

Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2

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Abstract

Cyclin-dependent kinase-5 (Cdk5) is required for neuronal survival, but its targets in the apoptotic pathways remain unknown. Here, we show that Cdk5 kinase activity prevents neuronal apoptosis through the upregulation of Bcl-2. Treatment of SH-SY5Y cells with retinoid acid (RA) and brain-derived neurotrophic factor (BDNF) generates differentiated neuron-like cells. DNA damage triggers apoptosis in the undifferentiated cells through mitochondrial pathway; however, RA/BDNF treatment results in Bcl-2 upregulation and inhibition of the mitochondrial pathway in the differentiated cells. RA/BDNF treatment activates Cdk5-mediated PI3K/Akt and ERK pathways. Inhibition of Cdk5 inhibits PI3K/Akt and ERK phosphorylation and Bcl-2 expression, and thus sensitizes the differentiated cells to DNA-damage. Inhibition of ERK, but not PI3K/Akt, abrogates Cdk5-mediated Bcl-2 upregulation and the protection of the differentiated cells. This study suggests that ERK-mediated Bcl-2 upregulation contributes to BDNF-induced Cdk5-mediated neuronal survival.

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Abbreviations: Cdk5, cyclin-dependent kinase-5; RA, retinoid acid; BDNF, brain-derived neurotrophic factor; PI3K, phosphatidylinositol-3 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; NGF, Nerve growth factor; siRNA, small interfering RNA

Introduction

Cyclin-dependent kinase-5 (Cdk5) was initially identified as a member of the cyclin-dependent kinase family of serine/threonine kinases based on sequence homology.¹ In contrast to other Cdk family members, however, Cdk5 is not involved in cell-cycle regulation. Cdk5 shows no enzymatic activity as a monomer, but Cdk5 becomes activated once it interacts and forms a heterodimer with its activator p35 (NCK5a, neuronal Cdk5 activator)² or p39 (NCK5ai, neuronal Cdk5 activator isoform).³ Cdk5 is expressed in all tissues, but its kinase activity is predominantly observed in postmitotic neurons of the nervous system⁴ because the Cdk5 activators p35 and p39 are expressed almost exclusively in the nervous system.⁵

Cdk5 kinase activity has been implicated in neuronal death and survival. Amyloid β -peptide⁶ and ischemic insults⁷ induce the conversion of p35 to its truncated form p25, which causes apoptotic death of neurons. In contrast, the association of p35 and Cdk5 is required for neuronal survival and is involved in many processes in neuronal differentiation and functions most notably neurite outgrowth,⁸ cortical lamination,⁹ neuronal migration,¹⁰ motility and adhesion.¹¹ Cdk5-mediated signals for neuronal survival appear to occur through the activation of phosphatidylinositol-3 kinase (PI3K)/Akt¹² and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway.¹³ However, the mechanisms by which Cdk5-mediated pathways modulate apoptotic signals for neuronal survival remain to be investigated.

There are two major signaling pathways that control the initiation of apoptosis: the extrinsic pathway through death receptors-mediated, caspase-8-initiated cleavage of caspase-3¹⁴ and the intrinsic pathway mediated by mitochondria.¹⁵ The intrinsic pathway is triggered in response to a wide range of death stimuli such as DNA damage, hypoxia and withdrawal of growth factors, resulting in the mitochondrial release of cytochrome *c*.¹⁶ In the cytosol, cytochrome *c* activates caspase-9, which in turn cleaves caspase-3, leading to apoptosis.¹⁷ The intrinsic pathway is regulated by Bcl-2 family proteins: Bax and Bak induce mitochondrial release of cytochrome *c*, whereas Bcl-2 and Bcl-X_L inhibit its release.¹⁵ DNA-damaging agents have been reported to induce apoptosis in neurons through the intrinsic mitochondrial pathway.¹⁸

Neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) regulate neuronal survival, development and function.¹⁹ Deprivation of neurotrophins activates mitochondrial apoptotic pathway.^{20,21} BDNF has been reported to induce neuronal differentiation of human SH-SY5Y cells through activation of p35/Cdk5 kinase activity.²¹ Here, we show that BDNF-induced p35/Cdk5 kinase activity upregulates Bcl-2 through the ERK pathway during neuronal differentiation, and thus protects the differentiated neuron-like cells from DNA damage-induced apoptosis. Inhibition of Cdk5 with small interfering RNA

(siRNA) inhibits ERK pathway and downregulates Bcl-2 in human neurons, thus rendering them vulnerable to DNA damage. These results suggest that ERK-mediated Bcl-2 upregulation provides one of the molecular pathways by which p35/Cdk5 mediates neuronal survival.

Results

The differentiated neuron-like cells become resistant to DNA-damaging agents

Sequential treatment of the human SH-SY5Y neuroblastoma cell line with retinoid acid (RA) and BDNF generates nearly pure populations of human neuron-like cells, thus providing a model for the study of neuronal differentiation and survival.²¹ Treatment of SH-SY5Y cells with 10 μ M RA in the culture medium containing 10% fetal bovine serum resulted in neurite outgrowth that appeared on day 3 and extended on days 4 and 5 (Figure 1a). The cells were then cultured in serum-free culture medium with 100 ng/ml BDNF for an additional 5 days.

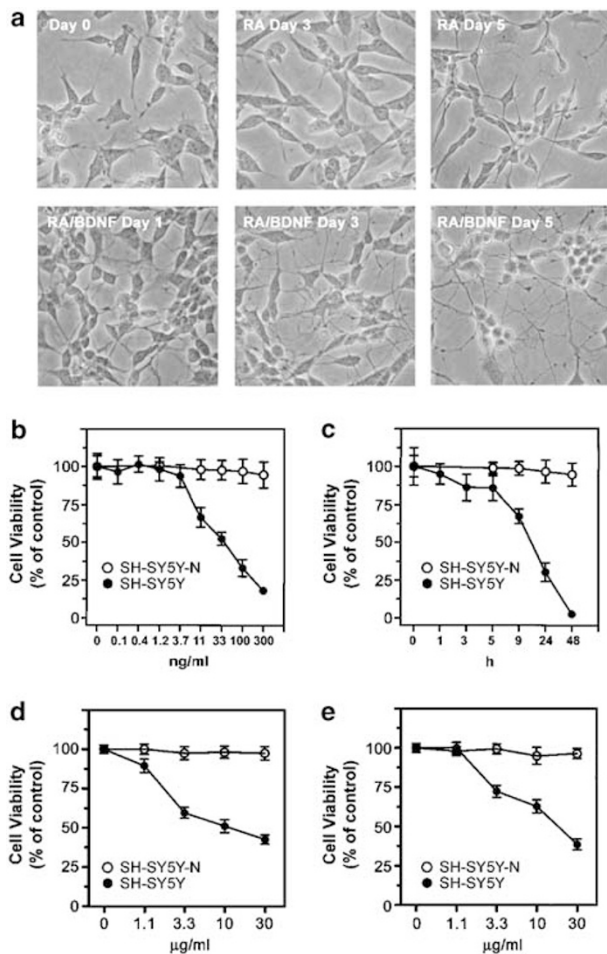


Figure 1 The differentiated SH-SY5Y-N cells are resistant to camptothecin-induced cell death. (a) SH-SY5Y cells were differentiated by sequential treatment with RA and BDNF. The undifferentiated SH-SY5Y (●) and the differentiated SH-SY5Y-N cells (○) were treated for 16 h with various doses of camptothecin (b), etoposide (d) and cisplatin (e) or 30 ng/ml camptothecin over a time course (c). Cell viability was analyzed by the trypan blue exclusion assay (mean \pm S.E.M.; $n = 6$)

