

# Silencing of long non-coding antisense RNA brain-derived neurotrophic factor attenuates hypoxia/ischemia-induced neonatal brain injury

LI-XING QIAO<sup>1\*</sup>, RUI-BIN ZHAO<sup>2\*</sup>, MING-FU WU<sup>3</sup>, LI-HUA ZHU<sup>4</sup> and ZHENG-KUN XIA<sup>2</sup>

<sup>1</sup>Department of Pediatrics, Zhongda Hospital, Southeast University, Nanjing, Jiangsu 210029;

<sup>2</sup>Department of Pediatrics, Jinling Hospital, Nanjing University School of Medicine, Nanjing, Jiangsu 210002;

<sup>3</sup>Department of Pediatrics, Affiliated Hospital of Yang Zhou University, Yangzhou, Jiangsu 225000;

<sup>4</sup>Institute of Clinical Science, Jiangsu Health Vocational College, Nanjing, Jiangsu 210029, P.R. China

Received February 25, 2020; Accepted May 12, 2020

DOI: 10.3892/ijmm.2020.4625

**Abstract.** Hypoxic/ischemic (HI) brain damage (HIBD) is a major cause of acute neonatal brain injury, leading to high mortality and serious neurological deficits. The antisense RNA of brain-derived neurotrophic factor (BDNF-AS) is transcribed from the opposite strand of the BDNF gene. The aim of the present study was to investigate the role of BDNF-AS in HI-induced neuronal cell injury *in vivo* and *in vitro*. Reverse transcription-quantitative PCR (RT-qPCR) assays indicated that BDNF-AS expression was significantly upregulated in HI-injured neonatal brains and hippocampal neurons. However, BDNF expression was downregulated in HI-injured neonatal brains and hippocampal neurons. Cell Counting Kit-8 assays, Hoechst staining, calcein-AM/PI staining, immunostaining, water maze tests and rotarod tests demonstrated that BDNF-AS silencing protected against hypoxia-induced primary hippocampal neuron injury *in vitro* and HI-induced brain injury *in vivo*. Mechanistically, RT-qPCR assays and western blotting indicated that BDNF-AS silencing led to increased expression of BDNF and activated the BDNF-mediated signaling pathway, as demonstrated by increased expression levels of BDNF, phosphorylated-Akt and phosphorylated-tropomyosin receptor kinase B. Collectively, the present study provides important insights into the pathogenesis of HIBD, and it was indicated that BDNF-AS silencing

may be a promising approach for the treatment of neonatal HIBD.

## Introduction

Hypoxic/ischemic (HI) brain damage (HIBD) is a serious complication of parturition caused by the deficiency of blood and oxygen supply in the neonatal brain (1). Moreover, HIBD is a major cause of acute neonatal brain injury, leading to high mortality and serious neurological deficits, such as behavioral, social and cognitive deficits (2). It has been reported that mortality occurs in ~20% of neonates with HIBD during the postnatal period, and an additional 25% of neonates with HIBD develop irreversible and lifelong mental and physical disabilities (3). Therapeutic hypothermia is the current method for the treatment of HI encephalopathy. However, mortality and significant neurologic disability after hypothermia treatment still occur in ~50% of patients (4,5). Thus, the underlying mechanism of HIBD requires further investigation in order to facilitate the development of novel therapeutic methods.

Long non-coding RNAs (lncRNAs), which are non-coding RNA transcripts, have gained increased attention due to their roles in several physiological and pathological processes (6). For example, lncRNAs play important roles in RNA processing, stabilization, metabolism and translation (7,8). Previous studies have reported that cerebral lncRNA expression profiles are extensively altered during brain damage. For instance, ischemia leads to temporal changes in the cerebral transcriptome that affects the neurologic outcome after brain damage (9,10). Moreover, lncRNAs such as Fos Downstream Transcript, Metastasis Associated Lung Adenocarcinoma Transcript 1 and growth arrest-specific 5 have been shown to be involved in ischemic stroke (11-13). Thus, it was speculated that lncRNAs are potential regulators of neonatal brain HI injury.

Brain-derived neurotrophic factor (BDNF) is a canonical nerve growth factor highly expressed in the brain, and the binding of BDNF protein to its receptor contributes to neuronal survival (14). It has also been revealed that BDNF plays an important role in stress responses and brain

---

*Correspondence to:* Dr Zheng-Kun Xia, Department of Pediatrics, Jinling Hospital, Nanjing University School of Medicine, 305 Zhongshan East Road, Nanjing, Jiangsu 210002, P.R. China  
E-mail: xiazhenkunn@aliyun.com

\*Contributed equally

**Key words:** hypoxia, ischemia, neonatal brain damage, long non-coding RNA, brain-derived neurotrophic factor/tropomyosin receptor kinase B/Akt signaling

disorders (15). lncRNA-BDNF-antisense (AS) is the antisense RNA transcript of BDNF (16). BDNF-AS is widely expressed in various human tissues, and may have crosstalk with BDNF expression in the regulation of neural cell function (17). Previous studies have revealed that inhibition of BDNF-AS exerts neuroprotective effects on ischemic damage in retinal ganglion neurons (18,19). However, direct evidence of the role of BDNF-AS in HI neonatal brain damage is still lacking.

Based on the potential crosstalk between BDNF-AS and BDNF, the present study hypothesized that BDNF-AS serves an important role in HI-induced brain injury. Therefore, the role of BDNF-AS in neonatal HIBD was investigated, as well as the underlying mechanism of BDNF-AS-mediated brain injury.

## Materials and methods

**HI neonatal brain injury model.** A total of 30 pregnant C57BL/6J (wild-type) mice (age, 14–16 weeks; weight, 30–40 g) were purchased from Nanjing Qinglongshan Experimental Animal Center. The day of birth was considered day 0. After birth, the pups were housed with their dam under a 12:12-h light-dark cycle at a temperature of  $23\pm 3^\circ\text{C}$  and relative humidity of  $50\pm 5\%$ , with food and water available *ad libitum* throughout the study. The male 7-day-old postnatal (P7) pups (weight, 10–15 g) were anesthetized with isoflurane (3% in a mixture of medical air and oxygen 70:30 ratio). A 5.0 silk surgical suture was used to ligate the right common carotid artery. The artery was cut between two ligation sites. After the surgery, the pups were recovered for 1 h and then placed in the hypoxic incubator containing 8% oxygen and 92% nitrogen at  $37^\circ\text{C}$  for 2 days, 5 days, 7 days or 8 weeks. Sham animals were selected as the control group, and underwent anesthesia and the common carotid artery was exposed without ligation and hypoxia. The normal brains from P7 mice without any treatment were used as the normal group. In total, six animals were used in each experimental group. All procedures were approved by the Animal Care and Use Committee of Nanjing University.

The male pups received a stereotaxic injection of BDNF-AS short hairpin (sh)RNA (5'-CCGGCCGGCATTGGAACCTCCAGTGTTCAAGACGCACTGGGAGTTCCAATGCCTTTT TTG-3') or scrambled shRNA (5'-AATGCCAGTTCCGGTTT TTTGGCCGGCCTGGGAACCTGGCATTGTTCAAGACC CCAGCAC-3') of 2  $\mu\text{l}$  concentrated BDNF-AS shRNA viral stocks ( $3\times 10^9$  particles/ $\mu\text{l}$ ) or scrambled shRNA viral stocks ( $3\times 10^9$  particles/ $\mu\text{l}$ ) into the hippocampus after HI treatment. The injection was performed using a Hamilton syringe and conducted unilaterally at the following coordinates calculated from Bregma and the skull surface: Anterior, -2.4; lateral, +1.5 (right side); ventral, -2.0 (20). Then, 48 h after HI, measurement of the brain infarct size and immunostaining of Glial fibrillary acidic protein (GFAP), CD11b/c or neuronal nuclei (NeuN) were performed in the cornu ammonis 1 hippocampal region. Subsequently, 8 weeks after HI, the neurobehavioral function of the brain was evaluated by water maze or rotarod tests.

