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ORIGINAL PAPER

Effects of Treadmill Exercise on Motor and Cognitive Function Recovery of MCAO Mice Through the Caveolin-1/VEGF Signaling Pathway in Ischemic Penumbra

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Abstract

Exercise has been regarded as an effective rehabilitation strategy to facilitate motor and cognitive functional recovery after stroke, even though the complex effects associated with exercise-induced repair of cerebral ischemic injury are not fully elucidated. The enhancement of angiogenesis and neurogenesis, and the improvement of synaptic plasticity following moderate exercise are conducive to functional recovery after ischemic damage. Our previous studies have confirmed the angiogenesis and neurogenesis through the caveolin-1/VEGF pathway in MCAO rats. As an essential neurotrophic factor, BDNF has multiple effects on ischemic injury. In this study, we attempted to determine an additional mechanism of treadmill exercise-mediated motor and cognitive functional recovery through the caveolin-1/VEGF pathway associated with BDNF in the ischemic penumbra of MCAO mice. We found that mice exposed to treadmill exercise after the MCAO operation showed a significant up-regulation in expression of caveolin-1, VEGF, BDNF, synapsin I and CYFIP1 proteins, numbers of cells positive for BrdU/CD34, BDNF, BrdU/NeuN, BrdU/Synapsin I and CYFIP1 expression were increased, which support the reduction in neurological deficit and infarction volume, as well as improved synaptic morphology and spatial learning abilities, compared with the non-exercise mice. However, the caveolin-1 inhibitor, daidzein, resulted in increase in neurological deficit and infarction volume. The selective VEGFR2 inhibitor, PD173074, significantly induced larger infarction volume and neurological injury, and decreased the expression of BDNF in the ischemic penumbra. These findings indicate that exercise improves angiogenesis, neurogenesis and synaptic plasticity to ameliorate motor and cognitive impairment after stroke partially through the caveolin-1/VEGF pathway, which is associated with the coregulator factor, BDNF.

Keywords Treadmill exercise · Caveolin-1/VEGF · BDNF · MCAO · Function recovery

Introduction

Stroke ranks the second leading cause of long-term disability-related deaths worldwide [1], and it is closely associated with an increased risk for subsequent dementia. 10% of patients suffered from pre-stroke dementia, 10% progressed to post-stroke dementia soon after their first stroke, and more than a third exhibit dementia after recurrent stroke [2]. Post-stroke cognitive impairment (PSCI) in the elderly is related to a high risk of functional decline, incapacitation and even death [3]. However, the mechanisms of motor dysfunction and cognitive impairment after stroke have not been fully elucidated, thus the therapeutic options for stroke are limited. Multiple pathophysiological changes following cerebral ischemia, including brain edema, neuronal loss and synaptic dysfunction, can result in motor and cognitive function decline [4, 5]. Brain tissue after ischemia is subjected to irreversible neuronal damage resulting from necrotic cell death; however, there are still salvageable and metabolically active cells in the tissue around the infarct, named ischemic penumbra, in which cell death is slower, and this region is a potential focus [6]. Our previous studies have illustrated that treadmill exercise can accelerate the recovery of neurological function by improving angiogenesis and neurogenesis in middle cerebral artery occlusion MCAO rats partially

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through the caveolin-1/VEGF signaling pathway [7–9], but what mediates their signaling remains unknown.

Evidently, the spatial learning in brain derived neurotrophic factor (BDNF) genetic knockout mice was significantly impaired. Moreover, a significant blunting of fear or spatial memories and attenuated hippocampal LTP were examined in the BDNF or TrkB gene deleted adult mice forebrain [10]. While BDNF level was up-regulated in the ischemic penumbra to aid rehabilitation recovery by enhancing angiogenesis [11] and neurogenesis [12], improving synaptic plasticity, and promoting axon-dendrite differentiation, growth and guidance [13, 14]. Nevertheless, few studies have focused on the relationship between BDNF and caveolin-1/VEGF signaling for facilitating motor and cognitive function recovery after stroke via these above mechanisms. BDNF was likely a new participator in this pathway.

Vascular endothelial growth factor (VEGF) is the most potent angiogenic and neurotrophic factor induced by ischemia and hypoxia via mediating the processes of post-ischemic brain and vessel repair, cerebral edema, neuroprotection [15, 16]. Mice treated with an intraventricular administration of VEGF-A before the MCAO operation is helpful for enhancing the survival of the penumbra, improving cerebral blood flow and balancing the brain energy state after ischemic injury due to facilitated mature, functional blood vessel formation [17]. On the other hand, in vivo and in vitro research on the cerebral ischemia model has shown that VEGF can stimulate neuronal viability and function by maintaining the membrane potential, the excitability of neurons and the spontaneous excitatory postsynaptic transmission, which improves synaptic plasticity and cognitive repair [18]. Vascular endothelial cells can synthesize and secrete bioactive BDNF, fostering axonal growth significantly [19, 20]. Up-regulation of both VEGF and BDNF by neuroprotective therapies after cerebral ischemic injury shows synergistic effects on angiogenesis, brain plasticity and functional recovery [21, 22]. Furthermore, in vitro, BDNF expression is significantly decreased in cultured mouse brain endothelial cells with VEGFR2 antagonist intervention [23]. VEGF stimulates BDNF by promoting angiogenesis for nerve cell development in the adult songbird brain, indicating that VEGF-induced vasculature may substantially increase the production and release of BDNF [24]. Overall, we inferred from these results that VEGF plays a partial regulatory role in the biological function of BDNF.

Caveolae/caveolins can mediate blood–brain barrier (BBB) permeability, prosurvival potency and the efficacy of neurotransmitter signaling, angiogenesis, neuroinflammation and preconditioning effects. Caveolin-1 has been defined as a major indispensable structure protein [25, 26]. Evidence suggests that caveolin-1 mediates synaptic plasticity during development and aging, as well as in the damaged adult CNS [27, 28]. Caveolin-1 knockout mice exhibit a distinct behavioral phenotype, such as cholinergic hypofunction and spatial memory impairment [29]. BDNF can regulate dendritic branching of hippocampal neurons via the caveolin-1-mediated endocytic pathway [30]. Caveolin-1 might play a neuroprotective role by NMDA- and BDNF-mediated signaling pathways with Cav-1 KO primary neuron after treatment with a SynCav1-containing lentivirus [31]. Additionally, P75NTR, which links with neurotrophins in lipid rafts, was coimmunoprecipitated with the truncated beta-caveolin-1 isoform. P75NTR-BDNF complexes were transferred from Trk via beta-caveolin-1-containing lipid rafts and possibly intracellular endosomes, concluding that this trafficking mechanism plays an essential role in neuronal development [32]. Thus, we postulate that BDNF may be an effective synergistic molecule of caveolin-1 signaling but little evidence has been established in the field of ischemia.

Moderate exercise training can trigger angiogenesis and neurogenesis and improve synapse formation in ischemic penumbra, contributing to motor and cognitive functional recovery after stroke. It confirmed that low-intensity exercise was beneficial to improve synaptic plasticity and spatial memory performance [33, 34]. Thus, in this present study, we further investigated the possible mechanisms underlying treadmill exercise-induced caveolin-1/VEGF mediated functional improvement in MCAO mice.

Materials and Methods

Animal Grouping

Two hundred and four adult male *C57BL/6* mice (weighing 22–25 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All mice were acclimated to the environmental conditions for at least 1 week before an operation. The mice were housed 3–4 mice per cage under the same animal care surroundings, under a 12-h/12-h light/dark cycle at a constant temperature (22 ± 1 °C) and humidity (50 ± 10%) and with ad libitum access to food and water. All mice were randomly assigned to five groups: Sham-operated group (S, $n = 24$); MCAO group (M: MCAO group, $n = 42$; VICM: VEGFR2 inhibitor control MCAO group, $n = 6$); exercise and MCAO group (EM, $n = 42$); inhibitor and MCAO group (IM: caveolin-1 inhibitor MCAO group, $n = 42$; VIM: VEGFR2 inhibitor MCAO group, $n = 6$) and inhibitor, exercise and MCAO group (IEM: caveolin-1 inhibitor, exercise MCAO group, $n = 42$). Subsequently, mice in the M, EM, IM, and IEM groups were divided randomly into 7-day and 14-day groups. All procedures were performed in strict accordance with recommendations established in the Guide for the National Science Council of the Republic of China. All experimental procedures were approved

by the Animal Research Committee of Wenzhou Medical University.

MCAO Model of Focal Cerebral Ischemic Stroke

The model of middle cerebral artery occlusion (MCAO) surgery for mice was performed as described previously [35]. Using a small animal anesthesia machine (Shen Zhen RWD Life Science Co., Ltd) and an operating microscope, mice were anesthetized with a nose cone providing continuous inhalation of 0.5–1% isoflurane (0.4 l/min), fixed on a foam board in a supine position, and then an approximately 1.0-cm incision was made on the neck to expose and strip the left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) without damaging the surrounding muscles and nerves. After clipping the CCA and ICA with microsurgical clips, a very small notch was cut in the ECA, and an appropriately sized 6-0 surgical monofilament nylon suture with a rounded tip (Guangzhou Jialing Biotechnology Co., Ltd., Guangzhou, China) was carefully inserted into the ICA from the bifurcation via the ECA and advanced approximately 10 mm past the carotid bifurcation until a slight resistance was felt, temporarily obstructing the MCA at its origin. The intra-operative and post-operative mice were all kept at 37 °C with a heating pad (awake in about 5 min). After 1 h of occlusion, the mice were anesthetized again and the filament was gently withdrawn to restore blood flow and allow reperfusion for 7 days or 14 days. The sham group mice were subjected to the same surgical procedure exactly as the experimental groups except for blocking or decreasing the blood flow of the MCA. For reducing errors as far as possible, we completed a batch of MCAO models almost at the same time (2–3 days) to detect each target indicator in different groups, and we followed the standard of Zea Longa and chose the ischemia mice which exhibited right hemiplegic symptom (unable to fully extend the right forelimb or circling to the side of the hemiplegia or fall to the side of the hemiplegia) as the successful models.

