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Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells

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Abstract

Neurofibrillary tangles (NFT) of hyperphosphorylated tau protein are a major pathological hallmark of Alzheimer's disease (AD). One of the tau phosphorylating kinases with pathological relevance in AD has been suggested to be the cyclin-dependent kinase 5 (Cdk5). The proposed mechanism leading to pathological Cdk5 activity is through induced cleavage of p35 to a proteolytic product, p25. To further study activation of Cdk5 and its role in tau phosphorylation in vitro, we used differentiated SH-SY5Y cells treated with neurotoxic stimuli or transfected with p25. We show that glutamate increased tau phosphorylation, concomitant with an increased Cdk5 activity achieved by upregulation of Cdk5 and p35 protein levels. Treatment with the calcium ionophore A23187 generated the calpain cleaved p25 fragment but only in toxic conditions that caused dephosphorylation and loss of tau. When p25 was transfected to the cells, increased tau phosphorylation was achieved. However, application of the Cdk5 inhibitor Roscovitine did not result in inhibition of tau phosphorylation possibly due to activation of extracellular regulated kinase 1/2 (Erk1/2), which also is capable of phosphorylating tau. Cdk5 and Erk1/2 kinases share some common substrates but impact of their cross talk on tau phosphorylation has not previously been demonstrated. We also show that p25 is degraded via the proteasome in Roscovitine treated cells.

Keywords: Cdk5; p35; p25; Glutamate; A23187; Transfection; Tau phosphorylation; Roscovitine; Erk

Neurofibrillary tangles (NFT) are together with amyloid plaques the major pathological hallmarks of Alzheimer's disease (AD). The major components of NFTs are paired helical filaments (PHF) of highly phosphorylated microtubule-associated protein tau [1,2]. The hyperphosphorylated tau is deleterious to microtubule organization and leads to disruption of cytoskeleton and neuronal cell death.

One of the tau phosphorylating kinases with pathological relevance in AD has been suggested to be the cyclin-dependent kinase 5 (Cdk5). To gain enzymatic activity, Cdk5 needs to bind to an activator protein, the most common of them being p35 [3]. Cdk5/p35 is involved in many physiological functions in the CNS, such as neuronal migration and neurite outgrowth, cellular motility and adhesion, and synaptic plasticity [4–8]. The proposed mechanism leading to a pathological kinase activity is through induced cleavage of p35 to a proteolytic product, p25 [9,10]. The dimeric Cdk5/p25 has been shown to possess prolonged enzymatic activity and potentially alter the substrate specificity of the kinase [9].

The published clinical findings on the pathological relevance of this mechanism are somewhat conflicting with both supportive and non-supportive evidence. Several authors, however, reported increased levels of p25 in AD as compared to control brain [9,10]. In addition, increased Cdk5 enzymatic activity has been documented in AD brain [9,11]. Further compelling support for a role of p25/Cdk5 in neurodegenerative processes has come from the p25

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transgenic mice described by Cruz et al. [12]. In contrast to previous p25 transgenic models, the transgene expression was inducible and targeted to the forebrain. These mice demonstrated both hyperphosphorylation of tau and neurofibrillary pathology derived from endogenous tau as a result of overexpression of p25 in postnatal mouse brain. Cruz et al. thereby provided strong evidence that aberrant Cdk5 kinase activity can lead to NFT formation.

In neuronal cells the calcium activated protease calpain has been shown to be responsible for the cleavage of p35 to p25 [13,14]. Induction of calpain mediated p35 cleavage to p25 can be achieved by acute exposure of cell cultures to Aβ peptides, oxidative stress, excitotoxins or to agents causing calcium influx [13–16], leading to increased Cdk5 kinase activity in the cultures [15,16]. This in turn may [15] or may not [16] result in increased tau phosphorylation.

The contradicting results from in vitro studies linking p25/Cdk5 to tau hyperphosphorylation prompted us to investigate these mechanisms further. The majority of previous in vitro studies have been performed using primary cells [13–16]. In an effort to identify a more easily accessible cell system to study Cdk5 and its role in tau phosphorylation, we used the human neuroblastoma cell line SH-SY5Y, which is a commonly used cellular model to study neuronal regulation and function. Even though differentiated SH-SY5Y cells express both Cdk5 and its neuron-specific activator p35 [17], basal activity of Cdk5 kinase in these cells is low (unpublished observation) and its involvement in tau phosphorylation minor [17]. We therefore used different neurotoxic stimuli and transfection with p25 to investigate whether SH-SY5Y cells could provide a model system to study the mechanisms of Cdk5 activation and Cdk5 mediated tau phosphorylation.

Materials and methods

Cell cultures and treatments. The human neuroblastoma cell line SH-SY5Y was purchased from European Collection of Cell Cultures (ECACC). Cells were grown in medium with equal amount of minimum essential medium (MEM, Gibco) and nutrient mixture Ham's F-12 (Gibco), supplemented with 1% non-essential amino acids (Gibco) and 0–10% heat-inactivated fetal calf serum (FCS) (HyClone). Cells were plated at a density of 4.0×10^3 cells/cm^2 in 6-well culture dishes (Corning) using cell medium with 10% FCS. All-*trans*-retinoic acid (RA, Sigma–Aldrich) was added the day after plating at the final concentration of 10 μM in the medium containing 1% FCS. After 6 days of RA pretreatment, the cells were cultured in the presence of 2 nM brain-derived neurotrophic factor (BDNF, Sigma–Aldrich) in serum-free medium for 48 h. Cells were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

Cell cultures were treated with calcium ionophore A23187 (Biomol), H_2O_2 (Sigma–Aldrich) or glutamate (Sigma–Aldrich). In some experiments calpeptin (ICN Biomedicals Inc.) was added together with A23187.

