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PEP-1-PEA-15 protects against toxin-induced neuronal damage in a mouse model of Parkinson's disease

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ABSTRACT

Background: PEA-15 is abundantly expressed in both neurons and astrocytes throughout the brain. It is a multifunctional protein with the ability to increase cell survival via anti-apoptotic and anti-proliferative properties. However, the function of PEA-15 in neuronal diseases such as Parkinson's disease (PD) remains unclear. In this study, we investigated the protective effects of PEA-15 on neuronal damage induced by MPP^+ in neuroblastoma SH-SY5Y and BV2 microglia cells and in a MPTP-induced PD mouse model using cell-permeable PEP-1-PEA-15.
Methods: PEP-1-PEA-15 was purified using affinity chromatography. Cell viability and DNA fragmentation were examined by MTT assay and TUNEL staining. Dopaminergic neuronal cell death in the animal model was examined by immunohistochemistry.
Results: PEP-1-PEA-15 transduced into the SH-SY5Y and BV2 cells in a time- and dose-dependent manner. Transduced PEP-1-PEA-15 protected against MPP^+-induced toxicity by inhibiting intracellular ROS levels and DNA fragmentation. Further, it enhanced the expression levels of Bcl-2 and caspase-3 while reducing the expression levels of Bax and cleaved caspase-3. We found that PEP-1-PEA-15 transduced into the substantia nigra and prevented dopaminergic neuronal cell death in a MPTP-induced PD mouse. Also, we showed the neuroprotective effects in the model by demonstrating that treatment with PEP-1-PEA-15 ameliorated MPTP-induced behavioral dysfunctions and increased dopamine levels in the striatum.
Conclusions: PEP-1-PEA-15 can efficiently transduce into cells and protects against neurotoxin-induced neuronal cell death in vitro and in vivo.
General significance: These results demonstrate the potential for PEP-1-PEA-15 to provide a new strategy for protein therapy treatment of a variety of neurodegenerative diseases including PD.

1. Introduction

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra (SN) and is second only to Alzheimer's disease (AD) as the most common age-related neurodegenerative disease. Affecting roughly 2% of elderly people PD symptoms include muscle rigidity, bradykinesia, and disabling motor abnormalities such as tremors and postural instability [1–4]. The pathogenesis of PD is related to a number of risk factors including aging, inflammation, exposure to a variety of chemicals, as well as genetic factors [5–7]. Although the exact mechanism of neuronal cell death is not fully understood, it is widely known that oxidative stress plays an important role in neurodegenerative disorders such as PD, AD, and amyotrophic lateral sclerosis (ALS) [8]. Reactive oxygen species (ROS) are the normal by-products of a number of cellular process and environmental factors which alter the DNA structures and function of a number of macromolecules including proteins and lipids so damaging them. The accumulation of ROS is associated with the pathology of a variety of diseases related to oxidative stress including PD [9].

Phosphoprotein enriched in astrocytes 15 (PEA-15) is a 15 kDa small protein that was initially identified as a phosphoprotein in astrocytes. It has subsequently been found to be widely expressed in a variety of tissues and is highly conserved among mammals [10]. The PEA-15 gene and protein are highly expressed in the nervous system, in particular, in astrocytes [10–13]. PEA-15 is composed of an N-terminal death effector domain (DED) and a C-terminal tail of irregular structure [14,15].

Abbreviations: PEA-15, phosphoprotein enriched in astrocytes 15; PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine; ROS, reactive oxygen species; MPP^+, 1-methyl-4-phenyl pyridinium; PTD, protein transduction domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

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Several studies have shown that PEA-15 regulates multiple cellular functions in various cancer cells through its interaction with extra-cellular signal receptors, activated kinases (ERK1/2) and ribosomal S6 kinase2 (RSK2), which are substrates of ERK1/2. PEA-15 over-expression has been shown to have contributed to increased patient survival in ovarian carcinoma whereas it increased survival of tumors in glioblastoma. Thus, PEA-15 has various functions including anti- and pro-tumorigenic properties [16–21]. Recent work has revealed that deletion of PEA-15 protein in mice causes specific defects in spatial learning abilities [22]. However, the physiology of PEA-15 functions in neuronal cells is not yet clearly understood.

Many studies, including those carried out by our group have established the use of protein transduction domains (PTDs) as an effective means to deliver therapeutic proteins into living cells in vitro and in vivo [23–32]. Although protein transduction efficiency depends on a number of factors including the target proteins, the types of cells and the nature of the PTD, protein transduction via PTDs has been demonstrated to be an effective approach to protein therapy [24,35–42]. In this study, we demonstrate that a PEP-1-PEA-15 protein can be directly transduced into cells and protects against cell death in vitro and in vivo leading us to suggest that PEP-1-PEA-15 protein may be a potential therapeutic agent for various diseases including PD.

2. Materials and methods

2.1. Materials

Ni^{2+}-nitrilotri-acetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). Human neuroblastoma SH-SY5Y cells were obtained from the Korean Cell Line Research Foundation, Seoul, Korea. 2′,7′-Dichlorofluorescein diacetate (DCF-DA), MPTP hydrochloride and probenecid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against cleaved caspase-3 and actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Human PEA-15 cDNA was isolated using the polymerase chain reaction (PCR) technique. All remaining chemicals and reagents used in this experiment were of the highest available analytic grade.

2.2. PEP-1-PEA-15 protein expression and purification

A cell-permeable PEP-1 expression vector was prepared in our laboratory as described previously [25]. The cDNA sequence for human PEA-15 was amplified by PCR using the sense primer 5′-CTCGAGATGGCTGA GTACGG-3′ and the antisense primer, 5′-GGATTCTCAGGCCTTCTTCG-3′. The resulting PCR product was excised, eluted, and ligated into a TA cloning vector. The purified TA vector containing human PEA-15 cDNA was ligated into the PEP-1 expression vector to produce a PEP-1-PEA-15 fusion protein. In a similar fashion, control PEA-15 was constructed that expressed the PEA-15 protein without PEP-1. To produce the PEP-1-PEA-15 and control PEA-15 proteins, the plasmid was transformed into *Escherichia coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of LB media at 37 °C to a D_{600} value of 0.5–1.0 and induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG; Duchefa, Haarlem, Netherlands) at 18 °C for 24 h. Harvested cells were lysed by sonication and purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen) and PD-10 column chromatography (Amersham, Braunschweig, Germany). To remove endotoxins, purified PEP-1-PEA-15 and control PEA-15 were treated with a Detoxi-Gel™ endotoxin removing gel (Pierce, Rockford, IL, USA). A *Limulus ameobocyte* lysate assay (BioWhitaker, Walkersville, MD, USA) showed that the endotoxin levels for the proteins were below detectable limits (<0.1 EU/ml). Purified protein concentration was estimated using the Bradford procedure with bovine serum albumin as a standard [43].

2.3. Cell culture and transduction of PEP-1-PEA-15 protein into the cells

SH-SY5Y human neuroblastoma cells were cultured in EMEM (Lonza, MD, USA) containing heat-inactivated fetal bovine serum (10% FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin). BV2 microglia cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 °C under humidified conditions of 95% air and 5% CO_2.

To assess the transduction of PEP-1-PEA-15, SH-SY5Y and BV2 cells were grown to confluence in wells of a 60 mm dish plate. The culture medium was replaced with 2 ml of fresh solution. The cells were treated with various concentrations of PEP-1-PEA-15 (0.5–3 μM) for various times (10–60 min). The cells were treated with trypsin-EDTA (Gibco) and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform Western blot analysis.

2.4. Western blot analysis

12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on equal amounts of cell lysates. The resolved proteins were electrotransferred to a nitrocellulose membrane, which was then blocked with 5% nonfat dry milk in PBS. The membrane was probed with the indicated antibodies, and the immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

2.5. Subcellular fractionation of the transduced cells

The nuclear and cytosolic fractions were prepared as previously described [27,31]. Transduced SH-SY5Y cells were washed with PBS, acid-washed with 0.2 M glycine-HCl, pH 2.2, and trypsinized for 10 min at 37 °C. The cells were harvested after washing with cold PBS and pelleted. The cells were then resuspended in 1 ml of NP-40 buffer by gentle pipetting and incubated on ice for 10 min. Cells were spun through a sucrose cushion at 1000 g for 10 min and the cytosolic fractions were collected from the supernatants. Pellets were washed with 1 ml of NP-40 buffer to completely remove cytosolic fractions. The nuclei were lysed in a lysis buffer (50 ml Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1% Triton X-100). The resulting nuclear and cytosolic lysates were analyzed by Western blotting.

2.6. Confocal microscopic analysis

Coverslips treated with 3 μM PEP-1-PEA-15 proteins were used to grow SH-SY5Y cells. After 1 h of incubation at 37 °C cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 5 min. Treated cells were permeabilized and blocked for 30 min with 3% bovine serum albumin, 0.1% Triton X-100 in PBS (PBS-BT) and washed with PBS-BT. After being permeabilized and blocked, cells were exposed to the primary antibody (His-probe, 1:2000; Santa Cruz Biotechnology) for 1 h at room temperature. The secondary antibody (Alexa flour 488, 1:15,000; Invitrogen) was applied for 1 h at room temperature in the dark and nuclei were stained for 3 min with 0.1 μg/ml 4′6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). Confocal microscopy using a model FV-300 microscope (Olympus, Tokyo, Japan) was used to analyze the distribution of fluorescence [27,28,32].

