Electroacupuncture improves cognitive function through Rho GTPases and enhances dendritic spine plasticity in rats with cerebral ischemia-reperfusion

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Abstract. The aim of the present study was to evaluate the effect of electroacupuncture (EA) on cognitive function following cerebral ischemia-reperfusion (I/R) injury, and to clarify the mechanism through which Rho GTPase is associated with EA analgesia modulation of dendritic spine plasticity. Rats were randomly divided into three groups: The sham surgery group, the middle cerebral artery occlusion (MCAO) model of ischemia group, and the MCAO with EA (MCAO+EA) treatment group. The MCAO+EA group received treatment with EA at points of Baihui (DU20) and Shenting (DU24) following surgery. It was demonstrated that treatment with EA significantly ($P<0.05$) protected the cognitive function of rats from impairment caused by cerebral I/R injury. Furthermore, EA treatment increased the density of dendritic spines in the hippocampus of cerebral I/R-injured rats. Simultaneously, EA increased the expression of cell division cycle 42, Ras-related C3 botulinum toxin substrate 1 and F-actin proteins. By contrast, EA treatment inhibited the expression of Ras homologous member A. Collectively, these findings suggest that Rho GTPases and dendritic spine plasticity are critical in mediating the effects of EA treatment at the points of Shenting and Baihui, and that EA protects against impairment of cognitive function following ischemic stroke.

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Introduction

Annually, ~795,000 individuals experience a new or recurrent stroke (1). Notably, stroke survivors exhibit various neurological deficiencies, including cognitive impairment (2). As the population ages, the incidence of individuals experiencing neurological damage caused by stroke is increasing, and cognitive impairment is correlated with an increase in the mean age at first onset of stroke (3). A recent analysis of a London registry of stroke patients revealed that 22% were cognitively impaired following stroke (4). Acupuncture has been used for treating numerous ailments in China, including stroke and memory deficiency. Numerous studies have shown the efficacy of electroacupuncture (EA) in the rehabilitation of patients with cognitive dysfunction in clinical and experimental settings, and Baihui (DU20) and Shenting (DU24) are common EA points selected for this type of treatment (5-8).

In neurons, the Rho GTPases are members of the Ras superfamily of small (±21 kDa) GTPases. Ras homologous member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42) are Rho GTPases that are best known for their effects on the actin cytoskeleton, which in dendritic spines are mainly comprised of F-actin (9-10). At the synapse Rac1, Cdc42 and RhoA have emerged as key regulators of dendritic spine formation and morphogenesis. These proteins have recently been implicated in synaptic plasticity, including the excitatory synapses, which are located on dendritic spines (11,12). RhoA and Racl/Cdc42 act as mutual antagonists on dendritic spines. Rac1 and Cdc42 have been shown to promote the formation, growth and maintenance of spines, whereas RhoA induces spine retraction and loss (13,14). Recent evidence indicates that variations in cognitive processes, in particular learning and memory, are associated with plastic changes in dendritic spines (15,16). Together these studies suggest that the regulation of the actin cytoskeleton by Rho GTPases in synaptic plasticity represents the cellular basis of cognition.

The precise mechanism of cognitive dysfunction remains unclear. In the present study, the therapeutic efficacy of EA against post-stroke cognitive dysfunction was evaluated and the underlying molecular mechanisms were investigated using a cerebral ischemia/reperfusion (I/R) rat model.

Materials and methods

Animals and study groups. Healthy adult male Sprague-Dawley rats (weight, 270-310 g; n=54) were purchased from the SLAC Laboratory Animal Co., Ltd., Shanghai, China [Laboratory Animal Use Certificate no. SCXK (SH) 2012-0002] and raised in a sterile environment at 22±1˚C and 50% humidity in a 12/12 h light/dark cycle with access to food and water *ad libitum*. All experiments were performed strictly in accordance with the International Ethical Guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The study was approved by the ethics committee of the College of Rehabilitation Medicine, Fujian University of Traditional Chinese Medicine (Fuzhou, China; no.: 2013047). Rats were randomly and evenly divided into three groups (n=18) as follows: i) Sham surgery control group (Sham group); ii) the middle cerebral artery occlusion (MCAO) model of ischemia control group (MCAO group); and (iii) the MCAO+EA treatment group (MCAO+EA group).