Injecting the Caveolin-1 Inhibitor, the VEGF Inhibitor and Bromodeoxyuridine

A caveolin-1 inhibitor, daidzein, was dissolved in DMSO (1 ml), diluted with phosphate buffer (49 ml) and then injected (0.4 mg/kg/day, s.c.) into the mice in the IM and IEM groups every 24 h following reperfusion for 7 or 14 days. At the same time, an equal volume of saline was injected into the mice in the S, M and EM groups following reperfusion. The selective, dose-dependent VEGFR2 inhibitor, PD173074, was also dissolved in DMSO (1 ml), diluted with phosphate buffer (49 ml) and then injected (2 mg/kg/day, i.p.) into the VIM group mice every 24 h following reperfusion for 7 days, and the VICM group mice received an equal volume of saline at the same time.

Mice in each group were subjected to injections of bromodeoxyuridine (BrdU; 50 mg/kg/day, i.p.) for 3 consecutive days before sacrifice to label dividing cells in vivo by double immunofluorescence techniques.

Physical Exercise

An animal electric treadmill machine (XR-PT-10A, Shanghai XinRuan Information Technology Co., Ltd. China) was used in this study. Mice in the exercise groups underwent a 3-day familiarization period of treadmill training at a speed of 5 m/min before the MCAO surgery. At 24 h after the MCAO operation, the formal exercise was conducted at 8–10 m/min for 30 min/day and continued 5 days/week for a total of 7 or 14 consecutive days until animals were sacrificed. During running, electrical or acoustic stimulation was administered to compel the mice to run forward. The other groups received the same intensity of stimulation on the treadmill for an almost identical time period but in static mode.

Neurological Functional Assessment

For neurological deficit assessment, all mice were scored on a modified neurological grading system with the standard of Zea Longa, a five-point scale (0–5), at 24 h, 7 days and 14 days after brain impairment. The scoring was described as follows: 0 = no neurological deficits and normal limb movements; 1 = unable to fully extend the right forelimb; 2 = circling to the side of the hemiplegia; 3 = fall to the side of the hemiplegia; 4 = no movement or spontaneous walking and disturbance of consciousness; 5 = death. Only mice with scores of 1–3 were admitted to the next experiments in the study.

Brain Infarct Volume Measurement

After the final neurologic functional scoring, MCAO-treated mice were deeply anesthetized with 4% chloral hydrate (10 ml/kg, i.p.), and the heart was perfused with precooled 0.9% saline to remove blood. Brains were quickly removed and placed in a refrigerator at − 20 °C for 10 min. One-millimeter coronal sections were obtained by sectioning the cooled brain tissues. The sections were rapidly placed into 2% TTC (2, 3, 5-triphenyltetrazolium chloride) solution (37 °C) for 30 min in the dark followed by fixation in 4% paraformaldehyde buffer for 10 min. Then, the infarct area and volume of the brain was measured and calculated as (contralateral hemispheric volume − ipsilateral non-infarcted volume/contralateral hemispheric volume) using Image-Pro Plus 6.0 image analyzer.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to detect the synaptic ultrastructure in the ischemic penumbra in each group mice. Briefly, fresh brain tissues were quickly removed after heart perfusion with precooled 0.9% saline and 2.5% glutaraldehyde (0.1 mol/l phosphate buffer, pH 7.4) sequentially, and then ischemic penumbra area of brain tissues (1 mm^3) was cut quickly and fixed in 2.5% glutaraldehyde immediately at 4 °C for at least 2 h. Fixed tissues were rinsed for 15 min in phosphate buffer three times and post-fixed in 1% osmic acid for 1 h at 37 °C. After rinsing with phosphate buffer and distilled water for 15 min twice each, tissues were stained with 1% uranyl acetate for 2 h then dehydrated in gradient acetone solutions (50%, 70%, 80%, 90% once for 15 min, and 100% two times for 10 min each), soaked in acetone: entrapped liquid (1:1 for 2 h at 37 °C and 1:4 at 37 °C overnight; in pure entrapped liquid for 2 h at 45 °C), and embedded for 3 h at 45 °C and 48 h at 65 °C. Finally, the tissues were cut into ultrathin sections, which were observed using TEM.

Immunofluorescence Staining

To evaluate whether the treadmill exercise and caveolin-1 inhibitor influenced angiogenesis, neurogenesis and synaptic plasticity in the mouse brains after ischemia, BDNF, BrdU/NeuN, BrdU/CD34, BrdU/Synapsin I, and CYFIP1 immunofluorescence staining were performed ($n = 6$ each group). Pre-fixed brain tissues were quickly removed after heart perfusion with precooled 0.9% saline and 4% paraformaldehyde sequentially. Then, the infarcts were separated and fixed in 4% paraformaldehyde for 24 h, and dehydrated in 15%, 20% and then 30% sucrose solution respectively until the tissues sank to the bottom. After embedding with OCT, we stored these tissues in the refrigerator at − 80 °C for making 8 μm frozen sections affixed to slides with freezing microtome. Sections were dried and restored to room temperature for at least 30 min then soaked with 0.01 M phosphate buffered saline (PBS, pH 7.6) three times for 5 min with slow shaking on a table concentrator. Subsequently, the sections were blocked with 10% goat serum or 5% bovine serum albumin (BSA) containing 0.3% Triton X-100 for 1 h (RT; 20–22 °C). For BrdU/NeuN, BrdU/CD34 and BrdU/Synapsin I double immunofluorescence staining, 2N HCL was used before blocking to denature DNA by incubating the sections for 30 min at 37 °C followed by borate saline buffer (pH 8.4) twice for 10 min. The sections were incubated in primary antibodies as follows: rat anti-BrdU (1:200, ab6326, Abcam) and rabbit anti-NeuN (1:300, ab177487, Abcam) mixture, rat anti- BrdU and rabbit anti-CD34 (1:200, ab81287, Abcam) mixture, rat anti-BrdU and rabbit anti-synapsin I (1:500, ab8, Abcam) mixture, rabbit anti-BDNF (1:500, ab108319, Abcam), and goat anti-CYFIP1 (1:50, sc-49935, Santa Cruz) overnight at 4 °C. Afterward, the sections were washed with 0.01 M PBS three times for 5 min and incubated with corresponding goat anti-rat-CY3, goat anti-rabbit-FITC and donkey-anti-goat-CY3 (1:500, Beyotime, China) secondary antibodies for 1 h at room temperature. All sections were examined by a fluorescence microscope (BX51, Olympus) to observe and analyze the fluorescent signals in four fields of ischemic core and border zone.

Western Blot

To analyze the expression levels of caveolin-1, VEGF, synapsin I, BDNF and CYFIP1 proteins, mice were sacrificed at 7 or 14 days after reperfusion ($n = 6$ each group). After heart perfusion with only precooled 0.9% saline, the fresh brains were removed immediately, frozen with liquid nitrogen quickly, and then the ischemic penumbra was dissected out on ice as described previously [7]. For the mouse, the midline is between the two hemispheres and we made a longitudinal cut approximately 1 mm from the midline through the ischemic ipsilateral hemispheres with a mice brain matrix based on previous experiments (Fig. 1a). The brain tissue was homogenized in cold RIPA buffer (50 mM Tris buffer, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; and 0.1% sodium dodecyl sulfate) containing 1 mM PMSF (10 μl/ml) and then centrifuged at 5000 rpm followed by 12,000 rpm at 4 °C for 5 min each. According to the Micro BCA kit procedure, standard bovine serum albumin was used to determine the total protein concentration of each sample. An equal amount of protein (30 μg) in each sample was separated on 8% (for synapsin I and CYFIP1) or 12% (for caveolin-1, VEGF and BDNF) SDS–polyacrylamide gels by electrophoresis and transferred onto PVDF membranes in cold buffer (wet conditions). Membranes were incubated in 5% skimmed milk diluted by 0.1% TBST to block the nonspecific binding sites for 2 h at room temperature. After washing with 0.1% TBST buffer three times for 5 min, the membranes were incubated with corresponding primary antibodies anti-caveolin-1 (1:10,000, ab192869, Abcam), anti-VEGFA (1:1000, ab46154, Abcam), anti-BDNF (1:1000, ab108319, Abcam), anti- synapsin I (1:1000, ab8, Abcam), anti-CYFIP1 (1:500, sc-49935, Santa Cruz) and anti-tubulin (1:1000, AP0064, Bioworld technology) at 4 °C overnight. HRP-conjugated goat anti-rabbit (1:5000, BL003A, Biosharp) and donkey anti-goat (1:5000), secondary antibodies were used to visualize the immunoreactivity after rinsing with 0.1% TBST buffer three times for 5 min. The relative expression levels of the immunocomplex signals were digitally quantified using a UVP gel-imaging system (Upland, CA, USA), normalized to β-tubulin expression and analyzed via AlphaEaseFC (version 4.0).

