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Cdk5 is a major regulator of p38 cascade: relevance to neurotoxicity in Alzheimer's disease

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Abstract

Cyclin-dependent kinase (Cdk) 5 and p38 activities are significantly increased in Alzheimer's Disease (AD). Both p38 and Cdk5 promote neurodegeneration upon deregulation. However, to date the mechanistic link between Cdk5 and p38 remains unclear. This study presents the first mechanism showing Cdk5 as a major regulator of p38 cascade in neurons and in transgenic mouse model of AD. Using β-amyloid and glutamate as the neurotoxic stimuli, our results show that deregulated Cdk5 induces p38 activation by increasing reactive oxygen species (ROS) in neuronal cells and in primary cortical neurons. Elimination of ROS inhibits p38 activation, revealing ROS as major stimuli of the p38 cascade. Importantly, Cdk5-mediated p38 activation increases c-Jun expression, thereby revealing a mechanistic link between deregulated Cdk5 and c-Jun level in AD brains. c-Jun is overexpressed in AD, and is believed to contribute significantly to neurodegeneration. Based on the proposed mechanism, Cdk5 inhibition is more neuroprotective relative to p38 and c-Jun, suggesting that Cdk5 is an upstream regulator of neurodegenerative pathways triggered by p38 and a preferable therapeutic target for AD.

Keywords: Alzheimer's disease, Cdk5, c-Jun, oxidative stress, p38.

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Alzheimer's Disease (AD) is a fatal neurodegenerative disorder that has no known cure, nor is there a clear mechanistic understanding of the disease pathology. AD brains consistently show a number of biochemical and molecular abnormalities including selective cell loss, impaired mitochondrial function, defective energy metabolism and various markers of oxidative stress and inflammation. However, an incomplete understanding of the underlying mechanisms has hindered the development of target-based therapies.

Several lines of evidence suggest that p38 MAPK signaling cascade plays an important role in AD pathology. Significant increase in level and activation of p38 and its upstream activator mitogen-activated protein kinase kinase 6 (MKK6) have been demonstrated in early stages of AD (Pei *et al.* 2001; Zhu *et al.* 2001). Amyloid precursor protein (APP)-transgenic mice exhibit aberrant β-amyloid (Aβ) accumulation and higher p38 activity (Koistinaho *et al.* 2002; Savage *et al.* 2002; Hwang *et al.* 2004). Aβ promotes activation of p38 (Zhu *et al.* 2005), p53, nuclear factor kappa B and cyclooxygenase-2, which induce apoptosis in neurons (Daniels *et al.* 2001; Tamagno *et al.* 2003a,b; Jang and Surh 2005; Kriem *et al.* 2005). Secreted forms of APP induce the release of interleukin-1β, which in turn initiates p38 cascade in activated microglia cells during neuroinflammation (Kim *et al.* 2004; Bodles and Barger 2005; Griffin *et al.* 2006). Immunohistochemical studies have revealed co-localization of phospho-p38 in neurons bearing neurofibrillary pathology in AD hippocampus. Several *in vitro* studies suggest that p38 dysregulation causes tau hyperphosphorylation leading to neurofibrillary tangles (NFT) formation in AD (Goedert *et al.* 1997; Reynolds *et al.* 1997, 2000; Sheng *et al.* 2001). p38 inhibition suppresses proinflammatory cytokine

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Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's Disease; APP, amyloid precursor protein; ATM, ataxia-telangiectasia mutated; Cdk, cyclin-dependent kinase; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; MKK, mitogen-activated protein kinase kinase 6; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TAT, 11-mer TAT sequence (residues 47–57 of HIV-Tat: YGRKKRRQRRR).

up-regulation and attenuates synaptic dysfunction and behavioral deficits in an AD mouse model (Munoz *et al.* 2007).

Similar to p38 kinase, cyclin-dependent kinase (Cdk) 5 is also highly deregulated in AD. Unlike other members of Cdk family, Cdk5 is not activated by cyclins but by specific regulatory binding proteins, p35 or p39. These proteins are cleaved into p25 and p29 (Patrick *et al.* 1999) under pathological conditions such as oxidative stress, calcium dysregulation, Aβ exposure, excitotoxicity, inflammation and mitochondrial dysfunction in AD (Lafon-Cazal *et al.* 1993; Schinder *et al.* 1996; Castilho *et al.* 1999; Lee *et al.* 2000; Cruz and Tsai 2004; Quintanilla *et al.* 2004; Kitazawa *et al.* 2005). These truncated proteins constitutively activate Cdk5 and change its subcellular localization from particulate to cytosolic and nuclear. Cdk5 can then access a variety of pathological substrates, triggering a cascade of neurotoxic pathways, culminating in neuronal death (Kanungo *et al.* 2009).

