Brain-derived neurotrophic factor and its related enzymes and receptors play important roles after hypoxic-ischemic brain damage

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Abstract
Brain-derived neurotrophic factor (BDNF) regulates many neurological functions and plays a vital role during the recovery from central nervous system injuries. However, the changes in BDNF expression and associated factors following hypoxia-ischemia induced neonatal brain damage, and the significance of these changes are not fully understood. In the present study, a rat model of hypoxic-ischemic brain damage was established through the occlusion of the right common carotid artery, followed by 2 hours in a hypoxic-ischemic environment. Rats with hypoxic-ischemic brain damage presented deficits in both sensory and motor functions, and obvious pathological changes could be detected in brain tissues. The mRNA expression levels of BDNF and its processing enzymes and receptors (Furin, matrix metallopeptidase 9, tissue-type plasminogen activator, tyrosine Kinase receptor B, plasminogen activator inhibitor-1, and Sortilin) were upregulated in the ipsilateral hippocampus and cerebral cortex 6 hours after injury; however, the expression levels of these mRNAs were found to be downregulated in the contralateral hippocampus and cerebral cortex. These findings suggest that BDNF and its processing enzymes and receptors may play important roles in the pathogenesis and recovery from neonatal hypoxic-ischemic brain damage. This study was approved by the Animal Ethics Committee of the University of South Australia (approval No. U12-18) on July 30, 2018.

Key Words: brain injury; brain-derived neurotrophic factor; enzyme; hypoxia-ischemia; receptors; recovery; repair

Introduction
Hypoxic-ischemic (HI) brain damage, which typically results from ischemia and hypoxia in the brain, can impact neuronal development and result in lifelong abnormal function (Sun et al., 2018). Neonatal HI encephalopathy represents a major cause of neonatal morbidity and mortality (Pimentel-Coelho and Mendez-Otero, 2010), often inducing severe asphyxia, seizures, and other severe neurological deficits in children. In addition, HI encephalopathy may also be associated with neurodevelopmental abnormalities, such as learning disorders and mental retardation (Gunn and Thoresen, 2019). Approximately 25% of HI encephalopathy infants have been
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reported to die during their first year, whereas those who survive present with permanent neurological disabilities (Logothetajah et al., 2009). An increasing number of studies have targeted neonatal HI encephalopathy treatments over the years.

Brain-derived neurotrophic factor (BDNF) has been considered to serve as a potent modulator of synaptic plasticity and is capable of regulating a wide range of brain functions, including motor functions, memory, and learning (Chen et al., 2013; Bae et al., 2020), which could promote the survival and growth of neurons in both the peripheral and central nervous systems (Zuccato et al., 2008; Lopes et al., 2017). BDNF, which is ubiquitously expressed in both the developing and adult mammalian brain, is synthesized in several regions of the hypothalamus and is expressed in almost all cortical regions (Tapia-Arancibia et al., 2004; Yu and Chen, 2011). Previous studies have shown a correlation between the early upregulation of BDNF and its neuroprotective effects after surgery using various ischemic models (Comelli et al., 1992; Rickhag et al., 2007; Madinier et al., 2013). However, studies examining the effects of BDNF expression levels following cerebral HI injury have been very limited. Thus, in this study, an HI injury model was established in neonatal rats to investigate changes in the expression levels of BDNF and its related enzymes and receptors.

Materials and Methods

Animals

Postnatal day 7 Sprague-Dawley (SD) rats (14–16 g, without gender selection) and their mothers were purchased from the Animal Center of the University of South Australia (UNISA) and were accommodated in a warm environment for the following operation. All procedures performed during this study were approved by the Animal Ethics Committee of the University of South Australia (approval No. U12-18) on July 30, 2018, and were in accordance with the Guide for the Care and Use of Laboratory Animals of National Institute of Health.

HI injury model establishment

Seven-day-old neonatal rats were selected and anesthetized by isoflurane (RWD Life Science, Shenzhen, Guangdong Province, China) inhalation (4% for induction, 2% for sustained inhalation anesthesia). Briefly, the midline of the ventral cervical skin was incised, followed by the blunt dissection of parenchyma to expose the right common carotid. Subsequently, the right common carotid was ligated using a monopolar microsurgery electrocoagulator (Chunguang Medical Cosmetology Instrument Co., Ltd., Wuhan, Hubei Province, China). Then, the subcutaneous tissues and skin were sutured, and the animals were returned to their mother for 1 hour before being moved to a hypoxic chamber on their own, containing 8% O₂ and 92% N₂ (air-flow rate maintained at 1 L/min) for 2 hours. Rats in the sham group underwent the same procedures without ligation of the right carotid artery. Animals were grouped randomly, as shown in Table 1.