Cerebral IR-injury rat model. Longa's suture-occluded method was used to establish the MCAO and reperfusion model (17). Briefly, rats were anesthetized with 10% chloral hydrate (0.03 ml/100 g body weight; Shanghai Chemical Reagent Co., Ltd., Shanghai, China) through intraperitoneal injection. Then, 18-22 mm of nylon surgical thread (Beijing Sunbio Biotech, Co., Ltd., Beijing, China) was inserted into the left internal carotid artery to block the MCA when the blunted distal end met resistance. After 2 h of occlusion, the thread was removed to allow complete reperfusion of the ischemic area. The rectal temperatures of the rats were maintained at 37˚C throughout the surgical procedures using heat pads. Following surgery, the rats were allowed to recover in pre-warmed cages for ~2 h. Sham surgery was conducted as above without the occlusion of the MCA.

Electroacupuncture treatment. In the MCAO+EA group, rats were administered EA for 30 min daily for 7 days ~4 h after MCAO was conducted. The acupuncture needles (0.3 mm diameter; Suzhou Medical Supplies Factory, Co., Ltd., Suzhou, China) were inserted at a depth of 2-3 mm into the Baihui (DU20) and Shenting (DU24) acupoints on the head. Stimulation was then generated using the EA apparatus (model G6805; Shanghai Chemical Reagent Co., Ltd.), and the stimulation parameters were set as disperse waves of 1 and 20 Hz. The Sham and MCAO groups did not undergo any EA treatment.

Measurement of cerebral infarct volume. Following completion of the experiment, 7 rats from each group were sacrificed by overdose of 10% chloral hydrate (Shanghai Chemical Reagent Co., Ltd.), the injection dosage was 0.03 ml/100 g body weight administered by intraperitoneal injection. Each rat was perfused transcardially with 0.9% NaCl and the brain was removed. The brain was sectioned in the coronal plane into 2-mm thick slices. The slices were stained with 2% tetrazolium chloride (TTC) solution (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C for 20 min and then fixed with 10% buffered formalin solution. The normal area of the brain was stained dark red based on intact mitochondrial function, whereas the infarct area remained unstained. Stained slices were scanned using a high-resolution digital camera (Canon SX20; Canon Lexus, Canon International Trading Shanghai, Co., Ltd., Shanghai, China). The infarct volume was determined using the Motic Med 6.0 system (Motic, Xiamen, China), and was expressed as the percentage of the total brain volume.

Morris water maze. Rats from each group (n=6) were subjected to the Morris water maze task from the 3rd day following surgery in order to investigate spatial learning and memory ability. The water maze (Chinese Academy of Sciences, Beijing, China) was a black circular pool with a diameter of 120 cm and a height of 50 cm, filled with 26±2˚C opaque water (obtained using black ink; Beijing Yidege Ink Industry, Co., Ltd., Beijing, China) to a depth of 30 cm. The maze was divided geographically into four equal quadrants, and four start positions were defined at the cardinal points of the pool. A MINTRON.1132C video camera (Chinese Academy of Sciences, Beijing, China) attached to a computer was placed above the center of the pool to record and analyze each trial. A submerged safe platform was located in the pool (2 cm below the water surface with a 6 cm diameter in a fixed position).

Morris water maze tasks predominantly included orientation navigation and space exploration trials. The orientation navigation trial consisted of four swims per day for 4 days, performed from days 3-6. During this trial, start positions were randomly selected each day, and each rat was allowed 90 sec to swim, find the platform and remain on it for 3 sec. Swimming duration and distances were measured. If the rat was unable to find the platform within 90 sec, it was gently placed on it and allowed to remain there for 10 sec. The average duration and distances covered by each rat over the four quadrants was assessed every day. The space exploration trial was performed 24 h after the last swim, on day 7. The platform was removed and each animal was allowed a free 90 sec swim. The time it took the rat to cross the location of the platform within 90 sec was measured, and this assessment tested their ability to remember the position of the platform. After the trials, the rats were dried thoroughly with a hair drier and returned to their cages.