This sequential treatment yielded a nearly pure population of differentiated neuron-like cells (SH-SY5Y-N) characterized by abundant neurite outgrowth (Figure 1a) and expression of neuronal markers such as neurofilaments and neuron-specific enolase.²¹ SH-SY5Y-N cells remained differentiated in the BDNF-containing medium for at least 3 weeks. In contrast, however, withdrawal of BDNF resulted in a rapid cell death of the differentiated SH-SY5Y-N cells. Approximately, 15 and 45% of cell death were observed in the differentiated cells 24 and 48 h, respectively, after the cells were left in BDNF-free medium, keeping in line with the previous study.²¹ These results indicate that the differentiated SH-SY5Y-N cells depend on BDNF for survival.

The differentiated cells were then examined for their vulnerability to DNA-damaging agents, as compared to the undifferentiated cells. Both the differentiated and undifferentiated cells were treated with various doses of camptothecin for 16 h and cell viability analysis showed that camptothecin killed the undifferentiated SH-SY5Y cells in a dose-dependent manner (0.1–300 ng/ml), with an EC₅₀ of 33 ng/ml (Figure 1b). In contrast, the differentiated SH-SY5Y-N cells were resistant to camptothecin (Figure 1b). SH-SY5Y and SH-SY5Y-N cells were then treated with 30 ng/ml camptothecin for various periods of time. The results showed a time-dependent cell death of SH-SY5Y but not SH-SY5Y-N cells (Figure 1c). Finally, the cells were treated with other DNA-damaging agents, etoposide (Figure 1d) and cisplatin (Figure 1e), and the results further confirmed that the differentiated cells are resistant to the DNA-damaging insults.

Mitochondrial apoptotic pathway is inhibited in the differentiated neuron-like cells

There are several lines of evidence that mitochondria play a crucial role for neuronal survival. Camptothecin has been reported to induce apoptosis in rat neurons through activation of mitochondrial pathway.¹⁸ The findings that the differentiated SH-SY5Y-N cells are resistant to camptothecin suggest that RA/BDNF treatment may result in the inhibition of the mitochondrial apoptotic pathways. We therefore conducted systematic experiments to illustrate signal events through which RA/BDNF may modulate the mitochondrial pathway.

It has been shown that p53 is involved in DNA damage-induced apoptosis through the mitochondrial pathway.²² Western blot analysis revealed an increase in the p53 expression in both SH-SY5Y and SH-SY5Y-N cells in response to camptothecin treatment, suggesting that the p53-mediated pathway may not play a direct role in the protection of the differentiated cells (Figure 2). Caspase-8 has been reported to cleave Bid, which then links death receptor-mediated extrinsic pathway to intrinsic mitochondrial pathway.²³ Western blots detected no caspase-8 protein in either the differentiated and undifferentiated cells (Figure 2), which is consistent with a report that human neuroblastoma cells lack caspase-8 expression.²⁴ Bid cleavage, suggested by the reduced levels of its protein expression (Figure 2), was not observed until other caspases were activated in SH-SY5Y cells. The results indicate that the caspase-8-mediated Bid pathway is inactive in the undifferentiated SH-SY5Y cells.

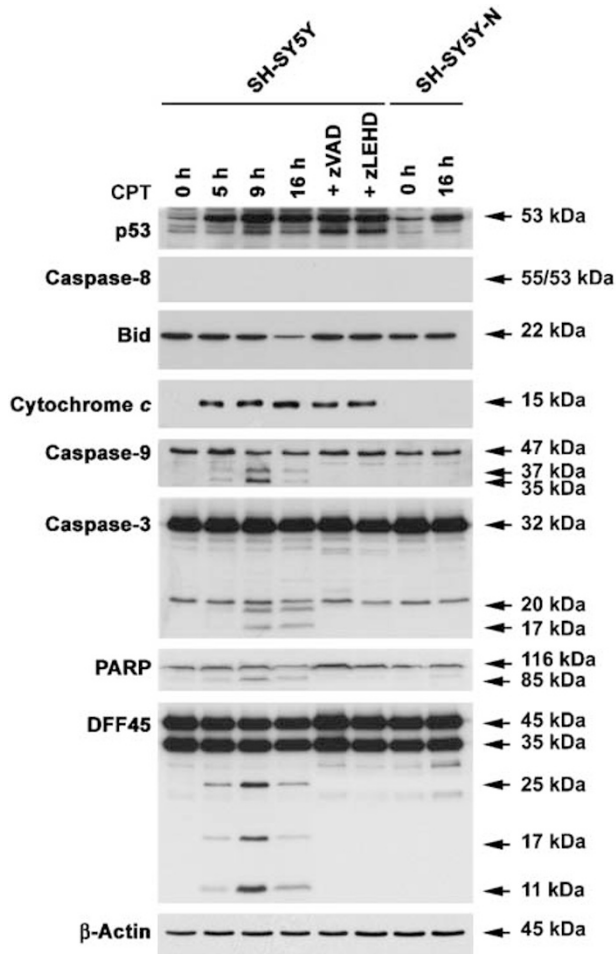


Figure 2 Mitochondrial pathway is inhibited in SH-SY5Y-N cells. SH-SY5Y and SH-SY5Y-N cells were treated with 30 ng/ml camptothecin for the times indicated. SH-SY5Y cells were also treated with 30 ng/ml camptothecin (CPT) for 16 h in the presence of 20 μ M pan-caspase inhibitor z-VAD-fmk (zVAD) or caspase-9 specific inhibitor z-LEHD-fmk (zLEHD). The cells were then subjected to Western blot analysis for expression of p53, Bid, and caspase-8, and for the cleavage of caspase-9, caspase-3, PARP and DFF45. Some of the cells were also subjected to subcellular fractionation. The cytosolic fractions that were free of mitochondria were examined by Western blots for the presence of cytochrome *c* (panel 4). β -actin was used as protein load control

We then examined mitochondrial release of cytochrome *c*, a crucial event in the activation of mitochondrial pathway. The cells were treated with 30 ng/ml camptothecin and then subjected to subcellular fractionation. Western blots detected cytochrome *c* in mitochondria-free cytosolic fractions obtained from SH-SY5Y cells, but not SH-SY5Y-N cells (Figure 2). Western blots also detected cleavage products of caspase-9, caspase-3, poly (ADP-ribose) polymerase-1 (PARP-1) and DNA fragmentation factor 45 (DFF45), a substrate of caspase-3²⁵ in SH-SY5Y, but not SH-SY5Y-N cells (Figure 2). Treatment of the undifferentiated cells with caspase-9 inhibitor carbobenzyloxy-leu-glu(*O*-methyl)-his-asp-(*O*-methyl)-fluoromethyl ketone (z-LEHD-fmk) or pan-caspases inhibitor carbobenzyloxy-Val-Ala-Asp(*O*me)-fluoromethyl ketone (z-VAD-fmk) eliminated camptothecin-induced cleavage of caspase-9, caspase-3, PARP-1 and DFF45, but not the upstream mitochondrial release of cytochrome *c* (Figure 2).

