Journal of Neurochemistry, 2004, **88**, 554–563 doi:10.1046/j.1471-4159.2003.02196.x

Amyloid-β-induced toxicity of primary neurons is dependent upon differentiation-associated increases in tau and cyclin-dependent kinase 5 expression

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Abstract

It has previously been reported that amyloid-β (Aβ) peptide is neurotrophic to undifferentiated but neurotoxic to differentiated primary neurons. The underlying reasons for this differential effect is not understood. Recently, the toxicity of Aβ to neurons was shown to be dependent upon the activation of cyclin-dependent kinase 5 (Cdk5), thought to promote tau phosphorylation that leads to cytoskeletal disruption, morphological degeneration and apoptosis. Here we report that Cdk5, tau, and phosphorylated-tau (P-tau) are expressed at very low levels in undifferentiated primary neurons, but that the expression of Cdk5 and tau and the phosphorylation of tau increase markedly between 4 and 8 days of differentiation *in vitro*. Tau expression decreased after this time, as did the level of P-tau, to low levels by 17 days. Aβ induced tau phosphorylation of neurons only after ≥4 days of differentiation, a time that coincides with the onset of Aβ toxicity. Blocking tau expression (and therefore tau phosphorylation) with an antisense oligonucleotide completely blocked Aβ toxicity of differentiated primary neurons, thereby confirming that tau was essential for mediating Aβ toxicity. Our results demonstrate that differentiation-associated changes in tau and Cdk-5 modulate the toxicity of Aβ and explain the opposite responses of differentiated and undifferentiated neurons to Aβ. Our results predict that only cells containing appreciable levels of tau are susceptible to Aβ-induced toxicity and may explain why Aβ is more toxic to neurons compared with other cell types.

Keywords: amyloid-β, antisense oligonucleotides, cyclin-dependent kinase 5, differentiation, tau, viability.

J. Neurochem. (2004) **88,** 554–563.

Alzheimer's disease (AD) neuropathology is characterized by the intracellular accumulation of neurofibrillary tangles (NFT) and the extracellular deposition of senile plaques. NFTs are composed primarily of highly phosphorylated tau in paired helical filaments (PHF) whereas senile plaques are composed primarily of fibrillar amyloid-β (Aβ; Glenner *et al.* 1984; Masters *et al.* 1985; Alvarez *et al.* 1999). Aβ is a 39–42 amino acid protein derived from the amyloid-β precursor protein (AβPP) via proteolytic cleavage at the β- and γ-secretase cleavage sites.

The physiological function of Aβ, and its role in AD, remains unclear. Early studies in primary cell culture indicated that Aβ was neurotrophic to undifferentiated hippocampal neurons at low concentration but neurotoxic to mature differentiated neurons at higher concentration (Whitson *et al*. 1989, 1990; Yankner *et al.* 1990; Kaltschmidt *et al.* 1999). In addition, we have shown that nanomolar concentrations of Aβ are anti-apoptotic to undifferentiated E18 rat neurons following trophic factor withdrawal

Received August 25, 2003; accepted September 18, 2003.

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Abbreviations used: Aβ, amyloid-β; AβPP, amyloid-β precursor protein; AD, Alzheimer's disease; Cdk5, cyclin-dependent kinase 5; JNK, c-Jun N-terminal kinase; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFT, neurofibrillary tangles; PHF, paired helical filaments; P-tau, phosphorylated-tau; SEK1, stress-activated protein kinase kinase-1.

(Chan *et al*. 1999), suggesting Aβ also has neuroprotective properties. These protective properties appear to be related to the protein's metal ion binding and redox/antioxidant capacity (Atwood *et al*. 1998, 2000; Bush *et al*. 1999; Chan *et al*. 1999; Kontush *et al*. 2001; Zou *et al*. 2002). Conversely, these same redox properties can promote Aβ toxicity at higher concentrations of Aβ (Cuajungco *et al*. 2000; Sponne *et al*. 2003).

