Up-regulation of *Idh3α* causes reduction of neuronal differentiation in PC12 cells

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The PC12 is the widely used cell line to study neuronal differentiation. We had extensively investigated the details of protein expression in differentiated PC12 cells by proteomic analysis. The cells were incubated at the presence of nerve growth factor. We had analyzed the expression changes in the differentiating PC12 cells by 2-dimensional electrophoresis and the identification of the proteins using MALDI-TOF MS. By comparing expression pattern in the time course, we identified the candidate genes which are associated with neuronal differentiation. Among these genes, we performed real-time PCR analysis to validate *Idh3α* expression by the time course. To identify the function of *Idh3α* in neuronal differentiation stage, the transfection of *Idh3α* to PC12 cells was performed. As a result, we proved that up-regulation of *Idh3α* causes reduction in neural differentiation of PC12 cells. Based on these data, we suggest that *Idh3α* plays a role to the neuronal differentiation. [BMB reports 2010; 43(5): 369-374]

INTRODUCTION

Recently, the field of neuronal differentiation has advanced rapidly, generating a rich and complex body of knowledge. However, there is still lack of details about neural differentiation and related genes. So, the identification of genes involved in neural development may lead to the therapeutic target for neural disease such as Alzheimer's disease. The rat pheochromocytoma cell line PC12 is widely used to study neuronal differentiation (1). The general shape of PC12 cells is circular in normal conditions. However, when nerve growth factor (NGF) is treated to the PC12 cells, PC12 undergoes neurite outgrowth, electrical excitability, and synaptic vesicles (2).

Neurite outgrowth (e.g. axonal or dendrite outgrowth) is necessary for the development and function of the central nervous system. Also, Neuronal loss or abnormal neurite outgrowth is associated with synaptic failure disease, Alzheimer's disease (3). It is well accepted that the divergence of neurons and neurite outgrowth are involved in alterations in gene expression (4). So the study of neural development is used as a not only basic research of development but also diagnosis of neural disease. Moreover finding out the genes related t neural differentiation can be used as a specific neural marker.

In specific developmental stage, proteomic analysis is an important approach to characterize the regulation of protein. It is a powerful screening method for detecting any alterations in protein expression that may reflect given phenotype precisely. Here, we performed a functional proteomic analysis of PC12 cells to indentify novel genes related to the neural development.

In this study, we report the results of a 2-DE analysis with high confidence for the PC12 cell line depending to the developmental stages. By proteomic approach, we selected some candidate genes which were up and down regulated during neuronal differentiation. Among these candidate genes, we focused on *Idh3α* gene, Isocitrate dehydrogenase (NAD+) subunit alpha [Mus musculus]. Three polypeptides, *Idh3α*, *Idh3β* and *Idh3γ*, encode enzymes utilizing NAD+ as a cofactor and presence in the mitochondrial matrix. These polypeptides form a heterotetramer including two *Idh3α* subunits, one *Idh3β* and one *Idh3γ* subunit (5). *Idh3α* is a catalytic subunit while *Idh3β* and *Idh3γ* regulate roles (6). And further *Idh3α* stands out in the isocitrate dehydrogenases, because it strongly expressed in almost all tissues in the CNS with transcripts (7). From these backgrounds and 2-DE analysis, *Idh3α* gene was further analyzed using Real-time PCR. Also we investigated the difference of expression pattern and influence of *Idh3α* in neuronal differentiation using Western blot of MAPK pathway. Because neuronal differentiation in PC12 cells induced by nerve growth factor (NGF) requires activation of ERK/MAP kinase pathway, such as Raf-MEK-ERK cascade (8), so we checked protein level of p-ERK in our samples. Therefore, we expect that this candidate gene proved by proteomic analysis can be used diagnostic and therapeutic markers for neuronal disease.

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RESULTS

Proteomic analysis of the expression changes during the neuronal differentiation in PC12 cell

We prepared PC12 cells each 0, 1, 3, 5 and 7 day after treatment of NGF, and the 0 day sample was harvested before NGF treatment, so there are no influences of NGF effect in this sample. Then, we analyzed protein expression of neuronal differentiation in PC12 cells by proteomics approach. We reproducibly detected more than 1,000 spots on the two-dimensional gels. The silver stained spots were detected by the Melanie III software with three different pH gradients. We analyzed two gels for each time point, and selected only those whose normalized volumes were similar to two gels and attempted to take as little of the surrounding gel as possible.