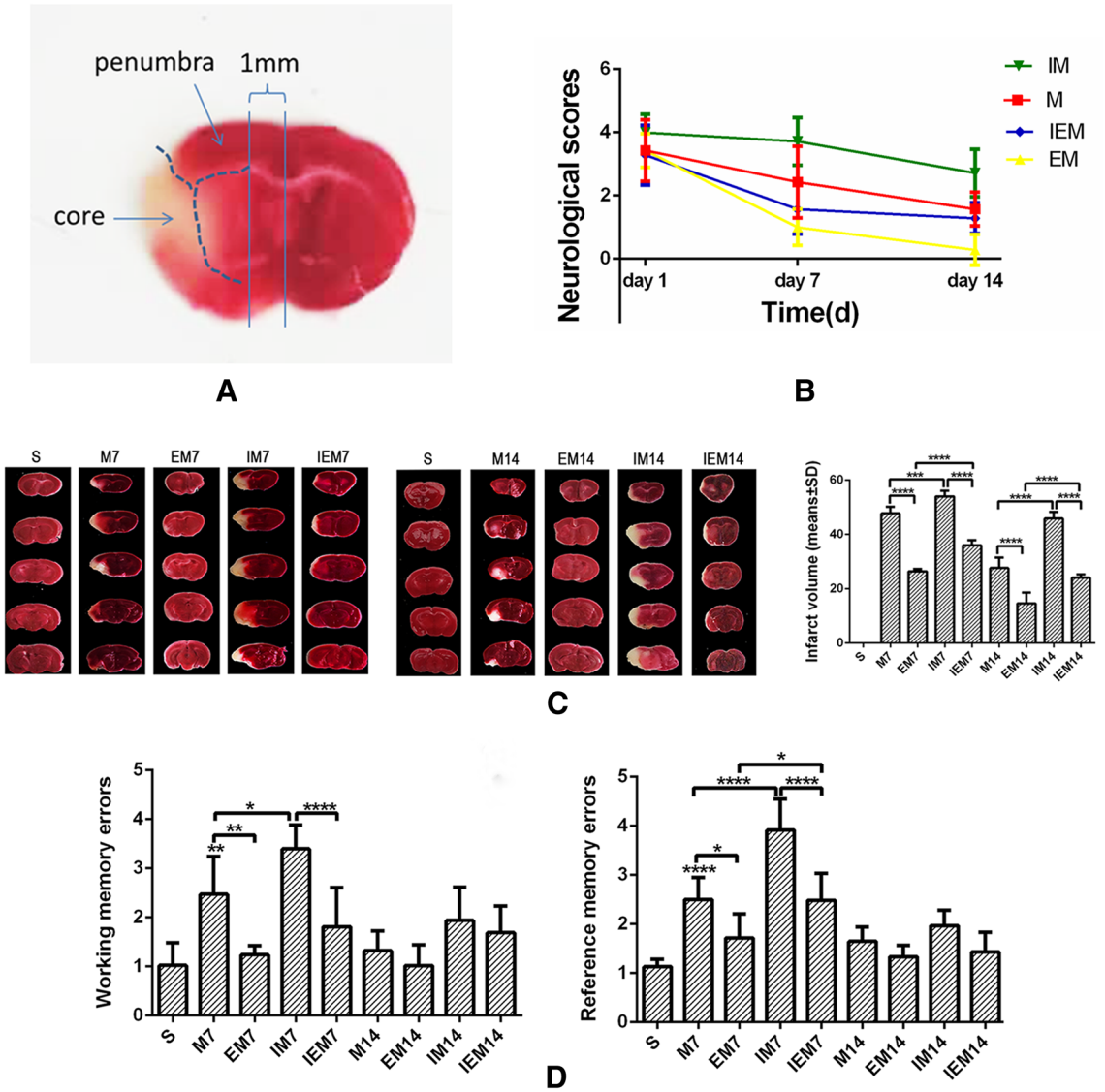

Fig. 1 Effects of treadmill exercise and caveolin-1 inhibition on neurological score and cerebral infarction volume. **a** The core and penumbra area of MCAO mouse. **b** Neurological scores in each group at 1, 7 and 14 days after MCAO followed by 7 and 14 days of treadmill exercise or caveolin-1 inhibitor or both of them ($n=21$). **c** Infarct volume stained by TTC in each group mice after MCAO followed by 7 and 14 days of treadmill exercise or caveolin-1 inhibitor or both of them. **d** Effects of treadmill exercise on spatial learning abilities reflected by working memory errors reference memory errors ($n=3$). Caveolin-1 inhibitor increased the volume of infarction and the neurological scores, and induced worse memory of MCAO mice, while treadmill exercise reduced the volume of infarction and the neurological scores, and enhanced memory. Data are expressed as the means ± SD and were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor exercise MCAO group

Eight-Arm Radial Maze Test

This behavioral test was conducted at 7 or 14 days after transient cerebral ischemia reperfusion. As in the previously described method, spatial learning ability was evaluated by using an 8-arm radial maze apparatus, which has a diameter of 22 cm across an octagonal arena with eight radiating arms, each measuring 30 cm in length, 10 cm in width and 100 cm above the floor. Prior to formal radial arm maze training, mice were fed restrictive diets to maintain each mouse at a weight no less than 85% of its free-feeding weight at the start of experiments to stimulate foraging for food in the maze. During the test, food bait was placed in the receptacles located at the end of each arm. Prior to the real test for spatial learning ability, consecutive training was performed three times. Mice were placed individually in the center of the maze and allowed to explore for food freely, and movement orbits were recorded during three sessions

every day for 5 days/week. A session finished when the mouse had entered into each of the eight arms and found the food at least once, or the maximum time limit of 5 min had elapsed. The number of times revisiting a previously visited arm with food bait was counted as a working memory error, and the number of times re-entering an arm without food bait was defined as a reference memory error, which were used to evaluate short-term memory and long-term memory, respectively, and learning and memory abilities were assessed after MCAO.

Statistical Analysis

Data are defined as the mean ± SD and were analyzed using GraphPad Prism 6.0 statistical software. Statistical significance was determined using one-way analyses of variance (ANOVAs) and Student's t-tests. Values were considered significant at $P < 0.05$.

Results

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on Neurological Score and Cerebral Infarction Volume

We assessed the neurological function of mice by Zea Longa scores after ischemic injury ($n = 21$, Table 1; Fig. 1b), and the sham-operated mice (S group) had no neurological symptoms and scored 0. On the first day after MCAO surgery, mice in other groups were scored closely without any significant differences in neurological statuses ($P > 0.05$). MCAO group mice exposed to caveolin-1 inhibitor (IM group) for 7 and 14 days exhibited significantly worse neurological statuses (3.71 ± 0.76 and 2.71 ± 0.76, respectively) compared with the MCAO group (M group: 2.43 ± 1.13 and 1.57 ± 0.53, respectively, $P < 0.05$), while the MCAO mice exposed to treadmill exercise (EM group) for 7 and 14 days exhibited a significantly better neurological outcome (1.00 ± 0.58 and 0.29 ± 0.49, respectively) compared with the ischemia without exercise group (M group: 2.43 ± 1.13 and 1.57 ± 0.53, respectively, $P < 0.05$). At 14 days after MCAO surgery, the IEM group (caveolin-1 inhibitor, exercise MCAO) mice scored significantly lower (1.29 ± 0.49) than the IM group (caveolin-1 inhibitor MCAO: 2.71 ± 0.76, $P < 0.05$).

Except for the sham group mice, mice in other groups exhibited different substantial areas of infarct in the ipsilateral hemispheres as determined by TTC staining at 7 and 14 days after MCAO ($n = 3$, Table 2; Fig. 1c). At 7 and 14 days after MCAO, compared with the M group mice (47.75% ± 2.43 and 27.70% ± 3.84, respectively), the IM group mice demonstrated a significantly greater volume of damage (54.03% ± 2.08 and 45.86% ± 2.41, respectively, $P < 0.05$). After 7 and 14 days treadmill exercise, EM group mice showed the smallest lesion in both cortical and subcritical areas (26.41% ± 0.86 and 14.60% ± 4.02, respectively, $P < 0.05$). The infarct volumes of mice in the IEM group (36.00% ± 1.91 and 24.09% ± 1.24, respectively) were significantly reduced compared with those in the IM group (54.03% ± 2.08 and 45.86% ± 2.41, respectively, $P < 0.05$) at 7 and 14 days after MCAO.

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on the Spatial Learning Abilities

Two different types of performance errors, including working memory errors and reference memory errors, were examined simultaneously using an 8-arm radial maze to measure spatial learning ability. A one-way ANOVA on the working memory errors revealed that mice in the S group made the fewest number of errors compared with the other mice exposed to cerebral ischemia $P < 0.05$). Mice in the IM group exhibited significantly more errors than the mice in the M and IEM groups ($P < 0.05$). Mice in the EM group made significantly fewer errors than the mice in the M group at 7 days after MCAO ($P < 0.05$). However, we did not find any significant differences among the groups at 14 days after MCAO ($P > 0.05$). The performance of reference memory errors across the 7-day

Table 1 Neurological scores in each group after MCAO (score)

Group	N	1 day	7 days	14 days
S	21	0	0	0
M	42	3.43 ± 0.98	2.43 ± 1.13	1.57 ± 0.53
EM	42	3.43 ± 0.53	1.00 ± 0.58[a]	0.29 ± 0.49[a]
IM	42	4.00 ± 0.58	3.71 ± 0.76[a]	2.71 ± 0.76[a]
IEM	42	3.29 ± 0.95	1.57 ± 0.79	1.29 ± 0.49[b]

Data are expressed as the means ± SD

ANOVA: [a]$P < 0.05$, versus the same period of M

[b]$P < 0.05$, versus the same period of IM

Table 2 Infarct volume in each group after MCAO (%)

Group	N	7 days	14 days
S	3	0	0
M	6	47.75 ± 2.43	27.70 ± 3.84
EM	6	26.41 ± 0.86[a]	14.60 ± 4.02[a]
IM	6	54.03 ± 2.08[a]	45.86 ± 2.41[a]
IEM	6	36.00 ± 1.91[b,c]	24.09 ± 1.24[b,c]

Data are expressed as the means ± SD

ANOVA: [a]$P < 0.05$, versus the same period of M

[b]$P < 0.05$, versus the same period of IM

[c]$P < 0.05$, versus the same period of EM

testing period was consistent with the working memory errors ($n=3$, Fig. 1d).

