

An enriched environment increases the expression of fibronectin type III domain-containing protein 5 and brain-derived neurotrophic factor in the cerebral cortex of the ischemic mouse brain

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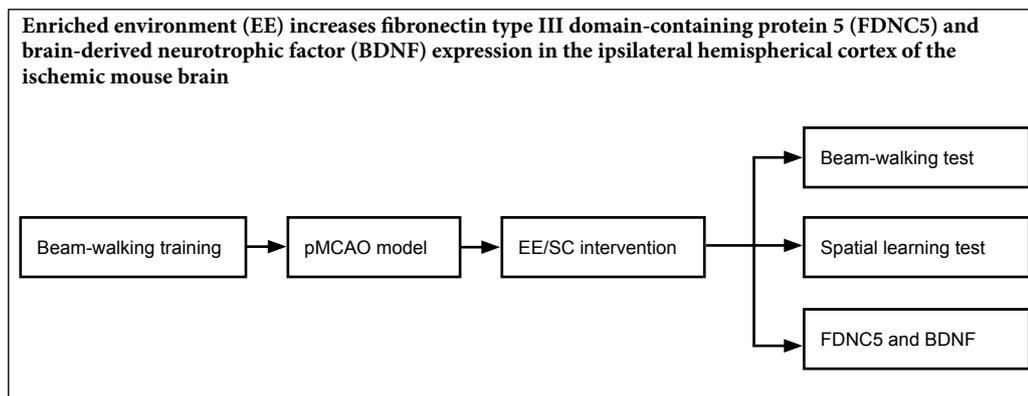
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Graphical Abstract



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Abstract

Many studies have shown that fibronectin type III domain-containing protein 5 (FDNC5) and brain-derived neurotrophic factor (BDNF) play vital roles in plasticity after brain injury. An enriched environment refers to an environment that provides animals with multi-sensory stimulation and movement opportunities. An enriched environment has been shown to promote the regeneration of nerve cells, synapses, and blood vessels in the animal brain after cerebral ischemia; however, the exact mechanisms have not been clarified. This study aimed to determine whether an enriched environment could improve neurobehavioral functions after the experimental induction of cerebral ischemia and whether neurobehavioral outcomes were associated with the expression of FDNC5 and BDNF. This study established ischemic mouse models using permanent middle cerebral artery occlusion (pMCAO) on the left side. On postoperative day 1, the mice were randomly assigned to either enriched environment or standard housing condition groups. Mice in the standard housing condition group were housed and fed under standard conditions. Mice in the enriched environment group were housed in a large cage, containing various toys, and fed with a standard diet. Sham-operated mice received the same procedure, but without artery occlusion, and were housed and fed under standard conditions. On postoperative days 7 and 14, a beam-walking test was used to assess coordination, balance, and spatial learning. On postoperative days 16–20, a Morris water maze test was used to assess spatial learning and memory. On postoperative day 15, the expression levels of FDNC5 and BDNF proteins in the ipsilateral cerebral cortex were analyzed by western blot assay. The results showed that compared with the standard housing condition group, the motor balance and coordination functions (based on beam-walking test scores 7 and 14 days after operation), spatial learning abilities (based on the spatial learning scores from the Morris water maze test 16–19 days after operation), and memory abilities (based on the memory scores of the Morris water maze test 20 days after operation) of the enriched environment group improved significantly. In addition, the expression levels of FDNC5 and BDNF proteins in the ipsilateral cerebral cortex increased in the enriched environment group compared with those in the standard housing condition group. Furthermore, the Pearson correlation coefficient showed that neurobehavioral functions were positively associated with the expression levels of FDNC5 and BDNF ($r = 0.587$ and $r = 0.840$, respectively). These findings suggest that an enriched environment upregulates FDNC5 protein expression in the ipsilateral cerebral cortex after cerebral ischemia, which then activates BDNF protein expression, improving neurological function. BDNF protein expression was positively correlated with improved neurological function. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Fudan University, China (approval Nos. 20160858A232, 20160860A234) on February 24, 2016.