2.7. Cell viability assay

The cell viability of SH-SY5Y cells treated with MPP^+ was measured in order to assess the biological role of transduced PEP-1-PEA-15 proteins. The cells were seeded into 96-well plates at 70% confluence having been pretreated with PEP-1-PEA-15 (1–3 μM) for 1 h prior to being

exposed to 1-methyl-4-phenylpyridinium (MPP^+, 4 mM) for 18 h. Cell viability was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT). The absorbance was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340) and cell viability was defined as the % of untreated control cells [27,28,32].

2.8. Measurement of reactive oxygen species (ROS)

Dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) is converted by ROS into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) and so, was used to assess intracellular levels of ROS in SH-SY5Y cells [27,28,32]. SH-SY5Y cells were incubated in the absence or presence of PEP-1-PEA-15 (3 μM) for 1 h before they were treated with MPP^+ (4 mM) for 40 min. Afterwards, SH-SY5Y cells were washed twice with PBS and incubated for 30 min with DCF-DA (10 μM). A Zeiss Axiovert S100 microscope with a MRC-1034ES confocal laser-scanning system was used to produce cellular fluorescence images. Under the same experimental conditions, the fluorescence intensity was quantified at excitation and emission wavelengths of 485 nm and 538 nm, respectively, using a Fluoroskan enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems Oy, Helsinki, Finland).

2.9. TUNEL assay

SH-SY5Y and BV2 cells were treated with MPP^+ (4 mM) for 24 h after having been incubated for 1 h in the presence or absence of PEP-1-PEA-15 (3 μM). A Cell Death Detection Kit (Roche Applied Science) was used to perform Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL) staining according to the manufacturer's specifications. An Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan) was used to produce images.

2.10. Measurement of apoptotic protein expression

SH-SY5Y cells were incubated in the absence or presence of PEP-1-PEA-15 (1–3 μM) for 1 h, and then treated with MPP^+ (4 mM) for various times (6 h, 18 h, and 24 h). The expression of Bcl-2 (6 h), Bax (6 h), caspase-3 (18 h) and cleaved caspase-3 (24 h) in whole cell lysates was analyzed by Western blotting using specific antibodies.

2.11. Experimental animals

Male, 8-week-old (22–25 g), C57BL/6 mice were obtained from the Hallym University Experimental Animal Center. The animals were provided with free access to food and water and were housed at a constant temperature of 23 °C and relative humidity of 60% with a fixed 12 h cycle of light and darkness. Experimental procedures involving animals and their care followed those specified in the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

In this study, we used a chronic MPTP-induced PD treated mouse model [44]. Mice were injected with a total of 10 doses of MPTP hydrochloride (25 mg/kg in saline, subcutaneous; s.c.) in combination with an adjuvant, probenecid (250 mg/kg in DMSO, intraperitoneally; i.p.). The 10 doses were administered on a 5-week schedule with an interval of 3.5 days between consecutive doses. Mice were sacrificed 3 weeks after the 5-week treatment schedule. In order to determine whether PEP-1-PEA-15 has protective effects against PD, mice were i.p. injected with PEP-1-PEA-15 (2 mg/kg) and control proteins 1 h prior to treatment with MPTP. Mice ($n = 5$ for each group) were divided into the following groups: 1) non-treated controls, 2) MPTP-treated, 3) MPTP + PEP-1-PEA-15 treated, 4) MPTP + control PEA-15 treated, and 5) MPTP + PEP-1 peptide treated. At the end of the experiment (8 weeks), behavioral tests were performed.

2.12. Immunohistochemistry

Immunohistochemistry was performed as previously described [32,45,46]. Sections of brain tissue were first incubated with 3% bovine serum albumin in PBS for 30 min at room temperature. Then the sections were incubated with a rabbit anti-His antibody (1:400) and anti-tyrosine hydroxylase (TH) IgG (1:500) primary antibody to detect PEP-1-PEA-15 and DA neurons, respectively. The sections were washed three times for 10 min with PBS, incubated sequentially in biotinylated goat anti-rabbit IgG and ABC complex diluted 1:200 in the same solution as the primary antiserum. Cresyl violet-immunostaining for Nissl bodies was conducted after TH immunostaining to detect viable cells. The sections were visualized with 3,3′-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides. Images were captured and analyzed using a model DP72 digital camera (Olympus, Tokyo, Japan) and DP2-BSW microscope digital camera software (Olympus). Figures were prepared using Photoshop 7.0 (Adobe, San Jose, CA, USA). Manipulation of images was restricted to threshold and brightness adjustments were applied to entire images.

For quantification of TH immunoreactive and cresyl violet stained neurons, we performed the cell count in the SN area according to a previous description [33]. Briefly, TH immunostaining and cresyl violet staining images (10 sections/mice) were captured in the same region (500 × 500 μm). Images were sampled from at least five different points within each SN section. Thereafter, the number of TH positive and cresyl violet positive neurons was actually counted within the sampled images. All immunoreactive cells were counted regardless of the intensity of labeling. Cell counts were performed by two different investigators who were blind to the classification of tissues. All data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine statistical significance.

2.13. Rotarod test

The motor function of mice was measured by Rotarod test as described previously [33,34,47]. To acclimate mice to the rotarod machine (IITC company, series 8 model, USA), mice were placed on the rotarod at 5 rpm for 600 s, every day for one week prior to MPTP treatment. Acclimated mice which could stay on the rod without falling were selected and randomly divided into experimental groups. 3 weeks after the last MPTP injection, mice received PEP-1-PEA15, control-PEA15, and PEP-1 peptide injection. Then, mice were placed in a separate compartment on the rotarod rod and tested at 25 rpm for 100 s. The length of time that mice were able to stay on the rod was measured automatically and the latency to fall was defined as the % of control mice.

2.14. Measurement of dopamine levels in the striatum

Dopamine levels in the striatum were measured by Liquid Chromatography (LC) with Electrospray Ionization Mass Spectrometry (Thermo Electron Corporation, USA) as previously described [33,34]. Mice striatal tissues were homogenized with 0.1 M perchloric acid and 0.1 mM EDTA buffer and centrifuged at 9000 rpm for 20 min. The supernatant was injected into an autosampler and eluted through a C18 column (4.5 × 1500 mm × 5 μM). The dopamine content was analyzed by LC/ESI-MS spectrometer.

2.15. Statistical analysis

Data represent the mean ± SD from three different experiments. Differences among those means were analyzed using one-way ANOVA and Student's *t*-test. Where differences in ANOVA testing were observed ($P < 0.05$) Newman–Keuls post hoc analysis was employed.

3. Results

3.1. Construction and purification of PEP-1-PEA-15

Human PEA-15 cDNA was subcloned into a pET-15b plasmid that had been produced to include the PEP-1 peptide in order to generate a cell permeable expression vector, PEP-1-PEA-15 which contained a consecutive cDNA sequence encoding the human PEA-15, PEP-1 peptide and six histidine residues at the amino-terminus. A PEA-15 expression vector was also constructed without the PEP-1 peptide as a control (Fig. 1A and B). After induction of expression, PEP-1-PEA-15 proteins were purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. Purified PEP-1-PEA-15 protein was confirmed by SDS-PAGE and Western blot analysis. The purification results are shown in Fig. 1C. The proteins were confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. PEP-1-PEA-15 proteins were detected at the corresponding bands in Fig. 1D.

3.2. Transduction of PEP-1-PEA-15 protein into SH-SY5Y and BV2 cells

In order to assess the ability of PEP-1-PEA-15 proteins to transduce into SH-SY5Y cells, various concentrations (0.5–3 μM) of proteins were added to the culture medium for various times (10–60 min), after which transduction levels were analyzed by Western blotting. Various concentrations (0.5–3 μM) of PEP-1-PEA-15 proteins were added to SH-SY5Y cells in culture for 60 min, and the levels of transduced proteins were measured by Western blotting. PEP-1-PEA-15 proteins transduced into the cells in a concentration dependent manner (Fig. 2A). The intracellular concentration of transduced PEP-1-PEA-15 (3 μM) proteins in cells was detected within 10 min and gradually increased at 60 min (Fig. 2B). The results revealed that PEP-1-PEA-15 proteins transduced into the cells in a dose- and time-dependent manner. However, control PEA-15 protein did not transduce into the cells. Also, we examined the stability of transduced PEP-1-PEA-15 proteins in SH-SY5Y cells. The intracellular level of transduced PEP-1-PEA-15 proteins in cells was initially detected after 1 h and it was nearly completely absent by 48 h. However, significant levels of transduced PEP-1-PEA-15 proteins persisted in the cells for 12 h (Fig. 2C).

Next, to investigate whether there is any significant difference between endogenous PEA-15 and transduced PEP-1-PEA-15, nuclear and cytosolic fractions were prepared from cells and Western blotting using subcellular specific marker antibodies were performed. As shown in Fig. 2D, the localization of transduced PEP-1-PEA-15 protein showed a similar pattern to that of endogenous PEA-15.

We also examined PEP-1-PEA-15 transduction efficiency in BV2 microglia cells. As shown in Fig. 2E and F, PEP-1-PEA-15 proteins transduced into the BV2 microglia cells in a dose- and time-dependent manner, similar to that of SH-SY5Y cells demonstrating that PEP-1-PEA-15 protein transduced into both SH-SY5Y neuroblastoma and BV2 microglia cells. To clarify the cellular localization of PEP-1-PEA-15 proteins in the SH-SY5Y cells, transduced cells were double-stained with the nucleus- and cytosolic markers DAPI and Alexa. PEP-1-PEA-15 protein was detected in the cytoplasm and in the nucleus of transduced cells (Fig. 3A). These results indicate that PEP-1-PEA-15 proteins transduced into the cells and were stable for at least 12 h.

