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Norepinephrine upregulates the expression of tyrosine hydroxylase and protects dopaminegic neurons against 6-hydrodopamine toxicity

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ABSTRACT

As a classic neurotransmitter in the brain, norepinephrine (NE) also is an important modulator to other neuronal systems. Using primary cultures from rat ventral mesencephalon (VM) and dopaminergic cell line MN9D, the present study examined the neuroprotective effects of NE and its effects on the expression of tyrosine hydroxylase (TH). The results showed that NE protected both VM cultures and MN9D cells against 6-hydroxydopamine-caused apoptosis, with possible involvement of adrenal receptors. In addition, treatment with NE upregulated TH protein levels in dose- and time-dependent manner. Further experiments to investigate the potential mechanisms underlying this NE-induced upregulation of TH demonstrated a marked increase in protein levels of the brain-derived neurotrophic factor (BDNF) and the phosphorylated extracellular signal-regulated protein kinase 1 and 2 (pERK1/2) in VM cultures treated with NE. In MN9D cells, a significantly increase of TH and pERK1/2 protein levels were observed after their transfection with BDNF cDNA or exposure to BDNF peptides. Treatment of VM cultures with K252a, an antagonist of the tropomyosin-related kinase B, blocked the upregulatory effects of NE on TH, BDNF and pERK1/2. Administration of MEK1 & MEK2 inhibitors also reversed NE-induced upregulation of TH and pERK1/2. Moreover, ChIP assay showed that treatment with NE or BDNF increased H4 acetylation in the TH promoter. These results suggest that the neuroprotection and modulation of NE on dopaminergic neurons are mediated via BDNF and MAPK/ERK pathways, as well as through epigenetic histone modification, which may have implications for the improvement of therapeutic strategies for Parkinson's disease.

1. Introduction

The locus coeruleus (LC) is the primary source of brain norepinephrine (NE), with its projections innervating the whole central nervous system. Many investigations revealed that the LC-NE system exhibits a neuronal protection on the nigra-dopaminergic system. This notion is based on the findings that while a functional LC-NE system facilitates the survival of the dopaminergic neurons, the disturbance of the LC-NE system influences both the onset and progression of neuronal damage to the dopamine (DA) nigrostriatal tract (Delaville et al., 2011; Isaias et al., 2011). For example, lesions of the LC with 6-hydroxydopamine (6-OHDA) resulted in more dopaminergic neuronal loss or activity reduction in the substantia nigra pars compacta and ventral tegmental areas of animals subsequently exposed to MPTP (Mavridis et al., 1991; Srinivasan and Schmidt, 2003; Fornai et al., 1995; Bing et al., 1994). Similarly, neurotoxin such as DSP-4-induced reductions in LC activities and functions worsens DA deficit caused by MPTP in animal models of Parkinson's disease (PD) (Marien et al., 1993). As a classic neurotransmitter of the LC-NE system, NE plays a critical role for such neuroprotection. It was reported that in vitro administration of NE confers substantial and long-term protection to dopaminergic neurons by reducing spontaneously occurring oxidative stress in primary cultured mesencephalic cells (Troadec et al., 2001). Elevation of extracellular NE levels by treatment with α2-adrenergic receptor antagonists (Martel et al., 1998) or by genetic methods (Kilbourn et al., 1998) protected dopaminergic neurons from neurotoxin-induced cell death. NE hyperinnervation of target areas or treatment of rats with NE exhibited the ability to resistance to experimental parkinsonism (Marien et al., 1994; Rommelfanger et al. 2004, 2007). In addition, a post-mortem examination of brains from PD patients revealed that the brain areas that were high in NE tended to be spared from DA loss, consistent to a neuroprotective role of NE (Tong et al., 2006). Nevertheless,

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Abbreviation

ARs	adrenoceptors
BDNF	brain-derived neurotrophic factor
ChIP	Chromatin immunoprecipitation assay
DA	dopamine
ECL	enhanced chemiluminescence
DIV	day in vitro
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
LC	locus coeruleus
MAPK	mitogen-activated protein kinase
NE	norepinephrine
ODs	optical densities
PBS	phosphate-buffer saline
6-OHDA	6-hydroxydopamine
PD	Parkinson's disease
pERK1/2	phosphorylated extracellular signal-regulated protein kinase 1/2
TH	tyrosine hydroxylase
TrkB	tropomyosin-receptor-kinase B
TUNEL	terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling
VM	ventral mesencephalon

despite these compelling evidences to support the neuroprotective effects of NE on dopaminergic neurons, whether NE affects dopaminergic phenotypes and the underlying mechanisms remain to be elucidated.

Tyrosine hydroxylase (TH, tyrosine 3-mono-oxygenase; EC 1.14.16.2) is a rate-limiting enzyme in the biosynthesis of DA, NE, and epinephrine. It is not only expressed in the noradrenergic neurons (Moore and Bloom, 1979), but also abundantly in the dopaminergic neurons (Raisman-Vozari et al., 1991; Blanchard et al., 1993). As such, TH is also considered as the dopaminergic phenotype. Owing to its physiological and pathophysiological importance, TH synthesis is under stringent regulation at several distinct and overlapping levels (Lenartowski and Goc, 2011; Kumer and Vrana, 1996); including by nerve growth factors (Thoenen et al., 1971). Changes in TH expression generally reflect alterations in activity of dopaminergic neurons. Therefore, measurement of TH can yield important information regarding the functional status of DA neurons (Zigmond et al., 1989). On the other hand, as a classic neurotransmitter of the LC-NE system, NE may play a critical role for the modulation and neuroprotection of the dopaminergic neurons. This modulation and neuroprotection of NE can be reflected through the expression of TH in the dopaminergic neurons. It has been suggested that the neuronal beneficial effects of NE on other neurons can be by directly working as a neurotrophic factor, and through indirectly facilitating the expression of other neurotrophic factors (Chen et al., 2007; Counts and Mufson, 2010; Aloyz et al., 1999). Examining the effects of NE on TH expression as well as the underlying mechanisms may be of pivotal importance to evaluate the modulation and neuroprotection of dopaminergic neurons by the noradrenergic system.

In this study, primary cultures of the rat ventral mesencephalon (VM) and dopaminergic neuronal cell line MN9D were used to examine the effects of NE-induced neuroprotection and regulation on TH expression, as well as potential mechanisms. The results showed that NE protected dopaminergic cultures and cells against 6-OHDA-caused cell death, and increased the expression of TH. This effect can be direct or through the induction of BDNF. Both of them are potentially mediated by activation of neuronal survival pathways and through epigenetic histone modifications. Understanding the molecular basis of the neuroprotective effects of NE on dopaminergic neurons and its regulation of TH expression will be helpful in developing future therapies for the neurodegenerative diseases associated with dopaminergic neurons.

2. Materials and methods

2.1. Cell cultures

2.1.1. Primary tissue cultures¶

Primary cultures from rat VM were carried out according to the reported method (Weinert et al., 2015) with minor modifications. Timed pregnant Sprague Dawley rats were purchased from Harlan Laboratories Inc. (Indianapolis, IN, USA). Rats were maintained on a 12 h light/dark cycle with ad-libitum access to food and tap water. All animal procedures were approved by the Animal Care and Use Committee of East Tennessee State University (approval reference number: P130701), and complied with the NIH Guide for the Care and Use of Laboratory Animals. Briefly, these rats at day 15 of gestation (ED 15; the day following nocturnal mating being considered as ED 1 as provided by Harlan Laboratories) were anaesthetized with ketamine/xylazine (100mg/10 mg/kg i.p.). After the embryos were removed, their brains were dissected under a stereomicroscope. Mesencephalic tissues were collected in ice-cold Hank's balanced salt solution (HBSS, Gibco-Invitrogen, Carlsbad, CA, USA) and digested for 15 min at 37 °C in a 15 ml-centrifuge tube containing 4.5 ml HBSS, 0.5 ml 0.25% trypsin-EDTA and 25 μl RQ1 DNase (0.1 mg/ml deoxyribonuclease). Digestion was terminated by addition of 5 ml HBSS containing 1 mM of pyruvate, 10 mM HEPES and 1 ml of fetal bovine serum (FBS). Subsequently, tissues were mechanically triturated with a fire-polished Pasteur pipette. After dissociation, the suspension was spun down and the pellet was suspended in the neurobasal medium, which was supplemented with serum free B-27 (Gibco-Invitrogen, Carlsbad, CA, USA), 0.5 mM glutamine, 25 μM glutamate, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were plated in 24-well plates coated with poly-L-lysine (Sigma, St Louis, MO, USA). At 4 day in vitro (DIV), the medium was replaced by fresh medium without glutamate. Thereafter, half of the medium was changed every 3 days. Cells were used for drug treatment at 10 DIV.

