RESEARCH ARTICLE

An enriched environment promotes synaptic plasticity and cognitive recovery after permanent middle cerebral artery occlusion in mice

Chuan-Jie Wang, Yi Wu*, Qun Zhang, Ke-Wei Yu, Yu-Yang Wang
Department of Rehabilitation Medicine, Huashan Hospital, Fudan University, Shanghai, China

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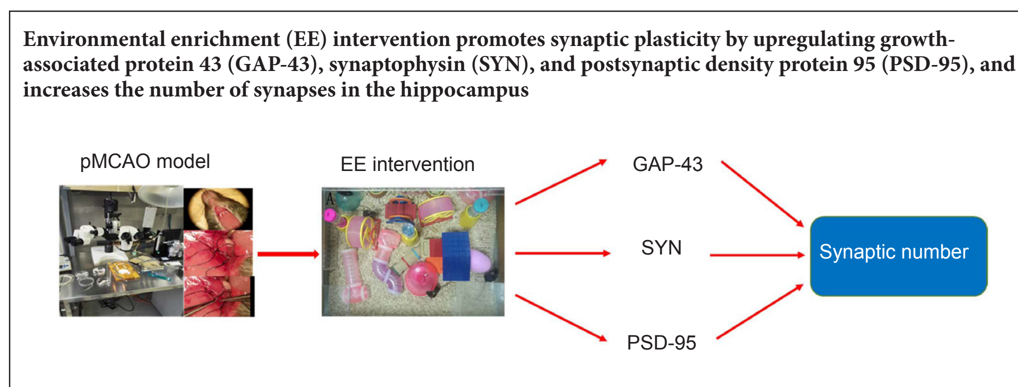
Graphical Abstract

Environmental enrichment (EE) intervention promotes synaptic plasticity by upregulating growth-associated protein 43 (GAP-43), synaptophysin (SYN), and postsynaptic density protein 95 (PSD-95), and increases the number of synapses in the hippocampus



Correspondence to:
Yi Wu, MD,
wuyi@fudan.edu.cn.

orcid:
0000-0003-4854-9898
(Yi Wu)

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Abstract

Cerebral ischemia activates an endogenous repair program that induces plastic changes in neurons. In this study, we investigated the effects of environmental enrichment on spatial learning and memory as well as on synaptic remodeling in a mouse model of chronic cerebral ischemia, produced by subjecting adult male C57BL/6 mice to permanent left middle cerebral artery occlusion. Three days post-operatively, mice were randomly assigned to the environmental enrichment and standard housing groups. Mice in the standard housing group were housed and fed a standard diet. Mice in the environmental enrichment group were housed in a cage with various toys and fed a standard diet. Then, 28 days postoperatively, spatial learning and memory were tested using the Morris water maze. The expression levels of growth-associated protein 43, synaptophysin and postsynaptic density protein 95 in the hippocampus were analyzed by western blot assay. The number of synapses was evaluated by electron microscopy. In the water maze test, mice in the environmental enrichment group had a shorter escape latency, traveled markedly longer distances, spent more time in the correct quadrant (northeast zone), and had a higher frequency of crossings compared with the standard housing group. The expression levels of growth-associated protein 43, synaptophysin and postsynaptic density protein 95 were substantially upregulated in the hippocampus in the environmental enrichment group compared with the standard housing group. Furthermore, electron microscopy revealed that environmental enrichment increased the number of synapses in the hippocampal CA1 region. Collectively, these findings suggest that environmental enrichment ameliorates the spatial learning and memory impairment induced by permanent middle cerebral artery occlusion. Environmental enrichment in mice with cerebral ischemia likely promotes cognitive recovery by inducing plastic changes in synapses.

Key Words: *nerve regeneration; environmental enrichment; cerebral ischemia; cognitive recovery; brain plasticity and reorganization; synaptic plasticity; electron microscopy; growth-associated protein 43; synaptophysin; postsynaptic density protein 95; permanent middle cerebral artery occlusion; neural regeneration*

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Introduction

Cerebral infarction is one of the most common debilitating diseases worldwide. The neuronal death in the infarcted region causes permanent neurologic deficits and significant disability (Go et al., 2014). Spontaneous functional recovery after cerebral ischemia has been shown to be related to brain plasticity and reorganization (Kreisel et al., 2006). Restoring neuronal function in the ischemic penumbra by activating endogenous repair mechanisms that induce neuronal plasticity and reorganization is considered a promising therapeutic strategy for treating ischemic injury. Recent studies suggest that activating endogenous repair mechanisms, as opposed to just reducing the area of the cerebral infarction, may lead to improved recovery of body functions post stroke in experimental animals (Chen et al., 2005; Cui et al., 2013). Understanding the underlying mechanisms of endogenous repair is important for developing new treatment strategies to improve functional outcomes.

