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Neural stem cells over-expressing brain-derived neurotrophic factor promote neuronal survival and cytoskeletal protein expression in traumatic brain injury sites

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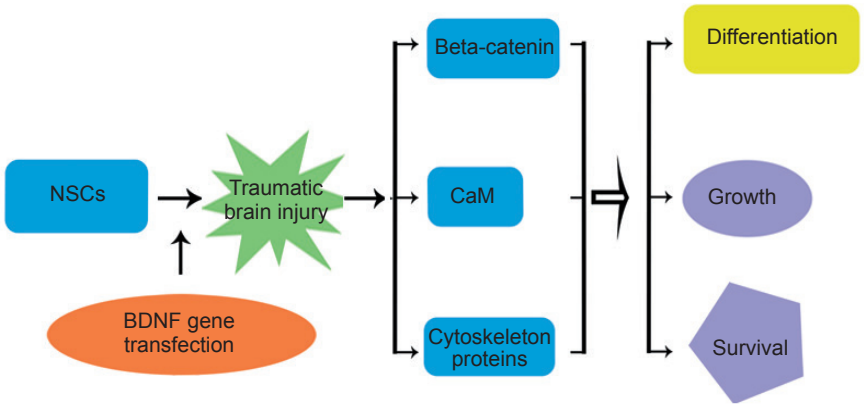
Graphical Abstract

Cytoskeleton protein and Wnt/catenin signaling pathway might be involved in the effects of neural stem cells (NSCs) genetically modified by brain-derived neurotrophic factor (BDNF) on improving regeneration



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Abstract

Cytoskeletal proteins are involved in neuronal survival. Brain-derived neurotrophic factor can increase expression of cytoskeletal proteins during regeneration after axonal injury. However, the effect of neural stem cells genetically modified by brain-derived neurotrophic factor transplantation on neuronal survival in the injury site still remains unclear. To examine this, we established a rat model of traumatic brain injury by controlled cortical impact. At 72 hours after injury, 2×10^7 cells/mL neural stem cells overexpressing brain-derived neurotrophic factor or naive neural stem cells (3 mL) were injected into the injured cortex. At 1–3 weeks after transplantation, expression of neurofilament 200, microtubule-associated protein 2, actin, calmodulin, and beta-catenin were remarkably increased in the injury sites. These findings confirm that brain-derived neurotrophic factor-transfected neural stem cells contribute to neuronal survival, growth, and differentiation in the injury sites. The underlying mechanisms may be associated with increased expression of cytoskeletal proteins and the Wnt/β-catenin signaling pathway.

Key Words: *nerve regeneration; brain-derived neurotrophic factor; neural stem cells; transfect; differentiation; traumatic brain injury; cytoskeleton; neurofilament; microtubule-associated proteins; calmodulin; Wnt/β-catenin; neural regeneration*

Introduction

Traumatic brain injury (TBI) is a major cause of death and disability globally, and is particularly associated with traffic accidents and disaster (Lin et al., 2016). Previous research has shown that the multi-directional differentiation potential of neural stem cells (NSCs) in the central and peripheral nervous systems plays an important role, and may also be involved in clinical treatment of nerve system injury and degeneration following NSC transplantation (McKay, 1997; Lee et al., 2015; Santamaria and Garica-Sanz, 2015; Lou et al., 2016; Yao et al., 2016). In different environments, embryonic NSCs from different areas of the central nervous system can be differentiated into neurons, astrocytes and oligodendrocytes (Kallur et al., 2006; Shetty and Hattiangady, 2013; Yuan et al., 2015; Ye et al., 2016). Survival, proliferation, and differentiation of transplanted NSCs are influenced by many factors including cytokines, endogenous gene regulation, and biochemical changes in the lesion microenvironment (Chodobski et al., 2011; Mendes Arent et al., 2014). An experimental study showed that NSCs transplantation recovered lost neurological function and cognitive disorder to a certain extent, as well as partly improving reconstruction of neural circuitry, and restoration and growth of cells in lesions of a rat model of TBI (Shear et al., 2011). Besides cell replacement therapy, the mechanisms might be associated with neurotrophic factor release and signal transduction (Castorina et al., 2015). However, the underlying mechanisms are still not clear. Further, the means by which lesions can be influenced to protect cells from loss has not been determined in TBI.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors that plays a critical role in improving differentiation, maturation, and survival of neurons, suppressing apoptosis in the central nervous system, and also exerting a neuroprotective effect under adverse conditions (Davies, 1994; Kang and Schuman, 1995; Wang et al., 2014; Lv et al., 2016). We previously found that NSCs over-expressing BDNF (BDNF/NSCs) significantly increased the number of surviving engrafted cells, proportion of engrafted cells with a neuronal phenotype, and expression of synaptic proteins and growth-associated protein 43 (Ma et al., 2012). Moreover, functional recovery following BDNF/NSCs transplantation was significantly improved in a rat model of TBI (Ma et al., 2012). We also found that expression of calmodulin (CaM) and the cytoskeletal protein, actin, were strongly associated with neuronal growth and maturation *in vitro* (Kitamura et al., 1995; Levinson et al., 2004; Schaloske et al., 2005; Larsson, 2006; Difato et al., 2011; Yu et al., 2011). BDNF can increase cytoskeletal protein expression during regeneration after axonal injury (Difato et al., 2011). Thus, these proteins may promote survival of transfected NSCs in TBI sites. Consequently, in this study we sought to determine the effect of BDNF/NSCs transplantation on the cytoskeleton and neuronal survival and growth in a rat model of TBI.