Aβ has been shown to exert its neurotoxicity through a mechanism that induces intracellular generation of $O_2^{-\cdot}$/H_2O_2 (Behl *et al*. 1994), lipid peroxidation (Mark *et al*. 1997; Rottkamp *et al*. 2001; Wei *et al*. 2002), calcium ion accumulation (Mattson *et al*. 1992), activation of c-Jun N-terminal kinase (JNK) (Wei *et al*. 2002) and subsequent neuronal or vascular endothelial cell death (Behl *et al*. 1994; Busciglio and Yankner 1995; Hensley *et al*. 1995; Thomas *et al*. 1996). Recently, the downstream mechanism of Aβ-induced toxicity has been linked to phosphorylated-tau (P-tau: Patrick *et al.* 1999; Zhang and Johnson 2000) and the disruption of microtubule networks (Sponne *et al*. 2003), characteristic changes evident as NFTs in vulnerable neurons of the AD brain. This mechanism involves Aβ-induced Ca(II) accumulation (Mattson *et al*. 1992) that activates calpain and subsequently the cleavage of p35 to p25 (Lee *et al.* 2000; Patzke and Tsai 2002; Town *et al.* 2002). p25 then binds with high affinity to cyclin-dependent kinase 5 (Cdk5), a small serine/threonine kinase and constitutively activates it, causing the hyperphosphorylation of tau that is thought to promote disruption of microtubules, collapse of the cytoskeleton and cell death (Patrick *et al.* 1999; Lee *et al.* 2000; Zhang and Johnson 2000; Alvarez *et al.* 2001; Town *et al*. 2002). That these pathways are involved in Aβ-induced toxicity is confirmed by the fact that blockage of any causative or signaling component of this pathway provides neuroprotection. For example, Aβ neurotoxicity can be abolished by the presence of superoxide dismutase (Thomas *et al*. 1996), catalytic synthetic $O_2^{-\cdot}$/H_2O_2 scavengers (Gsell *et al.* 1995), the spin-trap compound phenyl-*N*-*tert*-butyl-nitrone (Butterfield *et al.* 1996), or chain-breaking antioxidants, such as vitamin E or vitamin C (Mattson and Goodman 1995; Mark *et al.* 1996, 1997; Markesbery 1997; Varadarajan *et al.* 2000). Likewise, the Cdk5 inhibitor butyrolactone I, and a Cdk5 antisense probe, markedly decrease Aβ-induced death of rat hippocampal neurons (Alvarez *et al.* 1999), indicating that inhibition of tau phosphorylation prevents Aβ-induced neuronal death. Importantly, neurons from tau knockout mice are resistant to Aβ toxicity (Rapoport *et al.* 2002), indicating a key role for tau in the mechanisms leading to Aβ-induced neurodegeneration in the central nervous system. Likewise, Aβ-induced JNK activation and cell death are inhibited by overexpression of a dominant-negative stress-activated protein kinase kinase-1 (SEK1-AL) construct (Wei *et al.* 2002), an upstream signaling member of the JNK pathway. Butyrolactone I, a Cdk5 inhibitor, provided additional protection against Aβ toxicity in SEK1-AL-expressing cells, suggesting that Cdk5 and JNK activation independently contributed to this toxicity. Blockade of either calpain or Ca(II) channels also opposes p35-dependent tau phosphorylation induced by soluble Aβ (Town *et al.* 2002). The relevance of this mechanism to AD is shown by the fact that p25 accumulates in neurons (Patrick *et al.* 1999) and that there is persistent activation of Cdk5 in the brains of patients with AD (Iijima *et al.* 2000). Indeed, mice overexpressing AβPP have recently been shown to have increased p25 levels and display hyperphosphorylated tau in and around amyloid deposits (Otth *et al.* 2002).

Since Aβ is toxic to differentiated neurons, but not toxic to undifferentiated neurons, we hypothesized that the increased expression of components of the tau phosphorylation pathway during differentiation is critical to Aβ toxicity. We show for the first time that Aβ toxicity is modulated by differentiation-associated increases in tau and Cdk5 expression in rat primary neurons. These results indicate that Aβ is toxic only to differentiated neurons.

Materials and methods

Reagents

Human Aβ1–40 was synthesized and purified by HPLC and characterized by amino acid analysis and mass spectroscopy by W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA). The peptide was identified as a single peak upon HPLC and showed no chemical modification.

Tau sense and antisense oligonucleotides were obtained from IDT (Coralville, IA, USA). The tau antisense oligonucleotide sequence was 5′-G*T*T*CAGCCATGCTGCTTCAAAGC*C*T*G-3′ (* indicates phosphorothioate bond), which corresponds to nucleotide −16 to +10 in the rat tau gene sequence (Pizzi *et al.* 1994). Sense oligonucleotide was the reverse complement of the antisense.