2.1.2. MN9D cells

The mouse hybrid dopaminergic cell line MN9D (Choi et al., 1991) was obtained from the University of Pittsburgh (MT A000434). These cells, maintained in a Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Gibco-Invitrogen, Carlsbad, CA, USA), were grown at 37 °C in humidified air containing 5% CO_2. Cells were seeded into 6-well- or 100-mm plates. Cell viability was determined by exclusion of trypan blue dye; cell viability was 90–95% in the untreated cells. It is noteworthy that this cell line was differentiated before performing experiments for this manuscript. The western blotting results showed that there was no significant difference of their phenotypes such as TH and DAT protein expressions (data not shown) before and after differentiation. Therefore, this cell line was not differentiated in later experiments.

2.2. Western blot analysis

Performing of western blotting was the same as reported previously (Fan et al., 2011). Briefly, whole cell extracts were prepared by lysing cells, centrifugalizing and measuring protein concentrations step by step. Equally loaded protein samples (30 μg/each well) were electrophoretically separated on a 10% or 15% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK). Then the blots were probed with either monoclonal anti-TH antibody (1:1000 dilution, Sigma, USA),

monoclonal antibody anti-BDNF (1:100 dilution, Abcam, Cambridge, MA, USA), or phosphor-extracellular signal-regulated kinase 1 and 2 (pERK1/2, 1:2000, Cell signaling Technology, Danvers, MA, USA). A horseradish peroxidase-conjugated mouse/rabbit antibody (1:5000 dilution; Amersham Life Sciences, Buckinghamshire, UK) was used as the secondary antibody. The membranes were then subjected to enhanced chemiluminescence (ECL, Amersham Life Sciences, Buckinghamshire, UK) or super ECL substrate (Sigma Chemical Co., St Louis, MO, USA). Immunoreactive bands were visualized and detected by G:Box Imaging (Fyederick, MD, USA). To check for equal loading and transfer, the membranes were reprobed with a mouse IgG monoclonal anti-β-actin antibody (1:10,000 dilution, Amersham Life Sciences, Buckinghamshire, UK).

2.3. Immunofluorescence and TUNEL staining

To evaluate the neuronal cell death, terminal deoxynucleotidyl-transferase-mediated biotinylated UTP nick end labeling (TUNEL) was performed in situ, as previously reported from our laboratory (Wang et al., 2015). Cells were grown on coverslips in 24-well plates, followed by treatment with different chemicals as described in the text. After treatment, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 min. Coverslips were then incubated overnight in primary antibodies (anti-MAP2-H-300, 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; or anti-TH, 1:1000, Pel-Freez Biologicals, Rogers, AR, USA). On next day, after washing with PBS for three times, coverslips were incubated with the Alexa Fluor® 488 secondary antibodies (EMD Millipore Corporation, Billerica, MA, USA). Coverslips were then incubated with TUNEL reagent for 1 h at room temperature. After washing with PBS for three times, these coverslips were mounted onto microscope slides and viewed and photographed at 10x or 20x magnification using an EVOS inverted fluorescent microscope (Advanced Microscopy Group, Washington, USA).

To further identify the dopaminergic neurons in VM primary cultures, double immunofluorescence staining for TH and NeuN was performed in the coverslips of VM cultures. First antibody against rabbit TH (1:1000, Pel-Freez Biologicals, Rogers, AR, USA) and second antibody against mouse NeuN (1:100, MilliporeSigma, Burlington, MA, USA) were in turn incubated with coverslips. Other procedures were similar to the standard immunofluorescence staining. The results were viewed and photographed at 10x magnification in Olympus microscopy (BX41 7F, Olympus Optical Co. LTD, Tokyo, Japan) with a software Spot Imaging (Version 5.3, SPOT Imaging Solution Company, Sterling Heights, MI, USA).

2.4. Transient transfections in MN9D cells

Transient transfection of BDNF cDNA, which was constructed into the plasmid vector pcDNA3.1(+), and pcDNA3.1(+) vectors (for the

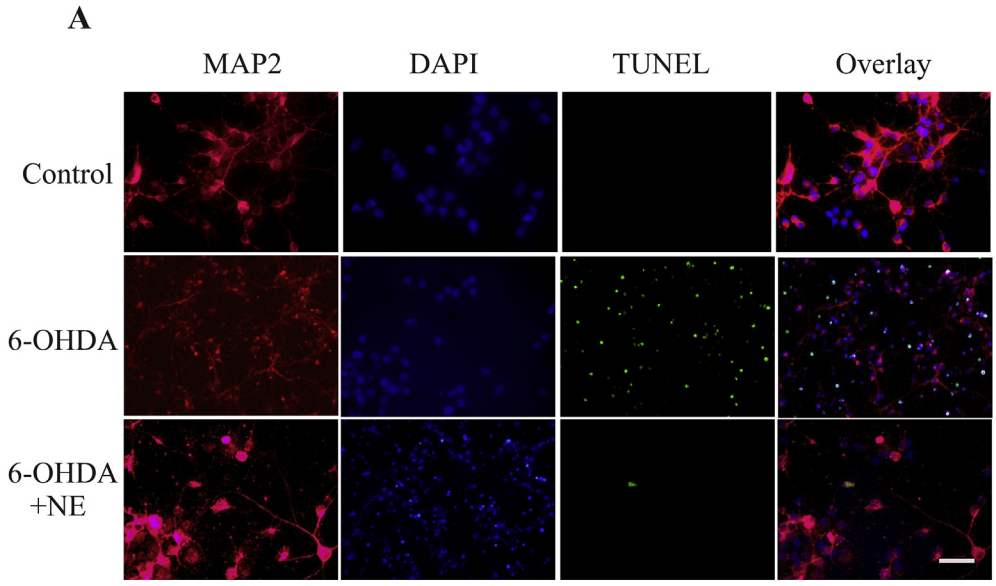


Fig. 1. NE protects VM neurons against cell damage caused by 6-OHDA. Cultured neuronal cultures from the VM were exposed to 6-OHDA (25 μM) or 6-OHDA + NE (100 nM) for 4 h. Immunochemical staining was performed with the neuronal marker MAP2 (red) and the nuclear marker DAPI (blue) and neuronal apoptosis was detected by TUNEL staining (green). Top panel (A) shows immunofluorescent images (taken with 20x magnification) and button panel (B) shows cell death analysis counted through trypan blue exclusion assay. Each bar from Fig. 1B represents data obtained from 6 separate experiments (N = 6 of independent cell culture preparations, which is the same in the following legends). *P < 0.05, compared to the control; †p < 0.05, compared to the group treated with 6-OHDA. Scale bar: 25 μm for all images.

control) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Briefly, cells (passage number below 10) were grown in the normal medium as described above to about 90% confluence on 6-well plates. Two solutions: A (5 μg of DNA in 250 μl serum-free Opti MEM I medium) and B (10 μl Lipofectamine in 240 μl serum-free Opti MEM I medium) were mixed and incubated at room temperature for 20 min. Then this mixture was added into the 6-well plate and incubated at 37 °C. Transfected cells were harvested 4 days after transfection for measurement of TH and pERK1/2 protein levels by western blotting.

2.5. Chromatin immunoprecipitation assay (ChIP)

ChIP was carried out with an EZ-Magna ChIP™ A kit according to the manufacturer's instruction (Millipore Biotechnology, Billerica, MA). Four aliquots of 1×10^7 nuclei were resuspended in 500 μl of cell lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS at pH 7.4) and chromatins were fragmented to 200–500 bp with 6 pulses of 10 s at 40% power with a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA, USA) at 4 °C. To remove insoluble components, samples were centrifuged at 13,600 g for 10 min and supernatants recovered. Rabbit polyclonal antibodies anti-acetyl-Histone H3 or anti-acetyl-Histone H4 (both from MilloporeSigma, Burlington, MA, USA) or normal rabbit IgG were conjugated to goat anti-rabbit IgG magnetic beads. For each aliquot of 1×10^7 nuclei, 50 μl of magnetic bead slurry were in turn added by 5 μg of primary antibody and the sheared chromatin in radioimmunoprecipitation assay buffer, and then incubated on a rotator overnight at 4 °C according to the manufacturer's recommendation. After purification, DNA was then subjected to quantitative real-time PCR using primers for the promoter regions of TH to detect the enriched genomic DNA fragments. ChIP-PCR-derived DNA was also electrophoresed through 2% agarose gels. Primers for quantitative real-time PCR were 5′- CTGTCTTCATGTCGTGTCTAGG and 5′- CTGCTTGGTATCCTGCTCTG.

