Cell Mol Neurobiol (2007) 27:943–957
DOI 10.1007/s10571-007-9224-3

ORIGINAL PAPER

# Role of Cyclin-Dependent Kinase 5 in the Neurodegenerative Process Triggered by Amyloid-Beta and Prion Peptides: Implications for Alzheimer's Disease and Prion-Related Encephalopathies

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Received: 19 June 2007 / Accepted: 5 October 2007 / Published online: 27 October 2007


**Abstract** Tau hyperphosphorylation, amyloid plaques, and neuronal death are major neuropathological features of Alzheimer's disease (AD) and Prion-related encephalopathies (PRE). Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase, active in post-mitotic neurons, where it regulates survival and death pathways. Overactivation of Cdk5 is conferred by p25, a truncated fragment of the p35 activator formed upon calpain activation. Cdk5 deregulation causes abnormal phosphorylation of microtubule-associated protein tau, leading to neurodegeneration. In this work we investigated the involvement of Cdk5 in the neurodegeneration triggered by amyloid-beta (A$\beta$) and prion (PrP) peptides, the culprit agents of AD and PRE. As a work model, we used cultured rat cortical neurons treated with A$\beta_{1-40}$ and $PrP_{106-126}$ synthetic peptides. The obtained data show that apoptotic neuronal death caused by both the peptides was in part due to Cdk5 deregulation. After peptide treatment, p25 levels were significantly enhanced in a pattern consistent with the augment in calpain activity. Moreover, A$\beta_{1-40}$ and $PrP_{106-126}$ increased the levels of tau protein phosphorylated at Ser202/Thr205. Cdk5 (roscovitine) and calpain (MDL28170) inhibitors reverted tau hyperphosphorylation and prevented neuronal death caused by A$\beta_{1-40}$ and $PrP_{106-126}$. This study demonstrates, for the first time, that Cdk5 is involved in PrP-neurotoxicity. Altogether, our data suggests that Cdk5 plays an active role in the pathogenesis of AD and PRE.

**Keywords** Cdk5 activators · Calpains · Tau hyperphosphorylation · Amyloid-beta · Prion

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## Introduction

Alzheimer's disease (AD) and Prion-related encephalopathies (PRE) are two progressive fatal types of neurodegenerative disorders, characterized by the cerebral deposition of amyloidogenic proteins, the amyloid-beta (A$\beta$) protein and the scrapie isoform of prion protein ($PrP^{Sc}$), respectively. These proteins aggregate and accumulate extracellularly in the form of amyloid plaques, having the same basic structure which consists of a core surrounded by degenerated neuritis, activated microglia, and astrocytes (Aguzzi and Haass 2003). Increasing evidences support that A$\beta$ and $PrP^{Sc}$ are key molecules in the pathogenesis of AD and PRE. Extensive neuronal death and hyperphosphorylation of tau are also features of these pathologies (Selkoe 1993; Prusiner 1998; Aguzzi and Haass 2003). In AD, hyperphosphorylated tau protein aggregates intracellularly forming paired helical filaments (PHFs), which are the major structural component of neurofibrillary tangles (NFTs) (Lee et al. 2001; Hardy 2003; Bautista et al. 2006). Although the presence of NFTs is not evident in PRE, the participation of hyperphosphorylated tau in these pathologies is widely recognized (Ishizawa et al. 2002; Bautista et al. 2006), and high levels of phospho-tau were found in the CSF of patients with prion diseases (Riemenschneider et al. 2003). Several evidences suggest a link between A$\beta$ accumulation and NFTs formation in AD (Oddo et al. 2003a, b; 2004), however, few studies have established a correlation between prion deposition and intracellular accumulation of hyperphosphorylated tau (Bautista et al. 2006).

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine–threonine kinase, is considered to have a major tau-phosphorylating function in the brain, with pathological relevance in AD. Hyperphosphorylated tau perturbs microtubule organization and leads to cytoskeleton disruption and neuronal death (Grundke-Iqbal et al. 1986; Tsai et al. 2004). Mass spectrometry studies have shown that Cdk5 phosphorylates tau on S202, T212, S396, and S404, sites that are phosphorylated in PHF preparations and in the brain of AD patients (Tsai et al. 2004). Similarly to other elements of the Cdk family, isolated Cdk5 does not display any enzymatic activity. In order to be activated, Cdk5 must bind to a regulatory subunit, p35 or p39 (Tsai et al. 1994; Humbert et al. 2000), which are cyclin-like proteins highly expressed in postmitotic neurons. The p35–Cdk5 complex has an important role in neurodevelopment and corticogenesis (Dhavan and Tsai 2001). Indeed, Cdk5 knockout mice display widespread disruption of cortical layering and prenatal mortality (Ohshima et al. 1996; Gilmore et al. 1998), whereas p35 null mice, although viable and fertile, have defects in cortical lamination (Chae et al. 1997). Recent evidence also shows that Cdk5 participates in synaptic plasticity and memory (Angelo et al. 2006). During neuronal injury and subsequent intracellular calcium homeostasis deregulation, membrane-associated Cdk5 activator p35 can be cleaved, by calcium-regulated calpains, into the cytosolic C-terminal fragment p25. This truncated Cdk5 activator, lacking the N-terminus, will relocalize to the cell soma and nucleus. Moreover, p25 is more stable and binds tightly to Cdk5 than p35, forming a hyperactive and mislocalized p25–Cdk5 complex (Tsai et al. 2004). Such cleavage of p35 into p25 is crucial for the alteration of Cdk5 substrate specificity and contributes to tau hyperhosphorylation, cytoskeletal disruption and neurodegeneration (Tsai et al. 2004; Shelton and Johnson 2004). Elevated levels of p25 have been reported in some neurodegenerative disorders, such as AD, Parkinson's disease, and amyothrophic lateral sclerosis (Nguyen et al. 2002; Tsai et al. 2004). However, there are no studies concerning a possible role for Cdk5 in PRE.

