

Potential protective effect of biphasic electrical stimulation against growth factor-deprived apoptosis on olfactory bulb neural progenitor cells through the brain-derived neurotrophic factor–phosphatidylinositol 3'-kinase/Akt pathway

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Abstract

Stem cell therapy may provide a therapeutic method for the replacement and regeneration of damaged neurons of the central nervous system. However, neural stem cells (NSCs) and neural precursor cells (NPCs) are especially vulnerable after transplantation due to a lack of sufficient growth factors at the transplant site. Electrical stimulation (ES) has recently been found to participate in the regulation of cell proliferation, growth, differentiation, and migration, but its underlying anti-apoptotic effects remain unclear. This study investigated the protective effects of biphasic electrical stimulation (BES) on olfactory bulb NPCs against growth factor-deprived apoptosis, examining the survival and apoptotic features of the cells. Differentiation was assessed by neuronal and glial markers. Brain-derived neurotrophic factor–phosphatidylinositol 3'-kinase (BDNF)-PI3K/Akt pathway activation was determined by enzyme-linked immunosorbent assay and Western blot. The chemical inhibitor wortmannin was used to inhibit the PI3K/Akt pathway. BES exerts a protective effect against growth factor-deprived apoptosis in the NPCs. BES enhanced cell survival and decreased the apoptotic/necrotic rate. Expression of phosphorylated Akt and BDNF secretion increased with BES for 12 h. Furthermore, the protective effects of BES were inhibited by blocking PI3K/AKT signalling. These results suggest that BES prevents growth factor-deprived apoptosis through the BDNF-PI3K/Akt signalling. This work strengthens the opinion that BES may be used as an auxiliary strategy for improving cell survival and preventing cell apoptosis in stem cell-based transplantation therapy.

Keywords: biphasic electrical stimulation, growth factor-deprived apoptosis, BDNF-PI3K/Akt pathway, neural precursor cells, stem cell therapy

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Introduction

Stem cell-based therapies may permit the repair and regeneration of damaged neurons and aid in the treatment of Parkinson's disease, Alzheimer's disease, and spinal cord injury.^{1–4} Neural stem cells/neural precursor cells (NSCs/NPCs) derived from the subventricular zone/olfactory bulb (OB) of mammals persist and proliferate throughout life,⁵ serving as ideal sources of stem cells for subsequent transplantation. However, the replacement of damaged neurons with exogenous stem cells faces a formidable challenge because the transplanted cells are particularly vulnerable to apoptosis in the brain or spinal cord due to a lack of adequate growth factors.^{6,7} Therefore, the development of

new technologies to prevent apoptosis due to growth factor deprivation is crucial to stem cell therapy.

Electrical stimulation (ES), a non-chemical procedure, can dramatically accelerate the speed of axonal regeneration and target innervation, and positively modulate the functional recovery of injured nerves.⁸ ES participates in the processes of proliferation, differentiation, and migration in NSCs/NPCs.^{9,10} The neuroprotective effects of ES have recently been observed in ischaemic stroke rats,¹¹ indicating that ES may be a novel therapeutic tool for combating apoptosis in diseases of the central nervous system. Therefore, ES may also provide insights into preventing growth factor deprivation-triggered apoptosis in NPCs.

Several signal transduction pathways, such as the phosphatidylinositol 3'-kinase (PI3K)/Akt, focal adhesion kinase, and extracellular signal-regulated kinase (ERK) pathways, are reportedly involved in ES regulation of specific cellular processes, such as cell growth and migration.¹²⁻¹⁴ The PI3K/Akt signalling pathway acts as a key modulator of neuronal development, degeneration, and death.¹⁵⁻¹⁷ ES activates the PI3K/Akt signalling pathway in the process of NPC migration,⁹ and recent studies on CNS diseases have demonstrated that the protective effects of ES against apoptosis are mediated by activation of the PI3K/Akt pathway.^{11,18} Thus, ES may play a role in mediating anti-apoptotic effects through PI3K/Akt signalling in NPCs.

In this study, an incubation system for the application of biphasic electrical stimulation (BES) was established. The potential protective effects of BES on NPCs in a growth factor-free environment were investigated, and apoptosis, differentiation, and brain-derived neurotrophic factor (BDNF)-PI3K/Akt signalling were studied.