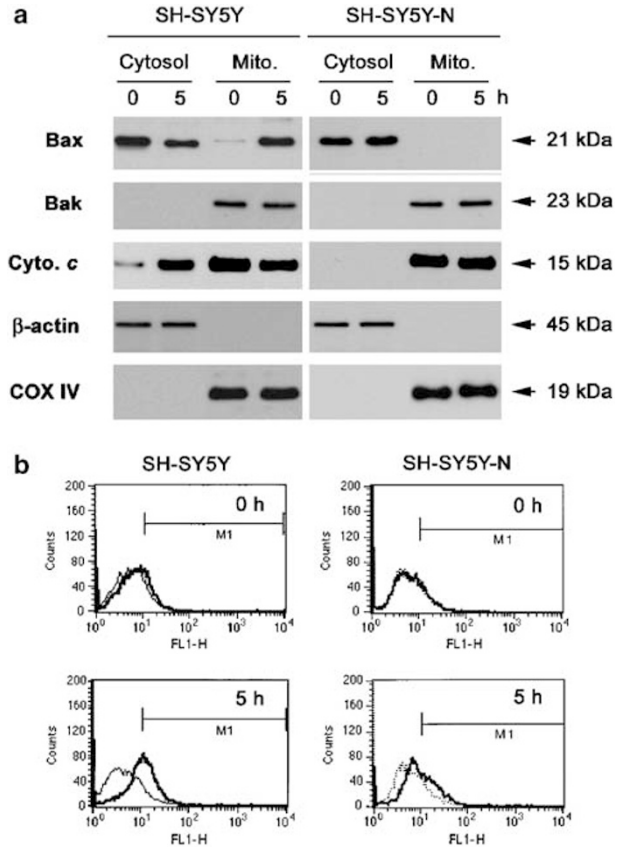


Figure 3 Bax translocation and Bak conformational changes are inhibited in SH-SY5Y-N cells. (a) Subcellular fractionation analysis of Bax and Bak. SH-SY5Y (left) and SH-SY5Y-N cells (right) were treated with 30 ng/ml camptothecin for the times indicated and subjected to subcellular fractionation. Cytosolic and mitochondrial (mito) fractions were subjected to Western blots using antibodies to Bax, Bak and cytochrome *c* (Cyto. *c*). β -actin and COX IV were used as loading controls for cytosolic and mitochondrial fractions, respectively. (b) Flow cytometry analysis of Bak conformational change. SH-SY5Y (left) and SH-SY5Y-N cells (right) were treated as described above and analyzed by flow cytometry using an antibody against the NH₂-terminus of Bak (dark line). Mouse IgG1 antibody was used as a negative control (grey line)

The results indicate that mitochondrial release of cytochrome *c* is inhibited in the differentiated SH-SY5Y-N cells.

Bcl-2 protects the differentiated neuron-like cells from camptothecin-induced apoptosis

To explore the molecular mechanisms involved in the mitochondrial inhibition, we examined the roles of Bcl-2 family proteins in camptothecin-induced apoptosis. Bax translocates from the cytosol to the mitochondrial membrane to trigger mitochondrial release of apoptotic factors.²⁶ Indeed, Western blot analysis of subcellular fractions revealed a decrease of Bax in the cytosol, but an increase of Bax in the mitochondrion in camptothecin-treated SH-SY5Y cells (Figure 3a). Bak resides in the mitochondrial membrane and Western blots detected Bak in mitochondrial but not cytosolic fractions in both cell types (Figure 3a). DNA damage-induced activation of the mitochondrial pathway occurs through induction of a conformational change in Bak, resulting in the exposure of the NH₂-terminus that is normally fold within the Bak protein.²⁷

Indeed, flow cytometry analysis with the antibody against the Bak NH₂-terminus revealed the conformational change of Bak in camptothecin-treated SH-SY5Y cells (Figure 3b). The Bax translocation and Bak conformational change were associated with mitochondrial release of cytochrome *c* in SH-SY5Y cells after exposure to camptothecin (Figure 3a).

In contrast, subcellular fractionation analysis revealed neither Bax translocation nor mitochondrial release of cytochrome *c* in SH-SY5Y-N cells treated with camptothecin (Figure 3a); the results suggest that RA/BDNF treatment may result in the inhibition of Bax and Bak expression and/or apoptotic functions. To test this, we first examined expression of Bcl-2 family proteins and showed a gradual decrease in Bak and increase in Bcl-2 expression during the SH-SY5Y differentiation (Figure 4a and b). Bcl-2 is well known for its role in inhibition of Bax and Bak apoptotic functions: one study has demonstrated that Bcl-2 inhibit Bax conformational change through interaction with Bak.²⁸ While another study has shown that Bcl-2 prevents Bax translocation from cytosol

to mitochondrial membrane.²⁹ We therefore examined the roles of Bcl-2 in the protection of the differentiated neuronal cells from camptothecin-induced apoptosis.

We first inhibited Bcl-2 expression in SH-SY5Y-N cells with small interfering RNA (siRNA). Two synthetic siRNA duplexes (Bcl-2-siRNA1, Bcl-2-siRNA2) targeting *Bcl-2* gene were generated; however, only Bcl-2-siRNA1 inhibited Bcl-2 expression in SH-SY5Y-N (Figure 4c). SH-SY5Y-N cells were transfected either with Bcl-2-siRNA1 or Bcl-2-siRNA2 as negative control. The results show that Bcl-2-siRNA1 transfection sensitized SH-SY5Y-N cells to camptothecin-induced apoptosis, as observed by the cleavage of caspase-3 and DFF45 (Figure 4c), cell death (Figure 4d) and cellular apoptosis (Figure 4e).

We then transfected Bcl-2 cDNA in SH-SY5Y cells to determine if Bcl-2 overexpression protects the undifferentiated cells. Once SH-SY5Y cells were transfected with Bcl-2 (Figure 5a), they became resistant to camptothecin-induced apoptosis, as Western blots detected no caspase-3 cleavage