Previous studies have demonstrated that brain ischemia can lead to learning and memory impairment (Calabresi et al., 2003; Chen et al., 2015; Gutierrez-Vargas et al., 2015). Environmental enrichment involves providing complex toys to stimulate mice with cerebral ischemic damage, increasing social interaction, and physical exercise (Ohline and Abraham, 2018; Zhang et al., 2018). The environmental enrichment paradigm has been shown to improve functional outcomes after focal cerebral ischemia (He et al., 2017; Sakalem et al., 2017).

The ability to adapt to a changing environment is one of the most important properties of the central nervous system (Pekna et al., 2012). In cerebral ischemic injury, the disruption of synaptic function is a major cause of memory impairment (Stuart et al., 2017; Djurisic et al., 2018; Mazzocchetti et al., 2018). Various medicines and other therapeutic strategies have been used to target the molecules and signaling pathways involved in synaptic remodeling (Zheng et al., 2017, 2018), in an effort to ameliorate symptoms. Numerous studies have focused on the effects of environmental enrichment on angiogenesis and nerve regeneration (Chen et al., 2017; Wu et al., 2018). Environmental enrichment improves angiogenesis, in part, by increasing the levels of CD31 and vascular endothelial growth factor, two specific markers of angiogenesis (Yu et al., 2014; Shilpa et al., 2017). Studies on the mechanisms underlying the effects of environmental enrichment on nerve regeneration have mainly focused on neurotrophic signaling molecules (Hirata et al., 2011; Paban et al., 2011) or neuronal differentiation (Matsumori et al., 2006). Environmental enrichment upregulates the expression of genes such as brain-derived neurotrophic factor and a subset of genes involved in signal transduction (Hirata et al., 2011; Paban et al., 2011). The effects of environmental enrichment on neurogenesis and synaptic plasticity have been reviewed previously (Nithianantharajah and Hannan, 2006).

Growth-associated protein 43 (GAP-43) is an important mediator of structural plasticity of axonal terminals, and it has been suggested to play a vital role in synaptic plasticity and synaptogenesis (Grasselli and Strata, 2013; Hou and Kang, 2016; Holahan, 2017). Several studies have shown that mice lacking one or both copies of the Gap43 gene exhibit defects in learning and memory (Rekart et al., 2005; Holahan et al., 2010).

Focal cerebral ischemia induces neuronal death and synaptic dysfunction in experimental cerebral ischemic models, resulting in cognitive decline (Li et al., 2013). In the present study, we investigated the effects of environmental enrichment on synaptic plasticity and functional outcomes in the mouse model of permanent middle cerebral artery occlusion (pMCAO). Synaptophysin (SYN), postsynaptic density protein 95 (PSD-95) and GAP-43 proteins are important markers of synaptic plasticity and synaptogenesis (Hirata et al., 2011; Pekna and Nilsson, 2012; Johnson et al., 2013; Shilpa et al., 2017). To evaluate synaptic plasticity, we examined the expression of the presynaptic marker SYN (Scheff et al., 2015) and PSD-95 (Li et al., 2009), as well as GAP-43, a representative marker of axonal terminal regeneration (Grasselli and Strata, 2013), using western blot assay. We examined the number of synapses in the hippocampal CA1 region by transmission electron microscopy. Furthermore, we evaluated spatial memory using the Morris water maze (MWM), as an index of function outcome.

Materials and Methods

Animals

Because estrogen has a protective effect following brain injury, only male mice were used in this study. A total of 60 clean male C57BL/6 mice, 8–12 weeks old and weighing 25–28 g, were provided by Jie Si Jie Lab Animal Ltd., Shanghai, China. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Fudan University, China (approval No. 20160858A232) on February 24, 2016.

