Abstract. Electroacupuncture (EA) may stimulate neurogenesis in animal models of ischemic stroke; however, the associated mechanisms are not clear. The present study aimed to evaluate the neurogenesis efficacy of EA on ischemic stroke and the underlying associated mechanisms. A model of middle cerebral artery occlusion (MCAO) was employed as the rat model of brain ischemia and reperfusion. EA treatment at the GV20 (Baihui) and GV14 (Dazhui) acupoints was conducted for 30 min daily following MCAO. Immunofluorescence was performed to measure the number of bromodeoxyuridine (BrdU)/nestin- or BrdU/doublecortin (DCX)-positive cells in the sham, MCAO and MCAO + EA groups. Results indicated that EA stimulation significantly decreased the neurological score and neuronal loss in rats in the MCAO group (both P<0.05). Furthermore, immunostaining assays indicated that BrdU/nestin- and BrdU/DCX-positive cells in EA-treated rats were significantly increased (P<0.05) when compared with the rats in the MCAO group, indicating EA may induce the proliferation and differentiation of endogenous neural stem cells (eNSCs) during cerebral ischemia-reperfusion. In addition, EA treatment significantly enhanced the protein expression levels of plasticity-related gene 5 (PRG5), a critical neurogenesis factor, and significantly decreased the protein expression levels of three neurogenesis inhibiting molecules, NogoA, lysophosphatidic acid and RhoA (all P<0.05). These results suggested that EA promotes the proliferation and differentiation of eNSCs, likely through modulating PRG5/RhoA signaling.

Introduction

Endogenous neural stem cells (eNSCs) have been proposed as a potential replacement of cells lost due to central nervous system injury, and the source of tropic molecules to minimize damage and promote recovery (1). eNSCs respond actively to injury stimuli, including brain ischemia-reperfusion (2-4). Furthermore, eNSCs may proliferate to maintain their self-renewal and differentiate into neurons, astrocytes and oligodendrocytes, and migrate from the subventricular zone (SVZ) or dentate gyrus (DG) to injured regions in order to repair damage (4). The rate of proliferation and differentiation will affect the number of eNSCs and neurons in different brain regions (5). The sufficient activation of neurogenesis and improving the neurological outcomes in a pathological condition are important for disease treatment.

Electroacupuncture (EA) has been used in East Asian countries, particularly in China, for >2,000 years to treat various diseases, including stroke (6). Recently, EA has been demonstrated as a good alternative therapeutic strategy compared with medicinal treatment, acupuncture, massage and physiotherapy to improve the symptoms of stroke (7) and has been indicated to effectively exert neuroprotective effects on patients who have experienced a stroke (8). A recent meta-analysis of a random, controlled study concluded that EA effectively improved ischemic stroke in patients (9). Additionally, animal studies have demonstrated that EA stimulation protects the brain against ischemia-reperfusion injury (10,11). Mechanistically, the neuroprotective role of EA is associated with anti-inflammatory, anti-oxidative and anti-apoptotic signaling pathways (11,12). Our previous study...
also identified that EA attenuated neuronal injury in the cervical spinal cord following brain ischemia in stroke-prone renovascular hypertensive rats (13).

EA has been indicated to stimulate neurogenesis, which may be another potential mechanism that elucidates why EA improves the neurological outcome in clinical and preclinical settings (14). EA has been reported to promote neurogenesis in the striatum (15), SVZ (16) and hippocampal DG (17) in animals with stroke. In-depth understanding of the time course of the neurogenesis effect induced by EA and the relevant mechanisms would benefit stroke therapy in the future.

NogoA is an inhibitor of neurite growth and is regulated by two inhibitory domains: 66-amino acid region (Nogo-66) and the N-terminal region (amino-Nogo) (18-20). Nogo-66 inhibits activity-dependent axonal growth by binding to the Nogo-66 receptor-1 (NgR1) (20). In turn, the NgR1 competitive antagonist, NEP1-40 (Nogo-66, residues 1-40), and antibodies against NogoA block this inhibitory effect and improve the neurological outcomes following ischemic stroke in adult rats (21,22). Lyosphosphatidic acid (LPA) is a bioactive lipid (23). LPA and NogoA are associated with the RhoA-Rho kinase signaling pathway, and regulate neurogenesis, although they act on different receptors (24). Plasticity-related gene 5 (PRG5) is regulated during brain and spinal cord development and is exclusively allocated within the nervous system (24,25). PRG5 has been demonstrated to impede LPA/RhoA and NogoA/RhoA signaling (24,25), which suggests that PRG5 may function as a positive regulator of neurogenesis. Previously, we have indicated that EA stimulation reduced brain ischemia-induced elevation of NogoA and NgR1 protein expression levels at day 14 and 28 in renal vascular hypertensive rats (13). However, whether PRG5/RhoA signaling is involved in the neurogenesis of EA is unknown.