Materials and methods

Reagents

Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12), basic fibroblast growth factor-2 (bFGF-2), epidermal growth factor (EGF), and trypsin were obtained from Invitrogen Corp. (CA, USA). Hoechst 33342 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO, USA). Monoclonal mouse anti-Nestin, monoclonal rabbit anti-Musashi, monoclonal mouse anti-NeuN, rabbit anti-glial fibrillary acidic protein (anti-GFAP), secondary antibodies conjugated with the fluorescent dye Cy3, total (rabbit polyclonal antibody), and a phosphorylated epitope (Thr308) of Akt were purchased from Chemicon (USA). BDNF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Millipore Systems (Canada). Streptomycin and penicillin were purchased from Amresco (USA).

Rat NPC cultures and characterization

NPCs were prepared from the OBs of neonatal Sprague-Dawley rats. All animals used in the experiments were provided by the animal centre of the Peking University. Procedures concerning animals reported in this study were approved by the Committee of Animal Use for Research and Education of Beihang University. All procedures were prepared as described in previous studies.^{19,20} After mechanical dissociation of dissected and pooled OBs and enzymatic digestion with 0.125% trypsin, the neurosphere populations were collected. All cells were seeded in DMEM/F12 that was supplemented with streptomycin (50 µg/mL), penicillin (50 U/mL), bFGF (20 ng/mL), and EGF (20 ng/mL). Neural progenitor cells were obtained from at least two passages neurospheres. In experimental conditions (BES or unstimulated), all culture media were free from growth factors. For the cell lineage analysis, the plates were fixed in 4% cold paraformaldehyde (15 min),

and washed (three times, 5 min each) with phosphate-buffered saline (PBS). The fixed cells were blocked for 30 min in PBS containing 5% normal goat serum and 0.25% Triton X-100, and then incubated overnight at 4°C with primary antibodies diluted in the PBS. Cell types were identified by monoclonal mouse anti-nestin and monoclonal rabbit anti-musashi. Cy3-conjugated secondary antibodies were used to detect the primary antibodies. Nuclei were counterstained with 2 µg/mL Hoechst 33342 diluted in PBS.

BES incubation system

Figure 2a and b details the BES incubation system used in this study. The upper and lower fluorine-doped tin oxide (FTO; an electrically conductive glass) plates were positioned parallel to each other and the incubation chamber was situated between them. The lower plate was seeded with NPCs, and a silicon gasket was used to seal the incubation chamber. Fluid inflow and outflow pipes were fixed on the upper glass plate to facilitate medium flow into and out of the incubation chamber. An air filter system was connected to the incubation chamber. A pair of electrode wires were introduced into the chamber and fixed at the edge of the FTO glass plates. The other ends of the electrodes were connected to the biphasic electrical stimulator (AFG 3000 series; Tektronix, Beaverton, OR, USA). The NPCs were exposed to 12 h of BES at 25 mV/mm and 50 mV/mm electric field strengths with a pulse-burst pattern and 8 ms pulses (20% duty cycle). Cells that were not exposed to BES served as controls.

Cell survival assay

NPCs were prepared and placed in a BES culture system free of growth factors for 12 h. Cell survival was determined by 3,2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) assay. Following the treatment, the treated and control NPCs were rinsed three times with PBS. The 200 µL aliquots of NPCs suspension (10^5 /mL) were seeded to three 96-well plates in eight replicates and 20 µL aliquots of MTT solution (5 mg/mL) were added to each well and incubated for 4 h in a humidified 5% CO₂ incubator at 37°C. The supernatant culture medium was carefully aspirated after centrifuge and 200 µL aliquots of dimethyl sulphoxide were added to each well to dissolve the formazan crystals. Optical densities (ODs) were read at 570 nm as a reference wavelength.

Cell apoptosis/necrosis assay

To distinguish between live and apoptotic/necrotic cells, a staining of nuclei with DNA dyes Hoechst 33342 and propidium iodide (PI) was applied as follows. After exposure, pretreated cells were trypsinized, washed with PBS, and stained with PI (5 µg/mL) and Hoechst 33342 (1 µg/mL; Sigma, USA) for 10 min at room temperature (RT). After rinsing in PBS, coverslips were examined using an Olympus BX60 fluorescence microscope equipped with a digital IX 71 camera (Olympus, Tokyo, Japan). Cells were counted, scoring at least 300 cells in five microscopic regions randomly selected on each coverslip. The experiments were performed in triplicates.

TEM evaluation

To analyse the ultrastructural changes of NPC, electron microscopy was applied. After culture, the cells were centrifuged, washed in PBS and fixed for 1 h in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate. They were then washed three times with 0.1 mol/L sodium cacodylate, postfixed in 1% osmium tetroxide for 2 h, and then were put in 2% tannic acid two times, each for 30 min. Cells were then dehydrated in graded ethanol. After absolute ethanol, they were dried in a critical point drier (hcp-2, Hitachi). Cells were fixed on a metal stage, gold-coated in a sputter coater (E102 Ion sputter, Hitachi) and observed under a transmission electron microscope (TEM; JEM-2000EX; JEOM, Tokyo, Japan).