Cdk5 increases p53 stability by phosphorylation, enhancing acetylation by p300 co-activator and by disrupting its interaction with murine double minute 2. Cdk5-stabilized p53 protein is transcriptionally active, resulting in the induction of pro-apoptotic genes and subsequent mitochondria-mediated apoptosis in response to genotoxic or oxidative stress (Lee *et al*. 2007; Lee and Kim 2007). Cdk5 and c-Abl also cooperatively regulate maximal activation of p53, resulting in neuronal death in response to oxidative stress (Lee *et al.* 2008). p25 binds to histone deacetylase catalytic domain of HDAC1 and inhibits its function, which causes aberrant expression of cell cycle proteins and DNA damage, ultimately provoking cell death (Kim *et al.* 2008). Cdk5 activates ataxia-telangiectasia mutated (ATM) by phosphorylation and regulates the function of the ATM targets p53 and H2AX. Interruption of the Cdk5-ATM pathway attenuates DNA-damage-induced neuronal cell cycle re-entry and expression of the p53 targets- p53 up-regulated modulator of apoptosis and Bax, protecting neurons from death (Tian *et al.* 2009). Cdk5 activation by Aβ and prion peptide triggers retinoblastoma phosphorylation leading to cell cycle re-entry and death (Lopes *et al.* 2009).

A mechanistic link between Cdk5 and p38 cascade has not been shown. Otth *et al.* (2003) showed that phospho-p38 co-localized with Cdk5 in Tg2576 mice, an animal model for AD; however, the consequences of the association were not analyzed. As deregulated Cdk5 promotes oxidative stress (Sun *et al.* 2008b), which is a prominent stimulus for p38 activation; we investigated mechanistic links between Cdk5 and p38 cascade in the present study.

Materials and methods

Materials

Glutamate, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and poly-L-lysine were obtained from Sigma. β-amyloid$^{25\text{-}35}$ (Aβ$^{25\text{-}35}$) and 2′,7′-dichlorofluorescein diacetate were purchased from Anaspec (Fremont, CA, USA). Roscovitine was purchased from LC laboratory (Woburn, MA, USA). SB203580 was from Calbiochem and MDL-28170 was obtained from Tocris (Ballwin, MO, USA). Antibodies against p-p38 (pT180/pY182), actin (C-2), Cdk5 (C-8), and c-Jun (G-4) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-MKK3/6 (Ser189/207) (No. 9231) antibody was from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

HT22 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Isolation of primary cortical cells

Time pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA, USA). Primary cortical neurons were isolated from E17 rat embryos as described previously (Behrens *et al.* 1999; Sun *et al.* 2008a,b). Under these conditions less than 5% of total cells were astrocytes. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. All experiments were conducted on 5 and 6 days *in vitro*.

Cdk5 kinase assay

Cdk5 immune complexes were isolated from glutamate or Aβ$^{25\text{-}35}$-treated cells and kinase assays were conducted as described before (Sun *et al.* 2008a,b, 2009).

Western blotting

Inhibitors of Cdk5 (roscovitine), p38 (SB203580) or calpain (MDL-28170) were added 30 min before glutamate or Aβ$^{25\text{-}35}$ treatments in HT22 and primary cortical neurons. Following the indicated treatments, cells were harvested and lysed in a ristocetin-induced platelet agglutination buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40 (Sigma-Aldrich, St. Louis, MO, USA), 0.25% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and incubated with primary and secondary antibodies as reported before (Sun *et al.* 2008a,b, 2009).

Expression and purification of 11-mer TAT sequence (residues 47–57 of HIV-Tat: YGRKKRRQRRR) (TAT)-fusion proteins

TAT-fusion proteins, TAT-Prx-II-(T89A) and TAT-green fluorescent protein (GFP), were expressed and purified as described previously (Sun *et al.* 2008a,b).

Transduction experiment

Two hundred nM of TAT-Prx-II (T89A) was added 0.5 h prior to glutamate or Aβ treatment. To sustain enough amount of Tat-Prx-II (T89A) in cells at the end of the cellular experiments, 100 nM of Tat-Prx-II (T89A) solution was also added every 3–4 h. 200 nM of TAT-GFP was used as a negative control.

Reactive oxygen species measurement

For reactive oxygen species (ROS) measurement, HT22 cells were seeded in a six-well plate for 12 h. After indicated treatments, ROS

production was measured by 2′,7′-dichlorofluorescein diacetate staining as described previously (Sun *et al.* 2008b).

MKK3/6 phosphorylation

Cells were treated with 10 μM roscovitine for 30 min, prior to glutamate (6 h) or $A\beta^{25-35}$ treatments (8 h). Cell lysates were prepared, separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and incubated with p-MKK3/6 (Ser189/207) antibody, followed by horseradish peroxidase-conjugated secondary antibody.