The mice were housed at 20°C, 45–50% humidity, under a 12-hour dark/light cycle (lighting from 8:00 to 20:00), with water and chow available *ad libitum*. Of the 60 mice, 16 were used as the sham group, and the remaining 44 mice were subjected to the pMCAO procedure. Of these 44 mice, five died, while the surgery failed in another seven. The remaining 32 mice were randomly divided into the environmental enrichment and standard housing groups. A diagram of the experimental design is shown in **Figure 1**.

pMCAO procedure

Before pMCAO surgery, each mouse was deeply anesthetized with 5% isoflurane, and maintained at 2% during surgery. pMCAO on the left side was performed as previously described (Doyle and Buckwalter, 2014). Briefly, a ventral midline incision was made in the neck, and the left common carotid artery was isolated and ligated with a 6-0 silk suture. Next, the internal and external carotid arteries were ligated with 6-0 silk sutures. A 4-0 surgical nylon monofilament with a silicone

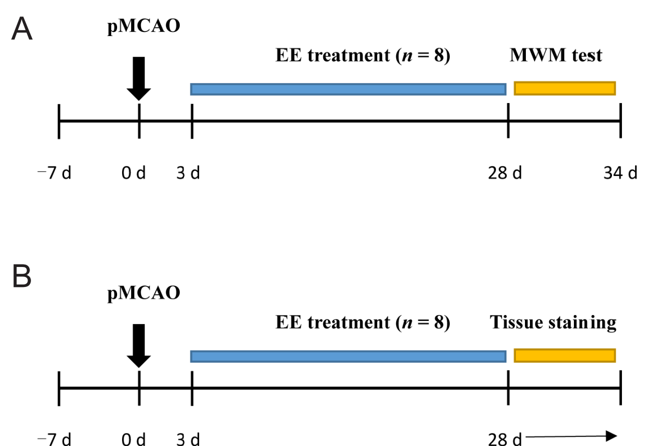

Figure 1 Study design.

All mice were adapted to their environment for 7 d, and then the pMCAO operation was performed. (A, B) Three days after the operation, mice in the EE group were exposed to EE for 28 d (n = 8), while mice in the standard housing group were maintained in standard (non-enriched) housing (n = 8). Mice in the sham group (n = 8) were also maintained in standard housing. Then, the Morris water maze (MWM) test (A) and histological examination (B) were performed. pMCAO: Permanent middle cerebral artery occlusion; EE: environmental enrichment; d: day(s).

tip was inserted into the internal carotid artery through an incision made in the proximal common carotid artery. The filament was advanced approximately 6.5 cm beyond the bifurcation of the internal carotid and external carotid arteries. An electric heating plate was used to maintain the temperature of the operating table at 36.5–37.5°C throughout the entire procedure. The mouse was returned to its cage after recovery from anesthesia. All sham-operated mice received the same procedure, but without artery occlusion.

Monitoring cerebral blood flow using a laser Doppler imaging system

To ensure that the ischemic insult was uniform among all experimental animals, cerebral blood flow was measured with a laser Doppler perfusion and temperature monitor (moorVMS-LDF2, Moor Instruments, Devon, UK) immediately after the filament was advanced into the middle cerebral artery. Briefly, the skull was exposed, and whole brain scans were conducted using the blood flow monitor in each cerebral hemisphere. Digital images were consecutively acquired, and the average instantaneous blood flow was calculated using LDF2 software (Moor Instruments). Cerebral blood flow decreased to 30–35% and remained relatively stable (**Figure 2**). Otherwise, the filament was advanced further into the MCA until cerebral blood flow decreased to the required target level.

Animal groups and environmental enrichment protocol

Three days later, mice were scored for their performance on the beam walk as previously described (Watanabe et al., 2004). Each mouse was placed on a beam (1.5 cm wide, 60 cm long) for 60 seconds. The mouse was then assigned a score between 0 and 6, as follows: good beam balance and walking freely = 0; grasping side of the beam with contralateral two paws = 1; grasped the beam, but with one limb falling off = 2; grasped the beam, but with two limbs falling off = 3; falling off the beam within 40–60 seconds = 4; falling off within 20–40 seconds = 5; unable to stay on the beam = 6. Only mice with a score of 2–4 were considered to successfully model cerebral ischemic injury and were included in the present study. These pMCAO mice were then randomly assigned to the standard housing (n = 8) and environmental enrichment (n = 8) groups. The third group consisted of sham-operated mice given standard housing (sham, n = 8).

In the environmental enrichment group, the home cage was 65 cm wide × 75 cm long × 25 cm high, and contained climbing ladders, plastic tubes and tunnels, running wheels, and small boxes (**Figure 3A**). Environmental enrichment also provided enhanced social stimulation because the mice were group-housed (10 mice; eight mice from the environmental enrichment group and two normal mice). The objects were changed every 3 days to maintain environmental novelty. Standard housing mice were housed in groups of four in standard accommodation (21 cm wide × 27 cm long × 16 cm high), with no objects (**Figure 3B**). The sham-operated mice were also housed in standard accommodation. During enrichment, chow and water were available ab libitum. The mice in the environmental enrichment group were maintained in the environmental enrichment cage for 4 weeks.