In order to determine the involvement of Cdk5 in the neuronal injury triggered by PrP and A$\beta$, we performed a comparative study in primary cultures of cortical neurons using the

synthetic peptides, $PrP_{106–126}$ and $A\beta_{1–40}$. $A\beta_{1–40}$ is one of the major components of amyloid plaques in AD, whereas $PrP_{106–126}$, is a peptide fragment that reproduces $PrP^{Sc}$ toxicity and forms fibrils in vitro (Forloni et al. 1996; Combs et al. 1999). Studies performed by our group have shown that aged $A\beta_{1–40}$ and $PrP_{106–126}$ form fibrils and can trigger apoptotic neuronal death (Resende et al. personal communication; Melo et al. 2007; Garção et al. 2006). Inhibitors of Cdk5 and calpains were used to investigate whether this kinase mediates tau phosphorylation and neuronal death triggered by these peptides. The obtained data show that $A\beta$ and PrP increased p25 levels, through the activation of calpains, causing Cdk5 deregulation and, consequently, tau hyperhosphorylation and apoptotic death. This is the first study showing the involvement of Cdk5 in neurotoxicity triggered by PrP peptides.

## Material and Methods

### Materials

Neurobasal medium and B-27 supplement were purchased from Gibco (Paisley, United Kingdom). Synthetic peptides of $A\beta_{1–40}$ and $PrP_{106–126}$ were from Bachem (Bubendorf, Switzerland). Alexafluor® IgG conjugate secondary antibodies and Hoechst 33342 were acquired from Molecular Probes (Leiden, The Netherlands). The fluorescent mounting medium was from DakoCytomation (Glostrup, Denmark). Reagents and apparatus used in immunoblotting assays were obtained from Bio-Rad (Hercules, CA, USA), whereas PVDF membranes, alkaline phosphatase-linked anti-mouse secondary antibody and the enhanced chemifluorescence (ECF) reagent were from Amersham Biosciences (Buckinghamshire, United Kingdom). The monoclonal antibodies against Cdk5 (C-8) and p35 (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the anti-tau (BT2) and anti-human PHF-tau (AT8) antibodies were from Pierce Endogen (Rockford, IL, USA). The monoclonal antibody anti-GAPDH (6C5) was from Chemicon-Milipore (Temecula, CA). Calpain activity assay kit was acquired from BioVision (Mountain View, CA, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

### Cell Culture

Primary cultures of cortical cells were prepared from 15 to 16-day embryos of Wistar rats according to previously described procedures (Agostinho and Oliveira 2003). Cortical cells were cultured in Neurobasal medium with 2 mM L-glutamine, 2% B27 supplement, penicillin (100 U/ml), and streptomycin (100 μg/ml). The neurons were seeded in poly-L-lysine (0.1 mg/ml)-coated plates or coverslips at a density of $0.4 \times 10^6$ cells/cm$^2$ and $0.05 \times 10^6$ cells/cm$^2$, respectively.

### Peptide Treatment

Cultured cortical neurons were treated with $A\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM) for different periods of time, ranging from 6 to 96 h, as indicated in figure captions. The peptides were added into culture medium at the 5th culturing day. The peptides were reconstituted according to the manufacturers' instructions, and $A\beta_{1–40}$ was aged in PBS buffer, in a stock concentration of 231 μM, for 7 days at 37°C.

### Neuronal Viability

To assess cell viability we used two methods: a modified MTT assay (Mosmann 1983) and the fluorescent DNA stain Hoechst 33342.

### MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced to formazan by metabolic active cells, and therefore this conversion is directly related to the amount of viable cells. Briefly, MTT was dissolved in Krebs medium, containing (in mM): NaCl 132, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1.4, $H_3PO_4$ 1.2, glucose 6 and HEPES-Na 10 (pH = 7.4), to a concentration of 5 mg/ml and then added to the neuronal culture medium for 2 h at 37°C. After this incubation, the medium was removed and the blue formazan crystals formed were dissolved in DMSO (Ankarcrona et al. 1995) and quantified by measuring absorbance at 570 nm in a Molecular Devices SpectraMax Plus 384 plate reader. Results were expressed as a percentage of the absorbance in control cells.

### Hoechst Assay

Neurons plated in glass coverslips were incubated in the dark for 5 min with 300 µl of Hoechst 33342 (10 µg/ml). After being washed with PBS, the cells were observed and scored in a fluorescence microscope (Zeiss, Axioskop2 Plus). Those cells showing irregular and relatively high blue fluorescence (dead cells) were identified from an average of 300 cells per treatment and cell batch. Each individual experiment was made in duplicate. The cells were examined by blinded counting. Four pictures from different fields (selected randomly) were taken from each individual experiment, in which all the cells (± 300) were counted. Data were expressed as the percentage of dead cells versus the total cells counted.