**Isolation of primary hippocampal neuron and stress induction.** Animal experiments were approved by the Animal Care and Use Committee of Nanjing University, and were conducted

according to the suggested method of euthanasia of fetuses by the University of California, Los Angeles (<https://rsawa.research.ucla.edu/arc/euthanasia-fetuses/>).

The pregnant mice at ~18 days post-fertilization were euthanized by cervical dislocation. The use of anesthesia to euthanize the pregnant female is not recommended, as anesthesia is known to cause brain cell death (21,22). The fetuses were euthanized by decapitation. Sterile scissors were used to open the cranium of pup from back of the neck to the nose. The entire brain was removed with the forceps. Then, a small section of meninges surrounding the hippocampus was grasped with the sterile forceps and pulled gently away. The hippocampi were treated with 0.25% trypsin (Sigma-Aldrich; Merck KGaA) supplemented with 100 ng/ml DNase (Roche Diagnostics) at  $37^\circ\text{C}$  for 30 min and dissociated using repeated passage through a series of fire-polished constricted Pasteur pipettes. The digestion was terminated with DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.). The hippocampal neurons were distributed in 8-well poly-L-lysine-treated chamber slide and cultured in the neurobasal medium (DMEM; 4.5 g/liter glucose; 100 U/ml penicillin; 100  $\mu\text{g}/\text{ml}$  streptomycin; 2 mM glutamine) at  $5\times 10^4$  cell/well. Cells were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and 95% relative humidity. Half of the medium was replaced once a week. Hippocampal cultures were used after a culturing period of 10–14 days. For hypoxic induction, cells were cultured in a NAPCO 7001 incubator (Precision Scientific Company) with 1% oxygen, 6%  $\text{CO}_2$  and balance nitrogen at  $37^\circ\text{C}$  for 24 and 48 h. For oxidative stress induction, cells were exposed to  $\text{H}_2\text{O}_2$  (150  $\mu\text{M}$ ) to mimic oxidative stress for 24 and 48 h.

**Detection of brain infarct size.** The male pups were anesthetized after neurological evaluation by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg) and euthanized by cervical dislocation. The death of mice was verified using the following criteria, including lack of pulse, breathing, corneal reflex, failure of a response to firm toe pinch, graying of mucus membranes and an inability to auscultate respiratory or heart sounds. The brain was quickly removed and placed in ice-cold sterile saline for 5 min. The infarct size of brain was detected with 2,3,5-triphenyltetrazolium chloride monohydrate (TTC; Sigma-Aldrich) staining (23). The slices of pup brains were cut into 2-mm thick sections and were immersed in 2% TTC solution for 5 min at  $37^\circ\text{C}$ . The slices were then fixed by 10% formaldehyde at  $4^\circ\text{C}$  overnight. The caudal and the rostral surfaces of each slice were imaged using a fluorescence microscope (magnification,  $\times 400$ ). The percentage of infarct size for each slice was traced and analyzed using the Image-Pro plus version 7.0 software (Media Cybernetics, Inc.).

**Reverse transcription-quantitative PCR (RT-qPCR) assay.** In HI neonatal brain injury model, the male pups were anaesthetized with 3% isoflurane, decapitated and the striatum, hippocampi and cortex were excised and stored in liquid nitrogen for RNA extraction. Total RNAs were extracted from the primary hippocampal neurons or infarcted brain tissues using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Ultraviolet analysis and formaldehyde gel electrophoresis were performed

to detect RNA quality. RT was performed using the miScript II RT kit (Invitrogen; Thermo Fisher Scientific, Inc.).

The temperature protocols for RT were as follows: 37°C for 60 min, 95°C for 5 min and maintenance at 4°C. Following this step, qPCR assays were performed using the Brilliant SYBR Green II RT-qPCR kit (Agilent Technologies, Inc.) according to the manufacturer's instructions. qPCR assays were performed in a final volume of 25  $\mu$ l and each PCR reaction mixture consisted of the specific primers and SYBR Green Supermix. The thermocycling protocols were as follows: Initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, followed by the final extension at 72°C for 60 sec. All qPCR assays were performed in triplicate and normalized to GAPDH. All reactions were carried out on the Applied Biosystems 7500 RT PCR system (Thermo Fisher Scientific, Inc.). Relative gene expression was analyzed using the  $2^{-\Delta\Delta C_q}$  method (24). The primers were designed as follows: BDNF-AS forward, 5'-CCGTGAGAAGATCTCATTGGG-3' and reverse, 5'-GGGTCACAAGTCACGTAGCA-3'; and GAPDH forward, 5'-GTC AAGGCTGAGAACGGGAA-3' and reverse, 5'-AAATGA GCCCAGCCTTCTC-3'.

**Cell Counting Kit (CCK)-8 assay.** CCK-8 assay kit was performed to detect cell viability (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Primary hippocampal cells were seeded onto 96-well plates at the density of  $5 \times 10^4$  cells/well. Then,  $\sim 10 \mu$ l CCK-8 solution was added to each well of the plate after hypoxic stress induction (1% oxygen, 6% CO<sub>2</sub> and balance nitrogen) with or without BDNF-AS expression intervention and incubated for 3 h at 37°C. Cell viability was detected by a microplate reader (Molecular Devices, LLC) at 450 nm.

**Calcein-AM/propidium iodide (PI) staining.** Calcein-AM/PI double staining was performed to detect the apoptosis of hippocampal cells. Hippocampal cells were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 10 min at 37°C, stained using Calcein-AM (10  $\mu$ mol/l; Biosharp Life Sciences) for 10 min at 37°C and then stained with PI (10  $\mu$ mol/l; BD Pharmingen; BD Biosciences) for 10 min at 37°C. The 490 nm excitation filter was used to observe the alive cells. The 545 nm excitation filter was used to observe the dead or dying cells. Calcein-AM/PI double staining was observed in ten selected visual fields under an IX71 inverted light microscope (Olympus Corporation) at magnification, x500.

**Hoechst staining.** Primary hippocampal cells were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 15 min at room temperature. After permeabilizing with Triton-X 100, cells were stained with Hoechst 33342 solution (10  $\mu$ g/ml; Biosharp Life Sciences) for an additional 10 min at 37°C. Hoechst staining was observed in ten selected visual fields under an IX71 inverted fluorescent microscope (Olympus Corporation) at magnification, x500.

**Immunofluorescence staining.** The male pups were anesthetized after neurological evaluation by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg) and euthanized

by cervical dislocation. The brains were quickly removed and the slices of brain were cut into 2-mm thick sections. The brain slices were fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 10 min at room temperature. Then, brain slices were blocked with 5% BSA (Beyotime Institute of Biotechnology) for 1 h at room temperature, incubated antibodies against CD11b/c (Abcam; 1:100; cat. no. ab226482), GFAP (Abcam; 1:100; cat. no. ab7260) and NeuN (Abcam; 1:100; cat. no. ab128886) overnight at 4°C, followed by the Alexa Fluor 594-conjugated secondary antibody (Invitrogen; Thermo Fisher Scientific, Inc.; 1:200; cat. no. R37117) for 1 h at room temperature. Fluorescent mounting medium was used to mount the slices. All slices were observed using a fluorescent microscope. CD11b/c and GFAP staining was observed in ten selected visual fields under an IX71 inverted fluorescent microscope (Olympus Corporation) at magnification, x200. NeuN staining was observed in ten selected visual fields at magnification, x500.

