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ER Stress Is Involved in Aβ-Induced GSK-3β Activation and Tau Phosphorylation

Rosa Resende, Elisabete Ferreiro, Cláudia Pereira,* and Catarina Resende Oliveira

Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Intracellular neurofibrillary tangles, one of the characteristic hallmarks of Alzheimer's disease (AD), are mainly composed of hyperphosphorylated tau. The abnormal tau phosphorylation seems to be related to altered activity of kinases such as glycogen synthase kinase-3β (GSK-3β). Tau pathology is thought to be a later event during the progression of the disease, and it seems to occur as a consequence of amyloid-beta (Aβ) peptide accumulation. The aim of this work was to investigate whether soluble Aβ1–42, particularly oligomers that correspond to the neurotoxic species involved early in the development of AD, triggers tau phosphorylation by a mechanism involving activation of tau-kinase GSK-3β. Several studies suggest that GSK-3β plays a central role in signaling the downstream effects of endoplasmic reticulum (ER) stress. Therefore, the involvement of ER Ca^{2+} release in GSK-3β activation and tau phosphorylation induced by Aβ1–42 oligomers was evaluated using dantrolene, an inhibitor of Ca^{2+} release through channels associated with ER ryanodine receptors. We observed that Aβ1–42 oligomers increase tau phosphorylation and compromises cell survival through a mechanism mediated by GSK-3β activation. We also demonstrated that oligomeric Aβ1–42 induces ER stress and that ER Ca^{2+} release is involved in oligomer-induced GSK-3β activation and tau phosphorylation. This work suggests that GSK-3β can be a promising target for therapeutic intervention in AD.

Key words: Alzheimer's disease; Aβ oligomers; tau phosphorylation; glycogen synthase kinase (GSK-3β); endoplasmic reticulum

The presence of intracellular neurofibrillary tangles (NFTs) composed of paired helical filaments of hyperphosphorylated tau is one of the hallmarks of Alzheimer's disease (AD; Price et al., 1998; Avila, 2006). Tau is a microtubule-associated protein whose main function is to bind and stabilize microtubules, allowing neurite extension and stabilization (Avila, 2006). Hyperphosphorylation of tau, associated with its detachment from microtubules (Alonso et al., 1994), perturbs axonal transport of synaptic vesicles and mitochondria, leading to neurodegeneration (Ebneth et al., 1998; Stamer et al., 2002; Mandelkow et al., 2004). Microtubule-dependent transport is impaired in AD (Terry, 1996). Tau protein can be phosphorylated through proline-directed kinases, namely, glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase-5 (cdk5) and non-proline-directed kinases such as protein kinase A and protein kinase C (Avila, 2006). Several in vitro (Ishiguro et al., 1992, 1993; Lovestone et al., 1994) and in vivo (Plattner et al., 2006) studies have demonstrated that GSK-3β phosphorylates tau. Overactivation of GSK-3β correlates with hyperphosphorylation of tau and the appearance of paired helical filaments (Liu et al., 2003), which are abolished by lithium chloride (LiCl), a selective inhibitor of GSK-3β. GSK-3β is found in the neurofibrillary tangles (NFTs) present in the brains of AD patients (Yamaguchi et al., 1996; Pei et al., 1999) and in transgenic mice overexpressing mutant human tau (Ishizawa et al., 2003). These studies support the idea that tau is a substrate for GSK-3β, which is a downstream element of the phosphoinositol-3 kinase (PI3K)/Akt cell survival pathway. PI3K is known to stimulate Akt, which in turn phosphorylates GSK-3β at the Ser9 residue, leading to enzyme inactivation (Grimes and Jope, 2001).

Several reports have demonstrated that amyloid-beta (Aβ) peptide leads to tau hyperphosphorylation (Ma et al., 2006; De Felice et al., 2007). Rapoport and colleagues

Abbreviations used: AD, Alzheimer's disease; Aβ, amyloid-beta peptide; BSA, bovine serum albumin; ER, endoplasmic reticulum; cdk5, cyclin-dependent kinase-5; DTT, 1,4-dithiotreitol; GSK-3β, glycogen synthase kinase 3β; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; RyRs, ryanodine receptors; RT, room temperature; NFTs, neurofibrillary tangles; PKA, protein kinase A; PKC, protein kinase C; ThS, thioflavin S.

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*Correspondence to: Cláudia Pereira, Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, 3004-504 Coimbra, Portugal. E-mail: cpereira@cnc.cj.uc.pt

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(2002) showed that tau-depleted neurons do not degenerate in the presence of Aβ, providing direct evidence of a role for tau in the mechanisms leading to Aβ-induced neurodegeneration. Accumulation of Aβ is one of the earliest events in AD, with tau pathology a later event in the development of the disease. Recent studies suggest that soluble oligomeric Aβ might be the main neurotoxic species early involved in AD (Deshpande et al., 2006, Lesné et al., 2006). In a triple transgenic mouse model ($PS1_{M146V}$, APP_{Swe}, and tau_{P301L}), it was shown that the injection of the antioligomer antibody A11 leads to reduction of the tau and Aβ pathologies, suggesting a link between Aβ oligomers and tau pathology (Oddo et al., 2006). Further, intraneuronal Aβ1–42 accumulation leads to down-regulation of Akt consistent with activation of GSK-3β and apoptosis (Magrané et al., 2005). Taken together, this evidence supports that GSK-3β has a key role in tau pathology induced by soluble Aβ oligomers. We have recently demonstrated that Ca^{2+} released from endoplasmic reticulum (ER) through ryanodine receptor (RyR) contributes to the early increase in intracellular Ca^{2+} levels on exposure of cortical neurons to Aβ oligomers and to the activation of apoptosis (Resende et al., unpublished results). The present work was aimed to investigate whether soluble oligomeric Aβ1–42 triggers tau phosphorylation and to evaluate the involvement of GSK-3β in this process. Because several studies suggest that GSK-3β plays a central role in signaling pathways downstream of ER stress (Song et al., 2002; Brewster et al., 2006), the involvement of Aβ oligomers-induced ER Ca^{2+} release was also evaluated. We observed that Aβ1–42 oligomers increase tau phosphorylation, decreasing cell survival, through a mechanism involving GSK-3β activation. We also demonstrated that Aβ1–42 oligomers increase GRP78 levels and activate caspase-12, two ER stress markers. In the presence of dantrolene, an inhibitor of RyR-mediated ER Ca^{2+} release, oligomer-induced GSK-3β activation and tau phosphorylation were decreased, demonstrating that ER Ca^{2+} release is involved in Aβ oligomer-induced GSK-3β-mediated tau phosphorylation and subsequent neuronal cell death.