### Western Blot

For the preparation of total cell extracts, untreated- or peptides treated-cultured neurons were scraped in 100 µl of ice-cold lysis buffer containing (in mM): HEPES-Na 25, $MgCl_2$ 2, EDTA 1, EGTA 1, supplemented with 100 µM PMSF, 2 mM DTT and protease inhibitor cocktail (containing 1 µg/ml leupeptin, pepstatin A, chymostatin, and antipain). Cell lysates were frozen three times in liquid $N_2$ and centrifuged at 14000× *g* to remove nuclei and large debris. Protein concentration in the supernatant was measured using the Bio-Rad protein dye assay reagent. Samples were denaturated at 95°C for 5 min in a sample buffer, containing (in mM): Tris 500, DTT 600, 10.3% SDS, 30% glycerol, and 0.012% bromophenol blue. Equal amount of each sample of protein was separated by electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE) and electroblotted onto PVDF membranes. The identification of proteins of interest was facilitated by the usage of a pre-stained precision protein standard (Bio-Rad), which was run simultaneously. The proteins in gel were electrophoretically transferred to membranes that were incubated for 1 h at room temperature (RT) in Tris buffer (TBS-T (in mM) NaCl 150, Tris-HCl 25, pH 7.6, with 0.1% Tween 20), containing 5% nonfat dry milk to block nonspecific binding. Then the membranes incubated with the primary antibodies overnight at 4°C in TBS-T containing 1% nonfat dry milk. The primary antibodies used were: (i) rabbit monoclonal anti-Cdk5 (1:500 dilution) (ii) rabbit monoclonal anti-p35 (1:500 dilution), (iii) mouse monoclonal anti-tau (1:500 dilution), and (iv) mouse monoclonal anti-phospho-tau (1:250 dilution). After this incubation, the membranes were washed and incubated

in TBS-T with 1% nonfat dry milk for 2 h at RT, with the appropriate alkaline-phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody at a dilution of 1:25000 or 1:20000, respectively. Immunoreactive bands were detected after incubation of membranes with ECF reagent for 5–10 min, on a Bio-Rad Versadoc 3000 Imaging System.

### Immunocytochemistry

Primary cortical neurons cultured in glass coverslips were treated with peptides and inhibitors. Then, the cells were washed with PBS and fixed with a 4% paraformaldehyde solution (pH 7.4) for 30 min at RT. The cells were permeabilized with 0.2% Triton X-100/PBS for 2 min at RT, and blocked with 0.1% bovine serum albumin (BSA) before incubation with a primary antibody anti-Human PHF-Tau (1:40) for 1 h at RT. After being washed in PBS to remove the unbound antibody, they were incubated with labeled anti-mouse Alexa Fluor 488 IgG antibodies (1:500) for 1 h at RT. Finally, the cells were mounted with the DakoCytomation fluorescent medium and visualized in a fluorescence microscope.

### Calpain Activity Assay

Calpain activity was determined according to the Calpain Activity Assay Kit (BioVision, CA, EUA) protocol. Cultured rat cortical neurons either untreated or treated with the peptides and/or inhibitors for 24 h were lysed using a buffer provided in the kit. Calpain substrate Ac-LLY-AFC was then added to the total cell extract and the mixture was incubated at 37°C for 1 h, in the dark. Fluorescence was measured at excitation/emission wavelengths of 400/505 nm in a Molecular Devices SpectraMax Gemini EM plate reader.

### Statistical Analysis

Results are expressed as means ± SEM. Statistical analysis was made using Graphpad Prism software. Significance was determined using an analysis of variance (ANOVA), followed by Dunnett's post-tests, or by the two-tailed Students' *t*-test.

## Results

In the present study, we used the synthetic peptides $A\beta_{1–40}$ and $PrP_{106–126}$ at concentrations previously defined by studies of our group (Garção et al. 2006; Melo et al. 2007; Resende et al. 2007). The cortical neurons used in this study were cultured in serum-free Neurobasal medium in the presence of B27 supplement, which supports the growth of neurons and minimizes glial cell proliferation, as confirmed by prior studies at our lab (Ferreiro et al. 2006).

### Cdk5 is Involved in Neuronal Death Induced by A$\beta$ and PrP

In order to evaluate the involvement of Cdk5 in neuronal death triggered by $A\beta_{1–40}$ and $PrP_{106–126}$, we used the Cdk5 inhibitor roscovitine. Changes in neuronal viability were assessed by determining modifications in the metabolic capacity of cells through the MTT test and by evaluating nuclear apoptotic morphology with the fluorescent dye Hoechst 33342. As can be seen in Fig. 1a, $A\beta_{1–40}$ and $PrP_{106–126}$ significantly ($P < 0.05$) decreased the capacity of