Rat (E18) primary cortical neurons were purchased from BrainBits (Dr Gregory Brewer at Southern Illinois University School of Medicine, Springfield, IL, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma (Saint Louis, MO, USA). Neurobasal medium without phenol red, B27, and glutamine were purchased from Invitrogen (Carlsbad, CA, USA). AT8 mouse anti-human PHF-Tau monoclonal antibody was purchased from Endogene (Woburn, MA, USA) and recognizes tau protein phosphorylated at both serine 202 and threonine 205 (Goedert *et al.* 1995); rabbit polyclonal antisera against (Perry *et al.* 1991). Cdk5 rabbit polyclonal antibody was obtained from StressGen Biotechnologies Corporation (Victoria, Canada) and recognizes the C-terminus of Cdk5. All other reagents were analytical grade or purer.

Primary rat cortical neuron culture

Neuronal cultures were taken from frontal cortices of Sprague-Dawley rat embryonic (E18) pups. The cells were dissociated by trituration in Hibernate E media (Brainbits, Southern Illinois

University School of Medicine, Springfield, IL, USA), undispersed tissue allowed to settle for 1 min, and the supernatant transferred to a new tube prior to centrifugation for 1 min at 200 *g*. The supernatant was then removed and the cells resuspended in serum-free Neurobasal medium (without phenol red) with B-27 supplement (Life Technologies, Inc., Rockville, MD, USA), 25 μM L-glutamate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 mM L-glutamine, counted using a hemocytometer and then equal volumes of mixed cells were plated into 6-well (1×10^6 cells/well) or 96-well (2×10^4 cells/well) poly-L-lysine-coated plates (BD Biosciences, Bedford, MA, USA). Cortical cultures were grown in 5% CO_2, 85% humidity in the above media. On the fourth day, the medium was replaced with the same media without L-glutamate and changed every 2–3 days. In cultures treated with Aβ, Aβ1–40 was filter sterilized prior to addition to the media at a concentration of 1 μM or 10 μM. The media of neurons treated with sense or antisense oligonucleotides (5 μM each) were changed daily. Experimental trials were performed in 6–9 replicate wells.

Amyloid-β preparation

Synthetic Aβ peptide was dissolved in sterile doubly deionized water at a concentration of 0.5–1.0 mg/mL, passed through a 0.22 μm filter (Millipore, Bedford, MA, USA) and the concentration determined by spectrophotometric absorbance at 214 nm. Peptide solutions were then incubated at 37°C for 4 days to fibrillize Aβ (modified from Wei *et al.* 2002).

Cell viability

Cell viability was assessed using the MTT assay. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. Briefly, neurons (and blank wells) were incubated with media (100 μL) containing 1.2 mM MTT for 4 h at 37°C. Following this, a 10% sodium dodecyl sulfate solution in 0.01 M HCl (100 μL) was added to the wells for a further 4 h at 37°C in a humidified chamber and the optical density was then read at 570 nm by using a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Sample collection and immunoblot analyses

Neurons were collected into sample buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% sodium dodecyl sulfate containing protease inhibitors: 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/mL leupeptins; 1 μg/mL pepstatin A). The protein concentration of samples was determined by using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Samples containing equal cell numbers (with roughly similar protein concentrations) were loaded, since the expression of traditional proteins used to control for loading, such as tubulin and actin, increases with differentiation (Drubin *et al*. 1988). Loaded samples were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Tricine gels, 10–20%; Novex, San Diego, CA, USA), transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA), fixed with glutaraldehyde (1%, v/v), blocked with milk (10%, w/v) and then probed with primary antibody overnight at 4°C. The blot was then incubated with corresponding horseradish peroxidase conjugate secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, and developed with enhanced chemiluminescence reagent (1 min; Amersham, Little Chalfont, UK) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiographs (Eastman Kodak Company, Aurora, IL, USA), and the signal quantified on a UVP BioImaging System using the LabWorks 4.0 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). Briefly, images were captured and the intensity of the autoradiograph signals (including a blank region) was determined. Control and treatment values were corrected for blank values and the results then expressed as a percentage or fold change over control levels. Molecular size markers were from Bio-Rad Laboratories.