MATERIALS AND METHODS

Materials

Neurobasal medium and B27 supplement were purchased from GIBCO BRL (Life Technologies, Paisley, UK). Trypsin, trypsin inhibitor type II–soybean, deoxyribonuclease I (DNase I), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and thioflavin T were obtained from Sigma Chemical Co. (St. Louis, MO). GSK3β inhibitor I was from Calbiochem (San Diego, CA). Alexa Fluor 488 goat antimouse IgG conjugate was purchased from Molecular Probes (Leiden, Netherlands). The synthetic Aβ1–42 peptide was from Bachem (Bubendorf, Switzerland). Phenol red-free Ham's F-12 medium was purchased from Cambrex Bio Science (Walkersville). The reagents and apparatus used in immunoblotting assays were obtained from Bio-Rad (Hercules, CA), whereas poly(vinylidene difluoride) (PVDF) membrane, goat alkaline phosphatase–linked antimouse secondary antibody, and enhanced chemifluorescence (ECF) reagent were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antihuman PHF-tau (clone AT8) and antitau monoclonal antibodies were obtained from Pierce Endogen (Rockford, IL). Anti-GSK-3β, anti-GRP78, and anti-pro–caspase 12 antibodies were purchased from BD Biosciences (San Jose, CA), whereas Phospho-GSK-3β (Ser9) was from Cell Signalling (Danvers, MA). The Glycergel Mounting Medium was purchased from DakoCytomation Inc. (Carpinteria, CA). All others chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or from Merck kgaA (Damstadt, Germany).

Primary Rat Embryo Cortical Neuronal Cultures

Cortical neurons were isolated from E15-E16 Wistar rat embryos according to the method described by Hertz and collaborators (1989) as slightly modified (Resende et al., 2007). Briefly, removed cortices were aseptically dissected and washed in Ca^{2+}- and Mg^{2+}-free Krebs buffer [120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 13 mM glucose, 10 mM Hepes (pH 7.4)] and then incubated in Krebs solution supplemented with BSA (0.3 g/L) containing trypsin (0.5 g/L) and DNase I (0.04 g/L) for 10 min at 37°C. Tissue digestion was stopped by the addition of trypsin inhibitor (type II-S; 0.75 g/L) in Krebs buffer containing DNase I (0.04 g/L), followed by a centrifugation at 140*g* for 5 min. After washing the pellet once with Krebs buffer, the cells were dissociated mechanically and resuspended in fresh Neurobasal medium with 2 mM glutamine, 2% B27 supplement, penicillin (100,000 U/L), and streptomycin (100 mg/L).

The cells were seeded on poly-L-lysine (0.1 g/L)–coated dishes at a density of 0.125×10^6 cells/cm^2 for the MTT assay or at 0.33×10^6 cells/cm^2 for Western blotting. For fluorescence studies, neurons were mounted on poly-L-lysine-coated glass coverslips at a density of 0.1×10^6 cells/cm^2. The cultures were maintained in serum-free Neurobasal medium supplemented with B27 at 37°C in a humidified atmosphere of 5% CO_2/95% air for 5–7 days before treatment in order to allow neuronal differentiation. Under these conditions, glial growth is less than 10% (Ferreiro et al., 2006).

Preparation of Amyloid-β Peptide Solutions and Treatment Protocols

Enriched oligomeric and fibrillar Aβ1–42 preparations. The synthetic peptide Aβ1–42, corresponding to neurotoxic amino acid residues of the human amyloid-beta protein (Aβ), was dissolved in sterile water or in a diluted ammonia solution in order to facilitate peptide solubilization at a concentration of 1 g/L (221.5 μM). Aβ1–42 aliquots were then stored at −20°C until use (enriched oligomeric Aβ1–42 preparation) or were incubated for 7 days at 37°C (enriched fibrillar Aβ1–42 preparation).

To study the effect of the aggregation state of Aβ1–42 peptide on GSK-3β activation and tau phosphorylation, enriched oligomeric or fibrillar Aβ1–42 (5 μmol/L) was added to the culture medium of cortical neuronal cells for 6–24 hr.

Aβ oligomers. Synthetic Aβ1–42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to 1 mM. The HFIP was then removed in a Speed Vac (İlshin Lab. Co., Ltd., Ede, The Netherlands), and the dried HFIP film was stored at −20°C. The peptide film was resuspended to make a 5 mM solution in anhydrous dimethyl sulfoxide (Dahlgren et al., 2002). Aβ1–42 oligomers were prepared by diluting the solution in phenol red–free Ham's F-12 medium without glutamine to a final concentration of 100 μM and incubated overnight at 4°C (Lambert et al., 1998). The preparation was centrifuged at 15,000*g* for 10 min at 4°C to remove insoluble aggregates, and the supernatant containing soluble oligomers was transferred to clean tubes and stored at 4°C. Protein concentrations of Aβ oligomers and fibrils were determined using the Bio-Rad protein dye assay reagent.

To study the effect of Aβ1–42 oligomers on GSK-3β activation and tau phosphorylation, 0.5 μmol/L of isolated oligomers was added to the culture medium of cortical neuronal cells for 1–24 hr.

Aggregation State Analysis of Aβ Peptide

The ultrastructural characteristics of Aβ1–42 in enriched oligomeric and fibrillar preparations and of the oligomers, prepared as described above, were examined as follows. Each Aβ1–42 sample was placed on Formvar-carbon-coated 400-mesh copper grids for 1 min, blotted with filter paper, and then negatively stained with 7.5 g/L uranyl acetate for 1 min. The specimens were examined on a Zeiss 902A electron microscope at an accelerating voltage of 50 kV.

The presence of β-sheet fibrils has also been assessed by thioflavin S (ThS) staining in cortical neuronal cultures treated for 24 hr with enriched oligomeric or fibrillar Aβ1–42 peptide preparations (0.5 μmol/L).

Western Blotting Analysis

Cortical neurons were scrapped in culture medium, and the recovered pellets were resuspended in ice-cold lysis buffer composed of 25 mM Hepes-Na, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA and supplemented with 2 mM 1,4-dithiotreitol (DTT), 100 μM PMSF, 2 mM ortovanadate, 50 mM sodium fluoride, and a protease inhibitor cocktail containing leupeptin, pepstatin A, chymostatin, and aprotinin (1 mg/mL). The cellular extract was then rapidly frozen and thawed three times. Protein content in the samples was measured using the Bio-Rad protein dye assay reagent. Whole cellular extracts containing 30 μg of protein were separated by electrophoresis on 10% SDS–polyacrylamide gel (SDS-PAGE) after denaturation at 100°C for 5 min in a 6 × concentrated sample buffer composed of 100 mM Tris, 100 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol. To facilitate the identification of proteins, a prestained precision protein standard (Bio-Rad) was used. Proteins were then transferred to PVDF membranes, which were further blocked for 1 hr at room temperature (RT) with 5% BSA in Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6) with 0.1% Tween 20 (TBS-T). The membranes were next incubated overnight at 4°C with mouse PHF-tau (clone AT8; 1:250), antitau (1:400), rabbit anti-GSK-3β phosphorylated at Ser9 residue (1:1,000), mouse anti-GSK-3β (1:2,500), polyclonal rabbit anti-caspase-12 (1:1,000) , or mouse monoclonal anti-GRP78 (1:250) antibodies. After washing in TBS-T, membranes were incubated for 1 hr at RT with an alkaline phosphatase–conjugated anti-rabbit or antimouse secondary antibody (1:20,000 dilution in TBS-T). Bands of immunoreactive proteins were visualized after membrane incubation with ECF reagent for 5 min on a VersaDoc Imaging System, and the density of protein bands was calculated using the Quantity One Program (Bio-Rad).