3.3. Effect of PEP-1-PEA-15 protein on MPP^+-induced cytotoxicity

To determine whether transduced PEP-1-PEA-15 proteins protect cells against neurotoxin-induced neuronal cell death, cell viability was measured after administration of MPP^+ using an MTT assay. The viability of cells treated with MPP^+ increased in a dose-dependent manner when pretreated with PEP-1-PEA-15 proteins. When the cells were exposed to 4 mM MPP^+ for 18 h, only 52% of the cells were viable. However, the viability of cells pretreated with PEP-1-PEA-15 protein increased in a dose-dependent manner, reaching over 75% at the maximum concentration used. However, control PEA-15 protein demonstrated no protective effects under the same conditions (Fig. 3B).

Also, we investigated whether PEP-1-PEA-15 protein inhibits intracellular reactive oxygen species (ROS) generation and DNA fragmentation induced by MPP^+. When the cells were exposed to 4 mM MPP^+ for 40 min, MPP^+ markedly increased the DCF signal. However, ROS generation was decreased by the presence of PEP-1-PEA-15 protein. ROS generation was quantified using a Fluoroskan ELISA plate reader. As shown in Fig. 4A, transduced PEP-1-PEA-15 protein significantly inhibited ROS production in SH-SY5Y cells. Next, we examined the protective effect of transduced PEP-1-PEA-15 protein against DNA fragmentation via TUNEL staining. As shown in Fig. 4B, the negative control cells were not stained. However, MPP^+ markedly increased the number of cells stained compared with the control, whereas cells treated with transduced PEP-1-PEA-15 protein were only slightly stained. Also, we measured DNA fragmentation in the BV2 microglia cells. Transduced PEP-1-PEA-15 protein efficiently protects against DNA fragmentation in BV2 cells in a similar fashion to SH-SY5Y cells (Fig. 4C). These results indicate that transduced PEP-1-PEA-15 protein efficiently inhibits MPP^+-induced ROS generation and DNA fragmentation.

GAP-43 is known to be a growth or plasticity marker protein and is expressed at high levels during neuronal cell development. As shown in Fig. 5, GAP-43 protein levels in SH-SY5Y cells treated with MPP^+ were significantly decreased by MPP^+, whereas pretreatment with PEP-1-PEA-15 protein prevented decreased GAP-43 expression levels. However, in BV2 microglia cells pretreated with PEP-1-PEA-15 protein, GAP-43 expression levels were only slightly decreased. These results indicate that PEP-1-PEA-15 protein effectively protects against MPP^+ induced decreases in neuronal cell differentiation.

Further, we performed Western blot analyses to assess Bax, Bcl-2, caspase-3, and cleaved caspase-3 expression levels using indicated antibodies. We observed that the expression levels of Bax and cleaved caspase-3 were significantly increased in MPP^+ treated cells, whereas the levels of Bax and cleaved caspase-3 expression were markedly reduced in the PEP-1-PEA-15 protein treated cells. However, the levels of Bcl-2 and caspase-3 expression showed a contrary pattern to Bax and cleaved caspase-3 expression (Fig. 6). In addition, we examined whether transduced PEP-1-PEA-15 protein was involved in Akt, ERK, and JNK phosphorylation which are well known to be involved with cellular processes including survival [48,49]. As shown in Figs. 7 and 8, transduced PEP-1-PEA-15 protein suppressed MPP^+-induced activation of Akt, ERK, and JNK in a dose-dependent manner in SH-SY5Y neuroblastoma cells and BV2 microglia cells. However, PEA-15 protein did not affect the phosphorylated Akt, ERK, and JNK. These results indicate that transduced PEP-1-PEA-15 proteins played a defensive role against MPP^+-induced cell death.

3.4. Transduction of PEP-1-PEA-15 protein into substantia nigra and protects against Parkinson disease animal model

To determine whether PEP-1-PEA-15 protein crossed the blood–brain barrier, we performed immunohistochemistry on the substantia nigra (SN) of sections of brain taken from mice treated with PEP-1-PEA-15 protein. The significantly elevated levels of PEP-1-PEA-15 throughout the SN of treated animals shown in Fig. 9A demonstrate that PEP-1-PEA-15 did go through the BBB. However, control PEA-15 protein did not transduce into the SN. These results indicate that PEP-1-PEA-15 protein efficiently transduced into the SN beyond the mice blood–brain barrier.

In order to evaluate the protective effect of transduced PEP-1-PEA-15 protein against dopaminergic neuronal cell death, dopaminergic neuronal cell levels were estimated by tyrosine hydroxylase (TH) and cresyl violet (CV) immunostaining in a chronic MPTP-induced PD mouse model. As shown in Fig. 9B, PEP-1-PEA-15 protein significantly protected against dopaminergic neuronal death caused by MPTP

treatment. However, control PEA-15 protein and PEP-1 peptide did not have the same protective effect compared to MPTP treated mice. In the MPTP-treated mice, the number of TH-positive cells in the SN was markedly reduced compared to the control mice. However, the number of neurons was significantly increased by transduced PEP-1-PEA-15 protein compared to those in the mice treated with MPTP, control PEA-15

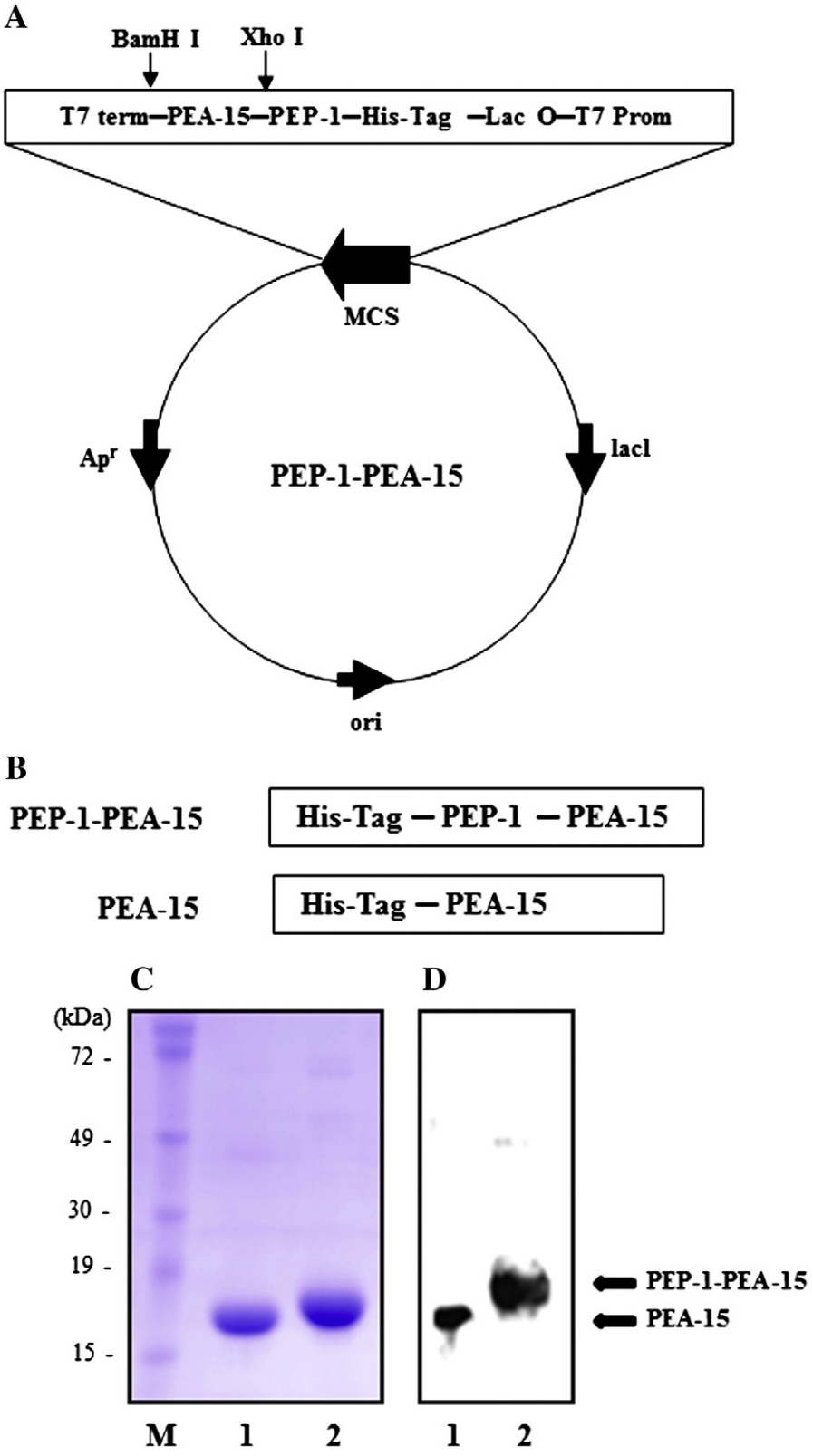

Fig. 1. Construction and purification of PEP-1-PEA-15 proteins. Construction of the PEP-1-PEA-15 expression vector system based on the vector pET-15b. A synthetic PEP-1 oligomer was cloned into the *Nde*I and *Xho*I sites, and human PEA-15 cDNA was cloned into the *Xho*I and *Bam*HI sites of pET-15b (A). Diagram of the expressed control PEA-15 and PEP-1-PEA-15 fusion proteins (B). Each contains a His tag consisting of six histidine residues. Expression was induced by adding IPTG. Purification of PEP-1-PEA-15 proteins. Protein extracts of cells and purified proteins were analyzed by 12% SDS-PAGE (C) and subjected to Western blot analysis (D) with antibody against rabbit polyhistidine. Lanes are as follows: lane 1, purified control PEA-15; and lane 2, purified PEP-1-PEA-15.

