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Role of CDK5 in neuroprotection from serum deprivation by μ-opioid receptor agonist

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Abstract

Cyclin-dependent kinase 5 (CDK5), a unique member of the CDK family of cyclin-dependent kinases, is predominantly expressed in postmitotic neurons with proposed roles in both cell survival and programmed cell death. To understand how CDK5 participates in such disparate cellular outcomes, we investigated whether activation of CDK5 could mediate neuroprotection from serum deprivation by μ-opioid receptor agonist in differentiated SH-SY5Y cells and primary hippocampal neurons. We found that CDK5 kinase activity decreased following serum deprivation in differentiated SH-SY5Y cells coincident with increased cell loss and activation of caspases cascade activation, which was reversed by opioid antagonist. Overexpression of CDK5 in serum-free medium reversed activation of caspase cascade and augmented DAMGO neuroprotection. Blocking CDK5 activity by pharmacologic inhibitor, roscovitine or overexpression of dominant negative CDK5 augmented activation of cell death markers and diminished μ-opioid receptor agonist protection. Reduction in CDK5 activity corresponded to reduction in protein levels of CDK5 activator p35 during serum deprivation which was also reversed by μ-opioid receptor agonist. Phosphorylation of STAT3 at Serine 727 by CDK5 decreased during serum deprivation, and partly recovered by μ-opioid agonist. PI3K signaling pathway was not required for CDK5-mediated μ-opioid neuroprotection against serum deprivation. These findings indicate that neuroprotection by μ-opioid receptor agonist against serum deprivation is mediated by activation of CDK5 through up-regulation of p35 and phosphorylation of STAT3 by CDK5 may contribute to the neuroprotection.

Keywords: Cyclin-dependent kinase 5; p35; μ-Opioid agonist; Neuronal survival; Serum deprivation; Neuroprotection; p-STAT3; PI3K

Introduction

Opium, which consists of more than 20 alkaloids, is an extract of the exudates derived from seedpods of the opium poppy. It is widely used as a powerful analgesic. Morphine, one alkaloid working through μ-opioid receptor, is extensively used in clinical medicine. DAMGO, an enkephalin-derived peptide, which improves the selectivity to 100-fold for the μ receptor over other opioid receptors is commonly used as a tool in opioid study. In addition to well characterized effects on regulation of intestinal mobility and immune system, several report studies suggest that opioids elicit other biological effects independent of their analgesic properties (Gross et al., 2004; Lim et al., 2004; Jafari et al., 2004). Of major interest here, opioids have been implicated in determining cell survival and neuroprotection (Zohar et al., 2006; Lee et al., 2004; Lim et al., 2004; Iglesias et al., 2003).

Activation of the opioid receptors has been shown to activate the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway which has been linked neuroprotection (Iglesias et al., 2003). However, other reports suggest that the PI3K/Akt pathway is a feature of cell survival and not specific for neuroprotection. This is supported by the observation that neuroprotection by opioids persists for 24 h, whereas Akt phosphorylation is transient in the serum deprivation model of opioid neuroproteciton (Iglesias et al., 2003). Thus, there must be some other molecules in neurons that are important for opioid neuroprotection.

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A neuron-specific signaling molecule implicated in determination of neuronal survival and cell death is cyclin-dependent kinase 5 (CDK5). CDK5 is a unique member of the cyclin-dependent kinase family of CDKs, activation of CDK5 kinase activity requires association with its neuron-specific activators, p35 and p39; however, these cofactors, which are believed to provide substrate specificity, differ markedly from other CDK activating subunits called cyclins in both their primary sequence and regulation. Active CDK5 is essential for neuronal migration, neurite outgrowth and laminar configuration of the cerebral cortex during development (Lew et al., 1994; Tsai et al., 1994; Humbert et al., 2000), modulation of dopaminergic and glutamatergic transmission (Chergui et al., 2004), and endocytosis of synaptic vesicles (Tomizawa et al., 2003). However, overactivation of CDK5 is toxic to cells. Interestingly, increased CDK5 activity can be induced by proteolytic cleavage of p35 to p25 by the calcium activated proteases called calpain. Increased conversion of p35 to p25 causes prolonged activation, abnormal cellular location and substrate specificity changes of CDK5 leading to neurotoxicity, hyperphosphorylation of tau, cytoskeletal disruption and promotion of apoptosis in primary and cultured neurons (Kusakawa et al., 2000; Lee et al., 2000; Patrick et al., 1999). Conversely, a critical role for CDK5 in mouse survival has also been described. In cortical neurons, CDK5 has been reported to play a key role in promoting survival by negative regulation of c-Jun N-terminal kinase 3 (Li et al., 2002a,b) and Akt activity through the neuregulin/PI3K signaling pathway (Li et al., 2003). In myoblasts, overexpression of dominant-negative-Akt reduced CDK5 activity, indicating that CDK5 might be a downstream molecule of Akt. Taken together, these studies suggest that CDK5 contributes to cell survival or apoptosis depending on the type of cell and or stimuli in question (Cheung and Ip, 2004).

Since CDK5 has been implicated in survival, we hypothesized that CDK5 may play a role in neuroprotection. To address this hypothesis, we assessed the role of CDK5 in neuroprotection from serum deprivation via a selective μ-opioid receptor in differentiated SH-SY5Y cells and primary hippocampal cultures. Here we report that CDK5 activity is required for μ-opioid receptor agonist, DAMGO, to protect primary and cell line-derived neurons from serum deprivation independent of AKT signaling.

Material and methods

Cell culture and treatments

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with and without 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in tissue culture dishes under a humidified incubator with 5% CO_2 at 37°C. For differentiation, the medium was replaced with fresh medium containing 10 μM *all-trans* retinoic acid (RA) the day after plating for 5 days (d) and changed every 2 d. RA differentiation of SH-SY5Y cells is associated with increased expression of μ-opioid receptor, as determined through binding experiments (Yu and Sadee, 1988; Zadina et al., 1994) and a constant cell number by [^{3}H]-thymidine incorporation assay (Iglesias et al., 2003; Datki et al., 2003), thus differentiated cells are both morphologically and physiologically very close to living neurons in the brain (Datki et al., 2003). For serum deprivation, adherent cells were rinsed three times with serum-free media and grown in serum-free media for 24 h or indicated time.