Immunocytochemistry in p25 transgenic mice

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-tTA and tet-o-p25 mice (Cruz *et al.* 2003) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and mated to generate p25 inducible transgenic mice. A doxycycline-containing diet (200 mg/kg; Bio-Serve, Frenchtown, NJ, USA) was provided before weaning (4–6 weeks after birth), and, thereafter, p25 expression was induced by replacing the doxycycline diet with a regular diet for 8 weeks. After induction, each mouse was perfused by phosphate-buffered saline/10% formalin and brains were extracted and processed further for the paraffin section (6 μm). Immunocytochemistry was performed by the ABC method according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA, USA). All slides were randomized and blinded with regard to genotype prior to staining and subsequent analysis. Briefly, slides were immersed in xylene, hydrated through graded ethanol solutions, and endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide for 30 min. For antigen retrieval, each slide was heated by a pressure cooker in Rodent Decloaker Buffer (Biocare Medical, Concord, CA, USA). To reduce non-specific binding, sections were incubated for 30 min in 10% normal goat serum in Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.6). After rinsing briefly with 1% normal goat serum in Tris-buffered saline, the sections were incubated overnight at 4°C with anti-phospho-p38 (Thr180/Tyr182) rabbit polyclonal antibody (Cell Signaling, Danvers, MA, USA). Antibodies were localized using 3-3′-Diaminobenzidine as a chromogen (Dako Corp., Carpinteria, CA, USA) after incubation with a secondary antibody. To exclude the possibility of non-specific reaction, all the immunocytochemistry experiments contained at least one sample without a primary antibody. Images were acquired through an AxioCam camera on an Axiophot microscope (Zeiss, Thornwood, NJ, USA).

MTT assay

HT22 cells were seeded onto six-well plates and were exposed to following treatments. For Cdk5 inhibition, TAT-Cdk5 inhibitory peptide was added every 5 h and TAT-GFP was used as a control. For c-Jun N-terminal kinase (JNK) and p38 inhibitions, SP600125 (2 μM) and SB203580 (2 μM) were added respectively, 30 min before glutamate treatment, and dimethylsulfoxide was used as a control. After 24 h following 5 mM glutamate treatment, MTT assay was conducted as described previously (Sun *et al.* 2008a,b).

Statistical analysis

Bar graph results were plotted as the average ± SEM. Probability values were calculated from one-way ANOVA followed by *post hoc* analysis and displayed as: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ when comparing data to the control.

Results

Inhibition of Cdk5 prevents p38 activation upon glutamate stimulation

HT22 cells

HT22 cells (immortalized mouse hippocampus cells) and primary cortical neurons were chosen for the present study. Glutamate and Aβ were chosen as the neurotoxic stimuli, both of which contribute significantly to pathological cell death in AD.

p38 activation was examined in glutamate-treated HT22 cells using a phospho-p38 (T180/Y182) antibody, which revealed a time-dependent increase in phospho-p38 level (Fig. 1a). As oxidative stress has shown to be an important stimulus for p38 activation, intracellular ROS levels were measured in parallel. ROS levels increased in 4–6 h following stimulation, consistent with the activation of phospho-p38 (Fig. 1a and b).

Our previous studies revealed that deregulation of Cdk5 triggers ROS accumulation in neuronal cells (Sun *et al.* 2008b), leading us to hypothesize that Cdk5 may act as an upstream regulator of p38. Thus, we initially measured Cdk5 kinase activity following glutamate treatment, which showed ~2-fold increase in 30 min, thereby preceding p38 activation (Fig. 1c). HT22 were then pre-treated with a Cdk5-specific inhibitor, roscovitine, followed by glutamate stimulation. Glutamate-induced p38 activation was completely prevented by Cdk5 inhibition (Fig. 1d), demonstrating that Cdk5 positively regulates p38 activation.

Primary cortical neurons

Next 5 days *in vitro* primary cortical neurons were subjected to glutamate treatment, which triggered phospho-p38 formation in ~6 h (Fig. 1e). Similar to HT22 cells, Cdk5 inhibition abolished p38 activation, suggesting an analogous pathway prevails in primary neurons.

Inhibition of Cdk5 prevents p38 activation upon β-amyloid stimulation

β-Amyloid$^{25–35}$ was next used as the neurotoxic stimulus, which is the biologically active and highly toxic core fragment of full-length Aβ ($A\beta^{1-42}$) (Pike *et al.* 1995), and is produced by enzymatic cleavage of naturally occurring Aβ in brains of AD patients (Kubo *et al.* 2002). $A\beta^{25-35}$ induces neurotoxic effects similar to those produced by $A\beta^{1-42}$ and generates neuropathological signs related to those of early stages of AD (Cheng *et al.* 2006; Klementiev *et al.* 2007). Cdk5 activation occurs within 2 h following $A\beta^{25-35}$ stimulation in HT22 (Fig. 2a).