**Western blotting.** Primary hippocampal cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and the concentration of protein was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). A total of 30  $\mu$ g total proteins were separated by 10-12% SDS-PAGE and transferred to PVDF membranes (Beyotime Institute of Biotechnology). The membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature and then incubated with the primary antibody overnight at 4°C using antibodies against BDNF (1:1,000; cat. no. ab108319; Abcam), tropomyosin receptor kinase B (TrkB; 1:1,000; cat. no. ab33655; Abcam), phosphorylated (p)-TrkB (phospho S479; 1:2,000; cat. no. ab228507; Abcam), Akt (1:2,000; cat. no. ab18785; Abcam), p-Akt (phosphor T308; 1:2,000; cat. no. ab38449; Abcam) or GAPDH (1:2,000; cat. no. ab181602; Abcam). Each PVDF membrane was washed three times in Tris buffered saline-0.1% Tween-20 for 10 min per wash and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 1 h. The membranes were washed three times in Tris buffered saline-0.1% Tween-20 for 10 min per wash and then visualized using enhanced chemiluminescence methods (Nanjing KeyGen Biotech Co., Ltd.). Quantity One software version 4.6.5 (Bio-Rad Laboratories, Inc.) was used to analyze protein bands, which were expressed as a relative level against the internal reference.

**Lentiviral overexpression of BDNF-AS.** The lentiviral vector expressing BDNF-AS cDNA (cat. no. LQP0010926) was purchased from Guangzhou RiboBio Co., Ltd. The controlled lentiviral vector expressing a non-specific cDNA (cat. no. LQP0010926-C) was also purchased from Guangzhou RiboBio Co., Ltd. The primary hippocampal cells were transduced with the lentiviral vector containing either BDNF-AS ( $1 \times 10^{13}$  U/ml) or non-specific cDNA ( $1 \times 10^{13}$  U/ml), in the presence of 8  $\mu$ g/ml polybrene (EMD Millipore) for 48 h. RT-qPCR assays were conducted to verify the transduction efficiency.

**Estimation of antioxidants and lipid peroxidation products.** Tissue lipid peroxide level was determined by thiobarbituric acid reactive substances (TBARS) using a reagent kit

(BioAssay Systems; cat. no. DTBA-100) according to the manufacturer's instruction. The activities of superoxide dismutase (SOD; cat. no. A001-3-2) and glutathione peroxidase (GPx; cat. no. A005-1-2) were measured using kits obtained from the Institute of Biological Engineering of Nanjing Jianchen, according to the manufacturer's protocol. The content of protein in the homogenate was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology).

**Neurobehavioral assay.** Rotarod test and water maze test was performed to detect the neurobehavioral function (25). To measure motor coordination, the mice were assessed on the rotarod apparatus. The rotarod consisted of the horizontal cylinder divided into four lanes. In total, three consecutive block trials were administered, in which the rotarod rotated at a constant speed of 5 RPM (rotations per min) for two trials for 300 sec, followed by two trials of acceleration by 3 RPM every 5 sec and finally two trials of acceleration by 5 RPM every 3 sec. The time that each mouse was able to remain on the rotating rod before falling was measured. All of the tests were repeated three times in a blinded fashion.

The Morris water maze test was performed for the assessment of spatial learning and memory. A circular pool (1.3 m in diameter; 50 cm in depth) was filled with room-temperature water. In addition, 1 cm below the surface of the water, a transparent platform was placed in the southeastern quadrant. The water maze tests consisted of cued learning, spatial learning and probe trial. In the cued learning trial, the mice were released from different starting points and allowed to swim for 60 sec to search for the platform. The escape latency time was defined by the time spent to find the hidden platform by the mice. In the spatial learning trial, the mice were allowed to find and climb onto the escape platform, and performed ten trials per day in five blocks of two consecutive trials (120 sec trial; 120 sec inter-trial interval during which time the mouse remained on the escape platform). The cumulative distance from the platform and escape latency to find the platform in each test was recorded. In the probe trial, the mice were released from a new start position in the pool without the platform. The amount of time spent in the southeastern quadrant was recorded. All behavioral tests were recorded by video tracking software (TopScan; version 2.0; CleverSys, Inc.).

**Statistics analysis.** Data are presented as the mean  $\pm$  SEM, and analyzed using SPSS statistics software (version 21.0; SPSS, Inc.). Each experiment was repeated three times. Comparisons between two groups were determined using Student's *t*-test (unpaired; 2-tailed). Comparisons between multiple groups were determined using one-way or two-way ANOVA followed by Tukey's test. As for two-way ANOVA, the parameters BDNF-AS expression and training location, were used for the interaction analysis of neurobehavioral function.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Detection of BDNF-AS and BDNF expression levels after HI stress *in vivo* and *in vitro*.** The normal brains were collected from P7 mice without any treatment. The P7 mice were anaesthetized with 3% isoflurane, decapitated and the

striatum, hippocampi and cortex were excised and stored in liquid nitrogen for RNA extraction. Absolute quantification of BDNF-AS expression demonstrated that BDNF-AS expression was higher in hippocampi compared with the cortex or striatum in the normal brain (Fig. 1A). P7 mice received ligation of the unilateral common carotid artery, and were exposed to 8% O<sub>2</sub> for ~30 min. Then, 5 and 7 days after HI stress, the striatum, hippocampi and cortex were excised. RT-qPCR assays demonstrated that BDNF-AS and BDNF expression levels were compared in the striatum, hippocampi and cortex between HI brains and normal brains. The results indicated that BDNF-AS expression was significantly increased in HI brains compared with Sham group (Control; Fig. 1B), while BDNF expression was reduced in these brains (Fig. 1C). The highest significant difference was detected in the hippocampal cells of HI brains compared with the control hippocampal neurons.

Primarily isolated hippocampal neurons were also exposed to hypoxia stress or oxidative stress. BDNF-AS expression was significantly upregulated after hypoxia stress or oxidative stress (Fig. 1D and E), while BDNF demonstrated an opposite expression pattern (Fig. 1F and G). Collectively, these results suggested that HI stress led to increased BDNF-AS expression *in vivo* and *in vitro*.

**BDNF-AS silencing protects hippocampal cells against HI stress *in vitro*.** To determine the role of BDNF-AS in hippocampal cells *in vitro*, BDNF-AS shRNA transfection was used to silence BDNF-AS expression in the primary hippocampal cells (Fig. 2A). CCK-8 results suggested that hypoxia-induced decrease in cell viability was partially rescued by BDNF-AS silencing (Fig. 2B).

Hoechst 33342 staining and Calcein-AM/PI staining demonstrated that compared with hypoxia group, BDNF-AS silencing significantly decreased the number of apoptotic nuclei (condensed or fragmented) and PI-positive cells (dying or dead cells), thus suggesting that hypoxia-induced cell apoptosis was reduced by BDNF-AS silencing (Fig. 2C and D). Therefore, these results indicated that BDNF-AS silencing protected primary hippocampal cells against HI stress *in vitro*.