Primary culture of rat embryonic hippocampal neurons

Cultures of primary dissociated neurons were prepared from embryonic rat hippocampus as described (Buchhalter and Dichter, 1991). Pregnant Sprague–Dawley rats were narcotized with CO_2 for 90 s and then killed by cervical dislocation. The embryos (gestational days 18–19) were removed; the brains were dissected and placed on ice, and the hippocampi were dissected under microscopic visualization. The hippocampi were incubated for 20 min in DMEM (Invitrogen, Carlsbad, CA, USA) containing 0.03% trypsin (Sigma, St. Louis, MO, USA) at 37°C and 5% CO_2. They were then resuspended in growth medium containing DMEM, supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 10% Ham's F12 containing glutamine (Sigma, St. Louis, MO, USA) and 50 units/ml penicillin–streptomycin (Sigma, St. Louis, MO, USA). The cells were triturated with a sterile Pasteur pipette and plated onto poly(L-lysine) (Sigma, St. Louis, MO, USA) precoated 35-mm Petri dishes (Nunc) at a density of 1500–2000 cells/mm^2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated bovine calf serum (Hyclone, Logan, UT). After 7 d, cells were treated and harvested for further analysis.

Western blot analysis

Cells were harvested by washing in phosphate-buffered saline (PBS) and then scraping in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and protease inhibitor cocktail (Sigma, St. Louis, MO, USA)). The lysate was centrifuged at 12,000×*g* for 15 min at 4°C. Quantity of protein content in the supernatant was measured using a protein assay kit (Pierce, Tattenhall, UK). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a NC membrane (Amersham Biosciences Corp., NJ, and USA). The membrane was blocked with 5% non-fat dry milk in Tris buffered saline (pH 7.6) containing 0.1% tween-20 and was incubated with the following antibodies: rabbit anti-CDK5 (polyclonal, 1:250 dilution, Santa Cruz Biotechnology, CA, USA), mouse anti-p-ERK (monoclonal, 1:500 dilution, Santa Cruz Biotechnology, CA, USA), rabbit anti-PARP, rabbit anti-phospho-Serine-727-STAT3 and rabbit anti-total STAT3 (1:500 dilution, Cell Signaling Transduction, Beverly, MA, USA), rabbit anti-Akt (1:200 dilution, Santa Cruz Biotechnology, CA, USA) and monoclonal anti-p-Akt (1:500 dilution, Cell Signaling

Transduction, Beverly, MA, USA) overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were immunodetected with an ECL detection system (Santa Cruz Biotechnology, CA, USA) according to the manufacturer's instructions. The intensity of the bands was assessed and analyzed by gel documentation system (ImageMaster, Pharmacia Biotech).

Cloning of CDK5 plasmids and mutagenesis of CDK5

For mammalian expression, the open reading frame of rat CDK5 (GenBank accession NO: NM_080885) was amplified by PCR and subcloned into a pEGFP-N1 vector (Clontech) using *Hin*dIII/*Bam*HI restriction sites, respectively. The Dominant negative (kinase inactive) D144N CDK5 was prepared by mutating an aspartic acid in the 144 position to an asparagine in the pEGFP-N1 CDK5 plasmid. Mutagenesis was performed with the Quick Change Site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutation was confirmed by sequencing performed by Shanghai Sangon Biological Engineering and Technology and Service CO. Ltd. (Shanghai, China).

Transfection experiments

Transient transfection of SH-SY5Y cells was carried out using Lipofectamine plus reagent as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). Briefly, 1 day before transfection, cells (5×10^5 cells/per well) in 2 ml of growth medium without antibiotics were plated to 35-mm Petri dishes (Nunc). During transfection, the complex which was formed by 2 μg plasmid mixed with 12 μl plus and 8 μl lipofectamine was added into each dish and incubated for 4 h. After the incubation, the transfection mix was removed and the normal serum-containing medium was added and incubated for further 48 h. Then cells were harvested for further analysis.

Immunoprecipitation and kinase assay

CDK5 kinase activity was measured as described using an immune complex kinase assay and histone H1 as substrate. Histone H1 phosphorylation with radioactive isotope ^{32}P is used to monitor CDK5 kinase activity. Thus, the amount of transferred radioactive ^{32}P to Histone H1 corresponds to the amount of CDK5 kinase activity present in the immune complex (Nikolic et al., 1998). Briefly, after treatments, SH-SY5Y cells were scraped and lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 10 mM EDTA, 0.5% NP-40, 10 μg/ml leupeptin, 1 mM PMSF, 4 mM NaF and protease inhibitor cocktail. Insoluble material was removed by centrifugation at 25,000×*g* for 30 min, and the cytoplasmic protein concentration of soluble supernatants was determined. Nuclear extraction was performed as described (Andrews and Faller, 1991). Briefly, cells were scraped into 1.5 ml of cold phosphate buffered saline (PBS). The cell suspension was then transferred to a microfuge tube. Cells were pelleted for 10 s and resuspended in 400 μl cold Buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mm $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 min, and then vortexed for 10 s. Samples were centrifuged for 10 s, and the supernatant fraction was discarded. The pellet was resuspended in 20–100 μl (according to starting number of cells) of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA binding proteins) is stored at −70°C. Protein determination was performed as above and 500 μg protein was incubated with CDK5 antibody (1:50) for 3 h at 4°C. Immune complexes were incubated with 30 μl protein A-Sepharose (Amersham Biosciences Corp, NJ, USA) overnight at 4°C, and washed four times with lysis buffer and twice with kinase buffer [20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA] without histone H1 and [γ-^{32}P] ATP. The washed beads were then incubated with kinase buffer containing 2 μg of histone H1 (Sigma, St. Louis, MO, USA) and 10 μCi of [γ-^{32}P] ATP at 30°C for 30 min in a final volume of 40 μl. The reaction was terminated by adding Laemmli sample buffer and the samples were separated on SDS-PAGE, and the dried gels were subjected to autoradiography. Negative controls were treated identically, containing 10 μl non-related antibodies instead of CDK5 antibody.