Immunocytochemistry

BES-treated neurospheres labelled with Hoechst 33342 were transferred to polyornithine-coated glass coverslips. The medium contained 10% fetal bovine serum. After being allowed to differentiate for seven days, the cells were verified by neuronal marker. Half of the medium was replaced every second day. The differentiated cells were fixed in 4% paraformaldehyde and incubated at 4°C overnight with monoclonal mouse anti-NeuN, monoclonal rabbit anti-GFAP. Ratios of differentiated cells versus total cells were calculated. The experiments were performed in triplicates.

Western blotting

Western blotting was used to evaluate proteins samples. Each protein sample was electrophoretically transferred onto nitrocellulose membranes (Millipore) and processed for immunoblotting. The membranes were first blocked with 5% non-fat dry milk powder for 2 h at RT and incubated with rabbit polyclonal antibody of Akt at 4°C overnight. Then the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G (Santa Cruz Biotechnology Inc.), which acted as a secondary antibody, for 2 h at RT. After detachment of previous primary antibodies, the membranes were also probed with β -actin antibodies which acted as an internal control.

Enzyme-linked immunosorbent assay

BDNF levels in culture media conditioned by ES were determined using a ChemikineTM BDNF ELISA kit following the manufacturer's protocol. Briefly, samples (100 μ L) were added to the microplates, incubated at 4°C overnight, and washed extensively, followed by a 3 h incubation with the biotinylated mouse anti-BDNF monoclonal antibody and 1 h incubation with streptavidin-HRP plus substrate for signal development. The OD of each well was detected using a microplate reader, and the readings were subtracted from those at 540 nm. The amount of BDNF in each sample was calculated based on the standard curve prepared in the same experiment.

Statistical analysis

The difference between groups was determined with one-way analysis of variance followed by Tukey's test using Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS, Chicago, IL). software. Differences were considered statistically significant at $P < 0.05$.

Results

Isolation and characterization of NPC

NPCs were obtained from OB tissue of newborn rats. After 3–7 days in culture, the NPCs displayed rounded spherical cells which were dividing and forming cell spheres or aggregates (Figure 1a). Cell spheres derived from OBs expressed nestin (+), musashi (+), Hoechst 33342 (+) (Figure 1b, c and d) and merged (Figure 1e). Markers of neuroepithelial stem cells were stained by nestin; early NSC were stained by musashi and nuclei were stained by Hoechst 33342.

BES incubation system

A schematic diagram of a longitudinal section of the incubation chamber is shown in Figure 2(a), which includes several detailed parts: the upper and lower electrically conductive glass plates (FTO glass), the cell chamber, silicone gasket, and stimulation electrode wires. A pair of electrode wires was introduced into the chamber and fixed at the edge of the FTO glass plates. The other end of the electrode wires was connected to the biphasic electrical stimulator for the supply of various ES modes. Figure 2(b) displays another longitudinal section of the incubation system with accompanying parts: an incubation chamber, a plexiglass cover and base, a culture medium perfusion system, an air filter system, and a biphasic electrical stimulator.

The NPCs were exposed to 12 h of BES at 25 mV/mm and 50 mV/mm electric field strengths with a pulse-burst pattern and 8 ms pulses (20% duty cycle). Cells that were not exposed to BES served as controls.

BES decreases apoptotic/necrotic rate of NPC in the absence of growth factor

In order to investigate the late apoptotic/necrotic properties of NPCs after 12 h of BES, staining with Hoechst 33342 and PI was used so that the number of necrotic/late apoptotic cells (PI-positive) could be expressed as a percentage of total cells. The percentage of apoptotic/necrotic NPCs was 54.98%, 28.75%, and 24.23% for the control, 25 mV/mm and 50 mV/mm BES-treated cells, respectively. Thus, the percentage of necrotic/late apoptotic cells of the BES-treated groups was decreased significantly compared with the control (Figure 3a and b).