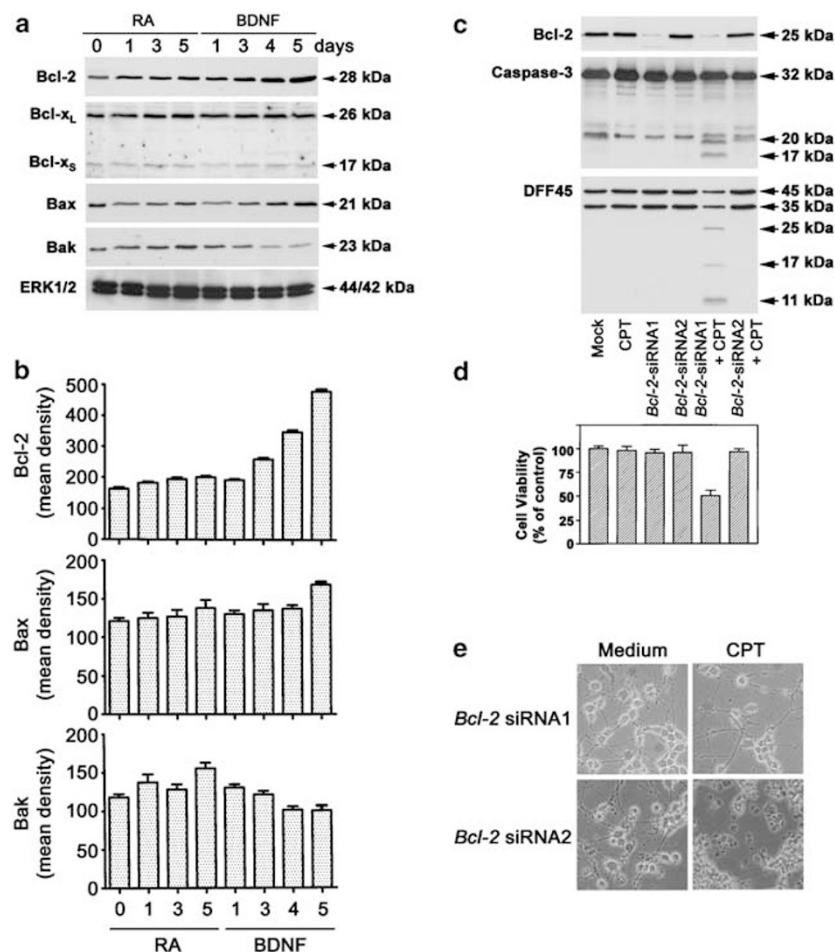


Figure 4 Inhibition of Bcl-2 in SH-SY5Y-N cells sensitizes the cells to camptothecin (CPT)-induced apoptosis. (a) Western blot analysis of the expression of Bcl-2 family proteins during SH-SY5Y differentiation. ERK1/2 was used as protein loading control. (b) The bar graphs show the densitometric analysis of Bcl-2, Bax and Bak expression levels during the SH-SY5Y neuronal differentiation. (c) Western blot detection of Bcl-2 expression and cleavage of caspase-3 and DFF45 in SH-SY5Y-N cells transfected with Bcl-2 siRNA for 24 h and then treated with 30 ng/ml camptothecin (CPT) for 5 h. (d) Cell viability analysis of SH-SY5Y-N cells following the treatments described above by trypan blue exclusion analysis. Bars represent mean \pm S.E.M. ($n = 6$). (e) Phase contrast microscopy of cellular apoptosis in SH-SY5Y-N cells transfected with Bcl-2 siRNA for 24 h and treated with camptothecin for 16 h

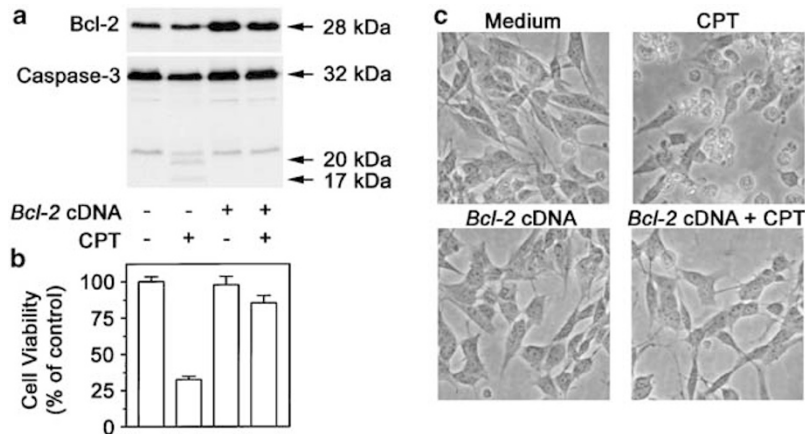


Figure 5 Bcl-2 overexpression protects SH-SY5Y cells from camptothecin (CPT)-induced apoptosis. (a) Western blot analysis of Bcl-2 expression and caspase-3 cleavage in SH-SY5Y cells after the cells were transfected with Bcl-2 expressing vector pUSEamp for 24 h and then treated with 30 ng/ml camptothecin (CPT) for 5 h. (b) Cell viability analysis of SH-SY5Y cells transfected with Bcl-2 expressing vector pUSEamp and treated with 30 ng/ml camptothecin for 16 h (mean \pm S.E.M.; $n = 6$). (c) Phase contrast microscopy of cellular apoptosis in SH-SY5Y cells transfected with Bcl-2 pUSEamp for 24 h and treated with camptothecin for 16 h

(Figure 5a), cell viability analysis revealed no significant cell death (Figure 5b) and phase contrast microscopy showed no cellular apoptosis (Figure 5c). Taken together, these studies with siRNA downregulation of Bcl-2 and Bcl-2 cDNA overexpression suggest that Bcl-2 may play a role in the survival of the differentiated and undifferentiated cells. This prompted us to examine BDNF-induced signal events that lead to Bcl-2 upregulation and neuronal survival during the SH-SY5Y cell differentiation.

RA/BDNF activates Cdk5-mediated PI3K/Akt and ERK pathways

NGF has been reported to upregulate the Cdk5 activator p35 and induce Cdk5 kinase activity in the differentiated PC12 cells.¹³ We therefore examined if BDNF induces p35 and Cdk5 protein expression and Cdk5 kinase activity during the SH-SY5Y differentiation. Western blot analysis showed an increased expression of p35, but not Cdk5 during the neuronal differentiation (Figure 6a). Cdk5 protein was then immunoprecipitated and Cdk5 kinase activity was examined using histon-H1 as a substrate. The activity of Cdk5 kinase was slightly increased by RA treatment, while the RA/BDNF treatment resulted in much higher Cdk5 kinase activity in SH-SY5Y-N cells (Figure 6b and c). The results indicate that RA/BDNF treatment upregulates p35 and thus activates Cdk5 kinase during the SH-SY5Y differentiation.