Transfection of the cells with p25 plasmid. For transfection experiments the cells were cultured as above but differentiated only with RA for 6 days. The cells were transfected with 4 or 8 μg p25 plasmid (pAPC227, made inhouse at AstraZeneca, R&D). The p25 gene was cloned into a mammalian expression vector, pcDNA3 and the expression was under the control of CMV promoter. Lipofectamine™ 2000 (Invitrogen) was used as a transfection reagent. Lipofectamine™ 2000 (10 μl/transfection) was first diluted in cell culture medium without FCS and incubated for 5 min at RT. The plasmid DNA diluted in medium was then combined with Lipofectamine and incubated further for 20 min at RT. The complexes were added to the cells and the transfection was carried out for 24 h. Transfected cells were treated with Cdk5 inhibitors Roscovitine (Sigma) or Olomoucine (Sigma) or the proteasome inhibitor Lactacystin (Calbiochem) together with Roscovitine for 4 h before cells were lysed 24 h posttransfection.

Western blot. The cells were lysed in buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, and 1 complete protease inhibitor cocktail tablet (Roche Diagnostics)/10 ml buffer. The cells were incubated with lysis buffer for 10 min on ice before scraping the cells from the dishes. Cell lysates were centrifuged at 14,000 rpm (Eppendorf 5417R) for 15 min. The protein content in the supernatants was measured using BCA Protein Assay kit (Pierce). Samples containing 50 μg protein were resolved in 10% NuPage® Bis–Tris gels (Invitrogen) and the proteins were transferred to Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked in PBS with 0.05% Tween 20 containing 5% non-fat dried milk for 1 h at room temperature (RT). Primary antibodies were diluted in either 5% BSA or 5% milk and incubations were carried out at 4 °C overnight. Primary antibodies were used at the following dilutions: Cdk5 (Santa Cruz Biotechnology Inc., C-8) 1:2000, p35 (Santa Cruz Biotechnology Inc., C-19) 1:2000, GSK3β [pS^9] (Cell Signaling) 1:1000, GSK3α/β [pY$^{279/216}$] (Biosource International) 1:1000, GSK3β (Santa Cruz Biotechnology Inc., H-76) 1:1000, phospho-p44/42 Map Kinase (pErk1/pErk2) (Cell Signaling) 1:1000, p44/42 Map Kinase (Erk1/Erk2) (Cell Signaling) 1:1000, β-actin (Sigma–Aldrich, clone AC-15) 1:20,000, Tau-5 (RDI) 1:200, Tau [pS^{202}] (Biosource International) 1:500, and Tau [pS^{404}] (Biosource International) 1:500. Horseradish-peroxidase (HRP) -conjugated secondary antibodies (Amersham Biosciences) were incubated 1 h at RT in 5% milk at the dilution of 1:5000 for anti-rabbit and 1:10,000 for anti-mouse antibody. Blots were developed using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences). When needed, membranes were stripped with Restore Western blot stripping buffer (Pierce) for 30 min at 50 °C.

Average density of the bands was measured in Fluor-S™ MultiImager (Bio-Rad) by using Quantity One software and was expressed as percent of untreated controls. Density of Cdk5, p35, Gsk3β, and Erk1/2 bands was normalized to β-actin levels whereas Gsk3β Y^{216} and Gsk3β S^9 were normalized to total Gsk3β levels and pErk1/2 to total Erk1/2 levels. The density of pTau was normalized to total tau levels detected with the phosphorylation-independent Tau-5 antibody.

Immunoprecipitation. The cells were scraped from the dishes in detergent-free buffer containing 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1 mM DTT, 20 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, and 1 complete protease inhibitor cocktail tablet (Roche Diagnostics)/10 ml buffer. Homogenization using the needles (23G) was performed. The cell lysates were centrifuged and the protein content in the supernatants was measured using BCA protein assay kit (Pierce). Five hundred micrograms of protein in 200 μl lysis buffer was precleared for 30 min at 4 °C with rabbit IgG (5 μg/ml, Vector Laboratories Inc.), 10 μl protein A/G Plus agarose beads (Santa Cruz Biotechnology Inc.) were added and the incubation was continued for an additional 30 min. In the Cdk5 scintillation proximity assay 50 ng of recombinant Cdk5/p25 enzyme complex (produced by Biotech Laboratories, AstraZeneca) was used as a positive control and was precleared and immunoprecipitated in a same manner as the samples. The samples were centrifuged at 14,000 rpm (Eppendorf 5417R) for 1 min and the supernatants were transferred to new tubes. Five microliters of Cdk5 (C-8) antibody and 20 μl protein A/G Plus agarose beads were added in each reaction and incubated at 4 °C for 1 h. The immunoprecipitates were centrifuged at 14,000 rpm (Eppendorf 5417R) for 1 min and washed twice in a lysis buffer supplemented with 0.1% NP-40 detergent.

Cdk5 scintillation proximity assay. Cdk5 kinase activity was assessed with scintillation proximity assay (SPA). The immunoprecipitates were washed once in a buffer consisting of 70 mM Hepes, 0.5 mM EDTA, 37.5 mM KCl, 30 mM β-glycerophosphate, 0.15% BSA, 0.05%

mercaptoethanol, 0.02% BRIJ 35 (w/v), and 2.5% glycerol (pH 7.35), and then diluted in 70 μl of this buffer. The immunoprecipitates were assayed for Cdk5 kinase activity in aliquots of 30 μl supplemented with 5 ng/ml biotinylated histone H1 peptide (OH-AKKPKTPKKAKKL-Bio, Bachem), 10 mM $MgCl_2$, 5 μM ATP, and 0.07 μCi [^{33}P]ATP (Amersham Biosciences). The incubations were carried out in clear-bottom microtiter plates for 50 min in room temperature. The reactions were stopped by addition of 30 μl of stop solution with the final concentration of 15 mM EDTA, 2.2 mM ATP, and 5 mg/ml of streptavidine coated SPA beads (Amersham Biosciences). The samples were centrifuged for 2 min at 800*g* and then counted in a 1450 MicroBeta TriLux liquid scintillation counter (Wallac).