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on the Caveolin-1/VEGF Signaling Pathway in the Ischemic Penumbra

The expression levels of caveolin-1 and VEGE protein in the ischemic penumbra were examined by western blot assays. After normalization with β-tubulin, M group mice expressed more caveolin-1 and VEGF than S group mice. Treadmill exercise elevated caveolin-1 expression significantly in the EM group mice at 7 and 14 days after ischemic injury compared with the M group and IEM group mice ($P<0.05$), and this significant difference in VEGF expression was shown only at day 7 ($P<0.05$). On the other hand, caveolin-1 and VEGE protein expression was significantly suppressed by the caveolin-1 inhibitor, daidzein, in the IM group mice at 7 and 14 days after MCAO ($P<0.05$) compared with the M and IEM group mice ($P<0.05$, $n=6$, Fig. 2a–c).

Effects of VEGFR2 Inhibitor on Neurological Score and Cerebral Infarction Volume

Except for the three sham group mice chose from the sham-operated group randomly, mice in the VEGFR2 inhibitor MCAO group (VIM) scored (2.33 ± 0.82 and 1.17 ± 0.75, respectively) more than the mice in VEGFR2 inhibitor control MCAO group (VICM: 2.17 ± 0.75 and 0.83 ± 0.75, respectively, $P<0.05$) at 3 days and 7 days after MCAO, but there is no significance at 1st day ($P>0.05$). On the other hand, VEGFR2 inhibitor induced more serious brain tissue destruction in VEGFR2 inhibitor group (VIM: $47.18\pm5.17\%$) than vehicle group (VICM: $28.13\pm2.21\%$, $P<0.05$) at 7 days after MCAO ($n=3$, Fig. 3a, b).

Effects of the VEGFR2 Inhibitor on VEGF and BDNF Expression in the Ischemic Penumbra

The changes in VEGF and BDNF expression were detected in the ischemic penumbra in animals treated with the VEGFR2 inhibitor, PD173074. We found that the VEGF and BDNF were significantly down-regulated in the VEGFR2 inhibitor group (VIM) mice at 7 days after MCAO, compared with the VEGFR2 inhibitor control MCAO group (VICM) mice ($P<0.05$). Thus, it suggested that VEGF was an effective mediator of BDNF synthesis and release ($n=3$, Fig. 4).

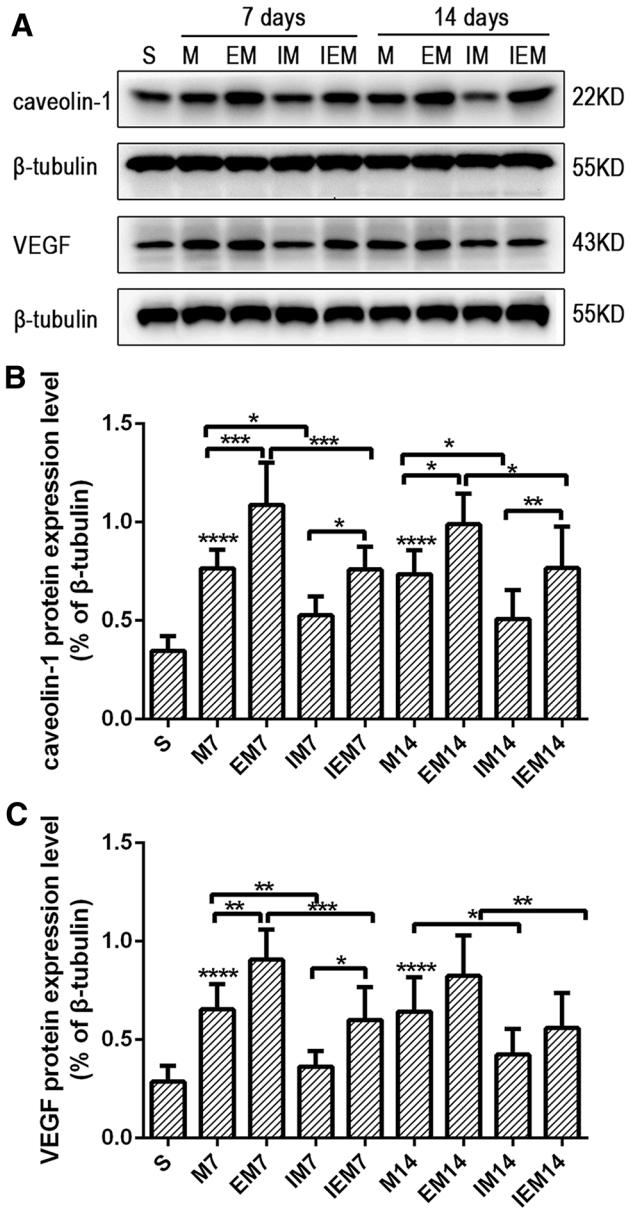

Fig. 2 **a** Caveolin-1 and VEGF expression level in the ischemic penumbra of each group mice after MCAO followed by 7 and 14 days of treadmill exercise or daidzein treatment ($n=6$). **b, c** Densitometry analysis of VEGF for quantified comparisons of each factor. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on BDNF, Synapsin I and CYFIP1 Expression in the Ischemic Penumbra

We found that BDNF expression was significantly higher while CYFIP1 expression was relatively lower in the M group than the S group in the ischemic penumbra ($P<0.05$),

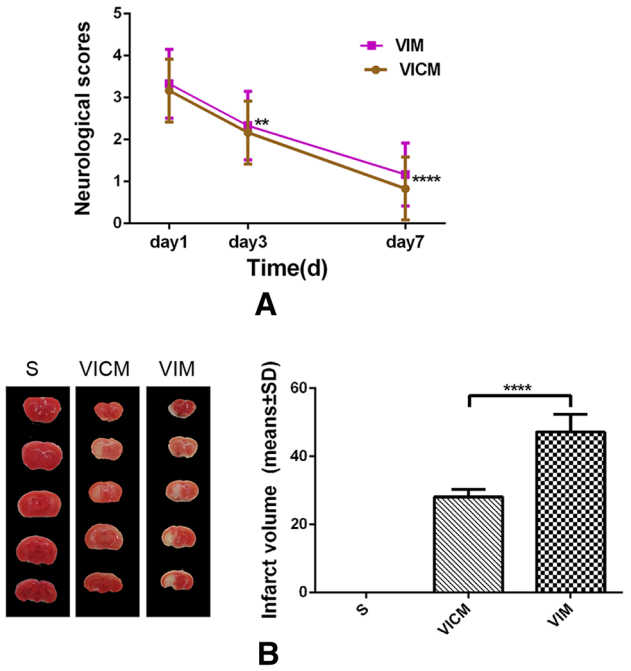

Fig. 3 Effects of VEGFR2 inhibitor on neurological score and cerebral infarction volume. **a** Neurological scores in VICM and VIM group at 1, 3 and 7 days after MCAO. **b** Consistent infarct in the ipsilateral hemispheres of VICM and VIM group mice stained by TTC at 7 days after MCAO followed by 7 days of VEGFR2 inhibitor or not ($n=3$). MCAO mice exposed to VEGFR2 inhibitor (VICM) exhibited larger volume of infarction and higher neurological scores than VEGFR2 inhibitor control MCAO group mice (VICM). Data are expressed as the means ± SD and were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; VICM: VEGFR2 inhibitor control MCAO group; VIM: VEGFR2 inhibitor MCAO group

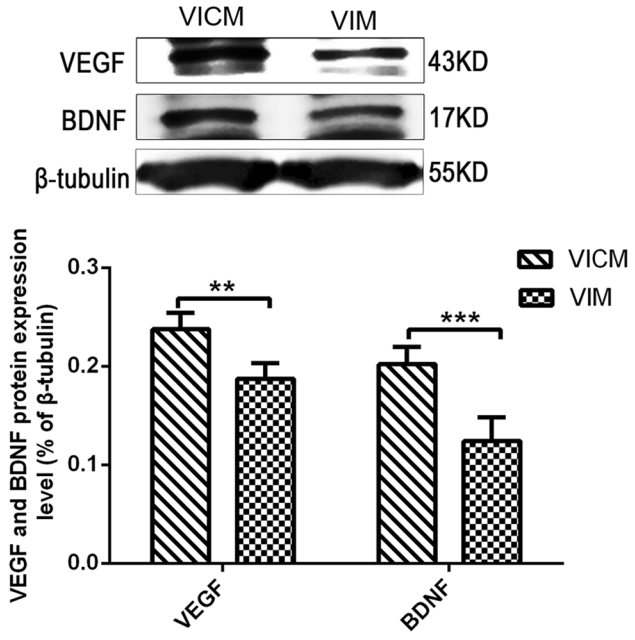

Fig. 4 VEGF and BDNF expression in the ischemic penumbra of VICM and VIM group mice after MCAO followed by 7 days of VEGFR2 inhibitor injections ($n=3$). Densitometry analysis of VEGF and BDNF for quantified comparisons of each factor. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). VICM: VEGFR2 inhibitor control MCAO group; VIM: VEGFR2 inhibitor MCAO group

and there was no difference in synapsin I expression. After detecting the BDNF protein isolated from the penumbra of caveolin-1 inhibitor daidzein-treated mice, we found that BDNF expression in IM group mice was decreased greatly at 7 and 14 days compared with the mice in the M and IEM groups ($P<0.05$), and synapsin I and CYFIP1 expression levels were almost consistent with the BDNF expression changes at same periods ($P<0.05$). Furthermore, compared with the M group mice at 7 and 14 days after MCAO, the three proteins were significantly up-regulated in EM group mice ($P<0.05$) ($n=6$, Fig. 5a–d).

