Interplay between MEK-ERK signaling, cyclin D1, and cyclin-dependent kinase 5 regulates cell cycle reentry and apoptosis of neurons

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ABSTRACT **In response to neurotoxic signals, postmitotic neurons make attempts to reenter the cell cycle, which results in their death. Although several cell cycle proteins have been implicated in cell cycle–related neuronal apoptosis (CRNA), the molecular mechanisms that underlie this important event are poorly understood. Here, we demonstrate that neurotoxic agents such as β-amyloid peptide cause aberrant activation of mitogen-activated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling, which promotes the entry of neurons into the cell cycle, resulting in their apoptosis. The MEK-ERK pathway regulates CRNA by elevating the levels of cyclin D1. The increase in cyclin D1 attenuates the activation of cyclin-dependent kinase 5 (cdk5) by its neuronal activator p35. The inhibition of p35-cdk5 activity results in enhanced MEK-ERK signaling, leading to CRNA. These studies highlight how neurotoxic signals reprogram and alter the neuronal signaling machinery to promote their entry into the cell cycle, which eventually leads to neuronal cell death.**

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INTRODUCTION

By definition, postmitotic neurons exit the cell cycle and are arrested in the G0 state. However, under neurotoxic stress and in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, a strong correlation between cell cycle reentry and neuronal apoptosis has been observed (Herrup and Busser, 1995; Herrup and Yang, 2007; Herrup, 2010; Hoglinger *et al.*, 2007). Neurotoxic agents such as β-amyloid 1-42 peptide ($A\beta_{42}$) or DNA-damaging agents reactivate the cell cycle machinery of neurons, resulting in their S-phase entry, which is indicated by DNA replication and an increase in the levels of S-phase markers such as proliferating cell nuclear antigen (PCNA). The alteration in levels or the activity of cell cycle proteins such as cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors may contribute to this process. For instance, levels of cyclin D1, activity of cdk4, and phosphorylation of retinoblastoma protein (Rb) are enhanced in response to trophic factor withdrawal or neurotoxic agents (Park *et al.*, 1998, 2000a,b; Giovanni *et al.*, 1999; Ino and Chiba, 2001). In addition, cdk inhibitors and other cell cycle blockers attenuate cell cycle–related neuronal apoptosis (CRNA; Park *et al.*, 1997a,b).

The molecular mechanisms that cause cell cycle reentry and control events such as aberrant expression of cell cycle proteins such as cyclin D1 are poorly understood. Cyclin D1 is a key regulator of the G1–S transition as it activates cdk4, which phosphorylates Rb protein. As a result, Rb protein dissociates from E2F1, which in turn facilitates the transcription of genes needed for S-phase progression. The increased expression of cyclin D1 in response to various neurotoxic agents is implicated in neuronal apoptosis (Kranenburg *et al.*, 1996; Sumrejkanchanakij *et al.*, 2003; Malik *et al.*, 2008). In addition, its nuclear localization correlates well with neuronal cell

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Abbreviations used: $A\beta_{42}$, β-amyloid 1-42 peptide; $A\beta_{42rev}$, control peptide with reverse sequence of $A\beta_{42}$; AD, Alzheimer's disease; BrdU, 5-bromo-2′-deoxyuridine; cdk, cyclin-dependent kinase; CRNA, cell cycle–related neuronal apoptosis; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IP, immunoprecipitate; MEK, mitogen-activated kinase kinase; NGF, nerve growth factor; PCNA, proliferating cell nuclear antigen; PC12, rat pheochromocytoma cell; STAT3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

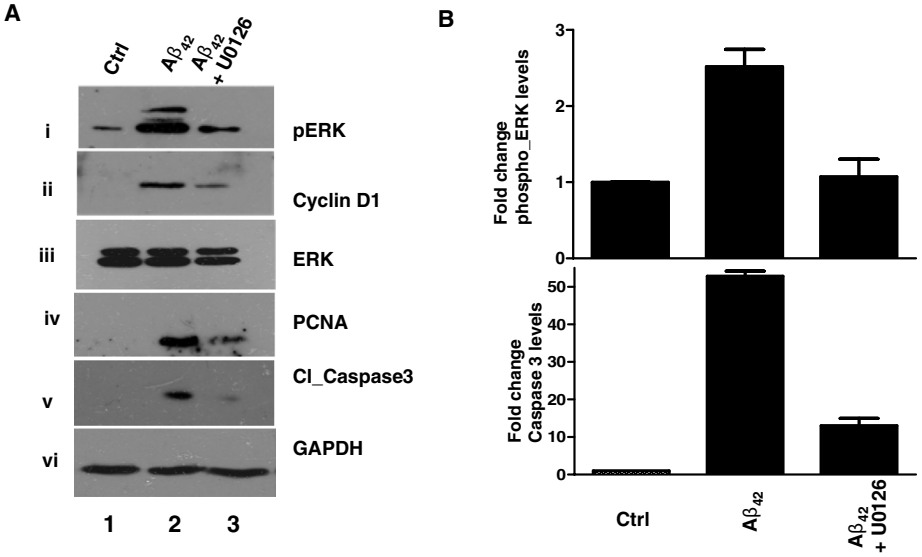

FIGURE 1: $A\beta_{42}$ induces aberrant MEK-ERK signaling and cyclin D1 expression in cortical neurons. (A) Rat cortical neurons were treated with $A\beta_{42}$ peptide for 48 h with DMSO (Ctrl) or 10 μM U0126. Cell lysates were prepared, followed by Western blotting with indicated antibodies. Because a time-course experiment suggested that cell death was evident 24–48 h posttreatment (Supplemental Figure S1A), treatments were performed for this duration. A representative of more than three independent experiments is shown. (B) The quantification of phospho-ERK and caspase 3 levels in experiments such as the one described in A and Supplemental Figure S1C was done. Densitometry of phospho-ERK and cleaved caspase 3 bands was performed, which was normalized with respect to total ERK and GAPDH levels, respectively. The fold change in phosphor-ERK levels with respect to untreated cells is illustrated. The mean of three independent experiments is shown, and error bars represent SEM.

death (Sumrejkanchanakij *et al.*, 2003). Therefore the understanding of cyclin D1 regulation in CRNA may provide useful insights into this important phenomenon.