| Table 1 | Animal grouping |
|-----------------|------------------|
| Experiments                               | Sample size (rats, N = 54) |
| 2,3,5-Triphenyltetrazolium chloride staining | n = 3/group (sham, HI) |
| Hematoxylin-eosin staining                 | n = 3/group (sham, HI) |
| Nissl staining                             | n = 3/group (sham, HI) |
| Geotaxis, Righting, and Climbing tests     | n = 7/group (sham, 6 h, 15 h, 24 h, 3 d, 7 d) |
| Quantitative real-time polymerase chain reaction | Western blot assay |

Zea-Longa score

The neurological functions of HI group rats were evaluated using the Zea-Longa score. The neurological scores of the rats were recorded at 6, 15, and 24 hours, and 3 and 7 days after HI. The Zea-Longa score was also used to determine that HI injury models were produced successfully. The 5-point Zea-Longa grading criteria are as follows (Longa et al., 1989): 0, no signs of nerve injury; 1, loss of the ability to fully stretch the contra-lateral forepaw; 2, the animal turns to one side while walking; 3, walking is unstable, the animal falls to one side; and 4, the animal is unable to walk or experiences a loss of consciousness.

Geotaxis test

The geotaxis test was used to assess motor coordination and vestibular sensitivity in neonatal rats 7 days after HI injury (Baharnoori et al., 2012; Ragaeva et al., 2017). The rats were placed in a 45° inclined grid, in a head downwards position, for 5 seconds. The grid provides a surface that can be gripped, allowing for the rodent to reorient itself towards an upwards position. After the rats were released, the time taken to reorient themselves was recorded. Each trial lasted for a maximum of 2 minutes, and each rat performed three trials.

Righting test

The righting test was used to evaluate the ability of newborn rats to roll out of the supine position, 7 days after HI injury (Li et al., 2019). The rats were placed on their backs on a cotton sheet and held in position for 5 seconds. After they were released, the direction in which they rolled to right themselves (left or right) and the time required to right themselves to a prone position was recorded. Each trial lasted for a maximum of 1 minute, and each rat performed three trials.

Climbing test

The climbing test was performed 7 days after HI. Rats were placed on a pad with a 45° slope, on which a line was drawn. The time taken for rats to climb across the line, with their heads facing downwards, was recorded by the researchers (Ennaceur et al., 2017). Each rat performed three independent trials.

Tissue collection

For morphological analysis, rats in the sham (n = 3 for 2,3,5-triphenyltetrazolium chloride (TTC) staining, n = 3 for hematoxylin-eosin (HE) and Nissl staining) and HI (n = 3 for TTC staining, n = 3 for HE and Nissl staining) groups (Table 1) were euthanized 24 hours after HI, under deep anesthesia using 4% isoflurane (sustained inhalation anesthesia) for 2 minutes. After the perfusion of 0.9% normal saline, followed by perfusion with 4% paraformaldehyde, the brain was harvested and incubated in 4% paraformaldehyde for more than 72 hours. After paraffin embedding, 5 µm brain sections were prepared for HE and Nissl staining, and 2 mm sections were prepared for TTC staining. For quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assays, neuronal rats were sacrificed at 6 (n = 7), 15 (n = 7), and 24 hours (n = 7), and 3 (n = 7) and 7 days (n = 7) after surgery (Table 1), under deep anesthesia conditions induced by continuous inhalation of 4% isoflurane for 2 minutes, followed by perfusion with 0.9% normal saline. The cortex was removed and stored at −80°C. In accordance with previously described coordinates (Paxinos and Watson, 1998), two incisions, approximately 0.7 mm deep, were made to the cerebral cortex at the ends of both hemispheres and 1.5–2 mm away from the ends of both hemispheres, along the ventral side of the brain to expose the hippocampus. Then, the hippocampus was separated from the surrounding tissues along the dorsal side, towards the ventral side. The entire ipsilateral and contra-lateral hippocampus were collected in a 1.5 mL centrifuge tube and stored for the later use.
TTC staining

To evaluate brain damage following HI injury in neonatal rats, TTC staining was performed to observe the infarction of brain tissues. Whole brains from rats in the sham and HI groups were quickly removed, 16 hours after rats were deeply anesthetized with 4% isoflurane, and brain tissues were subsequently removed (operating on ice) and placed in a refrigerator (Haier Group, Qingdao, Shandong Province, China) at −20°C for 10 minutes, before being cut into five coronal pieces (2 mm each). Afterward, the sections were incubated in 0.5% TTC solution (ServiceBio, Wuhan, Hubei Province, China) at 37°C for 30 minutes. The sections were then imaged using a digital camera (Huawei, Shenzhen, Guangdong Province, China). The infarction ratio (%) was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA) and was calculated as follows: (contralateral area – ipsilateral non-infarction area)/contralateral area × 100.

HE staining

The harvested cortical and hippocampal tissues were embedded in paraffin after being transparentized with xylene and dehydrated and were cut into 5 µm sections. After dewaxing and hydrating, the sections were soaked in hematoxylin for 1 minute, which resulted in tissues turning blue, and were then dehydrated and transparentized by xylene, and dehydrated and cut into 5 µm sections. The harvested cortical and hippocampal tissues were sealed with neutral gum. Images of the damaged cortex and hippocampal CA2 (0.3 mm²) were taken using a light microscope (CX40; Shunyu, Ningbo, Zhejiang Province, China).