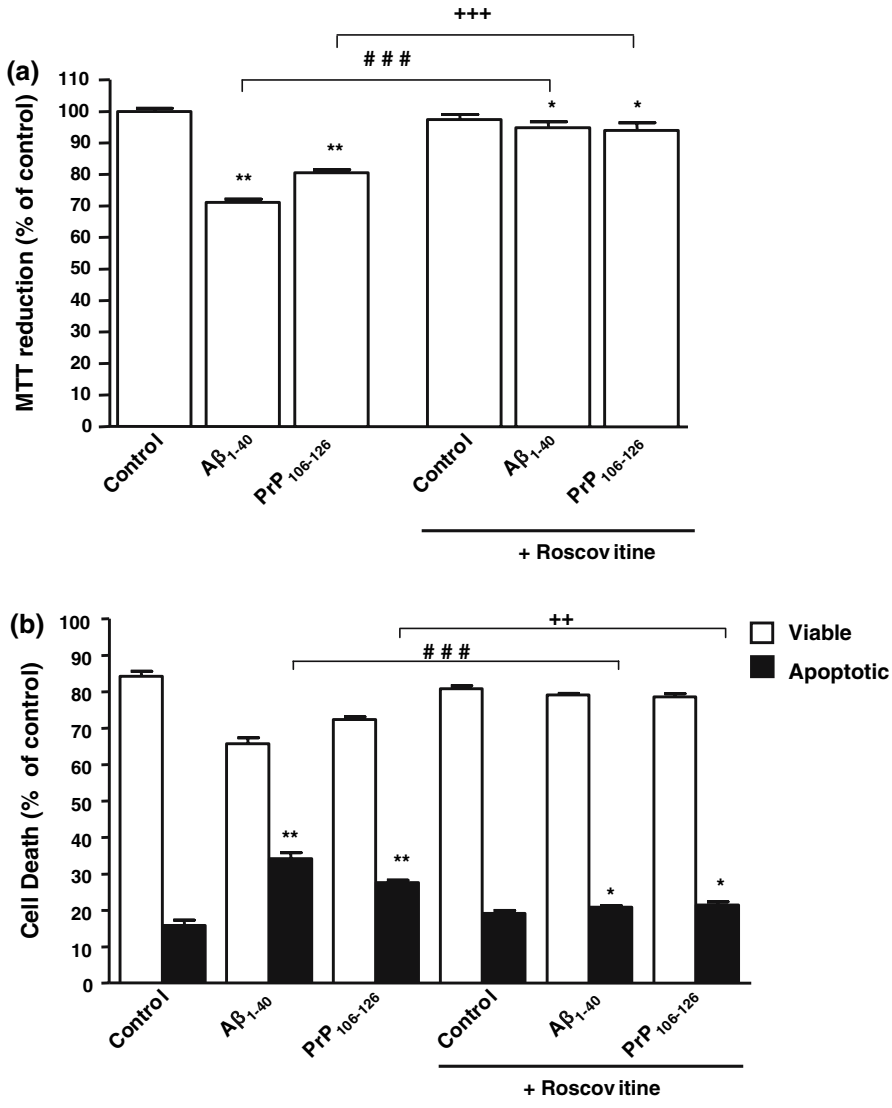


**Fig. 1** The Cdk5 inhibitor (roscovitine) partially prevents the neuronal death induced by A$\beta$ and PrP peptides**.** Cultured cortical neurons from rat brain were exposed to A$\beta_{1-40}$ (1 μM) or $PrP_{106-126}$ (2.5 μM) peptide in the presence or absence of the Cdk5 inhibitor roscovitine (0.5 μM) for 24 h. (**a**) Cell viability was assessed using the MTT assay. The viability of control (untreated) and treated cells was evaluated by measuring the capacity of the cells to reduce MTT. The assay evaluated the metabolic capacity of cells, and the values were expressed as percentage of the absorbance determined for control cells. (**b**) Quantification of apoptotic cell death was performed using the fluorescent nuclear dye Hoechst 33342, which identifies neurons undergoing DNA fragmentation and nuclear condensation. The values were expressed as a percentage of dead cells relative to the total number of cells counted (± 600 cells per treatment and cell batch). Data are means ± SEM of duplicates from three to six independent experiments for MTT assay and four to nine independent experiments for Hoechst test. * $P < 0.05$, ** $P < 0.01$, significantly different from control cells; $^{++}$ $P < 0.01$, ### and $^{+++}$ $P < 0.001$, compared with cells treated with the same peptide

neurons to reduce MTT by 28.9 ± 4.2% and 19.5 ± 5.0%, respectively. The neuronal injury caused by $A\beta_{1–40}$ and $PrP_{106–126}$ was partially reverted by the Cdk5 inhibitor, roscovitine. In fact, in the presence of this inhibitor, the viability of neurons treated with $A\beta$ and PrP peptides was not significantly different from control cells. Figure 1b shows that both $A\beta_{1–40}$ and $PrP_{106–126}$ significantly increased the number of apoptotic neurons, and this effect was once again reverted by roscovitine. These data suggest that Cdk5 was involved in apoptotic neuronal death triggered by $A\beta$ and PrP peptides. Therefore, we assessed if these peptides affect Cdk5 expression and/or activity.

## $A\beta$ and PrP Affect the Levels of Cdk5 Activators

The levels of Cdk5 were assessed in cortical neurons untreated or treated with $A\beta_{1–40}$ and $PrP_{106–126}$ for different time periods (8, 24, 48, and 96 h). The obtained data show that the peptides did not affect Cdk5 expression for all the incubation periods tested (Fig. 2). Since the peptides did not cause time-related changes in the levels of Cdk5, we determined if the levels