MTT Assay

After treatment of cortical neurons with different concentrations of Aβ peptides, metabolic activity was evaluated by the MTT assay (Mosmann, 1983), which measures the ability of metabolic active cells to form formazan through cleavage of the tetrazolium ring of MTT. Neurons were washed in sodium medium [132 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 6 mM glucose, 10 mM Hepes, and 1 mM $CaCl_2$ (pH 7.4)] and incubated with MTT (0.5 g/L) for 2 hr at 37°C. The blue formazan crystals formed were dissolved in an equal volume of 0.04 M HCl in isopropanol and quantified spectrophotometrically by measuring the absorbance at 570 nm using a microplate reader (Spectra Max Plus 384, Molecular Devices).

Immunocytochemistry

Cortical neurons grown in glass coverslips in the presence or the absence of Aβ1–42 were washed with PBS buffer (pH 7.4) and were fixed with 4% paraformaldehyde for 15 min at RT. Then the cells were permeabilized for 2 min with 0.2% Triton X-100 in PBS buffer (pH 7.4) and blocked for 1 hr and 30 min in PBS containing 3% BSA. The cells were incubated for 1 hr with mouse PHF-tau monoclonal antibody (1:30 dilution in 3% BSA/PBS) at RT and then washed and incubated with Alexa Fluor 488 goat antimouse IgG antibody conjugate (1:200 dilution in 3% BSA/PBS) for 1 hr. Finally, the cells were treated with mounting solution on a microscope slide, and neurons were visualized in an inverted fluorescence microscope Axiovert 200 (Zeiss, Germany).

Statistical Analysis

Data are expressed as means ± SEMs of the indicated number of experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Characterization of Enriched Oligomeric and Fibrillar Aβ1–42 Preparations

It is still controversial whether the fibrillar or the oligomeric Aβ peptide is the neurotoxic species in AD. In this work we have used different assemblies of synthetic Aβ1–42, namely, the oligomeric and fibrillar peptide samples that are enriched in oligomers and fibrils, respectively (Fig. 1). As evaluated by negative-stain electron microscopy (Fig. 1), the presence and the length of

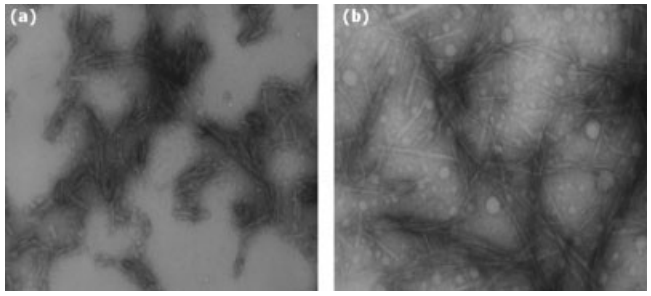

Fig. 1. Analysis of the aggregation state of Aβ1–42 peptide preparations (negative-stain electron microscopy). Representative images show increased number and length of fibrils in the sample incubated at 37°C for 1 week (**b**). The Aβ1–42 samples were placed on Formvar-carbon-coated 400-mesh copper grids, then negatively stained with 7.5 g/L uranyl acetate, and examined in an electronic microscope at an accelerating voltage of 50 kV; magnification ×30,000.

amyloid fibrils dramatically increased in enriched fibrillar Aβ1–42 (Fig. 1b) compared with those in enriched oligomeric Aβ1–42 (Fig. 1a).

When Aβ1–42 from stock solutions was added to cortical cultures, the absence or the presence of β-sheet-enriched fibrils was analyzed using ThS (Fig. 2). After 24 hr incubation with oligomer-enriched Aβ1–42 (0.5 μmol/L), amyloid fibrils were not detected (Fig. 2b), with the ThS staining similar to that observed in the control cells (Fig. 2a), which were not incubated with the Aβ peptide. On the other hand, after exposure to a similar concentration of enriched fibrillar Aβ1–42 for 24 hr, β-sheet-enriched structures were observed (Fig. 2c), demonstrating that preformed fibrils are still present when the peptide is diluted in culture medium. Similar results were obtained after 48 hr of incubation with both Aβ1–42 preparations (data not shown).