Statistics. All the results were from four to six separate experiments and the data presented as means ± SEM. Statistical significance for multiple variables was determined by using one way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc analysis. When only two variables, were compared Student's unpaired *t*-test was used. This applies also to transfection experiments since treatments were mostly performed at different time points and in these cases there was a corresponding control group to each treatment. The significance was set at $p < 0.05$.

Results

Calcium ionophore A23187 induces calpain mediated p35 cleavage to p25 and dephosphorylation and loss of tau

RA-BDNF differentiated SH-SY5Y cells were treated with 1, 2.5 or 5 μM A23187 for 1 h to increase intracellular levels of calcium. The p25 fragment could be detected only at 5 μM A23187 and therefore, instead of quantifying p25 for determining potential calpain activity, we measured p35 levels for all concentrations. At a concentration of 5 μM, A23187 decreased the p35 protein levels by 40% ($p < 0.05$) (Fig. 1A and B, $n = 4$). p35 was restored to control levels when 5 μM A23187 treatment was combined with 10 μM calpeptin (Fig. 1C). Cdk5 protein levels were not significantly affected by the exposure to A23187 (data not shown). Five micromolar A23187 was seemingly toxic to the cells causing loss of neurites (data not shown) and dephosphorylation and loss of tau (Fig. 1D).

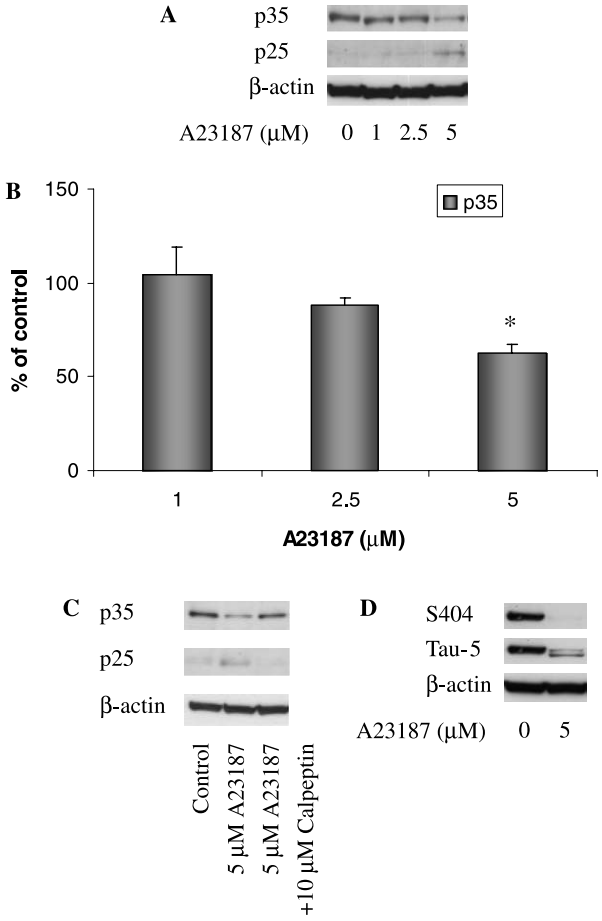

Fig. 1. Calcium ionophore A23187 induces calpain mediated p35 cleavage to p25 and dephosphorylation and loss of tau. RA-BDNF differentiated SH-SY5Y cells were treated with 1, 2.5 or 5 μM A23187 for 1 h and subjected to Western blot analysis. (A) A Western blot showing p35 and p25 levels in control and in cells treated with 1, 2.5 or 5 μM A23187 for 1 h. (B) Densitometric quantification of data as means ± SEM ($n = 4$). A23187 decreased p35 protein levels with 40% at 5 μM A23187 ($p < 0.05$). (C) A Western blot showing restored p35 levels when 5 μM A23187 was combined with 10 μM calpeptin treatment. (D) A Western blot showing dephosphorylation and loss of tau after treatment with 5 μM A23187. *$p < 0.05$, one way-ANOVA with Fisher's PLSD post hoc analysis.

H_2O_2 does not affect p35 and Cdk5 protein levels

The RA-BDNF differentiated SH-SY5Y cells were treated with 10 μM, 100 μM or 1 mM H_2O_2 for 2 h to induce oxidative stress. At the highest H_2O_2 concentration (1 mM), there was a tendency for reduced p35 levels. However, the reduction was non-significant and no p25 fragment could be detected (data not shown). H_2O_2 treatment did not affect the Cdk5 protein levels.

Glutamate increases p35 and Cdk5 protein levels, Cdk5 kinase activity and tau phosphorylation

The RA-BDNF differentiated SH-SY5Y cells were treated with 10 μM, 100 μM or 1 mM glutamate for 2 h to induce excitotoxicity. Protein levels of p35 were elevated by 30%, 50% ($p < 0.05$), and 40% at 10 μM, 100 μM, and 1 mM glutamate, respectively (Fig. 2A and B, $n = 4$). These concentrations elevated Cdk5 protein levels with 20%, 40% ($p < 0.01$), and 30% ($p < 0.01$), respectively (Fig. 2A and B, $n = 4$). 10 μM, 100 μM, and 1 mM glutamate elevated Cdk5 kinase activity by 40%, 60%, and 50%, respectively (Fig. 2C, $n = 4$). Ten micromolar Roscovitine reduced Cdk5 kinase activity to under the control levels (1 mM glutamate for 2 h with or without Roscovitine). Tau phosphorylation at the ser-404 epitope increased by 30%, 60% ($p < 0.05$), and 30% at 10 μM, 100 μM, and 1 mM glutamate, respectively, whereas phosphorylation at ser-202 epitope was not affected (Fig. 2D and E, $n = 4$). Glutamate treatment did not affect total tau levels as assessed with the Tau-5 antibody (data not shown).