MWM test

After environmental enrichment, mice were given the MWM test. The apparatus was a circular pool with a diameter of 150 cm filled with clear water at a temperature of 25 ± 1°C (Stoelting Co., Wood Dale, IL, USA). The pool had four equal quadrants; southwest, northwest, northeast and southeast. A transparent platform (diameter 8 cm) was positioned 1.5 cm below the water surface. The mice (n = 8 per group) were trained to find a hidden platform in the southwest quadrant that permitted escape from swimming, as previously described (Vorhees and Williams, 2006), but with some modifications. Briefly, the mice were placed individually into the pool in a random location to ensure they could not use visual cues on the wall of the pool. The mice were allowed 60 seconds to locate the submerged platform, and if found, were allowed to stay there for 15 seconds. If a mouse failed to locate the platform within 60 seconds, the researcher guided the mouse to the platform and allowed it to remain there for 15 seconds. Each mouse underwent four trials daily for 5 days. The mean escape latency per day was recorded for each mouse. After 5 consecutive days of continuous training, on the probe test day, the platform was removed, and the mouse was permitted to swim for 2 minutes. The distance and the time mice spent in the correct quadrant (northeast), and the frequency of crossings where the submerged platform was previously positioned were measured. The starting position for the probe trial was in the location most distal to the target quadrant. A computer tracking system (ANY-maze version 4.82; Stoelting, Chicago, IL, USA) was used to automatically record the swimming patterns. The escape latency over the 5-day period was used as an index of spatial learning (**Figure 4A**). The percent time, distance swam in the target quadrant and the total number of crossings of the platform location were used as indices of spatial memory (**Figure 4B–E**).

Western blot assay

Ipsilateral hippocampal tissue was collected 28 days after pMCAO. Protein extraction and western blot assay were carried out as previously described (Zhang et al., 2012). Briefly, the tissue was homogenized in radioimmunoprecipitation assay lysis buffer and centrifuged. The protein sample was concentrated and denatured at 95–100°C for 10 minutes. Then, 15 μg of the protein (20 μL) was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (BioRad, Berkeley, CA, USA) and then transferred onto 0.2 μm nitrocellulose membranes (Immobilon, EMD Millipore, Billerica, MA, USA). The membranes were incubated for 1 hour in blocking solution (Beyotime, Shanghai, China) at room temperature. The primary antibodies were as follows: mouse monoclonal anti-GAP-43 (1:2000), mouse monoclonal anti-synaptophysin (1:500), mouse monoclonal anti-PSD-95 (1:1000) and mouse monoclonal anti-β-tubulin (1:1000). The membranes were incubated overnight with

the primary antibodies at 4°C. The membranes were then washed and incubated with secondary antibody (anti-mouse IgG, 1:5000) for 1 hour at room temperature. All antibodies were purchased from Abcam (Cambridge, MA, USA). The intensity of the protein bands was determined using a scanning western blot imaging system and quantified with ImageJ 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 value for that protein. The researcher who performed the image acquisitions and quantifications was blinded to the experimental groupings.

Electron microscopy and synaptic counting

Four weeks after pMCAO, all mice were anesthetized with an overdose of chloral hydrate and intracardially perfused with 0.9% saline followed by 20 mL 4% paraformaldehyde. The brains were removed immediately, and samples (approximately 1 mm^3) were taken from the hippocampal CA1. The tissues were postfixed with 2.5% glutaraldehyde overnight and then processed for transmission electron microscopy as previously described (Zhang et al., 2013). In brief, the tissue samples were osmicated, dehydrated, stained with uranyl acetate, and embedded in araldite. Ultrathin sections (approximately 65 nm thick) were cut from one block of tissue, mounted on copper mesh grids, stained with lead citrate, and then examined using an electron microscope (CM120, Philips, Eindhoven, Netherlands). Micrographs of the neuropil in the hippocampal CA1 region were taken at a magnification of 6000×, and 15 micrographs of each mouse were observed and analyzed. The technique of Colonnier and Beaulieu was used to estimate the number of synapses (Colonnier and Beaulieu, 1985) using the formula $N_V = N_A/d$, where N_V is the number of synapses per unit volume, N_A is the number of synaptic junctions per unit area of an electron micrograph, and d is the mean length of densities associated with the synaptic junctions.