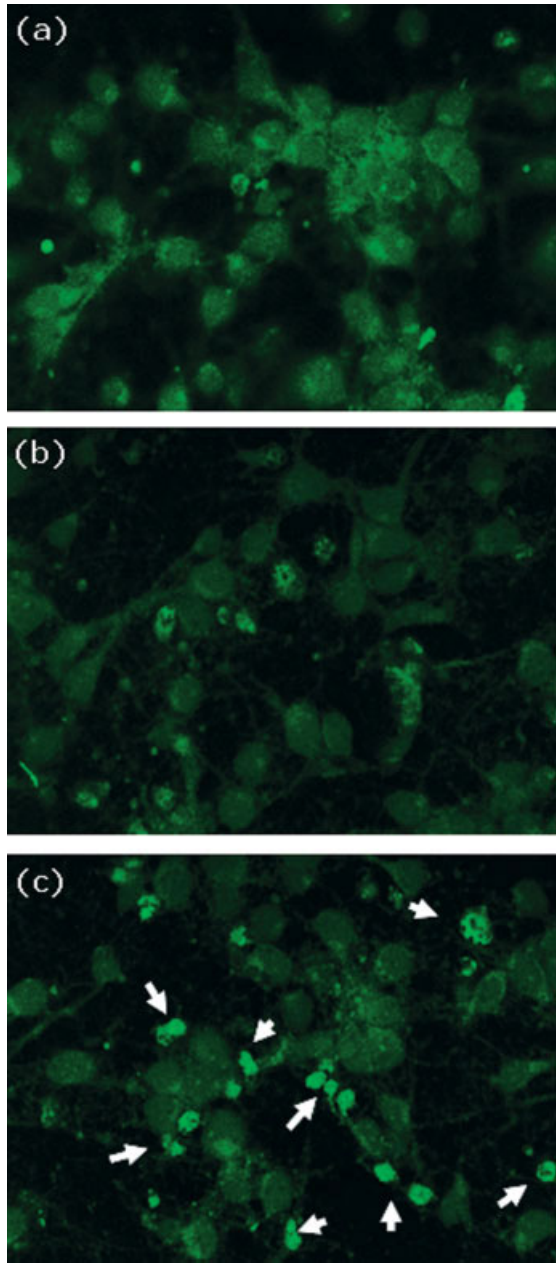

Fig. 2. Detection of β-sheet-enriched fibrils in primary cortical neuronal cultures exposed to different Aβ1–42 preparations. On incubation of cortical neurons for 24 hr with enriched oligomeric (**b**) or fibrillar Aβ1–42 preparations (**c**), the presence of β-sheet fibrils was analyzed by ThS staining and compared with control conditions in the absence of Aβ treatment (**a**). Cells were stained with ThS for 20 min in the dark and were subsequently washed with PBS buffer (pH 7.4), a 70% (vol/vol) ethanol solution, and finally with PBS. At the end, ThS-positive fibrils were visualized in a fluorescence microscope; magnification ×400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Aβ1–42 Induces Tau Phosphorylation and Decreases Cell Survival through Glycogen Synthase Kinase (GSK3β)

To investigate whether different assemblies of Aβ1–42 lead to tau phosphorylation, the level of phosphorylated tau at Ser202 residue was quantified by Western blotting in cortical neurons treated with 5 μM enriched oligomeric or fibrillar Aβ1–42. The oligomeric peptide significantly increased tau phosphorylation after 6 hr of treatment ($P < 0.05$), which persisted after 24 hr ($P < 0.01$), whereas the peptide mainly composed of fibrils had significantly increased tau phosphorylation only after 24 hr ($P < 0.05$; Fig. 3A). The increased tau phosphorylation observed on treatment with the oligomer-enriched Aβ1–42 sample (5 μM) after 24 hr was confirmed by immunocytochemistry (Fig. 3B). The level of GSK-3β phosphorylated at Ser9 residue, the inactive form of GSK-3β, significantly decreased in the presence of oligomeric ($P < 0.01$) and fibrillar ($P < 0.05$) Aβ1–42 peptide, suggesting that both Aβ preparations induce GSK-3β activation (Fig. 4A). Because oligomeric Aβ1–42 induced an early increase in tau phosphorylation (Fig. 3A) and a more significant effect on

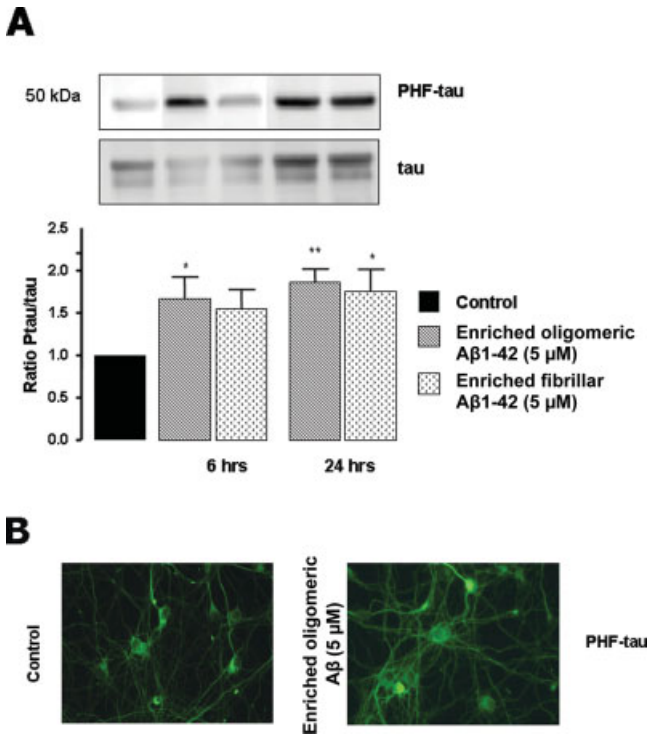

Fig. 3. Enriched oligomeric and fibrillar Aβ1–42 peptide induced tau phosphorylation. **A:** Cortical neurons were treated with enriched oligomeric or fibrillar samples of Aβ1–42 (5 μM) for 6 or 24 hr, and the levels of phosphorylated tau were quantified by Western blotting. Thirty mirograms of cell lysate protein was separated by SDS-PAGE and immunoblotted with PHF-tau or antitau antibodies. The ratio of P-tau/total tau was determined by densitometry analysis from three independent experiments ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from control). **B:** Neurons were treated with enriched oligomeric Aβ1–42 peptide (5μM) for 24 hr, fixed with 4% paraformaldehyde, and immunostained with a mouse PHF-tau antibody. Representative images of control and Aβ1–42-treated neurons were obtained from three independent experiments; magnification ×400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

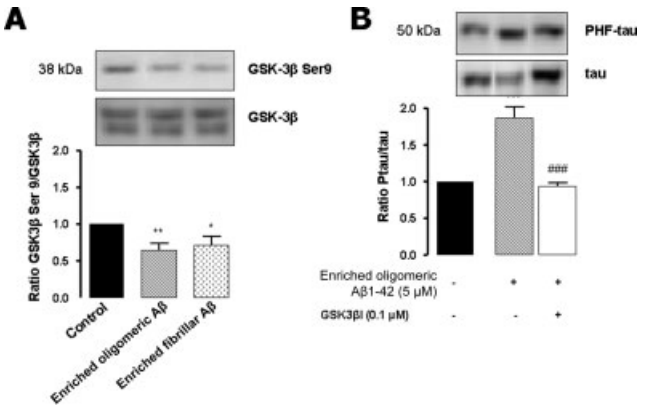

Fig. 4. GSK-3β inhibition decreased Aβ-induced tau phosphorylation. Cortical neurons were treated with 5 μM oligomer-enriched Aβ1–42 peptide alone or in combination with 0.1 μM GSK-3β inhibitor I (GSK3βI) for 24 hr, and the levels of GSK-3β phosphorylated on Ser9 residue (**A**) and phosphorylated tau (**B**) were quantified by Western blotting. Thirty micrograms of cell lysate protein were separated by SDS-PAGE and immunoblotted with PHF-tau, antitau, anti-phospho-GSK-3β (Ser9), or anti-GSK-3β antibodies. The ratio of P-protein/total protein was determined by densitometry analysis from three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from control; $^{\#\#\#}P < 0.001$, significantly different from the results obtained in control cortical neurons in the absence of GSK-3β inhibitor).