MTT assay

Cell survival was assayed by measuring the conversion of the yellow, water-soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the blue, water insoluble formazan derivative (Mosmann, 1983). For these assays, cells were seeded at an initial density of 2×10^4 cells/well in 96-well culture dishes (Corning, NY, USA). Before treatments, RA-differentiated cells were washed three times with serum-free DMEM and then cultured for 24 h in the basal DMEM with various treatments as indicated. At the end of the experiment, MTT was added at a final concentration of 0.5 mg/ml and incubated for 4 h at 37°C. Formazan crystals were dissolved with dimethyl sulfoxide (DMSO), and optical density was quantified by measurement at 570 nm with a wavelength of correction of 650 nm (Bio-Rad, Hercules, CA, USA). Optical blanks were generated by incubating the corresponding culture medium in the absence of cells. Values were expressed as percentage of survival taking as 100% the mean of the optical densities obtained from cells cultured in 10% serum containing media.

Data analysis

Data for multiple variable comparisons were analyzed by one-way or two-way analysis of variance (ANOVA). For the comparison of significance, Newman–Keuls or Bonferroni tests

were used as a post hoc test. The level of significance was $P<0.05$. The results are expressed as the mean±SEM from at least three independent experiments.

Results

Decreased CDK5 kinase activity correlates with cell death after serum deprivation in SH-SY5Y cells

To determine if CDK5 is a potential candidate for regulation of survival in response to serum deprivation, we examined cytoplasmic CDK5 kinase activity following serum deprivation of differentiated SH-SY5Y cells for 1 h, 3 h, 5 h and 24 h. CDK5 activity of the cells maintained in 10% serum was used as control. CDK5 kinase activity was determined by immune complex kinase assay using Histone H1 as a substrate. Phosphorylation of Histone H1 indicates the level of CDK5 kinase activity (Nikolic et al., 1998). While CDK5 kinase activity showed a trend toward decrease at all indicated time points assessed, a statistically significant reduction was only observed at 24 h following serum deprivation (Figs. 1A and B). Previous studies suggest that differentiated SH-SY5Y cells undergo cell death in response to withdrawal of serum. Using MTT assay as a measure of cell viability, our results suggest a similar loss of cells in this model coincident with decreased CDK5 kinase activity (Fig. 1C, 10% FBS vs. vehicle). Thus, cells cultured in serum-free medium for 24 h were used as the serum deprivation model in all subsequent experiments.

To confirm whether CDK5 activity was involved in serum deprivation induced cell death, serum-deprived SH-SY5Y cells were treated with increasing concentrations of a CDK5 inhibitor roscovitine. Our findings showed that roscovitine promoted cell death in a dose-dependent manner after serum deprivation for 24 h (Fig. 1C). Taken together, the above results indicate that decreased CDK5 kinase activity correlates with cell death following serum deprivation.

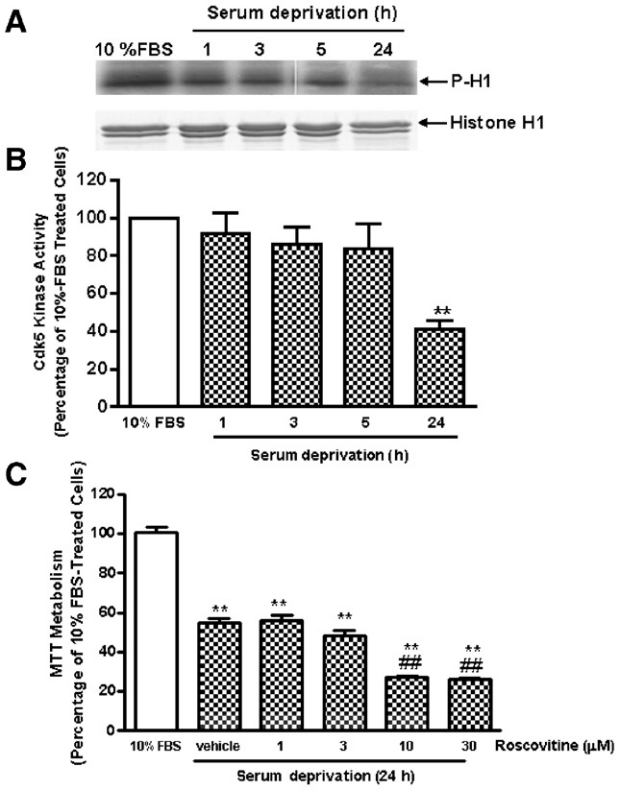

Fig. 1. Decreased CDK5 kinase activity correlates with cell death with cell death after serum deprivation in SH-SY5Y cells. (A) CDK5 kinase activity was measured as described using an immune complex kinase assay and histone H1 as substrate. Histone H1 phosphorylation was used to monitor CDK5 kinase activity. Autoradiographs (Upper) representing the CDK5 kinase activity and the corresponding Coomassie blue-stained gels of histone H1 (Lower) representing loading control are shown. (B) The level of CDK5 kinase activity was quantified by densitometry, and the data were normalized to values obtained from 10% FBS-treated cells. $^{**}P<0.01$ as compared to 10% FBS group. Data represent means±SEM for three separate experiments. (C) Roscovitine promoted cell death following serum deprivation for 24 h. Differentiated cells were exposure to different doses (1, 3, 10, 30 μM) roscovitine for 24 h following serum deprivation, cell survival was measured by MTT assay. Columns represent the mean±SEM of three independent experiments with five wells repeated and are expressed as percentage of the initial number of living cells. $^{**}P<0.01$ as compared to 10% FBS group, $^{\#\#}P<0.01$ as compared to vehicle group. Data were analysis of ANOVA followed by Newman–Keuls test.