Similar to glutamate, Aβ also increased phospho-p38 levels in 6–8 h following stimulation (Fig. 2b). Cdk5 inhibition abolished p38 phosphorylation both in HT22 cells

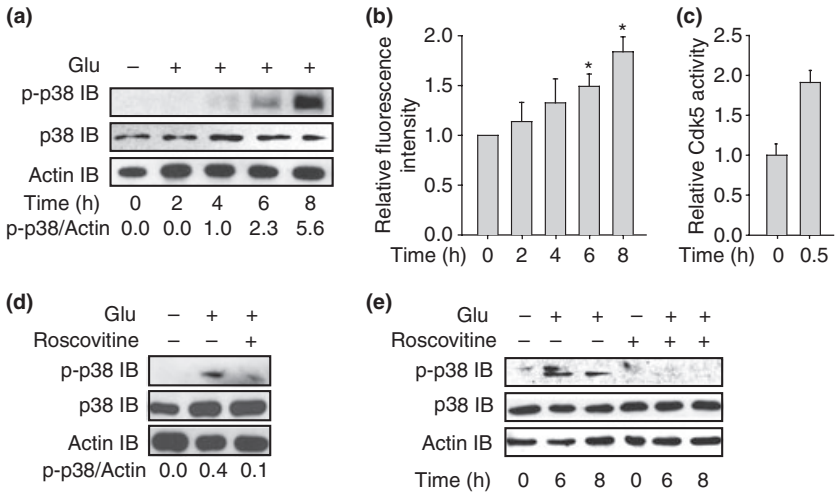

Fig. 1 Glutamate stimulation induces Cdk5 deregulation, followed by p38 activation in neuronal cells. Inhibition of Cdk5 prevents p38 activation. (a) Exposure of HT22 cells to glutamate activates p38. HT22 cells were treated with 5 mM glutamate for indicated times. Cells were then harvested, lysed, and subjected to western blot analysis using the indicated antibodies. (b) HT22 cells were treated with 5 mM glutamate for indicated times, followed by ROS measurement as described earlier (Sun *et al.* 2008a,b, 2009). (c) Exposure of HT22 cells to glutamate activates Cdk5 activity in 30 min. Cdk5 IP and kinase assay were conducted as described before (Sun *et al.* 2008a,b, 2009). (d) HT22 cells were exposed to 5 mM glutamate for 4 h in the presence or absence of roscovitine (10 μM). (e) Primary cortical neurons were exposed to glutamate for indicated times in the presence or absence of roscovitine. $^*p < 0.05$ when comparing data to the control.

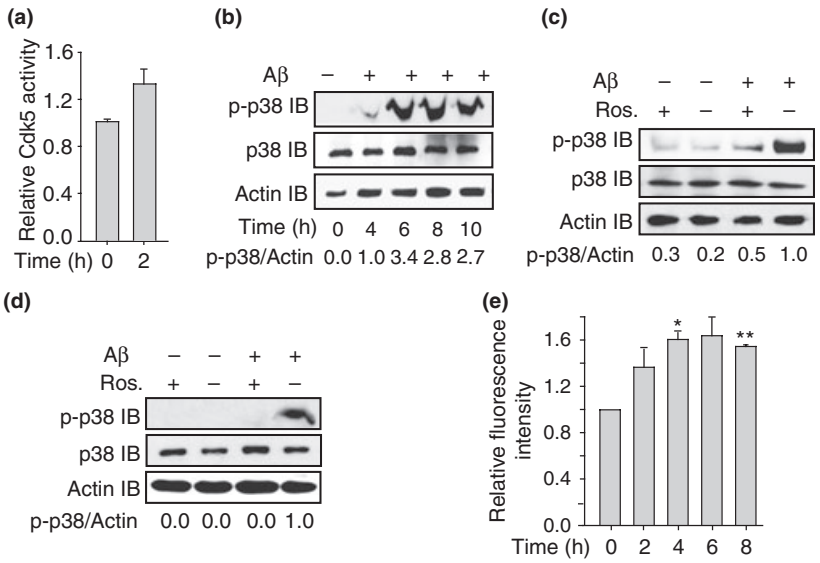

Fig. 2 Aβ stimulation induces p38 activation in a Cdk5-dependent manner in neuronal cells. (a) Exposure of HT22 cells to $A\beta^{25\text{-}35}$ (25 μM) activates Cdk5 activity in 2 h. (b) Exposure of HT22 cells to $A\beta^{25\text{-}35}$ activates p38. HT22 cells were treated with $A\beta^{25\text{-}35}$ for indicated times. Cells were then harvested, lysed, and subjected to western blot analysis using the indicated antibodies. (c) HT22 cells were exposed to $A\beta^{25\text{-}35}$ for 10 h in the presence or absence of roscovitine (10 μM). (d) Primary cortical neurons were exposed to $A\beta^{25\text{-}35}$ for indicated times in the presence or absence of roscovitine. (e) HT22 cells were treated with 25 μM $A\beta^{25\text{-}35}$ for indicated times, followed by ROS measurement as described earlier (Sun *et al.* 2008a,b, 2009). $^*p < 0.05$, $^{**}p < 0.01$ when comparing data to the control.