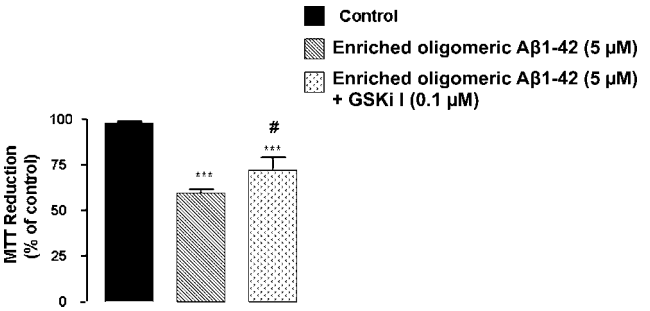

Fig. 5. Effect of GSK3β inhibition on Aβ1–42-induced toxicity. Cultured cortical neurons were treated for 6 hr with 5 μM enriched oligomeric Aβ1–42 preparation in the presence or in the absence of 0.1 μM GSK-3β inhibitor I. After that, metabolic activity was evaluated by measuring the capacity of the cells to reduce MTT. The results are presented as the percentage of absorbance determined for control conditions and represent the means ± SEMs of at least three independent experiments performed in duplicate ($^{\star\star\star}P < 0.001$, significantly different from control; $^{\#}P < 0.01$, significantly different from MTT reduction determined in cortical cultures exposed to oligomeric Aβ peptide).

GSK-3β activity (Fig. 4A), subsequent studies were performed in cortical neurons exposed to the peptide sample enriched in oligomeric species. In the presence of a GSK-3β inhibitor, GSK-3β inhibitor I (0.1 μM), a decrease ($P < 0.001$) in enriched oligomeric Aβ-induced tau phosphorylation was observed (Fig. 4B), demonstrating the involvement of this kinase in tau modification by soluble Aβ1–42. Similar results were obtained in the presence of 10 mM LiCl (data not shown).

In cortical neurons treated for 24 hr with 5 μM enriched oligomeric Aβ1–42, a significant decrease ($P < 0.001$) in cell survival was observed (Fig. 5), which was partially prevented ($P < 0.05$) in the presence of 0.1 μM GSK-3β inhibitor I, demonstrating that in addition to tau phosphorylation, GSK-3β is involved in soluble Aβ-induced toxicity.

Aβ1–42 Oligomers Induce ER Stress

Because the enriched oligomeric peptide, mainly composed of low-*n* oligomers (~14 kDa; Resende et al., unpublished results), was shown to induce GSK-3β activation, which is known to occur under ER stress condi-

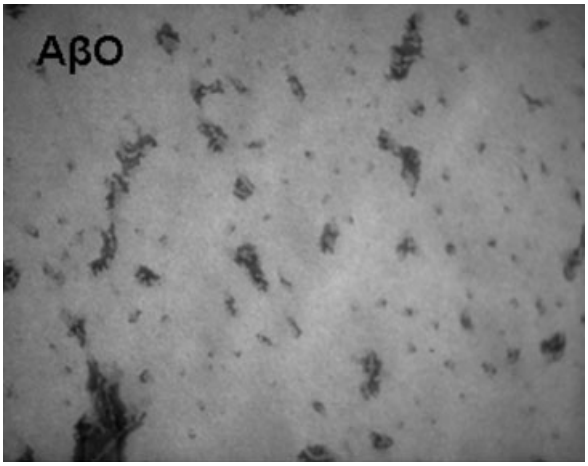

Fig. 6. Preparation of Aβ oligomers (negative-stain electron microscopy). Aβ1–42 oligomers (AβO) were placed on Formvar-carbon-coated 400-mesh copper grids, then negatively stained with 7.5 g/L uranyl acetate and examined at an accelerating voltage of 50 kV. Magnification ×30,000.

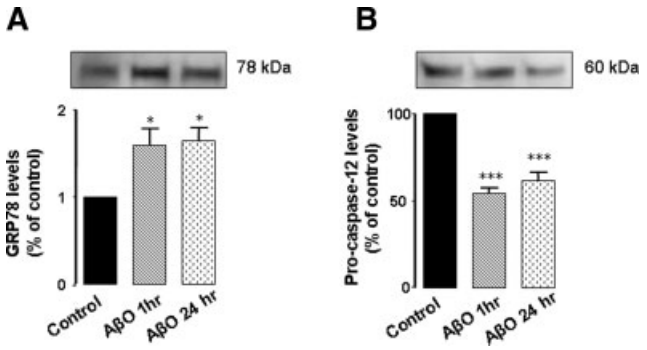

Fig. 7. Aβ1–42 oligomers induce caspase-12 activation and increase GRP78 levels. Cortical neurons were treated with 0.5 μM Aβ1–42 oligomers for 1 or 24 hr, and the levels of pro-caspase 12 (**A**) and GRP78 (**B**) were quantified by Western blotting. Thirty micrograms of cell lysate protein were separated by SDS-PAGE and immunoblotted with polyclonal rabbit anti-caspase-12 or with mouse monoclonal anti-GRP78 antibodies. Protein levels were determined by densitometry analysis from three independent experiments ($\star P < 0.05$, $\star\star\star P < 0.001$, significantly different from control).

tions (Song et al., 2002; Brewster et al., 2006), we decided to isolate Aβ1–42 oligomers (AβO; Fig. 6) and investigate whether they induce ER stress. For this purpose, we analyzed two known ER stress markers, namely, the ER chaperone GRP78 and the ER-resident caspase-12 (Nakagawa and Yuan, 2000; Rao et al., 2002), determining the levels of GRP78 and pro–caspase 12 by Western blotting. In cortical neurons treated with 0.5 μM Aβ1–42 oligomers for 1 or 24 hr, a significant increase ($P < 0.05$) in GRP78 levels was observed (Fig. 7A). Moreover, the level of pro–caspase 12 significantly decreased ($P < 0.001$) in treated neurons (Fig. 7B), suggesting that Aβ oligomers induce caspase 12 activation. These results demonstrate that Aβ1–42 oligomers induce ER stress in cortical neurons in culture.

ER Ca^{2+} release Is Involved in Aβ1–42 Oligomers-Induced Tau Phosphorylation

Tau phosphorylation that occurs in cortical neurons treated for 6 or 24 hr with Aβ1–42 oligomers (0.5 μM) was prevented ($P < 0.05$) in the presence of 10 μM dantrolene, an inhibitor of ER Ca^{2+} release through channels associated with RyRs (Fig. 8A). Aβ oligomer-induced GSK-3β activation, observed after 24 hr ($P < 0.01$), was also prevented ($P < 0.01$) in cells preincubated with dantrolene (Fig. 8B). On incubation of cortical neurons with Aβ1–42 oligomers for 6 hr, GSK-3β activity was not significantly affected (data not shown), suggesting the involvement of other mechanisms in tau phosphorylation after 6 hr of incubation. Together, these results suggest the involvement of ER Ca^{2+} release in Aβ1–42 oligomer–induced GSK-3β activation and subsequent tau phosphorylation.