μ-Opioid receptor agonist DAMGO partly attenuated cell death induced by treatment of Roscovitine in differentiated SH-SY5Y cells as well as in primary cultured hippocampal neurons following serum deprivation

To investigate whether activation of CDK5 mediated the neuroprotection of μ-opioid agonist against serum deprivation, differentiated SH-SY5Y cells were treated with roscovitine and μ-opioid receptor agonist DAMGO to verify whether CDK5 activity was involved in the neuroprotective effect of DAMGO. Cell survival was measured by MTT assay (Fig. 2A). The results indicated that 10 μM roscovitine promoted cell death following serum deprivation of differentiated SH-SY5Y. In contrast, DAMGO partly reversed the cells loss as compared with roscovitine alone control. Death of SH-SY5Y cells in response to serum deprivation has been well characterized and manifests itself with the hallmark features of apoptosis including changes in nuclear morphology, processing and activation of caspase 3 and cleavage of the caspase 3 substrates acetyl-Asp-Glu-Val-Asp-minomethyl-coumarin and poly (ADP-ribose) polymerase (PARP) (Macleod et al., 2001). PARP, which regulates DNA damage, apoptosis and genetic stability is a direct target of active caspase-3 which results in proteolytic cleavage of PARP from 116 kDa to 89 kDa (Li et al., 2002a,b). Thus, in the present study, we used the cleavage of PARP as an index of caspases activation preceding cell death. Serum deprivation for 24 h caused a significant increase in cleavage of PARP from 116 kDa to 89 kDa (Figs. 2B and C) which was partly attenuated by the addition of 0.1 μM DAMGO. Further, 10 μM roscovitine enhanced PARP cleavage as compared to vehicle control group. Interestingly, DAMGO partly reversed the enhanced cleavage of PARP by roscovitine. Similar results were seen in primary cultured hippocampal neurons (Figs. 2D and E). Taken together, these results indicate that CDK5

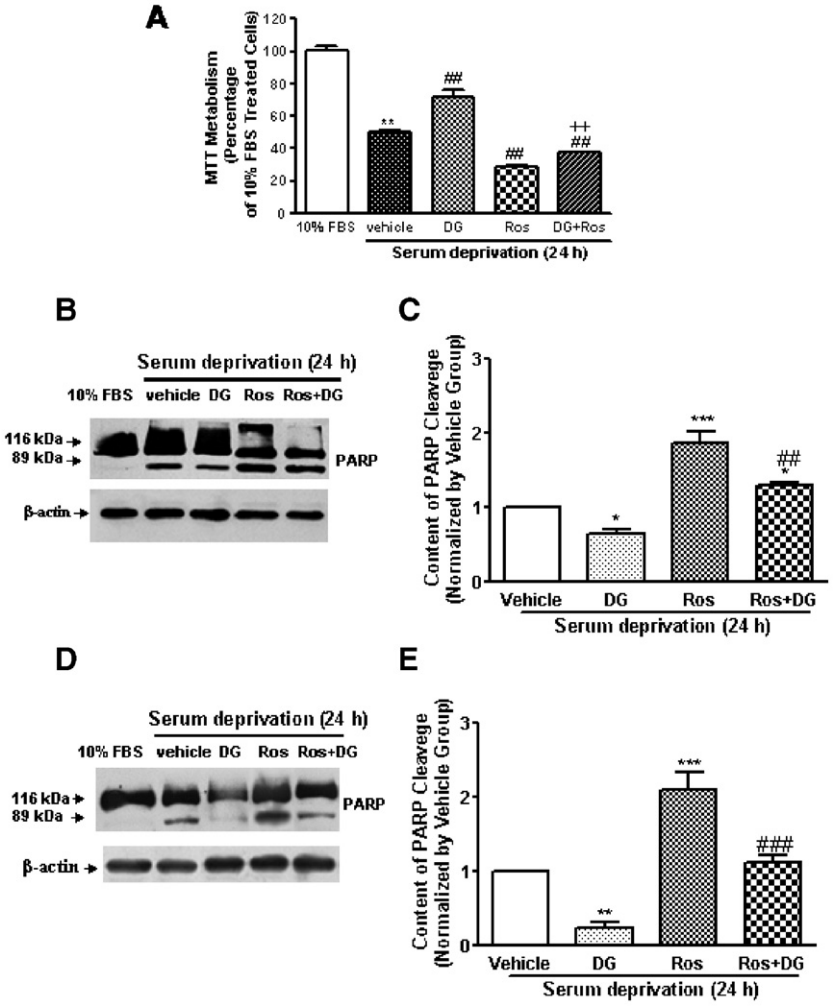

Fig. 2. μ-Opioid receptor agonist DAMGO partly attenuated the cell death induced by CDK5 inhibitor Roscovitine following serum deprivation. (A) Differentiated SH-SY5Y cells were cultured in DMEM with 10% FBS and without serum in the medium containing vehicle, 0.1 μM DAMGO (DG), 10 μM roscovitine (Ros) and 0.1 μM DAMGO plus 10 μM roscovitine (Ros+DG) for 24 h. Cell survival was measured by MTT assay. Columns represent the mean±SEM of three independent experiments with five wells repeated and are expressed as percentage of the initial number of living cells. $^{**}P<0.01$ as compared to 10% FBS group, $^{\#\#}P<0.01$ as compared to vehicle group, $^{++}P<0.01$ as compared to Ros group. Data were analysis of ANOVA followed by Newman–Keuls test. (B) Differentiated SH-SY5Y cells were cultured in DMEM with 10% FBS and without serum in the medium containing vehicle, 0.1 μM DAMGO (DG), 10 μM roscovitine (Ros) and 0.1 μM DAMGO plus 10 μM roscovitine (Ros+DG) for 24 h, the cells were then lysed and the lysates were analyzed by Western blotting for the 89 kDa fragment of the processed 116 kDa of intact PARP using a polyclonal antibody. The results are representative of three separate experiments. (C) The content of PARP cleavage was quantified by densitometry, and the data were normalized to values obtained from vehicle group. $^{*}P<0.05$, $^{***}P<0.001$ as compared to vehicle group, $^{\#\#}P<0.01$ as compared to roscovitine group. (D) Primary cultured hippocampal cells were cultured in DMEM with 10% FBS and without serum in the medium containing vehicle, 0.1 μM DAMGO (DG), 10 μM roscovitine (Ros) and 0.1 μM DAMGO plus 10 μM roscovitine (Ros+DG) for 24 h, the cells were then lysed and the lysates were analyzed by Western blotting for the 89 kDa fragment of the processed 116 kDa of intact PARP using a polyclonal antibody. The results are representative of three separate experiments. (E) The content of PARP cleavage was quantified by densitometry, and the data were normalized to values obtained from vehicle group. $^{**}P<0.01$, $^{***}P<0.001$ as compared to vehicle group, $^{\#\#\#}P<0.001$ as compared to roscovitine group.

activation was essential for cell survival during serum deprivation.