Although the mitogen-activated kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathway is important for neuronal survival (Ginty *et al.*, 1994; Bonni *et al.*, 1999; Sharma *et al.*, 2007), the hyperactivation or aberrant activation of this pathway caused by neurotoxic agents can lead to neuronal apoptosis (Stanciu *et al.*, 2000; Subramaniam *et al.*, 2004; Chong *et al.*, 2006). We report that the aberrant activation of the MEK-ERK MAP kinase pathway controls cyclin D1 expression and forces neurons into the S phase, resulting in CRNA. Cyclin D1, in turn, prevents the activation of cyclin dependent kinase 5 (cdk5) by its neuronal cyclin-like activator p35. Cdk5 is an atypical cdk, which controls a wide variety of neuronal functions, including neuronal migration (Ohshima *et al.*, 1996), differentiation, and survival (Dhavan and Tsai, 2001). Cdk5 is regulated by cyclin-like regulators p35 and p39, which are expressed in the brain and are essential for cdk5 function (Ko *et al.*, 2001). The conversion of its activator p35 to p25 is considered to be one of the reasons for cdk5 deregulation and activation in situations such as Alzheimer's disease (Patrick *et al.*, 1999). Recent studies have suggested that cdk5 may be important for cell cycle arrest of postmitotic neurons (Zhang *et al.*, 2008), and $A\beta_{42}$ alters the localization of cdk5, resulting in neuronal cell cycle reentry (Zhang *et al.*, 2010; Zhang and Herrup, 2011). The present work suggests that deregulation of p35-cdk5 by cyclin D1 may contribute to aberrant MEK-ERK signaling, which possibly provides an explanation for the role of cdk5 in CRNA.

RESULTS

$A\beta_{42}$ peptide causes aberrant MEK-ERK signaling, which results in neuronal cell cycle reentry and apoptosis

One of the hallmarks of Alzheimer's disease (AD) is the altered cleavage of amyloid precursor protein that leads to the generation of the 42–amino acid $A\beta_{42}$ peptide. The neurotoxic oligomeric form of $A\beta_{42}$ is a major constituent of amyloid plaques (Selkoe, 1998) and is also known to induce cell cycle reentry of neurons (Copani *et al.*, 1999; Yang *et al.*, 2001; Herrup and Yang, 2007). The treatment of cortical neurons (Figure 1A and Supplemental Figure S1, A and C) or nerve growth factor (NGF)–differentiated neuronal rat pheochromocytoma (PC12) cells (see discussion of Figure 3A later in the paper) with $A\beta_{42}$ led to an increase in the levels of cleaved caspase 3, which was indicative of apoptosis. A corresponding increase in the levels of the S-phase marker PCNA and cyclin D1 (Figures 1 and 3A [later in the paper] and Supplemental Figure S1, A and C) and enhanced DNA replication (discussed later) were indicative of attempts of neurons to enter the cell cycle. Because MEK-ERK signaling is important for neuronal survival and death (Bonni *et al.*, 1999; Chong *et al.*, 2006), we explored the involvement of this MAP kinase pathway. When cortical neurons (Figure 1A and Supplemental Figure S1, A and C) or neuronal PC12 cells (see discussion of Figure 5B later in the paper) were treated with $A\beta_{42}$, a significant increase in the levels of the phosphorylated form of ERK1/2 was observed in comparison to the untreated cells. The increase in ERK1/2 phosphorylation was at regulatory MEK1/2 target sites, and therefore these results were indicative of hyperactivated MEK-ERK. The treatment with U0126, a specific MEK inhibitor, attenuated the increase in cyclin D1, as well as in PCNA levels, suggesting that the neuronal cell cycle progression was blocked upon inhibition of MEK-ERK signaling (Figures 1 and 3A [later in the paper] and Supplemental Figure S1, A and C). In addition, the levels of the cleaved form of caspase 3, an indicator of apoptosis, were also reduced. A control peptide with reverse sequence of $A\beta_{42}$ ($A\beta_{42rev}$) did not cause any significant changes (Supplemental Figure S1C). A time-course experiment in which neurons were treated with $A\beta_{42}$ for 2–48 h revealed an increase in phospho-ERK levels within 2 h of treatment, accompanied by the expression of cyclin D1, which was followed by PCNA expression in the next 2–4 h. Interestingly, significant levels of cleaved caspase 3, which indicate apoptosis, were observed only after ~24 h of treatment (Supplemental Figure S1A). These results indicated that the neurons make attempts to first enter the cell cycle or S phase, which is followed by apoptosis after a few hours.

Next we probed whether MEK-ERK signaling was responsible for CRNA. To this end, we treated cortical neurons with $A\beta_{42}$ (Figure 2A) and labeled them with 5-bromo-2′-deoxyuridine (BrdU), followed by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect DNA replication and cell death, respectively. A significant number of neurons were both BrdU and TUNEL positive, which was a strong indication that these cells

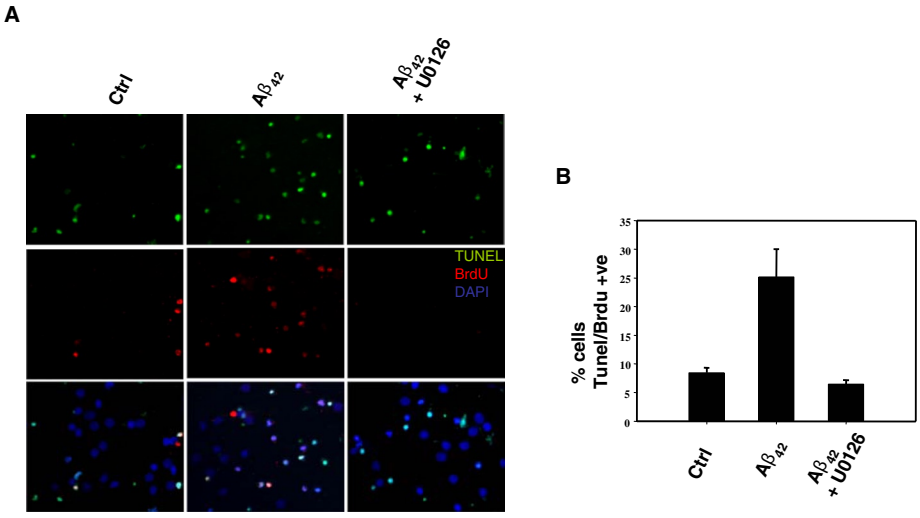

FIGURE 2: Aberrant MEK-ERK signaling induced by $A\beta_{42}$ causes cell cycle reentry and neuronal apoptosis (CRNA). (A) $A\beta_{42}$ peptide was added with DMSO or U0126 to cortical neurons, followed by incubation with BrdU. Immunofluorescence and TUNEL assays were performed to detect BrdU incorporation (red) or apoptotic (green) cells, respectively. (B) Percentage of cells that exhibited both BrdU and TUNEL staining. Mean of three experiments is shown, and error bars represent SE

underwent S-phase entry and cell death. Strikingly, U0126 treatment resulted in a significant decrease in BrdU/TUNEL$^+$ cells (Figure 2). Interestingly, the effect of U0126 on the number of TUNEL$^+$ cells that did not exhibit BrdU incorporation (TUNEL$^+$/BrdU$^-$) was significantly less (Supplemental Figure S2B), which suggested that aberrant MEK-ERK activation may regulate CRNA and may have lesser influence on $A\beta_{42}$-induced apoptosis of neurons that do not exhibit cell cycle reentry. As reported earlier (Bonni *et al.*, 1999), we also noticed that the inhibition of MEK-ERK signaling under physiological conditions led to neuronal apoptosis, whereas the cell cycle status of neurons was almost unaltered (Supplemental Figure S2, A and B). In contrast to terminally differentiated cells, $A\beta_{42}$ did not alter either the MEK-ERK signaling or cause apoptosis of dividing PC12 cells. However, U0126 treatment resulted in caspase 3 cleavage, suggesting that the MEK-ERK pathway is important for the survival of dividing cells (Supplemental Figure S6).