**BDNF-AS silencing attenuates HI-induced brain injury *in vivo*.** The present study further investigated whether BDNF-AS silencing protected against HI-induced brain injury *in vivo*. BDNF-AS shRNA or scrambled shRNA (used as the negative control) were administered before HI treatment. Compared with the scrambled shRNA group, BDNF-AS shRNA injection significantly decreased BDNF-AS expression (Fig. 3A). Furthermore, BDNF-AS silencing significantly attenuated HI-induced brain injury as demonstrated by a decreased brain infarct size (Fig. 3B).

The degree of astrocytosis was examined by GFAP staining, which is an astrocytic marker, and the degree of microgliosis by CD11b/c staining, which is a microglial marker (14). BDNF-AS silencing led to decreased GFAP and CD11b/c staining signaling in the hippocampus (Fig. 3C). NeuN staining was also performed to detect the neurons in the hippocampus. It was found that BDNF-AS silencing led to increased NeuN staining signaling in the hippocampus compared with control group (Fig. 3D). The neonatal brain is

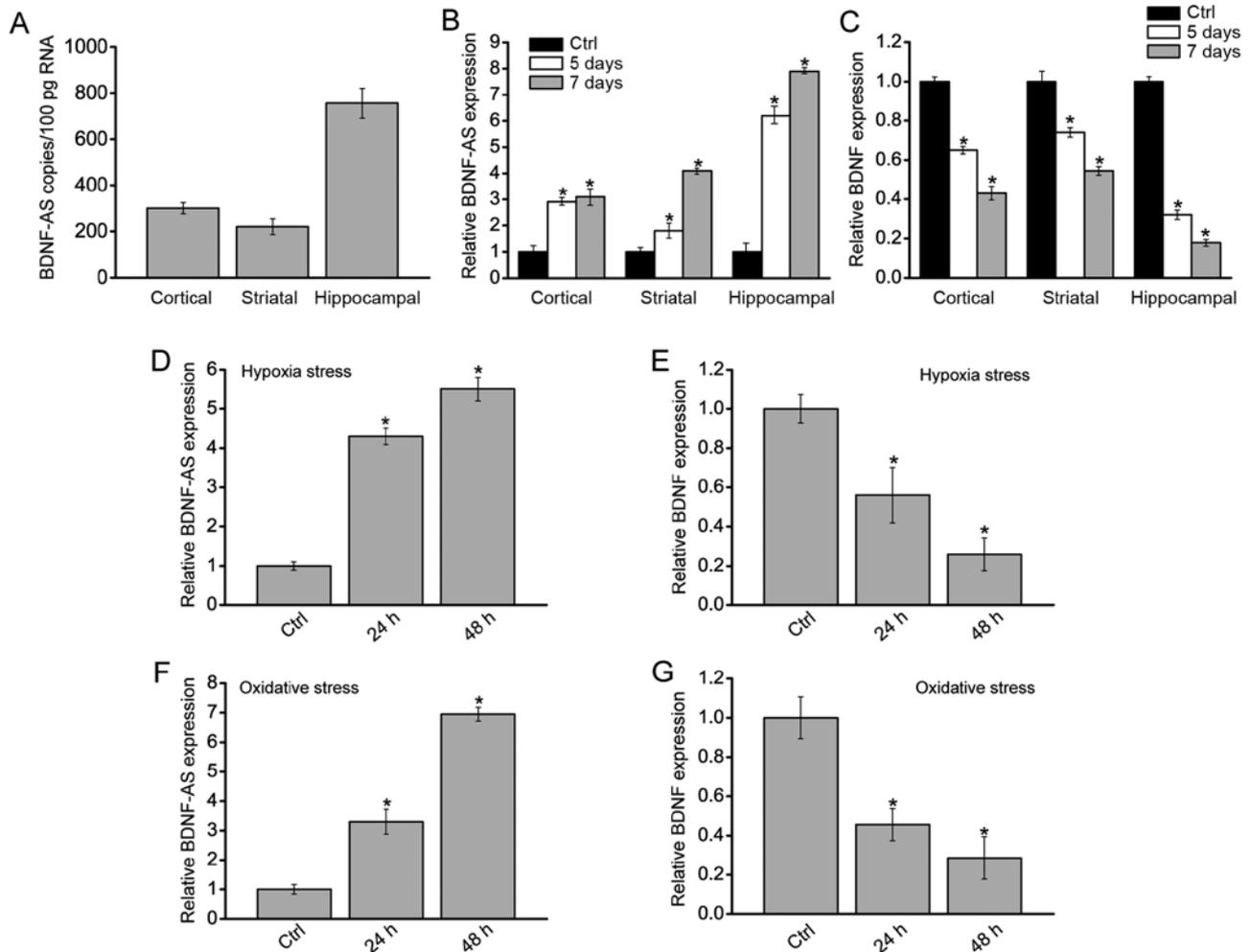


Figure 1. Detection of BDNF-AS and BDNF expression levels after hypoxic/ischemic stress *in vivo* and *in vitro*. (A) Absolute quantification of BDNF-AS expression in the striatum, hippocampi and cortex from the normal brain of P7 mice. The brains from P7 mice without any treatment were selected as the normal group. P7 mice received the unilateral common carotid artery ligation followed by exposure to 8% O<sub>2</sub> for 30 min. RT-qPCR was performed to detect (B) BDNF-AS and (C) BDNF expression levels in striatum, hippocampi and cortex after 5 and 7-day injury (n=6). Sham animals were used as the control group, and these underwent anesthesia and the common carotid artery was exposed without ligation and hypoxia. Primary hippocampal neurons were cultured in the hypoxia chamber (1% O<sub>2</sub>) for 24 and 48 h. RT-qPCR was then performed to detect (D) BDNF-AS and (E) BDNF expression levels (n=4). Primary hippocampal neurons were exposed to H<sub>2</sub>O<sub>2</sub> (150 μM) to mimic oxidative stress for 24 and 48 h. RT-qPCR was then performed to detect (F) BDNF-AS and (G) BDNF expression levels (n=4). Error bar represents the standard error of the mean. \*P<0.05 vs. Ctrl group. P7, postnatal day 7; RT-qPCR, reverse transcription-quantitative PCR; BDNF, brain-derived neurotrophic factor; AS, antisense; Ctrl, control.

usually vulnerable to oxidative stress (3). The results indicated that BDNF-AS silencing led to decreased biological activities of SOD and GPx, and reduced level of TBARS in the damaged hemispheres after HI injury (Table I).

*BDNF-AS silencing ameliorates brain neurological function in vivo.* Subsequently, whether BDNF-AS silencing was able to ameliorate brain neurological function *in vivo* was investigated. A total of 8 weeks after HIBD, the water maze experimental results demonstrated that BDNF-AS silencing did not affect the swimming distance to reach the platform in the cued learning task (Fig. 4A). However, BDNF-AS silencing improved the spatial learning ability as indicated by shorter swimming distance to reach the platform (Fig. 4B). A probe experiment was then conducted to determine the effects of BDNF-AS silencing on spatial memory. The results suggested that the animal injected with BDNF-AS shRNA did not show a preference for the target quadrant (Fig. 4C). Moreover,

BDNF-AS silencing improved the motor function during the constant and slow acceleration trials, but did not improve the motor function during the fast acceleration trials in the rotarod task (Fig. 4D).