Statistical analysis

All normally distributed data are expressed as the mean ± SEM. The data were analyzed with SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Contralateral and ipsilateral blood flow values after pMCAO were analyzed using the paired *t*-test. The results of western blot assay, the MWM test and the number of synapses were analyzed using one-way analysis of variance followed by Fisher's least significant difference post hoc test. The results of the correlation of cognitive functional outcomes with GAP-43, PSD-95 and SYN were analyzed using Spearman's correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Effects of environmental enrichment on spatial learning and memory

The MWM test revealed that there were no statistically significant differences in the total distance traveled in the four maze zones among the three different groups ($P > 0.05$) (**Figure 4B**). The environmental enrichment group spent less time finding the platform ($P < 0.05$) (**Figure 4A**), traveled significantly longer distances ($P < 0.05$) (**Figure 4C**), spent more time in the correct quadrant (northeast zone) ($P < 0.05$) (**Figure 4D**), and crossed the previous location of the platform more frequently compared with the standard housing group ($P < 0.05$) **(Figure 4E**). The differences between the environmental enrichment and sham groups were not statistically significant ($P > 0.05$) (**Figure 4A, C–E**).

Effects of environmental enrichment on SYN, GAP-43 and PSD-95 expression in the ischemic hippocampus

As shown in **Figure 5**, western blot assay demonstrated that SYN, GAP-43 and PSD-95 protein expression levels were significantly increased in the environmental enrichment group compared with the standard housing group ($P < 0.05$).

Effects of environmental enrichment on the number of synapses in the hippocampal CA1

The number of synapses (N_V) was significantly decreased in the hippocampal CA1 4 weeks after pMCAO ($P < 0.05$; **Figure 6**). Compared with the standard housing group, environmental enrichment attenuated the pMCAO-induced decrease in the number of synapses in the hippocampal CA1 ($P < 0.05$; **Figure 6**).

Correlation of cognitive functional outcomes with GAP-43, PSD-95 and SYN

We next examined whether cognitive functional outcomes correlated with synaptic protein expression. The number of crossings of the previous platform location, used as an index of cognitive function, was compared to the relative expression levels of GAP-43, PSD-95 and SYN using Spearman's correlation coefficient (r_s). The correlations between cognitive function and relative expression of GAP-43, PSD-95 and SYN are shown in **Figure 7**. The cognitive functional outcomes were positively correlated with the relative expression levels of GAP-43, PSD-95 and SYN (**Figure 7**).

Discussion

Our current findings show that environmental enrichment improves cognitive impairment induced by the pMCAO model of stroke. Environmental enrichment starting 3 days after pMCAO robustly upregulated the expression of the axonal terminal plasticity marker GAP-43 and the synaptic plasticity markers SYN and PSD-95 28 days post-stoke. The number of synapses in the hippocampal CA1 was also increased by environmental enrichment. Furthermore, we found that the recovery of cognitive function was positively correlated with the expression of GAP-43, a marker of post-stroke axonal terminal plasticity (Stokowska et al., 2017), and with the expression of SYN and PDS-95.

To study the effects of environmental enrichment in post-stroke synaptic plasticity and functional recovery, we used the pMCAO stroke model, which results in irreversible damage to the ischemic core in specific brain regions (Doyle and Buckwalter, 2014). This model produces a functional disorder that is repeatable and consistent, thereby facilitat-

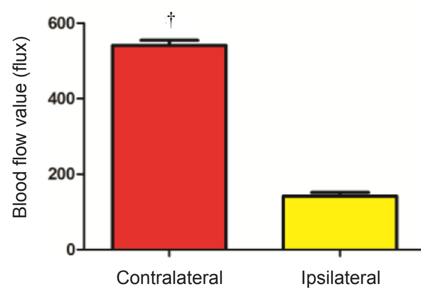

Figure 2 Contralateral and ipsilateral blood flow values.
Contralateral and ipsilateral blood flow values after permanent middle cerebral artery occlusion. †P < 0.05, *vs.* ipsilateral. Data are expressed as the mean ± SEM (n = 8; paired t-test).

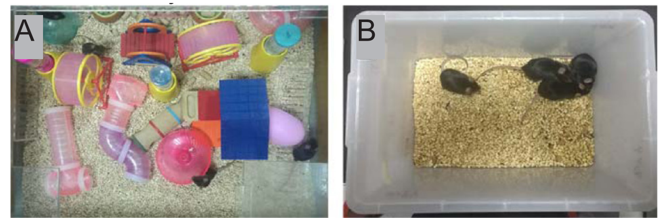

Figure 3 Environmental enrichment and standard housing conditions.
(A) Environmental enrichment; (B) standard housing.