The experiment was also run at a higher extracellular Ca^{2+}, 2.5 mM instead of 1 mM in normal culture medium and the cells were in this case treated with 10 μM, 100 μM, 1 mM or 3 mM glutamate for 2 h. In contrast to previous reports [13,16], we found no sign of p35 cleavage to p25

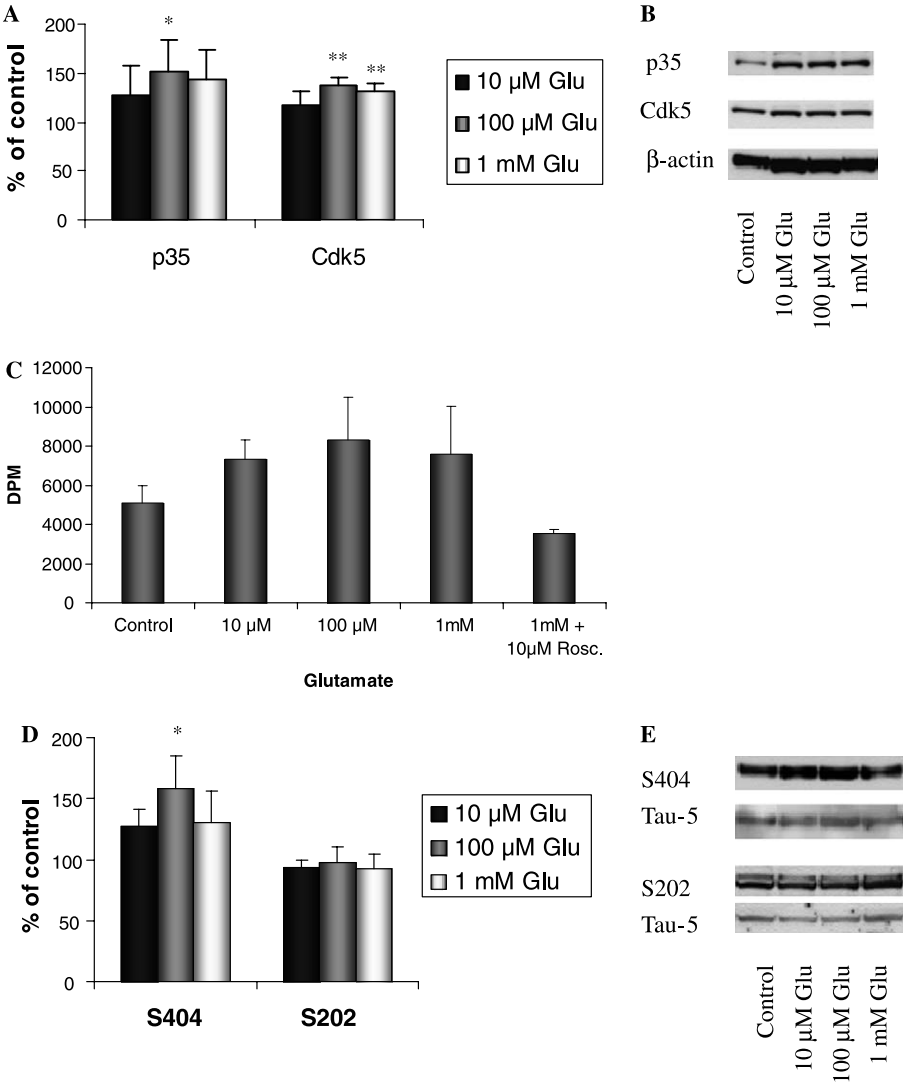

Fig. 2. Glutamate increases p35 and Cdk5 protein levels, Cdk5 kinase activity, and tau phosphorylation. RA-BDNF differentiated SH-SY5Y cells were treated with 10 μM, 100 μM or 1 mM glutamate for 2 h and subjected to Western blot analysis. p35/Cdk5 complex was immunoprecipitated using anti-Cdk5 antibody and Cdk5 kinase activity assessed with scintillation proximity assay (SPA). (A) Densitometric quantification of data as means ± SEM ($n = 4$). 10 μM, 100 μM, and 1 mM glutamate increase p35 protein levels with 30%, 50% ($p < 0.05$), and 40% and Cdk5 protein levels with 20%, 40% ($p < 0.01$), and 30% ($p < 0.01$), respectively. (B) A Western blot showing p35 and Cdk5 levels in control and in cells treated with 10 μM, 100 μM, and 1 mM glutamate for 2 h. (C) Cdk5 kinase activity presented as means ± SEM ($n = 4$). 10 μM, 100 μM, and 1 mM glutamate elevated Cdk5 kinase activity with 40%, 60%, and 50%, respectively. Ten micromolar Roscovitine reduced Cdk5 kinase activity under the control levels (1 mM glutamate for 2 h with or without Roscovitine). (D) Densitometric quantification of data as means ± SEM ($n = 4$). Tau phosphorylation at ser-404 epitope increased with 30%, 60% ($p < 0.05$), and 30% at 10 μM, 100 μM, and 1 mM glutamate, respectively, whereas phosphorylation at ser-202 epitope was not affected. (E) A Western blot showing ser-404 and ser-202 levels in control and in cells treated with 10 μM, 100 μM, and 1 mM glutamate for 2 h. *$p < 0.05$, **$p < 0.01$, one way-ANOVA with Fisher's PLSD post hoc analysis.

even at the higher extracellular Ca^{2+} levels and at the higher glutamate concentration (data not shown).

p25 transfection increases tau phosphorylation

RA-differentiated SH-SY5Y cells were transfected with 4 or 8 μg p25 plasmid. The cells were lysed 24 h after transfection and the levels of p25, p35, cdk5, and tau phosphorylated at ser-404 and ser-202 epitopes were assessed. Fig. 3A shows a dose-dependent increase in p25 levels and unchanged p35 and Cdk5 levels in the cells transfected with 4 and 8 μg p25 plasmid. Phosphorylation at the ser-404 epitope was increased by 40% after transfection with 4 μg p25 plasmid ($n = 6$) and 50% when cells were transfected with 8 μg p25 plasmid ($p < 0.05$, $n = 4$) (Fig. 3B and C). However, the Cdk5 inhibitor Roscovitine did not significantly reduce tau phosphorylation, the Roscovitine inhibition being only about 10% (data not shown). Phosphorylation at the ser-202 epitope was not affected by p25 transfection. Neither Lipofectamine nor transfection with p25 plasmid affected total tau levels as assessed with the Tau-5 antibody (data not shown).