Nissl staining

Paraffin-embedded tissues, after being sectioned and deparaffinized, were then stained with 30 µL of 1% cresyl violet solution (Solarbio, Beijing, China) in a wet incubator for 9 minutes. After washing with distilled water, the Nissl differentiation solution was added to the sections for 2 minutes. Subsequently, 95% ethanol was added for swift differentiation until Nissl bodies were purple, and the other tissues were colorless. Finally, specimens were dehydrated by absolute ethyl alcohol, transparentized by xylene, and sealed with neutral gum. Images of the damaged cortex and hippocampal CA2 (0.3 mm²) were taken using a light microscope (Leica) at 40× and 200× magnification.

qRT-PCR

Cortical and hippocampal tissues were collected, and total RNA was extracted using RNAiso plus (TaKaRa Bio Inc., Otsu, Japan). The concentration and purity of total RNA were determined using a microplate reader (BioTek, Shanghai, China), which was then reverse transcribed into complementary DNA using the Revert Aid™ First Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers were designed for the detected factors using Primer 5.0 software (Premier, San Francisco, CA, USA), as shown in Table 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Quantitative PCR reactions were performed using the QuantNova SYBR Green PCR Kit (QIAGEN, Louisville, KY, USA) according to the manufacturer’s instructions. The expression level of each gene was normalized to that for GAPDH using the 2-ΔΔCt method (Phan et al., 2018).

Western blot assay

The ipsilateral cortex and hippocampus from rats were collected and lysed with radiomunoprecipitation assay buffer containing a proteinase inhibitor cocktail (Roche Life Science, Shanghai, China) on ice for 30 minutes. The lysates were centrifuged at 12,000 r/min for 10 minutes at 4°C. Protein concentrations were determined by bicinchoninic acid protein quantification kit (Beyotime, Shanghai, China). The protein samples were boiled and denatured with a loading buffer containing a proteinase inhibitor cocktail (Roche Life Science, Shanghai, China). Western blot analysis was performed using a microplate reader (BioTek, Shanghai, China), and 40 µg of protein was run at 120 V for 90 minutes. The protein in the gel was transferred to a polyvinylidene difluoride membrane using 200 mA current. The polyvinylidene difluoride membrane was then blocked with 5% skim milk, which was prepared by dissolving 2.5 g skim milk powder in 50 mL phosphate buffered saline with Tween-20. After blocking for 90 minutes at room temperature, membranes were further incubated with a primary antibody against BDNF (rabbit; 1:500; Cat# bs-0248RB; BIORSS, Beijing, China) at 4°C overnight. The membranes were then incubated with secondary antibodies for 2 hours at room temperature (anti-rabbit; 1:100; Cat# Ba0565-1; Boster, Wuhan, Hubei Province, China). β-Actin was used as an internal control. Finally, the membranes were developed, and the bands were visualized using an enhanced chemiluminescent solution (Bio-Rad, San Francisco, CA, USA), as shown in Figure 1A. The expression levels of the detected factors were analyzed by two-tailed Student’s t-test for the comparison of neuronal counts between the sham and HI groups, in addition to comparisons of the behavioral evaluations between the two groups. A repeated-measures analysis of variance was used for the detected factors using Primer 5.0 software (Premier, San Francisco, CA, USA).

Table 2 | Primer sequences used for quantitative real-time polymerase chain reaction

| Gene     | Forward sequences | Reverse sequences |
|----------|------------------|------------------|
| BDNF     | 5'-GGT GTC GTA AGG TTC CAC T3' | 5'-GCG AAG TTT CCT TCG C3' |
| MMP-9    | 5'-GCC CCT ACT GCT GGT CTT-3' | 5'-TTG GCT TCC TGG ATT-3' |
| Furin    | 5'-GGC AAC CAG AAT GAG AAG CA-3' | 5'-ACA GGC CGT AGC CAT AGG AA-3' |
| tPA      | 5'-CTT TGT GGA GTG GCG TTC A-3' | 5'-CCC CAT TTT CTG TGC T3' |
| Sortilin | 5'-CAA ATG GAC ACC AAA CAA-3' | 5'-AGA GGC GAA GAG GAA ACG-3' |
| TrkB     | 5'-AAC CTC ACT GTG CAT TTT-3' | 5'-GGT GCA TCG TGA ATG-3' |
| PAI-1    | 5'-GCA GTA CAA AAG TTC A-3' | 5'-AAC CAC AAA GAG GAA A-3' |
| GAPDH    | 5'-CGT CAT TGT CAG CAA T-3' | 5'-CCA TCA ACA GTC TTC TGA GT-3' |

BDNF: Brain-derived neurotrophic factor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP-9: matrix metallopeptidase 9; PAI-1: plasminogen activator inhibitor 1; tPA: plasminogen activator, tissue type.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA), and results are expressed as the mean ± standard deviation (SD). The data between two groups were analyzed by two-tailed Student’s t-test for the comparison of neuronal counts between the sham and HI groups, in addition to comparisons of the behavioral evaluations between the two groups. A repeated-measures analysis of variance was used for the detected factors using Primer 5.0 software (Premier, San Francisco, CA, USA). The results were expressed as an optical density ratio between the protein of interest and β-actin.