Materials and Methods

NSCs culture

Primary NSCs were isolated from forebrain tissue of embryonic 14-day (E14) Wistar rats, and cultured as previously described (Ma et al., 2010). The plasmid recombinant pcDNA3.1-BDNF was constructed and BDNF/NSCs obtained (Ma et al., 2011, 2012). Cells were incubated at 37°C in a humid atmosphere with 5% CO_2. Culture medium consisted of Dulbecco's modified Eagle's medium and F12 (1:1; Sigma-Aldrich, St. Louis, MO, USA) with L-glutamine, hydroxyethyl piperazine ethanesulfonic acid, $NaHCO_3$, glucose, heparin (all from Sigma), N2 supplement (1%; Invitrogen, Carlsbad, CA, USA), basic fibroblast growth factor, and epidermal growth factor (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). NSCs were cultured in suspension and dissociated to single cell suspensions using Accutase (Thermo Fisher, Inc.) every 5–7 days.

TBI model establishment

Sixty specific-pathogen-free adult male Wister rats, weighing 200–220 g, were provided by the Experimental Animal Laboratory of Dalian Medical University, China, (license number SYXK (Liao) 2013-0006). Procedures were approved by the Institutional Animal Care and Use Committee of the Dalian Medical University, China.

A controlled cortical impact model of brain injury was performed, as previously described (Ma et al., 2012). Briefly, the skull was exposed between lambda and bregma. Next, a 3 mm craniotomy was performed over the left parietotemporal cortex. A controlled cortical impact was induced using a 2.0 mm diameter pneumatic impactor (Air-Power, Inc., High Point, NC, USA), which indented the exposed surface of the brain.

NSCs transplantation

Controlled cortical impact rats were randomly assigned to two groups for transplantation. At 72 hours after TBI, BDNF/NSCs or naive NSCs were harvested and dissociated to single cell suspensions with Accutase, washed again, and then resuspended in sterile phosphate-buffered saline (PBS) at a concentration of 2×10^7 cells/mL. PKH-26 (Sigma-Aldrich) was used to label these cells immediately before transplantation, as previously described (Fauza et al., 2008; Ma et al., 2012). Labeled cells were resuspended at a density of 1×10^5 cells/mL and maintained on ice for transplantation. TBI rats were anesthetized again. In the BDNF/NSCs group (n = 30), a Stoelting quintessential injector (Stoelting Co., Wood Dale, IL, USA) was used to provide a controlled injection of BDNF/NSCs suspension (3 mL per animal) to the cortex below the injury cavity on the ipsilateral hemisphere: anteroposterior, –3.0 mm from bregma; dorsoventral, 1.1 mm; and mediolateral, 1.0 mm. The injection rate was 1 μL/min for 3 minutes using a 10 μL Hamilton syringe (Hamilton Company, Reno, NV, USA). The NSCs group (n = 30) received an equivalent naive NSCs suspension.