Results

Amyloid-β is non-toxic to undifferentiated primary cortical neurons

Differentiation of primary neurons is seen by extensive neurite outgrowth and the up-regulation of microtubule-associated proteins over the first few days in culture (Drewes *et al*. 1992; Bramblett *et al*. 1993; Goedert *et al*. 1994). Aβ1–40 added to rat cortical primary neurons has been reported to significantly increase neuronal survival during the first 24 h of culture. This trophic effect became progressively less apparent when Aβ1–40 was added after 2 days of culture and was toxic after 3 days of culture (Yankner *et al*. 1990). To confirm the trophic properties of Aβ1–40 on undifferentiated neurons (Whitson *et al*. 1989; Yankner *et al*. 1990; Chan *et al*. 1999), primary rat cortical neurons cultured for 2 days were exposed to 0 or 10 μM Aβ1–40 and viability measured after 48 h. The viability of the treated

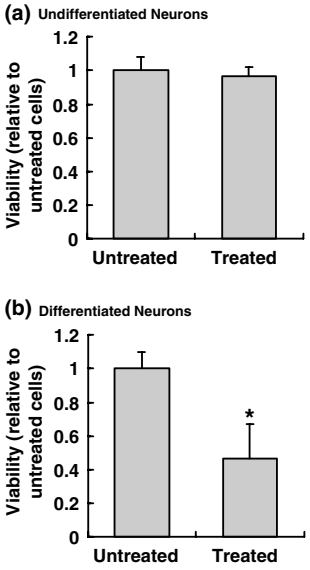

Fig. 1 Differentiation state modulates Aβ toxicity. Viability of undifferentiated neurons (a; 2 days in culture) and differentiated neurons from E18 rat embryos (b; 8 days in culture) in the absence (0 μM) and presence (10 μM) of Aβ1–40. Results are mean ± SD; $n = 6$; $^*p < 0.05$, *t*-test; Statview, SAS Institute Inc.

and untreated neurons was not significantly different (Fig. 1a). To confirm the toxic properties of Aβ1–40 on neurons that were more differentiated, primary rat cortical neurons cultured for 8 days were exposed to 0 or 10 μM Aβ1–40 and viability measured. As previously reported, by 24 h, the viability of treated differentiated neurons (46%) was significantly lower than that of untreated neurons (Fig. 1b).

Since it has been reported that Aβ may interfere with the MTT assay (Liu and Schubert 1997), primary neurons cultured for 10 days were incubated with 0, 1 or 10 μM Aβ1–40 for 2 days. Viability was then either measured immediately using the MTT assay or the media containing Aβ1–40 replaced with media containing no Aβ1–40 for 2 days (to allow time for Aβ clearance; Farris *et al*. 2003) before the viability was determined using the MTT assay. No significant difference in viability was detected between these two treatments at 1 μM Aβ1–40 (71.1 ± 9.6% vs. 70.5 ± 11.7%, $n = 6$) or 10 μM (52.6 ± 6.0% vs. 53.9 ± 8.8%, $n = 6$), indicating that Aβ1–40 was not interfering with MTT exocytosis and reduction.

P-tau levels increase with Aβ treatment

Since tau phosphorylation promotes microtubule disruption and neuronal death, we tested to determine if Aβ induced tau phosphorylation. Cells cultured for 1, 2 and 3 days were then treated for 25 h (i.e. ~2, 3 and 4 days in culture) with and without Aβ (10 μM) and the level of P-tau determined. No difference in the level of P-tau isoforms (43–61 kDa) was observed between treated and untreated neurons at 2 days (P-tau/actin ratio = 0.9 and 1.0, respectively) and 3 days (P-tau/actin ratio = 4.5 and 4.5, respectively) of culture, but an increase in the phosphorylation of these isoforms was observed following 4 days of treatment with Aβ1–40 (P-tau/actin ratio = 6.0 and 9.6, respectively; Fig. 2). Similar results have been reported in primary cells and cell lines (Greenberg *et al*. 1994; Busciglio *et al*. 1995). These results suggest a change associated with differentiation between 3 and 4 days of culture that allows for the increased phosphorylation of tau.

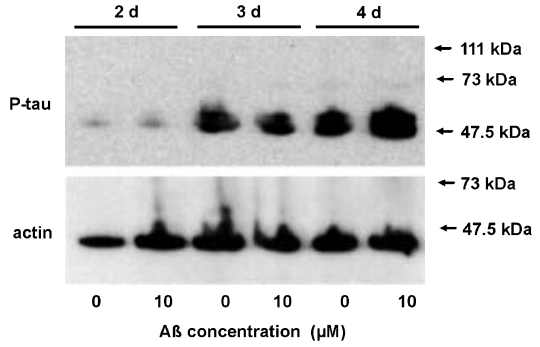

Fig. 2 Aβ-induced tau phosphorylation is dependent upon differentiation. Neurons were differentiated for 1, 2 or 3 days and then exposed to 0 or 10 μM Aβ1–40 for 25 h and the levels of tau phosphorylation determined using the AT8 mouse anti-human PHF-Tau monoclonal antibody.