The present study aimed to investigate the proliferation and differentiation of eNSCs following EA stimulation in brain ischemia-reperfused rats in the chronic phase. The dynamic expression levels of neurogenesis factors, including PRG5, RhoA, NogoA, LPA, were also detected to explore the potential mechanisms involved.

Materials and methods

Ethics statement. All animal treatments were conducted in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition, 2011). Experiments were performed with the approval of the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine (Foshan, China).

Animals. A total of 170 male Sprague-Dawley rats (weighing 200±20 g; 12 weeks old), were used. Rats were acclimatized for 3 days prior to experimentation to laboratory conditions (temperature, 25±1°C; humidity, 65±5%) and were maintained on a 12-h light/dark cycle and had free access to food and water. All efforts were made to minimize the number of animals used and their suffering. Animals were randomly divided into the sham, MCAO and MCAO + EA groups.

Focal cerebral ischemia-reperfusion and EA treatment. Middle cerebral artery occlusion (MCAO) was employed to induce focal ischemia-reperfusion, as we previously described (26). The rats were anesthetized with 5% isoflurane (Pharma Handelsgesellschaft mbH, Burgdorf, Germany) and maintained with 2% isoflurane in an oxygen/air mixture in a stereotaxic frame (flow rate, 2 l/min; Stoelting Co., Wood Dale, IL, USA). Once ischemia had been established for 2 h, the monofilament nylon suture was removed to induce reperfusion on days 1, 7 and 14. Rats in the sham-operated group underwent the same procedures without occluding the MCA. Notably 8 rats succumbed to surgical complications and were therefore not included in further analysis.

Rats were randomly divided into three groups (n=54 per group): The sham group, where rats received sham stimulation (no EA current stimulation following MCAO); the MCAO group, where rats received MCAO and sham stimulation; and the MCAO + EA group, where rats received MCAO and EA stimulation. Each group was further divided into three subgroups with reperfusion times of 1, 7 and 14 days following MCAO (n=18 in each group). EA treatment was conducted at the GV20 (Baihui) and GV14 (Dazhui) acupoints for 30 min daily following the induction of MCAO. During EA administration, rats were maintained within a cloth bag. Sterilized disposable stainless-steel needles (0.2x25.0 mm, Huan Qiu Brand, manufactured by Suzhou Medicine Co., Ltd., Suzhou, China) were inserted obliquely as deep as 5 mm at GV20 (Baihui) and GV14 (Dazhui) points. Electric stimulation was generated using an electrical stimulator (Hua Tuo SDZ-II; Hua Tuo Medical Instruments Co., Ltd., Suzhou, China). EA treatment commenced 30 min per session. The stimulation parameter exhibited disperse-dense waves of a frequency of 5/20 Hz (28.5/15 msec pulse duration) and a current density of 2-4 mA.

Evaluation of neurological deficits. Neurological deficits were scored 48 h following reperfusion by another investigator who was blinded to the experimental groups. The scoring criteria for neurological deficits were as follows: 0 points, no neurological deficit; 1 point, difficulty in fully extending the contralateral forelimb; 2 points, unable to fully extend the contralateral forelimb; 3 points, circling to the contralateral side; and 4 points, no consciousness or ambulation. The higher score of the neurological deficits represented greater impairment of motor function.