Results

HI injury induces brain damage and neurological dysfunction

TTC staining showed clear cerebral infarctions on the right sides of the brains in the HI group compared with those in the sham group (Figure 1A). The infarction rate of the HI group was significantly higher than that of the sham group (P < 0.001; Figure 1B). HE staining was examined to observe morphological variations in neurons in neonatal rats after HI injury. More cell cavities were observed in the HI group compared with the sham group, and the cell nuclei were compressed into the sides in the cortex in the HI group (Figure 1C). Nissl staining further confirmed HI-induced neuronal loss, as indicated by a significant decrease in total neurons in the cortex (P < 0.001) and the hippocampus (P < 0.001), as well as an increased number of dead neurons in the cortex (P < 0.01; Figure 1D, F, and G) and hippocampus (P < 0.001;
Changes in the mRNA and protein expression levels of BDNF in the cortex and hippocampus of HI rats

BDNF mRNA levels in the ipsilateral cortex increased in rats in the HI group compared with those in the sham group 6 hours after surgery ($P < 0.01$), but the levels were reduced at 15 and 24 hours relative to that at 6 hours, followed by a gradual increase over the next two days ($P < 0.01$). The mRNA expression levels of BDNF in the contralateral cortex decreased 6 hours after HI injury and remained at a low level compared with the levels in the ipsilateral hemisphere at the same time points ($P < 0.001$; Figure 2A). The mRNA expression level of BDNF in the ipsilateral hippocampus was increased at 6 hours and 3 days in the HI group compared with that in the sham group ($P < 0.001$). The mRNA levels of BDNF in the contralateral hippocampus were higher than those in the ipsilateral hemisphere at each time point ($P < 0.001$; Figure 2B). The protein expression levels of BDNF in the ipsilateral cortex exhibited marked reductions at 15 and 24 hours compared with the same time points in the sham group ($P < 0.005$; Figure 2C), whereas the protein levels in the ipsilateral hippocampus were significantly diminished at 24 hours relative to those in the sham group ($P < 0.05$; Figure 2D).

Changes in the mRNA expression level of BDNF-related enzymes and receptors in the cortex and hippocampus of HI rats

The mRNA expression level of *Furin* [a precursor of the BDNF converting enzyme (Zhu et al., 2018)] in the ipsilateral cortex of rats in the HI group increased at 6 hours compared with the sham rats, then decreased at 15 hours and was sustained at a low level for 7 days relative to that observed at 6 hours ($P < 0.05$; Figure 3A). *Furin* mRNA expression in the ipsilateral hippocampus of rats in the HI group was elevated at 6 hours and 3 days compared with that in the sham group ($P < 0.05$) and was lower than the expression levels observed in the contralateral hemisphere of rats in the HI group within 24 hours ($P < 0.001$; Figure 3B). Similarly, the mRNA expression level of matrix metallopeptidase 9 (MMP-9, an extracellular converting enzyme (Cai et al., 2017)] in the ipsilateral cortex was significantly increased at 6 and 24 hours ($P < 0.01$; Figure 3C), compared with those in the sham group, whereas the mRNA expression levels of MMP-9 in the ipsilateral hippocampus were notably depressed at 6 ($P < 0.001$) and 15 hours ($P < 0.01$) compared with those in the sham group (Figure 3D). The mRNA expression levels of *Furin* and MMP-9 were both increased at 6 hours in the contralateral hippocampus compared with those in the sham group ($P < 0.01$ for *Furin* and $P < 0.001$ for MMP-9).

The mRNA expression levels of plasminogen activator, tissue type [tPA, a precursor of BDNF converting enzyme (Rahman et al., 2018)] in the ipsilateral cortex were significantly increased at 6 hours compared with sham group ($P < 0.001$), followed by a gradual reduction and sustained lower levels at 15 hours and 24 days ($P < 0.01$; Figure 3E) relative to levels at 6 hours. In the hippocampus, the tPA mRNA expression level in the ipsilateral hemisphere was markedly elevated at 6 hours compared with that in the sham group ($P < 0.01$; Figure 3F). Similarly, the mRNA expression level of tissue plasminogen activator type [tPA, a precursor of BDNF converting enzyme (Zhu et al., 2018)] in the ipsilateral cortex was increased at 6 hours compared with that in the sham group ($P < 0.01$; Figure 3G), and the TrkB mRNA expression levels in the ipsilateral hippocampus were significantly increased at 6 ($P < 0.001$ for *Furin* and $P < 0.001$ for MMP-9).