DISCUSSION

Tau pathology has been described to be a later event in Alzheimer's disease (AD) development (LaFerla and Oddo, 2005). The abnormal tau phosphorylation that occurs in AD seems to be related to the altered activity of kinases such as glycogen synthase kinase-3β (GSK-3β). It has been previously demonstrated that amyloid-beta (Aβ) increases the activity of GSK-3β and the phosphorylation of tau (Takashima et al., 1993, 1996; Tomidokoro et al., 2001; De Felice et al., 2007; Koh et al., 2008). In addition, immunization with anti-Aβ antibodies reduces oligomer-induced activation of GSK-3β and tau pathology (Oddo et al., 2006; Ma et al., 2006), suggesting that Aβ peptides, in oligomeric form may lead to the hyperphosphorylation of tau through a mechanism involving GSK-3β activation. In the present study, using different assemblies of Aβ1–42 peptide, it was shown that Aβ oligomers activate GSK-3β and induce tau phosphorylation under conditions associated with ER stress. Moreover, it was demonstrated that the perturbation of ER Ca^{2+} homeostasis because of Ca^{2+} release through RyRs is involved in the activation of GSK-3β and the subsequent phosphorylation of the tau protein observed in cortical neurons on the addition of oligomeric Aβ1–42. We used two preparations of the synthetic Aβ1–42 peptide, oligomeric and fibrillar preparations, which are enriched in low-*n* oligomeric forms and fibrils, respectively. Even though both peptide samples induced tau phosphorylation, an earlier and more significant effect was observed in neurons incubated with the oligomeric peptide. In addition, an increase in the activity of GSK-3β was observed on treatment of cortical neuronal cultures with both oligomeric and fibrillar peptide. However, the soluble Aβ form was more effective than the fibril-enriched peptide. Taking these results into account and those we have pre-

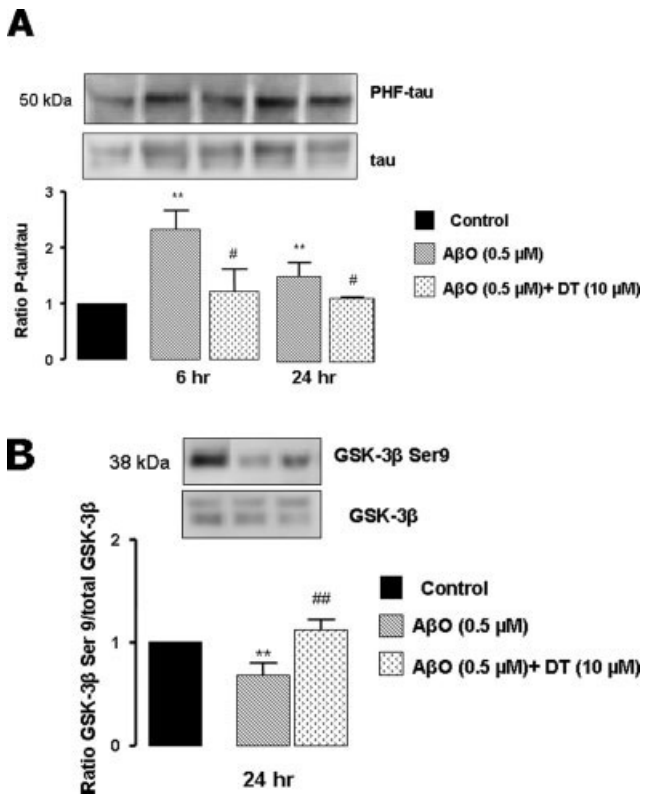

Fig. 8. Dantrolene prevents Aβ1–42-oligomer-induced GSK3β activation and tau phosphorylation. Cultured cortical neurons were treated for 6 or 24 hr with 0.5 μM Aβ1–42 oligomers (AβO) alone or in combination with 10 μM dantrolene (DT). Levels of phosphorylated tau (**A**) and GSK-3β phosphorylated on Ser9 residue (**B**) were quantified by Western blotting. Thirty micrograms of cell lysate protein was separated by SDS-PAGE and immunoblotted with PHF-tau, antitau, anti-phospho-GSK-3β (Ser9), or anti-GSK3β antibodies. The ratio of P-tau/total tau and P-GSK-3β/total GSK3β was determined by densitometry analysis from three independent experiments ($^{\star}P < 0.05$, $^{\star\star}P < 0.01$, significantly different from control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from neurons treated with AβO alone).

viously obtained demonstrating that oligomeric Aβ is more toxic than fibrillar Aβ (Resende et al., unpublished data), we decided to use the oligomeric peptide and isolated Aβ1–42 oligomers in most of the experiments. We have previously demonstrated that Aβ oligomers impair ER Ca^{2+} homeostasis, leading to early ER Ca^{2+} release, which is involved in Aβ-induced apoptotic neuronal death. Many studies have demonstrated that Aβ induces tau hyperphosphorylation (De Felice et al., 2007; Ma et al., 2006), and studies have also demonstrated that GSK-3β is involved (Ma et al., 2006; Koh et al., 2008). It has also been suggested that GSK-3β plays a central role in ER stress (Song et al., 2002; Brewster et al., 2006); however, there have been no reports stating that ER stress is involved in GSK-3β activation and tau phosphorylation induced by Aβ1–42 oligomers. In the present study, we have first established a link between oligomeric Aβ, GSK-3β activation, and tau phosphorylation. The involvement of ER Ca^{2+} release in GSK-3β-mediated tau phosphorylation on exposure to Aβ oligomers is another major finding of this work.

Several studies have demonstrated that tau is a GSK-3β substrate (Ishiguro et al., 1993; Lovestone et al., 1994; Plattner et al., 2006). However, tau can be phosphorylated by other kinases such as cyclin-dependent kinase-5 (cdk5), protein kinase A (PKA), or protein kinase C (PKC; Avila, 2006). The decrease in the inactive form of the enzyme, GSK-3β phosphorylated at the Ser9 residue, can be a result of down-regulation of the Akt/PI3K survival pathway, as was suggested by other authors (Magrané et al., 2005). There is also evidence that PKC inhibition causes a decline in GSK-3β levels (Liu et al., 2003). Using GSK-3β inhibitor I, we demonstrated that Aβ1–42 oligomer–induced tau phosphorylation is mediated by GSK-3β. Because tau phosphorylation occurred before GSK-3β activation, we cannot exclude the hypothesis that other kinases or other pathways may also be involved. It has been recently described that phosphorylation of tau by more than one kinase is required to convert it into the AD P-tau-like protein. Sequential phosphorylation of tau by PKA, CaMKii, and GSK-3β or by cdk5 and GSK-3β, converts normal tau into pathological tau, which self-assembles into paired helical filaments and tangles, leading to the inhibition of microtubule assembly (Wang et al., 2007). A recent work by Tseng and colleagues (2007) suggests that Aβ oligomers impair proteasome activity, contributing to the age-related pathological accumulation of Aβ and tau.