*BDNF-AS regulates hippocampal cell function by regulating BDNF-mediated signaling.* As BDNF-AS is the antisense RNA of BDNF (16), the present study determined whether intervention of BDNF-AS expression affected the expression of BDNF. RT-qPCR results suggested that BDNF-AS overexpression significantly decreased the expression of BDNF mRNA, suggesting that BDNF-AS affected BDNF expression at the transcriptional level (Fig. 5A). It was also determined whether the intervention of BDNF-AS expression affected the activation of BDNF-mediated signaling. BDNF-AS overexpression led to decreased expression levels of BDNF, p-Akt and p-TrkB (Fig. 5B), indicating that BDNF-AS affected hippocampal cell function via BDNF-mediated signaling.

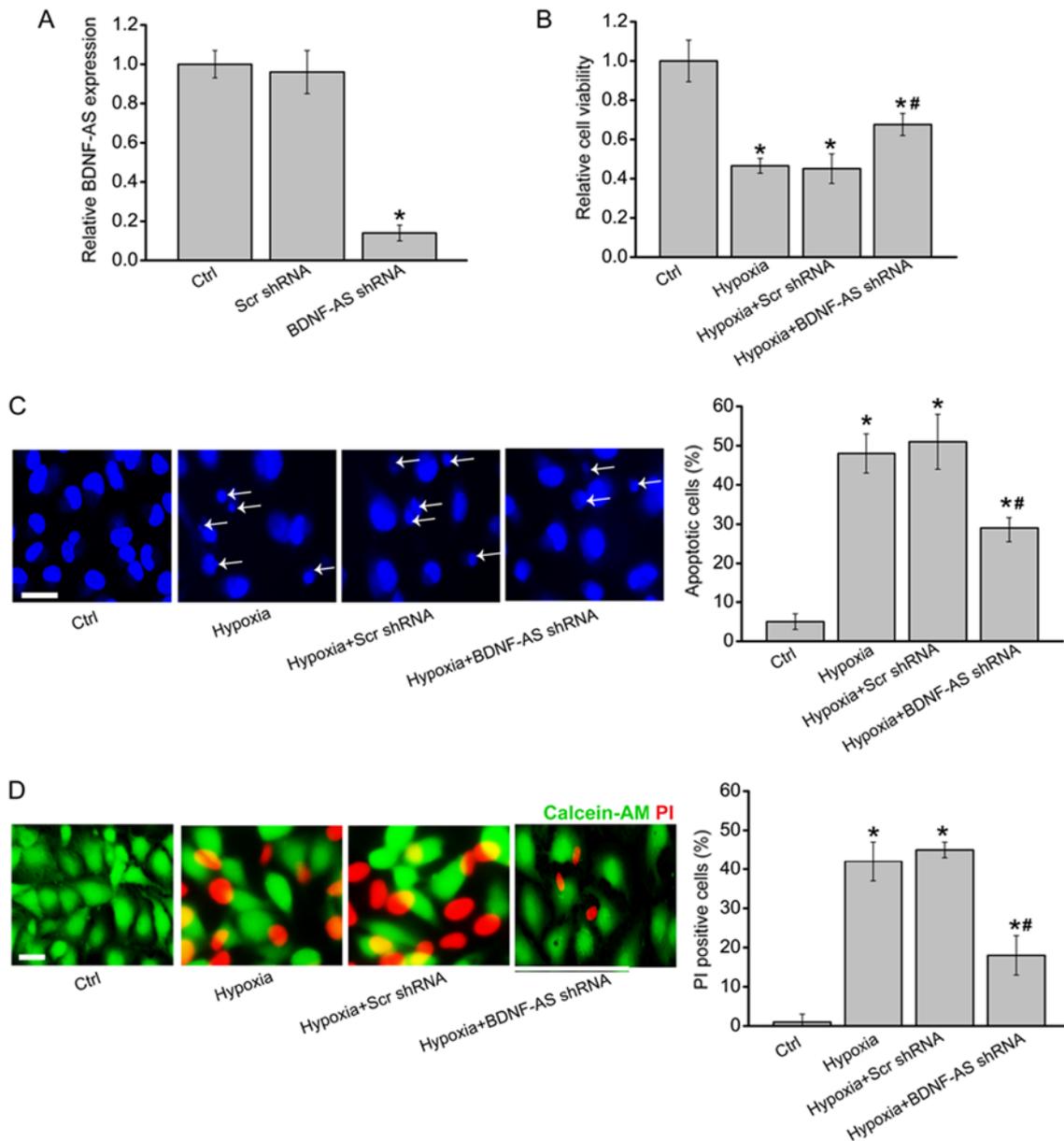


Figure 2. BDNF-AS silencing protects hippocampal cells against hypoxic stress *in vitro*. (A) Primary hippocampal cells were transfected with Scr shRNA, BDNF-AS shRNA or left untreated (Ctrl) for 48 h. Reverse transcription-quantitative PCR was performed to determine BDNF-AS expression (n=4). Primary hippocampal cells were transfected with Scr shRNA, BDNF-AS shRNA or left untreated, and then exposed to hypoxia chamber (1% O<sub>2</sub>) for 48 h. The untreated group was used as the Ctrl group. (B) Cell viability was determined by Cell Counting Kit-8 assays (n=4). (C) Apoptotic cells were detected by Hoechst staining. Scale bar, 50  $\mu$ m. Magnification, x500. Apoptotic nuclei are indicated by the white arrows (n=4). (D) Dead or dying cells were determined by Calcein-AM/PI staining. Green, live cells; Red, dead or dying cell. Scale bar, 50  $\mu$ m. Magnification, x500 (n=4). Error bars represent the standard error of the mean. \*P<0.05 vs. Ctrl group; #P<0.05 vs. hypoxia + Scr shRNA group. Scr, scrambled; shRNA, short hairpin RNA; PI, propidium iodide; BDNF, brain-derived neurotrophic factor; AS, antisense; Ctrl, control.

## Discussion

Previous studies have reported that lncRNAs are aberrantly dysregulated and play important roles in brain injury (26,27). The present study determined the role of BDNF-AS in HI-induced hippocampal cell injury *in vivo* and *in vitro*. It was found that BDNF-AS was significantly upregulated HI-induced neonatal brain injury and hippocampal cell injury. Furthermore, BDNF-AS silencing protected against HI-induced brain injury *in vivo* and primary hippocampal cell injury *in vitro*. Mechanistically, BDNF-AS affected neonatal brain injury via the inverse regulation of BDNF expression.

BDNF-AS is the antisense RNA of BDNF, which is expressed in various human tissues, and may have reciprocal neural functions to BDNF, such as supporting the survival of existing neurons, as well as encouraging growth and differentiation of new neurons and synapses (16). BDNF-AS protects local anesthetic-induced neurotoxicity in dorsal root ganglion neurons (18). Moreover, knockdown of BDNF-AS suppresses neuronal cell apoptosis in the acute spinal cord injury (28). Thus, these studies suggest that BDNF-AS regulates the apoptosis of neural cells. However, to the best of our knowledge, the current understanding of underlying role of BDNF-AS in neonatal brain injury is limited. The present results indicated that BDNF-AS

Table I. Effects of systemic BDNF-AS silencing on the activities of SOD and GPx, and TBARS levels.

Experimental group	SOD, U/mg protein	GPx, U/mg protein	TBARS, U/mg protein
Scr shRNA	4.73±0.52	0.063±0.023	1.98±0.37
BDNF-AS shRNA	2.08±0.28	0.036±0.029	1.12±0.54

SOD, superoxide dismutase; GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substance; shRNA, short hairpin RNA; Scr, scramble; BDNF, brain-derived neurotrophic factor; AS, antisense.