2.6. Statistics

All experimental data are presented in the text and graphs as the mean ± SEM. Statistical analysis were carried out using one way analysis of variance (ANOVA) when comparing multiple treatment groups (SigmaStat, Systat Software Inc., Richmond, VA). A repeated-measures analysis of variance (ANOVA) was performed for the time-course of the changes in Figs. 3–6 utilizing SigmaPlot 14 (SigmaStat, Systat Software Inc., Richmond, VA). Where necessary, post-hoc

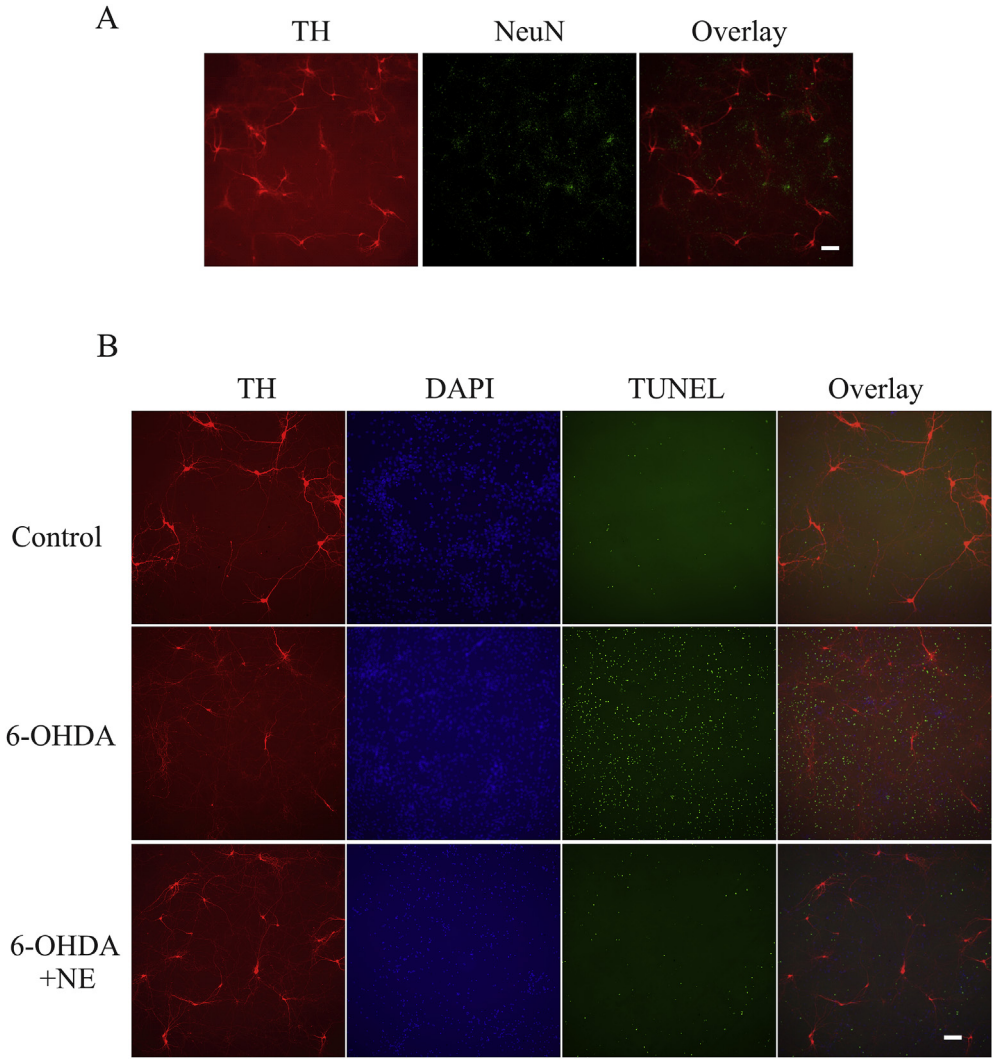


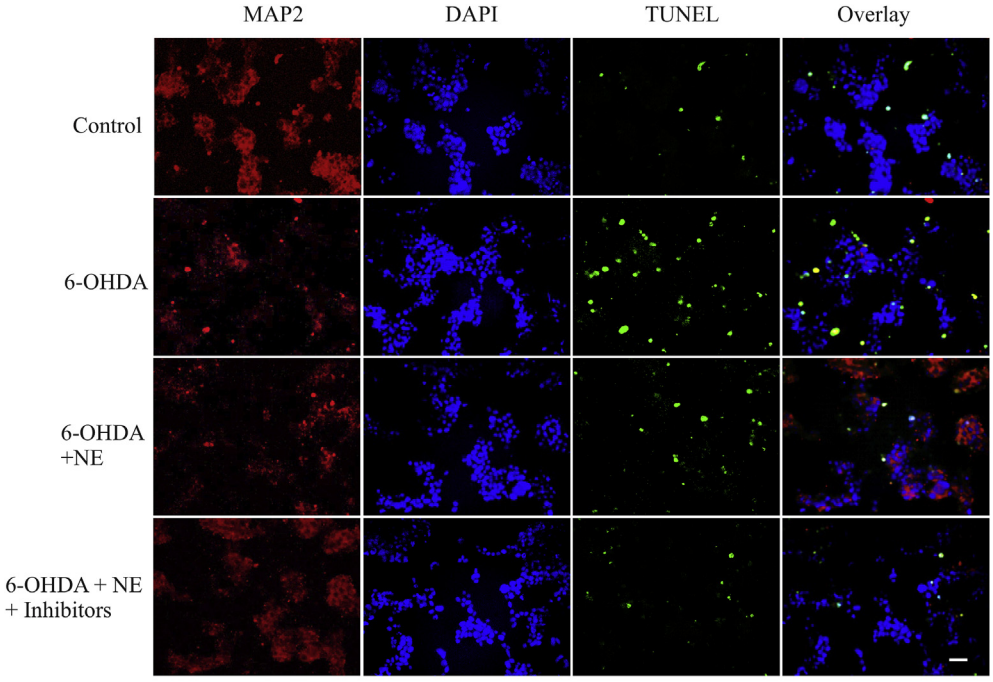
Fig. 2. NE protects dopaminergic neurons against cell damage caused by 6-OHDA. VM cultures and treatment procedures are the same as in Fig.1 above. Top panel (A) shows immunofluorescent staining using antibody against TH (red) and antibody against NeuN (green) (taken with 10x magnification). The bottom panel shows immunofluorescent staining using anti-TH (red) and the nuclear marker DAPI (blue) and neuronal apoptosis TUNEL staining (green). Each experiment from this figure was repeated for 6 times (N = 6 of independent cell culture preparations. Scale bar: 25 μm for all images.

Newman-Keuls tests were performed for planned comparisons. The unpaired Students t-test was used when there were only two groups to be compared such as the experiment to transfact BDNF cDNA for the expression of TH and pERK1/2 (Fig. 6).

3. Results

3.1. NE protected VM neurons and MN9D cells against cell damage caused by 6-OHDA

Neuronal cultures from rat VM in the coverslips or 6-well plates were exposed to 6-OHDA (25 μM) or 6-OHDA + NE (100 nM) for 4 h. NE was administrated 30 min in advance of 6-OHDA treatment. Immunofluorescence using antibody against MAP2 and TUNEL staining were performed in the coverslips to examine the effects of NE on 6-OHDA-induced cell damage. As shown in Fig. 1, while 6-OHDA caused significant neuronal death, 100 nM NE effectively blocked neuronal death. The trypan blue dye exclusion assays were carried out in the 6-well plates to account ratio of death/live cells after exposure to these chemicals. As shown in Fig. 1B, 6-OHDA caused significant cell death and treatment with NE markedly reduced cell death. The TH antibody was used further to quantify dopaminergic neurons in VM cultures. As shown in Fig. 2 A, in the primary culture from rat VM about 14% of TH-positive neurons are generally obtained, as compared to NeuN staining. This is consistent to the literatures (Takeshima et al., 1996). Also, while 6-OHDA badly damaged dopaminergic neurons with broken cell bodies, NE treatment effectively prevented 6-OHDA-caused dopaminergic neuron death (Fig. 2B). The similar experiments were carried out in dopaminergic MN9D cells for immunofluorescence/TUNEL and trypan blue dye exclusion assays. As shown in Fig. 3, exposure of MN9D cells to NE attenuated a 6-OHDA-caused cell death. However, such neuronal protection of NE was blocked by simultaneous administration of a cocktail of phentolamine (10 μM), a non-selective α-adrenoceptor (AR) antagonist, and pindolol (10 μM), a non-selective β-AR antagonist). It indicates that ARs may be involved in NE-caused neuronal protection.