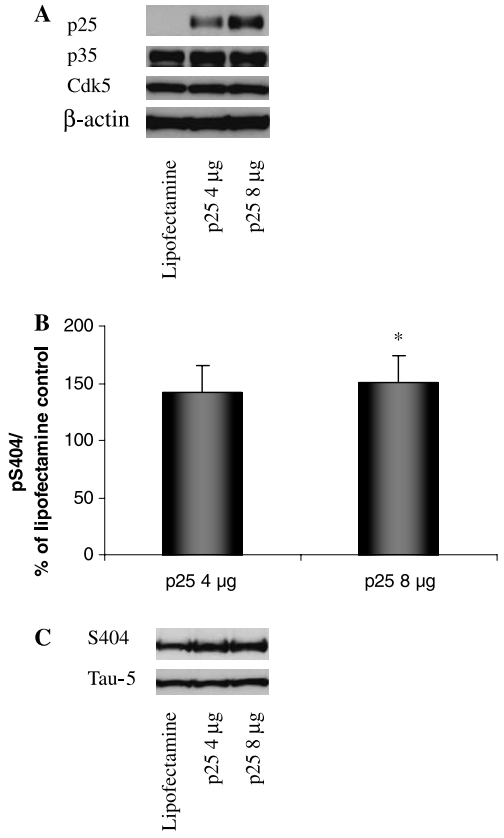

Fig. 3. p25 transfection increases tau phosphorylation. RA-differentiated SH-SY5Y cells were transfected with 4 or 8 μg p25 plasmid, the cells lysed 24 h after transfection and subjected for Western blot analysis. (A) A Western blot showing p25, p35, and Cdk5 levels after transfection with 4 or 8 μg p25 plasmid. (B) Densitometric quantification of data as means ± SEM. Four micrograms of p25 increased tau phosphorylation with 40% ($n = 6$) and 8 μg p25 with 50% ($n = 4$, $p < 0.05$) at ser-404 epitope. (C) A Western blot showing tau phosphorylation at ser-404 epitope in Lipofectamine control and after transfection with 4 and 8 μg p25 plasmid. *$p < 0.05$, Student's unpaired t-test.

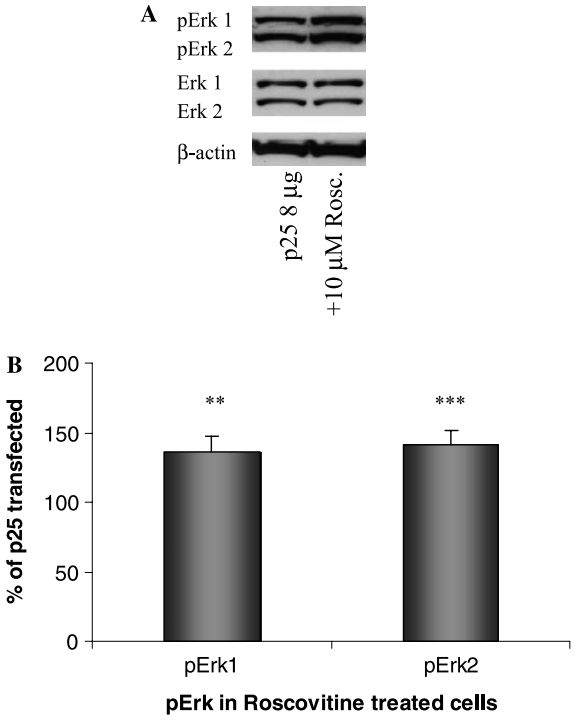

Fig. 4. Cdk5 inhibitor Roscovitine upregulates active Erk1/2. RA-differentiated SH-SY5Y cells were transfected with 8 μg p25 plasmid, the cells were treated with 10 μM Roscovitine for 4 h before they were lysed 24 h posttransfection and subjected to Western blot analysis. (A) A Western blot showing pErk1/2 and total Erk1/2 levels in cells transfected with 8 μg p25 plasmid with no Roscovitine and with 10 μM Roscovitine treatment. (B) Densitometric quantification of data as means ± SEM ($n = 4$). Treatment with 10 μM Roscovitine increased levels of pErk1 ($p < 0.01$) and pErk2 ($p < 0.001$) with 40% in the cells transfected with 8 μg p25. **$p < 0.01$, ***$p < 0.001$, Student's unpaired t-test.

Cdk5 inhibitor Roscovitine upregulates active Erk1/2

Next, we investigated whether the inability of Roscovitine to inhibit tau phosphorylation induced by p25 transfection was due to upregulation of other kinases. Inhibition of Cdk5 activity is reported to lead to activation of Gsk3β [18] and Erk [19]. In the present setting, no change in the levels of total Gsk3β, activation-associated phosphorylation at Gsk3β Y^{216} or inactivation-associated phosphorylation at Gsk3β S^{9} was detected (data not shown). However, levels of the phosphorylated forms of Erk kinases, pErk1/2, were elevated. The 10 μM Roscovitine treatment increased levels of pErk1 ($p < 0.01$) and pErk2 ($p < 0.001$) by 40% in the cells transfected with 8 μg p25 (Fig. 4A and B, $n = 4$). Levels of total Erk1 and Erk2 remained unchanged (Fig. 4A).

p25 is degraded via a proteasome in Roscovitine treated cells

In the p25 transfected SH-SY5Y cells, treatment with Roscovitine resulted in decreased levels of p25 as compared to p25 transfected non-treated cells. This reduction was dose-dependent as shown for 5 and 10 μM Roscovitine (Fig. 5A) and could be inhibited when 20 μM of the proteasome inhibitor Lactacystin was added (Fig. 5B). Reduction of the p25 levels was also achieved when cells were treated with another Cdk5 inhibitor, Olomoucine, at 30 μM concentration (Fig. 5C). The degradation of p25 by Roscovitine was also seen in undifferentiated SH-SY5Y cells (Fig. 5D).