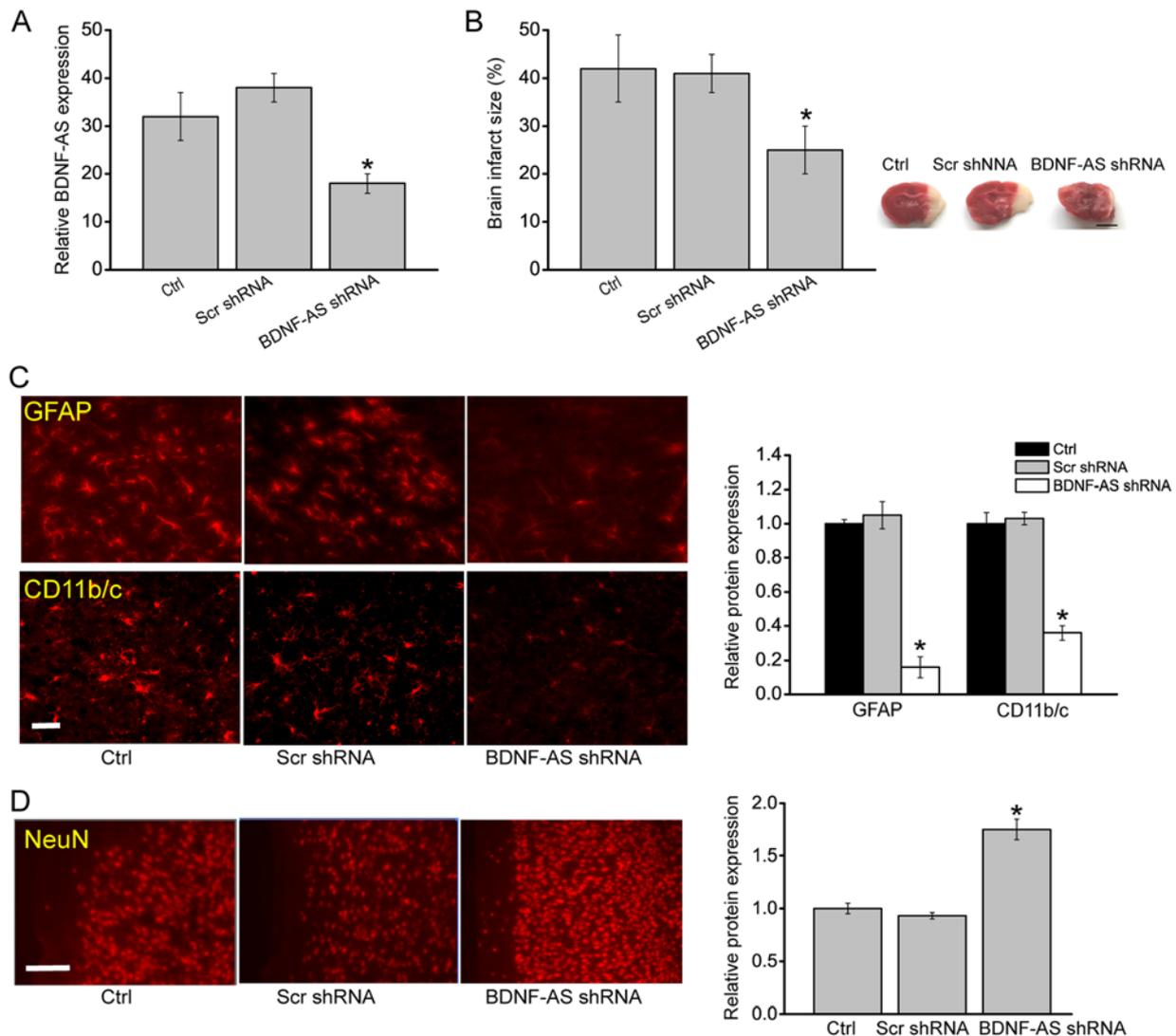


Figure 3. BDNF-AS silencing attenuates HI-induced brain injury *in vivo*. (A) Reverse transcription-quantitative PCR was performed to detect BDNF-AS expression in the hippocampus in Sham, Scr-shRNA-injected and BDNF-AS shRNA-injected C57Bl/6 pups (n=6). Sham animals were used as the Ctrl group, which underwent anesthesia and the common carotid artery was exposed without ligation and hypoxia. (B) P7 mice (C57Bl/6) received BDNF-AS shRNA or Scr shRNA prior to HI treatment, and brain infarct size was determined 48 h after HI (n=6). Scale bar, 2 mm. Immunostaining and quantification of (C) GFAP and CD11b/c, or (D) NeuN in the cornu ammonis 1 hippocampal regions of P7 mice (C57Bl/6) injected with BDNF-AS shRNA and Scr shRNA. Fig. 3C: Scale bar, 20  $\mu$ m; magnification, x200. Fig. 3D: Scale bar, 50  $\mu$ m; magnification, x500. (n=6). Error bars represent the standard error of the mean. \*P<0.05 vs. Ctrl group. HI, hypoxic/ischemic; Scr, scrambled; shRNA, short hairpin RNA; BDNF, brain-derived neurotrophic factor; AS, antisense; Ctrl, control; P7, postnatal day 7; GFAP, Glial fibrillary acidic protein; NeuN, neuronal nuclei.

knockdown protected hippocampal cells against HI-induced brain injury. The environment, together with the gene regulatory network, directs hippocampal cells to rest, proliferation, differentiate or undergo apoptosis (29). For instance, hypoxic

stress and oxidative stress are the two major pathological drivers during neonatal brain injury (3). In the present study, primary hippocampal cells were exposed to 1% oxygen or H<sub>2</sub>O<sub>2</sub> (150  $\mu$ M) to mimic hypoxic stress or oxidative stress, and it was found