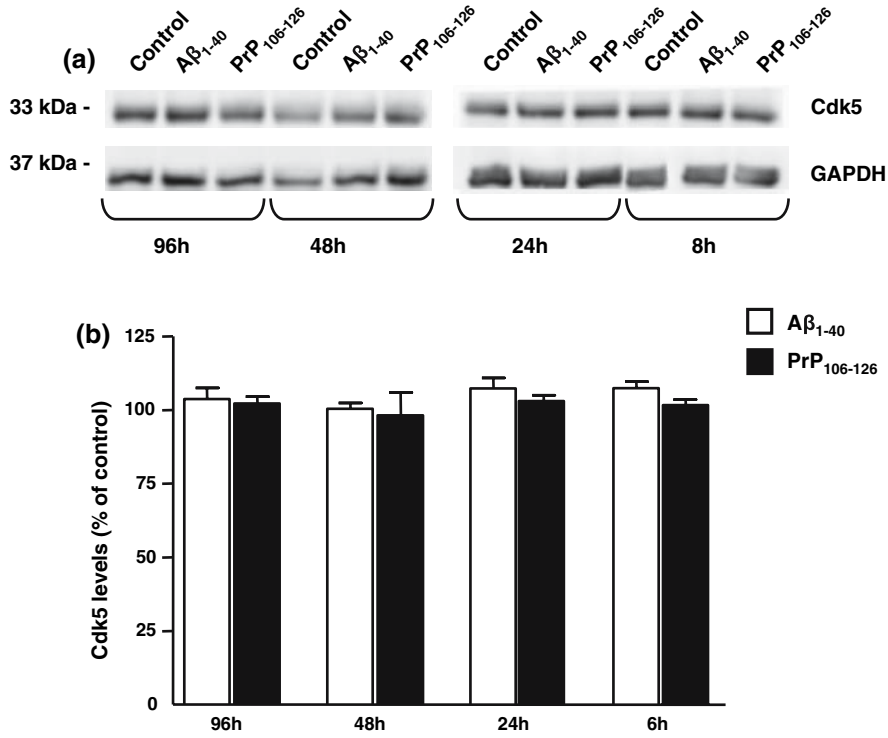


**Fig. 2** Cdk5 expression is not affected by $A\beta$ and PrP peptides. Cultured cortical neurons were treated for 8, 24, 48 or 96 hours with $A\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM). (**a**) Lysates from untreated and peptide-treated cells were resolved by SDS-PAGE and analyzed by Western Blotting with an anti-Cdk5 antibody. An antibody against GAPDH was used to estimate the total amount of protein loaded in the gel. The immunoreactive bands were visualized by scanning on a Versadoc Image System. (**b**) Quantitative analysis of immunoreactive bands was performed in the Quantity One program. The bars represent the relative levels of Cdk5 compared with GAPDH and are expressed as percentage of the control value. The data are means ± SEM of three to six independent experiments

of Cdk5 activators, p35 and p25, were affected by $A\beta_{1–40}$ and $PrP_{106–126}$. Our results show that the levels of p25, which can induce Cdk5 overactivation, were increased in A$\beta$ and PrP-treated neurons (Fig. 3). Since p35 can be cleaved to p25 by calpains (Lee et al. 2000), we further evaluated the activity of these cysteine proteases in neurons untreated or treated with the peptides. Figure 4 shows that calpain activity was significantly higher in A$\beta$- (about 50%) and PrP- (about 20%) treated cells than in control cells. This augment in calpain activity parallels the increase of p25 levels (see Figs. 3 and 4), suggesting a direct correlation between the increase in calpain activity induced by $A\beta_{1–40}$ and $PrP_{106–126}$ and the extent of p35 cleavage to p25. To confirm the involvement of calpains in the decrease of p35/p25 on our experimental conditions, we tested the effect of the calpain inhibitor, MDL28170, in the levels of the Cdk5 activators. We found that neurons treated with the peptides plus MDL28170 showed almost no p35 cleavage when compared with neurons exposed only to A$\beta$ and PrP (Fig. 3). Regarding calpain activity (Fig. 4), we also observed that co-incubation with MDL28170 prevented the increase induced by the treatment with A$\beta$ and PrP. Therefore, we decided to evaluate the effect of MDL28170 in preventing neuronal injury triggered by $A\beta_{1–40}$ and $PrP_{106–126}$. As can be seen in Fig. 5, the calpain inhibitor was able to significantly prevent neuronal death caused by $A\beta_{1–40}$ and $PrP_{106–126}$.

## Cdk5 is Involved in Tau Hyperphosphorylation Caused by A$\beta$ and PrP

To evaluate the impact of p25-induced Cdk5 deregulation on tau phosphorylation, we used the phospho-specific antibody AT8, which recognizes the Ser202/Thr205 phosphorylation site of tau protein. Consistently with the previous results, in A$\beta$-treated cells, there was a marked

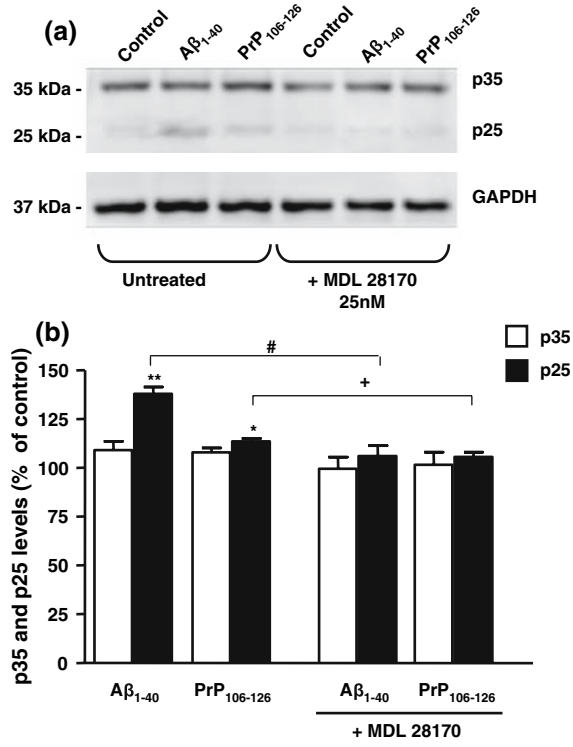


**Fig. 3** Changes in the levels of p35 and p25 in neurons treated with A$\beta$ or PrP peptides. Effect of calpain inhibitor. Cultured cortical neurons were treated with $A\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM) peptides for 24 h in the presence/absence of the calpain inhibitor MDL28170. (**a**) Lysates from treated cells were resolved by SDS-PAGE and analyzed by Western Blotting with an antibody against p35/p25. GAPDH was used to estimate the total amount of protein loaded in the gel. The immunoreactive bands were visualized by scanning on a Versadoc Image System. (**b**) Quantitative analysis of immunoreactive bands was performed in the Quantity One program. The bars represent the relative levels of Cdk5 compared with GAPDH levels and are expressed as percentage of the control value. The data are means ± SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from control cells; $^{\#}$ $P < 0.05$, $^{+}$ $P < 0.05$, compared with cells treated with the same peptide