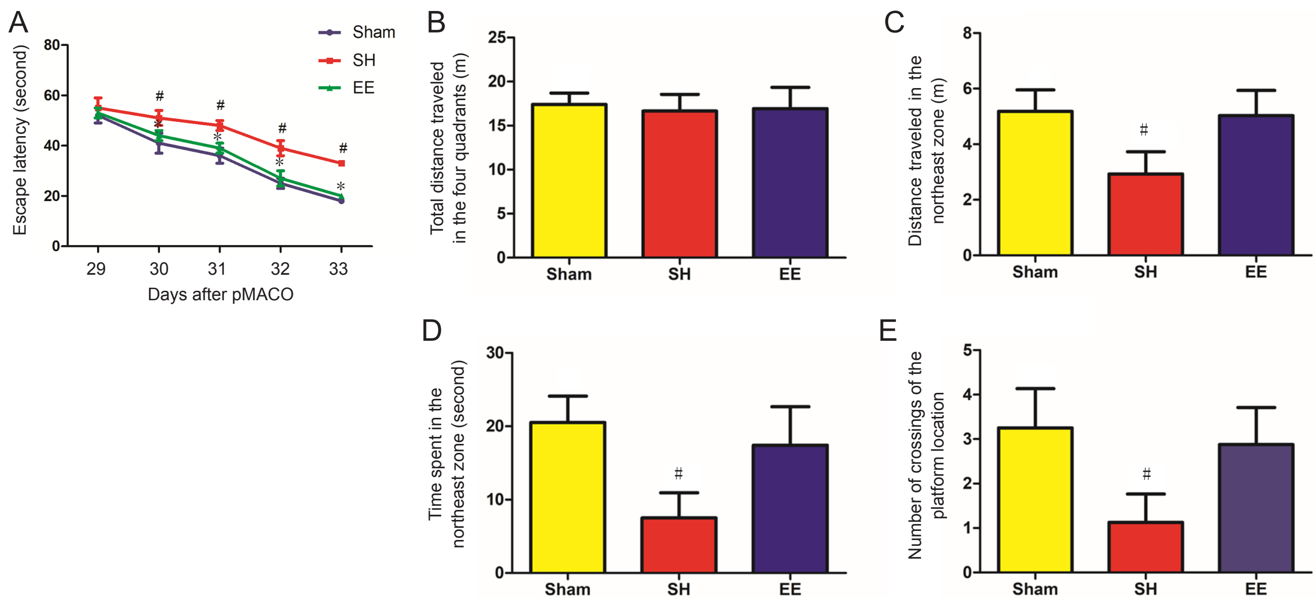

Figure 4 Effect of EE on spatial learning and memory (Morris water maze test).
(A) Escape latency. (B) In the probe test, there were no significant differences in the total distance traveled in the four quadrants (zones) among the groups. (C–E) The EE group traveled significantly longer distances, spent more time in the correct (northeast) quadrant, and crossed the platform location more frequently compared with the SH group. *P < 0.05, *vs.* sham group. #P < 0.05, *vs.* EE group. Data are expressed as the mean ± SEM (n = 8; one-way analysis of variance followed by Fisher's least significant difference *post-hoc* test). pMCAO: Permanent middle cerebral artery occlusion. EE: environmental enrichment; SH: standard housing; NE: northeast.