Key Words: beam-walking test; brain-derived neurotrophic factor; cerebral ischemia; correlation analysis; enriched environment; fibronectin type III domain-containing protein 5; Morris water maze task; neural plasticity; neuroprotection; permanent middle cerebral artery occlusion

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Introduction

Ischemic stroke is associated with a high death rate and a lack of effective treatment interventions. Recent studies have shown that enriched-environment (EE) training can have neuroprotective effects for brain function, including the promotion of neural plasticity (Jiang et al., 2016; Wang et al., 2019). Although EE training has been well-documented to improve neurobehavioral functions and to alter brain structures, the potential mechanisms underlying these beneficial effects have not been fully elucidated.

Neurotrophic factors are involved in the survival, proliferation, maturation, and growth of neurons in intact and damaged brains (Miller and Kaplan 2001; Bekinschtein et al., 2008; Cruz et al., 2018; Wagenaar et al., 2018). Previous studies have found that increased levels or the activation of brain-derived neurotrophic factor (BDNF) and related signaling pathways were associated with better recovery after stroke (Ploughman et al., 2007; de la Tremblaye et al., 2017). BDNF levels have been reported to increase in cultured neurons and brains following ischemic events (Zhao et al., 2013). Furthermore, BDNF levels increase in surrounding tissues after stroke (Clarkson et al., 2011), including dentate granule cells and the hippocampus (Wang et al., 2017). Some studies have shown that BDNF promotes nerve regeneration, ischemic penumbra angiogenesis, and the inhibition of inflammation, neurotoxicity, epilepsy, and apoptosis (Clarkson et al., 2011; Balaratnasingam and Janca, 2012; Zhao et al., 2014; Sanna et al., 2017).

Fibronectin type III domain-containing protein 5 (FDNC5), which was previously described as the peroxisome protein, is believed to be a transmembrane precursor protein that is processed into a soluble, secreted peptide, called irisin, which is highly conserved in mammals. FDNC5 plays a vital role in the neuronal differentiation of embryonic stem cells. Studies have claimed that irisin can regulate hippocampal neurogenesis. In the presence of irisin, the number of hippocampal neurons in the mouse brain increased. FDNC5 can also increase the expression level of BDNF (Zsuga et al., 2016). FDNC5 plays a vital role during the regulation of neuronal development.

In animal studies, EE has been associated with increased activity in the brain. Based on a recent review, EE represents an experienced experimental condition, which can be implemented by exposing experimental animals to improved habitats, raising animals in large cages among social groups, providing access to toys, running cycles, and periodically varying sensory stimuli, and EE exposure in animals increases the abilities of neurons to resist insults (Novkovic et al., 2015). EE is an active rehabilitation training mode that is not associated with stress response and is not harmful to animals. Moreover, this rehabilitation model is very suitable for group therapy. Although this type of rehabilitation therapy has been shown to be associated with curative effects, the specific mechanisms underlying these effects are not clear.

This study investigated the effects of EE exposure on the neurologic behavioral functions and the expression levels of FDNC5 and BDNF in an ischemic mouse model.

Materials and Methods

Animals

A total of 40 healthy, male, C57BL/6 mice, aged 8–12 weeks and weighing 25–28 g, were purchased from Jie Si Jie Lab Animal Co., Ltd., Shanghai, China (Animal license No. SCXK [Hu] 2018-004). Mice were housed at 20°C and 45–50% humidity, in a 12-hour light/dark cycle (lights from 8:00 to 20:00), and allowed free access to water and food. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Fudan University, China (approval Nos. 20160858A232 and 20160860A234) on February 24, 2016. The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Experimental design

All of the time points used for the subsequent operations and tests are described in **Figure 1**. A total of 26 mice were subjected to permanent middle cerebral artery occlusion (pMCAO) on the left side. Two mice died after the operation. On postoperative day one, the remaining mice were randomly assigned to either the EE group ($n = 12$) or the standard housing condition (SC) group ($n = 12$). The sham group contained 12 sham-operated mice. The EE group and two normal mice were maintained in an EE cage for 14 days. Mice in the SC and sham groups were maintained in cages under standard conditions for 14 days. Beam-walking training was conducted for three consecutive days before surgery. Beam-walking tests were performed on days 7 and 14 after surgery. Western blots were performed on samples collected 15 days after surgery from six mice in each of the EE and SC groups. The Morris water maze task was performed on days 16–20 after surgery in six mice from each of the EE and SC groups.