**Figure 1** | Effects of HI injury on brain damage and neurological dysfunction.

(A) 2,3,5-Triphenyltetrazolium chloride-stained images of brains from rats in the sham and HI groups at 24 hours post-HI injury. The non-ischemic tissue appears red, and the ischemic tissue appears white. Brain tissues in the HI group were obviously infarcted, whereas those in the sham group did not exhibit changes. (B) Quantitative evaluation of the infarction rate. (C) The morphology of cells in the cortex by hematoxylin-eosin staining. The number of vacuoles in the HI group increased relative to the sham group. The white arrows represent normal cells, and the red arrows represent vacuoles. (D, E) The morphology of neurons in the cortex (D) and hippocampus (E) by Nissl staining. The number of dead neurons in the HI group was greater than that in the sham group. Scale bars: 1 cm in A, 50 μm in C, and 100 μm in D. (F, G) Quantitative results of total neurons and dark neurons (dead neurons) in the cortex and hippocampus, as detected by Nissl staining. (H) Quantitative evaluation of the Zea-Longa scores. (I–K) Quantitative evaluations of geotaxis (I), righting (J), and climbing test (K) results 7 days after HI insult. Data are expressed as the mean ± SD (n = 7/group). **$P < 0.01$, ***$P < 0.001$ (two-tailed Student’s t-test). HI: Hypoxia-ischemia.
Table 1

| Sample          | Treatment       | mRNA Expression | Protein Expression |
|-----------------|-----------------|-----------------|-------------------|
| Cortex-contralateral | Hypoxia-ischemia | Increased       | Increased         |
| Cortex-ipsilateral | Hypoxia-ischemia | No change       | Decreased         |
| Hippocampus-contralateral | Hypoxia-ischemia | No change       | Decreased         |
| Hippocampus-ipsilateral | Hypoxia-ischemia | Increased       | Decreased         |

Discussion

In our study, we successfully established an HI injury model and verified HI-induced pathological brain damage and

0.001) and 24 hours (P < 0.001) and 3 days (P < 0.001; Figure 3H) compared with those in the contralateral hemisphere at the same time points. The mRNA expression levels of tPA and TrkB were notably augmented at 6 hours in the contralateral hippocampus compared with the levels in the ipsilateral hemisphere at the same time point (P < 0.01 for tPA and P < 0.001 for TrkB). The mRNA expression level of plasminogen activator inhibitor 1 (PAI-1, a tPA inhibitor [Thomas et al., 2016]) in the ipsilateral cortex showed an obvious increase at 6 hours in comparison with the sham group (P < 0.05), followed by a gradual reduction and sustained lower levels at 24 hours (P < 0.05) and 3 days (P < 0.05; Figure 3I) relative to those at 6 hours. In the hippocampus, the PAI-1 mRNA expression levels in the ipsilateral hemisphere were significantly increased at 6 and 24 hours compared with those of the sham group (both P < 0.01; Figure 3J). Similarly, the mRNA expression of Sortilin [a precursor of a BDNF signaling co-receptor (Vaeget et al., 2011)] in the ipsilateral cortex was significantly increased at 6 hours compared with that in the sham group (P < 0.01; Figure 3K), whereas the mRNA expression levels in the ipsilateral hippocampus were notably decreased at 6 hours (P < 0.001) compared with those in the contralateral hemisphere at the same time point (Figure 3L). The mRNA expression levels of PAI-1 and Sortilin were both increased at 6 hours in the contralateral hippocampus relative to those in the sham group (P < 0.01 for PAI-1 and P < 0.001 for Sortilin). Summarized mRNA expression level changes and differences in the expression levels of BDNF and its related factors between samples from the HI and sham groups at different time points can be found in Table 3 and Additional Table 1.

Figure 2  | Changes in the mRNA and protein expression levels of BDNF in the cortex and hippocampus after HI injury. (A, B) BDNF mRNA expression levels in the contralateral and ipsilateral cortex (A) and hippocampus (B), as detected by quantitative real-time polymerase chain reaction. The BDNF mRNA expression level was expressed relative to the level in the sham group. (C, D) BDNF protein expression level in the ipsilateral cortex (C) and hippocampus (D), as detected by western blot assay. The BDNF protein expression was expressed as the optical density relative to the optical density of β-actin. Data are expressed as the mean ± SD (C) and hippocampus (D), as detected by western blot assay. The BDNF protein expression was expressed as the optical density relative to the optical density of β-actin. Data are expressed as the mean ± SD (n = 7/group), analyzed by repeated-measures analysis of variance (A, B) or one-way analysis of variance (C, D). *P < 0.05, **P < 0.01, ***P < 0.001, vs. sham group; ###P < 0.001. BDNF: Brain-derived neurotrophic factor; HI: Hypoxia-ischemia.