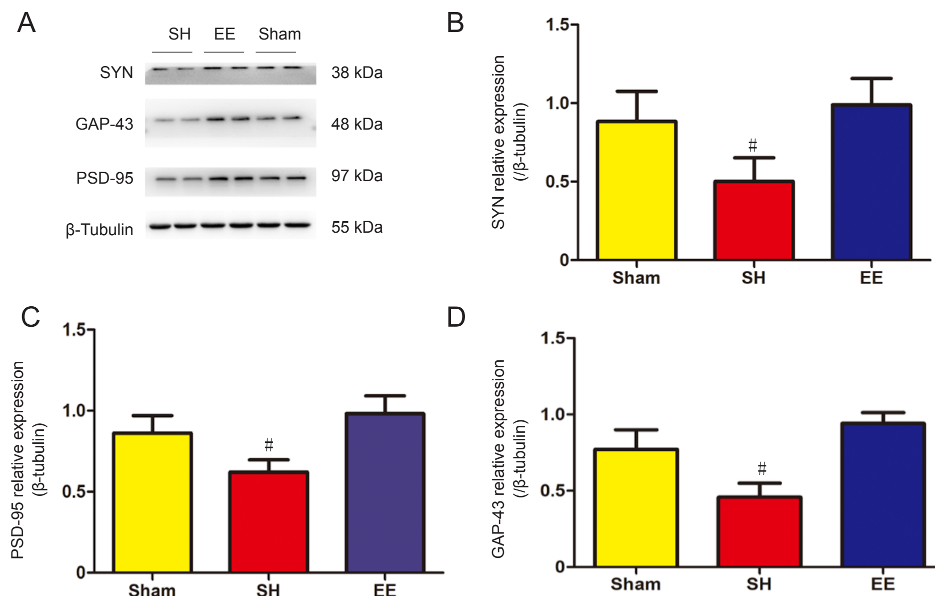

Figure 5 Effect of EE on SYN, GAP-43 and PSD-95 expression levels in the ischemic hippocampus.
(A) Representative western blots. (B–D) Quantitative analysis of western blots. Expression levels of GAP-43, SYN and PSD-95 were significantly increased in the ischemic hippocampus 4 weeks after permanent middle cerebral artery occlusion in the EE group. There was no significant difference between the EE and sham groups. #P < 0.05, *vs.* EE group. Data are expressed as the mean ± SEM (n = 8; one-way analysis of variance followed by Fisher's least significant difference *post-hoc* test). GAP-43: Growth-associated protein 43; PSD-95: postsynaptic density protein 95; SYN: synaptophysin; EE: environmental enrichment. SH: standard housing.

ing the study of post-stroke neuronal dysfunction (Lay and Frostig, 2014).

It has been reported that animals exposed to environmental enrichment have improved functional outcomes following ischemic injuries (Nygren and Wieloch, 2005; Sakalem et al., 2017). The mechanisms include enhanced neurogenesis (Komitova et al., 2005), dendritic restructuring (Johansson and Belichenko, 2002; Johansson, 2004), and angiogenesis (Yu et al., 2014; Shilpa et al., 2017). It is widely accepted that synaptic plasticity and functional remapping play important roles in functional recovery after stroke (Nithianantharajah and Hannan, 2006; Pekna et al., 2012; Li et al., 2017). However, only a few studies have focused on the regeneration of axonal terminals, the changes in presynaptic and postsynaptic proteins, and the simultaneous changes in the number of synapses.

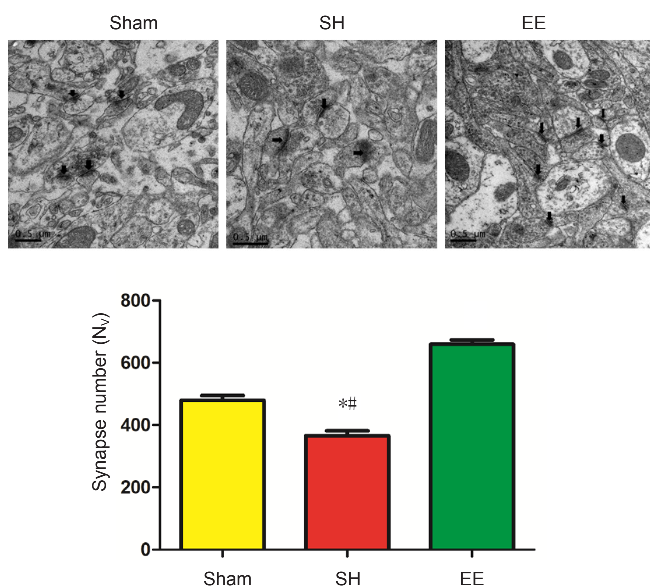

Figure 6 Effect of EE on the number of synapses in the hippocampal CA1.
The number of synapses (N_V, the number of synapses per unit volume) was markedly reduced in the hippocampal CA1 area 4 weeks after permanent middle cerebral artery occlusion. Black arrows indicate synapses in each micrograph. EE attenuated the occlusion-induced decrease in the number of synapses in the hippocampal CA1. Scale bars: 0.5 μm. Data are expressed as the mean ± SEM. $*P < 0.05$, *vs*. sham group; $\#P < 0.05$, *vs*. EE group ($n = 8$; one-way analysis of variance followed by Fisher's least significant difference *post-hoc* test). EE: Environmental enrichment; SH: standard housing.