pMCAO models

Model and cerebral blood flow

According to previous studies (Engel et al., 2011; Neher et al., 2011), pMCAO on the left side can cause permanent cerebral ischemia. All mice were anesthetized with isoflurane (3% induction, 1.5–2.0% operation maintenance). To determine the appropriate suture location, cerebral perfusion was monitored using a Moor VMS-LDF2 monitor (Moor Instruments, Devon, UK). If cerebral blood flow decreased to 30–35% and remained relatively stable, modeling was considered to be successful. pMCAO on the left side was performed, as previously described (Doyle and Buckwalter, 2014). Briefly, a needle probe, with a diameter of 1.9 mm, was placed directly on the skull above the MCA area (1 mm behind and 5 mm outside). The perfusion value of the carotid artery was recorded before and after the operation. The practical method was to perform a midline incision on the ventral surface of the neck. The left common carotid artery was separated and sutured with 6-0 silk threads. Both internal and external carotid arteries were sutured with 6-0 sutures. A 4-0 surgical nylon monofilament with a silicone tip was inserted into the internal carotid artery, through a proximal

carotid artery incision. The filaments were then pushed approximately 6.5 cm from the distal end of the proximal carotid artery incision. The mice were allowed to recover from anesthesia on a heating pad at $37.0 \pm 0.5^\circ\text{C}$ and returned to their home cages after full recovery from anesthesia. In addition, sham-operated mice underwent the same procedure, without the induction of pMCAO.

Housing conditions

All animals that survived surgery on postoperative day 1 were assessed for neurological deficits (Longa et al., 1989). The score criteria for neurological deficit assessment were as follows: 0, no neurological deficit; 1, the right front paw does not fully extend and the grip is reduced when the tail is stretched; 2, voluntarily movement in all directions and turning right when running; 3, walking or hovering to the right, sensitive to nociception when stimulated; 4, no response to stimuli or stroke-related deaths. The mice with scores between 1 and 3 were randomly divided into the EE and SC groups. SC group mice were placed in a standard cage (21 cm wide, 27 cm long, and 16 cm high) (Figure 2A). The EE group mice were placed in the EE home cage (65 cm wide, 75 cm long, and 25 cm high), which contained climbing ladders, sports wheels, plastic tubes, toys, tunnels, running wheels, huts, and various decorations (Figure 2B). Mice in the EE group were provided with enhanced social stimuli. A total of 14 mice were maintained in the EE home cage (12 EE mice and 2 normal mice). The above conditions were changed after 3 days to maintain environmental novelty. SC mice were divided into four groups ($n = 3$). Sham-operated animals were maintained in standard cages under standard conditions. During the enrichment period, food and water were freely available. Mice in the EE group were fed in cages under EE conditions for 2 weeks.

Neurological function tests

Beam-walking test

To assess the coordination and balance of movement, the beam-walking test was performed before cerebral ischemia and 7 and 14 days after cerebral ischemia. The beam-walking apparatus consisted of a square beam (1 cm wide, 30 cm long, 30 cm high) connected to a black box (20 cm \times 10 cm \times 10 cm). The mouse was placed on one end of the square beam and allowed to walk to the other end of the square beam, which was connected to a black box. The beam-walking test was performed three times and evaluated on a 0–3-point scale (3 points: walking distance > 20 cm; 2 points: walking distance < 20 cm; 1 point: walking distance < 10 cm; and 0 points: falling within a walking distance of 10 cm). The sum of points across the three evaluations was calculated for each mouse, as previously described (Li et al., 2017).

Morris water maze test

The Morris water maze test was used to test the spatial learning and memory of mice. As mentioned earlier, some modifications have been made (Vorhees and Williams, 2006). In short, the Morris water maze was performed in a black circu-

lar pool, filled with water, at $21\text{--}23^\circ\text{C}$, which was located in a room with obvious visual clues. Spatial learning was conducted from days 16 to 19 after the operation, for 4 consecutive days. Eight consecutive experiments (using randomly allotted beginning positions) were conducted daily to trace pathways taken by the mice to reach a platform submerged 1.5 cm beneath the water surface. The mice were allowed 60 seconds to locate the platform, which was maintained in a constant location. If they failed to locate the platform within 60 seconds, the mice were manually guided to the platform and maintained on the platform for 10 seconds. The average escape latency of each mouse was recorded and analyzed as an index of spatial learning. One day after the final acquisition training session (day 20 post pMCAO), all of the mice received a probe test with the escape platform removed. The mice were placed into the pool in the location most distal to the target quadrant (with the platform removed). The percent of time spent in the target quadrant was recorded and interpreted as spatial memory (Block, 1999).