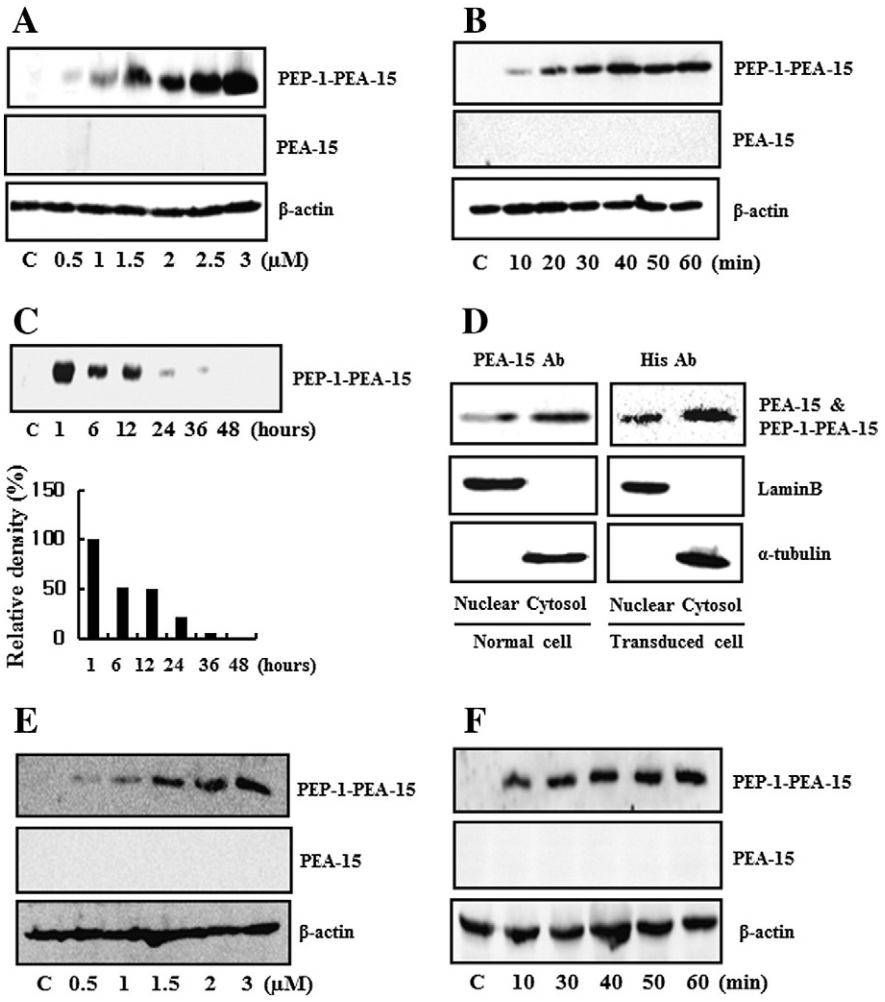

Fig. 2. Transduction of PEP-1-PEA-15 proteins into SH-SY5Y neuroblastoma and BV2 microglia cells. PEP-1-PEA-15 and control PEA-15 (0.5–3 μM) were added to the SH-SY5Y culture medium for 1 h (A). PEP-1-PEA-15 or control PEA-15 (3 μM) were added to the SH-SY5Y culture medium for 10–60 min (B). The transduction of PEP-1-PEA-15 proteins into the cells was analyzed by Western blotting. Intracellular stability of transduced PEP-1-PEA-15 (C). PEP-1-PEA-15 (3 μM) was transduced into SH-SY5Y cells for 1 h and the intracellular level of PEP-1-PEA-15 was measured over a 48 h period by Western blotting and band intensity was assessed by densitometer. Subcellular localization of PEP-1-PEA-15 (D). The nuclear and cytosolic extracts were prepared from transduced and normal SH-SY5Y cells and analyzed by Western blotting. PEP-1-PEA-15 and control PEA-15 (0.5–3 μM) were added to the BV2 culture medium for 1 h (E). PEP-1-PEA-15 and control PEA-15 (3 μM) were added to the BV2 culture medium for 10–60 min (F). The transduction of PEP-1-PEA-15 proteins into the cells was analyzed by Western blotting.

protein and PEP-1 peptide. These results indicate that PEP-1-PEA-15 protein efficiently transduced into the brain and strongly protected against neuronal cell death by detoxifying MPTP-induced neurotoxicity.

To assess the behavioral improvement of transduced PEP-1-PEA-15 protein on MPTP-induced PD mice, the rotarod test was performed. As shown in Fig. 9C, the amount of time mice stayed on the accelerating rotarod was markedly decreased in MPTP-treated mice (52 s) compared to control mice (300 s). However, the times of PEP-1-PEA-15 protein-treated mice were maintained (181 s) compared to those of MPTP-treated mice.

The motor symptoms of PD result from the death of dopaminergic neuronal cells in the SN [33,34]. Therefore, we confirmed mouse striatum dopamine levels. After the rotarod tests, mice were sacrificed and striatum dopamine levels were assessed. In a similar fashion with behavioral dysfunction, LC/ESI-MS analysis showed that in the group exposed only to MPTP striatum dopamine levels were 76.332 ng/mg. However, by comparison, PEP-1-PEA15 treated mice demonstrated markedly increased dopamine levels at 211.44 ng/mg. By contrast, control-PEA15 and PEP-1 peptide had no effect on dopamine levels (Fig. 9C). These results indicate that PEP-1-PEA-15 protein protected against MPTP-induced behavioral dysfunction and striatum dopamine levels.

To examine whether PEP-1-PEA-15 protein transduction is affected by MPTP, protein transduction of PEP-1-PEA-15 was examined in DA neurons in the SN in the absence and presence of MPTP. Mice were i.p. injected with PEP-1-PEA-15 and control proteins (PEA-15, GFP, PEP-1-GFP) after MPTP treatment. The brains of the mice were harvested 12 h and brain sections were stained with a His antibody. In both groups treated with MPTP and untreated groups, PEP-1-GFP and PEP-1-PEA-15 proteins were transduced into tissues (Fig. 10A). These results indicate that transduction was not facilitated by exposure to MPTP.

In addition, we examined the effect of PEP-1-PEA-15 protein on cell death in the absence or presence of MPTP 7 days after treatment. As shown in Fig. 10B, in the absence of MPTP, DA neuronal cell survival was elevated in the PEP-1-PEA-15 and control protein treated groups. However, in the presence of MPTP, DA neuronal cell death was significant in the control groups while cell survival remained high in the PEP-1-PEA-15 protein treated animals. These results indicate that PEP-

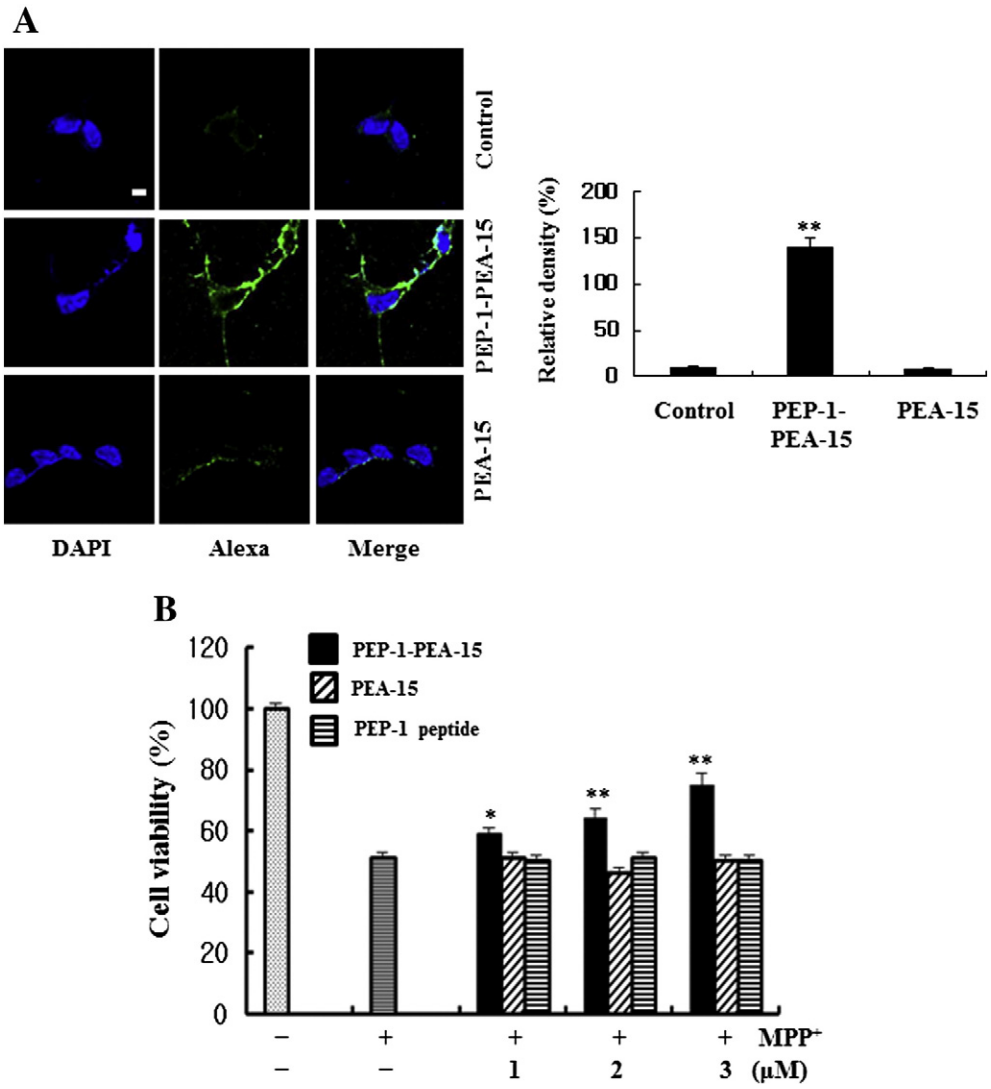

Fig. 3. Transduction of PEP-1-PEA-15 proteins into SH-SY5Y cells and cell viability. PEP-1-PEA-15 (3 μM) or control PEA-15 was added to the culture medium for 1 h, and the distribution of the transduced PEP-1-PEA-15 was observed by confocal microscopy. Fluorescence intensity was measured by a ZEN digital imaging calculator (A). Scale bar = 50 μm. MPP^+ (2 mM) was added for 18 h to SH-SY5Y cells that had been pretreated for 1 h with PEP-1-PEA-15 or control PEA-15 protein (1–3 μM). Cell viabilities were assessed by the MTT-based colorimetric assay (B). These results are representative of at least three separate experiments. $^*P < 0.05$ and $^{**}P < 0.01$, compared with MPP^+-treated cells.