Discussion

Activation of Cdk5 and its influence on tau phosphorylation are processes implicated in the pathology of Alzheimer's disease. To further study these mechanisms in vitro, we used the human neuroblastoma cell line SH-SY5Y. We have previously demonstrated that sequential differentiation of SH-SY5Y cells with RA and BDNF increased both the content and phosphorylation state of tau [17]. Moreover, p35 levels were elevated but this did not result in increased activation of the enzyme. Therefore, in the present study, different neurotoxic stimuli were used to activate Cdk5 in RA-BDNF differentiated SH-SY5Y cells. For the

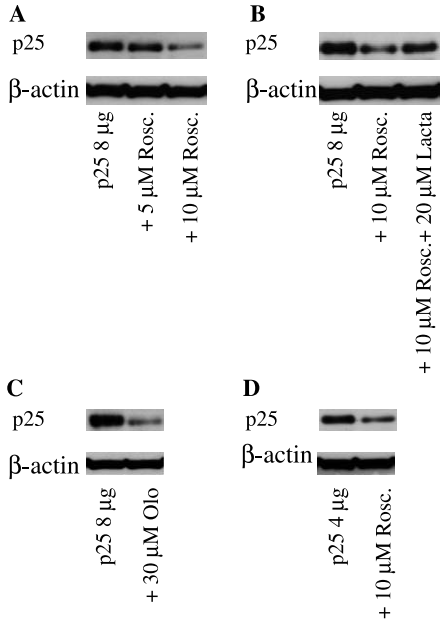

Fig. 5. p25 is degraded via a proteasome in Roscovitine treated cells. RA-differentiated and undifferentiated SH-SY5Y cells were transfected with 8 or 4 μg p25 plasmid, respectively. The cells were treated with 5 or 10 μM Roscovitine, 10 μM Roscovitine together with 20 μM Lactacystin or 30 μM Olomoucine for 4 h before they were lysed 24 h posttransfection and subjected to Western blot analysis. (A) A Western blot showing dose-dependent reduction of p25 levels after Roscovitine treatment in RA-differentiated p25 transfected SH-SY5Y cells. (B) A Western blot showing that degradation of p25 by Roscovitine could be inhibited with proteasome inhibitor Lactacystin in RA-differentiated p25 transfected SH-SY5Y cells. (C) A Western blot showing reduced p25 levels after treatment with another Cdk5 inhibitor Olomoucine in RA-differentiated p25 transfected SH-SY5Y cells. (D) A Western blot showing reduced p25 levels after Roscovitine treatment in undifferentiated p25 transfected SH-SY5Y cells.

p25 transfection experiments, p35 levels were not critical since p25 was transfected to the cells and in these experiments the SH-SY5Y cells were differentiated only with RA. The RA-treatment is also sufficient to increase endogenous tau levels to enable quantification of changes in tau phosphorylation.

In the present study using RA-BDNF differentiated SH-SY5Y cells, treatment with calcium ionophore A23187 to increase intracellular levels of calcium resulted in cleavage of p35 to p25, although this was only seen at concentrations that were toxic to the cells. Morphologically, A23187 caused significant damage to the neurite network of cells and this resulted in dephosphorylation and loss of tau, a finding that is in line with a previous report [16]. The ionophore-induced cleavage of p35 to p25 could be prevented by the calpain inhibitor calpeptin, indicating that the cleavage was indeed calpain mediated [13,14]. Treatment of the cells with H_2O_2 to cause oxidative stress slightly reduced the p35 levels but no p25 fragment could be detected.

We found that stimulation of RA-BDNF differentiated SH-SY5Y cells with the excitotoxic agent glutamate increased tau phosphorylation. We suggest that this increase in phosphorylation was caused by activation of Cdk5, which in turn was achieved by the glutamate treatment inducing an upregulation of the Cdk5 and p35 protein levels. Previously, increased expression of both Cdk5 and p35 with subsequent Cdk5 activation has been shown to occur after treatment with oxidative stress causing agents 4-hydroxynonenal (HNE) and ascorbate/$FeSO_4$ in human neuroblastoma IMR-32 cells [20]. Indeed, some evidence shows that glutamate can cause oxidative toxicity via a *N*-methyl-D-aspartic acid (NMDA) receptor-independent pathway [21]. In contrast to these findings, in primary cortical [13] and hippocampal [16] cultures, glutamate stimulation has been demonstrated to lead to a calpain mediated cleavage of p35 to p25 as a result of calcium influx via NMDA receptors.

Although there is some evidence of the existence of NMDA receptors in SH-SY5Y cells [22,23], the receptor density might be low, which could explain the lack of calcium mediated calpain cleavage of p35 to p25 as a result of glutamate treatment. NMDA receptor expression might also change depending on the time in the culture and the differentiation protocol used since at least in primary neuronal cells the response to glutamate is shown to be dependent of the maturation state of the neurons. Kerokoski et al. [24] show that in 13-day-old hippocampal neuronal cultures glutamate induces p35 cleavage, but this does not occur in the more immature 6-day-old cultures. These differences are proposed to be due to alterations in the NMDA receptor expression.