Study of PC12 cells has suggested that p35/Cdk5-mediated neuronal survival involves the ERK pathway¹³ and PI3K/Akt pathway.¹² ERK activation requires phosphorylation by an upstream activator MAPK/ERK kinase (MEK).³⁰ Indeed, Western blots detected a significant increase in expression of phosphorylated ERK1/2 proteins in both RA and RA/BDNF-treated cells (Figure 6a). We then examined the PI3K-mediated phosphorylation of Akt protein.³¹ The phosphorylated Akt protein was detected on Western blots in SH-SY5Y cells after RA treatment while the sequential treatment with RA/BDNF resulted in a higher and sustained

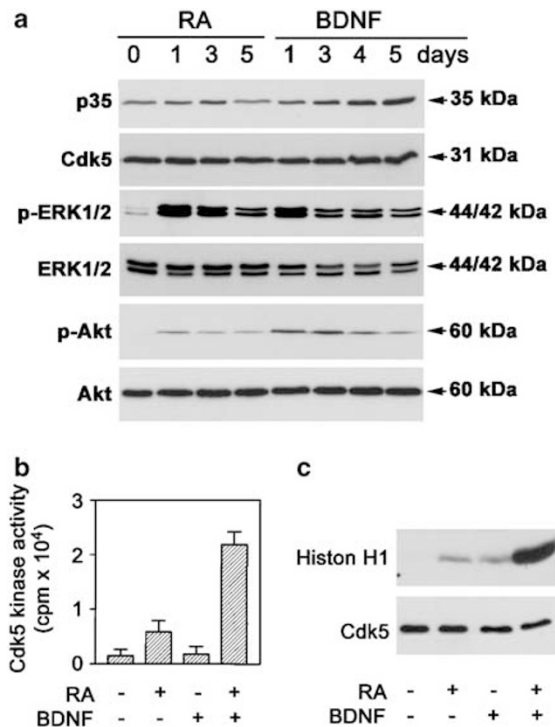


Figure 6 RA/BDNF treatment activates p35/Cdk5 kinase activity. (a) Akt and ERK phosphorylation. SH-SY5Y cells were treated first with RA for 5 days and then with BDNF for additional 5 days and examined by Western blot for expression of p35, Cdk5, phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated Akt (p-Akt), and Akt. (b, c) Cdk5 immunoprecipitation/kine assays. SH-SY5Y cells were treated with RA for 5 days or sequential RA/BDNF treatments for 10 days and then immunoprecipitated with anti-Cdk5 antibody. Cdk5 kinase activity was measured by radioactivity (b) and Western blot analysis of histon H1 (c)

expression of phosphorylated Akt (Figure 6a). These results indicate that RA/BDNF activates the ERK and PI3K/Akt pathways during the SH-SY5Y cell differentiation.

Cdk5-mediates Bcl-2 upregulation through ERK pathway

To define the roles of the PI3K and ERK pathways in Cdk5-mediated neuronal survival, we inhibited Cdk5 kinase activity first with the Cdk5 inhibitor roscovitine¹³ and then specifically silenced *Cdk5* gene expression with synthetic Cdk5 siRNA. Roscovitine treatment of SH-SY5Y-N cells inhibited Akt and ERK phosphorylation and Bcl-2 expression (Figure 7a), and sensitized the cells to camptothecin-induced apoptosis, as indicated by caspase cleavage (Figure 7b), cell death (Figure 7c) and cellular apoptosis (Figure 7d). Transfection of Cdk5 siRNA in SH-SY5Y-N cells inhibited Cdk5 kinase

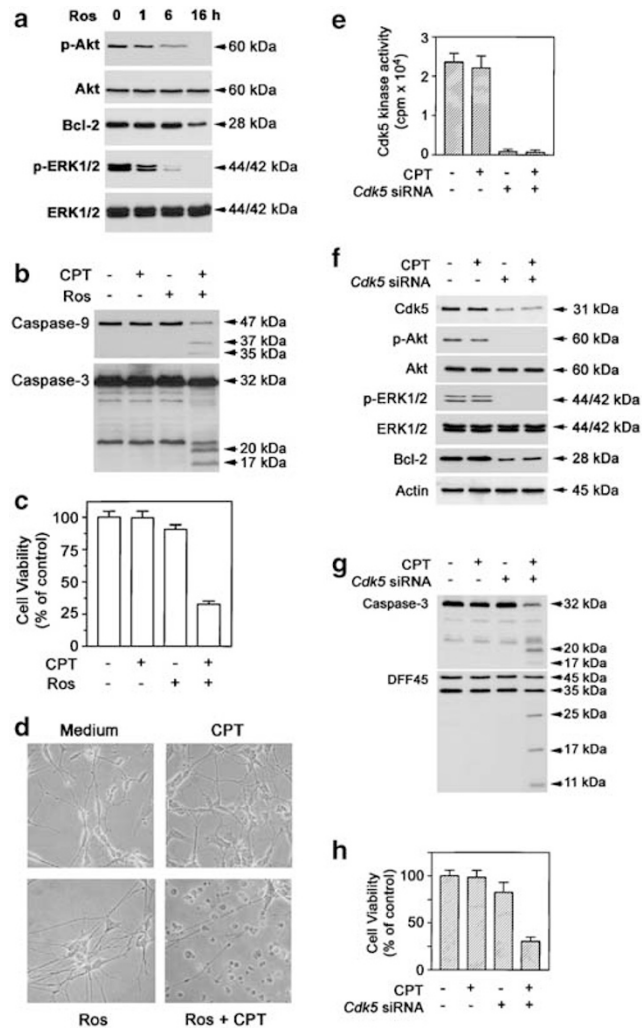


Figure 7 Inhibition of Cdk5 inhibits Akt and ERK phosphorylation and Bcl-2 expression. (a–d) Treatment with Cdk5 inhibitor roscovitine. SH-SY5Y-N cells were treated with 10 μ M roscovitine (Ros) and subjected to Western blots for detection of Akt and ERK phosphorylation and Bcl-2 expression (a). SH-SY5Y-N cells were treated with 10 μ M roscovitine for 16 h followed by camptothecin treatment for 5 h to detect cleavage of caspase-9 and caspase-3 by Western blots (b) or for 16 h to examine cell viability (mean \pm S.E.M.; $n = 6$) (c) and to examine cellular apoptosis under microscopy (d). (e–h) Cdk5 siRNA transfection. SH-SY5Y-N cells were transfected with Cdk5 siRNA for 24 h and examined for Cdk5 kinase activity using histon H 1 as a substrate (e). Cdk5 siRNA-transfected cells were treated with camptothecin and examined for protein expression and phosphorylation (f), cleavage of caspases (g) and cell viability (mean \pm S.E.M.; $n = 6$) (h).

activity (Figure 7e), Cdk5 and Bcl-2 protein expression and Akt and ERK phosphorylation (Figure 7f). Camptothecin treatment triggered apoptosis in the Cdk5 siRNA-transfected SH-SY5Y-N cells, as demonstrated by caspase-3 and DFF45 cleavage (Figure 7g) and cell death (Figure 7h). The results suggest that the p35/Cdk5-mediated Bcl-2 upregulation may occur through the PI3K and/or ERK pathway.

To differentiate the role of the PI3K and ERK pathway in Cdk5-mediated Bcl-2 upregulation and neuronal survival, we first treated the differentiated cells with the PI3K pharmacological inhibitor LY294002. LY294002 has been shown to have no inhibitory effect on NGF-induced p35/Cdk5 activity.¹³ Treatment of SH-SY5Y-N cells with 50 μ M LY294002 almost completely inhibited Akt phosphorylation but had no effect on Bcl-2 expression (Figure 8a). Combination treatment of SH-SY5Y-N cells with LY294002 and camptothecin failed to trigger apoptosis in the cells, as demonstrated by Western blot analysis of caspase cleavage (Figure 8b) and cell viability analysis (Figure 8c). These results suggest that Cdk5-mediated Bcl-2 upregulation occurs through PI3K/Akt-independent pathways.