We have recently demonstrated that ER Ca^{2+} release through RyRs contributes to the early Aβ oligomer–induced increase in intracellular Ca^{2+} levels and to the activation of apoptotic neuronal death (Resende et al., unpublished results). In the present study, we demonstrated that Aβ oligomer–induced ER stress and Ca^{2+} release lead to GSK-3β activation and tau phosphorylation, which corroborates the hypothesis that GSK-3β is a downstream event of ER stress (Song et al., 2002; Brewster et al., 2006). Dantrolene is an inhibitor of RyRs, which has been shown to prevent Aβ-induced neurotoxicity (Ferreiro et al., 2006) and to decrease GRP78 level (Yoshida et al., 2006).

Thapsigargin, a well-known ER stress inducer because of the perturbation of ER Ca^{2+} homeostasis, has also been shown to activate GSK-3β (Song et al., 2002; Takadera et al., 2006; Takadera and Ohyashiki, 2007). There are also evidences in the literature demonstrating that A23187 ionophore induces GSK-3β activation (Jing et al., 2008) and tau phosphorylation (Shea et al., 1997).

Several studies have demonstrated increased GSK-3β levels in AD brains. However, there is no evidence that the enzyme activity is also increased, and the in vivo role of GSK-3β in AD brains is still a controversial issue (Balaraman et al., 2006). Some therapeutic treatments currently used in AD, such as acetylcholinesterase

inhibitors and *N*-methyl-D-aspartate receptors, have been proved to increase levels of the inactive form of GSK-3β (De Sarno et al., 2006). GSK-3β plays a major role in many signaling pathways involved in brain function and development (Grimes and Jope, 2001), and deletion or inhibition of GSK-3β can be detrimental (Balaraman et al., 2006). Despite its interaction with proteins associated with AD, namely, tau and APP, GSK-3β also regulates metabolic and mitochondrial enzymes and transcription factors. Thus, the inhibition of GSK-3β can affect many physiological functions. The inhibition of GSK-3β by lithium can be a result of its competition with magnesium or occur through activation of the PI3K/Akt pathway (Balaraman et al., 2006). There are some evidence that lithium can be protective in the Aβ-related pathological changes observed in AD (Balaraman et al., 2006), but a very recent study revealed an increased risk of dementia in AD patients treated with lithium (Dunn et al., 2005). Caccamo and colleagues (2007) described that despite the reduction in phospho-tau, lithium treatment did not improve deficits in working memory. Other GSK-3β inhibitors have also been shown to be neuroprotective and could have more potential than lithium. In the present work, we used GSK-3β inhibitor I, which prevented Aβ-induced tau phosphorylation and toxicity.

In conclusion, our results suggest that Aβ oligomers are involved in the GSK3β-mediated tau phosphorylation and neuronal degeneration that occurs in AD by a mechanism involving early ER Ca^{2+} release. Pathological changes in AD are very complex and include numerous mechanisms such as amyloid plaque formation, NFTs, apoptosis, and oxidative stress, which means that an ideal therapy should target different processes. This work supports the idea that GSK-3β can be a primary target and that its inhibitors can be promising as a therapy for AD.

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REFERENCES

Alonso AC, Zaidi T, Grundke-Iqbal I, Iqbal K. 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. Proc Natl Acad Sci U S A 91:5562–5566.

Avila J. 2006. Tau protein, the main component of paired helical filaments. J Alzheimers Dis 9:171175.

Balaraman Y, Limaye AR, Levey AI, Srinivasan S. 2006. Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. Cell Mol Life Sci 63:1226–1235.

Brewster JL, Linseman DA, Bouchard RJ, Loucks FA, Precht TA, Esch EA, Heidenreich KA. 2006. Endoplasmic reticulum stress and trophic factor withdrawal activate distinct signaling cascades that induce glycogen synthase kinase-3 beta and a caspase-9-dependent apoptosis in cerebellar granule neurons. Mol Cell Neurosci 32:242–253.

Caccamo A, Oddo S, Tran LX, LaFerla FM. 2007. Lithium reduces tau phosphorylation but not A beta or working memory deficits in a transgenic model with both plaques and tangles. Am J Pathol 170:1669–1675.

Dahlgren KN, Manelli AM, Stine WB, Baker LK, Krafft GA, LaDu MJ. 2002. Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. J Biol Chem 277:32046–32053.

De Felice FG, Wu D, Lambert MP, Fernandez SJ, Velasco PT, Lacor PN, Bigio EH, Jerecic J, Acton PJ, Shughrue PJ, Chen-Dodson E, Kinney GG, Klein WL. 2007. Alzheimer's disease-type neuronal tau hyperphosphorylation induced by Abeta oligomers. Neurobiol Aging [Epub ahead of print].

De Sarno P, Bijur GN, Zmijewska AA, Li X, Jope RS. 2006. In vivo regulation of GSK3 phosphorylation by cholinergic and NMDA receptors. Neurobiol Aging 3:413–422.

Deshpande A, Mina E, Glabe C, Busciglio J. 2006. Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. J Neurosci 26:6011–6018.

Dunn N, Holmes C, Mullee M. 2005. Does lithium therapy protect against the onset of dementia? Alzheimer Dis Assoc Disord 19:20–22.

Ebneth A, Godemann R, Stamer K, Illenberger S, Trinczek B, Mandelkow E. 1998. Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. J Cell Biol 143:777–794.

Ferreiro E, Resende R, Costa R, Oliveira CR, Pereira CM. 2006 An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. Neurobiol Dis 23:669–678.

Grimes CA, Jope RS. 2001. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391–426.

Hertz E, Yua CH, Hertz L, Juurlink BHJ, Schousboe A. 1989. Preparation of primary cultures of mouse cortical neurons. In: Shahar A, De Vellis J, Vernadakis A, Haber B, eds. A dissection and tissue culture manual of the nervous system. New York: Alan R. Liss Inc. p 183–186.

Ishiguro K, Omori A, Takamatsu M, Sato K, Arioka M, Uchida T, Imahori K. 1992. Phosphorylation sites on tau by tau protein kinase I, a bovine derived kinase generating an epitope of paired helical filaments. Neurosci Lett 148:202–206.

Ishiguro K, Shiratsuchi A, Sato S, Omori A, Arioka M, Kobayashi S, Uchida T, Imahori K. 1993. Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. FEBS Lett 325:167–172.