Figure 3  | Changes in the mRNA expression levels of brain-derived neurotrophic factor-related enzymes and receptors in the cortex and hippocampus after HI injury. (A, B) Furin mRNA expression in the contralateral and ipsilateral cortex (A) and hippocampus (B). (C, D) MMP-9 mRNA expression in the contralateral and ipsilateral cortex (C) and hippocampus (D). (E, F) tPA mRNA expression in the contralateral and ipsilateral cortex (E) and hippocampus (F). (F, J) TrkB mRNA expression in the contralateral and ipsilateral cortex (G) and hippocampus (H). (I, J) PAI-1 mRNA expression in the contralateral and ipsilateral cortex (I) and hippocampus (J). (K, L) Sortilin mRNA expression in the contralateral and ipsilateral cortex (K) and hippocampus (L). Data are expressed as the mean ± SD (n = 7/group), analyzed by repeated-measures analysis of variance. *P < 0.05, **P < 0.01, ***P < 0.001, vs. sham group; ###P < 0.001. HI: Hypoxia-ischemia; MMP-9: matrix metalloproteinase 9; PAI-1: plasminogen activator inhibitor 1; tPA: plasminogen activator, tissue type; TrkB: neurotrophic receptor tyrosine kinase 2.
We believe that the early upregulation of BDNF and its related enzymes and receptors in the cortex and hippocampus after hypoxia-ischemia injury might result in the neuronal damage observed in our models. Thus, both beneficial and harmful effects could be induced by MMP-9 and Furin upregulation after injury, suggesting that sensorimotor impairments observed in neonatal rats after HI might be relevant to the reduced Furin and MMP-9 expression levels observed after 7 days. Evidence suggests that tPA upregulation could induce the conversion of early upregulated BDNF and its related enzymes and receptors, thereby leading to cell death and injury. The gene expression levels of BDNF converting enzyme precursors, including Furin, MMP-9, and tPA, were significantly upregulated 6 hours after HI injury in HI injury model rats compared with sham rats. The early upregulation of BDNF converting enzyme precursors may be occurring in the peri-infarcted areas, where mature BDNF is generated to facilitate neuronal survival. Furin, a member of the pro-protein convertase family, is a key cutting enzyme in the endocrine pathway of BDNF-expressing cells, and BDNF precursor protein can be cut by Furin to form the bioactive, mature form of BDNF. However, Furin also enhances the post-ischemic activation of MMP-2, which exerts damaging effects by weakening the blood-brain barrier and causing oxidative DNA damage in the brain. MMP-9 is involved in the brain injury process and is associated with persistent sensorimotor impairments observed in neonatal rats after HI. After HI insult, pathological changes and neuronal damage can be observed in rats (Zhang et al., 2018). The HI injury has previously been shown to have a harmful influence on the developing brain and has been associated with persistent motor, sensory, and cognitive impairments (Millar et al., 2017; Sanches et al., 2019; Jiang et al., 2020). The results of the behavioral experiments performed in this study showed that rats in the HI group displayed worse performance on the behavioral tests than the sham rats, indicating that the HI insult deteriorated the motor coordination and sensorimotor sensitivities of neonatal rats. The results of this study demonstrated altered neuronal morphologies and diminished neurons counts in the cortex and hippocampus of rats in the HI group, suggesting that aggravated neurological dysfunction may be attributed to HI-induced neuronal damage. Thus, the molecular alterations that occur within damaged neurons must be clarified.

BDNF has biological effects and has been shown to play a pivotal role in reducing the death of cells surrounding lesions, repairing central nervous system damage, and promoting nerve regeneration (Koshimizu et al., 2009; Numakawa et al., 2010a, b). In this study, we found the early upregulation of BDNF and its related enzymes and receptors in the ipsilateral cortex of HI injury model rats as early as 6 hours after HI induction. The early post-HI injury alterations of these factors might result in the neuronal damage observed in our models. We believe that the early upregulation of BDNF and its related enzymes and receptors may be associated with the infarcted tissues, and the elevated levels went through the injuring processes within 6 hours after HI insult.