1-PEA-15 protein crossed the BBB and protected against DA neuronal cell death in the SN in the MPTP-induced PD mice model.

4. Discussion

Phosphoprotein enriched in astrocytes 15 (PEA-15) is a multifunctional linker protein which controls cell survival, proliferation, and glucose metabolism [15]. The expression level of PEA-15 affects various cell types and plays important roles in cell survival. Recent studies have shown that overexpression of PEA-15 reduces neuroblastoma metastasis in patients and is a potential target for the development of new therapeutic agents in patients with neuroblastoma [50]. Also, in human breast and ovarian cancers, overexpression of PEA-15 is associated with improved overall survival in women with these cancers and PEA-15 expression is an important prognostic marker with therapeutic potential in the treatment of these cancers [21,51]. Further studies on PEA-15 protein are needed to determine its role in neuronal diseases including PD for it to be considered as a potential therapeutic agent. In this study, we investigated the protective effects of cell-permeable PEP-1-PEA-15 protein on neuronal damage induced by MPP^+ in SH-SY5Y neuronal cells, BV-2 microglia cells and in vivo in a MPTP-induced mouse model of PD.

Our data indicate that purified PEP-1-PEA-15 time and dose dependently transduced into SH-SY5Y cells and BV-2 cells efficiently. PEA-15 is known to localize in the cytoplasm [52]. However, other studies have shown that PEA-15 protein should be able to diffuse into the nucleus since it contains a leucine-rich nuclear export sequence at its N terminus [17,53,54]. In agreement with those results, we detected transduced PEP-1-PEA-15 protein in the nuclei and in the cytoplasm of transduced and normal cells. However, it was distributed more in the cytosol than in the nuclei.

1-Methyl-4-phenylpyridinium ion (MPP^+) is a neurotoxin which induces neuronal cell death by generating reactive oxygen species (ROS) and is widely used in PD experiments. The natural by-product of a variety of cellular processes involving interaction with oxygen, ROS plays important roles in neuronal death associated with PD [9,29]. Therefore, we examined whether transduced PEP-1-PEA-15 protein could inhibit the oxidative stress induced by MPP^+ using an MTT assay and the ROS sensitive dye DCF-DA. When the cells were pretreated with PEP-1-PEA-15 proteins, cell viability markedly increased compared to

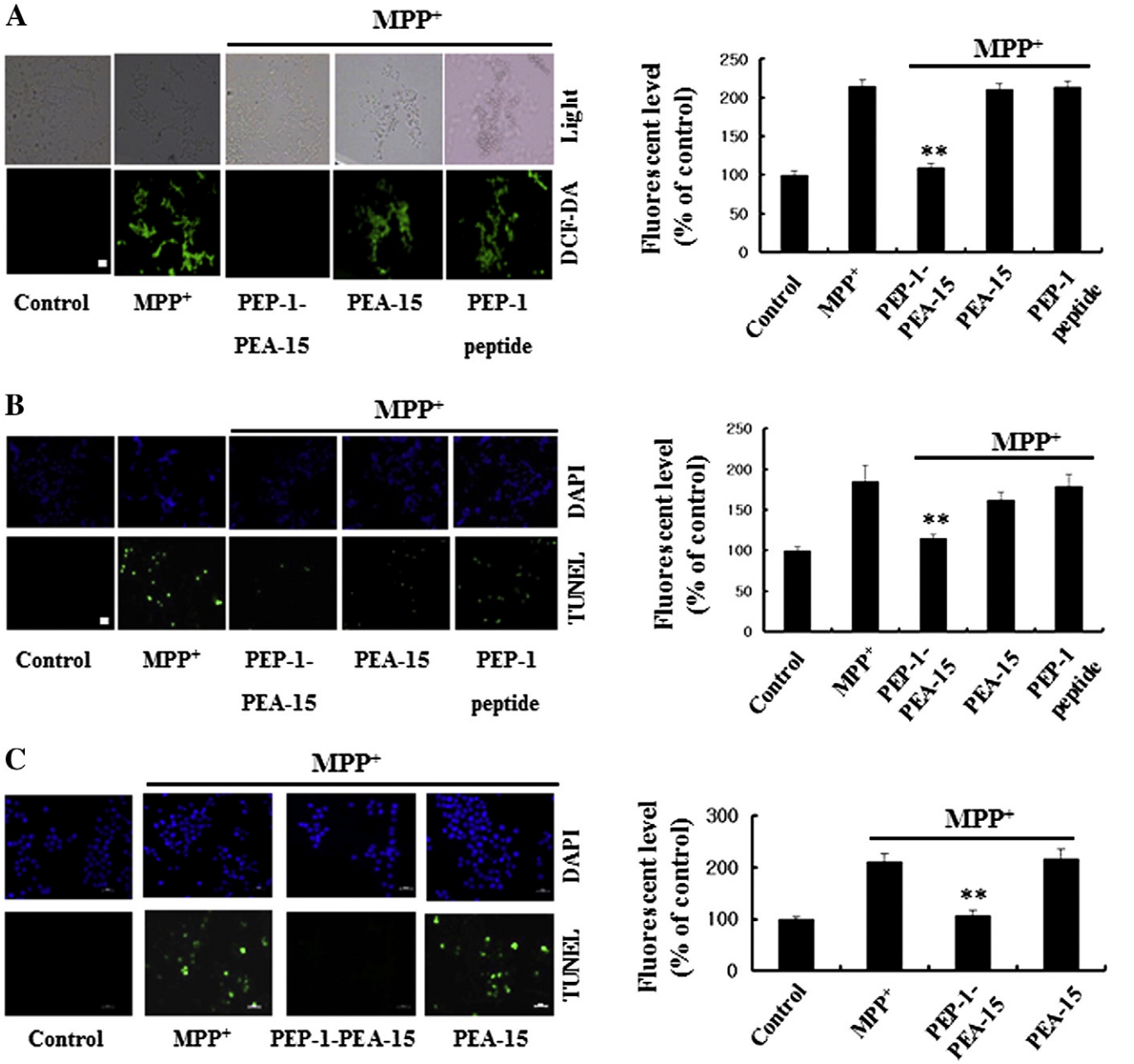

Fig. 4. Effect of transduced PEP-1-PEA-15 on MPP^+-induced ROS generation and DNA fragmentation in the SH-SY5Y and BV2 cells. Cells were treated with PEP-1-PEA-15 (3 μM) for 1 h, and then exposed to MPP^+ (4 mM) for 40 min. Intracellular ROS levels were measured after staining with the fluorescent dye, DCF-DA (A). The fluorescence intensity was measured by an ELISA plate reader. SH-SY5Y (B) and BV2 (C) cells were treated with PEP-1-PEA-15 (3 μM) for 1 h, and then exposed to MPP^+ (4 mM) for 18 h. DNA fragmentation was detected by TUNEL staining. These results are representative of at least three separate experiments. $^{**}P < 0.01$, compared with MPP^+-treated cells. Scale bar = 50 μm.

untreated MPP^+ exposed cells. Also, transduced PEP-1-PEA-15 protein significantly inhibited MPP^+ induced ROS production in SH-SY5Y cells.

Recent studies have demonstrated that GAP-43 mRNA and protein expression levels were decreased in SH-SY5Y cells treated with amyloid precursor protein (APP). However, decreases of GAP-43 levels were more significant in cells treated with retinoic acid [54,55]. Since GAP-43 is known to be a growth or plasticity marker protein and is expressed at high levels during neuronal cell development, we examined the relationship between GAP-43 and PEA-15 protein.