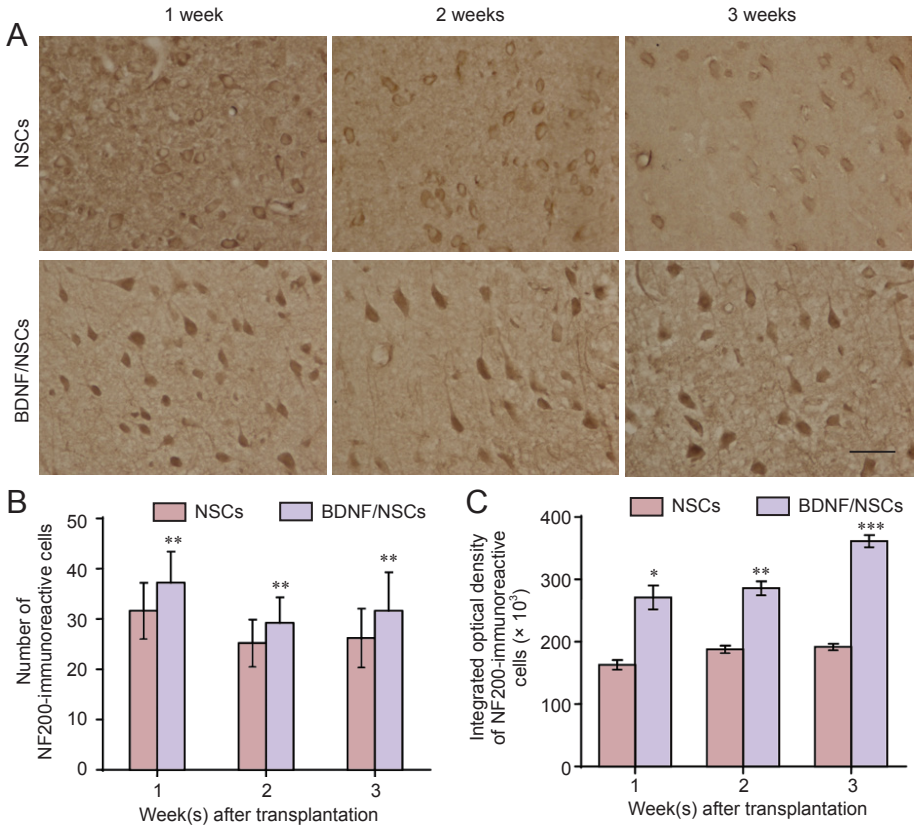

Figure 1 Effect of BDNF/NSCs on NF200 immunoreactivity and neuronal morphology in lesions following transplantation after traumatic brain injury.
(A) Immunohistochemical staining for NF200 in lesioned sections. Cell bodies were larger and axons longer and thicker in BDNF/NSCs-transplanted rats compared with naive NSCs-transplanted rats. Scale bar: 50 μm. (B) Quantitative analysis of NF200-positive cell number in 400-fold field. (C) NF200 immunoreactivity in lesions. NF200 immunoreactivity was expressed as integrated optical density ($\times 10^3$). Data are expressed as the mean ± SD (n = 5), and were analyzed by one-way analysis of variance followed by independent samples t-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs.* NSCs group. BDNF: Brain-derived neurotrophic factor; NSCs: neural stem cells; NF200: neurofilament 200.