Elevation of cytoplasmic and nuclear CDK5 activity after DAMGO treatment and reversion by naloxone and roscovitine

To determine if activation of CDK5 mediates neuroprotection by the μ-opioid receptor during serum deprivation, CDK5 activity was evaluated in cytoplasmic and nuclear fractions of SH-SY5Y cells treated with DAMGO with naloxone (opioid receptor antagonist) or roscovitine. By immune complex kinase assay, Histone H1 phosphorylation by CDK5 was increased by DAMGO (0.1 μM treatment in cytoplasmic fractions following serum deprivation for 24 h; Fig. 3A). This effect was antagonized by preincubation (15 min) with opioid receptor antagonist naloxone (1 μM) or CDK5 inhibitor roscovitine (10 μM), respectively (Figs. 3A and B). Moreover, DAMGO (0.1 μM) treatment elevated nuclear CDK5 activity by 40% in comparison with serum deprivation for 24 h as well (Figs. 3C and D). Taken together, these findings indicate that CDK5 activity is increased in both nuclear and cytoplamsic compartments by DAMGO stimulation.

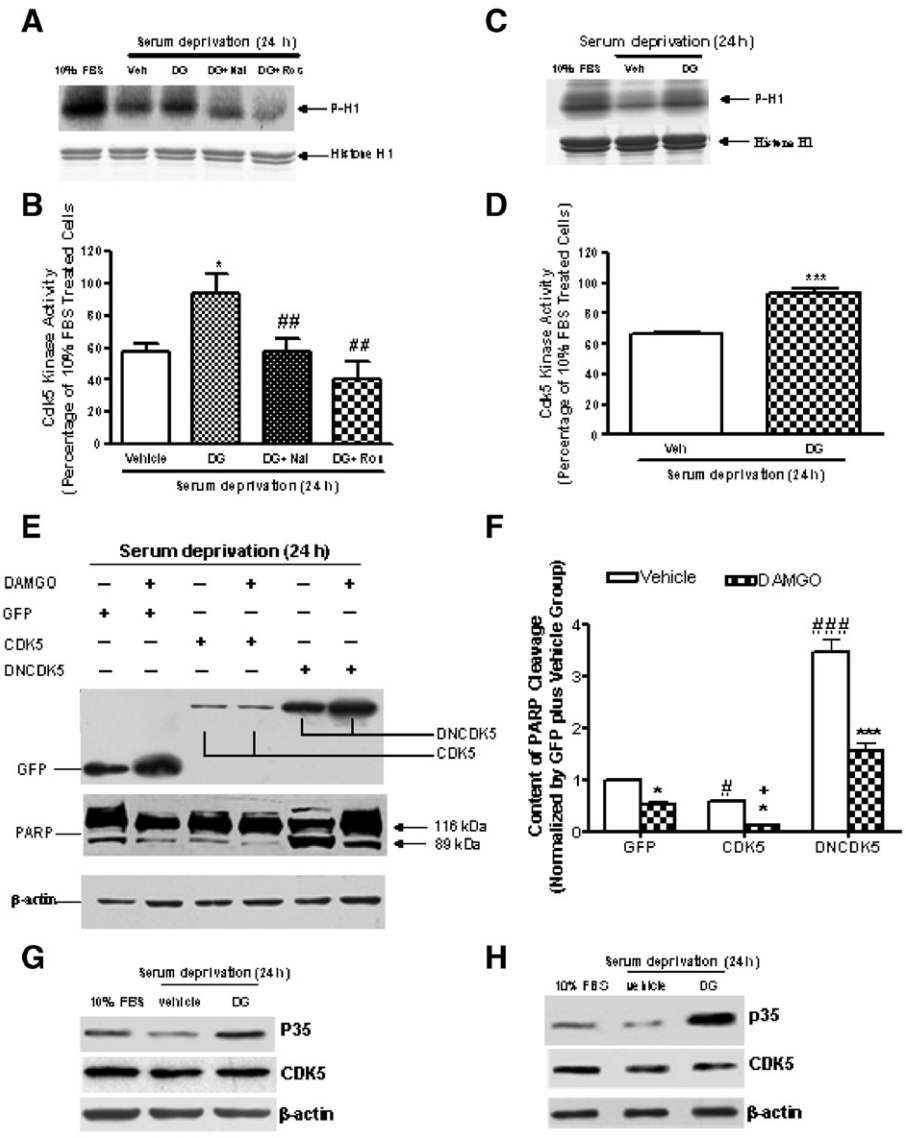

Fig. 3. Neuroprotection of μ-opioid receptor agonist against serum deprivation was mediated by activation of CDK5 through up-regulation of p35 in SH-SY5Y cells and primary cultured hippocampal neurons. (A–D) CDK5 kinase activity was measured as described using an immune complex kinase assay and histone H1 as substrate. Histone H1 phosphorylation was used to monitor CDK5 kinase activity. Thus, the intensity of Histone H1 bands represents CDK5 kinase activity. (A) Cytoplasmic CDK5 kinase activity increased after DAMGO treatment. Autoradiographs (Upper) representing the CDK5 kinase activity and the corresponding Coomassie blue-stained gels of histone H1 (Lower) representing loading control are shown. (B) The level of CDK5 kinase activity was quantified by densitometry, and the data were normalized to values obtained from 10% FBS-treated cells. $^{*}P<0.05$ as compared to vehicle group, $^{\#\#}P<0.01$ as compared to DG group. Data represent mean±SEM for three separate experiments. (C) Nuclear CDK5 kinase activity increased after DAMGO treatment. Autoradiographs (Upper) representing the CDK5 kinase activity and the corresponding Coomassie blue-stained gels of histone H1 (Lower) representing loading control are shown. (D) The level of CDK5 kinase activity was quantified by densitometry, and the data were normalized to values obtained from 10% FBS-treated cells. $^{***}P<0.001$ as compared to vehicle group. Data represent mean±SEM for three separate experiments. Veh: vehicle; DG: DAMGO 0.1 μM; DG+Nal: DAMGO 0.1 μM+Naloxone 1 μM; DG+Ros: DAMGO 0.1 μM+roscovitine 10 μM. The intensity of Histone1 bands represents CDK5 kinase activity. Data were analysis of ANOVA followed by Newman–Keuls test. (E) Overexpression of CDK5 promoted cell survival and potentiated the neuroprotection of μ-opioid agonist, while overexpression of dominant negative CDK5 drove cell to death and μ-opioid agonist DAMGO partly reversed the effect. Differentiated SH-SY5Y cells were transfected with GFP empty vector, GFP-CDK5 and GFP dominant negative CDK5 (GFP-DN-CDK5) for 24 h followed serum deprivation and treated with or without DAMGO for further 24 h, the cells were then lysed and the lysates were analyzed by Western blotting for the 89 kDa fragment of the processed 116 kDa of intact PARP using a polyclonal antibody. The results are representative of three separate experiments. (F) The content of PARP cleavage was quantified by densitometry, and the data were normalized to values obtained from GFP plus vehicle group. $^{*}P<0.05$, $^{***}P<0.001$ as compared to vehicle group; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ as compared to GFP plus vehicle group; $^{+}P<0.05$ as compared to GFP plus DAMGO group. Data were analysis by two-way ANOVA followed by Bonferroni post-tests. (G) Differentiated SH-SY5Y cells were cultured in DMEM with 10% FBS or in serum-free medium with treatment of vehicle and 0.1 μM DAMGO for 24 h. The expression level of p35 and CDK5 was detected by Western blotting. (H) Primary cultured hippocampal neurons were cultured in DMEM with 10% FBS or in serum-free medium with treatment of vehicle and 0.1 μM DAMGO for 24 h. The expression level of p35 and CDK5 was detected by Western blotting.