We investigated proteomic changes of neuronal differentiated PC12 cells. About 350 μg of protein from the differentiated PC12 cells by time points were applied the 2-DE analysis. Four changed protein spots were selected for MALDI-TOF mass spectrometry analysis. Because these four spots were single circular form, we expected that analysis of these spots is exact. Also, these clear spots represented definite patterns, so we focused on only these spots. We identified four differentially expressed proteins in Immobiline Drystrip pH 4-7, from which UCLH1 and L10E were up-regulated and *Idh3α* was down-regulated (Fig. 1A). One up-regulated protein, U3, is still unknown and showed high homology with mouse protein. The quantitative expression changes of these four proteins were listed in Table 1. Since Isocitrate dehydrogenase (NAD+) subunit alpha [Mus musculus] (*Idh3α*) is generally expressed in almost all tissues in the CNS (7), we further focused on *Idh3α*.

Idh3α gene is discovered by proteome profile changes of neuronal differentiation in PC12 cells

To verify the 2-DE data, we performed real-time quantitative RT-PCR analysis, and determined the changes in mRNA levels. Data were normalized by using GAPDH gene expression level. In correspondence with proteomic analysis, mRNA level of *Idh3α* was clearly reduced after 3 days (Fig. 1B). To investigate the function of *Idh3α*, we analyzed the effect of *Idh3α* up-regulation in PC12 cell. The *Idh3α* clone is transfected to PC12 cells with or without NGF treatment. Visual inspection applied respectively four cases (Fig. 2). In PC12 cells without NGF treatment, we can't show neural differentiation regardless of *Idh3α* expression level (lane 1 and 2). On the other extreme, when PC12 cells stimulated by NGF, cells begin to differentiate (lane 3). Otherwise, PC12 cells with over-expression of *Idh3α* tend to delay of neuronal differentiation compare to control cells. These data suggested that *Idh3α* can inhibit the neural differentiation although the presence of NGF stimulation.

Idh3α is an up-regulator of ERK in ERK/MAP kinase pathway

Whether *Idh3α* is involved in ERK/MAP kinase pathway, we performed Western blot. As noted, classical ERK are activated by NGF signaling, and phosphorylation of ERK is essential to neuronal differentiation. Therefore, we monitored both total form of both p44ERK and p42ERK in differentiation process. Both p44ERK and p42ERK isoforms were rapidly increased at
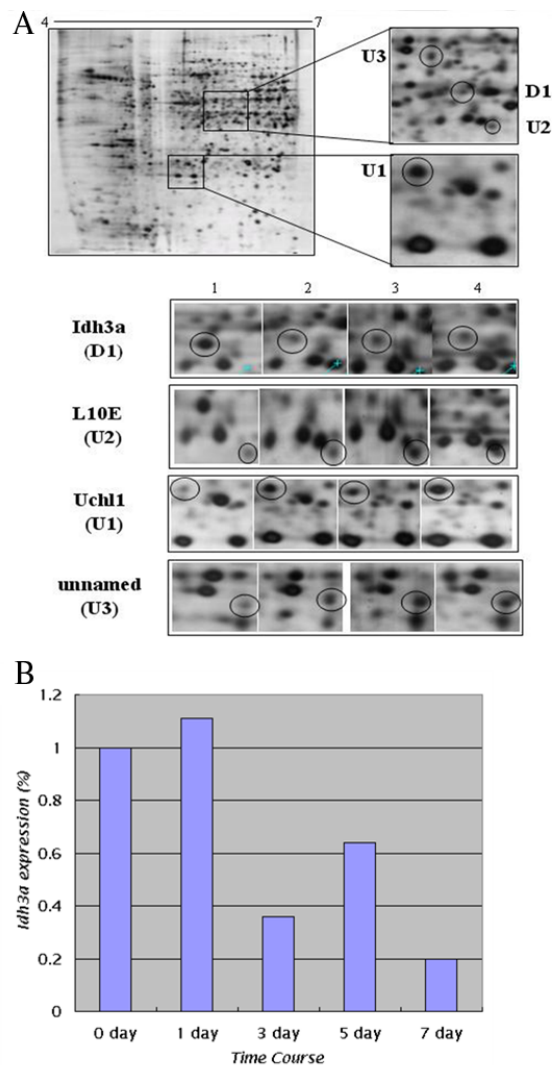