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on Angiogenesis in the Ischemic Penumbra as Reflected by BrdU/CD34-Positive Cells with Immunofluorescence

Per unit of brain microvessels area in the ischemia penumbra was assessed by recording the number of BrdU/CD34-positive cells, which peaked at 7 days after MCAO. The IM group mice showed significantly sparse microvessels compared with the mice in the M and IEM groups at the same period. After treadmill exercise, there was more intensive BrdU/CD34 labeling of cells in the EM group mice than the M group mice ($P<0.05$, $n=6$, Fig. 6).

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on Neurogenesis in the Ischemic Penumbra as Reflected by BDNF- and BrdU/NeuN-Positive Cells with Immunofluorescence

A significant difference between groups was observed in terms of BDNF- and BrdU/NeuN-positive cells stained by immunofluorescence. Compared with the mice in the M and IEM groups after MCAO, the number of BDNF-positive cells was significantly decreased in the IM group mice, especially at 14 days, while the number was greatly increased in the EM group mice ($P<0.05$). We observed that the number of BrdU/NeuN-positive cells in the M group mice was increased compared to the S group mice ($P<0.05$). There were much fewer positive BrdU/NeuN cells in the IM group mice than the M and IEM group mice at 14 days after MCAO ($P<0.05$), and we found that EM group mice exhibited a markedly higher number of BrdU/NeuN-positive

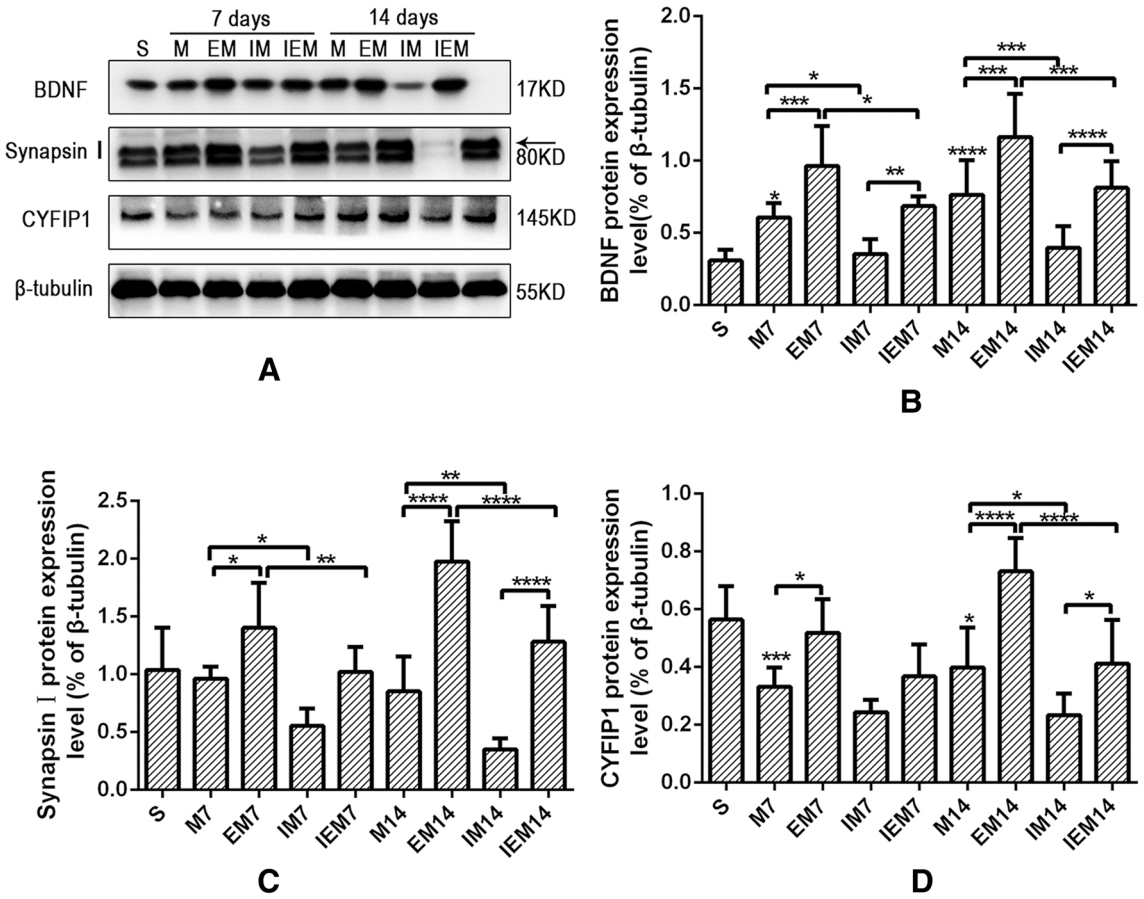

Fig. 5 **a** BDNF, synapsin I and CYFIP1 expression in the ischemic penumbra of each group mice after MCAO followed by 7 and 14 days of treadmill exercise or caveolin-1 inhibitor or both of them ($n=6$). **b–d** Densitometry analysis of BDNF, synapsin I and CYFIP1 for quantified comparisons of each factor. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group

cells than the M and IEM group mice at 14 days after MCAO ($P<0.05$, $n=6$, Figs. 7, 8).

Effects of Treadmill Exercise on and Caveolin-1 Inhibitor Synaptic Plasticity in the Ischemic Penumbra as Reflected by BrdU/Synapsin I- and CYFIP1-Positive Cells with Immunofluorescence

Newly formed synapses around the infarct were labeled as BrdU/Synapsin I-positive cells, which were fewer observed in the IM group mice than the M and IEM group mice at 7 or 14 days after MCAO, and more abundant in the EM group mice exposed to the treadmill exercise ($P<0.05$, $n=6$, Fig. 9).

The CYFIP1-positive cells in the IM group mice exposed to 14 days of caveolin-1 inhibitor drug injection were decreased compared with the M group, while EM group mice after 14 days of treadmill exercise exhibited an active outcome and more positive cells than M group ($P<0.05$). However, we did not observe any difference in CYFIP1 among the groups at day 7 ($P>0.05$). Notably, the mice in the M group showed much fewer CYFIP1-positive cells than the S group mice ($P<0.05$, $n=6$, Fig. 10).

Effects of Treadmill Exercise and Caveolin-1 Inhibitor on the Morphology of Cortical Neurons and the Ultrastructure of Synapses in the Ischemic Penumbra

The S group mice showed normal neurons with clear and complete synaptic structures. However, in the other groups, synapses, mitochondria and neurons were swelling, and the outline of synaptic connections was partially disordered. M group mice appeared to exhibit fewer clear and irregular synaptic vesicles and narrower synaptic clefts compared with the EM group mice at the same period. Notably, more serious damage was observed in the IM group mice, such as uneven in thickness of synaptic membrane, formation of

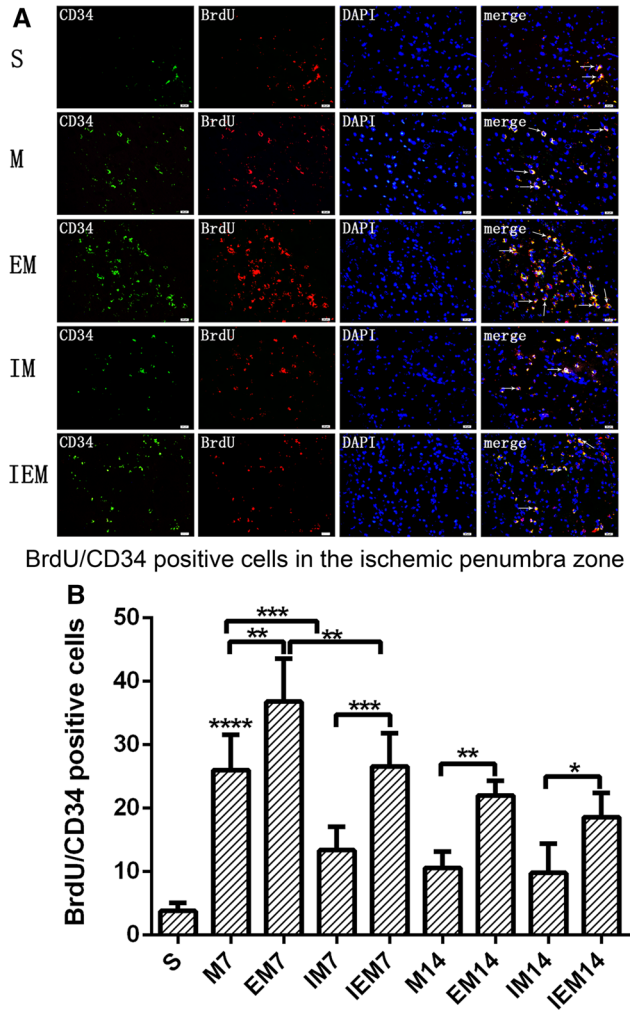

Fig. 6 **a** Immunofluorescence of BrdU/CD34-positive cells in the ischemic penumbra of each group of mice at 14 days after MCAO ($n=6$). Red: BrdU; green: CD34; blue: DAPI; merge: BrdU/CD34; scale bar, 20 μm. **b** The number of BrdU/CD34-positive cells in the ischemic penumbra of each group of mice at 7 and 14 days after MCAO. Data as the means ± SD. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group (×400). (Color figure online)