Tau and Cdk5 expression change with differentiation

To determine if changes in the expression of tau or Cdk5 during differentiation could explain the increase in tau phosphorylation following Aβ treatment, we measured tau and Cdk5 expression changes during the differentiation of E18 primary rat cortical neurons grown in 6-well plates for 1, 4, 8, 10, 14 and 17 days. Morphological changes such as progressive neurite extension were observed consistent with differentiation of these neurons (data not shown). Immunoblot analyses indicated an approximately three-fold increase in the concentration of the 43–61 kDa isoforms of tau between 1 and 4 days that further increased to seven-fold by 8 days (Fig. 3). After 8 days, the concentration of these tau isoforms decreased through to 17 days while the concentration of a higher molecular weight tau isoform (~120 kDa) progressively increased (~30-fold) in intensity through to 17 days. These results indicate an increase in the cellular concentration of both low- and high-molecular weight tau isoforms during cortical neuron differentiation. The expression of Cdk5 also increased, by five-fold between 4 days to 8 days and continued to increase until 10 days (13-fold) after which it plateaued to 17 days of culture (Fig. 3). The 43–61 kDa isoforms of tau displayed increased phosphorylation in parallel with their expression, increasing 48-fold to 10 days and thereafter decreasing to low levels by 17 days of culture. The maintenance of Cdk5 expression after 10 days but the decline of tau phosphorylation suggests that phosphorylation was more dependent upon the level of tau expression (or Cdk5 activity) after 10 days rather than the level of Cdk5 expression. No phosphorylation of the higher molecular weight isoform of tau was observed.

Tau antisense oligonucleotide blocks Aβ-induced toxicity

To confirm that increases in cellular tau were responsible for mediating the toxicity of Aβ, primary neurons grown for 1 day were then treated with an antisense or sense tau oligonucleotide to block tau expression. Treatment with the tau antisense oligonucleotide for 5 days, but not sense oligonucleotide, markedly decreased the expression of tau (81% vs. 20%, respectively) compared to untreated neurons (Fig. 4a). When these cultures treated with antisense oligonucleotide were exposed to Aβ1–40 for 24 h, there was a significant dose-dependent increase in MTT signal, indicating that Aβ1–40 was neurotrophic to tau-deficient neurons (Fig. 4b). Conversely, when these cultures treated with sense oligonucleotide were exposed to Aβ1–40 for 24 h, there was a significant decrease in MTT signal at 10 μM Aβ1–40. The lower Aβ1–40-induced toxicity of the sense-treated neurons (19%; Fig. 4b) compared with our previous experiment

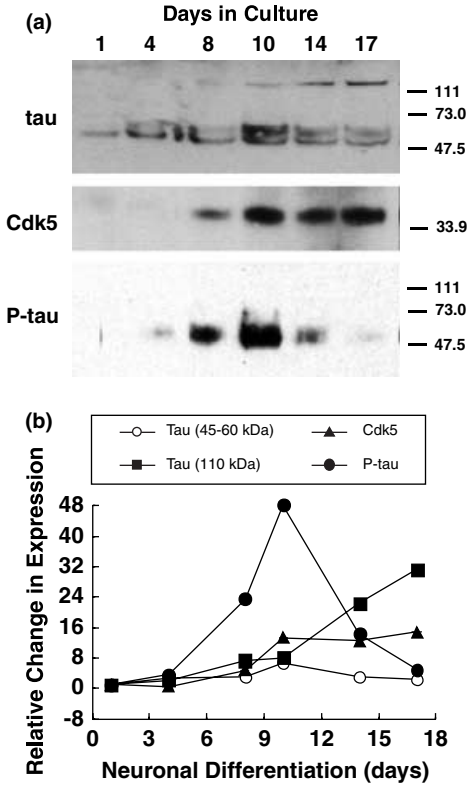

Fig. 3 Tau, Cdk5 and phosphorylated-tau expression changes during differentiation. (a) E18 rat cortical neurons were cultured for different times, collected and analysed by immunoblot for tau, Cdk5 and phopho-tau expression during differentiation. Molecular weight markers are shown. (b) Quantification of results in (a) expressed relative to day 1 expression level.