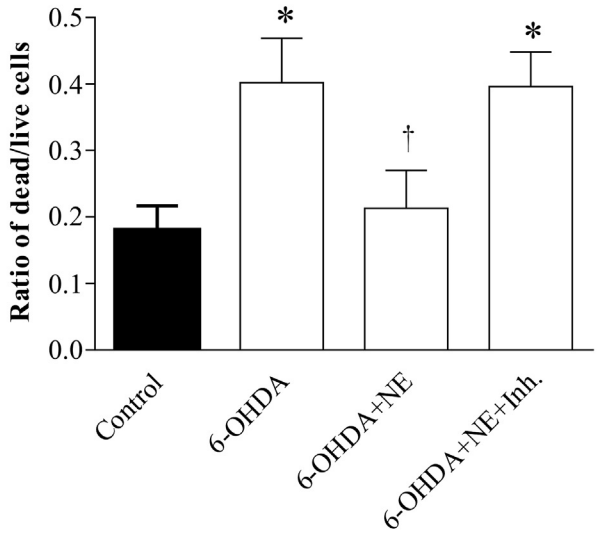


Fig. 3. NE protects MN9D cells against cell damage caused by 6-OHDA. MN9D cells were exposed to 6-OHDA (25 μM), 6-OHDA + NE (100 nM) or 6-OHDA + NE (100 nM) + Antagonists of ARs for 4 h. Immunochemical staining was performed with the neuronal marker MAP2 (red) and the nuclear marker DAPI (blue) and neuronal apoptosis was detected by TUNEL staining (green). Top panel (A) shows immunofluorescent images (taken with 10x magnification) and button panel (B) shows cell death analysis counted through trypan blue exclusion assay. Each bar from Fig. 2B represents data obtained from 6 separate experiments (N = 6 of independent cell culture preparations, which is the same in the following legends). *P < 0.05, compared to the control; †p < 0.05, compared to the group treated with 6-OHDA. Inh.: phentolamine + pindolol. Scale bar: 25 μm for all images.

3.2. NE upregulated TH protein levels in neuronal cultures from rat VM

It has been reported that NE may act as a modulator to affect other neuronal circuits especially the dopaminergic neurons (Henry et al., 1999; Lundblad et al., 2002; Rascol et al., 2001). Therefore, the neuroprotective effects of NE may be consistent to its modulatory effects on dopaminergic phenotypes. As such, whether NE can affect the expression of TH was examined. Neuronal cultures from rat VM were exposed to different concentrations of NE for 4 h. Western blotting results showed that NE treatment significantly affected TH protein levels ($F_{4, 19} = 3.62$, $p < 0.05$, Fig. 4A). Time course treatments also showed that exposed of neuronal cultures to 100 nM NE for 1–6 h markedly affected TH expression ($F_{4, 19} = 3.26$, $p < 0.05$, Fig. 4B). The post-hoc Newman-Keuls tests further revealed that upregulated TH protein levels by NE were in a concentration- and time-dependent manner.

3.3. NE upregulated BDNF protein levels in neuronal cultures from rat VM

It has been suggested that the beneficial effects of NE on other neurons can be accounted either by its direct role as a neurotrophic factor, or by an indirect role through facilitating the expression of other neurontrophic factors. BDNF is a member of the neurotrophin family (Barde et al., 1982; Leibrock et al., 1989). This led us to perform next experiments to examine the concentration and time effects of NE on BDNF expression. Neuronal cultures from rat VM were exposed to different concentrations of NE for 4 h, or 100 nM NE for 1–6 h, respectively. Western blot analysis showed that NE significantly affected BDNF protein levels in these cultures (the concentration course: $F_{4, 19} = 4.96$, $p < 0.01$, Fig. 5A; the time course: $F_{4, 19} = 2.93$, $p < 0.05$, Fig. 5B). The post-hoc tests showed that 10 ($p < 0.05$), 100 ($p < 0.01$) and 500 nM ($p < 0.01$) NE significantly upregulated BDNF proteins in VM cultures, respectively. Exposure of the neuronal cultures to 100 nM NE for 1, 2 and 4 h markedly increased BDNF proteins, although 6 h exposure did not affect BDNF protein levels significantly.

3.4. NE upregulates pERK1/2 protein levels in neuronal cultures from rat VM

To examine whether signal transduction pathways are possibly involved in the NE-induced TH upregulation, the pERK1 and pERK2 protein levels were measured in the VM neuronal cultures that were treated similarly as above. Western blot results showed that exposure of VM neuronal cultures to different concentrations of NE for 4 h ($F_{4, 19} = 3.02$, $p < 0.05$ for pERK1; $F_{4, 19} = 3.44$, $p < 0.05$ for ERK2), and 100 nM NE for different times significantly affected pERK1 and pERK2 protein levels ($F_{4, 19} = 3.35$, $p < 0.05$ for pERK1; $F_{4, 19} = 3.00$, $p < 0.05$ for pERK2). The post-hoc tests revealed that all 1–500 nM concentrations of NE significantly increased pERK1 and pERK2 proteins ($p < 0.05$). The time course treatments also showed that 100 nM NE significantly increased pERK1 protein levels ($p < 0.05$ or $p < 0.01$, Fig. 6B) for all exposure times (1–6 h). However, 100 nM NE-induced increase of pERK2 only occurred in 1–4 h exposure, not for 6 h exposure.

3.5. Effects of BDNF cDNA transfection or exposure of cells to BDNF on TH and pERK1/2 expression

To examine the effects of BDNF on TH and pERK1/2 expression, MN9D cells were transfected with BDNF cDNA for 4 days. Protein levels of TH and pERK1/2 were examined by western blotting. As shown in Fig. 7A and B, transfection of BDNF cDNA significantly increased protein levels of TH ($p < 0.05$), pERK1 ($p < 0.01$) and pERK2 ($p < 0.05$). Moreover, MN9D cells were treated with 25 μM BDNF peptide for 1–6 h. Western blot analysis showed that treatment with BDNF for 4 and 6 h markedly enhanced TH protein levels ($F_{4,19} = 4.56$, $p < 0.05$) (Fig. 7C).

3.6. NE upregulates TH, BDNF and pERK1/2 through BDNF receptors in neuronal cultures from rat VM

To examine the possible role of BDNF receptor tropomyosin-receptor-kinase B (TrkB) in NE-induced upregulation of TH, BDNF and pERK1/2, VM neuronal cultures were exposed to 100 nM NE or NE plus 100 nM K252a, a TrkB receptor antagonist for 4 h. The protein levels of TH, BDNF and pERK1 and pERK2 were measured by western blotting. Analysis showed that simultaneous administration of k252a almost