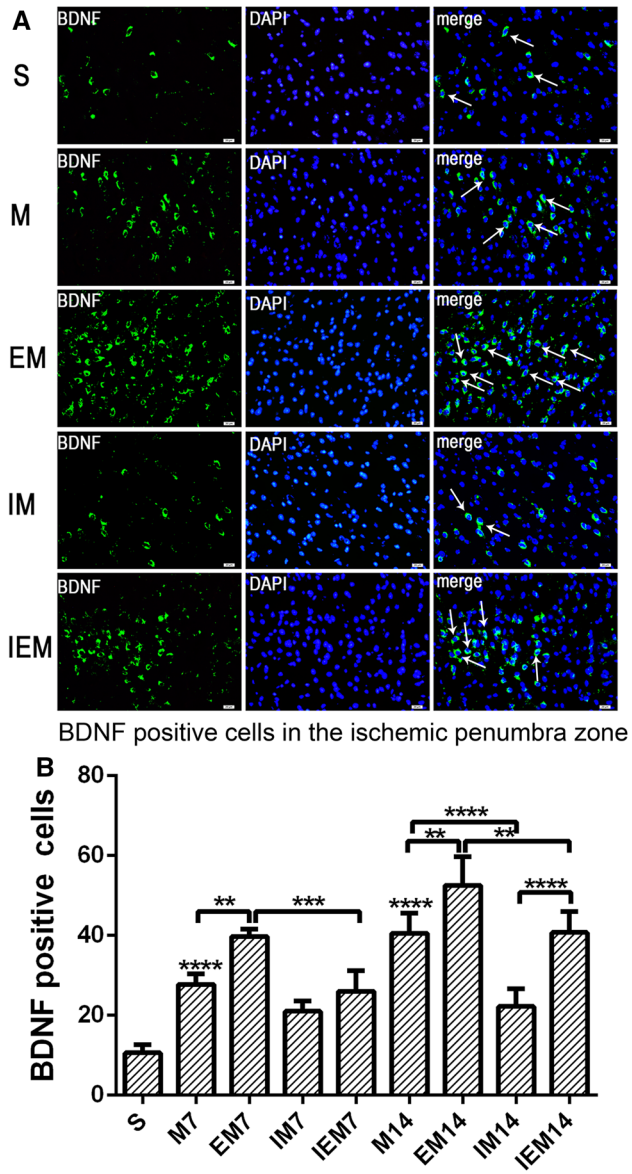

Fig. 7 **a** Immunofluorescence of BDNF-positive cells in the ischemic penumbra of each group of mice at 14 days after MCAO ($n=6$). Green: BDNF; blue: DAPI; scale bar, 20 μm. **b** The number of BDNF-positive cells in the ischemic penumbra of each group of mice at 7 and 14 days after MCAO. Data are expressed as the means ± SD. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group (×400). (Color figure online)

synaptic vesicle lysis cavities and completely invisible synaptic structures ($n=3$, Fig. 11).

Discussion

Recent evidence has demonstrated that mechanisms of brain injury and neurodegeneration are affiliated with the multicellular interactions within the neurovascular unit (NVU), consisting of pericytes, microglia, astrocytes, neurons, and the basal lamina. Disruptions in the NVU induced by ischemia/reperfusion lead to the evolution of BBB damage, neuronal cell death or degeneration, glial reaction, and immune cell infiltration [36, 37]. In recent years, treadmill exercise is an effective way to ameliorate the ischemic injury in rehabilitation. Undoubtedly, exercise-induced angiogenesis and neurogenesis compensate for ischemic brain damages to advance motor function recovery, which coincides with our previous results through the caveolin-1/VEGF pathway in MCAO

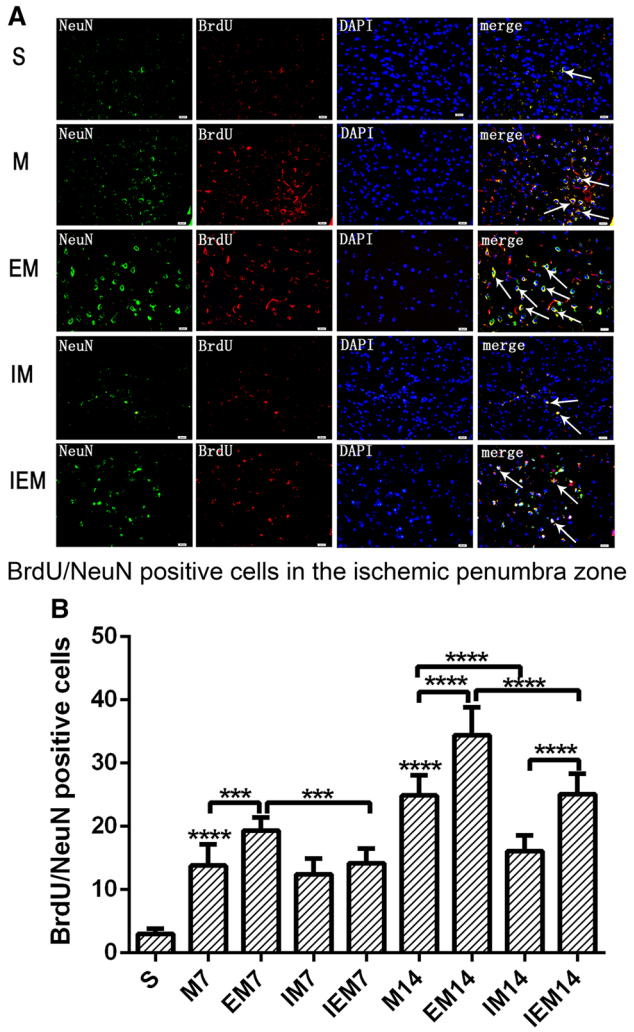

Fig. 8 **a** Immunofluorescence of BrdU/NeuN-positive cells in the ischemic penumbra of each group of mice at 14 days after MCAO ($n=6$). Red: BrdU; green: NeuN; blue: DAPI; merge: BrdU/NeuN; scale bar, 20 μm. **b** The number of BrdU/NeuN-positive in the ischemic penumbra of each group of mice at 7 and 14 days after MCAO. Data are expressed as the means ± SD. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group (×400). (Color figure online)

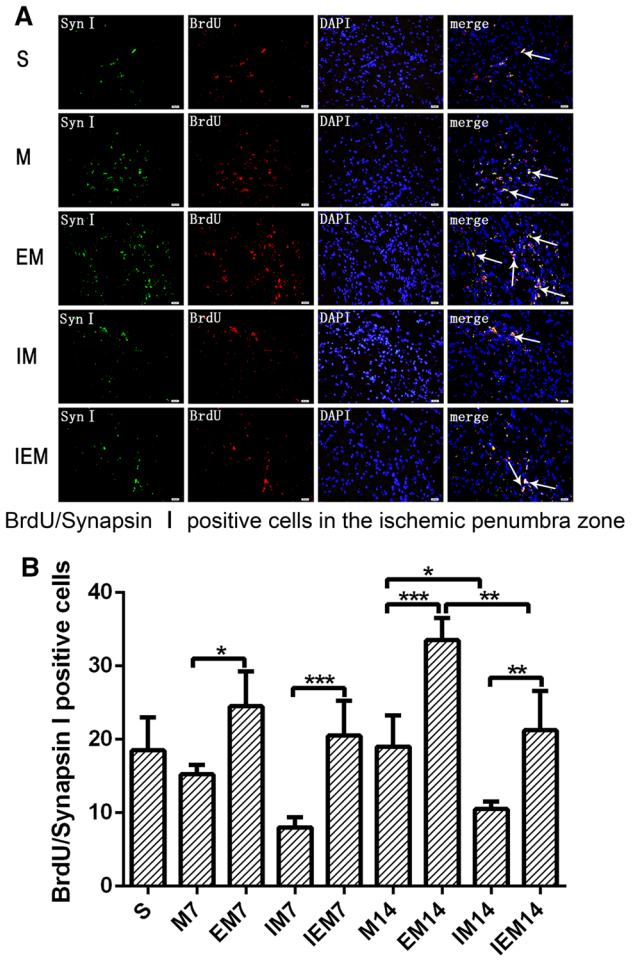

Fig. 9 **a** Immunofluorescence of BrdU/Synapsin I-positive cells in the ischemic penumbra of each group of mice at 14 days after MCAO ($n=6$). Red: BrdU; green: synapsin I; blue: DAPI; merge: BrdU/Synapsin I; scale bar, 20 μm. **b** The number of BrdU/Synapsin I-positive in the ischemic penumbra of each group of mice at 7 and 14 days after MCAO. Data are expressed as the means ± SD. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group (×400). (Color figure online)

rats. Nevertheless, in this vivo study with the MCAO mouse model, we also found further mechanisms of exercise-mediated angiogenesis and neurogenesis and the cognitive repair partially through this pathway after ischemic injury.