CDK5 is necessary and sufficient for DAMGO-mediated neuroprotecion from μ-opioid agonist

To demonstrate that CDK5 activity mediates μ-opioid neuroprotecion, SH-SY5Y cells were transfected with CDK5 (to increase CDK5 activity) or dominant negative CDK5 (to compete with endogenous CDK5 and decrease CDK5 activity), then treated with μ-opioid agonist DAMGO or vehicle. PARP cleavage was used as a read out for activation of caspases-mediated death associated with serum deprivation as described above. As previously shown, DAMGO decreased PARP cleavage during serum deprivation for 24 h in SH-SY5Y cells transfected with GFP (Fig. 3E, Lane 2 and Lane1). Overexpression of CDK5 by transient transfection reduced PARP cleavage, our indicator of cell death (Fig. 3E; Lane 3 and Lane 1) and potentiated the neuroprotection of μ-opioid agonist DAMGO (Fig. 3E; Lane 4 and Lane 3). In contrast, overexpression of dominant negative CDK5 promoted PARP cleavage (Fig. 3E; Lane 5 and Lane 1), which was partially reversed by treatment with μ-opioid agonist DAMGO (Fig. 3E; Lane 6 and Lane 5). The quantitative analysis is shown in Fig. 3F. These findings suggest that CDK5 activity is necessary and sufficient to mediate μ-opioid agonist DAMGO neuroprotection against serum deprivation.

μ-Opioid agonist induces p35 expression, but not CDK5 expression, in SH-SY5Y cells and primary cultured neurons following serum deprivation for 24 h

Since CDK5 kinase activity is dependent on the expression of its coactivator p35, we determined p35 protein levels by Western blot using a p35 antibody (c-19, recognize both p35 and p25 protein; Fig. 3G). Serum deprivation for 24 h reduced p35 levels as compared to differentiated SH-SY5Y controls (Fig. 3G); while treatment with μ-opioid agonist DAMGO increased p35 levels over untreated serum deprived controls (Fig. 3G). However, no significant difference in CDK5 protein level was observed. Further, we did not observe the presence of the p35 cleavage product, p25 which has been shown to increase CDK5 activity due to increased p25 protein half life as compared to p35. Similar results were observed in primary neuronal hippocampal cultures (Fig. 3H).

Phosphorylation of neuroprotective CDK5 target, STAT3 is increased in SH-SY5Y cells treated with μ-opioid agonist DAMGO

Signal transducer and activator of transcription (STAT) 3 can protect cells from apoptosis and promote cell survival. In the present study, we detected decreased phosphorylation of STAT3 in nuclear fraction using the phospho-Serine-727-STAT3 antibody following serum deprivation for 24 h (Fig. 4, Vehicle vs. 10% FBS). However, μ-opioid agonist treatment increased phospho-STAT3 as compared to serum deprivation and 10% FBS treated cultures (Fig. 4, DG vs. Vehicle). Phosphorylation level of STAT3 was even lower

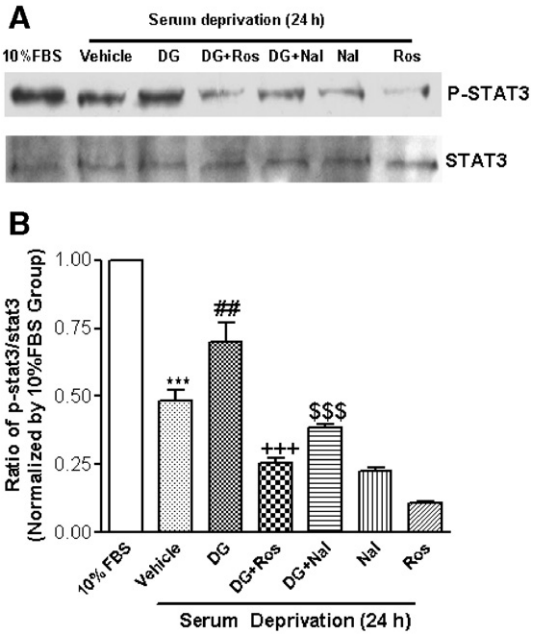

Fig. 4. μ-Opioid agonist induced phosphorylation of STAT3 at Serine 727 in nucleus following serum deprivation for 24 h. (A) DAMGO enhanced Phospho-STAT3 at Ser727 following serum deprivation for 24 h in differentiated SH-SY5Y cells. Phospho-STAT3 at Serine 727 was induced by μ-opioid agonist and the phosphorylation level was even lower with the treatment of roscovitine and naloxone, respectively following serum deprivation for 24 h, and was partly recovered by DAMGO. Veh: Vehicle; DG: 0.1 μM DAMGO; Ros: 10 μM roscovitine; DG+Ros: 0.1 μM DAMGO+10 μM roscovitine; Nal: naloxone 1 μM; Nal+Ros: naloxone 1 μM+roscovitine 10 μM. (B) The level of phosphorylation of STAT3 was quantified by densitometry, and the data were normalized to values obtained from 10% FBS-treated cells. $^{***}P<0.001$ as compared to 10% FBS group, $^{\#\#}P<0.01$ as compared to roscovitine group, $^{+++}P<0.001$ as compared to roscovitine group, $^{\$\$\$}P<0.001$ as compared to naloxone group. Data represent means±SEM for three separate experiments.

than that of vehicle group upon serum deprivation by roscovitine and naloxone, respectively (Fig. 4, Nal or Ros vs. vehicle). Treatment with DAMGO reversed effects of roscovitine and naloxone (Fig. 4, DG+Ros vs. Ros or DG+Nal vs. Nal).