Fig. 1. Proteomic changes of differentiated PC12 cells using Immobiline Drystrip pH 4-7. (A) U means up-regulated gene and D means down-regulated gene. Each lane has different time point. The lane 1 is undifferentiated PC12 cells, lane 2 is three days after induction, lane 3 is five days after induction, lane 4 is seven days after induction with NGF in PC12 cells. (B) The mRNA level of *Idh3α* was determined by real-time quantitative RT-PCR. The experiments were conducted in duplicate. *Idh3α* down-regulation pattern in PC12 cells are showed by time course.

Table 1. Selected proteins of PC12 cells

	Accession number	Protein name	Seq.cov	pI	Mass	FC ratio 1 (0 day)	FC ratio 2 (3 day)	FC ratio 3 (5 day)	FC ratio 4 (7 day)
D1	gi|18250284	Isocitrate dehydrogenase 3 (NAD+) alpha [Mus musculus] (Idh3α)	17	6.3	39.62	1	0.42	0.52	0.58
U2	gi|18203410	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL-1)	43	5.1	24.82	1	1.71	1.30	1.91
U1	gi|27923993	60S acidic ribosomal protein P0 (L10E)	26	5.9	34.17	1	0.77	1.07	1.20
U3	gi|26328873	Unnamed protein product [Mus musculus]	19	6.2	77.32	1	1.11	1.44	1.59

FC: fold change

1X71 by olympus, X400
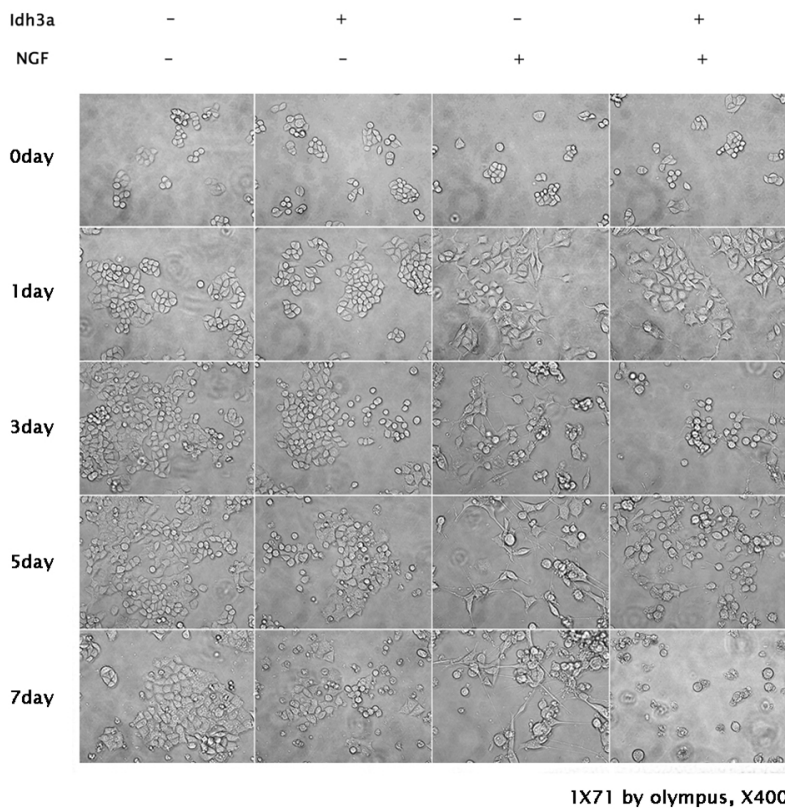
Fig. 2. Morphological changes in neuronal differentiation induced by NGF (100 ng/ml) with time course. To over-expression of *Idh3α*, we make a clone of *Idh3α* and transfect the clone into PC12 cells. Each horizontal line indicates different time point while each vertical line displays different treatment; Lane1 is PC12 cells without any treatments. Lane 2 displays PC12 cells with transfection of *Idh3α* at 1 day. Lane 3 indicates NGF is treated in PC12 cells. Lane 4 shows both NGF and cloned *Idh3α* are treated in PC12 cells. The figures were photographed at each time point.