BES enhances NPC survival in the absence of growth factor

The effect of 12 h of BES on NPC viability in the absence of growth factor media was assessed. The results of an MTT assay indicated that BES significantly promoted the

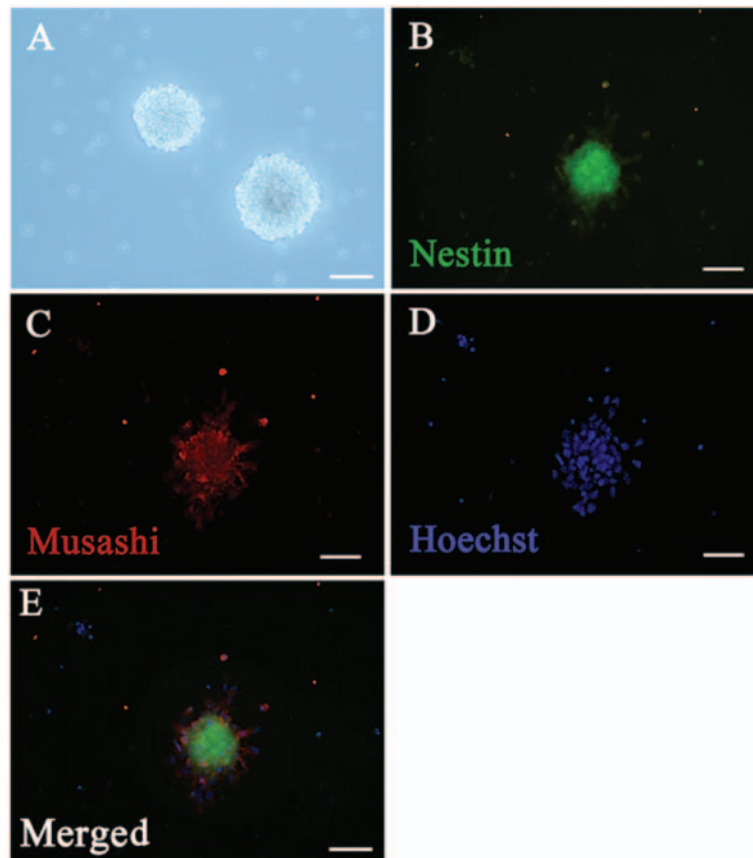


Figure 1 Isolation and characterization of NPCs from neonatal rat OB. (a) Isolated NPCs developed into cell spheres after seven days in culture; (b–e) Immunocytochemistry analyses revealed that OB NPCs formed neurospheres and stained with nestin and musashi. The scale bar corresponds to 100 μm . (A color version of this figure is available in the online journal)

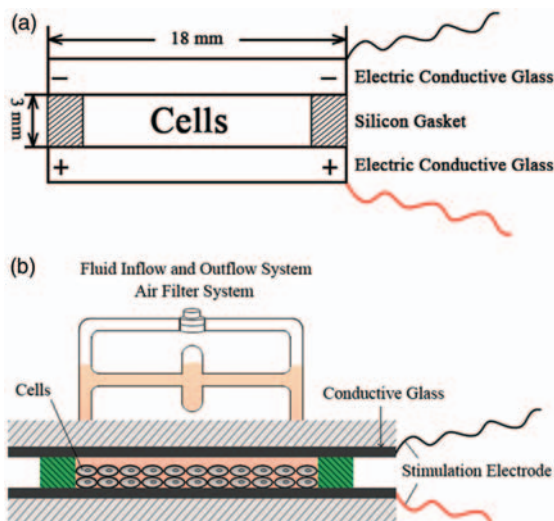


Figure 2 The BES incubation system. (a) Schematic diagram of a longitudinal section of the incubation chamber including: the upper and lower electric conductive glass plates (FTO glass), a closed silicone gasket, the incubation chamber, and a pair of electrode wires; (b) Schematic diagram of a longitudinal section of the entire BES incubation system including the incubation chamber, the fluid inflow-outflow system, the air filter system, a pair of electrode wires, and a fixed cover and base. Conditions of BES: the NPCs were exposed to 12 h of BES at 25 mV/mm and 50 mV/mm electric field strengths with a pulse-burst pattern and 8 ms pulses (20% duty cycle). Cells that were not exposed to BES served as controls. (A color version of this figure is available in the online journal)

survival of NPCs. Cells cultured in 50 mV/mm BES showed greater cell viability than other groups (Figure 3c).

BES changes the ultrastructure of NPCs

As shown in Figure 4, the ultrastructure of cells was investigated using TEM (Figure 4). In the control group, most cells lost their normal cellular structure, with disaggregation of the cellular membrane and nuclear membrane, expulsion of cell contents, organelle damage, and some cell necrosis. In 25 mV/mm and 50 mV/mm BES-treated groups, some NPCs showed apoptotic morphology-nuclear fragmentation and condensation.