Western blot assay

On postoperative day 15, the ipsilateral cortex surrounding the ischemic zone was harvested. The cortex protein extracts were analyzed by western blot assay. In brief, cortex tissue was homogenized in RIPA lysis buffer. The samples were heated at $95\text{--}100^\circ\text{C}$ for 10 minutes, after centrifugation and concentration. Using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (Biorad, Berkeley, CA, USA), 50 μg of protein were electrophoretically separated and transferred to a nitrocellulose membrane. The membrane was then sealed at room temperature and incubated in a 5% bovine serum albumin liquid (Beyotime, Shanghai, China), for 1 hour. The primary antibodies used were anti-FDNC5 (1:2000; rabbit monoclonal; Abcam, London, UK) and anti-BDNF (1:500; rabbit monoclonal; Abcam). Anti- β -tubulin (1:1000; rabbit polyclonal; Abcam) was used as a loading control. The membranes were incubated overnight with primary antibodies, at 4°C . Within an hour, the membrane was cleaned, washed, and incubated with secondary antibody (anti-mouse IgG, 1:5000; Abcam), at room temperature, for 2 hours. The intensity of protein bands was measured using a western imprinting image system and quantified by ImageJ analysis software (NIH, Bethesda, MD, USA). The ratio of the optical density of the target protein to that of β -tubulin was used as the relative expression level for that protein.

Statistical analysis

The data are presented as the mean \pm standard error of the mean (SEM). SPSS 18.0 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis. Cerebral cortical blood flow value after pMCAO was analyzed using the paired Student's *t*-test. The one-way analysis of variance, followed by a least significant difference *post hoc* test, was used to determine multiple comparisons between groups. The correlations between neurological function outcomes and FDNC5 and BDNF expression levels were analyzed using Pearson's correlation coefficient. $P < 0.05$ was considered to

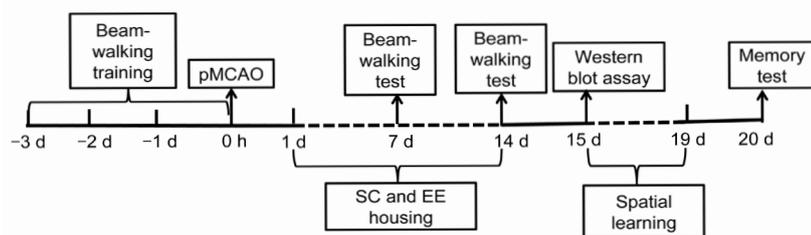


Figure 1 A schematic diagram of the experimental design.

Beam-walking training was conducted for 3 consecutive days before surgery. Beam-walking tests were conducted on days 7 and 14 after surgery. Brain samples analyzed by western blots were collected on day 15 after pMCAO. Spatial learning was tested from days 16 to 19 after surgery. A memory test was performed on day 20 after the operation. EE: Enriched environment; pMCAO: permanent middle cerebral artery occlusion; SC: standard housing conditions.

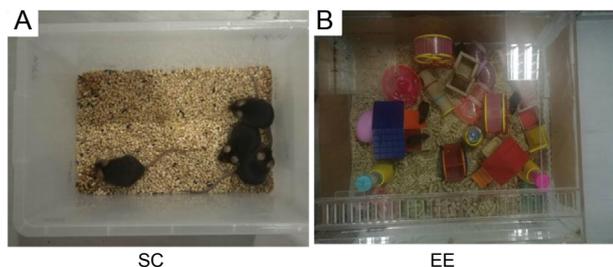


Figure 2 SC and EE housing conditions. (A) SC pMCAO mice; (B) EE pMCAO mice. EE: Enriched environment; pMCAO: permanent middle cerebral artery occlusion; SC: standard housing conditions.

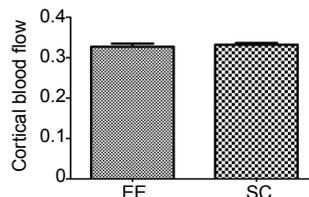


Figure 3 Mean values of cerebral blood flow in EE and SC mice. No significant difference was found between the EE and SC groups. The cerebral cortical blood flow decreased to 30–35% ($n = 6$). EE: Enriched environment; SC: standard housing conditions.