In the current study, we found that treadmill exercise could reduce infarction volumes and neurological scores in MCAO mice, but caveolin-1 inhibitor, daidzein, produced adverse results. However, given the IEM group mice, we also saw that exercise was still able to significantly decrease infarct volume and improve neurological function in the presence of the caveolin-1 inhibitor. In accordance with this, we measured the caveolin-1, VEGF and BDNF expression levels by western blot assays. Analysis showed that treadmill exercise significantly up-regulated caveolin-1, VEGF and BDNF levels. Additionally, daidzein had a negative impact on these protein expressions at 7 and 14 days after MCAO. Similarly, the promotion effect still existed in the view of exercise and caveolin-1 inhibitor in IEM group, which indicated that caveolin-1/VEGF pathway might be only one of signaling pathways in the exercise-mediated processes after ischemic injury. Rehabilitation training after stroke is a long process. We also found a further reduction of cerebral infarct volume in each group after 14 days especially in treadmill

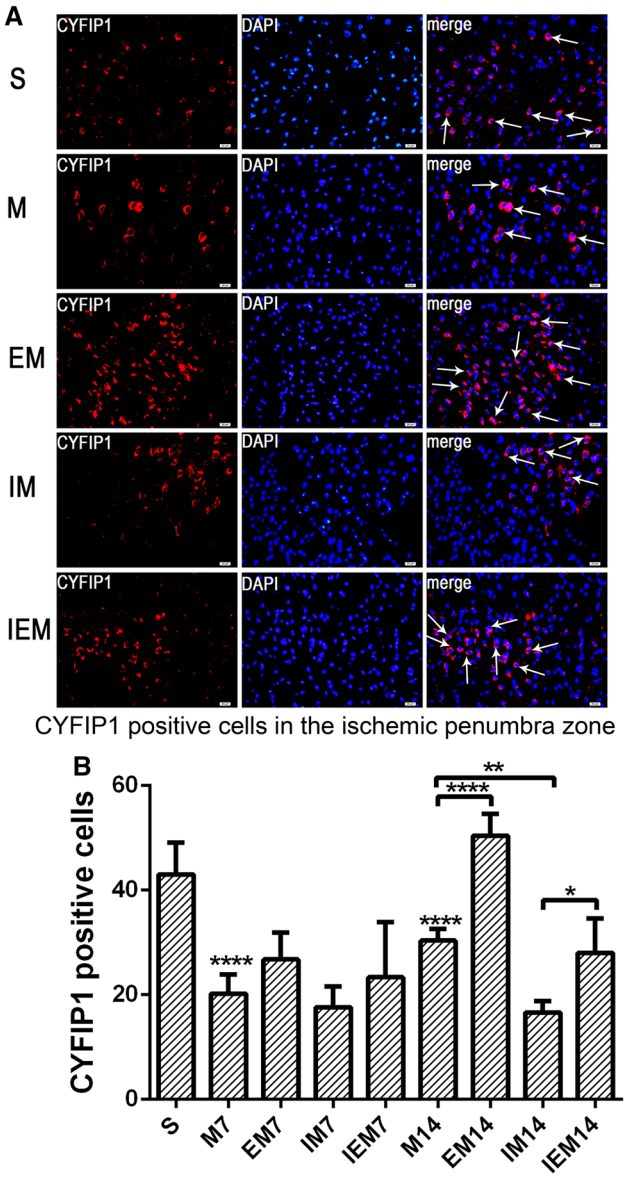

Fig. 10 **a** Immunofluorescence of CYFIP1-positive cells in the ischemic penumbra of each group of mice at 14 days after MCAO ($n=6$). Red: CYFIP1; blue: DAPI; scale bar, 20 μm. Data are expressed as the means ± SD. **b** The number of CYFIP1-positive in the ischemic penumbra of each group of mice at 7 and 14 days after MCAO. Data were analyzed by ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). S: Sham-operated group; M: MCAO group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group (×400). (Color figure online)

exercise MCAO group, which was consistent with the neurological scores, indicating that 14 days may be a suitable period for post stroke recovery via some protective reactions, abundant collateral circulation, and favorable external intervention factors.

A study concluded that tanshinone IIA (TIIA) enhanced the dose-dependent neuronal differentiation partially through MAPK42/44-mediated BDNF and NGF signals, and it is worth mentioning that activated caveolin-1 was able to trigger this differentiation effect by facilitating TIIA transport across the cell membrane, while a caveolin-1 deficit led to a cessation of the effect [38]. Transient receptor potential C3 channel (TRPC3) can substantially increase BDNF release under the regulation of caveolin-1 in terms of Ca^{2+} influx [39]. Moreover, knockdown of store-operated Ca^{2+} entry (SOCE) proteins, such as STIM1 and Orai1 or plasma membrane caveolin-1, could reduce secreted BDNF significantly in airway smooth muscle (ASM) [40]. On the other hand, researchers found that an appropriate form and intensity of physical movement has a beneficial effect on the expression level of BDNF, which is beneficial to neuron-specific protein up-regulation, synaptic development and motor/cognitive performance after cerebral ischemia [41, 42]. Increased expression and secretion of VEGF and BDNF was detected with the transplantation of Ad-VEGF-BMSC (adenovirus carried VEGF into BMSC) into the peri-infarct area of an ischemic rat brain, and microvascular density and behavioral function were improved [43]. In this study, in the case of administering VEGF inhibitor to MCAO mice, we found that BDNF was significantly decreased, as well as worse cerebral infarction and neurological deficit. These cases suggest that both caveolin-1 and VEGF are connected with BDNF that play a synergistic biological regulation role, and BDNF is likely to be a further mechanism in the caveolin-1/VEGF pathway for neurological recovery. Moreover, a decreased level of VEGF was observed after the use of VEGFR inhibitor. The reason may be that signal pathway exits negative regulation, VEGFR inhibitor interrupts VEGF/VEGFR signaling, inducing a down-regulation of VEGF level. We speculate that this negative regulation will gradually weaken as VEGFR becomes less sensitive to inhibitors.

BDNF has crucial effects on the regulation of both vascular development and response to injury by activating receptors (VEGFR1 and VEGFR2) localized in most endothelial cell populations. Evidence demonstrates that BDNF-induced TrkB activation of PI3-kinase and Akt may mediate endothelial survival to promote angiogenesis [44]. In the current study, inhibition of the caveolin-1/VEGF pathway with a caveolin-1 inhibitor after injury induced severe vascular damage in the ischemic penumbra, while the microvessel density significantly increased following treadmill exercise in MCAO mice, reflected by BrdU/CD34 double-stained cells, and the number of which reached its peak at day 7. We speculated that the caveolin-1/VEGF pathway and BDNF had greater effects against acute ischemic injury. This finding confirms the quantifiable measure of VEGF and BDNF protein levels, as does our previous studies. Thus, the data indicate that treadmill exercise can enhance the process of angiogenesis after stroke through caveolin-1/VEGF