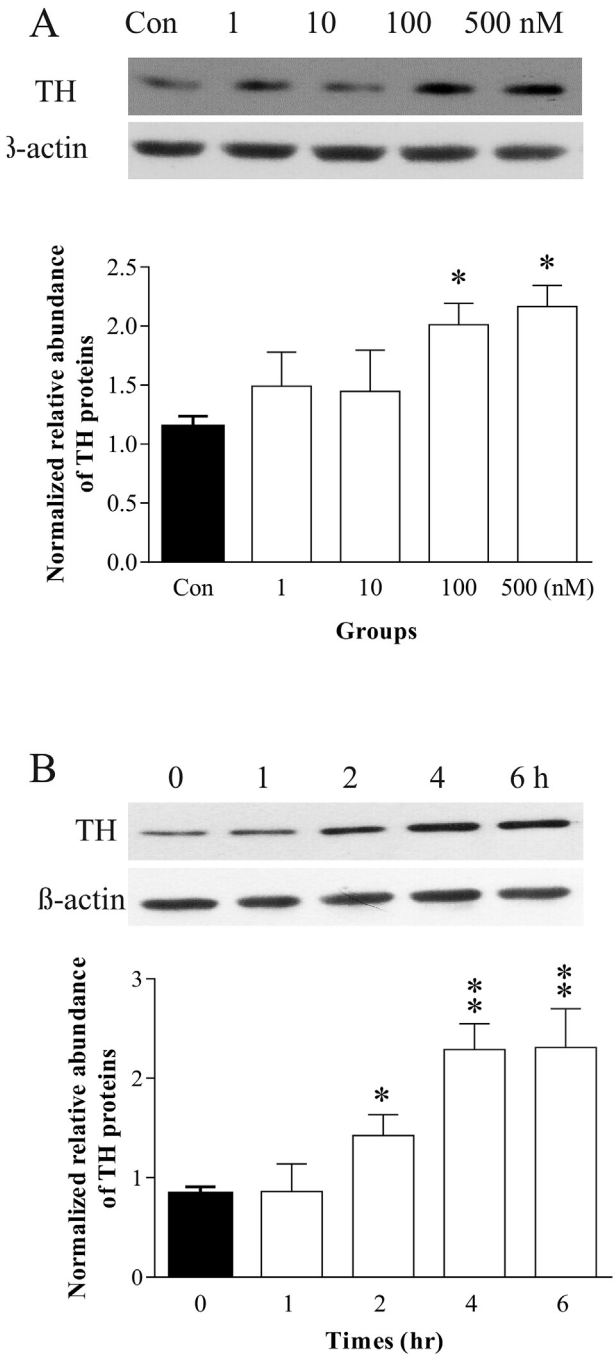


Fig. 4. The concentration- (A) and time-courses (B) for effects of NE on TH protein levels in VM cultures, which showed an upregulation of TH by NE in a concentration- and time dependent manner. The upper figures in A and B show autoradiographs obtained by western blotting. The lower graphs in A and B show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). *P < 0.05, **P < 0.01, compared to the control (con) (in B, referred as 0).

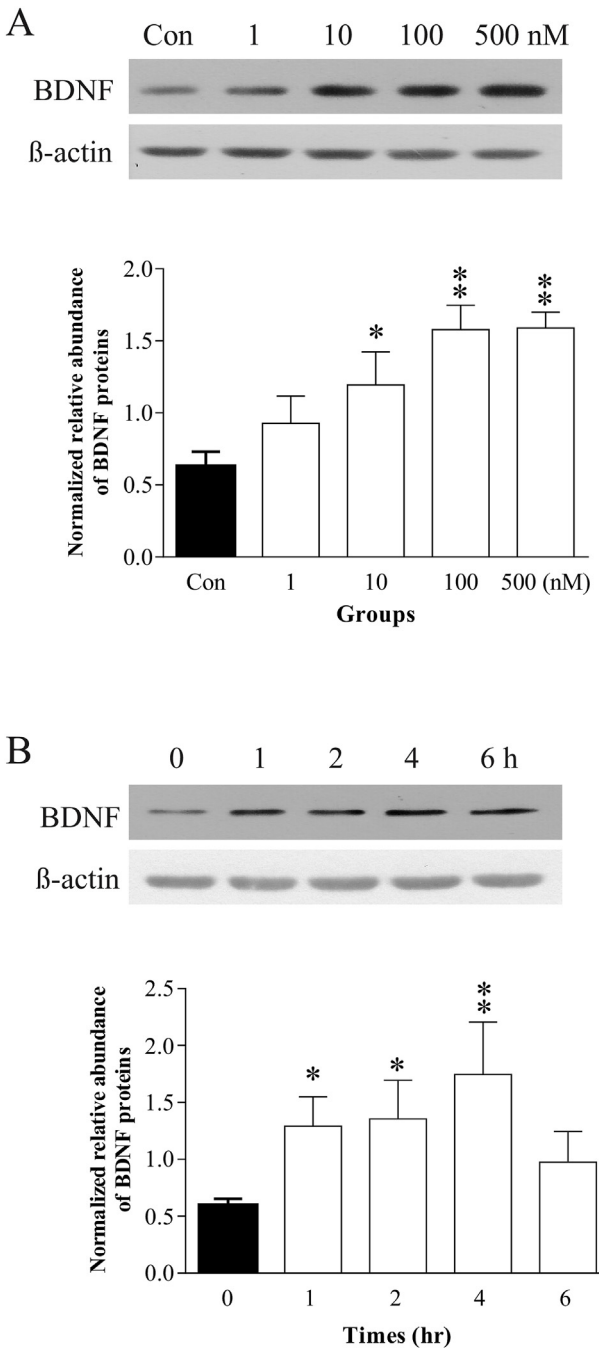


Fig. 5. NE upregulates BDNF protein levels of VM cultures in concentration-dependent manner, demonstrated by the concentration- (A) and time-courses (B) of NE effects. The upper figures in A and B show autoradiographs obtained by western blotting. The lower graphs in A and B show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). *P < 0.05, **P < 0.01, compared to the control (con) (in B, referred as 0).

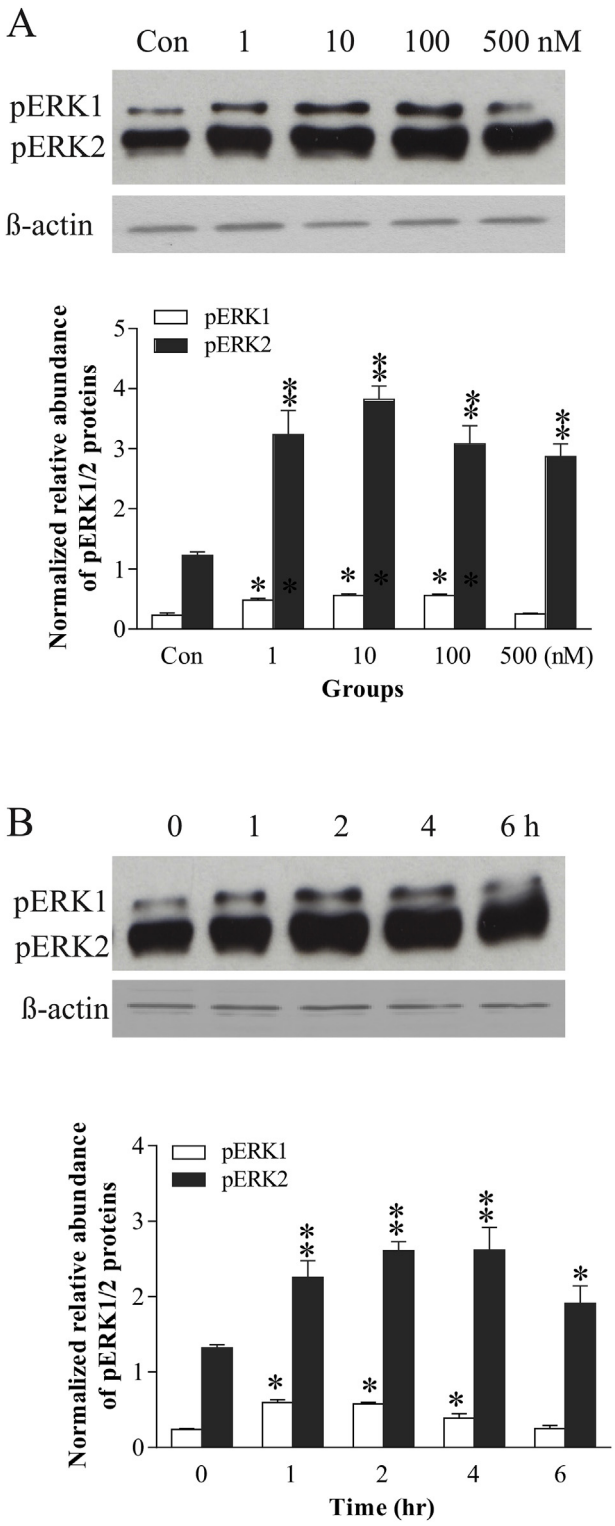


Fig. 6. Effects of NE on pERK1/2 protein levels in VM cultures, as demonstrated by the concentration- (A) and time-courses (B) of NE effects. The upper figures in A and B show autoradiographs obtained by western blotting. The lower graphs in A and B show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). *P < 0.05, **P < 0.01, compared to the control (con) (in B, referred as 0).

completely abolished NE-caused upregulation of protein levels of TH (Fig. 8A), BDNF (Fig. 8B) and pERK1/2 (Fig. 8C), indicating TrkB is involved in the NE-induced upregulation of these proteins.