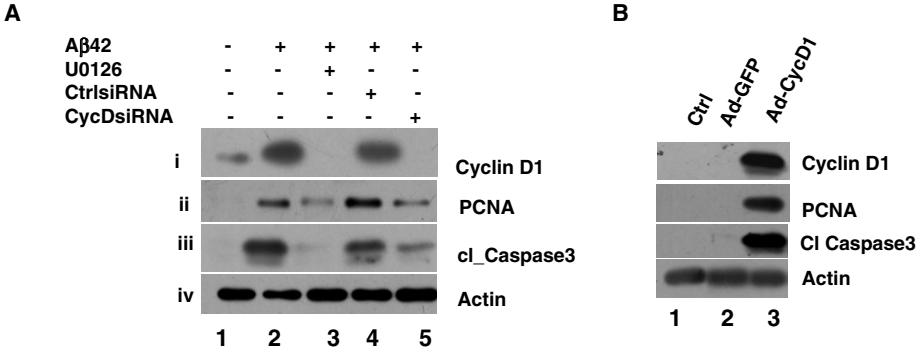

FIGURE 3: Increased levels of cyclin D1 may cause CRNA. (A) NGF-differentiated neuronal PC12 cells were transfected with cyclin D1 siRNA or a control siRNA. Subsequently, cells were incubated with $A\beta_{42}$. The Western blotting of cell lysates revealed that the knockdown of cyclin D1 (i) caused a significant decrease in levels of PCNA (ii) and cleaved caspase 3 (iii), respectively. (B) Rat cortical neurons were infected with recombinant adenovirus for cyclin D1 (Ad-cyc D1) or GFP alone (Ad-GFP). After 48 h, Western blotting was performed using antibodies against PCNA and cleaved caspase 3.

MEK-ERK signaling controls cyclin D1 expression via signal transducer and activator of transcription 3 during CRNA

Next we addressed whether cyclin D1 is directly involved in CRNA and investigated mechanisms by which its levels are elevated during this process. Cyclin D1 knockdown by specific small interfering RNA (siRNA) reversed the effects of $A\beta_{42}$, which included the inhibition of PCNA and cleaved caspase 3 levels (Figure 3A). Furthermore, when recombinant adenovirus was used to overexpress cyclin D1 in primary neurons, an increase in the levels of mature caspase 3 and PCNA was observed (Figure 3B). Collectively these observations indicated that the hyperactivation of MEK-ERK results in an increase in cyclin D1, which may promote CRNA.

$A\beta_{42}$ induces cyclin D1 levels by regulating its transcription (Supplemental Figure S1B). To gain insights into the mechanisms involved in this process, we studied the effect on cyclin D1 promoter. $A\beta_{42}$ induced the promoter activity, which was prevented by U0126 (Figure 4A, first and second bars), suggesting MEK-ERK–dependent regulation. Signal transducer and activator of transcription 3 (STAT3) is known to promote cyclin D1 transcription during G1–S transition in dividing cells (Klein and Assoian, 2008). Therefore a previously described STAT3 binding site (Leslie *et al.*, 2006) on cyclin D1 promoter was disrupted by site-directed mutagenesis. $A\beta_{42}$ failed to enhance the activity of the mutant promoter (Figure 4A, third bar). Furthermore, the knockdown of STAT3 expression by a specific siRNA in neuronal PC12 cells resulted in a significant decrease in cyclin D1 levels (Figure 4B). Collectively these results suggested that STAT3 may control cyclin D1 expression in response to $A\beta_{42}$.

Phosphorylation of STAT3 at S727 and Y705 is crucial for maximal transcriptional activation of STAT3 (Wen *et al.*, 1995) and may involve several pathways, including the MAPK pathway (Ng *et al.*, 2006; Wan *et al.*, 2010). $A\beta_{42}$ caused an increase in the phosphorylation of both S727 and Y705, which was reduced upon U0126 treatment (Figure 4C). Based on these data, it is reasonable to propose that $A\beta_{42}$-induced MEK-ERK signaling may control cyclin D1 levels via STAT3 phosphorylation.

Elevated cyclin D1 levels may contribute to sustained MEK-ERK activation via a positive feedback loop

Even though $A\beta_{42}$ hyperactivates the MEK-ERK MAP kinase pathway, resulting in increased cyclin D1 levels, the molecular mechanisms by which cyclin D1 contributes to CRNA remained unclear. One of the possibilities was that it influences the MEK-ERK pathway, which regulates several neuronal functions, including survival and apoptosis (Ginty *et al.*, 1994; Bonni *et al.*, 1999). Surprisingly, overexpression of cyclin D1 levels caused a significant enhancement