In SH-SY5Y cells, p25 could only be detected after a treatment that was apparently toxic to the cells. Therefore, we chose to transfect the cells with p25. After transfection, two epitopes on the tau protein, ser-404 and ser-202, were examined but increased tau phosphorylation was detected only at ser-404. Ser-202, ser-235, and ser-404 are the major reported phosphorylation sites for Cdk5 in vitro and in neurons [25] but in previous studies with SH-SY5Y cells we have been able to demonstrate Cdk5 mediated phosphorylation only at the ser-404 epitope [17]. Furthermore, glutamate-induced tau phosphorylation was detected at ser-404 but not at ser-202 epitope. Hamdane et al. [26] demonstrated a different phosphorylation pattern in SH-SY5Y cells transfected with a p25 inducible expression vector. The AD2 epitope (ser-396/ser-404) was found to be unchanged whereas increased phosphorylation was detected at the AT8 (ser-202/thr-205) and AT180 epitopes. A possible explanation for these differences might be the fact that Hamdane et al. used SH-SY5Y cells constitutively expressing an adult tau isoform (2+3−10−), in contrast to our cell model that only expresses fetal tau [17].

In the present study, the increase in tau phosphorylation induced by p25 transfection could not be inhibited with the Cdk5 inhibitor Roscovitine. This finding was somewhat unexpected and prompted us to investigate the potential involvement of additional kinase signalling pathways. Inhibition of Cdk5 activity has been reported

to lead to activation of Gsk3β [18] but in the present study, we could not detect any change in either activation-associated phosphorylation at Gsk3β Y^{216}, inactivation-associated phosphorylation at Gsk3β S^{9} or the levels of total Gsk3β in the SH-SY5Y cells. However, levels of the phosphorylated form of Erk kinases, pErk1/2, were elevated in the Roscovitine treated cells. Furthermore, detectable levels of pErk1/2 were identified in both controls as well as in transfected cells, which can be explained by the RA differentiation being able to activate Erk1/2 in SH-SY5Y cells [27]. There are previous reports showing cross talk between Cdk5 and the MAP kinase pathways, probably by feedback downregulation of the MAP kinase pathway by Cdk5 inactivation of MEK1. Vice versa, when Cdk5 is inhibited, Erk1/2 seems to increase its activity. Furthermore, Sharma et al. [19] demonstrated higher Erk1/2 phosphorylation in p35−/− mice brain when compared to p35+/+ mice and in vitro, treatment with Roscovitine has been shown to increase Erk1/2 phosphorylation in PC12 cells [19] and in carcinoma cells [28]. It has been demonstrated that Roscovitine is capable of inhibiting the Erk1/2 kinases but with a significantly poorer potency than Cdk5. The reported IC_{50} value for Erk1 is approximately 170 times higher and for Erk2 70 times higher than that for Cdk5 [29] and thus in the present setting, Erk1/2 inhibition should be marginal. Cdk5 and Erk1/2 share some common substrates such as the NF-M subunit of neurofilament as shown by Sharma et al. [19] but impact of their cross talk on tau phosphorylation has not previously been demonstrated. Erk1/2 has, however, been shown to be capable of phosphorylating tau [30] and ser-404 is reported as an Erk phosphorylation site in SH-SY5Y cells [30]. This could explain the fact that after Roscovitine treatment, tau phosphorylation at ser-404 does not decrease even though Cdk5 should be inhibited both due to a Roscovitine action and degradation of p25. The increased tau phosphorylation after p25 transfection is most likely caused by Cdk5 activation since up to date, the p25 fragment has not been connected with activation of other systems than Cdk5.

p25 was degraded after Roscovitine treatment both in differentiated and undifferentiated p25 transfected SH-SY5Y cells. This effect was almost completely blocked by the proteasome inhibitor Lactacystin, indicating that p25 was degraded via a proteasome. Olomoucine, an additional Cdk5 inhibitor, was also able to degrade p25 in the present system. This phenomenon was not seen in Roscovitine treated p25/Cdk5 transfected HEK293 cells (unpublished observation) that do not have p35 and thus lack endogenous Cdk5 activity, indicating that the degradation is not compound related but rather cell specific. p35 has previously been shown to be degraded by a proteasome [31] but this has not been reported for p25. In the present study, p25 was transfected to the cells and it remains to be investigated whether the calpain-produced p25 fragment is also degraded via the proteasome.

Our results with the SH-SY5Y cells show that glutamate increases tau phosphorylation, which we suggest to be caused by an increase in Cdk5 activity, which in turn is achieved by upregulation of Cdk5 and p35 protein levels. Treatment with calcium ionophore A23187 generates the calpain cleaved p25 fragment but only in conditions that are toxic and result in dephosphorylation and loss of tau. When p25 is transfected to the cells, increased tau phosphorylation can be achieved but application of Cdk5 inhibitor Roscovitine does not result in inhibition of tau phosphorylation possibly due to activation of an additional kinase, Erk1/2, that also is capable of phosphorylating tau. Furthermore, this study demonstrates that p25 is degraded via the proteasome in Roscovitine treated cells.

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References

[1] I. Grundke-Iqbal, K. Iqbal, Y.C. Tung, M. Quinlan, H.M. Wisniewski, L.I. Binder, Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology, Proc. Natl. Acad. Sci. USA 83 (1986) 4913–4917.

[2] K.S. Kosik, C.L. Joachim, D.J. Selkoe, Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease, Proc. Natl. Acad. Sci. USA 83 (1986) 4044–4048.

[3] L.-H. Tsai, I. Delalle, V.S. Caviness Jr, T. Chae, E. Harlow, p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5, Nature 371 (1994) 419–423.

[4] T. Ohshima, J.M. Ward, C.G. Huh, G. Longenecker, Veeranna, H.C. Pant, R.O. Brady, L.J. Martin, A.B. Kulkarni, Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death, Proc. Natl. Acad. Sci. USA 93 (1996) 11173–11178.

[5] M. Nikolic, H. Dudek, Y.T. Kwon, Y.F. Ramos, L.H. Tsai, The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation, Genes Dev. 10 (1996) 816–825.

[6] M. Nikolic, M.M. Chou, W. Lu, B.J. Mayer, L.H. Tsai, The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity, Nature 395 (1998) 194–198.

[7] Y.T. Kwon, A. Gupta, Y. Zhou, M. Nikolic, L.H. Tsai, Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase, Curr. Biol. 10 (2000) 363–372.