Golgi stain. The FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc., Columbia, MD, USA) was developed using an improved Golgi-Cox impregnation method to provide stable, sensitive, and convenient staining of mature neurons (18). The Golgi-Cox stain was performed on 150-μm frozen brain sections (a uniform random sample of sections between +3.7 mm anterior and -5.8 mm posterior relative to the bregma) obtained from rats in each group (n=5). The staining process was performed according to the manufacturer's protocol. The number of dendritic spines was observed under an optic microscope (Leica DM2500; Leica Microsystems, Wetzlar, Germany). To measure spine density,

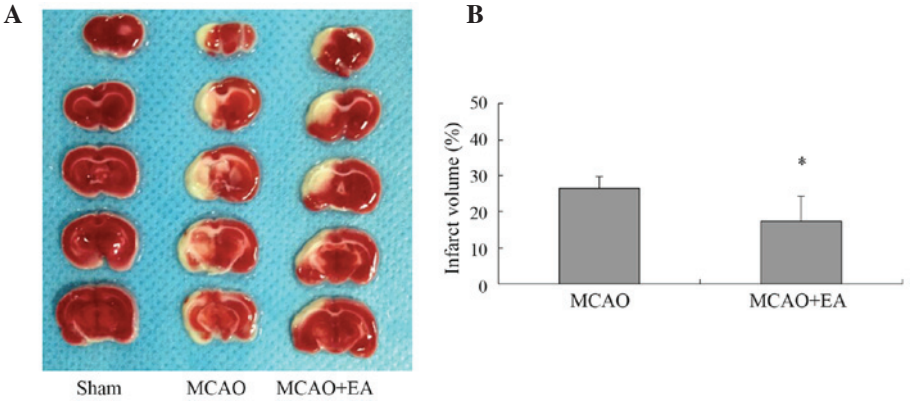

Figure 1. Effect of EA on infarct volume. (A) Tetrazolium chloride staining indicated cerebral infarct volume in MCAO and MCAO+EA groups. (B) Bar graph shows the percentage of total infarct volume in each group (n=7). *P<0.05, vs. the MCAO group. EA, electroacupuncture; MCAO, middle cerebral artery occlusion.

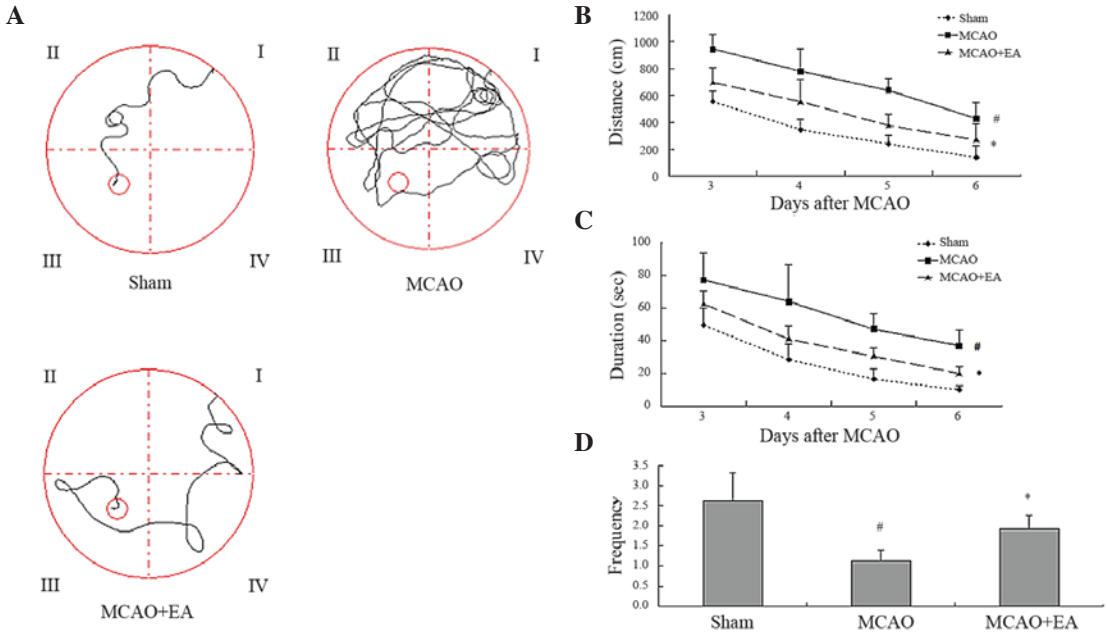

Figure 2. Effects of EA on cognitive impairment. (A) Tracing images from the Morris water maze test of Sham, MCAO and MCAO+EA groups (n=6). (B) Distances and (C) durations of swimming to find the platform (within 90 sec). (D) The frequencies of the rats that passed through the area in which the platform was located. #P<0.05 vs. Sham; *P<0.05, vs. MCAO. EA, electroacupuncture; MCAO, middle cerebral artery occlusion.