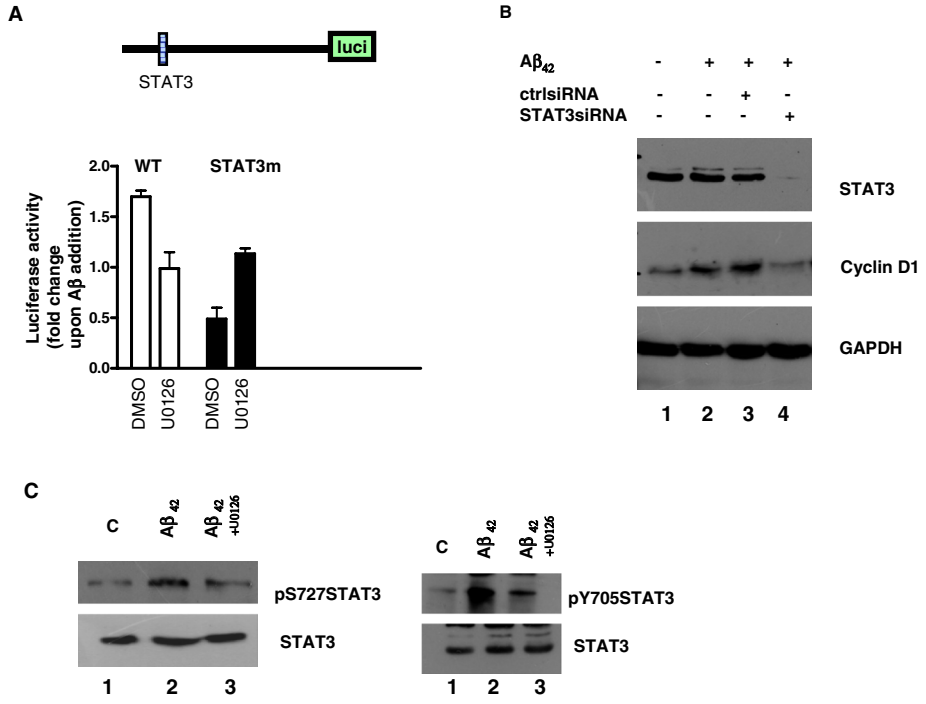

FIGURE 4: $A\beta_{42}$-triggered MEK-ERK signaling regulates cyclin D1 expression via STAT3. (A) Cyclin D1 promoter or its variant with a mutation in STAT3-binding site (–984 base pair), which was fused to luciferase reporter, was transfected in rat cortical neurons, followed by treatment with $A\beta_{42}$ in the presence of DMSO or U0126. After 48 h, luciferase activity was determined, and the fold change upon $A\beta_{42}$ treatment is indicated. $A\beta_{42}$ caused a significant increase in the activity of wild-type promoter but not of a STAT3–binding site mutant (STAT3m). (B) Neuronal PC12 cells were transfected with siRNA against STAT3 or a control siRNA, followed by the addition of $A\beta_{42}$. Western blot was performed to determine the levels of cyclin D1 as a result of STAT3 knockdown. C. $A\beta_{42}$ treatment of cortical neurons was followed by Western blot with an antibody that recognizes STAT3 phosphorylated at S727 (left) or Y705 (right).

in MEK-ERK activity (Figure 5A) in cortical neurons. The cell cycle reentry and cell death caused as a result of cyclin D1 overexpression, which was indicated by the change in levels of PCNA (Supplemental Figure S3) and cleaved caspase 3 levels (Figure 5A), was prevented by U0126. Furthermore, the reverse experiments revealed that cyclin D1 knockdown in $A\beta_{42}$-treated neuronal PC12 cells caused a significant decrease in phospho-ERK levels (Figure 5B), confirming the positive effect of increased cyclin D1 expression on MEK-ERK signaling. Although the earlier observations suggested that the MEK-ERK pathway promotes the expression of cyclin D1 (Figures 1, 3, and 4), these results (Figure 5) were indicative of a positive feedback loop by which cyclin D1 may cause sustained MEK-ERK activation, which may contribute to CRNA.

$A\beta_{42}$-mediated increase in cyclin D1 attenuates p35-dependent cdk5 activation

Next efforts were made to gain insight into events that regulate CRNA downstream of cyclin D1. We explored the involvement of cdk5 in this process, as it keeps the cell cycle of neurons suppressed (Cicero and Herrup, 2005; Zhang *et al.*, 2010). We questioned whether an increase in cyclin D1 levels regulates cdk5, which in turn may have an impact on neuronal apoptosis. p35 activates cdk5 in neurons, and $A\beta_{42}$ and other neurotoxic agents promote its cleavage to p25. Aberrant activation of cdk5 by p25 may lead to the hyperphosphorylation of proteins such as Tau and result in neuronal cell death (Patrick *et al.*, 1999; Lee *et al.*, 2000). The effect of $A\beta_{42}$ on p35-cdk5 activity is poorly understood. Because we found that p35-associated cdk5 activity is significantly reduced upon $A\beta_{42}$ treatment (Figure 6B, discussed later in detail), we investigated the role of cyclin D1 in this process. To address this, we used a p35 antibody for immunoprecipitation. Because it is raised against the N-terminal of p35, it does not recognize p25 (N-20; Santa Cruz Biotechnology). Cyclin D1 overexpression in cortical neurons resulted in a significant reduction in p35-immunoprecipitate (IP)–associated cdk5 activity (Figure 6A). Independent transfection studies revealed that the overexpression of cyclin D1 prevented p35-mediated activation of cdk5, whereas p25-cdk5 activity was not reduced (Supplemental Figure S4), suggesting that cyclin D1 may compete with p35 and not p25 for cdk5.

Having demonstrated that increased cyclin D1 negatively regulates p35-cdk5 activity, we further dissected the cross-talk between p35-cdk5 and cyclin D1 in cortical neurons exposed to $A\beta_{42}$. $A\beta_{42}$ caused a decrease in p35-associated cdk5 activity (Figure 6B), and the inhibition of MEK-ERK signaling restored the activity significantly (Figure 6B, third bar). The coimmunoprecipitation experiments revealed a significant decrease in the amount of cdk5 associated with p35-IP (Figure 6C, i) in $A\beta_{42}$-treated cells, which was the likely cause for the decrease in p35-cdk5 activity (Figure 6B). Not only did the levels of cyclin D1 increase upon $A\beta_{42}$ treatment, the amount associated with cdk5 was also higher (Figure 6C, ii). The treatment with U0126 caused a decrease in cyclin D1 levels (Figure 6C, iii, lane 3). As a result, the amount immunoprecipitated with cdk5 was also reduced significantly (Figure 6C, ii, lane3). The inhibitor had an opposite effect on p35-cdk5 association; the amount of cdk5 bound to p35-IP was reinstated (Figure 6C, i, lane 3), which corroborated well with the reversal in the loss of p35-cdk5 activity upon $A\beta_{42}$ treatment (Figure 6B). As reported earlier (Lee *et al.*, 2000), $A\beta_{42}$ treatment caused an increase in p25 levels and total cdk5-IP–associated activity (Supplemental Figure S5A). In contrast to p35-IP activity, cdk5-IP activity was not influenced by U0126 (Supplemental Figure S5B). Furthermore, cyclin D1 siRNA transfection was performed to ascertain whether MEK-ERK–dependent regulation of p35-cdk5 was via cyclin D1. The knockdown of cyclin D1 in $A\beta_{42}$-treated neuronal PC12 cells significantly prevented $A\beta_{42}$-mediated suppression of p35-cdk5 activity (Figure 6D, second vs. fourth bar). Collectively these data indicated that the increase in cyclin D1 levels as a result of $A\beta_{42}$ treatment may block p35-cdk5 activation by competing with p35 for cdk5.