Axonal plasticity is an important mechanism that leads to the formation of new synapses after ischemic injury. Previous studies have demonstrated that this phenomenon is associated with robust upregulation of the membrane phosphoprotein GAP-43, which is critically involved in axonal terminal growth (Benowitz and Routtenberg, 1997; Carmichael et al., 2005; Allegra Mascaro et al., 2013). GAP-43 is upregulated in neuronal regeneration, and is a sensitive marker of axonal regeneration in the hippocampus (Bomze et al., 2001). Recently, GAP-43 was shown to be involved in glial cell plasticity and to enhance neuronal plasticity (Hung et al., 2016). Here, we found that the expression of GAP-43 is increased in the hippocampus 28 days after pMCAO in mice exposed to environmental enrichment. The enhanced cognitive recovery in these mice suggests that environmental enrichment facilitates post-stroke synaptic plasticity, possibly associated with axonal terminal regeneration.

Synaptogenesis is considered essential for learning and memory. SYN and PSD-95 are two important markers associated with synaptogenesis. SYN is a transmembrane glycoprotein that plays a fundamental role in synaptic plasticity and synaptogenesis (Jahn et al., 1985; Ujike et al., 2002). A previous study showed that SYN is involved in hippocampus-dependent cognition after cerebral ischemic injury (Dandi et al., 2018). SYN knockout mice exhibit impaired spatial learning and memory without limb function impairment (Schmitt et al., 2009). PSD-95 is another important synaptic marker related to synaptogenesis. It is the most representative protein member of the postsynaptic density protein family (Niethammer et al., 1996), which plays an important role in synaptic plasticity and in learning and memory (Xu et al., 2009). Several previous studies have demonstrated that PSD-95 expression is downregulated in the hippocampus after cerebral ischemia (Watanabe et al., 2004; Yan et al., 2013). Our present results show that environmental enrichment prevents the reduction in PSD-95 expression, thereby helping to maintain synaptic plasticity

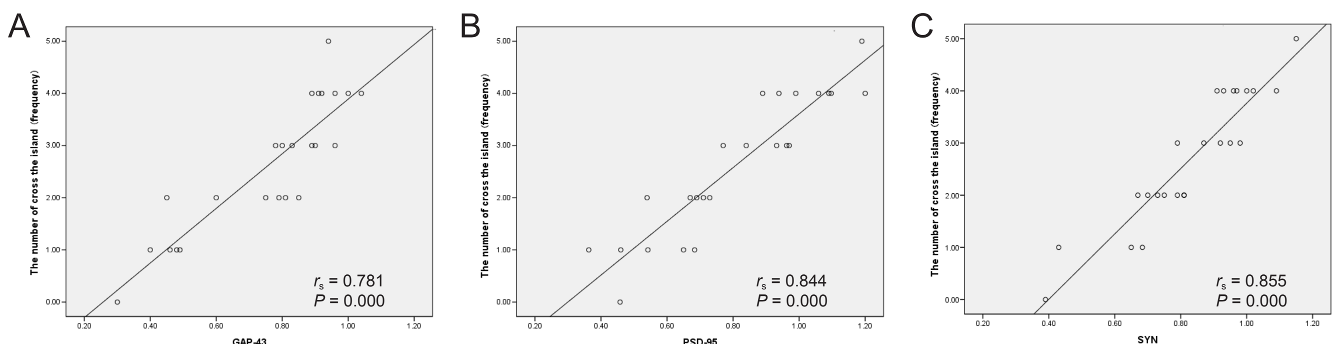

Figure 7 Correlation of cognitive function with the relative expression levels of GAP-43, PSD-95 and SYN.
(A–C) GAP-43, PSD-95 and SYN, respectively. r_s: Spearman's correlation coefficient. $P < 0.001$ was considered to indicate a significant correlation. GAP-43: Growth-associated protein 43; PSD-95: postsynaptic density protein 95; SYN: synaptophysin.

in the hippocampus. Furthermore, transmission electron microscopy showed that environmental enrichment increased the number of synapses in the hippocampal CA1 4 weeks after pMCAO. Therefore, environmental enrichment not only increases the expression of synaptic remodeling-related proteins, but also increases the number of functional synapses in the hippocampus. These findings are novel and have not previously been reported. Further study is needed to more fully elucidate the cell and molecular mechanisms mediating the functional improvement induced by an enriched environment. For example the roles of GAP-43, SYN and PSD-95 in this process can be examined using transgenic methods and viral vectors to up- or downregulate their levels in specific brain regions.

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