Effect of BES on NPC differentiation

The differentiation of NPCs was studied through the use of two markers of differentiated cells, NeuN (mature neurons) and GFAP (glial cells), and Hoechst 33342 labelling (nuclei; Figure 5a). The number of GFAP-positive double-labelled cells was greater than NeuN-positive cells (Figure 5b). In 50 mV/mm BES group, differentiated cells were verified by the expression of GFAP and NeuN. Approximately 74.2% of cells were labelled with GFAP and 24.4% of cells were labelled with NeuN (percentage of NeuN/GFAP-positive cells versus Hoechst-labelled cells).

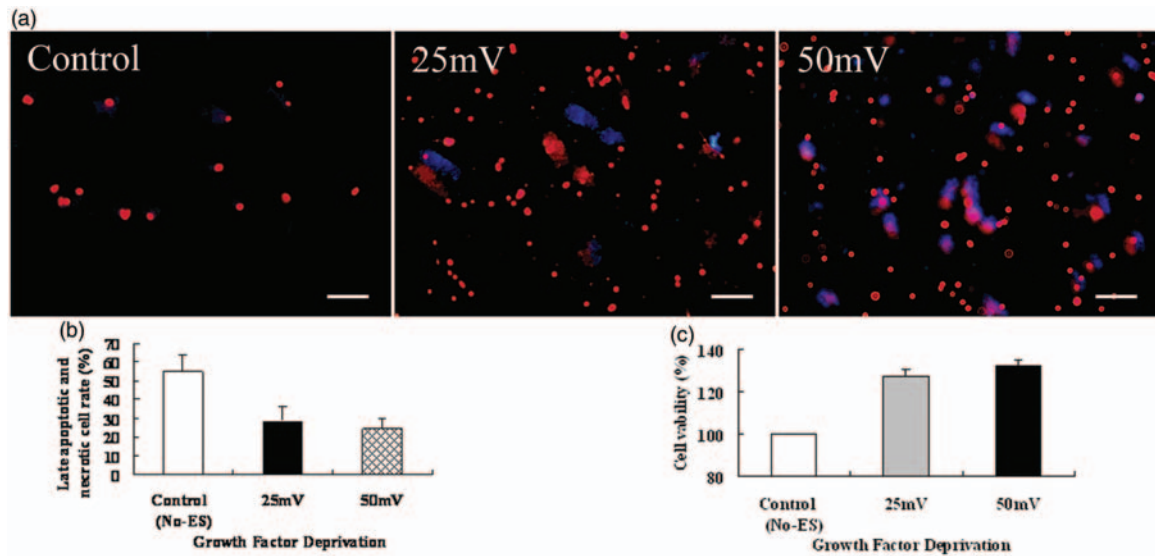


Figure 3 BES decreases the apoptotic/necrotic rate and enhances NPC survival in the absence of growth factor. (a and b) Hoechst 33342/PI staining was used to score the number of necrotic/late apoptotic cells. The necrotic/late apoptotic rate of the BES-treatment groups decreased significantly compared with the control (unstimulated) cells. (c) BES significantly enhances the survival of NPCs in the absence of growth factor. (A color version of this figure is available in the online journal)

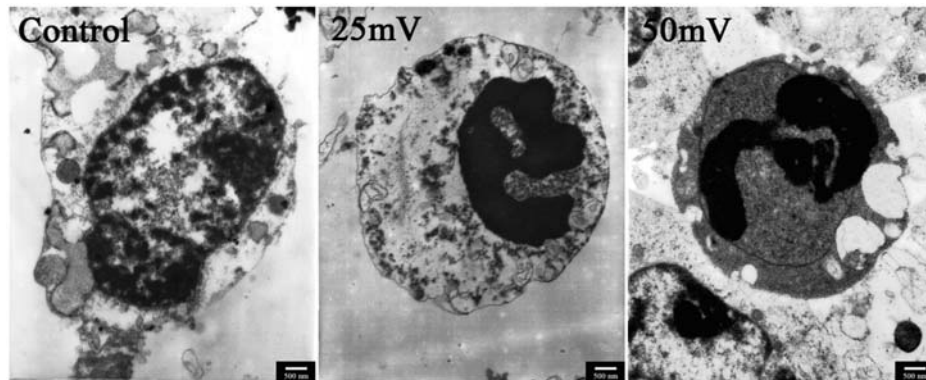


Figure 4 BES alters the ultrastructure of NPCs. The ultrastructural morphological changes of cells were investigated by TEM. In the control group (unstimulated), cells had a necrotic appearance: most cells lost the normal cellular structure with a consequent release of cell contents. In the 25 mV/mm and 50 mV/mm BES groups, the NPCs showed an apoptotic morphology with nuclear fragmentation and condensation