(Fig. 2c) and primary cortical neurons (Fig. 2d), suggesting Cdk5 deregulation triggers p38 activation. As Aβ stimulation also causes oxidative stress, ROS levels were measured in Aβ-treated HT22 cells, which increased upon Aβ stimulation (Fig. 2e).

Cdk5-mediated p38 activation is ROS-dependent

To examine whether oxidative stress acts as the stimulant for p38 activation upon Aβ and glutamate treatments, ROS was eliminated in neurotoxins-treated HT22 cells and primary cortical neurons using 'TAT-fused peroxiredoxin-II-T89A'

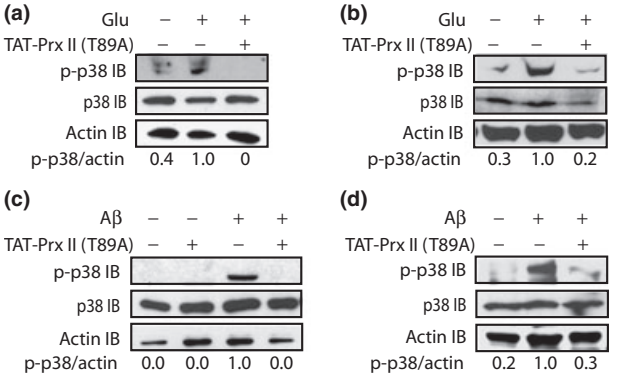

Fig. 3 Transduction of TAT-Prx-II-T89A prevents p38 activation upon neurotoxic stimulation in neuronal cells. (a) HT22 cells were exposed to 5 mM glutamate for 6 h with or without sustained addition of TAT-Prx-II-T89A. (b) Primary cortical neurons were exposed to 5 mM glutamate for 6 h with or without sustained addition of TAT-Prx-II-T89A. (c) HT22 cells were exposed to 25 μM $A\beta^{25-35}$ for 8 h with or without sustained addition of TAT-Prx-II-T89A. (d) Primary cortical neurons were exposed to 25 μM $A\beta^{25-35}$ for 8 h with or without sustained addition of TAT-Prx-II-T89A. In all these cases, 200 nM TAT-Prx-II-T89A was initially added together with the neurotoxin, and re-added every 3–4 h. After indicated incubation times, cells were harvested, lysed, and subjects to western blot analysis using the indicated antibodies. All experiments were conducted at least three independent times. Representative results are shown.

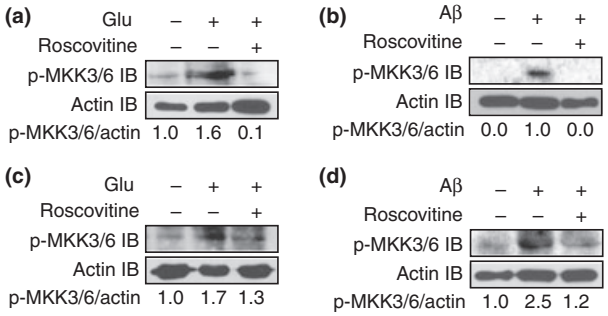

Fig. 5 Cdk5 deregulation triggers MKK6 phosphorylation in neuronal cells. (a) HT22 cells were treated with glutamate for 6 h in the presence or absence of roscovitine (10 μM). Cells were then harvested, lysed, and subjected to western blot analysis using the indicated antibodies. (b) HT22 cells were treated with $A\beta^{25-35}$ (25 μM) for 8h in the presence or absence of roscovitine. (c) Primary cortical neurons were exposed to glutamate for 6h in the presence or absence of roscovitine. (d) Primary cortical neurons were exposed to $A\beta^{25-35}$ for 8 h in the presence or absence of roscovitine and MKK3/6 phosphorylation analyzed using phospho-MKK3/6 antibody.

(TAT-Prx-II-T89A) (Sun *et al.* 2008a,b). Prx-II is a major antioxidant protein that is responsible for eliminating cytosolic ROS. As Prx-II antioxidant activity is eliminated by phosphorylation at Thr89 by Cdk5 (Qu *et al.* 2007; Sun *et al.* 2008b), Prx-II-T89A was used. TAT-Prx-II-T89A transduction successfully reduces ROS in neuronal cells (Sun *et al.* 2008a,b, 2009).