Overexpression of p35 reverts the effect of $A\beta_{42}$ on p35-cdk5 regulation and neuronal apoptosis

We next tested whether p35 overexpression is able to revert some of the foregoing effects of $A\beta_{42}$. To this end, we overexpressed p35 and p25 in neuronal PC12 cells, followed by treatment with $A\beta_{42}$, and we measured the kinase activity associated with p35-IP or cdk5-IP. As observed earlier, $A\beta_{42}$ treatment caused an increase in total cdk5-IP activity and a decrease in p35-IP–associated activity

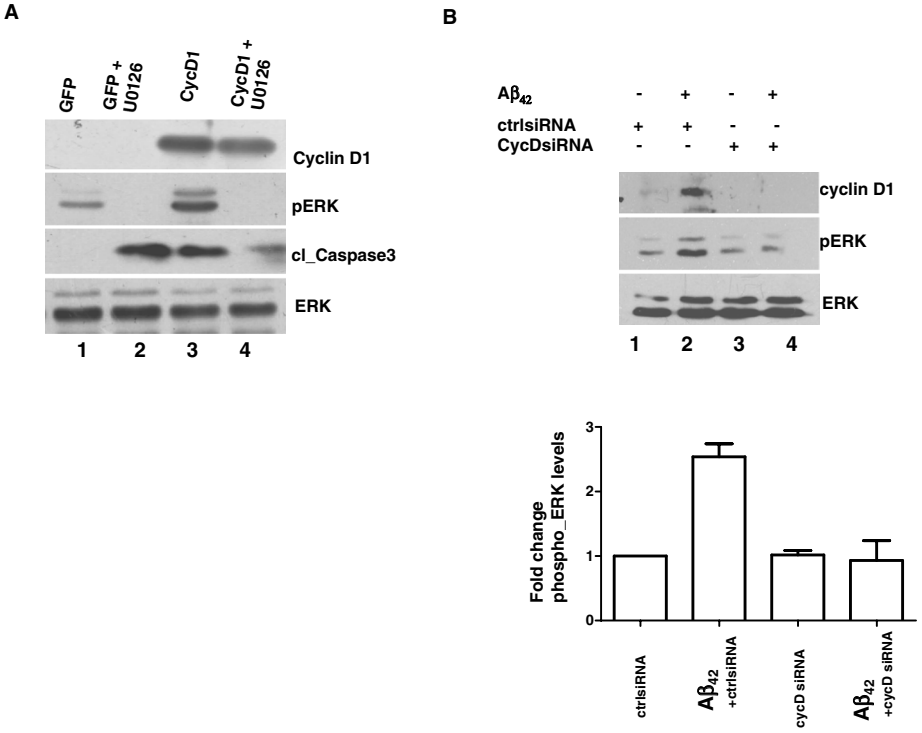

FIGURE 5: Cyclin D1 contributes to an increase in MEK-ERK signaling via a positive feedback loop. (A) Cyclin D1 was overexpressed using Ad-cyc D1 or a control Ad-GFP, followed by U0126 treatment. Western blotting was performed to assess the levels of phospho-ERK and cleaved caspase 3. (B) NGF-differentiated neuronal PC12 cells were transfected with cyclin D1 siRNA or a control siRNA and incubated with $A\beta_{42}$. The Western blotting of cell lysates revealed a significant decrease in the levels of phospho-ERK1/2. Bottom, densitometry of phospho-ERK bands was performed, which was normalized with respect to total ERK levels, and the fold change in comparison to untreated cells was determined.

(Figure 7A). The cdk5-IP kinase activity significantly increased upon overexpression of both p35 and p25. In the case of p35-IP, the increase was observed only in p35-overexpressing cells. These data indicated that the loss in p35-cdk5 activity that occurs as a result of increased cyclin D1 overproduction (Figure 6) may be overcome by p35 overexpression. Interestingly, the aberrant MEK-ERK signaling indicated by higher phospho-ERK levels was significantly curbed by p35 overexpression (Figure 7B, v). Of importance, $A\beta_{42}$-mediated apoptosis as indicated by levels of mature caspase 3 and PCNA (Figure 7B, iii and iv) was significantly prevented by p35 and not p25 overexpression. Therefore it is reasonable to conclude that the loss of the interaction between p35 and cdk5 as a result of an increase in cyclin D1 may lead to CRNA.

Collectively these studies demonstrate that neurotoxic stimuli such as amyloid peptide cause aberrant activation of MEK-ERK signaling and promote cell cycle reentry by increasing cyclin D1 levels. Subsequently, cyclin D1 may attenuate p35-cdk5 association and activity, which may further contribute to aberrant MEK-ERK signaling, resulting in neuronal cell death (Figure 8). These results may also help to explain the mechanism by which cdk5 may suppress the neuronal cell cycle and prevent cell cycle reentry by keeping the MEK-ERK pathway in check.

DISCUSSION

The evidence for the connection between cell cycle reentry of postmitotic neurons and neurodegeneration is very strong (reviewed in Herrup and Yang, 2007). CRNA is reported in vitro and in animal models for neurodegenerative disorders such as Parkinson's disease (Hoglinger *et al.*, 2007) and Alzheimer's disease (Yang *et al.*, 2001; Malik *et al.*, 2008). Cell cycle proteins may play a crucial role in this process and may promote S-phase entry of neurons, which leads to cell death (Park *et al.*, 1998, 2000a,b; Giovanni *et al.*, 1999; Malik *et al.*, 2008). For example, it has been demonstrated that cyclin D1 knockdown prevents CRNA in neurons from an AD mouse model (Malik *et al.*, 2008). In this study, we described the link between cell cycle machinery and neuronal signaling pathways. $A\beta_{42}$ can induce hyperactivation of MEK-ERK signaling (Harper and Wilkie, 2003) and cause cell death by possibly mediating Tau hyperphosphorylation (Veeranna *et al.*, 1998; Zheng *et al.*, 2007). We found that an $A\beta_{42}$-mediated aberrant MEK-ERK signaling pathway results in increased expression of cyclin D1 and causes S-phase entry and neuronal cell death. These results were surprising, as MEK-ERK signaling is known to promote neuronal differentiation and survival (Bonni *et al.*, 1999; Riccio *et al.*, 1999) by promoting the transcription of genes important for this process (Riccio *et al.*, 1999; Lonze and Ginty, 2002). The $A\beta_{42}$-triggered MEK-ERK signaling promotes cyclin D1 expression, which is reminiscent of its role during cell division, in which it induces cyclin D1 expression via transcription factors such as STAT3 (Lavoie *et al.*, 1996). STAT3 is phosphorylated in response to $A\beta_{42}$ in a MEK-ERK–dependent manner and controls cyclin D1 expression. Interestingly, during neuronal differentiation STAT3 promotes cyclin D1 expression and its knockdown prevents withdrawal of neurons from the cell cycle (Ng *et al.*, 2006). For maximal activation of STAT3, phosphorylation of both S727 and Y705 is needed (Wen *et al.*, 1995), and we found that $A\beta_{42}$ stimulates the phosphorylation of both these sites.