Role of the PI3K/Akt pathway in mediating BES effects

The PI3K/Akt signalling pathway mediates protective effects against apoptosis, which is induced by serum deprivation in neuronal cells.²¹ BES has been found to increase the proliferation and migration of NPCs,¹⁰ and may activate several signalling pathways that are important for NPC survival, including the PI3K/Akt pathway. Therefore, an important consideration is whether the protective effect of BES in cells grown in the absence of growth factors involves activation of the PI3K/Akt signalling pathway. The effect of BES on the activity of the PI3K/Akt signalling cascade was investigated by Western blot analysis and antibodies that specifically recognize the activated (phosphorylated) forms of Akt. NPCs treated with BES (25 mV/mm or 50 mV/mm) showed an increase in phospho-Akt levels compared with the control (unstimulated)

group after 12 h. In comparison with the control, 50 mV/mm BES produced a significant threefold increase in the phospho-Akt level in NPCs (Figure 6a and b). Administration of the potent PI3K inhibitor wortmannin (1 μ mol/L) markedly blocked the expression of phospho-Akt (Figure 6a and b). Wortmannin is a specific and direct inhibitor of PI3K, and it has an *in vitro* half maximal inhibitory concentration of around 5.8 nmol/L, making it around 2.1 μ mol/L more potent than LY294002.²²

In order to determine whether activation of this pathway is important for NPC survival, NPCs were pretreated with wortmannin (1 μ mol/L) and conditioned with 50 mV/mm BES. The results showed that the survival of NPCs stimulated with 50 mV/mm BES was dramatically decreased in the presence of wortmannin (Figure 6c). These results coincide with those obtained from Western blotting analysis of