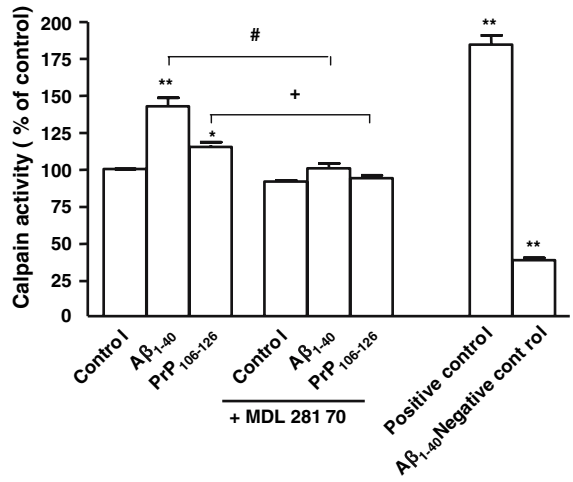


**Fig. 4** A$\beta$ and PrP peptides cause calpain activation. Cultured cortical neurons were incubated for 24 h with A$\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM) in the presence/absence of the calpain inhibitor MDL28170. It were also used a positive control, corresponding to the extraction buffer plus active calpain I, and a negative control consisting of A$\beta$-treated cells plus calpain inhibitor Z-LLY-FMK. The values of calpain activity were expressed as a percentage of the value observed in untreated (control) cells and are means ± SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from control cells; $^{\#}$ $P < 0.05$, $^{+}$ $P < 0.05$, compared with cells treated with the same peptide

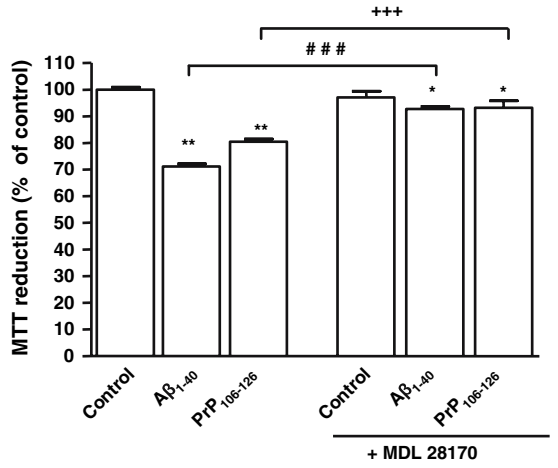


**Fig. 5** MDL28170 partially reverts the cell death caused by A$\beta$ and PrP peptides**.** The calpain inhibitor MDL28170 (25 nM), was incubated simultaneously with the peptides A$\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM) for 24 h. Cell viability was then assessed using the MTT assay. The viability of control (untreated) and treated cells was evaluated by measuring the capacity of the cells to reduce MTT. The assay evaluated the metabolic capacity of cells, and the values were expressed as percentage of the absorbance determined for control cells. The data are means ± SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from control cells; $^{\#\#\#}$ $P < 0.001$, $^{+++}$ $P < 0.001$, compared with cells treated with the same peptide

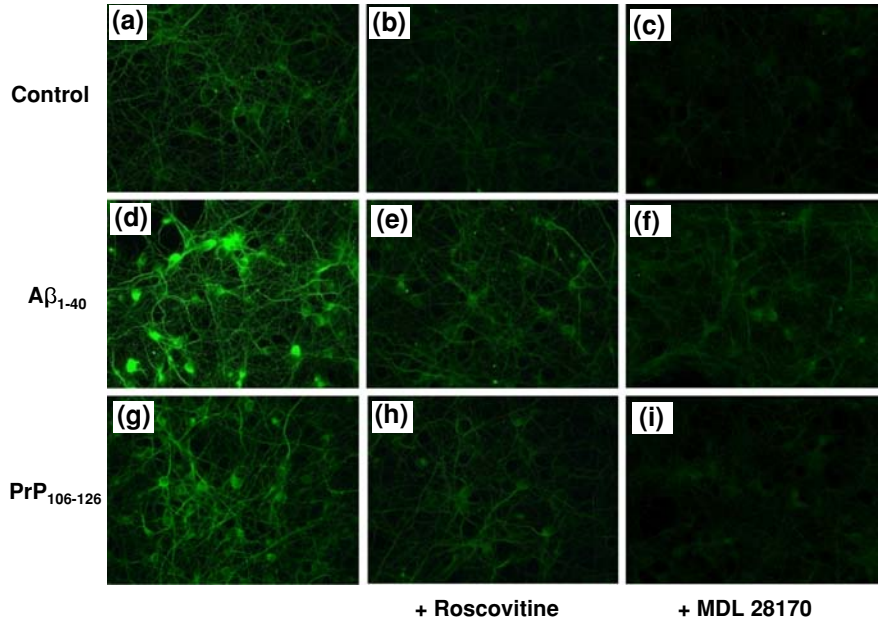


**Fig. 6** Effect of A$\beta$ and PrP peptides on tau protein phosphorylation**.** Cultured rat primary cortical neurons were treated with $A\beta_{1–40}$ (1 μM) or $PrP_{106–126}$ (25 μM) for 24 h in the absence (**a,d,g**)/presence of the Cdk5 inhibitor roscovitine (0.5 μM) (**b,e,h**) or calpain inhibitor MDL28170 (25 nM) (**c,f,i**). The cells were immunostained using anti-human PHF-tau (clone AT8) antibody and observed in a fluorescence microscope. The images are representative for each experimental condition

increase in tau hyperphosphorylation at the AT8 site (Fig. 6d and Fig. 7). The effects induced by PrP were milder than those obtained with A$\beta$, although still significantly ($P < 0.01$) different from control cells (Fig. 6g and Fig. 7). In the presence of roscovitine, the levels of hyperphosphorylated tau in A$\beta$- and PrP-treated cells were similar to those in control cells (Fig. 6e, h and Fig. 7a). MDL28170 also led to a similar shift of tau phosphorylation in A$\beta$- and PrP- treated cells, with a decrease to control-like levels (Fig. 6f, i and Fig. 7b). In addition, we analyzed total tau levels and observed that $A\beta_{1–40}$ and $PrP_{106–126}$ did not affect the levels of this protein (data not shown). Taken together, the results suggest that the increase in hyperphosphorylated tau levels, caused by A$\beta$ and PrP, was correlated with Cdk5 overactivation due to calpain cleavage of p35.