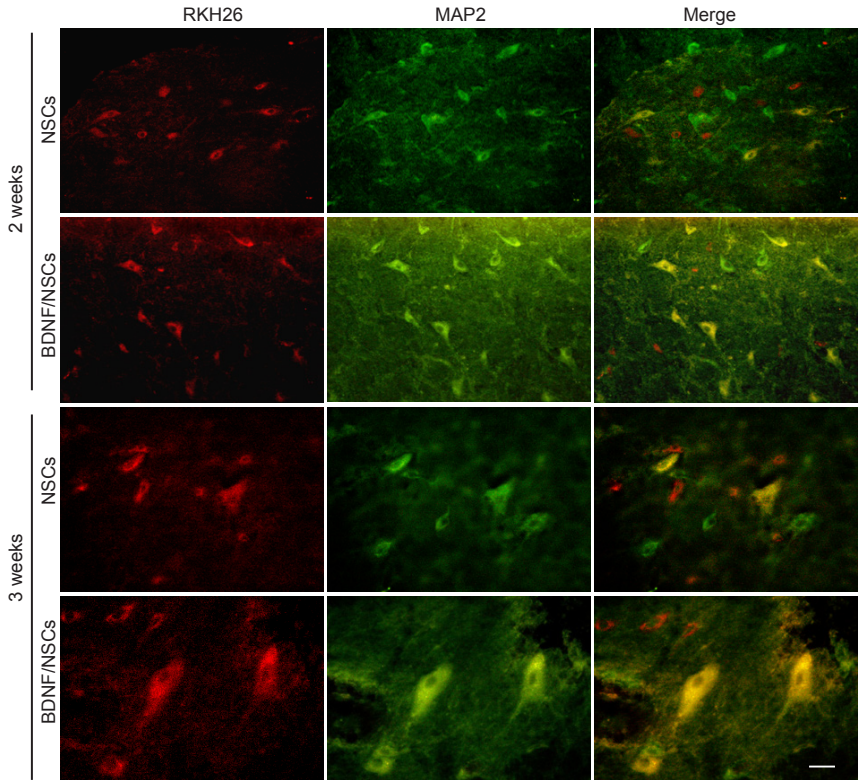

Figure 2 Effect of BDNF/NSCs on MAP2 expression in host cells and neuron-like cells with concomitant PKH26 at 2 and 3 weeks after BDNF/NSCs or NSCs transplantation (immunofluorescence staining).
Transplanted cells were labeled using PKH26 dye (red), with MAP2 labeled by FITC (green). Merged cells show MAP2 expression in transplanted neuron-like cells with concomitant PKH26. Scale bar: 50 μm. BDNF: Brain-derived neurotrophic factor; NSCs: neural stem cells; MAP2: microtubule-associated protein 2; FITC: fluorescein isothiocyanate.

Immunohistochemical method
At 1, 2, and 3 weeks after transplantation, rats (n = 5 in each group at different time points) were killed by paraformaldehyde perfusion. Brains were harvested and cryosectioned at 16 μm thickness along the coronal plane. To determine neuronal survival and growth within lesions, immunohistochemistry was performed using neurofilament 200 (NF200) antibody. Immunofluorescence was used to assess microtubule-associated protein 2 (MAP2) expression in host cells and neuron-like cells with concomitant PKH26.

Three sections per rat (n = 5) were immunostained, and three regions per section observed. Sections were washed twice with PBS and preincubated in 10% normal goat serum. Sections were incubated at 4°C overnight with one of the following primary antibodies: mouse monoclonal anti-NF200 (1:500; Sigma-Aldrich) or MAP2 (1:200; Boster, Wuhan, China). After washing three times with PBS, sections were incubated with an appropriate secondary antibody for 1 hour at room temperature: goat anti-mouse IgG (1:1,000; ZSGB-Bio, Beijing, China) for NF200 and avidin-biotin complex (Boster), and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:1,000; Sigma-Aldrich) for MAP2. Digital color images were obtained using a Nikon C1 Plus confocal microscope (Nikon, Tokyo, Japan). NF200-positive cells were counted in three random regions/section at 400× magnification, with integrated optical density of NF200 immunoreactivity calculated using Image-Pro Plus 5.1 software (Media Cybernetics, Rockville, MD, USA).

Western blot assay

Western blot assay was performed as previously described (Ma et al., 2012). The remaining rats (n = 5 in each group at different time points) were sacrificed. Brain tissue encompassing the transplant site was immediately collected onto a bed of ice. Samples were lysed and protein quantified using a KeyGen assay (KeyGen biotech, Nanjing, China). Protein (50 μg protein per lane) was boiled and separately loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini-gels, and then transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat dry milk in Tris buffered saline with Tween (TBST) for 2 hours at room temperature, and then incubated overnight at 4°C with rabbit anti-actin antibody (1:1,000; Sigma-Aldrich), mouse monoclonal anti-CaM (1:2,000; Sigma-Aldrich), mouse monoclonal anti-β-catenin (1:1,000; Sigma-Aldrich), or mouse monoclonal anti-GAPDH (1:1,000; ZSGB-Bio). Blots were washed three times for 10 minutes each with TBST, incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5,000; ZSGB-Bio), washed three times for 10 minutes each, and then bound antibodies detected by chemiluminescence using an ECL western blotting detection system kit (Amersham Biosciences, Piscataway, NJ, USA), before exposure to ChemiDOC™ XRS+ with Image Lab™ Software (BIO-RAD Laboratories, Inc., Hercules, CA, USA). The relative optical density of each band was standardized to optical density of the GAPDH band.