[8] B.S. Li, M.K. Sun, L. Zhang, S. Takahashi, W. Ma, L. Vinade, A.B. Kulkarni, R.O. Brady, H.C. Pant, Regulation of NMDA receptors by cyclin-dependent kinase-5, Proc. Natl. Acad. Sci. USA 98 (2001) 12742–12747.

[9] G.N. Patrick, L. Zukerberg, M. Nikolic, S. de la Monte, P. Dikkes, L.H. Tsai, Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration, Nature 402 (1999) 615–622.

[10] H.C. Tseng, Y. Zhou, Y. Shen, L.H. Tsai, A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains, FEBS Lett. 523 (2002) 58–62.

[11] K.Y. Lee, A.W. Clark, J.L. Rosales, K. Chapman, T. Fung, R.N. Johnston, Elevated neuronal Cdc2-like kinase activity in the Alzheimer disease brain, Neurosci. Res. 34 (1999) 21–29.

[12] J.C. Cruz, H-C. Tseng, J.A. Goldman, H. Shih, L.-H. Tsai, Aberrant Cdk5 activation by p25 triggers pathological events leading to

neurodegeneration and neurofibrillary tangles, Neuron 40 (2003) 471–483.
[13] M.S. Lee, Y.T. Kwon, M. Li, J. Peng, R.M. Friedlander, L.H. Tsai, Neurotoxicity induces cleavage of p35 to p25 by calpain, Nature 405 (2000) 360–364.
[14] G. Kusakawa, T. Saito, R. Onuki, K. Ishiguro, T. Kishimoto, S. Hisanaga, Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25, J. Biol. Chem. 275 (2000) 17166–17172.
[15] G. Li, A. Faibushevich, B.J. Turunen, S.O. Yoon, G. Georg, M.L. Michaelis, R.T. Dobrowsky, Stabilization of the cyclin-dependent kinase 5 activator, p35, by paclitaxel decreases β-amyloid toxicity in cortical neurons, J. Neurochem. 84 (2003) 347–362.
[16] P. Kerokoski, T. Suuronen, A. Salminen, H. Soininen, T. Pirttilä, Cleavage of the cyclin-dependent kinase 5 activator p35 to p25 does not induce tau hyperphosphorylation, Biochem. Biophys. Res. Commun. 298 (2002) 693–698.
[17] A. Jämsä, K. Hasslund, R.F. Cowburn, A. Bäckström, M. Vasänge, The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau phosphorylation, Biochem. Biophys. Res. Commun. 319 (2004) 993–1000.
[18] G. Morfini, G. Szebenyi, H. Brown, H.C. Pant, G. Pigino, S. DeBoer, U. Beffert, S.T. Brady, A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons, EMBO J. 23 (2004) 2235–2245.
[19] P. Sharma, Veeranna, M. Sharma, N.D. Amin, R.K. Sihag, P. Grant, N. Ahn, A.B. Kulkarni, H.P. Pant, Phosphorylation of MEK1 by Cdk5/p35 down-regulates the mitogen-activated protein kinase pathway, J. Biol. Chem. 277 (2002) 528–534.
[20] P. Strocchi, A. Pession, B. Dozza, Up-regulation of cDK5/p35 by oxidative stress in human neuroblastoma IMR-32 cells, J. Cell. Biochem. 88 (2003) 758–765.
[21] D. Schubert, D. Piasecki, Oxidative glutamate toxicity can be a component of the excitotoxicity cascade, J. Neurosci. 21 (2001) 7455–7462.
[22] J. Naarala, P. Nykvist, M. Tuomala, K. Savolainen, Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells, FEBS Lett. 330 (1993) 222–226.
[23] V.D. Nair, H.B. Niznik, R.K. Mishra, Interaction of NMDA and dopamine D2L receptors in human neuroblastoma SH-SY5Y cells, J. Neurochem. 66 (1996) 2390–2393.
[24] P. Kerokoski, T. Suuronen, A. Salminen, H. Soininen, T. Pirttilä, Both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors mediate glutamate-induced cleavage of the cyclin-dependent kinase 5 (cdk5) activator p35 in cultured rat hippocampal neurons, Neurosci. Lett. 368 (2004) 181–185.
[25] F. Sakaue, T. Saito, Y. Sato, A. Asada, K. Ishiguro, M. Hasegawa, S. Hisanaga, Phosphorylation of FTDP-17 mutant tau by cyclin-dependent kinase 5 complexed with p35, p25, or p39, J. Biol. Chem. 280 (2005) 31522–31529.
[26] M. Hamdane, A.-V. Sambo, P. Delobel, S. Bégard, A. Violleau, A. Delacourte, P. Bertrand, J. Benavides, L. Buée, Mitotic-like tau phosphorylation by p25-Cdk5 kinase complex, J. Biol. Chem. 278 (2003) 34026–34034.
[27] J. Pan, Y.-L. Kao, S. Joshi, S. Jeetendran, D. DiPette, U.S. Singh, Activation of Rac1 by phosphatidylinositol 3-kinase in vivo: role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic-acid induced neuronal differentiation of SH-SY5Y cells, J. Neurochem. 93 (2005) 571–583.
[28] S.R. Whittaker, M.I. Walton, M.D. Garrett, P. Workman, The cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase pathway, Cancer Res. 64 (2004) 262–272.
[29] L. Meijer, A. Borgne, O. Mulner, J.P. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.G. Delcros, J.P. Moulinoux, Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5, Eur. J. Biochem. 243 (1997) 527–536.
[30] F.J. Ekinci, T.B. Shea, Hyperactivation of mitogen-activated protein kinase increases phospho-tau immunoreactivity within human neuroblastoma: additive and synergistic influence of alteration of additional kinase activities, Cell. Mol. Neurobiol. 19 (1999) 249–260.
[31] G.N. Patrick, P. Zhou, Y.T. Kwon, P.M. Howley, L.-H. Tsai, p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway, J. Biol. Chem. 273 (1998) 24057–24064.