Ishizawa T, Sahara N, Ishiguro K, Kersh J, McGowan E, Lewis J, Hutton M, Dickson DW, Yen SH. 2003. Co-localization of glycogen synthase kinase-3 with neurofibrillary tangles and granulovacuolar degeneration in transgenic mice. Am J Pathol 163:1057–1067.

Jing P, Jin Q, Wu J, Zhang XJ. 2008. GSK3beta mediates the induced expression of synaptic acetylcholinesterase during apoptosis. J Neurochem 140:409–419.

Koh SH, Noh MY, Kim SH. 2008. Amyloid-beta-induced neurotoxicity is reduced by inhibition of glycogen synthase kinase-3. Brain Res 118:254–262.

LaFerla FM, Oddo S. 2005. Alzheimer's disease: Abeta, tau and synaptic dysfunction. Trends Mol Med 11:170–176.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL. 1998. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 98:6448–6453.

Lesné S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH. 2006. A specific amyloid-β protein assembly in the brain impairs memory. Nature 440:352–357.

Liu SJ, Zhang AH, Li HL, Wang Q, Deng HM, Netzer WJ, Xu H, Wang JZ. 2003. Overactivation of glycogen synthase kinase-3 by inhibition of phosphoinositol-3 kinase and protein kinase C leads to hyperphosphorylation of tau and impairment of spatial memory. J Neurochem 87:1333–1344.

Lovestone S, Reynolds CH, Latimer D, Davis DR, Anderton BH, Gallo JM, Hanger D, Mulot S, Marquardt B, Stabel S, et al. 1994. Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. Curr Biol 4:1077–1086.

Ma QL, Lim GP, Harris-White ME, Yang F, Ambegaokar SS, Ubeda OJ, Glabe CG, Teter B, Frautschy SA, Cole GM. 2006. Antibodies against beta-amyloid reduce Abeta oligomers, glycogen synthase kinase-3beta activation and tau phosphorylation in vivo and in vitro. J Neurosci Res 83:374–384.

Magrané J, Rosen KM, Smith RC, Walsh K, Gouras GK, Querfurth HW. 2005. Intraneuronal beta-amyloid expression downregulates the Akt survival pathway and blunts the stress response. J Neurosci 25: 10960–10969.

Mandelkow EM, Thies E, Trinczek B, Biernat J, Mandelkow E. 2004. MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. J Cell Biol 167:99–110.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival. J Immunol Meth 65:55–63.

Nakagawa T, Yuan J. 2000. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150:887–894.

Oddo S, Caccamo A, Tran L, Lambert MP, Glabe CG, Klein WL, LaFerla FM. 2006. Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. A link between Abeta and tau pathology. J Biol Chem 281:1599–1604.

Pei JJ, Braak E, Braak H, Grundke-Iqbal I, Iqbal K, Winblad B, Cowburn RF. 1999. Distribution of active glycogen synthase kinase 3beta (GSK-3beta) in brains staged for Alzheimer disease neurofibrillary changes. J Neuropathol Exp Neurol 58:1010–1019.

Plattner F, Angelo M, Giese KP. 2006. The roles of cyclin-dependent kinase 5 and glycogen synthase kinase 3 in tau hyperphosphorylation. J Biol Chem 281:25457–25465.

Price DL, Sisodia SS, Borchelt DR. 1998. Genetic neurodegenerative diseases: the human illness and transgenic models. Science 282:1079–1083.

Rao RV, Peel A, Logvinova A, del Ri G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE. 2002. Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. FEBS Lett 514:122–128.

Rapoport M, Dawson HN, Binde LI, Vitek MP, Ferreira A. 2002. Tau is essential to beta -amyloid-induced neurotoxicity. Proc Natl Acad Sci U S A 99:6364–6369.

Resende R, Pereira C, Agostinho P, Vieira AP, Malva JO, Oliveira CR. 2007. Susceptibility of hippocampal neurons to Abeta peptide toxicity is associated with perturbation of Ca^{2+} homeostasis. Brain Res 1143:11–21.

Shea TB, Prabhakar S, Ekinci FJ. 1997. Beta-amyloid and ionophore A23187 evoke tau hyperphosphorylation by distinct intracellular pathways: differential involvement of the calpain/protein kinase C system. J Neurosci Res 49:759–768.

Song LP, Jope RS. 2002. Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. J Biol Chem 277:44701–44708.

Stamer K, Vogel R, Thies E, Mandelkow E, Mandelkow EM. 2002. Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. J Cell Biol 156:1051–1063.

Takadera T, Ohyashiki T. 2007. Caspase-dependent apoptosis induced by calcineurin inhibitors was prevented by glycogen synthase kinase-3 inhibitors in cultured rat cortical cells. Brain Res 1133:20–26.

Takadera T, Yoshikawa R, Ohyashiki T. 2006. Thapsigargin-induced apoptosis was prevented by glycogen synthase kinase-3 inhibitors in PC12 cells. Neurosci Lett 408:124–128.

Takashima A, Noguchi K, Michel G, Mercken M, Hoshi M, Ishiguro K, Imahori K. 1996. Exposure of rat hippocampal neurons to amyloid beta peptide (25–35) induces the inactivation of phosphatidyl inositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3 beta. Neurosci Lett 203:33–36.

Takashima A, Noguchi K, Sato K, Hoshino T, Imahori K. 1993. Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity. Proc Natl Acad Sci U S A. 90:7789–7793.

Terry RD. 1996. The pathogenesis of Alzheimer disease: an alternative to the amyloid hypothesis. J Neuropathol Exp Neurol 55:1023–1025.

Tomidokoro Y, Ishiguro K, Harigaya Y, Matsubara E, Ikeda M, Park JM, Yasutake K, Kawarabayashi T, Okamoto K, Shoji M. 2001. Abeta amyloidosis induces the initial stage of tau accumulation in APP(Sw) mice. Neurosci Lett 299:169–172.

Tseng BP, Green KN, Chan JL, Blurton-Jones M, Laferla FM. 2007. Abeta inhibits the proteasome and enhances amyloid and tau accumulation. Neurobiol Aging [Epub ahead of print].

Wang JZ, Grundke-Iqbal I, Iqbal K. 2007. Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. Eur J Neurosci 25:59–68.

Yamaguchi H, Ishiguro K, Uchida T, Takashima A, Lemere CA, Imahori K. 1996. Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau protein kinase (TPK) I/glycogen synthase kinase-3 beta and cyclin-dependent kinase 5, a component of TPK II. Acta Neuropathol (Berl) 92:232–241.

Yoshida I, Monji A, Tashiro K, Nakamura K, Inoue R, Kanba S. 2006. Depletion of intracellular Ca^{2+} store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells. Neurochem Int 48:696–702.