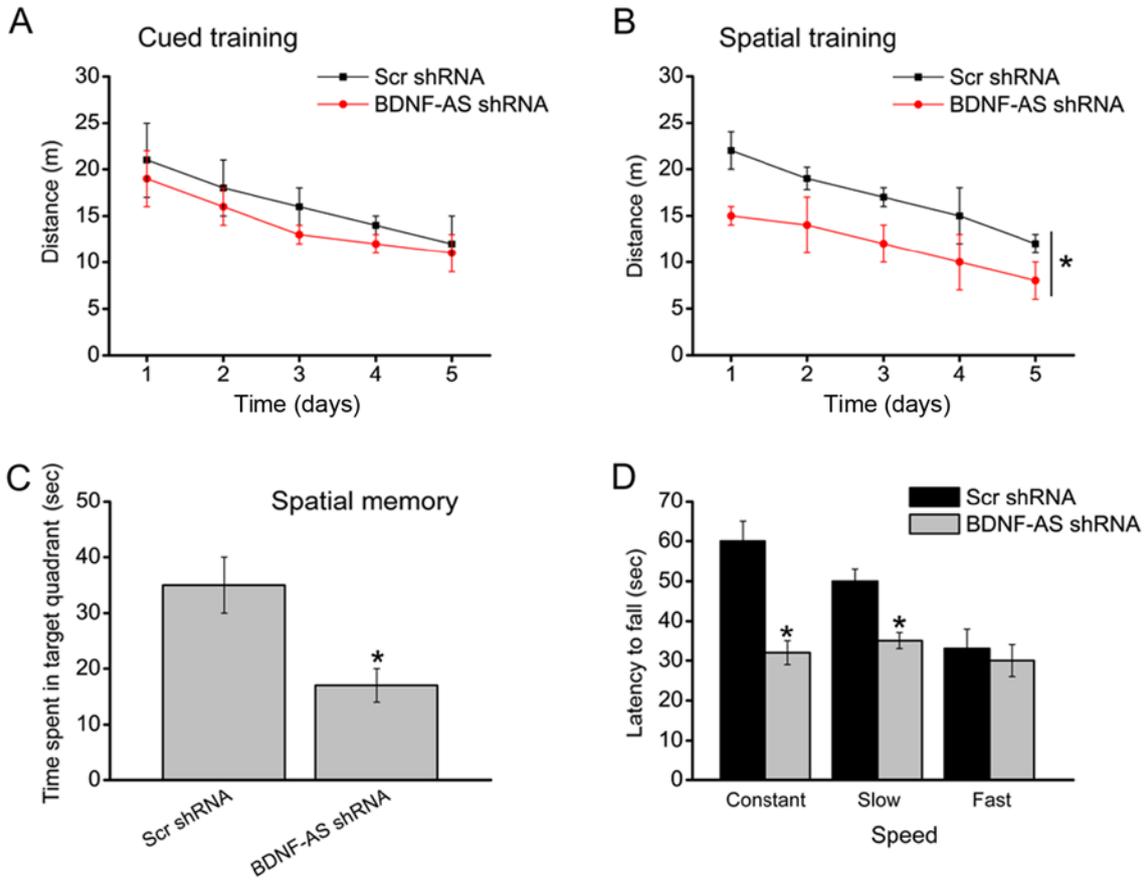


Figure 4. BDNF-AS silencing ameliorates brain neurological function *in vivo*. P7 mice (C57Bl/6) received Scr shRNA or BDNF-AS shRNA after HI treatment, and neurological function was evaluated 8 weeks after HI. Water maze tests of (A) cued training, (B) spatial leaning and (C) spatial memory. (D) Rotarod tests (n=6). Error bars represent the standard error of the mean. \*P<0.05 vs. Scr shRNA. HI, hypoxic/ischemic; Scr, scrambled; shRNA, short hairpin RNA; BDNF, brain-derived neurotrophic factor; AS, antisense; P7, postnatal day 7.

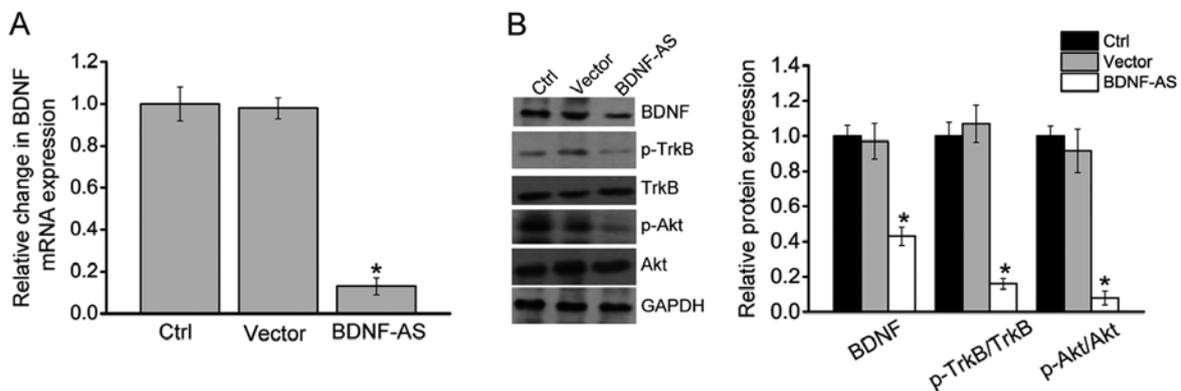


Figure 5. BDNF-AS regulates hippocampal cell function by regulating BDNF-mediated signaling. Hippocampal cells were transfected with pcDNA 3.0 (vector), pcDNA3.0- BDNF-AS or left untreated (Ctrl). (A) Reverse transcription-quantitative PCR was conducted to detect BDNF mRNA expression (n=4). (B) Western blotting was performed to detect the expression levels of BDNF, p-Akt, Akt, TrkB and p-TrkB (n=4). Error bars represent the standard error of the mean. \*P<0.05 vs. Ctrl group. Scr, scrambled; shRNA, short hairpin RNA; BDNF, brain-derived neurotrophic factor; AS, antisense; Ctrl, control; p-, phosphorylated; TrkB, tropomyosin receptor kinase B; OE, overexpression.

that hypoxic and oxidative stress led to increased expression of BDNF-AS. Thus, BDNF-AS may direct hippocampal cells to adapt hypoxic or ischemic conditions by regulating oxidative stress or hypoxic stress-responsive genes.

BDNF is important for neuronal proliferation, maturation, differentiation and maintenance (14). Furthermore, BDNF synchronizes neuronal and glial maturation and enhances

neuronal cell survival (30). BDNF upregulation is speculated to have beneficial effects in a number of neurological disorders, such as Alzheimer's disease, Huntington's disease and Parkinson's disease (31). Since BDNF-AS is transcribed oppositely by the BDNF gene, the present study investigated whether BDNF-AS regulates BDNF expression in hippocampal cells. BDNF-AS overexpression decreased the expression of BDNF

mRNA. Moreover, BDNF-AS knockdown affected the activation of the BDNF-mediated signaling pathway as indicated by decreased expression levels of BDNF, p-Akt and p-TrkB. Therefore, the present results suggested that BDNF-AS knockdown induced the activation of the BDNF/TrkB/PI3K/Akt signaling pathway after HI-induced neurotoxicity (32).

The neonatal brain is usually characterized by a low concentration of anti-oxidants and high level of oxygen consumption, which is why the neonatal brain is vulnerable to oxidative stress injury (33). As a result, it is beneficial to decrease oxidative damage and increase the anti-oxidant defense during neonatal brain injury. The present results indicated that BDNF-AS knockdown affected the activities of anti-oxidant enzymes, SOD and GPx, and the level of TBARS, which is a lipid peroxidation index (34). In addition, newborns and premature babies experience free radical oxidative injury (35). Thus, it was demonstrated that BDNF-AS silencing plays a neuroprotective role in HI brain injury at least partially via the modulation of anti-oxidant enzyme activity.

In conclusion, the present study identified a potential role of BDNF-AS in the pathogenesis of HI-induced neonatal brain injury and its underlying molecular mechanism. It was demonstrated that BDNF-AS knockdown could improve brain function by reducing the infarct size and improving the neurological function, suggesting that BDNF-AS may be a promising target for the treatment of HIBD.

#### Acknowledgements

The authors would like to thank Dr Li-Hua Zhu (Jiangsu Health Vocational College) for the helpful discussion for paper revision.

#### Funding

The present study was supported by the Project of Clinical Advanced Techniques, Primary Research & Development Plan of Jiangsu Province (grant no. BE2017719), the Pediatric Medical Innovation Team of Jiangsu Province (grant no. CXTDA2017022), the Project of National Youth Found (grant no. 81601355) and the Project of Postdoctoral Fund of Jiangsu Province (grant no. 1701162C).