(48%; Fig. 1b) is consistent with the shorter time period of differentiation of the neurons in these experiments (6 days vs. 8 days in Fig. 1b). In all our experiments, Aβ1–40 was toxic to neurons cultured for greater than or equal to 4 days, and toxicity increased as a function of time in culture (i.e. differentiation). Taken together, these results indicate that Aβ1–40 is toxic to neurons once tau reaches a critical threshold level.

Discussion

These results demonstrate that differentiation-associated changes in cellular tau (and Cdk5) regulate the viability of cortical neurons to Aβ (Figs 1–4). Aβ1–40 was toxic only to primary neurons containing high concentrations of tau, but not toxic or neurotrophic to primary neurons containing low concentrations of tau (Figs 1, 2 and 4). Our results confirm and explain why in previous studies Aβ was neurotrophic to undifferentiated neurons but toxic to more differentiated neurons (Whitson *et al*. 1989, 1990; Yankner *et al*. 1990; Behl *et al*. 1992).

The microtubule-associated protein (MAP) tau is mainly found in the brain and spinal cord, but is also found at relatively high levels in heart, skeletal muscle, lung, kidney and testis and at much lower levels in adrenal gland, stomach, liver, spleen and fibroblasts (Montejo *et al*. 1986; Tucker 1990; Ashman *et al*. 1992; Lee and Rook 1992; Lubke *et al*. 1994; Matsuyama and Bondareff 1994; Gu *et al*. 1996). Tau is highly enriched in axonal compartments (especially in mature neurons; Fig. 3) but is not present in microglia (Binder *et al*. 1985; Trojanowski *et al*. 1989). Interestingly, tissues with high levels of cellular tau (i.e. brain and muscle) are the only reported tissues that accumulate Aβ (Glenner and Wong 1984; Masters *et al*. 1985; Kuo *et al*. 2000) and display cell loss (Murakami *et al*. 1995a,b; Gomez-Isla *et al*. 1996, 1997). Our results may explain why glia (Binder *et al*. 1985; Trojanowski *et al*. 1989) with low concentrations of cellular tau is more resistant to Aβ toxicity (DeWitt *et al*. 1998; Casal *et al*. 2002). Our results would further predict that only those cell types that contain appreciable levels of tau, and Cdk5 activity, would be susceptible to Aβ-induced toxicity. That astrocytes have been reported to contain tau (Papasozomenos and Binder 1987; Papasozomenos 1989) but are not susceptible to Aβ-induced toxicity (Kerokoski *et al*. 2001; Malchiodi-Albedi *et al*. 2001) suggests they may contain low Cdk5 activity (Tsai *et al*. 1993; Nikolic *et al*. 1996).

Division competent cells (such as microglia) that do not contain tau may not be as susceptible to Aβ-induced toxicity. Conversely, nerve growth factor-induced differentiation of pheochromacytoma (PC12) cells, which induces neurite process formation and parallel increases in the levels of the microtubule-associated proteins, tau, MAP1, and tubulin (Drubin *et al*. 1988), greatly potentiates the toxicity of amyloid-β peptides (Behl *et al*. 1992).

Previous workers (Alvarez *et al*. 1999) have shown that tau phosphorylation via the p35/p25/Cdk5 pathway is required for Aβ-induced neuron death (Fig. 5). Indeed, Aβ induces Cdk5 activity in rat hippocampal neurons. We show that the expression of Cdk5 increases during differentiation of primary cortical neurons (Fig. 3; Wu *et al.* 2000). Given that previous studies have shown Cdk5 inhibitors and Cdk5 antisense prevent hyperphosphorylation and neuronal death (Alvarez *et al*. 1999; Lee *et al*. 2000; Town *et al*. 2002), it is likely that differentiation-associated increases in tau and the activity of Cdk5/p35 are essential for Aβ toxicity of neurons. Interestingly, enzymatically active Cdk5 is only present in neurons (Tsai *et al*. 1993; Nikolic *et al*. 1996; Chae *et al*. 1997).

