuropathology can predict the progression from MCI to AD (Petersen 2004). Studies showed that Aβ accumulation is associated with enhanced OS in both MCI and AD specimens (Butterfield and Sultana 2007). In our study, we confirmed an increase in Aβ (1–42) in both AD and MCI subjects that might interact with the PI3K/Akt/mTOR pathway inducing the overactivation of this axis (O'Neill *et al.* 2012). The deregulation of this signaling pathway could conceivably represent a crucial mechanism by which Aβ instigates a pathophysiological effect on synaptic function and tau, contributing to the development of AD pathology *In vitro* and *in vivo* studies showed that that Aβ oligomer production induces kinases, like PI3K and extracellular-regulated kinase 2 (ERK2), which phosphorylate Akt and mTOR in AD brain (Bhaskar *et al.* 2009 and Talbot *et al.* 2012). Consistent with the previous studies, we found an increased phosphorylation of Akt and PI3K (p85subunit), respectively at Ser^{473} and Tyr^{508} in both MCI and AD in IPL compared to healthy subjects.

PI3K/Akt signaling is attenuated by PTEN through dephosphorylation of PIP. The lipid and protein phosphatase PTEN dephosphorylates PI3K and is a vital negative regulator of Akt activation (Leslie and Downes 2002); thus reduced PTEN levels resulted in hyperactivation of the Akt signaling. A significant loss of neurons immunoreactive to PTEN reportedly was evident in AD hippocampus and temporal cortex, and PTEN correlated negatively with the severity of NTFs and with the levels of SPs (Griffin *et al.* 2005). The impairment of PI3K/Akt/PTEN in the IPL samples of both AD and MCI of this study could reflect the diversity of its downstream targets. The main downstream effector of this pathway is mTOR. mTOR acts mainly through two mTOR complexes: mTORC1 and mTORC2 in which mTOR associates with its regulatory proteins. The components of mTORC1 currently include mTOR, which is the catalytic subunit of the complex, and Raptor. Raptor is mTOR-binding protein that also binds to 4EBP1 and p70S6K, two main downstream targets of mTOR. The binding of Raptor to mTOR is essential to induce the phosphorylation of 4EBP1 *in vitro*, and it strongly enhances the mTOR kinase activity toward p70S6K (Hara *et al.* 2002). Moreover, the PI3K/Akt signaling pathway, in concert with mTOR, induces the phosphorylation of 4EBP1 and p70S6K (Hara *et al.* 2002; An *et al.* 2003). Our results showed a strong activation in both downstream targets of the PI3K/Akt/mTOR pathway in AD and MCI. The activation of p70S6K and 4EBP1, downstream of mTORC1, has been identified as a contributor to hyperphosphorylated tau. Both dramatically increase in AD and are positively correlated with tau phosphorylation (Li *et al.* 2005a,b).

A recent study establishes a relationship between mTOR signal activation and AD and also a possible correlation of mTOR activation with the degree of cognitive impairment in AD (Yates *et al.* 2013). Our results confirmed the up-regulation of mTOR phosphorylation in IPL of AD patients, and for the first time, we report a significant increase in mTOR phosphorylation in IPL of MCI subjects. In addition, the hyperphosphorylation of mTOR in AD and MCI subjects, is positively and significantly correlated with increased amounts of Aβ (Fig. 2).

In the current study, we also demonstrated that mTOR hyperactivation might alter autophagy activation in subjects with AD and MCI IPL. Indeed, decreased levels of the LC-3 complex, an index of autophagosome formation, coupled with decreased levels of Beclin, another marker of autophagy activity, occur in PCAD, MCI, and AD brain. Considering the data on amounts of Aβ obtained, we suggest that the overactivation of the PI3K/Akt/mTOR axis, which results in autophagy reduction, might be involved in the decrease in protein degradation pathways leading to Aβ deposition since early in AD progression.

Different Aβ species, including monomers and soluble oligomers, not only impact the impairment of the PI3K/Akt/mTOR axis, but also are able to induce insulin resistance (Butterfield *et al.* 2014b). Indeed, Aβ monomers and soluble oligomers bind to IRs, induce IR internalization in neurons and remove IR from dendrites thus leading to insulin resistance in AD (Townsend *et al.* 2007; Zhao *et al.* 2008; De Felice *et al.* 2009; Zhao and Townsend 2009; Moloney *et al.* 2010; De la Monte 2012; Butterfield *et al.* 2014b). An increased phosphorylation of the IRS1 inhibitory serine residue (Ser^{307}) in both AD and MCI IPL when compared to their control was observed. This result correlates with the impairment of the PI3K/Akt/mTOR pathway shown in both MCI and AD subjects. Significant evidence indicates that IRS1 is phosphorylated at Ser^{307} by increased Akt/mTOR/p70S6K activity in AD neurons leading these cells to become totally resistant to both insulin and IGF-1 (Craft 2005; Moloney *et al.* 2010; De la Monte 2012). In our previous study, we described an increase in IRS1 inhibitory phosphorylation at serine residue (Ser^{307}) in frontal cortex of DS subjects compared to their controls that is maintained in DS with AD pathology (Butterfield *et al.* 2014a; Perluigi *et al.* 2014).