Statistical analysis

Data are expressed as the mean ± SD, and were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses were performed using one-way analysis of variance and independent samples t-tests. A value of $P < 0.05$ was considered statistically significant.

Results

BDNF/NSCs promoted neuronal survival, neurite growth, and NF200 immunoreactivity in TBI lesions

Immunostaining showed more NF200-positive cells in lesions from the BDNF/NSCs group compared with the NSC group at different time points after transplantation (**Figure 1B**). Further, the cell bodies of surviving neurons (NF200-positive cells) were larger, and with longer neurites in BDNF/NSCs-transplanted rats than NSCs-transplanted rats, even in the first week after transplantation. NF200 was mainly distributed in the cytoplasm of neurons in NSCs-transplanted rats (**Figure 1A**). NF200 immunoreactivity was significantly increased ($P < 0.05$ or $P < 0.01$; **Figure 1C**) and extended into neurites in many neurons in BDNF/NSCs-transplanted rats compared with NSCs-transplanted rats (**Figure 1A**).

MAP2 expression in host cells and neuron-like cells with concomitant PKH26

To determine the effect of BDNF on differentiation of surviving transplanted cells, microtubule protein expression of MAP2 was determined in neuron-like cells with concomitant PKH26 as well as host cells (MAP2-positive cells except for marked engrafted cells). MAP2 expression was greater in the BDNF/NSCs group compared with the NSCs group at 2 and 3 weeks. Additionally, MAP2 expression in neuron-like cells with concomitant PKH26 dye was also greater in the BDNF/NSCs group compared with the NSCs group, especially at 3 weeks. MAP2-positive cells coupled with PKH26 displayed a star-like morphology with more processes, and MAP2-positive fluorescence was also enhanced in cell bodies and processes in the BDNF/NSCs group at 2 and 3 weeks (**Figure 2**).

BDNF increased β-catenin, actin, and CaM expression in TBI rats after NSCs transplantation

Western blot assays were performed to examine β-catenin, actin, and CaM expression at different time points after transplantation. Expression levels of β-catenin, actin, and CaM were all significantly increased in the BDNF/NSCs group compared with the NSCs group ($P < 0.05$ or $P < 0.01$; **Figure 3**).

Discussion

Our previous studies have shown that NSCs over-expressing BDNF significantly improve survival and neural growth in transplanted BDNF/NSCs after TBI (Ma et al., 2012). In this study, we further investigated the correlation between increased survival and cytoskeletal protein expression following transplantation of NSCs over-expressing BDNF. We found that after TBI, neuron survival and neurite growth in lesions significantly improved in BDNF/NSCs-transplanted rats compared with naive NSCs-transplanted rats. Correspondingly, cytoskeletal proteins (specifically, NF200, MAP2, and actin) were increased by BDNF over-expression in BDNF/NSCs-transplanted rats. Thus, we predict that improved survival and neural growth are associated with enhanced cytoskeletal protein expression by BDNF. Furthermore, β-catenin, a key factor and Wnt signaling pathway downstream target, was also increased in BDNF/NSCs-transplanted rats, suggesting that the Wnt/catenin sig-

naling pathway might be involved in improved regeneration due to NSCs genetically modified by BDNF.