Although we and other workers have shown that Aβ-induced tau phosphorylation is required for neuron death, an intriguing observation from our present results was that although there was a large increase in P-tau during differentiation, this did not affect the viability of the neurons. Indeed, the increase in tau expression and phosphorylation

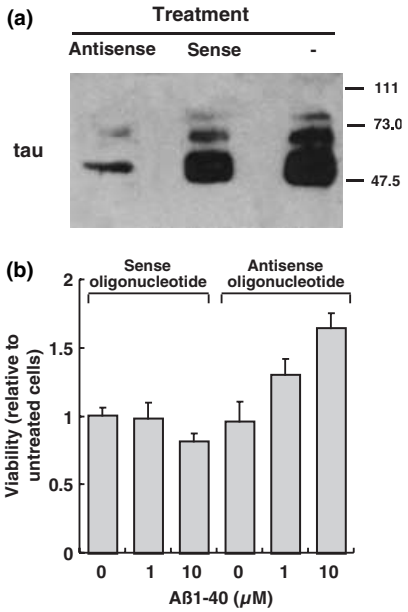

Fig. 4 (a) Antisense oligonucleotides lower neuronal tau expression. Neurons were cultured for 1 day and then treated with antisense (5 μM), sense (5 μM) or no treatment for a further 4 days prior to western analyses for tau. (b) Aβ1–40 is neurotropic in the absence of tau but toxic in the presence of tau. E18 rat neurons were treated with sense or antisense oligonucleotide or no treatment from day 2 after plating for 4 days and then exposed to fibrillized Aβ1–40 (0, 1 or 10 μM) for 24 h. Results are mean ± SD; $n = 4$–6.

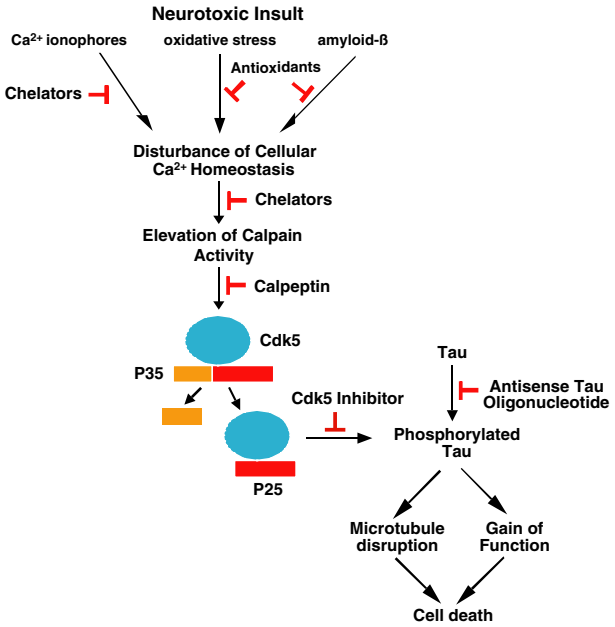

Fig. 5 Schematic diagram of the calpain–p35/Cdk5–tau pathway of Aβ toxicity. Neurotoxic insults, such as Aβ, promote calpain (a Ca(II)-dependent cysteine protease) activation and cleavage of p35 to p25. The resultant p25 fragment binds and activates Cdk5, which in turn promotes tau phosphorylation and cell death. Inhibition of calpain or Cdk5 activity, tau expression, calcium disruption or oxidation prevents Aβ-mediated toxicity of neurons. p25 accumulates in ischemic brains and in brains of patients with Alzheimer's disease. Cdk5 activity is increased in AD brain.

during differentiation suggests these processes are important for normal neuritic outgrowth that occurs in the first 10 days of culture, whereas the decline in tau expression and phosphorylation after this time suggests lower levels of tau and P-tau are required for the maintenance of formed neurites. Therefore, Aβ may induce its toxicity by phosphorylation of amino acids on tau other than those detected with our antibody (phosphorylated serine 202 and threonine 205).

Neuroblastoma cells display low levels of tau phosphorylation during interphase, but tau becomes highly phosphorylated during mitosis (Pope *et al*. 1994; Illenberger *et al*. 1998; Preuss and Mandelkow 1998), as also is seen following Aβ treatment (Fig. 2). That tau is hyperphosphorylated in neurons vulnerable to AD pathology strengthens the argument that there is a reactivation of cell cycle machinery in the AD brain. Intriguingly, Aβ-induced tau hyperphosphorylation suggests the real possibility that the accumulation of Aβ in the AD brain triggers this aberrant cell cycle activity (Raina *et al*. 2000).