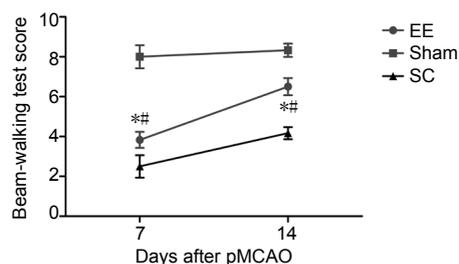


Figure 4 Beam-walking test scores in pMCAO mice. * $P < 0.05$, vs. SC group; # $P < 0.05$, vs. sham group. Data are expressed as the mean \pm SEM ($n = 6$; one-way analysis of variance followed by the least significant difference *post hoc* test). EE: Enriched environment; SC: standard housing conditions; pMCAO: permanent middle cerebral artery occlusion.

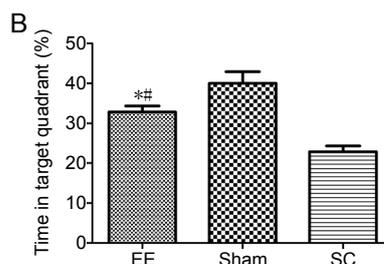
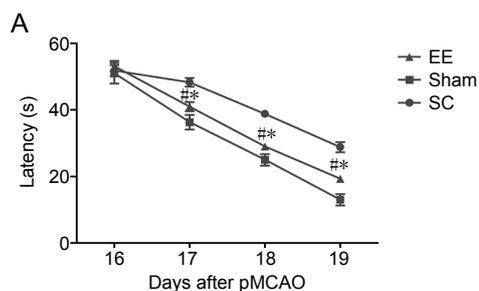


Figure 5 Effects of EE on spatial learning and memory of pMCAO mice. (A) Evaluation of spatial learning, using the Morris water maze test, following pMCAO: The three groups showed no significant differences on day 16 post pMCAO; however, mice in the EE group required less time to find the platform on days 17, 18, and 19 post pMCAO compared with the SC group on those days. (B) Determination of spatial memory using the probe test on day 20 post pMCAO. Compared with the SC group, mice in the EE group spent significantly more time in the correct quadrant. Data are expressed as the mean \pm SEM ($n = 6$; one-way analysis of variance followed by the least significant difference *post hoc* test). * $P < 0.05$, vs. SC group; # $P < 0.05$, vs. sham group. EE: Enriched environment; pMCAO: permanent middle cerebral artery occlusion; SC: standard housing conditions.

be statistically significant.

Results

Alterations of cerebral cortical perfusion in experimental mice

As shown in **Figure 3**, cerebral cortical blood flow in the sham group showed no significant changes after the operation. In contrast, the cerebral cortical blood flow was

decreased to 30–35% and remained stable after surgery in the pMCAO mice. The mean values for cerebral blood flow showed no significant differences between EE and SC pMCAO mice.

Neurological function

Effects of EE on motor coordination and balance

The beam-walking test was chosen to evaluate deficiencies

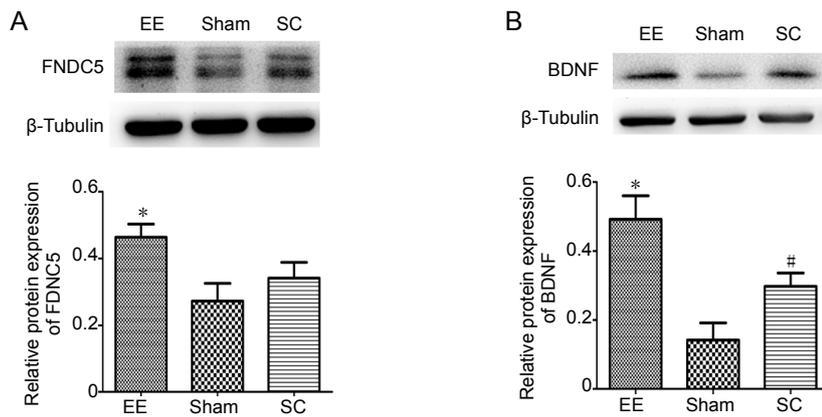


Figure 6 Western blot assay for FDNC5 (A) and BDNF (B) proteins in the cortex surrounding the ischemic border zone, using samples collected on day 15 after pMCAO.