### Table 3

| Gene     | Position       | 6 h | 15 h | 24 h | 3 d | 7 d |
|----------|----------------|-----|------|------|-----|-----|
| BDNF     | Contralateral cortex | ↓   | ↓   | ↓   | No change | ↓ |
|          | Ipsilateral cortex | ↑   | No change | No change | ↑ | ↑ |
|          | Contralateral hippocampus | ↑ | ↑ | ↑ | ↑ | ↑ |
|          | Ipsilateral hippocampus | ↑ | ↑ | ↑ | ↑ | ↑ |
| Furin    | Contralateral cortex | ↓   | ↓   | ↓ | ↓ | ↓ |
|          | Ipsilateral cortex | No change | ↓ | ↓ | ↓ | ↓ |
|          | Contralateral hippocampus | ↑ | ↓ | ↓ | No change | ↓ |
|          | Ipsilateral hippocampus | ↑ | ↓ | ↓ | ↑ | No change |
| MMP-9    | Contralateral cortex | ↓   | ↓   | ↑ | ↑ | ↑ |
|          | Ipsilateral cortex | ↓ | No change | No change | No change | ↓ |
|          | Contralateral hippocampus | ↑ | ↓ | No change | ↓ | No change |
|          | Ipsilateral hippocampus | ↑ | No change | No change | No change | No change |
| tPA      | Contralateral cortex | ↓   | ↓   | ↓ | ↓ | ↓ |
|          | Ipsilateral cortex | ↑ | No change | ↓ | ↓ | No change |
|          | Contralateral hippocampus | ↑ | ↑ | ↑ | ↑ | ↑ |
|          | Ipsilateral hippocampus | ↑ | No change | No change | ↑ | ↑ |
| Sortilin | Contralateral cortex | ↓   | ↓   | ↓ | ↓ | ↓ |
|          | Ipsilateral cortex | ↑ | ↓ | ↓ | ↓ | ↓ |
|          | Contralateral hippocampus | ↑ | No change | No change | ↑ | No change |
|          | Ipsilateral hippocampus | ↑ | No change | No change | ↑ | ↑ |
| TrkB     | Contralateral cortex | ↓   | ↓   | ↓ | ↓ | ↓ |
|          | Ipsilateral cortex | ↑ | No change | No change | No change | ↓ |
|          | Contralateral hippocampus | ↑ | ↑ | ↓ | No change | ↓ |
|          | Ipsilateral hippocampus | ↑ | ↓ | ↑ | ↑ | ↑ |
| PAI-1    | Contralateral cortex | ↑   | ↓   | No change | No change | No change |
|          | Ipsilateral cortex | ↑ | No change | No change | ↑ | ↑ |
|          | Contralateral hippocampus | No change | No change | ↑ | No change | ↑ |
|          | Ipsilateral hippocampus | No change | No change | ↑ | No change | ↑ |

BDNF: Brain-derived neurotrophic factor; MMP-9: matrix metallopeptidase 9; PAI-1: plasminogen activator inhibitor 1; tPA: plasminogen activator, tissue type.

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Additional file: Additional Table 1: Repeated measurement design analysis of variance of mRNA expression levels of BDNF and its related enzymes and receptors in the cortex and hippocampus after hypoxia-ischemia.

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Additional file: Additional Table 1: Repeated measurement design analysis of variance of mRNA expression levels of BDNF and its related enzymes and receptors in the cortex and hippocampus after hypoxia-ischemia.
### Additional Table 1

Repeated measurement design analysis of variance of mRNA expression levels of BDNF and its-related enzymes and receptors in the cortex and hippocampus after hypoxia-ischemia