20 pyramidal neurons in the CA1 region of the hippocampus from each animal were selected. Matching regions of distal branch dendrites were photographed using a x1,000 objective. Numbers of spines were counted in 50 μm segments, with results expressed as the number of spines/10 μm.

Western blotting analysis. The left cerebral hippocampal tissues were collected and triturated in a radioimmunoprecipitation assay buffer (Fans Bio, Guangdong, China), and the proteins were quantified using a bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal quantities of protein (50 μg), obtained from the hippocampus tissue in the left side of brain of rats in each group (n=6), were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The blots were blocked with 5% non-fat milk for 2 h at room temperature and then incubated with the following mouse monoclonal primary antibodies overnight at 4˚C: Anti-Cdc42 (cat. no. ab41429), anti-Rac1 (cat. no. ab33186), anti-RhoA (cat. no. ab86297), anti-F-actin (cat. no. ab205), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. ab9485) and anti-β-actin (cat. no. ab189073) (1:1,000; Abcam, Cambridge, MA, USA). The membranes were washed in TBST three times for 5 min per wash. Subsequently, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. ab131368; 1:5,000; Cell Signaling Technology, Inc.) for 60 min. After washing again in TBST, the blots were detected with Clarity Western Enhanced Chemiluminescence Substrate (Bio-Rad Laboratories, Inc.) for 1 min using a camera along with the ChemiDoc XRS$^+$ system (Bio-Rad Laboratories, Inc.). The

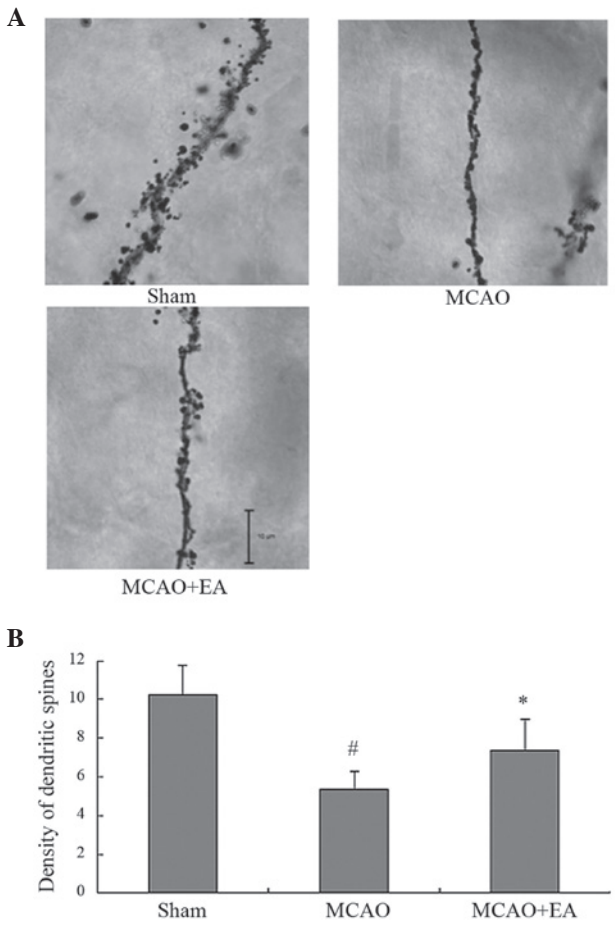

Figure 3. Effect of EA on the density of dendritic spines. (A) Golgi stained tissue from the hippocampus of Sham, MCAO and MCAO+EA groups (n=5). Photomicrograph at x1,000 magnification of a pyramidal cell from CA1 demonstrating complete impregnation (scale bar, 10 μm). (B) Bar graph shows the density of dendritic spines from each group. $^{\#}$P<0.05 vs. Sham; *P<0.05, vs. MCAO. EA, electroacupuncture; MCAO, middle cerebral artery occlusion.

pixel intensities of the immunoreactive bands were quantified using the percentage adjusted volume feature of Image Lab 3.0 software (Bio-Rad Laboratories, Inc.). β-actin served as an internal control.