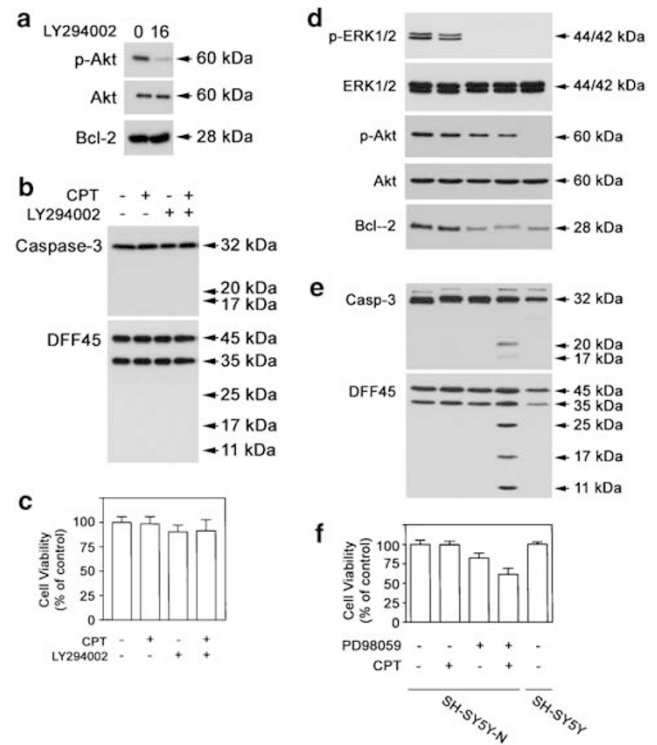


Figure 8 Inhibition of ERK1/2 but not PI3K downregulates Bcl-2 expression. (a–c) Inhibition of PI3K. SH-SY5Y-N cells were treated with 20 μ M PI3K inhibitor LY294002 for 16 h and examined for p-Akt, Akt and Bcl-2 expression on Western blots (a). LY294002 treated cells were then treated with 30 ng/ml camptothecin (CPT) for 5 h and subjected to Western blot analysis of caspase-3 and DFF45 cleavage (b) or for 16 h for cell viability analysis (mean \pm S.E.M.; $n = 6$) (c). (d–f) Inhibition of ERK1/2. SH-SY5Y-N cells were treated with 50 μ M the MEK inhibitor PD98059 for 16 h and then with 30 ng/ml camptothecin for 16 h to examine protein expression and phosphorylation by Western blots (d), for 5 h to detect cleavage of caspase-3 and DFF45 (e) and for 16 h to examine cell viability (mean \pm S.E.M.; $n = 6$) (f).

We then treated SH-SY5Y-N cells with PD98059, a specific inhibitor of the MEK1³² to determine if Cdk5-mediated Bcl-2 upregulation occurs through the ERK pathway. Treatment of SH-SY5Y-N cells with 50 μ M PD98059 for 16 h inhibited ERK1/2 phosphorylation as well as Bcl-2 protein expression (Figure 8d), causing a synergistic effect on camptothecin-induced apoptosis, as evident by caspase-3 and DFF45 cleavage (Figure 8e) and significant cell death (Figure 8f). Collectively, the results indicate that RA/BDNF-induced Cdk5-mediated upregulation of Bcl-2 expression occurs through the activation of the ERK1/2 pathway. This ERK1/2-mediated Bcl-2 upregulation may provide one of the molecular pathways for Cdk5-mediated neuronal survival.

Cdk5 contributes to BDNF neuroprotection of primary human neurons

These studies of SH-SY5Y cell line suggest that the Cdk5-mediated Bcl-2 expression occurs through ERK pathway during neuronal differentiation. To determine if the Cdk5-

mediated Bcl-2 pathway also plays role in the survival of primary human neurons, we generated enriched (over 90% purity) cultures of human neurons (Figure 9a), based on the protocols as we previously reported.³³ The human neurons were first treated with various doses of camptothecin and the cell viability assay showed that camptothecin killed primary human neurons in a dose dependent manner with toxicity manifesting at camptothecin concentrations of more than 100 ng/ml (Figure 9b). Next, we examined the human neurons to determine if Cdk5-mediated ERK and Bcl-2 signal pathway contributes to BDNF-induced neuroprotection of the primary human neurons.

BDNF has been reported to activate ERK1/2 in cortical rat neurons and ERK1/2 activation reaches its peak at 7 h and maintained for 12 h under the BDNF treatment.³⁴ To examine the role of BDNF-mediated Cdk5 pathway in the survival of human neurons, we transfected the human neurons with Cdk5 siRNA for 24 h to inhibit Cdk5. The Cdk5 siRNA-transfected neurons were then treated with 300 ng/ml BDNF for 6 h and subjected to Western blots analysis. Western blots