Cdk5 is activated by its regulators p35 and p39 in the nervous system. Although $p35^{-/-}$ mice survive embryonic lethality (Chae *et al.*, 1997), they exhibit cortical migration defects like the $cdk5^{-/-}$ animals and also exhibit seizures and adult lethality. $p35^{-/-}$ $p39^{-/-}$ mice exhibit prenatal lethality and other features of $cdk5^{-/-}$ animals. However, $p39^{-/-}$ animals do not reveal any significant abnormalities, suggesting that p39 may compensate for some but not all the functions of p35 (Ko *et al.*, 2001). Cdk5 is involved in controlling both neuronal survival and death (Cheung and Ip, 2004; Hisanaga and Endo, 2010). It cross-talks with other signaling and apoptosis pathways and prevents apoptosis of neurons (Li *et al.*, 2002; Zheng *et al.*, 2007; Cheung *et al.*, 2008). Even though the kinetics of Tau phosphorylation by p35-cdk5 or p25-cdk5 is not very different (Peterson *et al.*, 2010), the cleavage of p35 to p25 has been attributed to deregulation of cdk5 in AD brain and $A\beta_{42}$-mediated neuronal apoptosis (Patrick *et al.*, 1999). Although p25 levels are elevated in AD brain and in $A\beta_{42}$-treated cortical neurons, a significant amount of p35 expression is still observed (Lee *et al.*, 2000; Taniguchi *et al.*, 2001; Tandon *et al.*, 2003). Consistent with this, we also noted the presence of p35 in cortical neurons treated with $A\beta_{42}$. Although p25 causes sustained cdk5 activation (Patrick *et al.*, 1999; Cruz *et al.*, 2003), which may contribute to neurodegeneration, there is little

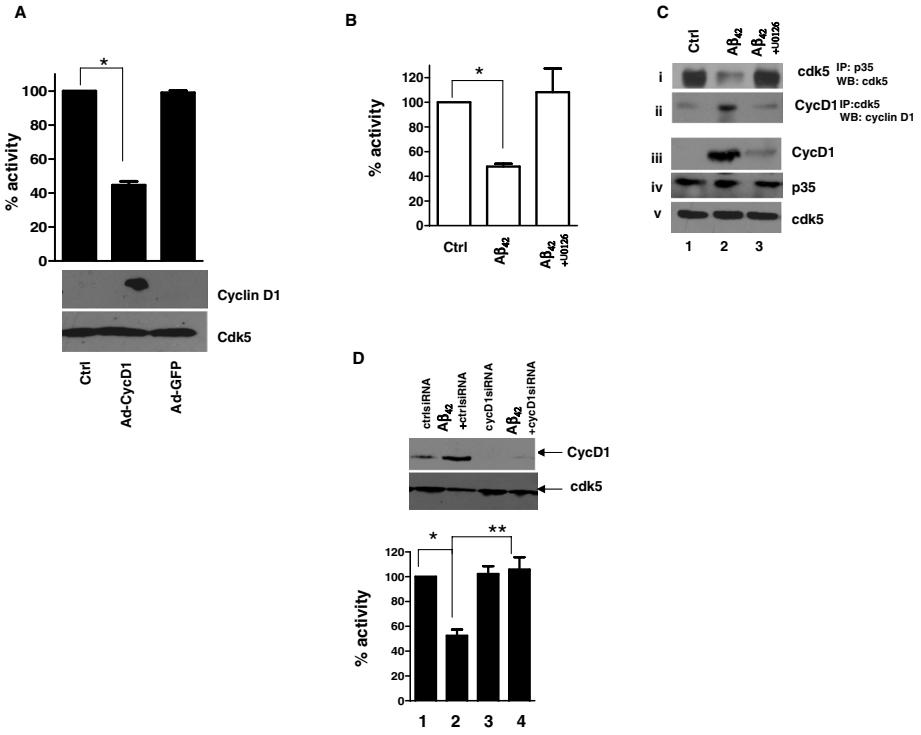

FIGURE 6: Aberrant MEK-ERK signaling and enhanced cyclin D1 expression prevent p35-mediated cdk5 activation. (A) Cortical neurons were infected with Ad-cyc D1 (lane 2) or Ad-GFP (lane 3). p35-IP associated cdk5 activity was determined by using a histone H1–derived peptide (PKTPKKAKKL) as substrate. Phosphate incorporation in the peptide was determined by scintillation counting. Western blots were performed with antibodies against indicated proteins. The percentage activity in comparison to the control uninfected cells (100%) is shown. *$p < 0.001$ by ANOVA ($n = 4$). (B) Cortical neurons were treated with $A\beta_{42}$ for 48 h in the presence of DMSO or U0126. p35 was immunoprecipitated with N-20 antibody raised against the N-terminal of p35 (Santa Cruz Biotechnology). p35-IP associated cdk5 kinase activity was determined as described for A. The percentage activity with respect to the control DMSO-treated cells (100%) is shown. Error bars reflect SE. *$p < 0.01$ by ANOVA ($n = 4$). (C) As described in B, cortical neurons were treated with $A\beta_{42}$ for 48 h in the presence of DMSO (Ctrl) or U0126. p35 (i) or cdk5 (ii) was immunoprecipitated, followed by Western blotting with cdk5 (i) or cyclin D1 (ii). Whereas the amount of cdk5 associated with p35 was significantly reduced (i, lane 2) upon $A\beta_{42}$ treatment, a concomitant increase in cyclin D1 binding to cdk5 (ii, lane 2) was observed. Western blotting was performed on whole-cell lysate using indicated antibodies (iii–v). (D) NGF-differentiated neuronal PC12 cells were transfected with cyclin D1 siRNA or a control scrambled siRNA, followed by treatment with $A\beta_{42}$. Western blotting was performed with antibodies against cyclin D1 or cdk5. Anti-p35 antibody (N-20) was used for immunoprecipitation, and p35-IP was used to assay the associated cdk5 kinase activity as described for B. The mean percentage activity in comparison to the control siRNA–transfected cells (100%) is shown. Error bars reflect SE. *,**$p < 0.001$ by ANOVA ($n = 5$).

information about the regulation of cdk5 by p35 in the presence of $A\beta_{42}$. We found that $A\beta_{42}$ selectively prevented the activation of cdk5 by p35, whereas the total cdk5 activity, which would include p35- as well as p25-associated activity, was stimulated. $A\beta_{42}$ may achieve this by elevating the levels of cyclin D1, which competes with p35 for cdk5, resulting in an inactive cdk5–cyclin D1 complex. These results are consistent with previous studies that indicated that the D-type cyclins interact with cdk5 but fail to stimulate its activity (Xiong *et al.*, 1992; Guidato *et al.*, 1998; Zhang *et al.*, 2010). We observed that cyclin D1 feeds back positively into the MEK-ERK pathway, which also contributes to CRNA. Because cdk5 negatively regulates MEK1-ERK signaling (Sharma *et al.*, 2002; Zheng *et al.*, 2007), the decrease in p35-cdk5 activity may result in the hyperactivation of MEK-ERK. Indeed, when p35 was overexpressed, $A\beta_{42}$-mediated aberrant MEK-ERK signaling was attenuated, which resulted in significant protection from neuronal apoptosis as indicated by a decrease in cleaved caspase 3 levels (Figure 7B). The neurons from cdk5$^{-/-}$ mice exhibit signs of cell cycle reentry and are more susceptible to $A\beta_{42}$ (Cicero and Herrup, 2005), implicating cdk5 in the suppression of the neuronal cell cycle (Zhang *et al.*, 2008). The nuclear localization of cdk5 (Zhang *et al.*, 2008) and its ability to regulate E2F-1/DP1 complex have been suggested to play a role in neuronal cell cycle arrest (Zhang *et al.*, 2010). Our present findings indicate that the down-regulation of p35-cdk5 caused by aberrant activation of MEK-ERK-cyclin D1 may contribute to CRNA. Although further studies are needed, our findings may aid development of new strategies to prevent cell cycle reentry of neurons in situations such as Alzheimer's disease.