In the adult central nervous system, neurofilaments are the most abundant cytoskeletal protein in neurons and axons, and are important intermediate filaments involved in regulating cellular function. Neurofilaments contribute to dynamic properties of the axonal cytoskeleton during neuronal differentiation, axon outgrowth, regeneration, and guidance (Perrot et al., 2008). Neurofilaments consist of nestin, three neurofilament subunits (NFL, NFM, and NFH), α-internexin, peripherin, and synemin. Nevertheless, only NF200 is found in axons under normal conditions (Portier et al., 1983-1984; Lendahl et al., 1990; Liu et al., 2011). It was previously suggested that TBI mainly causes loss of cytoskeletal proteins, including NF66, NF200, and MAPs. Loss of cytoskeletal proteins leads to more pronounced neuronal loss, which suggests that decayed NF200 adversely affects survival of injured neurons (Posmantur et al., 1994; Galvin et al., 2000; Huh et al., 2002; Meng et al., 2008). NF200 is commonly used as an axonal marker protein (Portier et al., 1983-1984; Liu et al., 2011). Here, we show significantly increased NF200 in lesions in the BDNF/NSC group compared with the NSC group. In addition, neural growth and neurites were particularly enriched in surviving cells in the BDNF/NSC group compared with the NSC group during the experimental period. These results suggest that BDNF may promote NF200 protein expression, which potentially contributes to growth and differentiation of transplanted NSCs. While alternatively, Wang et al. (2014) demonstrated that BDNF was capable of improving the traumatically injured brain microenvironment and promoting axonal regeneration after TBI. Therefore in this study, increased NF200 expression stimulated by NSCs genetically modified by BDNF might protect host neuronal loss and promote axonal regeneration in lesions (Perrot et al., 2008; Liu et al., 2011). Indeed, it is possible that TBI-induced primary injury and secondary inflammation may suppress regeneration of axons with lower NF200 levels in the early stage of TBI. Although some cells would continue to be lost during the first two weeks, survival will be ongoing (modified by increased levels of important cytoskeletal proteins due to transplantation), leading to neuronal growth and partially repaired or regenerated neurites in lesions, which in turn may lead to further increased NF200 expression, especially in BDNF/NSCs-transplanted rats (Shetty and Turner, 1995; Huh et al., 2002).

To investigate the effect of BDNF on transplanted cell differentiation and microtubules in lesions, PKH26 staining was performed to label transplanted cells, with MAP2 expression detected. MAP2 is an important cytoskeletal element that connects microtubules and actin filaments. Microtubules are polymerized under control of MAP2, which also shapes microtubule networks and confers their distinct functional properties, thereby regulating microtubule stability *in vitro*. Microtubules are required for dendrite and axon formation in neurons (Bonnet et al., 2001; Ikegami et al., 2006), and subsequently regulate neuronal growth and restoration. Our data show that BDNF significantly promotes MAP2 expression not only in neuron-like cells differentiated from transplanted cells, but also in host cells after transplantation. Our previous study showed that BDNF increases expression of β-tubulin III, which is an important microtubule component (Ma et al., 2012). Accordingly, this demonstrates that BDNF increases these cytoskeletal proteins, and may play an important role in improving neuronal growth and differentiation, and axonal regeneration.

Our data also show that BDNF increases CaM and actin expression. Some studies have shown that growth-associated protein 43 is involved in growth and regeneration of neurons and axons, and improves behavioral functional recovery after transplantation of NSCs. The underlying mechanism is that growth-associated protein 43 binds to CaM and actin fibrils (Mosevitsky, 2005; Ma et al., 2011; Chung et al., 2016a). Difato et al. (2011) reported that after axonal lesions, BDNF regulates growth and favors actin wave formation during regeneration. Thus, improved growth and differentiation by BDNF following BDNF/NSCs transplantation may be directly associated with increased CaM and actin protein levels.

β-Catenin is a multifunctional protein extensively involved in neuronal growth, proliferation, and differentiation. Wnt/β-catenin signaling regulates proliferation and differentiation of neural progenitor cells, and subsequently, timely sequential cortical neurogenesis (Chung et al., 2016b). During axonal remodeling, Wnt affects looped microtubule formation, and acts in the β-catenin pathway, directly resulting in microtubule growth. The Wnt signal also promotes actin cytoskeleton growth (Hall et al., 2000; Purro et al., 2008), and stimulates NSCs growth and proliferation by shortening cell cycle length (Piccin and Morshead, 2011; Ortega et al., 2013). Yang et al. (2015) demonstrated that BDNF promotes neuronal growth *in vitro* through crosstalk with the Wnt/β-catenin signaling pathway. They showed that Wnt signaling factors are upregulated by BDNF in human neurons. Our data show significantly increased β-catenin protein expression in the BDNF/NSCs group compared with the NSCs group. Therefore, we suggest that a possible mechanism underlying BDNF accelerated growth, regeneration, and stability of cytoskeletal proteins might involve the Wnt/β-catenin signaling pathway. Indeed, the underlying molecular mechanisms are still worthy of further study.