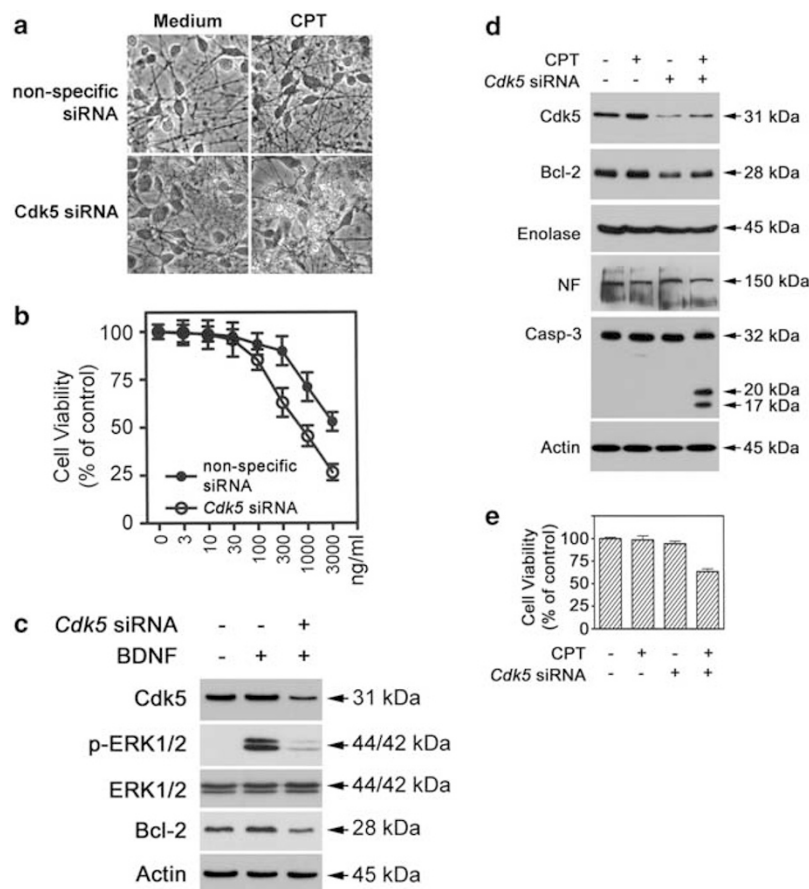


Figure 9 Cdk5 siRNA inhibits ERK1/2 phosphorylation and Bcl-2 expression and enhances camptothecin-induced apoptosis in human neurons. **(a)** Primary cultures of human neurons. The human neurons were cultured in the medium (medium) and treated with camptothecin (CPT) after transfected with nonspecific siRNA or Cdk5 siRNA. **(b)** Camptothecin dose response curve. Human neurons, either nontransfected or transfected with Cdk5 siRNA, were treated with various doses of camptothecin for 24 h and subjected to cell viability analysis. **(c)** Cdk5 siRNA transfection. Human neurons were transfected with Cdk5 siRNA for 24 h and then treated with 300 ng/ml BDNF for 6 h before subjected to Western blot analysis of ERK1/2 phosphorylation and Bcl-2 expression. Actin was used as protein loading control. **(d)** Caspase-3 cleavage. The Cdk5 siRNA-transfected human neurons as described above **(c)** were treated with 100 ng/ml camptothecin for 10 h and then subjected to Western blot analysis for expression of Cdk5, Bcl-2, enolase and neurofilament (NF) proteins and cleavage of caspase-3. **(e)** Cell death. The Cdk5 siRNA-transfected human neurons were further treated with 100 ng/ml camptothecin for 16 h and examined by cell viability analysis (mean \pm S.E.M.; $n = 4$)

indeed detected a significant decrease of the expression of Cdk5 and Bcl-2 and phosphorylated ERK1/2 (Figure 9c and d). Transfection of Cdk5 siRNA did not affect expression of neuronal proteins such as enolase and neurofilament (NF) (Figure 9d). The Cdk5 siRNA-transfected human neurons were then treated with various doses of camptothecin to determine if the inhibition of Cdk5 renders the human neurons vulnerable to camptothecin. Phase contrast microscopy showed cellular apoptosis (Figure 9a), Western blots detected cleavage of caspase-3 (Figure 9d) and cell viability assay revealed enhanced cell death (Figure 9b and e) in camptothecin-treated human neurons-transfected with Cdk5 siRNA. The results validate the finding observed in the study of SH-SY5Y cells that Cdk5-mediated ERK1/2 and Bcl-2 pathway contributes to BDNF neuroprotection of human neurons against DNA damage-induced apoptosis.

Discussion

Transgenic mice provided the first evidence that p35/Cdk5 kinase activity is required for neuronal survival during the development of the nervous system.⁹ Studies of rat neurons in culture have further demonstrated that NGF and BDNF induce p35/Cdk5 kinase activity for neuronal survival.¹³ Through a systematic analysis of the interaction between BDNF-induced p35/Cdk5-mediated pathway and camptothecin-induced apoptotic pathway during SH-SY5Y neuronal differentiation, we report here that BDNF-induced p35/Cdk5 activity upregulates Bcl-2 through ERK1/2 pathway during neuronal differentiation and the ERK1/2-mediated Bcl-2 expression contributes to Cdk5-mediated neuronal survival (Figure 10).

NGF and BDNF induce p35/Cdk5 kinase activity during neuronal differentiation in culture.^{13,20} We have further shown that BDNF treatment upregulates p35 and activates Cdk5 kinase, thus protecting the differentiated neuron-like cells from DNA damage-induced apoptosis. Inhibition of Cdk5

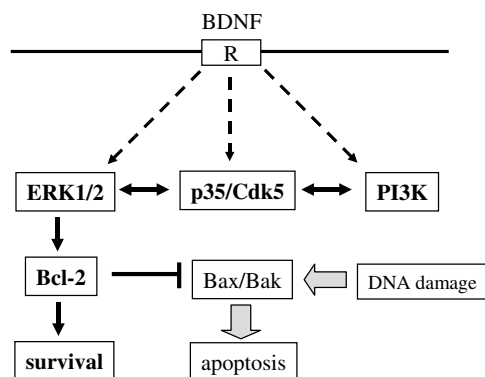


Figure 10 Schematic diagram of ERK1/2-mediated Bcl-2 expression for BDNF-induced Cdk5-mediated neuroprotection. The BDNF treatment induces p35 expression and thus Cdk5 kinase activation. BDNF activates ERK1/2 and PI3K pathway through Ras and Raf pathway for neuronal survival.¹⁹ Inhibition of p35/Cdk5 prevents ERK1/2 and PI3K activation; the results suggest that Cdk5 is required for ERK1/2 and PI3K activation during the BDNF-induced neuronal differentiation. Inhibition of ERK1/2, but not PI3K, downregulates Bcl-2, suggesting that ERK1/2 pathway mediates Bcl-2 expression. Bcl-2 inhibits Bax and Bak apoptotic functions and thus protects neurons from DNA damage-induced activation of mitochondrial pathway

kinase activity with either its pharmacological inhibitor or synthetic siRNA resulted in vulnerability of the differentiated cells to DNA damage agents such as camptothecin, providing clear evidence that Cdk5 activity is required for neuronal survival during neuronal differentiation. Mature murine cortical neurons¹⁸ and human fetal neurons, as demonstrated in this study, undergo apoptosis after exposure to high toxic doses of camptothecin; however, inhibition of Cdk5 renders the human neurons more vulnerable to camptothecin-induced apoptosis. Clearly, the results presented here demonstrate that Cdk5-mediated signal events play an important role in protection of primary human neurons from DNA damage insults.

NGF activates the PI3K/Akt pathway during PC12 cell differentiation³⁵ perhaps through Ras and Raf pathway.¹⁹ Cdk5 knockout mice show inhibition of the PI3K/Akt pathway in neurons.¹² Cdk5 phosphorylates the neuregulin receptor ErbB2/3 and thus is involved in neuregulin-induced Akt activity and neuregulin-mediated neuronal survival.¹² An earlier study indicated that Akt phosphorylates Bad, a Bcl-2 family member, for cell survival.³⁶ In this study, we have shown that BDNF-induced Cdk5 kinase activity upregulated Bcl-2 during the neuronal differentiation. Inhibition of PI3K in the differentiated cells, however, neither prevented Cdk5-mediated Bcl-2 upregulation nor sensitized the cells to the DNA damage-induced apoptosis. Therefore, the BDNF-induced Cdk5-mediated Bcl-2 pathway for neuronal survival occurs through a PI3K/Akt-independent pathway.

Studies of PC12 cells have shown that MAPK/ERK1/2 mediates NGF-induced Cdk5 kinase activity.¹³ The finding in this study that inhibition of Cdk5 activity inhibits ERK1/2 phosphorylation further supports the notion that the ERK1/2 pathway involves in the Cdk5-mediated cell signal events. NGF has been reported to activate the Bcl-2 promoter through MEK/MAPK in PC12 cells,³⁷ while NGF induces Bcl-2 expression in sympathetic neurons through cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-dependent transcriptional mechanisms.³⁸ Here, we demonstrate that Cdk5-mediated Bcl-2 upregulation occurs through the ERK1/2 pathway during neuronal differentiation and ERK1/2-mediated Bcl-2 expression contributes to Cdk5-mediated neuronal survival. Clearly, further investigation of the interactions between Cdk5-mediated signaling pathways and apoptotic pathways will shed more light on the molecular mechanisms that control neuronal death and survival during neuronal differentiation and disease.