MATERIALS AND METHODS

Plasmids, transgenic animals, siRNA, antibodies

For p35 and p25 overexpression, enhanced green fluorescent protein (EGFP) N1-p35 and EGFP N1-p25 plasmids were used. For STAT3 and cyclin D1 knockdown, 25nt duplexes were designed and custom synthesized. The sequence of siRNA against STAT3 (Ng *et al.*, 2006) is as follows: STAT3siRNA, 5′GGAAAUUUAACAUUCUGGGCACGAA 3′; STAT3 control scrambled siRNA, 5′ GGAUUUCAAUUAGUCCGGCAAAGAA 3′. The knockdown of cyclin D1 was achieved with siRNA of following sequence: 5′ GCGAGGAGCAGAAGUGCGAUG3′; cyclin D1 scrambled siRNA, 5′ GCGAGGAGAAGCAGUGCGAUG 3′.

For cyclin D1 overexpression, human cyclin D1 was cloned in pAdTrack adenovirus expression vector as described later. The cyclin D1 promoter construct in PD1-Luc was a gift from Ramin Massoumi, Lund University.

The following antibodies were used for the described studies: cyclin D1 (sc-753, 1:500), PCNA (sc-56, 1:500), phospho-ERK (sc-7383, 1:500), ERK (sc-94, 1:1000), phospho-STAT 3 (Ser-727; sc-80001R, 1:500), STAT3 (sc-483, 1:5000) (all Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase 3 (9664, 1:500; Cell Signaling, Beverly, MA), actin (CP-01; Calbiochem, La Jolla, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-25778, 1:1000; Santa Cruz Biotechnology), GFP (2555, 1:1000; Cell Signaling), p35 (N-20, which recognizes only p35; sc-821, 1:500; Santa Cruz Biotechnology), p35/p25 (C-19, which recognizes both p35 and p25; sc-820, 1:500; Santa Cruz Biotechnology), anti-BrdU (RPN202, Amersham-Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ), and cdk5 (sc-173, 1:500; Santa Cruz Biotechnology).

Primary neuronal and PC12 cultures

Embryonic cortical neurons were isolated from Sprague Dawley rats by using a previously published and standardized protocol.

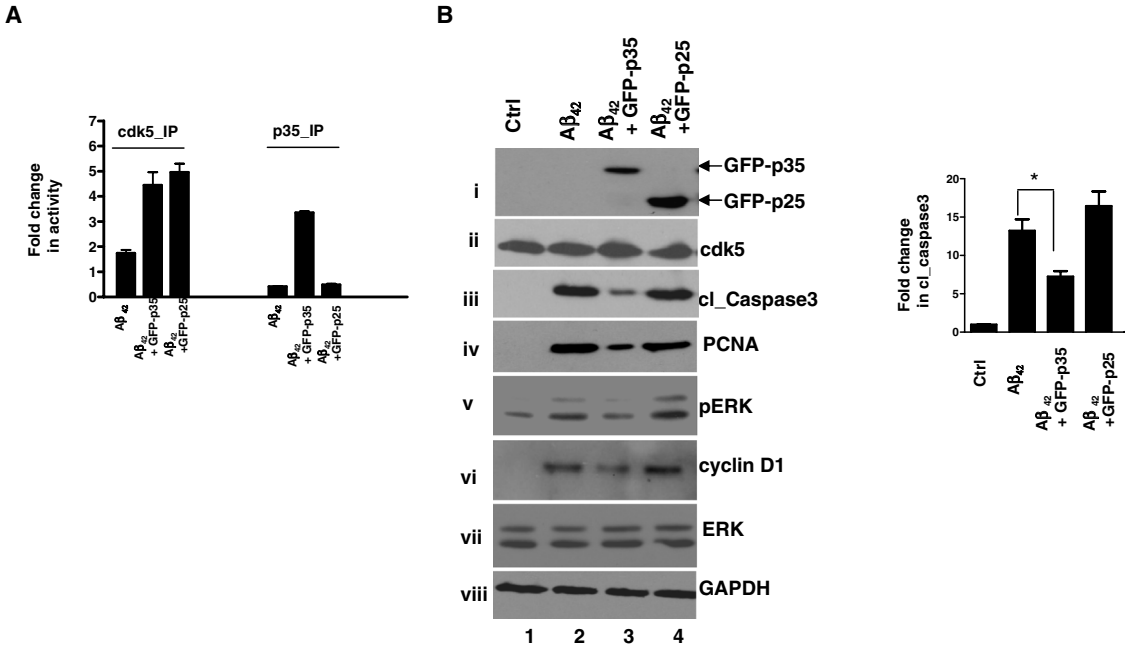

FIGURE 7: p35 overexpression prevents neuronal apoptosis. GFP-p35 or GFP-p25 were transfected in neuronal PC12 cells, followed by $A\beta_{42}$ treatment. (A) p35 or cdk5 was immunoprecipitated, and p35-IP– or cdk5-IP–associated activity was determined and normalized with respect to cdk5 protein levels. Fold increase in the activity compared with untransfected cells was determined, and the average of three independent experiments is shown. Error bars represent SE. (B) Western blotting was performed using the indicated antibodies. Anti-GFP was used to detect the expression of GFP-p35 or GFP-p25. Right, densitometry of the band corresponding to cleaved caspase 3 was performed and normalized with respect to GAPDH, and the fold change in comparison to untreated cells was determined. *$p < 0.05$ by ANOVA ($n = 3$).

Briefly, cortical lobes were dissected from day 18 rat embryos, and the tissue was minced finely before removing excess buffer and trypsinized with Trypsin-DNase solution. Subsequently, DMEM containing 10% fetal bovine serum (FBS) was added. The cells were gently resuspended in DMEM containing 10% FBS and 1% penicillin/streptomycin and plated on poly-L-lysine–coated 35-mm culture plates. After 12 h, cortical neurons were washed with pre-warmed Tyrode's CMF-PBS supplemented with glucose and $NaHCO_3$ and were maintained in Neurobasal media supplemented with B-27, N2, penicillin–streptomycin (1×), L-glutamine, and glucose. Sometimes plasmosin was also used as a precaution against mycoplasma. Typically, cortical neurons were used after 5 d in vitro for experiments.