#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

RBZ and ZKX were the major contributors to the experimental design. RBZ established the animal models. LXQ, MFW and LHZ performed the western blot analysis and cell culture. RBZ and ZKX were involved in writing the manuscript, the analysis and interpretation of data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All experimental procedures adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals

(updated 2011; National Institutes of Health) and were approved by the Animal Care and the Use Committee of Nanjing University.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

- Zhu C, Kang W, Xu F, Cheng X, Zhang Z, Jia L, Ji L, Guo X, Xiong H, Simbruner G, *et al*: Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. *Pediatrics* 124: e218-e226, 2009.
- Rocha-Ferreira E and Hristova M: Antimicrobial peptides and complement in neonatal hypoxia-ischemia induced brain damage. *Front Immunol* 6: 56, 2015.
- Johnston MV, Trescher WH, Ishida A and Nakajima W: Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatr Res* 49: 735-741, 2001.
- Blackmon LR and Stark AR; American Academy of Pediatrics Committee on Fetus and Newborn: Hypothermia: A neuroprotective therapy for neonatal hypoxic-ischemic encephalopathy. *Pediatrics* 117: 942-948, 2006.
- Tagin MA, Woolcott CG, Vincer MJ, Whyte RK and Stinson DA: Hypothermia for neonatal hypoxic ischemic encephalopathy: An updated systematic review and meta-analysis. *Arch Pediat Adol Med* 166: 558-566, 2012.
- Quinn JJ and Chang HY: Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 17: 47-62, 2016.
- Mercer TR, Dinger ME and Mattick JS: Long non-coding RNAs: Insights into functions. *Nat Rev Genet* 10: 155-159, 2009.
- Fatica A and Bozzoni I: Long non-coding RNAs: New players in cell differentiation and development. *Nat Rev Genet* 15: 7-21, 2014.
- Zhang J, Yuan L, Zhang X, Hamblin MH, Zhu T, Meng F, Li Y, Chen YE and Yin KJ: Altered long non-coding RNA transcriptomic profiles in brain microvascular endothelium after cerebral ischemia. *Exp Neurol* 277: 162-170, 2016.
- Zhao F, Qu Y, Liu J, Liu H, Zhang L, Feng Y, Wang H, Gan J, Lu R and Mu D: Microarray profiling and co-expression network analysis of lncRNAs and mRNAs in neonatal rats following hypoxic-ischemic brain damage. *Sci Rep* 5: 13850, 2015.
- Mehta SL, Kim T and Vemuganti R: Long noncoding RNA FosDT promotes ischemic brain injury by interacting with REST-associated chromatin-modifying proteins. *J Neurosci* 35: 16443-16449, 2015.
- Zhao RB, Zhu LH, Shu JP, Qiao LX and Xia ZK: GAS5 silencing protects against hypoxia/ischemia-induced neonatal brain injury. *Biochem Biophys Res Commun* 497: 285-291, 2018.
- Zhang X, Tang X, Liu K, Hamblin MH and Yin KJ: Long noncoding RNA Malat1 regulates cerebrovascular pathologies in ischemic stroke. *J Neurosci* 37: 1797-1806, 2017.
- Rossi C, Angelucci A, Costantin L, Braschi C, Mazzantini M, Babbini F, Fabbri ME, Tessarollo L, Maffei L, Berardi N and Caleo M: Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur J Neurosci* 24: 1850-1856, 2006.
- Angelucci F, Mathe AA and Aloe L: Neurotrophic factors and CNS disorders: Findings in rodent models of depression and schizophrenia. *Prog Brain Res* 146: 151-165, 2004.
- Li Y, Xu F, Xiao H and Han F: Long noncoding RNA BDNF-AS inversely regulated BDNF and modulated high-glucose induced apoptosis in human retinal pigment epithelial cells. *J Cell Biochem* 119: 817-823, 2018.
- Zheng X, Lin C, Li Y, Ye J, Zhou J and Guo P: Long noncoding RNA BDNF-AS regulates ketamine-induced neurotoxicity in neural stem cell derived neurons. *Biomed Pharmacother* 82: 722-728, 2016.
- Zhang Y, Yan L, Cao Y, Kong G and Lin C: Long noncoding RNA BDNF-AS protects local anesthetic induced neurotoxicity in dorsal root ganglion neurons. *Biomed Pharmacother* 80: 207-212, 2016.

19. Guo CC, Jiao CH and Gao ZM: Silencing of lncRNA BDNF-AS attenuates A $\beta$ 25-35-induced neurotoxicity in PC12 cells by suppressing cell apoptosis and oxidative stress. *Neurol Res* 40: 795-804, 2018.
20. Cetin A, Komai S, Eliava M, Seeburg PH and Osten P: Stereotaxic gene delivery in the rodent brain. *Nat Protoc* 1: 3166-3173, 2006.
21. Klaunberg BA, O'malley J, Clark T and Davis JA: Euthanasia of mouse fetuses and neonates. *Contemp Top Lab Anim Sci* 43: 29-34, 2004.
22. Artwohl J, Brown P, Corning B and Stein S: Report of the ACLAM task force on rodent euthanasia. *J Am Assoc Lab Anim Sci* 45: 98-105, 2006.
23. Kramer M, Dang J, Baertling F, Denecke B, Clarner T, Kirsch C, Beyer C and Kipp M: TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses. *J Neurosci Methods* 187: 84-89, 2010.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
25. Merritt JR and Rhodes JS: Mouse genetic differences in voluntary wheel running, adult hippocampal neurogenesis and learning on the multi-strain-adapted plus water maze. *Behav Brain Res* 280: 62-71, 2015.
26. Yin KJ, Hamblin M and Chen YE: Non-coding RNAs in cerebral endothelial pathophysiology: Emerging roles in stroke. *Neurochem Int* 77: 9-16, 2014.
27. Xin JW and Jiang YG: Long noncoding RNA MALAT1 inhibits apoptosis induced by oxygen-glucose deprivation and reoxygenation in human brain microvascular endothelial cells. *Exp Ther Med* 13: 1225-1234, 2017.
28. Zhang H, Li D, Zhang Y, Li J, Ma S, Zhang J, Xiong Y, Wang W, Li N and Xia L: Knockdown of lncRNA BDNF-AS suppresses neuronal cell apoptosis via downregulating miR-130b-5p target gene PRDM5 in acute spinal cord injury. *RNA Biol* 15: 1071-1080, 2018.
29. Hagg T: Molecular regulation of adult CNS neurogenesis: An integrated view. *Trends Neurosci* 28: 589-595, 2005.
30. Waterhouse EG, An JJ, Orefice LL, Baydyuk M, Liao GY, Zheng K, Lu B and Xu B: BDNF promotes differentiation and maturation of adult-born neurons through GABAergic transmission. *J Neurosci* 32: 14318-14330, 2012.
31. Lu B, Nagappan G and Lu Y: BDNF and synaptic plasticity, cognitive function, and dysfunction. *Handb Exp Pharmacol* 220: 223-250, 2014.
32. Yoshii A and Constantine-Paton M: Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Dev Neurobiol* 70: 304-322, 2010.
33. Vannucci SJ and Hagberg H: Hypoxia-ischemia in the immature brain. *J Exp Biol* 207: 3149-3154, 2004.
34. Kumral A, Gonenc S, Acikgoz O, Sonmez A, Genc K, Yilmaz O, Gokmen N, Duman N and Ozkan H: Erythropoietin increases glutathione peroxidase enzyme activity and decreases lipid peroxidation levels in hypoxic-ischemic brain injury in neonatal rats. *Biol Neonate* 87: 15-18, 2005.
35. Vexler ZS and Ferriero DM: Molecular and biochemical mechanisms of perinatal brain injury. *Semin Neonatol* 6: 99-108, 2001.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.