3.7. NE upregulates TH and pERK1/2 through mitogen-activated protein kinase (MAPK) pathways

It is well known that neuronal viability is maintained through a complex interacting network of signaling pathways, which plays principal roles in promoting gene synthesis and plasticity (Heerssen and Segal, 2002). To examine whether these signal pathways are involved in the neuronal protection and modulation of NE, VM primary cultures were exposed to 100 nM NE alone, NE plus U0126 (10 μM, a dual

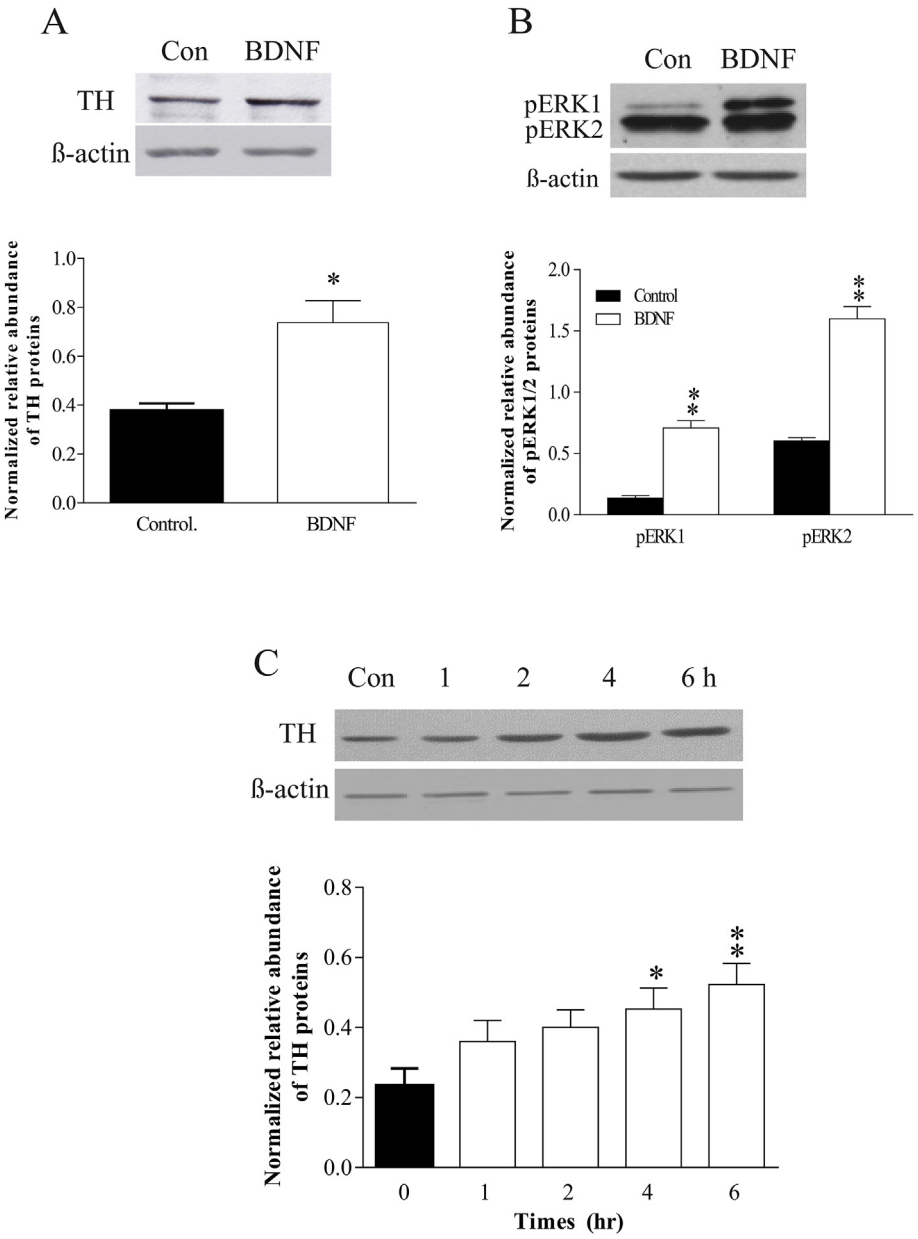


Fig. 7. Effects of BDNF on TH and pERK1/2 expression. A and B: MN9D cells were transfected with BDNF cDNA for 4 days. C: MN9D cells were exposed to 100 nM BDNF peptide for indicated times. The upper figures in A, B, and C show autoradiographs obtained by western blotting. The lower graphs in A, B, and C show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). *P < 0.05, **P < 0.01, compared to the control (con).

MEK1/2 inhibitor), and PD098059 (10 μM, a MEK1 inhibitor), or NE plus both U0126 and PD098059 for 4 h, respectively. The protein levels of TH, BDNF and pERK1/2 were measured by western blotting. The results showed that these MAPK inhibitors significantly altered the effects of NE on expression of these proteins, except for BDNF ($F_{4, 20} = 4.61$, $p < 0.01$ for TH; $F_{4, 19} = 18.98$, $p < 0.001$, for pERK1, and $F_{4, 19} = 30.33$, $p < 0.001$ for pERK2, respectively). The post-hoc tests showed that while 100 nM NE significantly increased protein levels of TH and pERK1/2, either U-126, PD098059, or both effectively blocked NE's upregulative effects on TH and pERK1/2 (Fig. 9A and C). Nevertheless, there was no synergistic effect when both U-126 and PD098059 were used in combination. In contrast to NE's effects on TH and pERK1/2, neither U-126, PD098059, nor their combination altered NE's effects on BDNF (Fig. 9B), indicating MAPK pathway is not involved in the regulation of BDNF by NE.

3.8. NE and BDNF upregulated TH through epigenetic histone modification

One of the results of histone acetylation is to transcriptionally upregulate gene expression. Therefore, TH upregulation facilitated by NE and BDNF in VM culture and MN9D cells may also be a result of chromatin remodeling. To examine whether an epigenetic mechanism is involved in NE- and BDNF-induced upregulation of TH, ChIP assay was carried out. As shown in Fig. 10A, treatments with NE or BDNF caused increased H4 acetylation in the TH promoter, although a significant alteration in H3 acetylation was not found. With normal rabbit IgG, there was no binding of the TH promoter, which demonstrated the specificity of the ChIP assay. In addition, quantitative real-PCR confirms the increase in NE- and BDNF-treated samples over the vasa, level of H4 acetylation in the control cells (Fig. 10B).