PC12 cells were maintained in DMEM containing 10% horse serum, 5% FBS, and 1% antibiotic-antimycotic. Neuronal PC12 cells were obtained by differentiation with NGF in low-serum media containing 1% FBS and 1% antibiotic-antimycotic for 5–7 d.

Treatment and transfection of cells

The 42–amino acid version of Aβ peptide ($A\beta_{42}$; rPeptide) or $A\beta_{42rev}$ (Tocris Biosciences, Ellisville, MO) was reconstituted in sterile 1% ammonium hydroxide, sonicated for 30 s, and incubated at 37°C for 5 d and was used at end concentration of 0.5–1 μM. Typically, cells were treated with $A\beta_{42}$ for 48 h and U0126 or 0.1% dimethyl sulfoxide (DMSO; for control experiments) was added 30 min before the addition of $A\beta_{42}$. Typically, fresh media and reagents were added after every 24 h. The transfection of cells with siRNA or plasmid DNA constructs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent, and manufacturer's instructions were followed.

Promoter luciferase assays

Cortical neurons were transfected with 1 μg of cyclin D1 promoter-luciferase or its mutant plasmid along with 100 ng of β-gal-pCMV plasmid, which was used as an internal control, using Lipofectamine 2000. The first two bases of STAT3 site (Leslie *et al.*, 2006) at base pair –984 (TTCCAGCAA) in the cyclin D1 promoter were changed by site-directed mutagenesis. After 48 h, lysates were prepared, and luciferase activity was determined by using the luciferase assay system from Promega (Madison, WI), following the manufacturer's instructions. β-Galactosidase was assayed using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate; the release of chromogen was determined by measuring absorbance at 420 nm. The luciferase activity was normalized with respect to β-galactosidase activity.

BrdU labeling and TUNEL assay

To determine the cell cycle status of neurons upon $A\beta_{42}$ treatment and to correlate it with neuronal apoptosis, we performed BrdU labeling along with the TUNEL assay. Cells were incubated with 10 μM BrdU in fresh media for 48 h, and a fresh pulse of BrdU was given every 4 h. After fixing and permeabilizing the cells, TUNEL assay was performed using the Dead End Fluorometric TUNEL kit from Promega, following the manufacturer's instructions. After the TUNEL procedure, blocking was done with 2% bovine serum albumin (BSA). The incorporated BrdU was detected using anti-BrdU antibody (Amersham-Pharmacia Biotech), and Hoechst 33342 was used as a nuclear stain. The fluorescently labeled cells were visualized and imaged as described later, and the number of BrdU- and TUNEL-positive cells was determined by counting at least 400 cells from a minimum of five different fields.

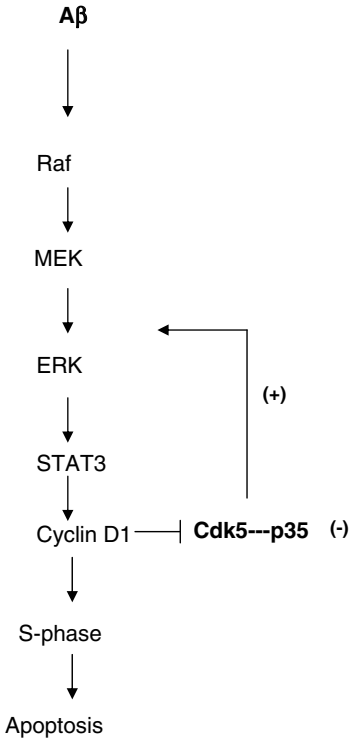

FIGURE 8: A model for cell cycle reentry–mediated apoptosis. Neurotoxic signals might trigger aberrant activation of the MEK-ERK pathway, resulting in an increase in cyclin D1 levels, which can be by transcription factor STAT3. Cyclin D1 prevents p35-mediated cdk5 activation, which might facilitate the increase in MEK-ERK signaling activity. These events can contribute to cell cycle reentry of neurons, which results in their death.

Generation of recombinant adenovirus and transduction

Human cyclin D1-coding region was subcloned in *Xba*I and *Hin*dIII sites of pAdTrack vector. After digestion of this construct with *Pme*I, the DNA was mixed with pAdEasy vector and electroporated in *Escherichia coli* BJ5138. The recombinant clones were digested with *Pac*I and transfected in HEK293 cells. The virus was harvested, amplified, and purified using standard procedures. For infection of neuronal cells, ~10 multiplicity of infection was used for transduction, and GFP fluorescence was observed 24–48 h postinfection.

Immunoblotting and immunofluorescence

After SDS–PAGE, proteins were transferred to nitrocellulose membrane and immunoblotting was performed. The Super Signal West Pico and Super Signal West Dura extended duration chemiluminescence substrate from Pierce (Rockford, IL) were used, following the manufacturer's instructions.

For immunofluorescence assays, cells were plated on poly-L-lysine–coated glass coverslips, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Tween-20 for 20 min. After blocking with 2% BSA for 12 h at 4°C, incubations with primary antibodies and appropriate secondary antibodies (labeled with Alexa Flour 594 or 488) were performed for 2–3 h. The nuclei were stained with Hoechst 33342. Cells were visualized using an AxioImager microscope (Zeiss, Jena, Germany) equipped with MRm camera, and AxioVision software was used for image acquisition and manipulation. Photoshop (Adobe, San Jose, CA) was used for preparing images for illustrations.

Immunoprecipitation and kinase assay

From 50 to 70 µg of protein lysate was incubated with ~1 µg of the desired primary antibody for 12 h at 4°C on an end-to-end shaker in a 200-µl reaction volume. Subsequently, 50 µl of protein A+G Sepharose (Amersham-Pharmacia Biotech) was incubated with the antibody–protein complex for 4–6 h at 4°C. The beads were washed and resuspended in 1× kinase assay buffer. The catalytic activity of immunoprecipitated kinase was assayed in a buffer containing 50 mM Tris, pH 7.5, 10 mM magnesium chloride, 1 mM dithiothreitol, and 100 µM [γ-^{32}P]ATP (6000 Ci/mmol) using 2.5 µg of histone H1 or 100 µM of a peptide (PKTPKKAKKL) derived from H1. Reactions were typically carried out for 40 min at 30°C and were terminated by boiling the assay mix in Lammeli's buffer for 5 min at 100°C, followed by SDS–PAGE. Phosphate incorporation in histone H1 was judged by autoradiography of SDS–PAGE gels, and scintillation counting was done to assess phosphorylation of the peptide substrate.

Image and statistical analysis

Densitometric analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD). For statistical comparisons, the data were analyzed by one-way analysis of variance (ANOVA) using Prism software (GraphPad Software, La Jolla, CA). $p < 0.05$ was considered statistically significant.

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