PI3K pathway was not required for the increased CDK5 coactivator, p35, by μ-opioid agonist

Recent studies suggest that μ-opioid receptor activation prevented apoptosis following serum deprivation in differentiated SH-SY5Y cells via activation of phosphatidylinositol 3-kinase pathway (Iglesias et al., 2003). To determine whether PI3K pathway was involved in the neuroprotection of μ-opioid agonist mediated by CDK5 during serum deprivation for 24 h, we assessed phosphorylation of PI3K substrate, AKT in our paradigm. AKT phosphorylation by PI3K pathway was not increased by μ-opioid agonist at 24 h after serum deprivation (Fig. 5A) suggesting that the PI3K pathway was not active at this time point. To determine whether CDK5 activation is a target of PI3K/AKT signaling pathway in our paradigm, we inhibited PI3K with LY294002 in SH-SY5Y cells treated with DAMGO or not during serum deprivation and assessed

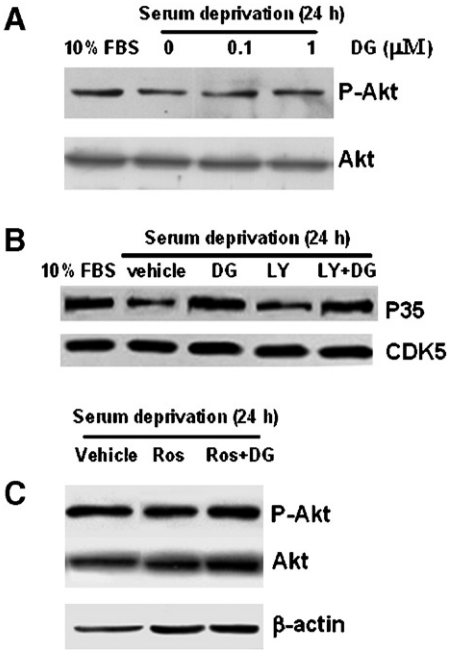

Fig. 5. PI3K pathway was not required for CDK5-mediated neuroprotection of μ-opioid agonist against serum deprivation for 24 h. (A) Differentiated SH-SY5Y cells were cultured in DMEM with 10% FBS or in serum-free medium with treatment of vehicle and 0.1 μM DAMGO (DG), 10 μM PI3K inhibitor LY294002, 10 μM PI3K inhibitor LY294002 plus 0.1 μM DAMGO (LY+DG) for 24 h. The expression level of p35 and CDK5 was detected by Western blotting. (B) Differentiated SH-SY5Y cells were cultured in DMEM with 10% FBS or in serum-free medium with treatment of vehicle and 0.1 μM DAMGO for 24 h. The expression level of p-Akt was detected by Western blotting. (C) Differentiated SH-SY5Y cells were cultured in serum-free medium with treatment of vehicle, 10 μM roscovitine (Ros) and 0.1 μM DAMGO (DG) for 24 h. The expression level of p-Akt was detected by Western blotting. The results are representative of three separate experiments.

expression of p35 or CDK5. The PI3K inhibitor did not alter induction of p35 by μ-opioid agonist following serum deprivation for 24 h (Fig. 5B). Further, CDK5 inhibitor roscovitine did not induce PI3K activation as determined by AKT phosphorylation (Fig. 5C). Taken together, these results suggest that PI3K pathway is neither stimulated nor responsible for the observed increase in CDK5 activity in response to μ-opioid receptor agonist treatment for 24 h during serum deprivation.

Discussion

CDK5 activity has been implicated in both cell death and cell survival; however, its role in preventing neuronal loss in response to challenge has not been elucidated. In the present study, we demonstrate that CDK5 activity plays an important role in maintaining cell survival following serum deprivation and mediates neuroprotection provided by the μ-opioid agonist, DAMGO. The former premise is supported by our observations that CDK5 kinase activity is decreased with cell death following serum deprivation while overexpression of CDK5 prevented this mode of cell death. Further, overexpression of dominant negative CDK5 [CDK5−D144N] prevented protection by DAMGO and hastened cell death in the serum deprivation model (Wang et al., 2003). Moreover, we have demonstrated that CDK5 activity mediates neuroprotection of μ-opioid agonist against serum deprivation as supported by the following observations. First, CDK5 kinase activity increased after treatment with μ-opioid receptor agonist DAMGO, which was reversed by μ-opioid receptor antagonist naloxone. Treatment with CDK5 inhibitor, roscovitine, attenuated DAMGO neuroprotection suggesting that CDK5 activity was necessary for preventing cell loss. Finally, overexpression of CDK5 was sufficient to protect cells from death by serum deprivation. Taken together, these findings demonstrate that CDK5 is both necessary and sufficient to protect neurons from serum deprivation induced death and that this is the pathway by which DAMGO mediates such protection.

Our studies go on to demonstrate that induction of CDK5 activity by DAMGO is due, at least in part, to an increase in protein levels of its coregulator, p35. This increase was not accompanied by cleavage of p35 to p25 indicating that increased kinase activity was not due to calpain cleavage. As calpain activation is associated with necrotic death, these findings are consistent with activation of CDK5 via a survival pathway. Further, our results demonstrate that phosphorylation of STAT3 by CDK5 occurs in response to μ-opioid receptor agonist treatment during serum deprivation suggesting that this known survival pathway may contribute to the neuroprotection seen with μ-opioid receptor agonist (Ji et al., 2004). These data underscore the importance of CDK5 in a model for neuroprotection and point to potential mechanisms by which protection is achieved by opiates during serum deprivation.