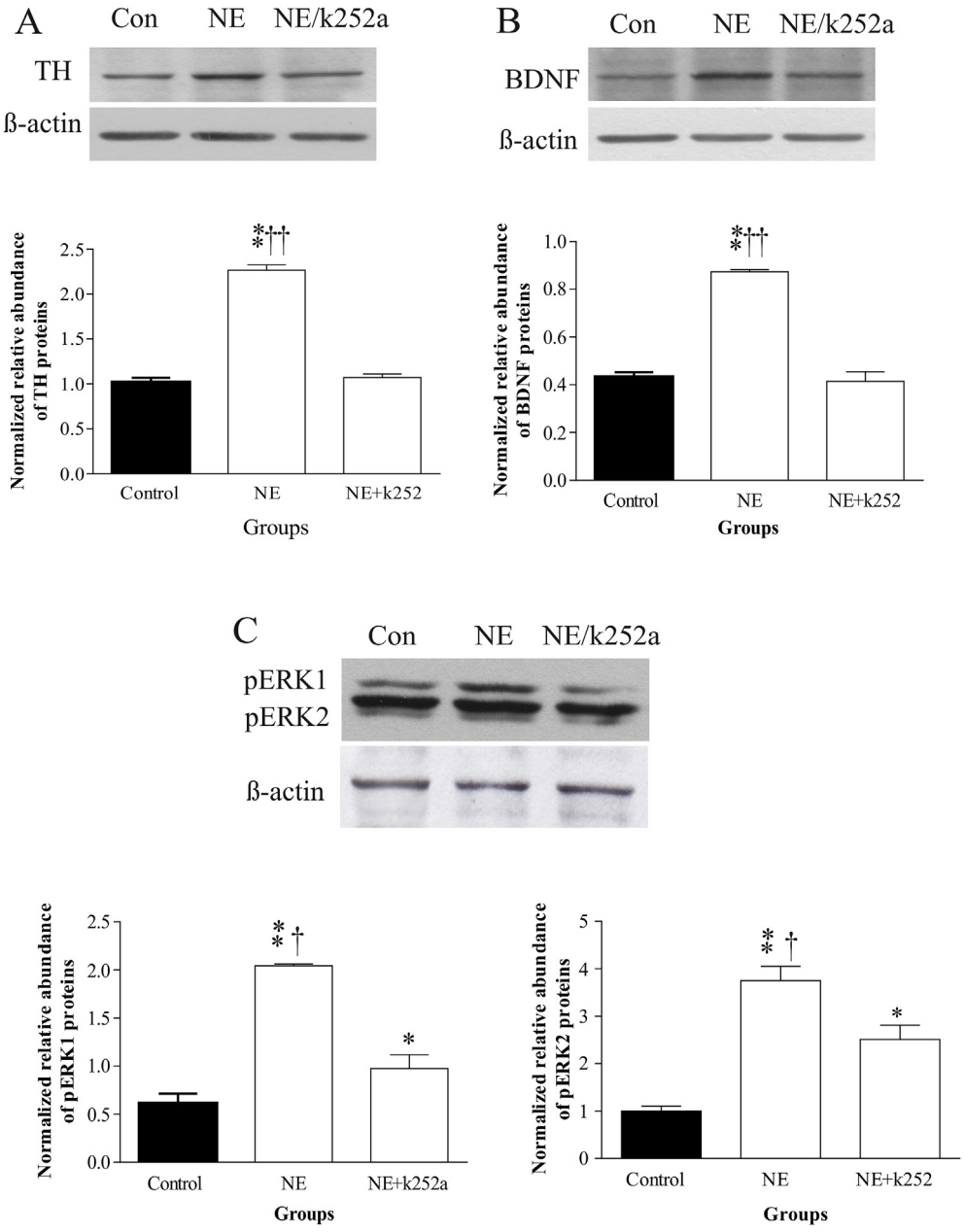


Fig. 8. NE upregulates TH (A), BDNF (B), and pERK1/2 (C) through BDNF receptors in VM cultures. The upper figures in A, B, and C show autoradiographs obtained by western blotting. The lower graphs in A, B, and C show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). *P < 0.05, **P < 0.01, compared to the control. †p < 0.05, ††p < 0.01, compared to the group treated with NE + k252a. K252a: the TrkB receptor antagonist.

4. Discussion

In the present study, primary cultures from rat VM and dopaminergic cell line MN9D were used to examine neuroprotection of NE on dopaminergic neurons and its effects on TH and BDNF expression. The underlying mechanisms were also explored. The results showed that NE administration blocked 6-OHDA-caused apoptosis in VM neuronal cultures and MN9D cells. Furthermore, treatment of VM neuronal cultures with NE significantly upregulated TH protein levels in a concentration- and time-dependent manner. Further experiments showed that similar treatment of VM neuronal cultures with NE markedly increased protein levels of BDNF and pERK1/2. Transfection with BDNF cDNA or exposure of MN9D cells to BDNF significantly increased protein levels of TH and pERK1/2. Moreover, NE's modulatory effects on the expression of TH, BDNF and pERK1/2 were mediated by TrkB receptors. MAPK pathways were also involved in the NE's regulation of TH and pERK1/2, but not for BDNF expression. Finally, treatment with NE and BDNF significantly enhanced H4 acetylation on the TH promoter. The present study reveals that NE has neuroprotective effects, which is possibly related to its direct action through ARs and its ability to facilitate the expression BDNF, which lead to upregulation of dopaminergic phenotype TH through activating MARK/ERK pathways. In addition, acetylation of histone proteins may also participate this modulation.

The present study showed that the neuroprotection of NE on MN9D cells involves the ARs as combination of α- and β-AR antagonists abolished NE-caused protection. This result is consistent with some previous reports that the activation of β-ARs ameliorated amyloid-β toxicity and amyloid-β-induced cell death in vivo and in vitro (Counts and Mufson, 2010; Heneka et al., 2002). Also, some in vivo studies showed that β2-AR agonists reduced ischemic brain damage in models

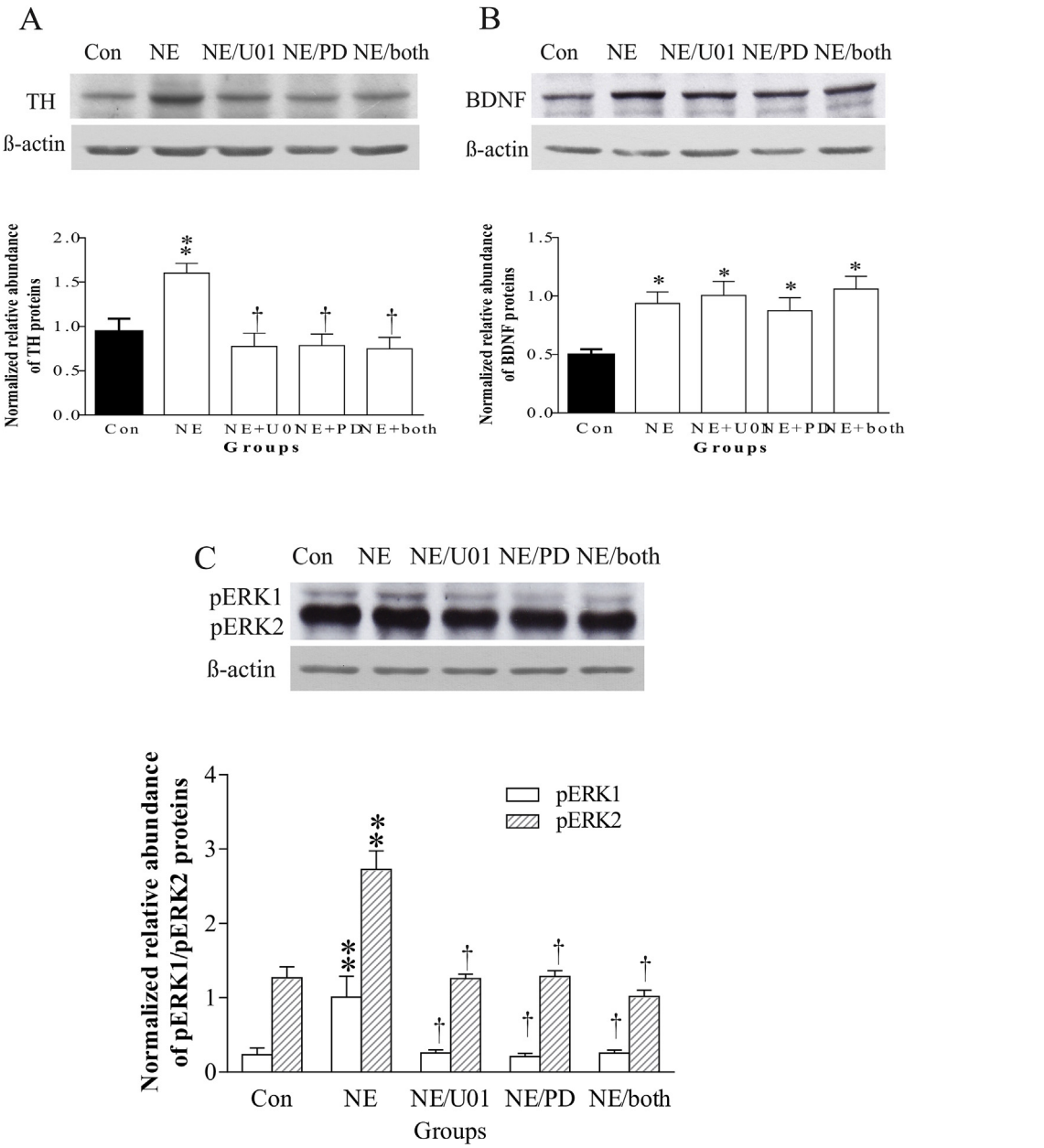


Fig. 9. NE upregulates TH (A), BDNF (B) and pERK1/2 (C) through MAPK pathways. The upper figures in A, B, and C show autoradiographs obtained by western blotting. The lower graphs in A, B, and C show quantitative analysis of band densities. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5). **P < 0.01, compared to the control. †p < 0.01, compared to group treated with NE. U01: U0126, a dual MEK1/2 inhibitor; PD: PD098059, a MEK1 inhibitor.

of focal cerebral ischemia and neuronal death in primary cultures (Zhu et al., 1998; Junker et al., 2002). Similarly, stimulation of α2-ARs induced neuroprotection against cerebral ischemia through its anti-apoptotic effects (Ma et al., 2004; Goyagi and Tobe, 2014). Compatible with these studies, the compounds which possess a catechol moiety, such as the β- and β_1-AR agonists isoproterenol and dobutamine, as well as o-catechol (pyrocatechol), mimicked the protective effects of NE in dopaminergic and other cultures (Noh et al., 1999; Ancerewicz et al., 1998; Troadec et al., 2001). However, on the contrary, a cocktail of α- and β-AR antagonists failed to block the neuronal protection of NE against amyloid-β-mediated oxidative stress and toxicity in SK-N-SH cells or primary cultures of rat cortex (Jhang et al., 2014; Liu et al., 2015). The neuroprotection of NE on cholinergic (Traver et al., 2005) or dopaminergic (Troadec et al., 2002) neurons could neither be mimicked by AR agonists, nor prevented by AR antagonists. A likely explanation for the discrepancy between these findings including the present study is that the involvement of ARs in NE-induced neuroprotection is complex, with some through the activation of ARs, and some by AR-independent mechanisms.