GAP-43 expression levels were markedly decreased in cells treated with MPP^+, whereas cells treated with PEP-1-PEA-15 protein didn't demonstrate lower GAP-43 expression. These data indicate that PEP-1-PEA-15 protein is associated with the preservation of growth or differentiation mechanisms in neuronal cells. Further, we found that transduced PEP-1-PEA-15 protein protects against MPP^+-induced apoptotic cell death in SH-SY5Y cells. Mitochondria dysfunction has been reported to play a crucial role in mammalian cells. The Bcl-2 family of proteins, such as apoptotic activator (Bax) and apoptotic inhibitor (Bcl-2), play important roles in cell death [56,57] and the Bax to Bcl-2 ratio is a key factor in cell death regulation [58]. Also, caspase-3 activation is well known as a maker of apoptotic cell death [59]. In this study, we observed that transduced PEP-1-PEA-15 protein protected against cell death by increasing the expression of Bcl-2 and caspase-3. We further investigated whether PEP-1-PEA-15 protein was involved in the biological functions of cell survival. We found that transduced PEP-1-PEA-15 protein inhibits MPP^+-induced cell death by regulating the

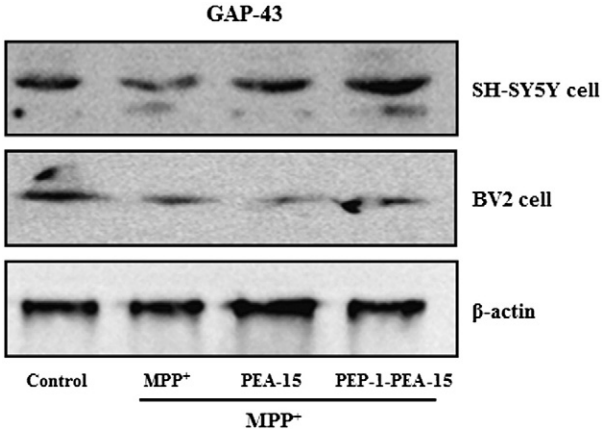

Fig. 5. Effect of transduced PEP-1-PEA-15 on MPP^+-induced GAP-43 expression in the cells. SH-SY5Y and BV2 cells were treated with PEP-1-PEA-15 (3 μM) for 1 h, and then exposed to MPP^+ (2 mM) for 36 h. Cells were lysed, and total protein extracts were separated by SDS-PAGE followed by immunoblotting with GAP-43 antibody. GAP-43 expression levels were analyzed by Western blotting.

phosphorylation of Akt, ERK, and JNK. Also, PTD-PEA-15 proteins were delivered to islets and pancreatic tissue, without impairing islet function, and prevented stress-induced protein kinase [60]. Although the detailed mechanism remains to be further examined, these results indicate that transduced PEP-1-PEA-15 protein mitigates MPP^+-induced oxidative stress and helps promote cell survival by regulating apoptotic molecular events.

Some studies have demonstrated that deletion of PEA-15 protein in mice causes specific defects in spatial learning abilities. Also these mice showed impaired ERK or RSK2 functions. Since PEA-15 protein binds to ERK and regulates ERK signaling which is involved in learning and memory, PEA-15 may be an essential regulator of ERK-dependent pathways and play a critical role in the mediation of spatial learning [22]. In addition, other groups have demonstrated that PEA-15 promotes TGF-β1 mediated autophagy on differentiation in skeletal muscle cells [61]. Autophagy is known to play a role in homeostasis and cell-size regulation in various organs under both normal and under stressful conditions [62]. In this study, we have shown that transduced PEP-1-PEA-15 protein regulated ERK, MAP kinase and inhibited oxidative stress. Further pretreatment with PEP-PEA-15 protein prevented decreases of

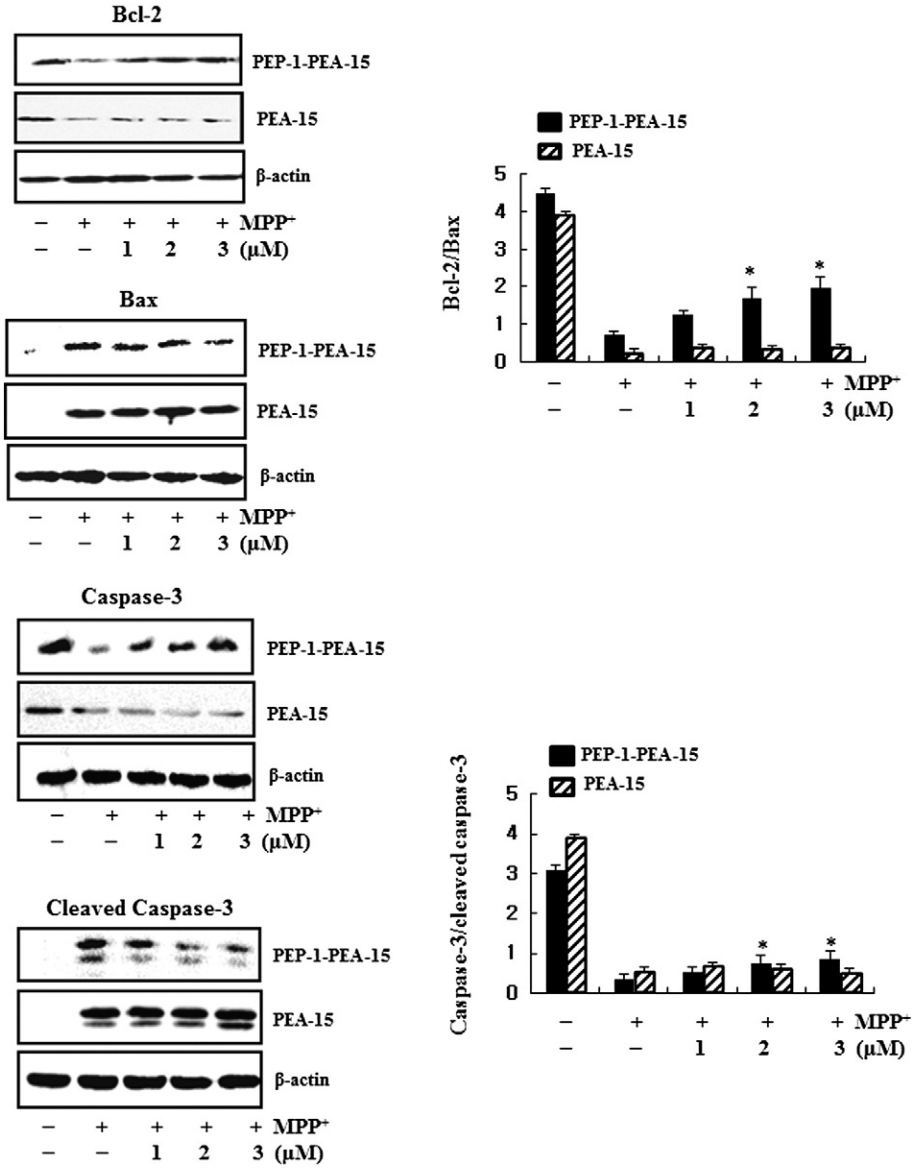

Fig. 6. Effect of transduced PEP-1-PEA-15 on the expression of Bcl-2, Bax, caspase-3 and cleaved caspase-3 in SH-SY5Y cells. The cells were treated with PEP-1-PEA-15 (1–3 μM) for 1 h, and then exposed to MPP^+ (4 mM) for 6 h, 18 h and 24 h, respectively. The expression of Bcl-2, Bax, caspase-3 and cleaved caspase-3 levels were measured by Western blotting and band intensity by densitometer. $^*P < 0.01$, compared with MPP^+-treated cells.

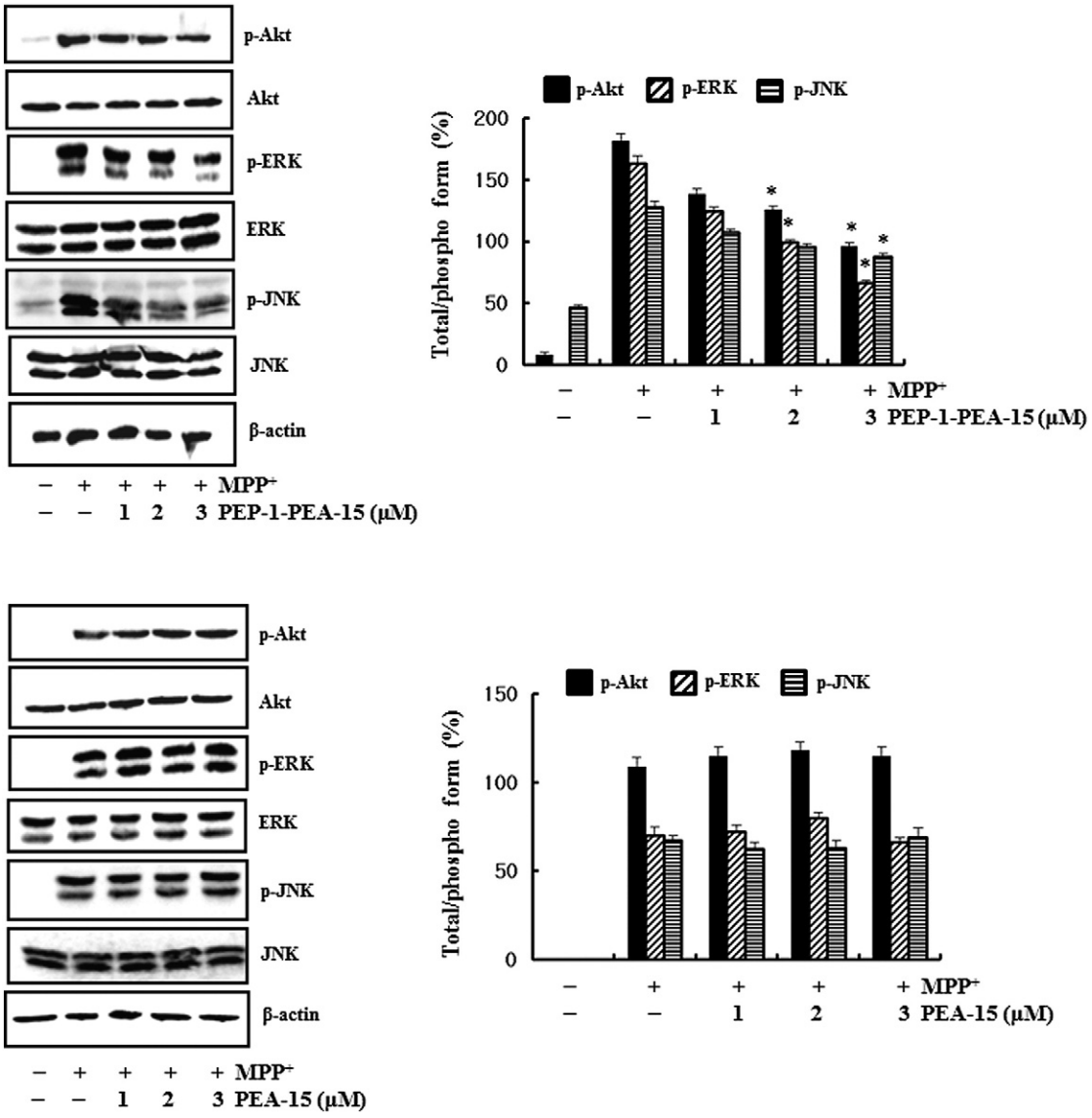

Fig. 7. Effect of transduced PEP-1-PEA-15 on the expression of phosphorylation of Akt, ERK, and JNK in SH-SY5Y neuroblastoma cells. The cells were treated with PEP-1-PEA-15 (1–3 μM) for 1 h, and then exposed to MPP^+ (4 mM). The expression of phosphorylation of Akt, ERK, and JNK levels was measured by Western blotting and band intensity by densitometer. $^*P < 0.01$, compared with MPP^+-treated cells.