Bromodeoxyuridine (BrdU) labeling. The S-phase marker BrdU (50 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (27) was intraperitoneally injected twice a day for 5 consecutive days prior to ischemic injury. Sham surgery rats received the same dose of BrdU, which was also administered 5 days before rats were subjected to the same procedures without occluding the MCA. Following injection, BrdU was incorporated into the DNA of dividing cells and its signaling was observed using a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Immunofluorescence. Brains were removed and hippocampal tissues were fixed with formaldehyde-sucrose for 24 h at 20°C. Brains were removed and fixed with 4% paraformaldehyde at 4°C for 24 h. Following dehydration in graded ethanol and
xylene, brain slices were embedded in paraffin, sectioned to 4 µm in thickness, dewaxed, rehydrated and stained with 1% toluidine blue at 37°C for 30 min. Following rinsing, sections were dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted with Permount cover slips. Brain sections were washed twice with PBS, fixed with 4% paraformaldehyde at 4°C for 1 h, permeated with 0.1% Triton for 30 min at 4°C. Samples were then blocked with 10% normal goat serum (Vector Laboratories, Inc, Burlingame, CA, USA) for 30 min at 4°C and incubated with primary antibodies overnight at 4°C. The primary antibodies used included mouse anti-BrdU (1:100; EMD Millipore, Billerica, MA, USA), mouse anti-Nestin (1:200; EMD Millipore), mouse anti-Doublecortin (DCX; 1:200; Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA). The sections were then incubated with fluorescein-conjugated secondary antibodies rhodamine-conjugated donkey anti-mouse IgG antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) used as secondary antibodies. The secondary antibody solution (1:200) was then added and incubated at room temperature for 1 h. Immunostained sections were visualized using an inverted Olympus fluorescence microscope (LSM 700; Carl Zeiss GmbH; Jena, Germany). For immunohistochemistry, the number of positive cells or staining intensity in the peri-ischemic zone was measured at magnification of x200. In all of the slices, five fields per sample and three to six tissues were quantified in each group. Expression was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics Inc, Buckinghamshire, UK). For BrdU, DCX and Nestin, positive cells were counted in five different fields per rat by an observer blind to the present study, and the quantitation was performed by calculating the positive cells per mm².

**Nissl staining.** Isolated brain sections from rats in each group were dehydrated, dipped into paraffin and, using a microtome, sliced into 5 µm tissue sections. Samples were sectioned into three slices, starting 3 mm from the anterior tip of the frontal lobe in the coronal plane. The second set of slices was used to evaluate neurologic deficits of rats at 1, 7 and 14 days after induction of ischemia-reperfusion. The neuroprotective role of EA stimulation was evaluated. The modified neurological score was used to compare multiple western blot and BrdU labeling datasets. P<0.05 was considered to indicate a statistically significant difference.

### Results

**Western blot analysis.** The rat ischemic cerebral hemispheres and bilateral hippocampi were isolated at day 1, 7 and 14. Protein concentration was determined using a BCA Protein Assay Reagent kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer’s instructions. Once proteins were denatured in SDS-loading buffer, protein samples (50 µg/lane) were separated using 8-12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (EMD Millipore). Following blocking in PBS blocking solution containing 3% non-fat powdered milk and 0.3% Tween for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies, including mouse NogoA monoclonal antibody (AB5888; 1:1,000; EMD Millipore), mouse PRG5 monoclonal antibody (AB15172; 1:500; EMD Millipore), mouse RhoA monoclonal antibody (sc-28565; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-β-actin (TA-09; 1:500; ZSGB-Bio, Inc., Beijing, China), followed by incubation with anti-rabbit or anti-mouse horse-radish peroxidase-conjugated secondary antibodies (sc-2371; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Bands were detected by chemiluminescence technology using an ECL advanced western blot analysis detection reagent (EMD Millipore), quantified and normalized to β-actin using ImageJ software (version 1.38, National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Statistical analysis was performed using SPSS for Windows (v. 14.0; SPSS, Inc., Chicago, IL, USA). Experiments were repeated in triplicate independently. Data were expressed as the mean ± standard error of the mean. One-way analysis of variance with Dunnett’s post hoc testing was used to compare multiple western blot and BrdU labeling datasets. P<0.05 was considered to indicate a statistically significant difference.
agreement with previous findings that indicated EA stimulation has a positive neuroprotective effect in a model of cerebral ischemia-reperfusion (28).