HT22 cells and primary cortical neurons were transduced with TAT-Prx-II-T89A following neurotoxic stimulation, and phosphorylation of p38 was analyzed upon glutamate or Aβ stimulation. TAT-Prx-II-T89A transduction significantly decreased p-p38 levels (Fig. 3a–d). These results demonstrate that oxidative stress is the major mechanism by which Cdk5 up-regulates p38 activity upon glutamate and Aβ stimulation in neuronal cells.

p38 activation upon glutamate and β-amyloid is calpain-dependent

As Cdk5 activation upon glutamate and Aβ is mediated by calpain-induced p25 formation, effect of calpain inhibition was examined on p38 activation (Camins *et al.* 2009). HT22 cells and primary cortical neurons were treated with either glutamate or Aβ in the presence or absence of MDL-28170 and p38 phosphorylation analyzed. Calpain inhibition decreased phospho-p38 levels significantly (Fig. 4a–d).

Cdk5 deregulation induces MKK6 phosphorylation

To further delineate the mechanism leading to p38 activation, we next investigated whether Cdk5 deregulation causes MKK6 phosphorylation, one of the major upstream regulators of p38. Phospho-MKK6 levels are increased in AD brains as compared to control cases (Zhu *et al.* 2001). Glutamate and Aβ-treatments in HT22 cells and primary cortical neurons triggered MKK6 activation, which was mediated by Cdk5 (Fig. 5a–d).

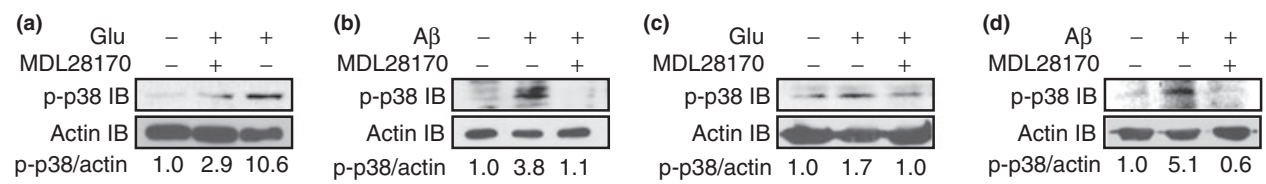

Fig. 4 Calpain inhibition abrogates p38 activation upon glutamate and Aβ stimulations in neuronal cells. (a) HT22 cells were treated with glutamate for 6h in the presence or absence of MDL-28170 (10 μM). Cells were then harvested, lysed, and subjected to western blot analysis using the indicated antibodies. (b) HT22 cells were treated with $A\beta^{25-35}$ (25 μM) for 8 h in the presence or absence of MDL-28170. (c) Primary cortical neurons were exposed to glutamate for 6 h in the presence or absence of MDL-28170. (d) Primary cortical neurons were exposed to $A\beta^{25-35}$ for 8 h in the presence or absence of MDL-28170 and p38 phosphorylation analyzed using phospho-p38 antibody.

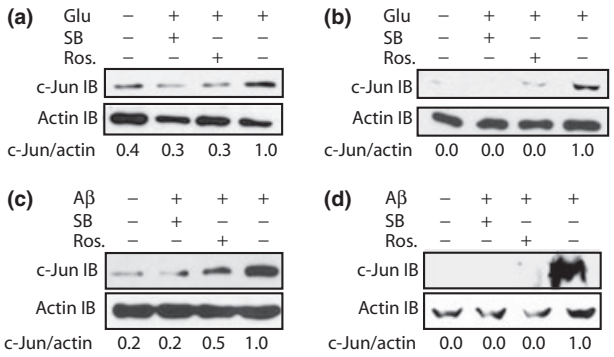

Fig. 6 c-Jun levels are regulated both by Cdk5 and p38 upon neurotoxic stimulation. (a) HT22 cells were exposed to 5 mM glutamate for 6 h with or without either roscovitine (10 μM) or SB203580 (2 μM). (b) Primary cortical neurons were exposed to glutamate (5 mM) for 6 h in the presence or absence of roscovitine (10 μM) or SB203580 (2 μM). (c) HT22 cells were exposed to 25 μM $A\beta^{25-35}$ for 8 h with or without either roscovitine (10 μM) or SB203580 (2 μM). (d) Primary cortical neurons were exposed to 25 μM $A\beta^{25-35}$ for 8 h in the presence or absence of roscovitine (10 μM) or SB203580 (2 μM). All experiments were conducted at least three independent times. Representative results are shown.