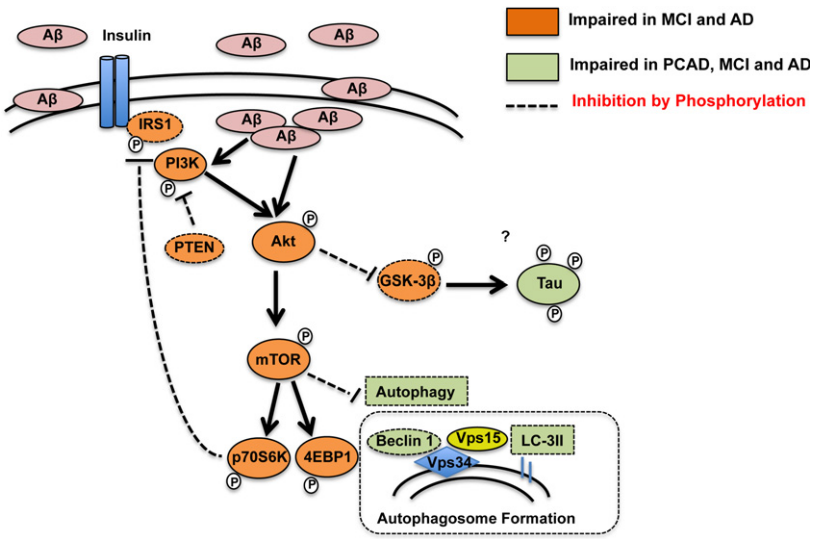

Fig. 6 Role of the PI3K/Akt/mTOR pathway in IPL in three clinical stages of AD. The PI3K/Akt/mTOR axis is represented; increased Aβ induces an impairment of this axis and its downstream targets in MCI and AD (orange), but not in PCAD compared to control, except p-tau and autophagy markers (green). Black arrow and dashed lines indicate, respectively, phosphorylation-induced stimulatory and inhibitory proteins.

Further, PI3K/Akt/mTOR signaling is considered a primary candidate to transmit pathophysiological responses from Aβ to tau. Up-regulation of p70S6K was found to be associated with accumulation of hyperphosphorylated tau in NTFs in AD (An *et al.* 2003; Li *et al.* 2005a,b). In our study, we confirmed the hyperphosphorylation of tau (Ser^{404}) in AD and extended these results to PCAD and MCI. GSK-3β is one of the major candidate kinases involved in tau hyperphosphorylation, Akt inhibits GSK-3β, (Bhat *et al.* 2004), and consistent with this notion we evaluated the phosphorylation of GSK-3β at Ser^{9}, its inhibitory residue and at Tyr^{216}, its stimulatory residue (Hong and Lee 1997; Baki *et al.* 2004). Our data emphasize a link between Akt hyperactivation and GSK-3β inhibition through increased phosphorylation at Ser^{9} only in IPL of the AD group. To confirm a decreased activity of GSK-3β, we measured the phosphorylation on its stimulatory residue (Tyr^{216}). Our results showed a strongly decreased GSK-3β activation in the AD groups. Thus, the finding of increased phosphorylation and decreased trends of GSK-3β phosphorylation and potential loss of the GSK-3β kinase activity suggest that Akt induces the inactivation of GSK-3β in AD but not in MCI. In contrast, different studies described an increased GSK-3β activity in AD, which lead to tau hyperphosphorylation (Lovestone and Reynolds 1997), strategies to inhibit GSK-3β has been developed for AD treatments but showed no efficacy so far (Bhat *et al.* 2004). Our group showed that down-regulation of GSK-3β led to decreased phosphorylated tau in brain of senescence accelerated prone mice-8 (SAMP8) mice, a model of Aβ-related AD (Farr *et al.* 2014). In our previous study we found the inhibition of brain GSK-3β only in DS patients who had developed AD and not in young DS; thus, we could speculate that the imbalance in the activity of GSK-3β occurs only in the late stage of AD but not earlier, similar to what we observed in the present study.

In summary (Fig. 6), our results show a hyperactivation of the PI3K/Akt/mTOR axis in the IPL region of AD and MCI subjects in comparison to control but no differences between PCAD compared to controls. Such alterations might lead to the decreased autophagy as demonstrated by the reduction in autophagy markers (Beclin-1 and LC-3). Interestingly, autophagy also is predicted to be impaired in PCAD subjects, suggesting this as an early event in AD progression that might contribute to Aβ deposition into the brain. Furthermore, increased IRS1 inhibitory phosphorylation was demonstrated in IPL of MCI and AD subjects supporting a link between Aβ and PI3K/Akt/mTOR axis and providing new insights into the relationship between AD pathology and insulin resistance. Indeed, we showed a positive correlation of Aβ and mTOR. Akt hyperphosphorylation might be involved in the reduction in GSK-3β, thus raising doubt on the effective role of GSK-3β in early stages of AD as tau kinases. In addition, this study suggests alteration of mTOR signaling and related molecular pathways in the IPL of AD and MCI subjects, but not in PCAD, supporting a role for OS in the dysregulation of those pathways.

Acknowledgments and conflict of interest disclosure

The authors thank the faculty of Sanders-Brown Center of Aging for well-documented specimens used in this study. The authors did not

receive external funding for this study and have no conflicts of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

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