**EA treatment promotes the proliferation and differentiation of eNSCs in rats with MCAO.** To investigate the neurogenesis effects of EA treatment, BrdU incorporation and nestin staining assays were conducted to detect the proliferation of eNSCs. BrdU is a marker of newly formed cells but not undivided cells. Nestin is a specific marker protein for neural stem cells (29). BrdU-positive cells (red) represented the new dividing nerve cells, whereas nestin-positive immunoreactive cells (green) indicated eNSCs. The double-stained cells (yellow) in the merged Brdu/Nestin images revealed the newly generated immature neurons and indicated eNSCs that were in the proliferous period (Fig. 3A-C). Results demonstrated cell cycle-specific nestin was increasingly expressed in a time-dependent manner, until day 14, where expression appeared to decrease (Fig. 3D). Following focal cerebral ischemia at day 1, the number of nestin immunoreactive cells increased in the DG zone. Furthermore, an increased expression of immunoreactive cells was indicated in the infarct area of the DG zone at day 7 compared with day 1 in the MCAO and the MCAO + EA group. In addition, the number of nestin immunoreactive cells in the MCAO + EA group was significantly higher than that in the MCAO group, especially on
day 7. Double staining of BrdU and nestin in the MCAO + EA group demonstrated that a significantly increased number of the proliferous eNSCs were expressed compared with those in the MCAO group on days 1, 7 and 14 (P<0.05), suggesting EA stimulation increased eNSC proliferation in rats with MCAO.

In response to injurious stimuli, eNSCs may differentiate into neurons (30). To investigate whether EA stimulates neurogenesis during brain ischemia-reperfusion, brain sections were co-stained with BrdU and antibody for DCX, a marker of newborn neurons (31), as indicated in Fig. 4A-C. The number of DCX immune-reactive cells increased at day 1, peaked at day 7 and declined at day 14 (Fig. 4) in the DG zone of rat hippocampus. The DCX immunoreactive cells were located in the infarct DG zone. No DCX immunoreactive cells were detected in the contralateral hemisphere of the brain in the sham group. Few DCX immunoreactive cells were indicated in the MCAO group. Notably, the number of DCX immunoreactive cells in the MCAO + EA group was significantly higher than that in the MCAO group, particularly on day 7 (P<0.05; Fig. 4D). These results suggest that EA stimulation may effectively induce neurogenesis during brain ischemia and reperfusion.

**EA treatment increases PRG5 expression and reduces NogoA-LPA/RhoA signaling in the brain of rats.** To study the underlying mechanism of EA-mediated neurogenesis in the established model of stroke, neurogenesis-regulated signaling molecules were detected with western blot analysis. Results in Fig. 5A revealed that MCAO lowered the protein expression levels of PRG5 and increased RhoA, NogoA and LPA in comparison with the sham group at day 7, and quantification of these results indicated the differences were statistically significant (all P<0.05; Fig. 5B-E). However, compared with the MCAO group, treatment with EA significantly elevated the protein expression levels of PRG5, whose expression was even higher compared with the sham group (P<0.05; Fig. 5B). Notably, EA treatment significantly decreased RhoA, LPA and NogoA protein expression levels, compared with the MCAO group (all P<0.05; Fig. 5C-E, respectively). These data suggest that PRG5/NogoA-LPA/RhoA signaling likely mediates EA-induced neurogenesis in rats with stroke.

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**Figure 3.** EA treatment promotes proliferation of endogenous neural stem cells in rats with MCAO. Representative DG zone from (A) sham, (B) MCAO and (C) MCAO + EA groups were subjected to immunofluorescence labeling with BrdU (red) and nestin (green) staining. Results suggested that the number of BrdU/Nestin positive stained cells (yellow) was attenuated in the MCAO group compared with that in sham group. However, EA treatment increased the number of BrdU/Nestin positive cells compared with that in the MCAO group. Scale bar=20 µm. (D) The quantified data of nestin/BrdU positive cells. Data were represented as the mean ± standard error of the mean (n=6 in each group). *P<0.05 vs. Sham group; #P<0.05 vs. MCAO group. MCAO, middle cerebral artery occlusion model; EA, electroacupuncture; BrdU, bromodeoxyuridine.