The optical density values were normalized to their corresponding tubulin loading controls. Data are expressed as the mean \pm SEM ($n = 6$; one-way analysis of variance followed by the least significant difference *post hoc* test). * $P < 0.05$, vs. SC group, # $P < 0.05$, vs. sham group. BDNF: Brain-derived neurotrophic factor; EE: enriched environment; FDNC5: fibronectin type III domain-containing protein 5; pMCAO: permanent middle cerebral artery occlusion; SC: standard housing conditions.

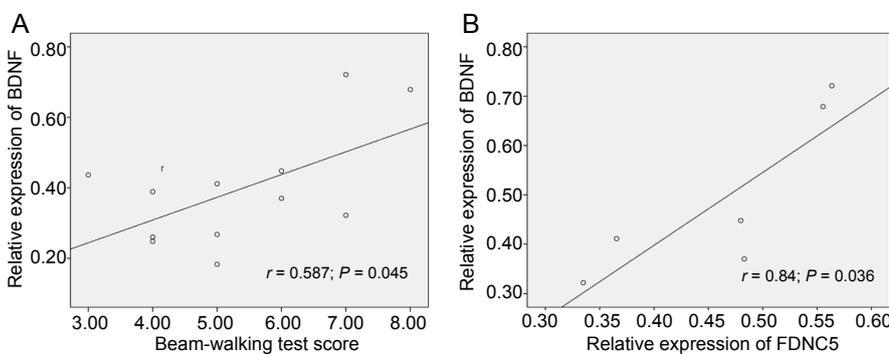


Figure 7 Correlation between the beam-walking test scores and the expression levels of BDNF (A) and the correlation between the expression levels of FDNC5 and BDNF (B) in the EE group.

r represents Pearson's correlation coefficient. $P < 0.05$ was considered to have a significant correlation. BDNF: Brain-derived neurotrophic factor; FDNC5: fibronectin type III domain-containing protein 5.

in motor coordination and integration in ischemic mice. As exhibited in **Figure 4**, beam walking scores were better for ischemic mice in the EE group than those in the SC group.

Effects of EE on spatial learning and memory

According to the location and severity of the injury, cerebral ischemia may lead not only to motor deficits, but also to spatial learning and memory deficits. Therefore, the protective effects observed for EE on motor function were encouraging. Consequently, the Morris water maze was used to study whether EE has protective effects for learning and memory after ischemic injury. Preliminary assessments of escape latency to identify the underwater platform did not show any significant differences among these three groups during the first day of training (the 16th day after pMCAO). However, mice in the SC group demonstrated a significant increase in the latency to find the platform on days 2, 3 and 4 of training (days 17, 18 and 19 post pMCAO, respectively) compared with both the sham and EE groups (**Figure 5A**). Spatial learning was assessed on the day after the last escape latency test (20 days after pMCAO). During this test, the platform was removed and the time spent by the mouse in the quadrant where the platform had previously been located (the correct quadrant) was measured. The results showed that the EE group spent more time in the correct quadrant than the SC group (**Figure 5B**).

Western blot assay of FDNC5 and BDNF proteins

As displayed in **Figure 6**, the western blot assay demon-

strated that FDNC5 and BDNF protein expression levels increased significantly in the EE group compared with the SC group ($P < 0.05$). These results indicated that EE promoted neural plasticity in the ischemic cortical zone.

Relationship between neurological function outcomes and BDNF protein expression

To evaluate the possible relationship between BDNF protein expression and neurological function, Pearson's correlation coefficient (r) was used to compare beam-walking test scores and BDNF protein expression levels 15 days after cerebral ischemia, as shown in **Figure 7A**. The beam-walking test scores were positively correlated with BDNF protein expression levels.

Correlation between the protein expression levels of FDNC5 and BDNF

Pearson's correlation coefficient (r) was used to analyze the relationship between the relative expression levels of FDNC5 and BDNF proteins in the EE group. As shown in **Figure 7B**, the FDNC5 protein expression level was positively correlated with the BDNF expression level.