Statistical analysis. All data were analyzed using the SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) software package. Data are expressed as the mean ± standard deviation. Student's t-test, the Mann-Whitney U test and one-way analysis of variance were used to assess statistical differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of EA treatment on infarct volume in rats with cerebral IR injury. The effect of EA treatment at the Baihui (DU20) and Shenting (DU24) acupoints after cerebral infarction was investigated using TTC staining. As shown in Fig. 1, EA treatment reduced the cerebral infarct volumes. The total infarct volumes were 26.53±3.32 and 17.36±7.04% of the total brain volume in the MCAO and MCAO+EA groups, respectively (P<0.05).

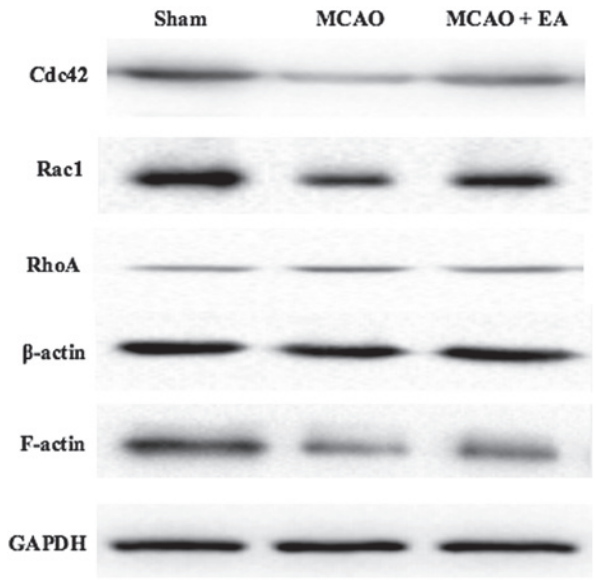

Figure 4. Effect of EA on Rho GTPases and F-actin. Western blot analysis of levels of Cdc42, Rac1, RhoA and F-actin in hippocampus tissue of Sham, MCAO and MCAO+EA groups (n=6). β-actin and GAPDH were used as internal controls. EA, electroacupuncture; MCAO, middle cerebral artery occlusion; RhoA, Ras homologous member A, Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, cell division cycle 42; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Effect of EA treatment on cognitive impairment in rats with cerebral IR-injury. In order to investigate the effect of EA treatment on cognitive impairment, a Morris water maze test was performed from the 3rd day after MCAO. As shown in Fig. 2, the duration and distances of swimming achieved by rats in the MCAO group increased as they sought the hidden platform during the trial, whereas the frequency of rats actually crossing the location of the platform was significantly decreased compared with rats in the Sham group (P<0.05). These results indicated that cerebral I/R injury led to cognitive impairment in the MCAO group. EA treatment significantly decreased the duration and distances swum by rats in the MCAO+EA group, as well as increased their frequency of crossing the location of the platform, when compared with rats in the MCAO group (P<0.05). These results suggest that EA treatment at the Baihui and Shenting acupoints protects cognitive function against impairment from cerebral IR-injury to a certain extent.

Effects of EA on dendritic spines in rats with cerebral IR-injury. Images of Golgi-stained neurons are shown in Fig. 3. In the Sham, MCAO and MCAO+EA groups, the majority of the dendritic spines were mushroom-shaped. The dendritic spines were sparse in the MCAO group. Compared with the Sham group, the MCAO group had a lower spine density (P<0.05). The density of dendritic spines in the MCAO+EA group was significantly increased compared with that in the MCAO group on day 7 (P<0.05).

Effect of EA treatment on the expression of Cdc42, Rac1, RhoA and F-actin. In order to investigate the mechanism underlying the protective effect of EA treatment on cognitive function, the expression of Cdc42, Rac1, RhoA and F-actin was examined

in the hippocampus of the left brain using western blotting. Figure 4 shows that MCAO injury decreased the expression of Cdc42, Rac1 and F-actin expression, but increased the expression of RhoA. By contrast, EA treatment significantly increased the expression of Cdc42, Rac1 and F-actin ($P<0.05$), and decreased the expression of RhoA at the protein level compared with the MCAO group. According to these results, EA treatment may protect cognitive function in MCAO rats through a mechanism that is closely associated with the regulation of Cdc42, Rac1, RhoA and F-actin expression.