In conclusion, NSCs transplantation modified by the BDNF gene significantly promotes neuronal survival and growth in lesions, and differentiation of transplanted NSCs. This is associated with increased cytoskeletal protein expression by BDNF. We predict that the mechanism underlying correlation between neuronal growth and increased cytoskeletal proteins modified by BDNF might involve the Wnt/β-catenin signaling pathway.

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Author contributions: *TC and YY conceived and designed the study, analyzed data and wrote the paper. LJT and HYC participated in cell culture, immunofluorescence and western blot assay. LK provided reference data and technical support. CHZ participated in cell culture and TBI*

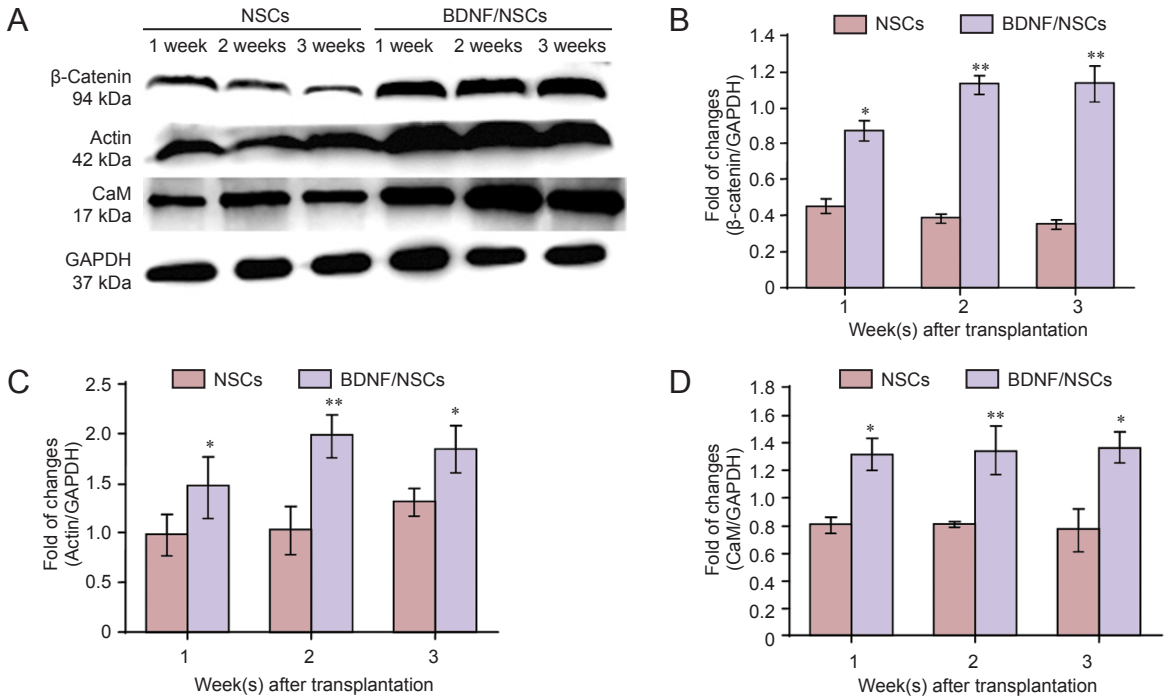

Figure 3 Effect of BDNF/NSCs on actin, CaM, and β-catenin expression in lesions at 1, 2, and 3 weeks after BDNF/NSCs or NSCs transplantation (western blot assay).

(A) Representative western blot results of lesion samples from BDNF/NSCs-transplanted rats or NSCs-transplanted rats. Relative optical density of each band was standardized to optical density of the GAPDH band. Densitometric analysis showed statistically significant increased expression of β-catenin (B), actin (C), and CaM (D) in the BDNF/NSC group compared with the NSC group. Data are expressed as the mean ± SD (n = 5 in each group), and were analyzed by one-way analysis of variance followed by independent samples t-test. *P < 0.05, **P < 0.01, *vs.* NSCs group. BDNF: Brain-derived neurotrophic factor; NSCs: neural stem cells; CaM: calmodulin.

model establishment. LWY participated in transplantation, ensured the integrity of the data and obtained the funding. HYM served as a principle investigator, obtained the funding, conceived and designed the study. All authors approved the final version of the paper.

Conflicts of interest: *None declared.*

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