Discussion

BDNF is a member of the neurotrophin family and is involved in the differentiation, development, and survival of neurons. Some studies have suggested that elevated levels of BDNF may be beneficial during the recovery from acute and chronic stroke (Ploughman et al., 2005; Vaynman and

Gomez-Pinilla, 2005; Rosenblum et al., 2015). The most probable mechanisms underlying these benefits involve the prevention of acute ischemic injury (Ferrer et al., 2001), increased angiogenesis (Kermani and Hempstead 2007; Pikula et al., 2013), increased brain repairs (Wang et al., 2013; Luo et al., 2015) and neurogenesis (Schabitz et al., 2007), and enhanced synaptic plasticity (Kuczewski et al., 2010; Balaratnasingam and Janca, 2012). Ischemic brain injury, a primary cause of mortality and morbidity, results from reduced oxygen and blood flow to the brain. EE training can reduce nerve injury and promote BDNF protein expression. Furthermore, some studies have suggested that FDNC5 expression is associated with BDNF expression, and FDNC5 plays a vital role during neuronal development (Lecker et al., 2012; Hashemi et al., 2013; Forouzanfar et al., 2015) and is expressed in the brain (Dun et al., 2013). A study observed significantly higher levels of BDNF and FNDC5 in quercetin-treated integrated synapses, as observed by transmission electron microscopy, and the improved performance by rats during the Morris water maze test highlighted the maintenance of neuronal function in quercetin-treated rats (Liu et al., 2015). Compared with SC rats, EE training was able to promote the expression of BDNF and FDNC5 after pMCAO. The levels of BDNF and FDNC5 in the brains of EE mice increased significantly, and these mice were able to maintain nerve function, as demonstrated by improved Morris water maze test results.

The direct injection of exogenous BDNF into the damaged brain is unsafe and impractical because it may cause additional damage. The intravenous infusion of exogenous BDNF is a relatively non-invasive treatment method. However, the permeability of BDNF through the blood-brain barrier is weak, dramatically reducing its application prospects and clinical value (Zhao et al., 2014). Consequently, determining safe, efficient and clinically applicable strategies for the administration of BDNF is necessary. Activating the expression of endogenous BDNF in the area surrounding the cerebral infarction may represent a better solution (Peng et al., 2008; He et al., 2018) than exogenous BDNF administration.

EE is an effective rehabilitation program for rodents and animals, implemented by exposing the animals to larger social groups, in larger cages, with many new objects to promote increased social interactions, activities, and exploration (Xie et al., 2013). EE provides various sensory, motor, and cognitive stimuli, improving the morphological and biochemical changes in the adult brain, and promotes the functional recovery of brain-injured animals (Yu et al., 2014). Scholars have suggested that EE stimuli may induce some varieties of plasticity in the brains of adults, which range from biochemical parameters to dendritic growth, gliogenesis, and neurogenesis (Nithianantharajah and Hannan 2006; Zhang et al., 2017). An enriched environment was shown to induce significant increases in the cell body and nucleus sizes, dendritic branch numbers, dendritic spine densities, and synapse numbers (Mataga et al., 2004; Leggio et al., 2005; Alvarez and Sabatini, 2007; Karpova, 2014; Moreno-Jimenez et al., 2019).

Some studies have shown that EE can enhance brain plasticity and promote the release of BDNF (Nithianantharajah and Hannan, 2006; Shono et al., 2011; Moradi-Kor et al., 2019). Some experimental data have suggested that the protective effects of EE are associated with increased BDNF expression levels (Lu, 2003), which are consistent with our results. Therefore, this treatment strategy for cerebral ischemia treatment may be more promising than neurotrophic therapy alone.

In summary, EE treatment should translate into a rehabilitation strategy for poststroke patients in the clinical setting. However, many factors must be examined prior to the routine clinical use of this therapy. Many studies include multiple stimulation modalities during EE, and no quantitative indicator exists for EE. In our previous study, we only quantified the amount of exercise received by mice in an EE environment (Yu et al., 2013), whereas this study used multiple stimuli. Therefore, most research must be performed to elaborate on the specific role played by EE. The mechanisms underlying the EE training effects observed for neuroprotection after cerebral ischemia require further investigations.

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Conflicts of interest: The authors declare that there are no conflicts of interest associated with this manuscript.

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