In the present study, exposure of EM primary cultures to NE significantly increased TH protein levels. While NE markedly enhanced BDNF expression, transfection of BDNF cDNA in MN9D cells or exposure of MN9D cells to BDNF peptide significantly increased TH protein levels. These results are coincident with the notion that the neuronal beneficial effects of NE on other neurons can be direct by acting as a neurotrophic factor, and indirect by its ability to facilitate the expression of other neurotrophic factors such as BDNF (Chen et al., 2007; Counts and Mufson, 2010; Aloyz et al., 1999). It is suggested that the neurotrophic effect of NE is related to its ability to drive DNA synthesis (Lauder, 1993) and increase gene expression (Day et al., 2014) in

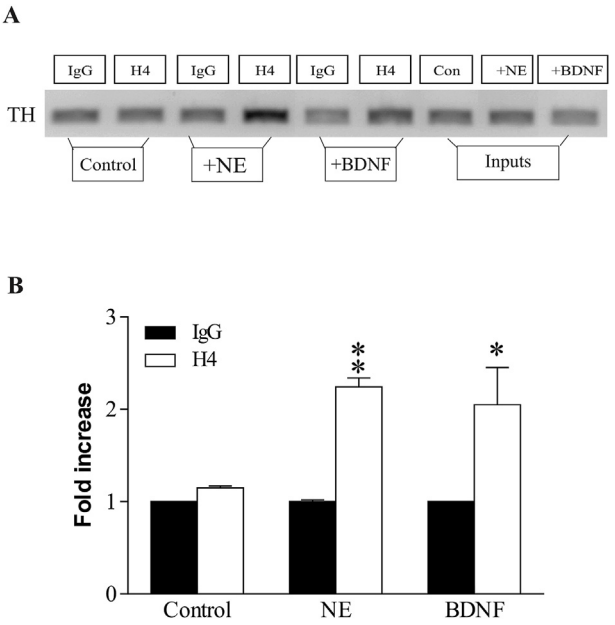


Fig. 10. A: ChIP assay showed acetylation of histone (H4) in MN9D cells in response to NE (+NE) or BDNF (+BDNF) treatments. "Input" serves as a loading control (Con) and rabbit IgG immunoprecipitation serves as a negative control. Band indicates the TH promoter in response to NE and BDNF treatment. B: Quantitative real-time PCR of the TH promoter regions from immunoprecipitation with antibody against H4. The fold enrichment value is shown as the normalized ChIP signal divided by the normalized input signal. Each bar from both pictures represent data obtained from 5 separate experiments (N = 5).

nervous system, resulting in neurite outgrowth in cultured neurons (Day et al., 2014). As to the facilitative effects of BDNF on TH expression, it was reported that overexpression of BDNF exhibited a 52% increase of TH-positive neurons in the substantial nigra in vivo (Alonso-Vanegas et al., 1999), and BDNF increased the number of TH-positive cells in cultured cortical cells (Zhou et al., 1994). Further, BDNF can activate the TH gene transcription and increase TH expression in vitro (Fukuchi et al., 2010; Theofilopoulos et al., 2001). Therefore, the present study provides further evidence for directly and indirectly regulatory effects of NE on the dopaminergic phenotype.

NE and BDNF are not transcriptional factors and cannot directly act on transactivation of related genes (Ruiz et al., 2014). As such, neuronal pathways should be involved to mediate the neurotrophic effects of NE and BDNF. As one of the consequences, activation of these signaling pathways leads to the binding of transcription factors with cis-acting elements on the TH promoter, and transactivation of the TH gene (Kalashnikova EV, 2006; Lim et al., 2000; Nagamoto-Combs et al., 1997; Suzuki et al., 2004; Fukuchi et al., 2010). It is well known that the MAPK/ERK pathways promote neuronal growth and neuroplasticity, influence gene expression through activation of transcription factors (Chen et al., 2007; Troadec et al., 2002; Cottingham et al., 2012). In the present study both NE and BDNF increase the expression of pERK1/2 proteins, and MEK1/2 inhibitors abolished NE-induced upregulation of TH and pERK1/2. ERK1/2 is the member of MAPK family and plays an important role in multiple cellular functions (Pearson et al., 2001; Kyriakis et al., 1994). In line with the present study, it was reported that NE promoted viability of pulmonary artery smooth muscle cells and increased expression of cell nuclear antigen and other proteins by activation of ERK1/2, which was attenuated by U0126 (Liu et al., 2017). Furthermore, BDNF can activate the TH gene promoter through its specific receptor TrkB and the ERK/MAPK pathway (Fukuchi et al., 2010). Therefore, one potential mechanisms underlying the upregulation of TH expression by NE and BDNF in the present study is through activation of pERK1/2, and MAPK pathway.

One of the consequence of activation of signal transduction pathways can be the increase of gene expression, which can involve epigenetic modulation and transactivation of target genes. Epigenetic modifications to histone proteins such as acetylation can alter the structure of chromatin, allowing the transcriptional machinery to access gene promoters (Narlikar et al., 2002; Gibbons, 2005; Roeder, 2005) and result in transcriptional activation (Grunstein, 1997). It was reported that NE can activate transcription of target genes through increasing histone H3 phosphorylation (Chik et al., 2007; Ho et al., 2007) and acetylation (Maity et al., 2016), and elevated NE levels epigenetically modulated PKCε gene promoter in the heart (Xiong et al., 2012). It indicates that NE can activate signaling pathways coupled to epigenetic mechanisms and the subsequent transcriptional control of gene expression. Similarly, BDNF triggers gene transactivation by increasing H3 acetylation at K9 and K14 (Sen and Snyder, 2011; Calfa et al., 2012), as well as its inductive effects on gene activation can be enhanced by inhibiting HDAC (Nott et al., 2008; Fukuchi et al., 2015). The present study demonstrated that exposure of MN9D cells to NE or BDNF significantly increased H4 acetylation, although there is no markedly alteration in H3 acetylation. It suggests that this NE- and BDNF-induced enhancement of H4 acetylation may contribute to the increased TH expression in the cells. However, given the role of histone acetylation in TH gene regulation is not fully established (Lenartowski and Goc, 2011), more studies are needed to clarify this point.

In the present study, TrkB is considered to be involved in the NE-induced upregulation of TH, BDNF and pERK1/2, as simultaneous administration of NE and k252a almost completely abolished NE-caused upregulation of these proteins. This observation is consistent with the previous report that NE treatment increased expression of BDNF and activation of TrkB in the hippocampal neuronal culture, which was blocked by co-incubation of NE and k252a (Chen et al., 2007). Similarly, in other studies, TrkB is required for the neuroprotective effect of NE in cultured hippocampal, cortical and LC neurons against amyloid-β neurotoxicity, which was fully blocked by administration of k252a (Counts and Mufson, 2010; Liu et al., 2015). Based on these studies, there are several explanations for the relationship between NE and TrkB. First, as simultaneous administration of NE and k252a also abolished NE-induced activation PI-3K immunoreactivity, the TrkB receptor may be activated more directly following NE stimulation of the G-protein-coupled receptor (Chen et al., 2007) and activation of the PI-3K/Akt pathway, even in the absence of neurotrophins (Lee et al., 2002). Secondly, considering ARs were also involved in NE-induced neuroprotection (Fig. 3), canonical G-protein-coupled ARs and TrkB may act in concert to transduce NE-mediated effects on upregulation of genes and neuroprotection. Finally, NE may transactivate TrkB via Src family kinase activity. However, more studies are needed for clarifying their correlative effects.

In conclusion, our findings demonstrated that NE treatment not only exhibited neuroprotective effects on rat VM dopaminergic cultures and MN9D cells against 6-OHDA caused cell death, but also upregulated protein levels of TH and BDNF in a time- and concentration-dependent manner. These effects are possibly related to its neurotrophic ability and its facilitative induction of BDNF. Both are mediated through its actions on pERK1/2, and MAPK pathways, as well as altering on histone acetylation on TH promoter. This study provides further evidence for the importance of noradrenergic neurons to the functional integrity of dopaminergic system. Further work to examine the molecular mechanisms underlying these modulations will contribute to a better understanding of the regulatory role of NE on dopaminergic phenotypes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104549.

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