Cdk5 inhibition down-regulates p38-induced c-Jun expression upon glutamate or Aβ stimulation

Previous studies have revealed that p38 activation enhances c-Jun expression by activation of the activating transcriptional factor 2 (Marinissen *et al.* 1999, 2001). c-Jun levels were next investigated upon Aβ or glutamate treatments. Glutamate and Aβ treatments increased c-Jun levels in both HT22 cells and primary cortical cells, which was dependent both on Cdk5 and p38 (Fig. 6a–d), further supporting an upstream role of Cdk5 in activating p38 cascade.

The induction of phospho-p38 in p25 inducible transgenic mice

To examine whether level of phospho-p38 is also induced by Cdk5 *in vivo*, we examined the level of p-p38 in forebrain neuron specific p25 inducible transgenic mice (Cruz *et al.* 2003) in which neurodegenerative phenotypes including neuronal cell loss and tau-associated pathology are significant after the induction of p25. After 8 weeks of induction of p25, the number of phospho-p38 (Thr180/Tyr182) positive cells is significantly increased in cerebral cortex (Fig. 7) compared with control mice which have the same genotype but no induction of p25. This result confirms our *in vitro* studies and suggests that the activation of Cdk5 in p25 inducible mice induces the phosphorylation of p38 in cortical neurons.

Inhibition of Cdk5 confers a higher degree of neuroprotection than p38 inhibition

Our recent study revealed that Cdk5 deregulation triggers JNK activation and c-Jun phosphorylation at Ser63 and Ser73 in neuronal cells (Sun *et al.* 2009). Importantly, this study

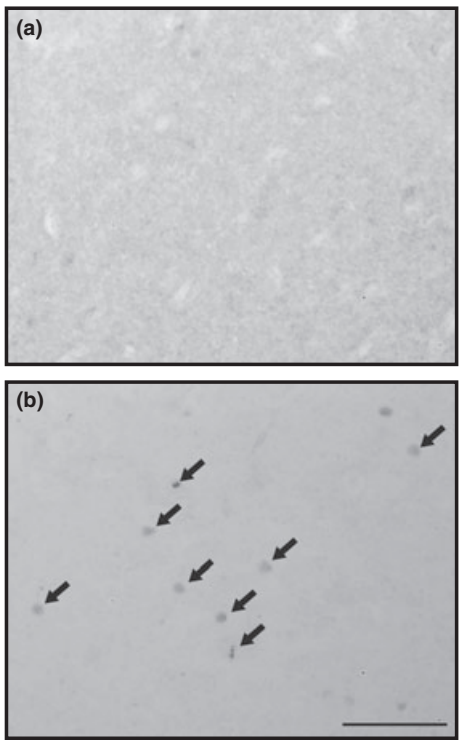

Fig. 7 Induction of p38 phosphorylation by Cdk5 activation *in vivo*. The phosphorylation of p38 (Thr180/Tyr182) is significantly induced in the nuclei of cortical neurons 8 weeks after induction of p25 (b, arrows) while the level of phospho-p38 is very low in non-induced mice (a). Scale bar = 50 μm.

demonstrated that c-Jun is also directly phosphorylated by Cdk5 following neurotoxic stimulation, which is JNK-independent. Accordingly, c-Jun inhibition was found to be more neuroprotective than JNK (Cdk5 > c-Jun > JNK, Sun *et al.* 2009). Thus, to further examine p38 contribution in glutamate-mediated neurotoxicity, p38, JNK and Cdk5 were independently inhibited in glutamate-treated cells and cell viability quantified. TAT-Cdk5 inhibitory peptide was used for Cdk5 inhibition, as roscovitine causes appreciable toxicity upon prolonged exposure (> 24 h; Sun *et al.* 2008a,b), presumably because of Cdk1 and Cdk2 inhibition. p38 was inhibited using SB203580, and JNK using SP600125. Cdk5 inhibition almost completely protected glutamate-treated HT22 cells significantly from cell death (> 95%) whereas p38 and JNK inhibitions were partially protective (Fig. 8a). As Cdk5 deregulation activates many neurodegenerative pathways and is upstream of both p38 (this study) and JNK (Sun *et al.* 2009), it is not surprising that Cdk5 inhibition was most neuroprotective.