Our experiments support the idea that aged (differentiated) neurons are more vulnerable to Aβ toxicity. It has been reported that fibrillar Aβ injected into aged (25–27 years) monkey cerebral cortex results in tau phosphorylation, neuronal loss and microglial proliferation. However, young monkeys (5 years) were not sensitive to such injections (Geula *et al*. 1998), suggesting that the level of tau and/or level or activity of Cdk5 (and regulatory subunit p35) in neurons of these monkeys was insufficient to allow Aβ-induced toxicity. This is supported by the finding that tau expression increases from embryonic day 13–14 in rats and is maximal between E20 and P8, corresponding to times of maximal axonal growth, before decreasing to adult levels (Kosik *et al*. 1989). Likewise, Cdk5 activity increases with neuronal differentiation (Nikolic *et al*. 1996; Munoz *et al*. 2000) between E18 to postnatal day 14 but then decreases in the adult and aged rat brain (Wu *et al*. 2000). Moreover, the pattern of p35 and p25 expression and Cdk5 activity correlates well with neuronal death in AD, i.e. they are significantly higher in the cerebral cortex and hippocampus, but lower in the cerebellum and striatum (Patrick *et al*. 1999; Wu *et al*. 2000; Tseng *et al*. 2002). Future studies will be required to confirm if age- or disease-related changes in cellular tau and activities of Cdk5 or regulatory subunits (p35 and p39) increase the susceptibility of neurons to Aβ toxicity *in vivo*.

Morphological and biochemical changes (Fig. 3) that we report in our aging cultured neurons are consistent with differentiation-associated changes previously reported in

differentiating cultured cortical neurons (Drewes *et al*. 1992; Arioka *et al*. 1993; Bramblett *et al*. 1993; Brion *et al*. 1993; Goedert *et al*. 1993; Pizzi *et al*. 1995; Smith *et al*. 1995). Neurite outgrowth was associated with an increase in tau isoforms between 43 and 61 kDa (Gu *et al*. 1996). A larger tau isoform (~120 kDa), formed by alternative splicing and normally associated with peripheral neurons (Couchie *et al*. 1992; Goedert *et al*. 1992) and skeletal muscle, heart and adrenal gland (Gu *et al*. 1996), increased from day 8 of differentiation (Fig. 3). The relevance of this larger tau isoform to the differentiation process and whether there are functional differences between isoforms remains to be determined.

Tau's major cellular role is thought to involve regulation of neuronal microtubule assembly and stabilization of microtubules against depolymerization *in vivo* (Drubin and Kirschner 1986; Drubin *et al*. 1986; Kanai *et al*. 1989). Such a role is supported by the time course of tau expression during process formation where neurite extension is paralleled by an increase in the amount of expressed tau (Fig. 3 and references above). Gene transfer experiments have shown that the level of expressed tau influences microtubule assembly, neurite outgrowth, and neuritic stability in cultured neural cells (Pelech 1995). Tau also has been shown to have a binding site for AβPP (Giaccone *et al*. 1996) suggesting tau may have alternative activities. One such function may involve axonal transport, since tau (and perhaps P-tau) competes with the kinesin/dynesin binding site of microtubules to regulate transport (Stamer *et al*. 2002). Although tau antisense experiments have been shown to reduce microtubule levels and slow neurite outgrowth (Esmaeli-Azad *et al*. 1994; data not shown), tau knockout mice appear to be histologically normal and display no phenotype indicating tau is not essential for the development and maintenance of axons (Harada *et al*. 1994). Indeed, blocking tau expression with antisense oligonucleotides is unlikely to influence the functional differentiation of primary neurons. The function of tau, other than as a mediator of toxicity, remains to be fully elucidated.

From our results and others, it is apparent that Aβ-induced cell death occurs via the calpain/p35/Cdk5/tau phosphorylation pathway (Fig. 5). The increased expression of proteins in this pathway is positively related to the cellular sensitivity to Aβ1–40 toxicity. Whether Aβ-induced tau phosphorylation promotes toxicity via microtubule depolymerization or a toxic gain of function via other mechanisms remains to be determined. Nevertheless, redundancies in this cellular signaling pathway that express themselves later in life and lead to tau phosphorylation may promote the neuronal loss and pathology associated with AD.

Acknowledgements

This work is supported by funds from the National Institute of Health (RO1 AG19356) and the Alzheimer's Association to CSA.

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