1day as shortly after stimulated by NGF. In NGF stimulation, both p44ERK and p42ERK tend to constantly expressed (Fig. 3A), but dropped suddenly with over-expressed *Idh3α* by time course (Fig. 3B). It would be thus expected that *Idh3α* has a dramatic effect in cell differentiation and resides up-stream of ERK in the ERK/MAP kinase pathway.

DISCUSSION

The nervous system regulates the differentiation pathway, which controls molecular signaling. PC12 cells are preferred to understand the different aspects of neuronal differentiation such as mitotic arrest. It proliferates until further stimulates while neuronal growth factor excites it to differentiate such as complete mitotic arrest (9).

In this study, *Idh3α* highly expressed in the undifferentiated PC12 cells, and decreased with the starting of neuronal differentiation. Because the *Idh3α* gene was down-regulated in neuronal differentiation, we suggest that *Idh3α* up-regulation cause to reduce the neuronal differentiation in PC12 cells. Differentiation of PC12 cells requires sustained activation of ERK pathway (10). Activation of ERK pathway is induced by

A

B
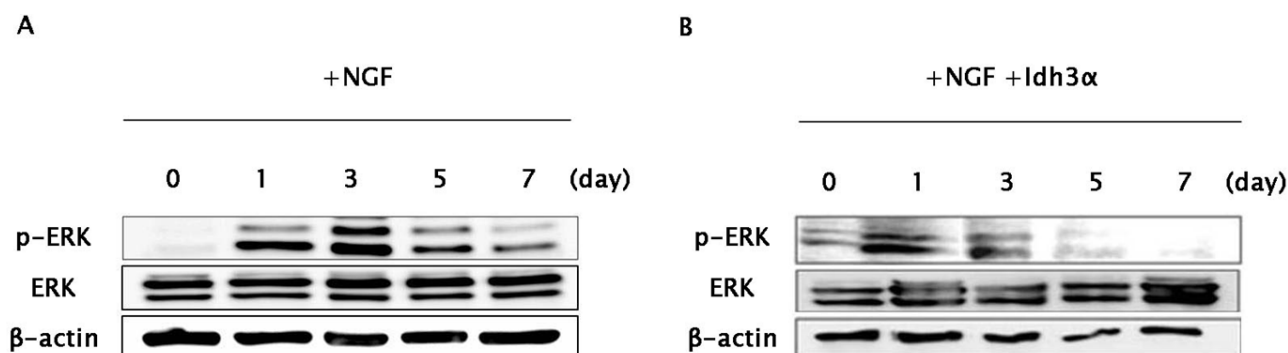
Fig. 3. ERK expression pattern during differentiation is analyzed by Western blot analysis. (A) PC12 cell is constantly stimulated to differentiate by NGF. (B) *Idh3α* is transfected at 1 day in PC12 cell. ERK phosphorylation was detected by Western blot analysis in 0, 1, 3, 5, 7 days in culture. Activation of p44ERK and p42ERK were determined by normalization of the band density from the phosphorylated form with that of the total form. Representative Western blots of ERK activity on 0, 1, 3, 5, 7 days are shown under each panel.

NGF. When NGF binds its TrkA (Tropomycin-related kinase A) receptors, M-Ras which subsequently causes transient activation of ERK pathway is activated (11). In this pathway, Overexpressed *Idh3α* would cause inactivation of NGF-induced sustained ERK phosphorylation activity and neurite outgrowth.

The study of PC12 cell-mediated neuronal differentiation allows elucidating the mechanism of neuronal differentiation. It has raised hopes that we can find ways to actually repair the damage in the nervous system. In this paper, certain proteins were extensively investigated the details of an expression profiling in the level of protein using and proteomic techniques during the differentiation of PC12 cells.

Based on protein work, we discover a novel gene, *Idh3α*, and its function. These results suggest that *Idh3α* is related to neuroendocrine differentiational system, and this novel gene can be used to investigate some of the other mechanism that involve in neuronal diseases or study.