## Discussion

Several studies have addressed the mechanism of A$\beta$- or PrP-toxicity in different models (Forloni et al. 1996; Combs et al. 1999; White et al. 2001; Agostinho et al. 2003; Fereiro et al. 2006; Garcao et al. 2006), but no work so far has tried to assess the role of Cdk5 in the neurotoxicity caused by $PrP_{106–126}$, a peptide that mimics the toxic effects of $PrP^{Sc}$. Since AD and PRE share analogous clinical and neuropathological characteristics (Aguzzi and Haass 2003; Veerhuis et al. 2005; Barnham et al. 2006), in the present work, we perform a comparative study, using A$\beta$ and PrP synthetic peptides in order to determine the involvement of

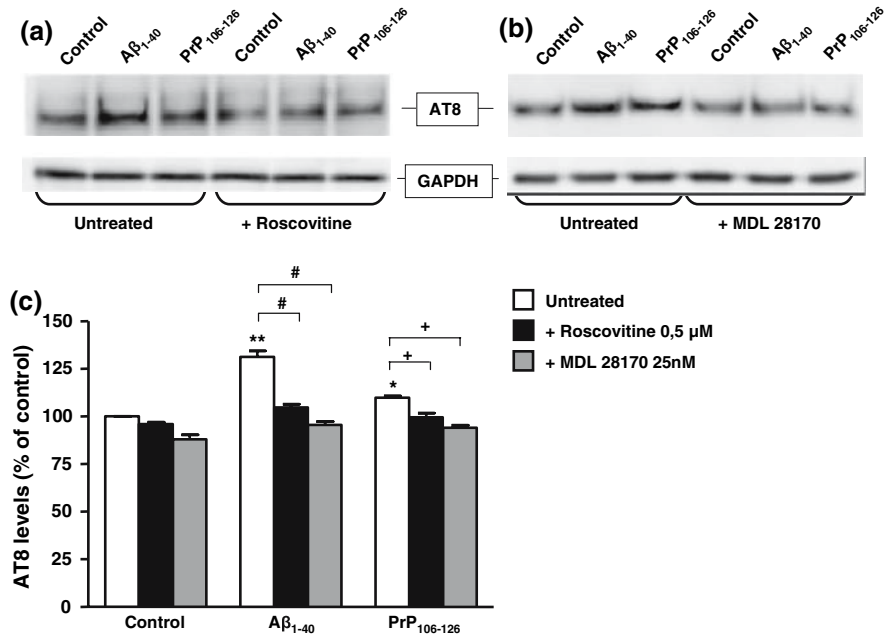


**Fig. 7** A$\beta$ and PrP peptides increase tau protein hyperphosphorylation in cultured cortical neurons. Neuronal cultures were incubated with $A\beta_{1-40}$ and $PrP_{106-126}$ for 24 h in the presence/absence of Cdk5 inhibitor roscovitine (0.5 μM) or calpain inhibitor MDL 28170 (25 nM). (**a, b**) Lysates from treated cells were resolved by SDS-PAGE and analyzed by Western Blotting with an antibody for human PHF-Tau (clone AT8). (**c**) Quantitative analysis of immunoreactive bands was performed in the Quantity One program. The bars represent the relative levels of PHF-tau compared with GAPDH levels and are expressed as percentage of the control value. The data are means ± SEM of three to six independent experiments. * $P < 0.05$, ** $P < 0.01$ significantly different from control cells; $^{\#}$ $P < 0.05$, $^{+}$ $P < 0.05$ compared with cells treated with the same peptide

Cdk5 in tau phosphorylation and neuronal death under AD and PRE conditions. Using the selective Cdk5 inhibitor roscovitine, we observed that, when Cdk5 is not active, there is a significant reduction in the neuronal apoptotic death caused by $PrP_{106-126}$ or $A\beta_{1-40}$ (Fig. 1a, b). Since this decrease was only partial, we can speculate that there are other cell death pathways acting simultaneously and independently from Cdk5, and thus not inhibited by roscovitine (Agostinho et al. 2003; Ferreiro et al. 2006). Although several other studies have shown that Cdk5 mediates the neurotoxicity triggered by A$\beta$peptides (Alvarez et al. 2001; Liu et al. 2004), this is the first study showing that Cdk5 is in part responsible for the neurotoxic effect of PrP. In our experimental conditions, $PrP_{106-126}$ and $A\beta_{1-40}$ did not affect Cdk5 levels (Fig. 2). Since the activity of this kinase depends on the levels of p35 or p25, we determined their levels in cortical neurons treated with the peptides. Our results showed that A$\beta$ and PrP significantly increase the levels of p25, which is responsible for Cdk5 overactivation. This is consistent with other studies that report an increase in p25 levels when cells are exposed to A$\beta$ peptides and in AD brains (Patrick et al. 1999; Tseng et al. 2002), and also confirmed our hypothesis that PrP causes Cdk5 deregulation in a manner similar to A$\beta$. However, the effects of PrP treatment are less pronounced than those of the A$\beta$ peptide. The increase in p25 triggered by A$\beta$ and PrP was prevented by the calpain inhibitor MDL28170 (Fig. 3), indicating that this augment in p25 levels is due to calpain activation. Indeed, we found that A$\beta$ and PrP