CDK5 kinase activity is essential for several physiologic neuronal processes including neuronal migration, neurite outgrowth, and laminar configuration of the cerebral cortex and neuronal survival (Lew et al., 1994; Tsai et al., 1994; Humbert et al., 2000), modulation of dopaminergic and glutamatergic transmission (Chergui et al., 2004), and endocytosis of synaptic vesicles (Tomizawa et al., 2003). However, CDK5 activity has also been reported to mediate neuronal cell death. It has been suggested that these opposing activities of CDK5 are linked to duration of CDK5 activation. Prolonged activation of a protein with normally positive impact on the cells can have negative results when over-stimulated or overexpressed (Fischer et al., 2005). CDK5, in association with p35, is active predominantly under physiologic conditions. In contrast, CDK5 associated with the proteolytic product of p35, p25, has been linked to neuronal dysfunction and cell death due to pathologic stresses (Fischer et al., 2005; Cruz et al., 2003; Nguyen et al., 2002). While both p35 and p25 activate CDK5, p25 is more stable than p35 leading to prolonged activation of CDK5. Increased longevity of kinase activity may also broaden the array of CDK5 target substrates. In addition, p25 exhibits different subcellular localization from p35 and results in redistribution of CDK5 activity within the cell provides access to new substrates (Cruz et al., 2003; Noble et al., 2003). Taken together, these studies suggest that the role of CDK5 in neuronal survival or neuronal death depends on the coactivator. Consistent with this

supposition, we found that p35 was down-regulated under conditions of neuronal loss, 24 h serum deprivation, while p35 levels, and consequently CDK5 activity, was increased by addition of μ-opioid receptor agonist DAMGO. These changes were not accompanied by alterations in CDK5 expression level or detectable p25 expression. As similar results were obtained in primary cultured hippocampal neurons, our SH-SY5Y model reflects results seen in untransformed, primary cells. Taken together, our results indicated that the neuroprotection of μ-opioid receptor agonist against serum deprivation for 24 h was mediated by activation of CDK5 through up-regulation of p35, providing a novel role for CDK5 activity in neuroprotection.

CDK5 is a multifunctional protein kinase in the central nervous system, and the biological activity has been ascribed to its phosphorylation of cytoplasmic substrates. However, CDK5 is located in both cytoplasm and nucleus (Ino and Chiba, 1996) and its roles in the latter are still being elucidated. Here we have shown that, CDK5 kinase activity is increased in the nuclear compartment by the μ-opioid agonist DAMGO, suggesting that nuclear CDK5 substrates promote cell survival. For example, STAT3, an anti-apoptotic factor with potential to regulate Fas and Bax expression (Shen et al., 2001; Stephanou et al., 2001; Ivanov et al., 2002), is activated by phosphorylation in our model of neuroprotection. Phosphorylation of STAT3 on Serine 727 has been shown to block activity of the pro-apoptic Bcl-2 family member, Bax (Vogel, 2002; Sarkar et al., 2003; Korsmeyer, 1995) and pro-apoptotic death receptor, Fas, leading to increased survival in p38α-deficient cells (Porras et al., 2004). Serine 727 of STAT3 has also been shown to be phosphorylated by CDK5 (Fu et al., 2004). Our data show that phosphorylation of STAT3 on Serine 727 is reduced following serum deprivation for 24 h and recovered by μ-opioid agonist treatment as compared to 10% FBS cultured condition. Further, inhibition of CDK5 with roscovitine blocked phosphorylation of STAT3 in response to DAMGO suggesting STAT3 phosphorylation is a direct or indirect target of CDK5 activity. Another CDK5 target with a role in the nucleus is the transcription factor p53 which has pro and anti-apoptotic activities(Lassus et al., 1996; Zhang et al., 2002). Though p53 did not show significant changes in non-neuronal cells after serum deprivation (Alexandre et al., 2000; Huang et al., 1997), its role in neuronal cells remains to be investigated. In addition to transcription factors, CDK5 also regulates activity of other kinases that regulate cell death such as members of the c-Jun N-terminal kinase/stress-activated protein (JNK/SAP) kinase signaling pathway. CDK5 regulation of JNK/SAP pathway protects against neuronal loss in response to UV irradiation (Li et al., 2002a,b). Therefore, further investigation of the role of CDK5 targets in neuroprotection of μ-opioid receptor during serum deprivation is warranted to elucidate the complex mechanisms regulating neuronal survival.

Surprisingly, a known pathway with strong implications for neuronal survival, the PI3K pathway, did not appear to contribute to CDK5-mediated neuroprotection by DAMGO during serum deprivation in our model. The PI3K pathway has been reported to constitute the primary survival mechanism for BDNF protection against serum deprivation (Hetman et al., 1999). Further, CDK5 has been shown to phosphorylate and activate PI3K to mediate neuronal survival against serum deprivation in cortical neurons (Li et al., 2003). However, we have observed no significant changes in phosphorylation of PI3K target, Akt, in serum-deprived, differentiated SH-SY5Y cells with or without the treatment of μ-opioid agonist DAMGO. These results support a previous report that transient activation of Akt by DAMGO after serum deprivation lasted for 5 min returning to basal level within 1 h (Iglesias et al., 2003). Since some transcription factors, such as FOXO (Brunet et al., 1999), NF-κB (Burow et al., 2000; Madrid et al., 2000) would mediate the actions of PI3K following its early activation, therefore, though our result clearly showed that Akt activation was not observed by DAMGO at 24 h after serum deprivation, it still could not exclude the possibility that downstream targets of Akt might also contribute to DAMGO protection against serum deprivation for 24 h. Thus, we performed further experiment to verify this possibility. The result showed that no significant influence of PI3K inhibitor on the elevated p35 induced by μ-opioid agonist following serum deprivation for 24 h suggested that PI3K signaling pathway was not the upstream molecule of CDK5 activation induced by μ-opioid agonist against serum deprivation. Likewise, CDK5 inhibitor roscovitine had no influence on PI3K pathway, either. Taken together, it suggested that CDK5 was a PI3K-independent pathway in mediating the neuroprotection of μ-opioid agonist against serum deprivation.

In summary, we have shown for the first time that neuroprotection of μ-opioid receptor agonist against serum deprivation was mediated by activation of CDK5 through up-regulation of p35 and phosphorylation of STAT3 by CDK5. In addition, we further demonstrated that PI3K signaling pathway was not required for μ-opioid neuroprotection mediated by CDK5 activation against serum deprivation. These findings further elucidate the multifaceted role of the neuron-specific cyclin-dependent kinase CDK5 in neuronal survival.

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