**Figure 4.** EA treatment promotes neuron differentiation of endogenous neural stem cells in rats with MCAO. Representative images of BrdU- and DCX-positive cells in the DG Zone of the (A) Sham, (B) MCAO and (C) MCAO + EA groups at day 14. Groups were subjected to immunofluorescence labeling with BrdU (green), DCX (red) staining. Double-stained cells (yellow) represent newly generated immature neurons. White arrows indicate BrdU and DCX stained cells. Scale bar=20 µm. (D) Quantified results of nestin/DCX-positive cells. Data were represented as the mean ± standard error of the mean (n=6 in each group). *P<0.05 vs. Sham group; #P<0.05 vs. MCAO group. MCAO, middle cerebral artery occlusion model; EA, electroacupuncture; BrdU, bromodeoxyuridine; DCX, doublecortin.
Discussion

The present findings indicated that EA effectively ameliorated neuronal injury and activated the proliferation and differentiation of eNSCs, which may contribute to the repair of the central nervous system and improvement of neurological function in a rat model of brain ischemia-reperfusion.

EA has been used as a treatment for stroke for >2,000 years in East Asia (32). The neuroprotective and neurogenesis roles of EA in animals with stroke have been previously reported in various studies (8,9,11-13). The present study focused to elucidate EA-stimulated eNSC proliferation and neuronal differentiation at day 1, 7 and 14 following reperfusion in rats with MCAO. The time points of EA-induced neurogenesis were in line with the length of EA treatment, which suggests the neurogenesis-inducing role of EA contributes to brain repair during the chronic phase of ischemic stroke.

BrdU was used to label newly-divided cells, which were observed in the DG zone and ipsilateral hemisphere of the ischemic brain; however, the proliferation sequence and absolute BrdU-labeled cell counts were significantly different (33). The DG is a potential source of neural precursors for neuronal injury (34). BrdU-labeled cells were identified in the DG zone, which was thought to be the source of neural precursor cells, and the ipsilateral cortex. Furthermore, the absolute number of BrdU-labeled cells in the ipsilateral cortex was five times higher compared with the peak cell count in the DG zone (data not shown). The migration speed of DG cells in the rostral migration stream has been indicated to be slow in reaching the lesion (35). The proximity of stroke injury to the lateral ventricle wall may determine whether there is a stem/progenitor cell response from the DG. Alternatively, focal (mild) cortical stroke may not induce neuroblast migration from the DG because it does not directly injure the DG itself. The MCAO model produces stroke by disruption of blood flow to cortical surface arteries. Other MCA occlusion models are generated by occlusion of the proximal MCA that disrupts blood flow to various areas, including the lateral ventricle. Because the neurovascular relationship is a key factor in maintaining the neurogenic niche at the DG, DG may impact neurogenesis. In proximal MCA occlusion models, newly born mature neurons in the striatum, radial glial-like cells at the DG, and/or migration of neuroblasts to the infarct zone have been reported (36), suggesting that the disruption of blood flow in neurogenic niches may induce neurogenesis. This suggests that the DG zone is the dominant neural stem cell niche during cerebral ischemia. Furthermore, this finding suggests the possibility of intraparenchymal progenitors in the neocortex, which is consistent with a previous report (37).

RhoA inhibits survival and growth of newly developed neurons, and functions as a negative regulator in neuronal survival and maturation (38). RhoA (and other small GTPase family proteins) is the convergent signal of multiple upstream molecules, including PRG5, LPA and NogoA (39). Furthermore, PRG5 is a negative regulator, whereas LPA and NogoA are the positive regulators of RhoA signaling (24). In the present study, EA stimulation upregulated PRG5 and downregulated the protein expression levels of RhoA, LPA and NogoA, which suggests these neurogenesis molecules may participate in EA-induced neurogenesis activity, although gene loss-of-function studies are required to further elucidate their roles in the future.

In conclusion, the present study indicated that EA improves neuronal function, reduces neuronal loss and promotes the proliferation and differentiation of eNSCs. Furthermore, the present findings suggested that the neurogenesis activating role of EA is likely through modulating PRG5/RhoA signaling. These preclinical investigations may provide the experimental basis for the treatment of ischemic stroke using EA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

FT and YG conceived and designed the study. JW and JXL and CW and ML performed the experiments. JW and CW and JXL wrote the paper. FT and YG and JW reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine (Foshan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