Discussion

Stroke is associated with a higher risk of dementia and cognitive impairment without dementia, and ischemic stroke accounts for 80% of stroke cases (19,20). Acupuncture is based on traditional Chinese medicine, with proven efficacy in numerous health conditions, such as stroke and memory deficiency (21,22). According to the theory of traditional Chinese medicine, Baihui (GV20) and Shenting (DU24) acupoints belong to the Du Meridian, which is considered to affect the diseases of the nervous system. It has been established that electroacupuncture at the Baihui and Shenting acupoints exerts a therapeutic effect on post-stroke cognitive impairment (5). Therefore, the MCAO model was used to test the efficacy of EA. In the present study, EA treatment at Baihui and Shenting reduced infarct volume, and improved learning ability and memory in MCAO+EA rats compared with the MCAO group as determined by the Morris water maze test.

Learning and memory have a certain functional location in the brain. The hippocampus is an important storage structure for learning and memory. It has been extensively investigated in terms its physiological function as well as its involvement in pathological models (23,24). Dendritic spines are small mushroom-like protrusions arising from neurons where the majority of excitatory synapses reside. Dendritic spines are critical in cognitive and motor function, as well as memory formation. Memory formation is hypothesized to lead to an increase in dendritic spine density, and an increased spine density has been proposed to enhance learning ability after exposure to an enriched environment (25,26). The cytoskeleton of the dendritic spines predominantly consists of F-actin. It serves as a structural framework and as the principal regulator of protein and vesicular trafficking. In the present study, it was demonstrated that cerebral I/R injury decreased dendritic density, and treatment with EA was shown to significantly increase spine density and the expression of F-actin in the hippocampus.

Cognitive impairment is strongly associated with synaptic plasticity, which is regulated by various intracellular mechanisms, such as fluctuating expression levels of Rho GTPases. Over the past few years, it has become clear that Rho GTPases and related molecules are important in various aspects of neuronal development, including neurite outgrowth and differentiation, and the formation and maintenance of dendritic spines through its effect on the actin cytoskeleton (27-29). A recent imaging study detected persistent activation of Rho GTPases in the dendritic spine following long-term potentiation induction. The pharmacological blockade affected several downstream signaling pathways of these proteins, including p21-activated kinase (PAK) and Rho-associated, coiled-coil containing protein kinase (ROCK), and this effectively inhibited spine enlargement (30).

Rho GTPases act on several downstream effectors involved in the stabilization, contraction, polymerization and capture of cytoskeletal building blocks. Protein assemblies required for actin polymerization are induced by several Rho GTPases. For example, RhoA binds to mDia; Rac1 binds to WAVE; and Cdc42 binds to N-WASP (31). Microtubule stabilization is regulated by RhoA, Rac1 and Cdc42 through the actions of mDia, PAK or PAR6 (32). Regulation of the actin cytoskeleton by Rac and Cdc42 controls local dendritic spine growth, and its stability is associated with the Rac or Cdc42/PAK3/LIMK1 pathways. PAK3 is a downstream effector for Rac/Cdc42 Rho GTPases. Activation of PAK3 by Rac1 or Cdc42 leads to the phosphorylation of LIMK. In turn, activated LIMK phosphorylates and inactivates cofilin, resulting in actin depolymerization (33,34).

RhoA activates several other effector proteins, among them the Rho-associated coiled-coil-containing protein kinases, ROCKI and ROCKII. These proteins phosphorylate the myosin light chain and myosin phosphatase-targeting subunit 1, resulting in enhanced actomyosin-based contractility (35,36). The introduction of constitutively active RhoA decreases spine density and length, indicating that RhoA has a negative effect on spine formation and maintenance (37,38). Conversely, inhibition of Rho using C3 exoenzyme has been shown to increase the density and length of spines of certain mouse cortical and hippocampal pyramidal neurons (39). It is hypothesized that the extension of the dendritic spines and the maintenance of several pathways of the nervous system require the positive effects of Racl/Cdc42 and a negative effect of RhoA. In the present study, it was demonstrated that EA treatment upregulated the levels of Rac1 and Cdc42, and downregulated the expression of RhoA, compared with the MCAO group.

In conclusion, these results demonstrate the positive effect of EA on Baihui and Shenting acupoints, which lead to the improvement of cognitive function following cerebral I/R injury. In addition, the possible mechanisms through which the Rho family of GTPases act to enhance dendritic spine plasticity during EA analgesia was investigated. These data suggest that EA is a promising solution for the treatment of cognitive impairment after stroke. However, the long-term effects of EA on Baihui and Shenting are yet to be determined.

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