| Variables                  | DF  | SS     | MS   | F     | P     |
|----------------------------|-----|--------|------|-------|-------|
| **BDNF in cortex**         |     |        |      |       |       |
| Intervene                  | 1   | 55.786 | 55.786 | 1357.966 | <0.001 |
| Intergroup error           | 12  | 0.493  | 0.041 |       |       |
| Time                       | 2.961 | 37.208 | 12.567 | 67.914  | <0.001 |
| Time × Intervene           | 2.961 | 37.550 | 12.682 | 68.538  | <0.001 |
| Repeated measurement error | 35.531 | 6.574  | 0.185 |       |       |
| **BDNF in hippocampus**    |     |        |      |       |       |
| Intervene                  | 1   | 105.018 | 105.018 | 408.284 | <0.001 |
| Intergroup error           | 12  | 3.087  | 0.257 |       |       |
| Time                       | 2.056 | 71.447 | 34.754 | 51.638  | <0.001 |
| Time × Intervene           | 2.056 | 1.447  | 0.722 | 48.265  | <0.001 |
| Repeated measurement error | 24.670 | 16.603 | 0.673 |       |       |
| **Furin in cortex**        |     |        |      |       |       |
| Intervene                  | 1   | 3.715  | 3.715 | 75.125  | <0.001 |
| Intergroup error           | 12  | 0.593  | 0.049 |       |       |
| Time                       | 1.585 | 15.022 | 9.480  | 61.647  | <0.001 |
| Time × Intervene           | 1.585 | 11.761 | 7.422  | 48.265  | <0.001 |
| Repeated measurement error | 19.015 | 2.924  | 0.154 |       |       |
| **Furin in hippocampus**   |     |        |      |       |       |
| Intervene                  | 1   | 4.518  | 4.518 | 25.177  | <0.001 |
| Intergroup error           | 12  | 2.153  | 0.179 |       |       |
| Time                       | 2.120 | 75.233 | 35.484 | 186.055 | <0.001 |
| Time × Intervene           | 2.120 | 34.712 | 16.372 | 85.844  | <0.001 |
| Repeated measurement error | 25.442 | 4.852  | 0.191 |       |       |
| **MMP-9 in cortex**        |     |        |      |       |       |
| Intervene                  | 1   | 161366.853 | 161366.853 | 216.546 | <0.001 |
| Intergroup error           | 12  | 8942.235 | 745.186 |       |       |
| Time                       | 1.001 | 836216.193 | 835450.050 | 223.008 | <0.001 |
| Time × Intervene           | 1.001 | 849030.666 | 848252.782 | 226.425 | <0.001 |
| Repeated measurement error | 12.011 | 44996.629 | 3746.284 |       |       |
| **MMP-9 in hippocampus**   |     |        |      |       |       |
| Intervene                  | 1   | 1.947  | 1.947 | 59.656  | <0.001 |
| Intergroup error           | 12  | 0.392  | 0.033 |       |       |
| Time                       | 3.046 | 11.729 | 3.851  | 143.417 | <0.001 |
| Time × Intervene           | 3.046 | 13.792 | 4.528  | 168.641 | <0.001 |
| Repeated measurement error | 36.553 | 0.981  | 0.027 |       |       |
| **PAI-1 in cortex**        |     |        |      |       |       |
| Intervene                  | 1   | 11.457 | 11.457 | 242.113 | <0.001 |
| Intergroup error           | 12  | 0.568  | 0.047 |       |       |
| Time                       | 1.349 | 22.308 | 16.535 | 84.569  | <0.001 |
| Time × Intervene           | 1.349 | 21.615 | 13.632 | 81.940  | <0.001 |
| Repeated measurement error | 16.190 | 3.165  | 0.196 |       |       |
| **PAI-1 in hippocampus**   |     |        |      |       |       |
| Intervene                  | 1   | 2.854  | 2.854 | 26.247  | <0.001 |
| Intergroup error           | 12  | 1.305  | 0.109 |       |       |
| Time                       | 1.795 | 250.344 | 139.495 | 286.018 | <0.001 |
| Time × Intervene           | 1.795 | 107.396 | 59.842  | 122.699 | <0.001 |
| Repeated measurement error | 21.536 | 10.503 | 0.488 |       |       |
|                      | Intervene | Intergroup error | Time | Time × Intervene | Repeated measurement error |
|----------------------|-----------|------------------|------|------------------|---------------------------|
| **Sortilin in cortex** |           |                  |      |                  |                           |
|                      | 1         | 0.81             | 0.81 | 23.975           | <0.001                    |
|                      | 12        | 0.405            | 0.034|                  |                           |
|                      | 1.925     | 15.732           | 8.172| 101.121          | <0.001                    |
|                      | 1.925     | 16.371           | 8.504| 105.228          | <0.001                    |
|                      | 23.101    | 1.867            | 0.081|                  |                           |
| **Sortilin in hippocampus** |          |                  |      |                  |                           |
|                      | 1         | 15.721           | 15.721| 49.677          | <0.001                    |
|                      | 12        | 3.798            | 0.316|                  |                           |
|                      | 1.169     | 333.997          | 285.594| 193.870       | <0.001                    |
|                      | 1.169     | 182.769          | 156.282| 106.089       | <0.001                    |
|                      | 14.034    | 20.673           | 1.473|                  |                           |
| **TPA in cortex**    |           |                  |      |                  |                           |
|                      | 1         | 12.793           | 12.793| 204.557        | <0.001                    |
|                      | 12        | 0.75             | 0.063|                  |                           |
|                      | 1.577     | 31.791           | 20.153| 107.549       | <0.001                    |
|                      | 1.577     | 31.82            | 20.172| <0.001        |                           |
|                      | 18.93     | 3.547            | 0.187|                  |                           |
| **tPA in hippocampus** |          |                  |      |                  |                           |
|                      | 1         | 26.223           | 26.223| 57.468         | <0.001                    |
|                      | 12        | 5.476            | 0.456|                  |                           |
|                      | 1.406     | 244.098          | 173.587| 90.627       | <0.001                    |
|                      | 1.406     | 117.113          | 83.283| 43.481        | <0.001                    |
|                      | 16.874    | 32.321           | 1.915|                  |                           |
| **TrkB in cortex**   |           |                  |      |                  |                           |
|                      | 1         | 8.998            | 8.998| 428.852         | <0.001                    |
|                      | 12        | 0.252            | 0.021|                  |                           |
|                      | 1.96      | 27.859           | 14.213| 227.932      | <0.001                    |
|                      | 1.96      | 25.224           | 12.869| 206.374      | <0.001                    |
|                      | 23.521    | 1.467            | 0.062|                  |                           |
| **TrkB in hippocampus** |          |                  |      |                  |                           |
|                      | 1         | 0.677            | 0.677| 5.503           | 0.037                     |
|                      | 12        | 1.476            | 0.123|                  |                           |
|                      | 1.337     | 67.601           | 50.556| 147.812    | <0.001                    |
|                      | 1.337     | 27.225           | 20.361| 59.529        | <0.001                    |
|                      | 16.046    | 5.488            | 0.342|                  |                           |

BDNF: Brain-derived neurotrophic factor; DF: degree of freedom; MMP-9: matrix metallopeptidase 9; MS: mean square; PAI-1: plasminogen activator inhibitor 1; SS: sum of squares; tPA: plasminogen activator, tissue type.