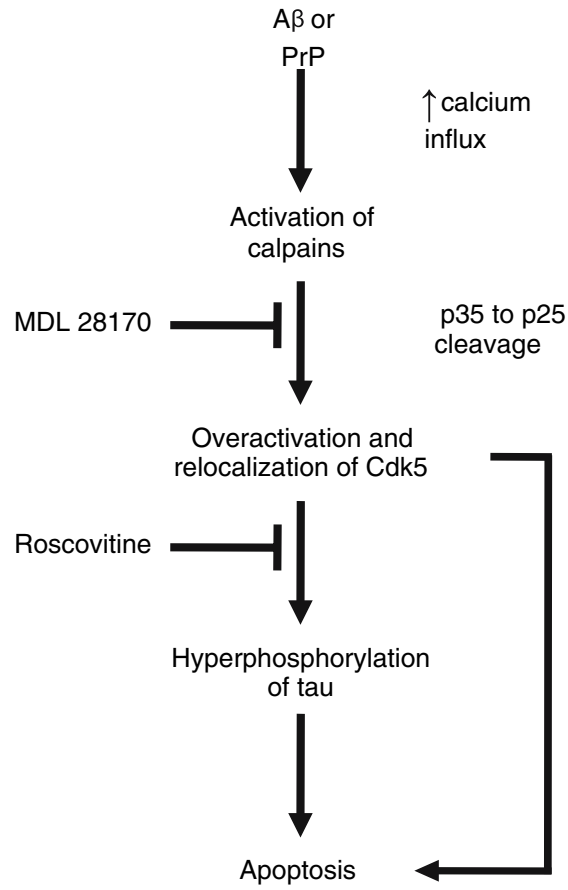


**Fig. 8** Hypothetic model for A$\beta$ and PrP neurotoxicity involving Cdk5. The treatment of cortical neurons with A$\beta$ or PrP causes a deregulation in calcium homeostasis, leading to an increase of the intracellular $Ca^{2+}$ level. Overactivation of calpains will consequently increase the cleavage of the Cdk5 activator p35 to p25. The augment in p25 levels promotes the formation of a hyperactive p25/Cdk5 complex, responsible for tau hyperphosphorylation and apoptotic neuronal death. By halting this pathway, either by inhibiting calpains (using MDL28170) or by blocking Cdk5 itself (with roscovitine), the neurotoxicity triggered by A$\beta$ and PrP can be prevented

significantly increase calpain activity, although once again the effects were more pronounced in cells treated with A$\beta$ (Fig. 4). Other studies have also reported the activation of calpains in neurons exposed to A$\beta$ peptides (Boland and Campbell 2003; Raynaud and Marcilhac 2006) and in human neuroblastoma cells treated with $PrP_{106–126}$ (O'Donovan et al. 2001). This enhancement in calpain activity is probably due to intracellular $Ca^{2+}$ homeostasis deregulation, which has been shown to occur in cortical neurons treated with A$\beta$ or PrP peptides (Agostinho and Oliveira 2003; Ferreiro et al. 2006). To determine the involvement of this calpain/Cdk5-p25 pathway in the neuronal death caused by these peptides, we then analyzed the effect of the calpain inhibitor MDL28170 on neuronal viability. The obtained data show that this inhibitor prevented A$\beta$- and PrP-neurotoxicity in a pattern similar to that of roscovitine (Fig. 5). These observations strongly support the idea that p35 cleavage by calpains is a crucial event in Cdk5-mediated neurotoxicity triggered by A$\beta$ or PrP peptides.

Another consequence of p35 cleavage into p25 is the loss of a membrane targeting sequence, causing qualitative changes in Cdk5 activity (Dhavan and Tsai 2001). Increasing evidence suggests that the p25/Cdk5 complex is responsible for tau protein phosphorylation in several sites associated to AD (Cruz et al. 2003; Tsai et al. 2004). Our data show that both A$\beta$ and PrP treatments increased the levels of phosphorylated tau at the AT8 sites (Figs. 6 and 7). Although the increase in phospho-tau levels caused by PrP was more moderate than that

observed in the presence of A$\beta$, in PrP-treated neurons the levels of phospho-tau were significantly different from untreated cells (control). This is somehow consistent with the fact that, in PRE, even though tau hyperphosphorylation can be observed, the formation of NFTs does not occur (Bautista et al. 2006). In rat brain cortex, it was shown that Cdk5 is co-localized with tau and glycogen synthase kinase 3$\beta$ (GSK3$\beta$, which also mediates tau phosphorylation at sites characteristic for tauopathies and AD (Li et al. 2006). This study also shows that Cdk5 primes the tau protein for subsequent phosphorylation by GSK3$\beta$. Since the AT8 site (corresponding to S202/T205) does not require priming, phosphorylation can occur independently by both kinases (Li et al. 2006). On the other hand, a study performed in mice that overexpress p25 reported that GSK3$\beta$ is directly involved in tau hyperphosphorylation, whereas Cdk5 acts as an inhibitory modulator of GSK3$\beta$ (Plattner et al. 2006), suggesting that Cdk5 overactivation may avoid tau hyperphosphorylation. On the contrary, our data showed that Cdk5 inhibition, as well as the blockage of p25 formation, prevents tau hyperphosphorylation and neuronal death.

In conclusion, our results showed that A$\beta$ and PrP peptides increase the levels of p25, due to calpain overactivation. This augment in calpain activity may be due to an increase in intracellular $Ca^{2+}$ concentration, which was shown to occur at an early phase in cortical neurons treated with A$\beta$ and PrP peptides (Ferreiro et al. 2006). The increase in p25 levels can promote the formation of a hyperactive p25/Cdk5 complex leading to tau hyperphosphorylation and apoptotic neuronal death (Fig. 8). Our work is the first of demonstrating a connection between Cdk5 deregulation and PrP-induced tau hyperphosphorylation and neuronal death. Since our data revealed that the direct or indirect inhibition of Cdk5 activity prevents the neuronal damage caused by A$\beta$ and PrP peptides, we can speculate that therapeutic strategies directed to the calpain/p25/Cdk5 pathway may be useful for Alzheimer's and Prion diseases.

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