GAP-43 protein levels in neuronal cells. Although further studies are necessary to understand the protective mechanisms of PEA-15 in the brain, transduced PEP-1-PEA-15 protein plays an important role in protecting against neuronal cell damage and may be useful as a therapeutic agent against various diseases related to neuronal cell growth and differentiation.

PEA-15 is also known to be associated with various diseases including Alzheimer's disease (AD) and diabetes [63,64]. Amyloid deposits, neurofibrillary tangles, and neuronal loss are well known in AD [63]. Studies have demonstrated that PEA-15 was elevated in AD model mouse brains, suggesting that PEP-15 may be used as a biomarker of AD. Although further study is needed to identify PEA-15 functions in AD, enhancement of PEA-15 expression may have beneficial effects on glial pathology and protect against astrocyte apoptosis induced by TNF or Aβ plaque. As AD symptoms are similar to PD, including disabling motor abnormalities and neuronal loss, PEP-1-PEA-15 protein also may play a protective role in AD. Therefore, further studies to identify the function of PEA-15 in AD are necessary in order to explore those potential roles.

In addition, peroxisome proliferator-activated receptor-γ (PPAR-γ) represses transcription of the PEA-15 gene and this repression impairs glucose tolerance in mice. And also PPAR-γ regulates the inflammatory network via the PEA-15 gene [64]. It is well known that inflammation plays an important role in the pathogenesis of PD and the MAPK pathway is known to be associated with inflammation [6,17]. Our study showed that PEP-1-PEA-15 protein is involved in MAPK and cell survival pathways in neuronal cells. Therefore, PEP-1-PEA-15 protein may be applied to diabetes and inflammation studies by reducing PEA-15 gene expression in the PPAR-γ regulated inflammatory network.

Owing to its similarity to the pathophysiology of human PD a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT)-induced mouse model has been used extensively to investigate the pathological mechanism of PD [65,66]. It is well known that exposure of C57BL/6 mice to the neurotoxin MPTP is one of the most valuable approaches to analyze critical aspects of PD in animal models. MPTP, a selective neurotoxicant, is known to deplete striatal dopamine and is initiated by the MPP^+

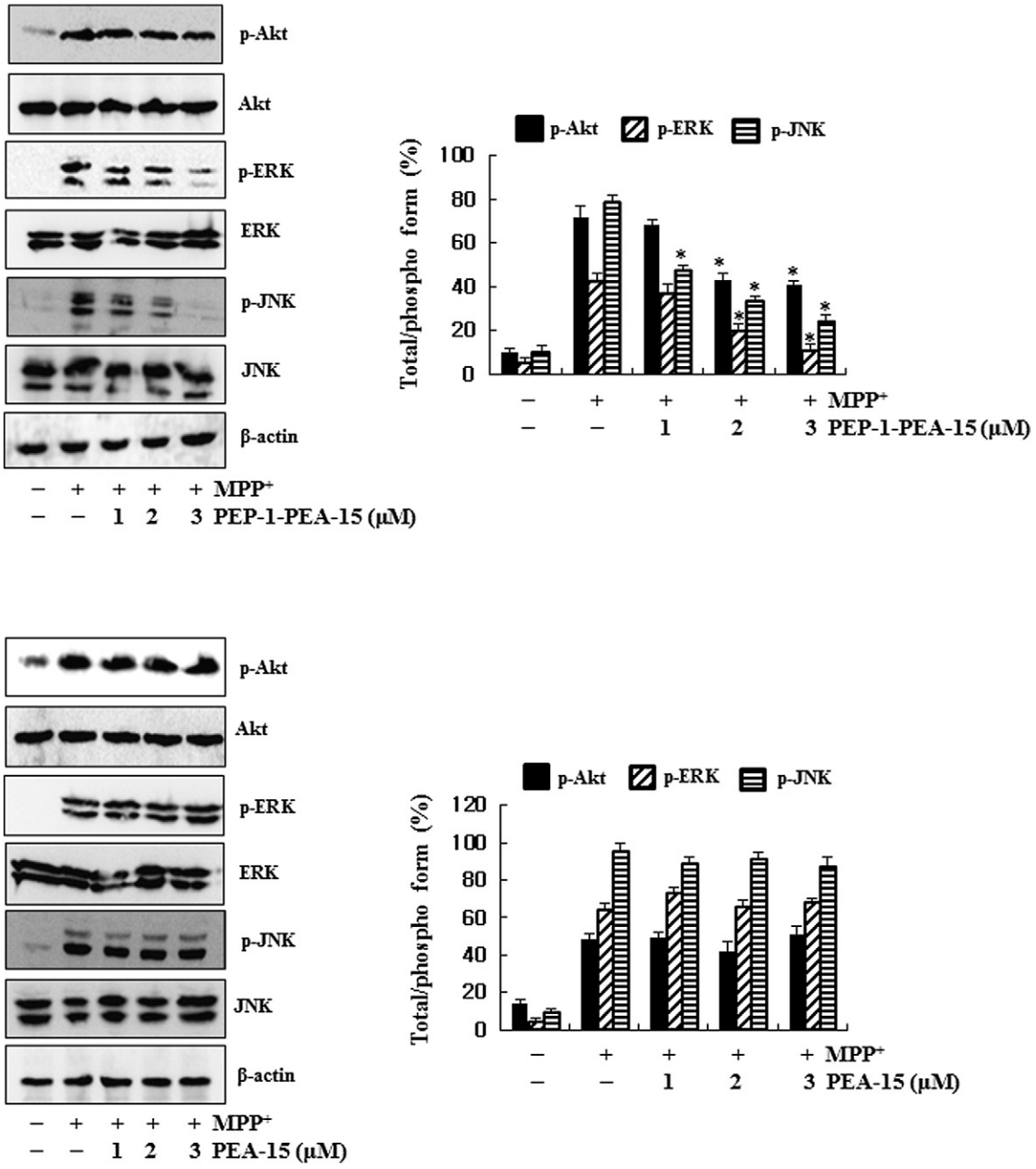

Fig. 8. Effect of transduced PEP-1-PEA-15 on the expression of phosphorylation of Akt, ERK, and JNK in BV2 microglia cells. The cells were treated with PEP-1-PEA-15 (1–3 μM) for 1 h, and then exposed to MPP^+ (4 mM). The expression levels of phosphorylation of Akt, ERK, and JNK were measured by Western blotting and band intensity by densitometer. $^*P < 0.01$, compared with MPP^+-treated cells.

[67,68]. Several studies have demonstrated that MPTP injected mice demonstrate decreased the levels of striatal dopamine which induces motor dysfunction leading to increased latency to fall on the rotarod. However, antidepressants such as paroxetine and fluoxetine prevent MPTP-induced loss of dopaminergic neurons. In this study, MPTP injection also reduced dopamine levels and increased latency to fall off the rod. However, PEP-1-PEA15 protein protected against MPTP-induced motor dysfunction caused by decreased levels of dopamine in the striatum. The significant biochemical change in the striatum resulting from MPTP injection is a decrease in the levels of striatal dopamine. MPTP induced biochemical insufficiency in C57/BL mice causes a motor dysfunction that leads to increased latency to fall on the behavior test machine. These results agree with the present evidence that motor performance on a rotarod decreases with the loss of striatum TH stained cells indicating a reduction of dopamine levels in the MPTP injected striatum. Thus, PEP-1-PEA-15 protein protected against MPTP-induced motor dysfunction by increasing the levels of dopamine in the striatum.

In this study, we examined the effect of MPTP on transduction efficiency of proteins. We used GFP protein as a control to measure the differences of transduction efficiency in the absence and presence of MPTP. PTD fusion proteins such as PEP-1-PEA-15 and PEP-1-GFP protein were transduced into the SN in mice brains in the presence and absence of MPTP. These results indicate that PEP-1-PEA-15 transduction protein was not affected by MPTP. Also, the number of neurons was significantly increased in the transduced PEP-1-PEA-15 protein group.