MATERIALS AND METHODS

Cell culture and neuronal differentiation
PC12 cell line was derived from a transplantable rat pheochromocytoma (KCLB No. 21721) and show neuronal differentiation upon NGF treatment. Undifferentiated PC12 cells were cultured in RPMI 1640 (WelGENE Inc. South Korea) supplement with 10% heat inactivated (56°C, 30 min) fetal bovine serum (FBS) (WelGENE Inc.), 100 U/ml penicillin, and 100 ug/ml streptomycin in humidified air (CO_2, 5%) at 37°C. Culture medium was replaced every 1-2 days. PC12 cells was starved that HS decreased by 1% and removed FBS before 24 hours of NGF-induced differentiation. After that, cells were cultured with 100 ng/ml NGF, 1% HS. And when clone of *Idh3α* were transfected to PC12 cells, RPMI 1640 media contain FBS 1% were used.

Two-dimensional polyacrylamide gel electrophoresis
Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and then harvested into lysis buffer (9 M urea and 4% CHAPS containing protease inhibitor cocktail Roche, Switzerland) following 5 minutes incubation. The cell lysate was centrifuged in a Beckman TL-100 table ultracentrifuge. The supernatant was taken as the total cell extract and stored at −70°C until use. Protein extracts were normalized by bicinchoninic acid (BCA) assay (Pierce, USA) or Bradford protein assay (Bio-Rad, USA). Isoelectric focusing (IEF) was carried out using Multiphor II(GE Healthcare, USA). Immobilized pH gradient (IPG) strips were used according to manufacturer's instructions. Samples containing appropriate amounts of protein were diluted to 350 µl with rehydration solution (9 M urea, 4% CHAPS, 100 mM dithiothreitol, 0.5% (v/v) IPG buffer, trace bromophenol blue), and applied to strips (pH 3-10 (NL), 4-7, 4.5-5.5, and 5.5-6.7) by overnight rehydration in a rehydration tray two-DE was performed using 9-16% second-dimension gels (200 × 250 × 1.0 mm) in a IsoDALT apparatus (Hoefer Scientific Instruments, USA) until the tracking dye reached the anode end of the gels.

Protein visualization and image analysis
Silver-stained protein spots were excised from the stained gels. The gels were fixed with methanol: acetic acid : water (40 : 10 : 50) for 30 minutes, followed by sensitizing in 30% methanol, 5% sodium thiosulfate, 6.8% (w/v) sodium acetate for 30 minutes. This was followed by three 5 minutes washes in deionized water. Proteins were stained in a solution 2.5% silver nitrate for 20 minutes, and washed twice in deionized water for 1 minute. Subsequently, gels were developed with 2.5% (w/v) sodium carbonate, 0.04% formaldehyde. When the desired intensity was attained, the developer was discarded and stopped with 1.46% (w/v) EDTA solution for 10 minutes. Finally, the gels were washed with deionized water and stored in the sealed plastic bags at 4°C. Protein patterns in the gels

were recorded as digitalized images using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer, Bio-Rad, USA). Scanned images were analyzed using two-DE program, Melanie III (SIB, Switzerland)

In-gel digestion

Silver-stained protein spots were excised from the stained gel and destained with freshly (Lee et al., 2006) with minor modification. Gels of protein spots were put into 1.5 ml microtubes and destained 1 : 1 mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate with gentle shaking for 10 minutes. Gels were washed twice with deionized water with gentle shaking for 15 minutes. Followed by washing with 50% acetonitrile/25 mM ammonium bicarbonate, pH 7.8, with shaking for 10 minutes. Liquid was removed, and acetonitrile (50 μl) was added and incubated for 5 minutes. All liquid was removed and gel particles were dried in a vacuum centrifugal concentrator for 5 minutes. Prior to enzymatic digestion, the gel particles were incubated on ice in 10 μl of 0.02 μg/μl sequencing grade trypsin solution. After 45 minutes, the supernatant was discarded and replaced with 20 mM ammonium carbonate. After 12 hours, digestion at 37°C, 10 μl of 0.5% (v/v) trifluoroacetic acid in 50% acetonitrile was added and the solutions were subjected to sonication twice in an ultrasonic water bath. The extraction of protein was performed with 0.1% formic acid in 2% acetonitrile for furthers MALDI-TOF MS analysis.