Discussion

Oxidative stress, Cdk5 deregulation, JNK and p38 activations are early events in the pathogenesis of AD. Our

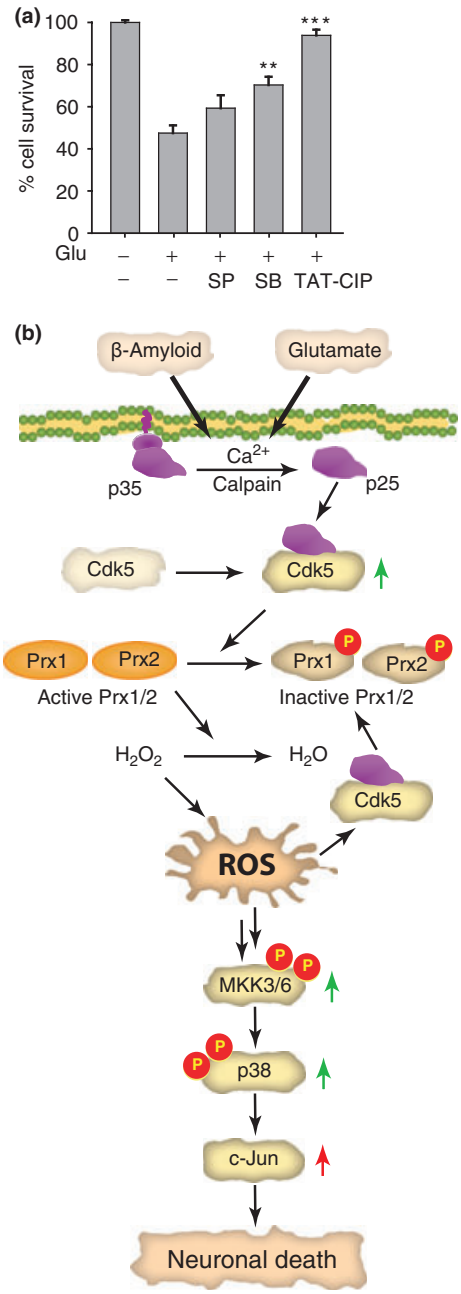

Fig. 8 (a) Role of Cdk5 in regulating p38 cascade in neurons upon neurotoxic insults. Inhibition of Cdk5 or p38 activity confers higher degree of neuroprotection as compared to JNK inhibition. Inhibition of Cdk5, JNK, or p38 was performed in HT22 cells as described in Materials and Methods, followed by 5 mM glutamate stimulation. After additional 24 h incubation, cell viability was analyzed by MTT assay. This assay was conducted three independent times. Representative results are shown. SB denotes p38 inhibitor SB203580, SP is JNK inhibitor SP600125 and TAT-Cdk5 inhibitory peptide was used to inhibit Cdk5 activity. 200 nM TAT-Cdk5 inhibitory peptide was initially added together with glutamate, and re-added every 4–6 h. (b) Proposed model of Cdk5 in regulation of p38 cascade. Yellow squares highlight four targets that are deregulated in AD. Cdk5 deregulation upon β-amyloid and glutamate stimulations increases oxidative stress *via* inactivation of two antioxidant enzymes, Prx-I and Prx-II. Increase in ROS levels activates MKK3/6 and p38, which leads to increase in c-Jun levels causing neuronal death. Green arrows show increase in kinase activities. Red arrow shows increase in protein level. $^{**}p < 0.01$, $^{***}p < 0.001$ when comparing data to the control.

previous studies have shown that Cdk5 deregulation promotes oxidative stress by inactivating the peroxidase activities of Prx-I and Prx-II, leading to JNK activation, mitochondrial dysfunction and cell death. As oxidative stress is also a major stimulant for p38 activation in AD, a link between Cdk5 and p38 was examined in this study. p38 regulates a variety of transcription factors that control the expression of genes involved in cell survival or cell death signaling pathways. Our study indicates that glutamate/Aβ-induced Cdk5 deregulation promotes p38 activity and thus causes up-regulation of c-Jun level, suggesting a role of p38 in apoptotic cell death in AD.

c-Jun expression is up-regulated in AD (Anderson *et al.* 1994; Sajan *et al.* 2007). Intense c-Jun immunoreactivity was found to be co-localized with paired helical filaments-Tau in AD (Anderson *et al.* 1994; Marcus *et al.* 1998). c-Jun promotes formation of abnormal Aβ in AD by the activation of β-APP gene (Trejo *et al.* 1994; Ferrer *et al.* 1996). Activated c-Jun stimulates the expression of Fas ligand, which triggers a cascade of events of programmed cell death (Morishima *et al.* 2001). c-Jun promotes apoptosis in Aβ-treated hippocampal neurons (Anderson *et al.* 1995). Inhibition of c-Jun expression protects neonatal hippocampal and sympathetic neurons from cell death, and neurons from c-Jun knockout mice are resistant to Aβ-toxicity (Ham *et al.* 1995; Kihiko *et al.* 1999). Thus, our results showing an upstream role of Cdk5 in enhancing c-Jun levels via p38 cascade suggest that Cdk5 inhibition should confer neuroprotection in AD. In conclusion, deregulation of Cdk5 triggers diverse events that are fatal to neurons. One such event is mediated via the p38 cascade (Fig. 8b). Therefore, our study suggests that Cdk5 is a preferable therapeutic target for AD relative to p38 and c-Jun.

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