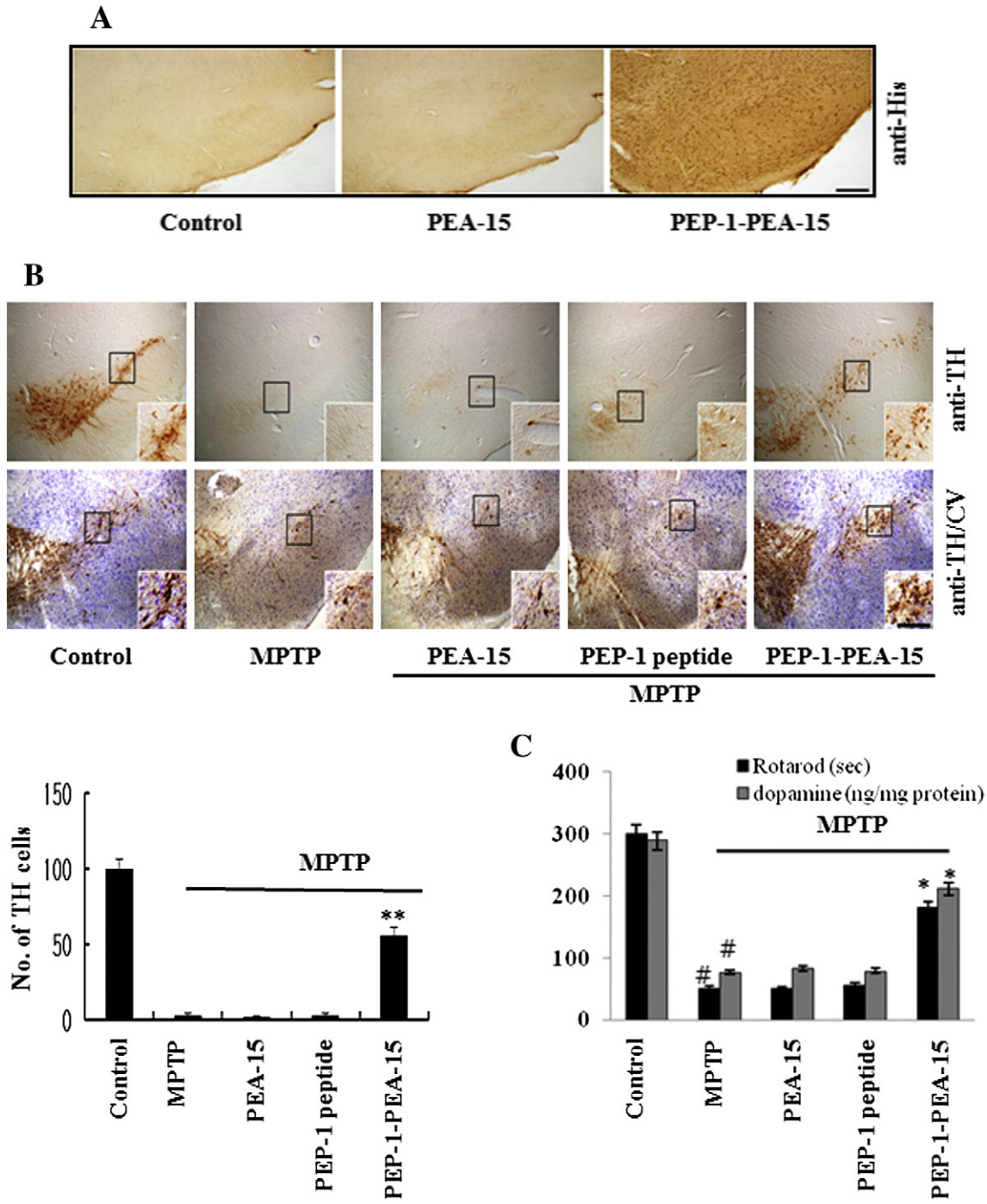

Fig. 9. Effects of transduced PEP-1-PEA-15 on dopaminergic neuronal cell viability. Transduction of PEP-1-PEA-15 protein across the blood–brain barrier. Transduction of PEP-1-PEA-15 protein in mouse brain was analyzed by immunohistochemistry using anti-histidine antibody. Animals were treated with a single injection of PEP-1-PEA-15 and killed after 8 h (A). Scale bar = 100 μm. Representative photomicropraphs of the tyrosine hydroxylase (TH) and double staining with cresyl violet (CV) and TH-immunoreactivity (B). Scale bar = 100 and 50 μm. The number of TH-immunoreactivity neurons in 250 × 250 μm^2 is shown in the graph. $^{**}P < 0.01$, compared with MPTP-treated mice. After mice were positioned on a Rotarod and the time that the mice stayed on the rod without falling was recorded, dopamine levels in the striatum were assessed (C). Five mice were used for each experimental group. $^{\#}P < 0.01$, compared to control group. $^{*}P < 0.01$, compared to MPTP-treated group.

These results indicate that PEP-1-PEA-15 protein protects against dopaminergic neuronal cell death in the SN region, and attenuates neuronal damage.

In conclusion, this study has demonstrated that human PEA-15, when fused with a cell permeable PEP-1 peptide (PEP-1-PEA-15) can efficiently transduce into cells and protects against neurotoxin-induced neuronal cell death in vitro and in vivo. Although further study is required to understand the precise mechanism of that protection, these results suggest that PEP-1-PEA-15 may provide a new therapeutic strategy for protecting against the neuronal cell death associated with PD and other human neuronal diseases.

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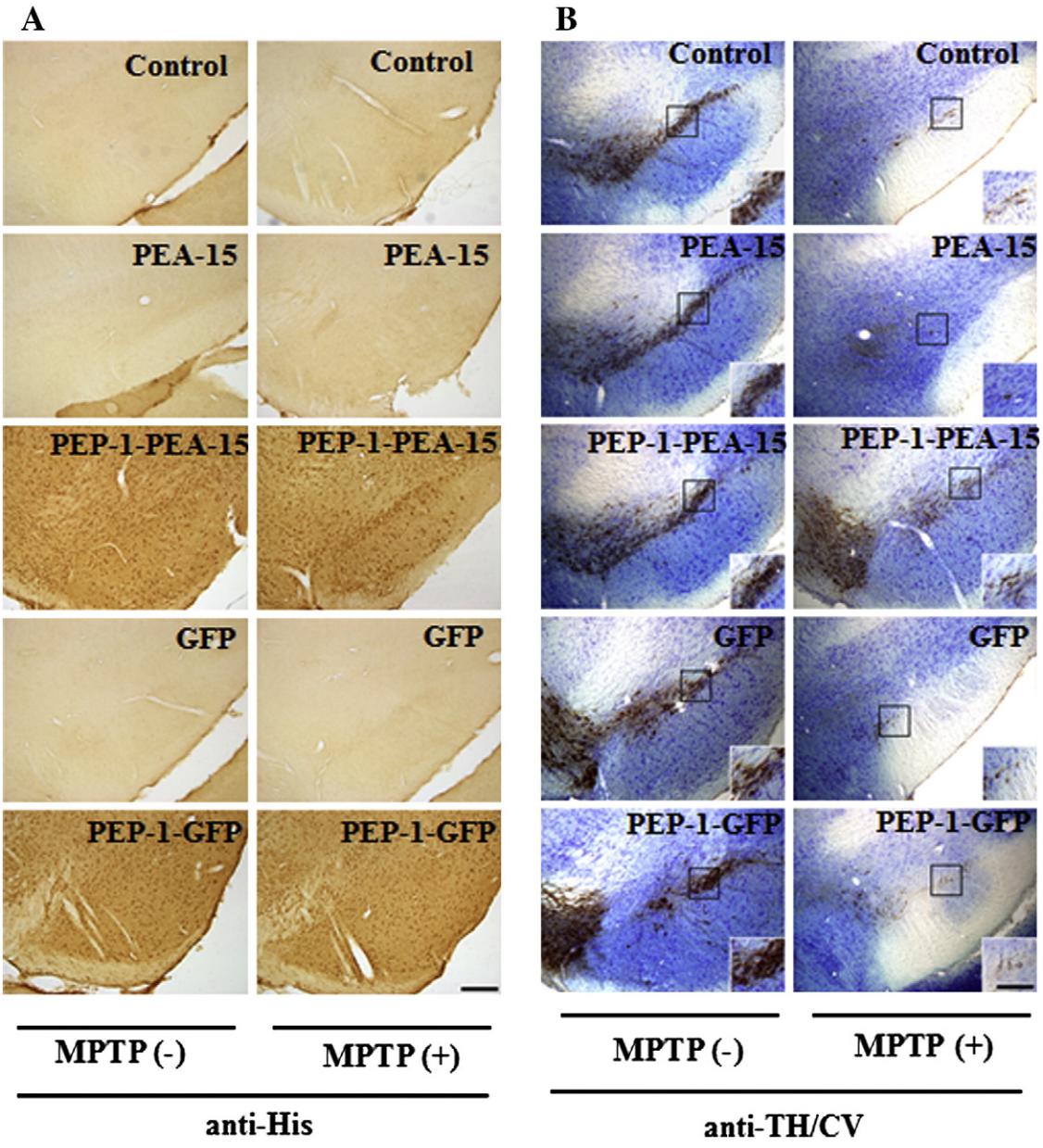

Fig. 10. Effects of MPTP on PEP-1-PEA-15 protein transduction. Transduction of PEP-1-PEA-15 into the SN in the absence and presence of MPTP. Brain tissues were taken 12 h after treatment with PEP-1-PEA-15 and control proteins. Representative photomicropraphs of the His-immunoreactivity (A). PEP-1-PEA-15 affects cell death by MPTP. PEP-1-PEA-15 and control proteins on cell death in the absence and presence of MPTP 7 days after treatment. Then the brains were analyzed by immunohistochemistry. Representative photomicropraphs of the double staining with TH and cresyl violet (CV)-immunoreactivity (B). Scale bar = 100 and 50 μm.

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