MALDI-TOF of protein MS and data analysis

Mass analysis was performed on a Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystem, USA) in reflector mode. The spectrometer was run with the settings: accelerating voltage, 20 kV; grid voltage, 65%; and a DELAY, 100 NS. Half of one μl in-gel digested tryptic peptide extracts were dispensed on to a MALDI sample plate along with 0.5 μl of matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% TFA and 50% acetonitrile. External peptide calibrants, angiotensin I (monoisotopic mass, 1296.6853), rennin substrate (1758.9331), ACTH (18-39) (2465.1989), were used for mass calibration. Spectra were internally calibrated using autolytic fragments from trypsin. Under conditions where the auto-digested fragments of trypsin were abundant, the monoisotopic peak m/z 1020.5030, m/z 2163.0564 and m/z 2273.1594 were used as mass calibrants. For each sample, the average of 18-20 spectra was acquired in the delayed extraction and reflector mode. Besides, proteins were identified by peptide mass fingerprinting with search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5). All mass searches were performed using a mass window between 0 and 100 kDa and included *Mus musculus*, *Rattus* and *Rodentia* sequence. The search parameters allowed for N-terminal acetylation and carboxyamidomethylation of cycteine. The criteria for positive identification of proteins were set as follows; (i) at least 4 matching peptide masses, (ii) 50 ppm or better mass accuracy, and (iii) molecular weight and pI of identified proteins should match estimated values obtained from images analysis.

Real time RT-PCR

Level of *Idh3α* was quantified by Real-Time Reverse Transcription. Total RNA was isolated from PC12 cells. Confluent cells were trypsinize with trypsin, washed 10 ml PBS, and centrifuge at 1500 rpm for 5 minutes. Total RNA was prepared by using TRIZOL as directed by the manufacturer (Invitrogen, USA). 5 ug of total RNA was reverse transcribed by using superscript II reverse transcriptase (Invitrogen, USA). For the reverse transcription, RNA was mixed with 0.5 ug oligo (dt) and 10 mM dNTP mixture, incubated at 65°C for 5 minutes, and placed in ice for 1 minute. To the reaction solution, 200 units of superscript II reverse transcriptase was added (Invitrogen, USA) at the final volume 20 ul containing 4 units RNase inhibitor, 5× reaction buffer and 0.1 M DTT. The mixture was incubated at 42°C for 60 minutes and inactivated by heating to 70°C for 10 minutes for enzyme inactivation. Real-time PCR was perfomed on obtained cDNA in the presence of 1·SYBR Green Mastermix (Applied Biosystems, France) containing preset concentrations of dNTPs, MgCl2 and buffers, along with adequate concentrations of forward and reverse primers.

Id3hα expression cloning and cell transfection

The *Idh3α* was amplified using primers (5'- ACGCGTCGACAT GGCCGGGTCCGCGTGGGT-3' and 5'-GATCTCTAGACTAAT CTAAGTCTTTGACTC-3'). This primer set generated full coding sequence of the *Idh3α*. The PCR product, with *XbaI* and *SalI* linkers, was purified and inserted into the *XbaI/SalI* restriction enzyme site. And then using vector enzyme (*SalI*, *XbaI*), the *Idh3α* full length was inserted into *pCMV-flag2*. And then 2.0 x 10^6 PC12 cells were placed in 100 pie plates and cultured for 24 hr. The medium was RPMI1640 (JBI, Australia) supplemented with 10% Horse serum (HS; Invitrogen, USA) and 5% FBS (JBI, Australia). For transfection, the medium were changed with RPMI1640 (JBI, Australia) supplemented with 1% FBS (JBI, Australia) before 2 hr transfection.

Protein extraction and Western blot analysis

Each PC12 cell was homogenized on ice in 40 minutes with pro-prep protein extraction solution (Intron). For study of ERK/MAP kinase pathway, a total 100 ug of proteins from the each PC12 cells run on 12% SDS-PAGE, and transferred onto nitrocellulose membranes. The primary antibody was phosphor-p44/42 MAPK (Thr202/Tyr204) (Rabbit, Cell signaling, USA), and secondary antibody was goat anti-rabbit IgG (Sigma, USA). Labled bands were detected by ECL plus Western blotting detection system (Amersham Biosciences, UK) and images were captured. The intensity of the bands was measured by the Luminescent image analyzer (LAS-3000, Fusifilm life science, Japan). The relative expression of certain protein was de-

termined by dividing the densitometric value of the test protein by that of the control β-actin.

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