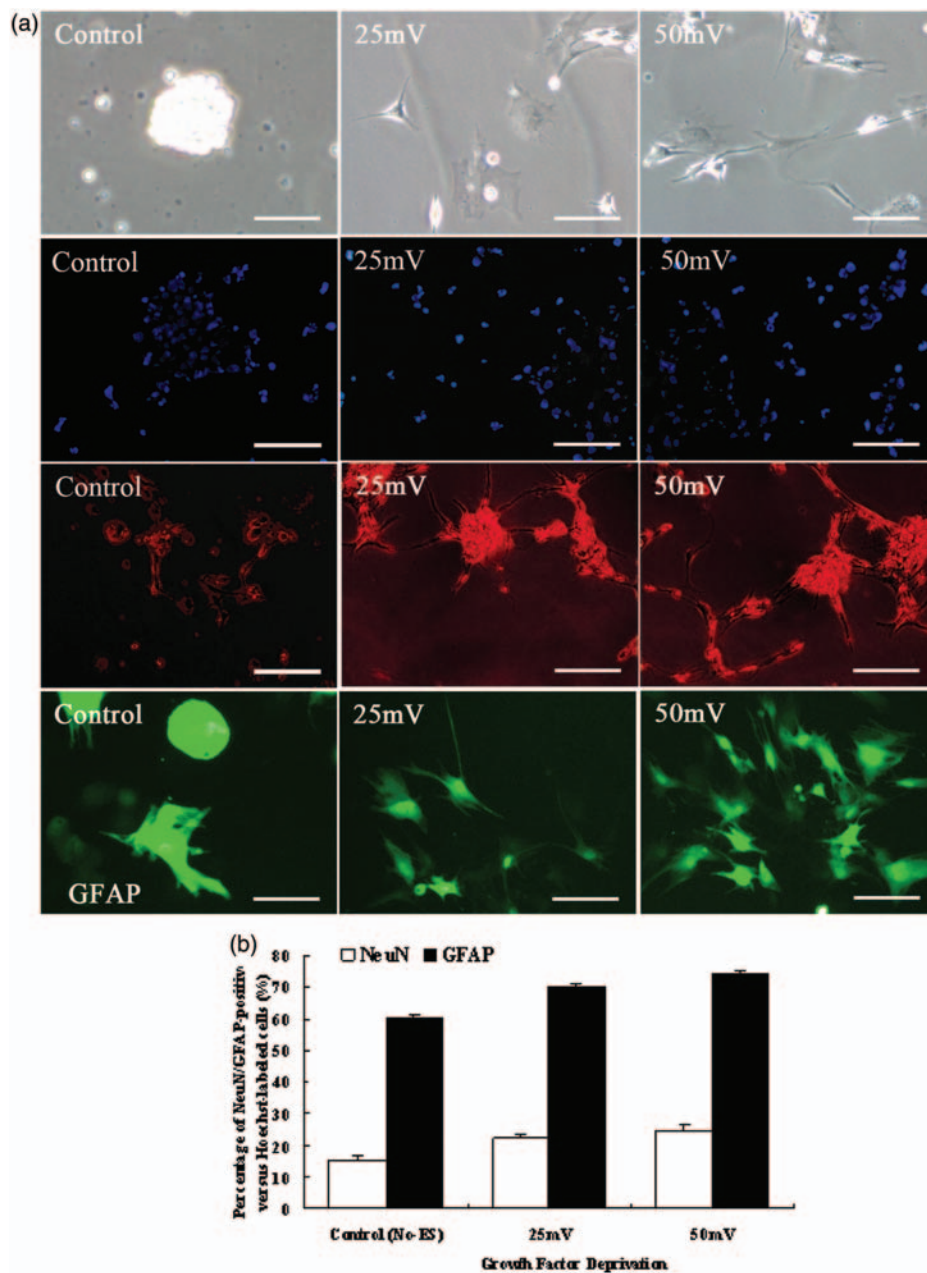


Figure 5 Effect of BES on NPC differentiation. (a) The numbers of differentiated NPCs were calculated based on the presence of NeuN (mature neurons), GFAP (glial cells), and Hoechst 33342-stained nuclei. (b) The number of GFAP-positive double-labelled cells was greater than that of NeuN-positive cells. (A color version of this figure is available in the online journal)

phospho-Akt levels. Therefore, these results confirmed that BES can act against apoptosis and increase cell survival through the activation of the PI3K/Akt pathway.

Effect of BES on BDNF levels in culture media

Neurotrophins, such as BDNF, have been recognized as important factors in neuronal survival and differentiation during neuronal development.^{23–25} The PI3K/Akt pathway

can play a role in mediating neuroprotective effects by altering the secretion of trophic factors in NPCs.²⁶ We proposed that the BES promotion of NPC survival may involve BDNF production and a subsequent activation of the PI3K/Akt signalling pathway. The levels of BDNF in NPC culture media were assessed with ELISA analysis. As shown in Figure 7, an increase in the BDNF protein level was found in the BES-treated groups compared with the untreated control group. Furthermore, the BDNF level was markedly decreased by suppressing Akt activity with the PI3K

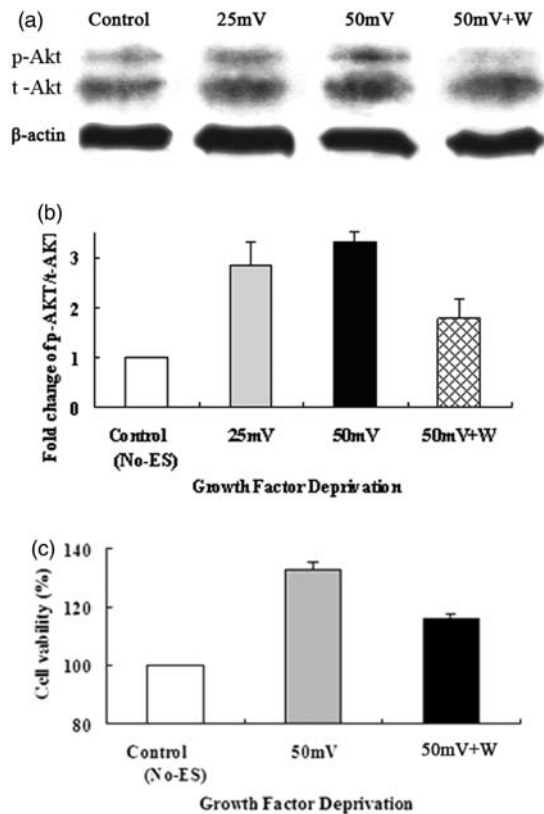


Figure 6 Role of PI3K/Akt pathway in mediating BES effects. (a and b) Western blotting revealed the activated/phosphorylated forms of Akt. NPCs treated with BES (25 mV/mm and 50 mV/mm) showed an increase in phospho-Akt levels compared with the control group (unstimulated) after 12 h. Administration of wortmannin (1 μ mol/L), a potent PI3K inhibitor, markedly blocked the expression of phospho-Akt. Data are expressed as the ratio of phosphorylated Akt-1 to total Akt-1. (c) MTT results indicated that the survival of NPCs stimulated by 50 mV/mm BES was strongly decreased in the presence of wortmannin