Materials and Methods

SH-SY5Y cell culture and differentiation

Human SH-SY5Y neuroblastoma cell line (kindly provided by Dr JX Comella, Lleida, Spain) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μ g/ml), and 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO) at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂. Cells were differentiated into neuronal phenotype as described previously.²¹ Briefly, cells were seeded at an initial density of 10⁴ cells/cm² in culture dishes and incubated for five days in the medium with 10 μ M 13-*cis*-retinoic acid (RA, Sigma). The cells were then incubated in serum-free DMEM with 100 ng/ml BDNF (Alomone

Labs, Har Botzvim Hi-Tech Park, Jerusalem, Israel) for an additional 5 days to generate the differentiated SH-SY5Y-N cells. The differentiated and undifferentiated cells were treated with camptothecin, cisplatin and etoposide (Sigma) in the presence or absence of the caspase-9 inhibitor z-LEHD-fmk (R&D) or the pan-caspase inhibitor z-VAD-fmk (R&D). Cell viability was determined using the trypan blue exclusion assay. The cells were also treated with the Cdk5 inhibitor roscovitine (Sigma), the PI3K inhibitor LY294002 and the MEK1 inhibitor PD98059 (Cell Signaling).

Human neuron culture

Human neuron cultures were prepared from brains of human embryos of 10–18 weeks gestation that were obtained at therapeutic abortions as approved by local institutional ethics committees.³³ In brief, 5–15 g of brain were diced into fragments of 1 mm and incubated for 15 min at 37°C in 40 ml PBS containing 0.25% trypsin and 200 µg/ml DNase I. The suspension was washed through a filter of 130 µm pore size and the filtrate was centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in PBS and centrifuged again. The cell pellet was suspended and cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 0.1% dextrose and antibiotics, and cells were plated directly either into 96 well plates or culture flasks coated with 10 µg/ml polylysine. The cells were cultured for 2 days in cytosine arabinoside-containing medium and an additional 3 days in the medium free of cytosine arabinoside at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂.

Transfection of cDNA constructions and siRNA duplexes

SH-SY5Y cells grown to 50% confluency were transfected with Bcl-2 expressing pUSEamp plasmid (*Bcl-2/pUSEamp*, Upstate, Lake Placid, NY) using LipofectAMINE™ Plus (Invitrogen). Two Bcl-2 siRNA duplexes (Bcl-2-siRNA1: 5'-GACUCUGCUCAGUUUGGCC-3', 3'-GGCCAAACU GAGCAGAGUC-5'; Bcl-2-siRNA2: 5'-CAUCGCCUCUGGAUGACU-3', 3'-AGUCAUCCACAGGGCGAUG-5') were synthesized, purified and annealed by Qiagen-Xeragon (Germantown, USA). The siRNA duplex targeting Cdk5 (Smartpool™) and nonspecific control of siRNA were purchased from Upstate Biotechnology (Dharmacon). SH-SY5Y-N cells and human fetal neurons were transfected for 24 h with 2 µg of each siRNA using the TransMessenger transfection reagent following the manufacturer's protocol (Qiagen). The cultures were then treated with chemotherapy agents and subjected to Western blot and cell death analyses.

Cdk5 kinase assay

Cdk5 proteins were immunoprecipitated from cells using polyclonal rabbit anti-Cdk5 antibody (c-8; Santa Cruz Biotechnology, Santa Cruz, USA). Briefly, a total volume of 50 µl of kinase assay mixture containing 10 µl washed Cdk5 protein beads (Protein G-Sepharose; Amersham Biotech) in 20 mM MOPS (pH 7.4) with 1 mM EGTA, 1 mM dithiothreitol, 20 mM MgCl₂ and 100 µM ATP plus 10 µg of histon-H1 and 5 µCi [³²P]ATP was assembled. After incubation for 30 min at 30 °C, the reaction was terminated by spotting 30 µl of the reaction mixture on P81 phosphocellulose pads (Upstate) that were washed five times with 0.75% phosphoric acid followed by rinsing with acetone. Radioactivity was measured with a liquid scintillation counter.

Flow cytometry

Cells (10⁶) treated with camptothecin were harvested, washed and incubated for 40 min at 4°C with either mouse IgG1 antibody as a negative control or a mouse monoclonal antibody against Bak N terminus (AB-1, Oncogene; diluted 1:50 in 100 µg/ml digitonin in PBS). After being washed with PBS, the binding of antibody was visualized with FITC-conjugated antimouse-IgG (1:200) (Sigma), and 10 000 cells were analyzed. The data were processed using Cell Quest™ software (Becton and Dickinson).

Subcellular fractionation

Subcellular fractionation was performed based on our protocol.³⁹ Subconfluent cells treated as indicated in the RESULTS were harvested, and the cell pellet was suspended in 5 volumes of isotonic buffer (10 mM HEPES-KOH (pH 7.5), 210 mM mannitol, 70 mM sucrose, 1 mM Na-EDTA, and 1 mM Na-EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture. The cells were incubated on ice for 15 min and passed through a 22-gauge needle 15 times. After centrifugation twice at 700 × *g* for 10 min at 4°C, the supernatant was collected and centrifuged at 13 000 × *g* for 10 min at 4°C. The resulting mitochondrial pellets were suspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton-X 100, 1 mM PMSF, and 0.2% protease inhibitor cocktail). The supernatants of the 13 000 × *g* spin were further centrifuged at 100 000 × *g* for 1 h at 4°C, and the resulting supernatants were designated as the S-100 cytosolic fractions. The protein concentrations in the cytosolic and mitochondrial fractions were determined by the Bradford assay (Bio-Rad), and equal amounts of mitochondrial and cytosolic proteins were subjected to Western blot analysis.

Western blots

For Western blot analysis,³⁹ cells were harvested and lysed in a cold lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF and 1% protease inhibitor mixture). The cell lysates were centrifuged for 15 min at 18 000 × *g* at 4°C, supernatants were collected and protein concentrations in the supernatant were determined by the Bio-Rad protein assay (Bio-Rad Laboratories). The cell lysates (50 µg), as well as the cytosolic or mitochondrial fractions (10 µg) from the subcellular fractionation were subjected to SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in TBS-T (Tris-buffered saline with 0.05% Tween-20) containing 5% milk and blotted overnight with various primary antibodies. The membranes were washed and incubated for 1 h with either horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit antibodies (Southern Biotech, Birmingham). The membranes were washed and developed by chemiluminescence. Mouse monoclonal antibodies used in this study included anti-human caspase-8 (Medical & Biological Laboratories, Nagoya, Japan), and cytochrome *c* oxidase IV (Molecular Probes, Eugene, USA). Rabbit polyclonal antibodies included anti-human caspase-3, DFF45, ERK1/2 (StressGen, Victoria), Bid (Biosource International Inc., Camarillo), Bak, cytochrome *c* (Upstate Biotechnology, Lake Placid), Bcl-2, Bax, Cdk5, p35, β-actin (Santa Cruz Biotechnology, Santa Cruz), p53, p-ERK1/2, p-Akt, Akt (Cell Signaling), Bcl-X_L and PARP-1 (Biomol, Plymouth Meeting). Densitometric analysis of protein expression levels was performed with UVP Bioimaging Systems (UVP Inc., Upland, CA).

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