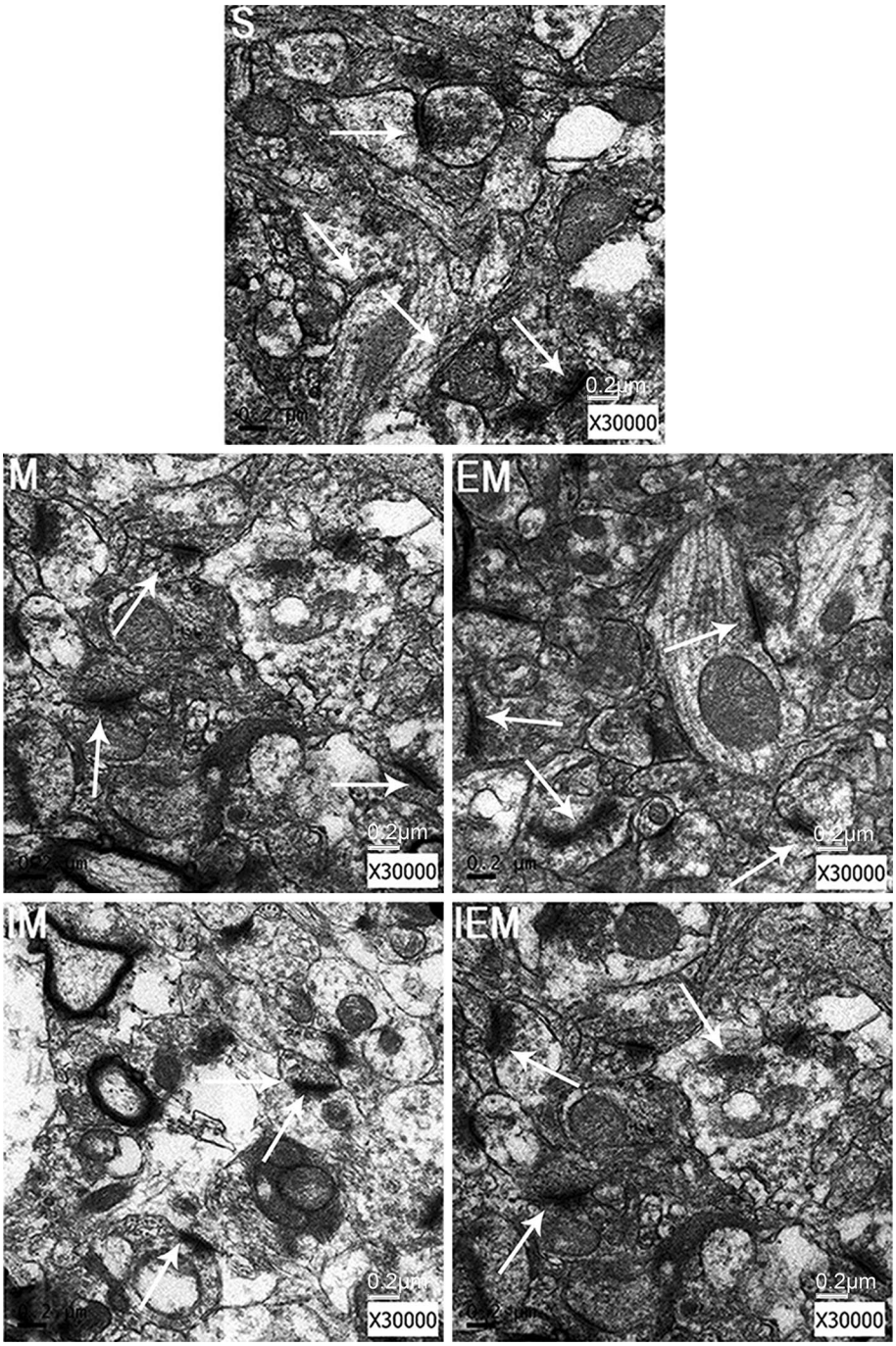

Fig. 11 Morphology of cortical neurons and the ultrastructure of synapses in the ischemic penumbra of each group mice after MCAO ($n=3$). S: Sham-operated group; M: model group; EM: exercise and MCAO group; IM: caveolin-1 inhibitor MCAO group; IEM: caveolin-1 inhibitor, exercise MCAO group

signaling pathway, and BDNF is one of the crucial further factors in synergistic repair.

Our previous study demonstrated that treadmill exercise enhanced neurogenesis through the caveolin-1/VEGF signaling pathway possibly by promoting subventricular zone (SVZ)-derived nerve progenitor cells (NSC) proliferation, migration to the ischemia penumbra and differentiation into mature neurons [8]. NSC derived from SVZ migrated along the vessel closely to the surrounding area of demyelinating lesions of the rat brain, while the microvessel density decreased significantly in the injured SVZ and area around it after anti-VEGF treatment, as well as a reduction of 41% nerve progenitor cells compared with control group. It indicated that angiogenesis played a regulatory role on neurogenesis in brain demyelination injury [45]. In rats, the number of adult-born neurons was markedly increased after direct infusion of BDNF or adenoviral expression of BDNF to the dentate gyrus or SVZ [46, 47]. Additionally, deletion of BDNF in mice inhibited the differentiation of interneurons [48]. Consistent with this, a statistically significant

increase of BDNF positive cells and BrdU/NeuN double-labeled cells in the ischemic penumbra of MCAO mice after 7 or 14 days of treadmill exercise was observed in this study, while the caveolin-1 inhibitor resulted in reduction in both positive cells. Therefore we suggested that caveolin-1/VEGF signaling, as one of potential upstream pathways of BDNF, stimulated BDNF expression to promote neurogenesis after treadmill exercise following ischemic injury.

Synaptic strengthening is another vital process of repairing ischemic damage besides angiogenesis and neurogenesis. It is now firmly established that cerebral ischemic injury impacts the development of synapses, including variability in synaptic structure and function, neurotransmitter receptors and their coupling to the intracellular signaling machinery at the presynaptic and postsynaptic level. Exercise-induced remodeling of synapses can improve neuronal activity and synaptic plasticity after cerebral ischemia and hypoxic injury, which can relieve brain damage and effectively ameliorate motor and cognitive dysfunctions [49–51].

Synapsin I is a terminal-specific phosphoprotein, involved in spatial and emotional memory, and mainly participates in synaptic vesicle aggregation, release and fusion at the presynapse terminal [52]. The level of synapsin I expression or phosphorylation for mammalian synapse terminal can be modulated via BDNF, which is established as an upstream molecule of synapsin I [53]. CYFIP (Cytoplasmic FMRP interacting protein) is a type of Fragile X mental retardation protein (FMRP), including CYFIP1 and CYFIP2, which are enriched in the terminal of axons and motor neurons, especially at excitatory synapses in dendritic spines. Data have shown that CYFIP1 can promote CNS maturation and neuronal connectivity by regulating dendrite morphology, synaptic structural plasticity and neurotransmitter receptor mobility [54]. Recent work has shown that exogenous BDNF in hippocampal neuronal cultures or cortical neurons plays a role in the enhancement of miRNA-mediated translation in dendrites through the release of CYFIP1 from eIF4E, benefiting to regulate synaptic structure, function, and plasticity [55, 56]. In addition, retrolinkin directly interacts with the CYFIP1/2 subunits of WAVE1 to act on dendrite outgrowth induced by BDNF, while WAVE1 depletion or double knockdown of CYFIP1/2 impairs dendritic development [57, 58]. The evidences imply that CYFIP1 is also a possible downstream molecule of BDNF-TrkB signaling.

Exercise can improve cerebrovascular plasticity, as well as neuronal plasticity, and enhance brain health against cognitive dysfunction and neurodegenerative diseases, indicating that the neuroprotective effect by angiogenesis and neurogenesis is associated with cognitive repair after stroke [59]. In many cases, the critical role of neurotrophic factors in neuronal development and synaptic plasticity has been extensively confirmed, including stimulating neuronal proliferation, differentiation and survival and promoting neurite outgrowth and regeneration, which are necessary to enhance synapse formation and synaptic plasticity that correlate with cognitive function [12, 23]. Caveolin-1 [28], VEGF [18] and BDNF are all involved in the brain plasticity processes associated with cognitive recovery. Furthermore, BDNF is considered an upstream factor to influence synapsin I and CYFIP1 expression [53, 60]. In our study, we found that synapsin I and CYFIP1 protein expression levels at 14 days were significantly higher in the EM group mice while the IM group mice exhibited lower expression levels than the M group mice; the difference in 7 days groups was not very obvious, suggesting that 14 days or more might be a suitable time for exercise-mediated synaptic remodeling after ischemia. These results were consistent with the new formation synapses as represented by BrdU/Synapsin I-positive cells, axon numbers represented by CYFIP1-positive cells and the morphology of cortical neurons and ultrastructure of synapses in the ischemic penumbra of each group. In addition, working memory errors and reference memory errors in MCAO mice after treadmill exercise or inhibitor treatment coincided with neurological scores and changes in the expression levels of BDNF, synapsin I and CYFIP1 at 14 days after stroke, and the motor and cognitive functions were significantly alleviated in the EM group, but the IM group mice showed worse cognitive impairment. However, at 7 and 14 days after MCAO, both synapsin I and CYFIP1 were down-regulated in the M group mice exhibited more memory errors, compared with the sham-operated group, which suggested that synapsin I and CYFIP1 were probably associated with the early cognitive impairment. On the other hand, working memory errors and reference memory errors were increased obviously at 7 days after stroke in all MCAO group even though both of synapsin I and CYFIP1 were up-regulated in the EM group compared with M group, thus, we conjectured that the two proteins were only part of mechanisms of cognitive function recovery after ischemic injury. We therefore hypothesized that caveolin-1/VEGF was one of potential signaling pathways conducive to strengthening exercise-mediated synaptic plasticity via BDNF for improving post-stroke cognitive impairment (PSCI), but CYFIP1 was firstly studied in ischemic injury and further work should be carried out in genetic level.

Sincerely speaking, the main limitations of our study are necessary to discuss. First, although we testified the role of the caveolin-1/VEGF pathway on functional recovery after stroke in wild-type mice, caveolin-1 knockout mice were not evaluated in this study. Second, as a valuable factor, BDNF was verified elementarily as a further potential mechanism in the regulation of angiogenesis and neurogenesis through the caveolin-1/VEGF pathway, but we couldn't be sure whether the caveolin-1/VEGF/BDNF pathway existed because no further study was available to corroborate these findings by exogenous incorporation of VEGF or the use of VEGF

KO mice or a BDNF-specific inhibitor. Third, except for the morphology of cortical neurons and the ultrastructure of synapses in the ischemic penumbra observed by TEM, and synapsin I and CYFIP1 expression level, no other proteins related to synaptic plasticity were studied, or the long-term potential and long-term depression were examined for synaptic function. Fourth, we just used three mice per group to test the spatial learning abilities. Thus, much larger group sizes should be carried out for more persuasive in the further study. At last, we only investigated two time periods (7 days and 14 days) after stroke, and it should be valuable to study a longer time period, especially for synaptic plasticity.

Conclusions

In summary, angiogenesis and neurogenesis interact with each other, and both of them have impact on synaptic plasticity. Caveolin-1, VEGF and BDNF are associated with angiogenesis, neurogenesis and synaptic plasticity closely. Our results indicate that treadmill exercise improves the motor and cognitive function recovery after ischemic injury through the caveolin-1/VEGF pathway partially by enhancing angiogenesis, neurogenesis and synaptic plasticity. In addition, BDNF is a costimulatory molecule of caveolin-1/VEGF pathway to participate in the above biological processes.

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Author Contributions All authors contributed equally to this work.

Compliance with Ethical Standards

Conflict of interests We declare that all authors have no financial or other conflict of interests in connection with the submitted article.

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