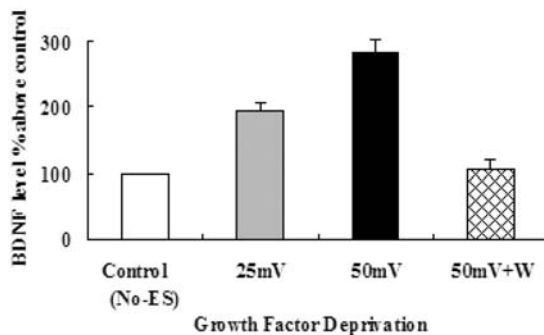


Figure 7 Effect of BES on BDNF levels in culture media. The levels of BDNF in BES-treated and control groups were assessed with ELISA. The levels of secreted BDNF in BES-treated cell culture media were significantly increased compared with the control group. Furthermore, suppressing Akt activity with wortmannin markedly decreased the BDNF level in the 50 mV/mm BES-treated group

inhibitor wortmannin. These results suggest that there may be an existing regulatory network involving BES, the PI3K/Akt pathway, and neurotrophic factors during the process of stem cell survival in this culture system.

Discussion

This study investigated the protective effects of BES on growth factor-deprived apoptosis in OB NPCs. We found that BES enhanced cell survival and prevented the apoptosis of NPCs caused by growth factor deprivation. The anti-apoptotic effect of BES was dependent on the activation of the PI3K/Akt signalling cascade and an increase in BDNF production. These findings suggest that BES effectively prevented growth factor deprivation-induced apoptosis and helped to regulate BDNF-PI3K/Akt signalling. BES may thus be used as an auxiliary strategy to improve cell survival and prevent cell apoptosis in stem cell-based transplantation therapies.

Transplantation of NSCs/NPCs into the damaged brain or spinal cord has been performed in previous studies.^{27,28} However, the therapeutic prospects are limited by multiple factors.²⁹ ES, as a non-chemical strategy for improving stem cell survival, is not yet thoroughly understood. ES can dramatically accelerate the speed of axonal regeneration and reinnervation and can positively modulate the functional recovery capability following peripheral nerve injury.^{30,31} ES activates PI3K/Akt and ERK signalling pathways, and participates in multiple cellular processes. ES can regulate the proliferation of neural cells and potentially contribute to relevant cellular responses.^{9,32} In ischaemic stroke rat model, ES protective effect against apoptosis is mediated by activation of the PI3K/Akt pathway.¹¹ In this study, BES was shown to enhance cell survival and decrease the apoptotic rate of NPCs grown in the absence of growth factor media. Furthermore, BES was found to activate the PI3K/Akt signalling pathway, which was shown with the PI3K/Akt inhibitor wortmannin to be essential for cell survival and apoptosis. These findings indicated that the anti-apoptotic effect of BES is mediated through PI3K/Akt activation.

Several neurotrophic factors, such as glial cell line-derived neurotrophic factor, vascular endothelial growth factor, and BDNF, participate in the repair of injured nerves and vasculature.³³⁻³⁵ BDNF is also thought to be involved in stem cell proliferation and differentiation. However, the mechanisms underlying these growth factor-mediated neuroprotective effects remain unclear. Thus, another major goal of this study was to clarify the relationship between BES activation of PI3K/Akt and BDNF production. BDNF is a member of the neurotrophin family, which plays important roles during neuronal development. It has recently been reported that BDNF knock-down within NSCs/NPCs could decrease cognitive benefits in a transgenic model of Alzheimer disease.^{36,37} The present study found a new relationship between the production of BDNF, the enhancement of NPC survival, and BES. The levels of BDNF secreted from BES-treated cells were increased compared with the control group. Wortmannin suppressed Akt activity and markedly decreased the BDNF level, which is consistent with the notion that BES stimulation of PI3K/Akt pathway activation is a key step in the production of BDNF. These results revealed that BDNF-induced NPC survival was mediated through the PI3K/Akt pathway.

In addition, BES was found to increase the number of GFAP-positive cells. GFAP is widely recognized as an astrocyte differentiation marker, forming the major intermediate filament protein of mature astrocytes. Neuron-astrocyte interactions play a leading role in the differentiation of NPCs,³⁸ and a recent study showed that GFAP-positive astrocytes can promote neurite outgrowth, respond to growth factors, and regulate neurogenesis.³⁹ Region-specific cues are important in the neuronal differentiation of naïve NPCs. The present study revealed that certain factors 'guide' NPCs towards certain differentiation and that the process may be promoted by BES.

The present study demonstrates that activation of the PI3K/Akt pathway and an increase in BDNF production are essential for the pro-survival and anti-apoptotic effects of BES. Further studies are needed to clarify the precise mechanism of BES-mediated NPC protection.

Author contributions: MW and YF conceived and designed the experiments. MW, PL, ML, WS, and QW performed the experiments. MW, YF, and PL analysed the data. MW, YF, and PL wrote the paper.

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