Electroacupuncture Regulates Hippocampal Synaptic Plasticity via Inhibiting Janus-Activated Kinase 2/Signal Transducer and Activator of Transcription 3 Signaling in Cerebral Ischemic Rats

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Objective: To determine the mechanism(s) involved in electroacupuncture (EA)-mediated improvements in synaptic plasticity in a rat model of middle cerebral artery occlusion and reperfusion (MCAO/R)-induced cognitive deficits. *Methods:* Focal cerebral ischemic stroke was induced by (MCAO/R) surgery. Rats were randomly split into 4 groups: control group (sham operation control), MCAO group, *Baihui* (GV 20) and *Shenting* (GV 24) acupoint EA group (verum acupuncture, MCAO + VA), and nonacupoint EA group (control acupuncture, MCAO + CA). EA treatment was administered for 14 consecutive days in MCAO + VA and MCAO + CA groups. Neurological assessment, behavioral performance testing, and molecular biology assays were used to evaluate the MCAO/R model, EA therapeutic effect and potential therapeutic mechanism(s) of EA. *Results:* Significant amelioration of neurological deficits was found in MCAO + VA rats compared with MCAO rats ($P < .01$). Moreover, learning and memory significantly improved in EA-treated rats compared with MCAO or MCAO + CA rats ($P < .05$) together with an increase in the number of PSD-95$^+$ and SYN$^+$ cells and synapses in the hippocampal CA1 region ($P < .05$). MCAO + VA rats also showed amelioration of pathological synaptic ultrastructural changes compared with MCAO or MCAO + CA groups ($P < .001$). In contrast, EA decreased the levels and phosphorylation of JAK2 (Janus-activated kinase 2) and STAT3 (signal transducer and activator of transcription 3) in the hippocampal CA1 region compared with MCAO or MCAO + CA group ($P < .01$). *Conclusion:* EA at GV 20 and GV 24 acupoints improved cognitive deficits in cerebral ischemic rats via the JAK2/STAT3 signaling pathway and mediated synaptic plasticity in the peri-infarct hippocampal CA1 region of rats following ischemic stroke.

Key Words: Electroacupuncture—ischemic stroke—cognitive deficit—synaptic plasticity—JAK2/STAT3

Abbreviations: IR, ischemia/reperfusion; MCAO/R, middle cerebral artery occlusion and reperfusion; EA, electroacupuncture; SYN, synaptophysin; PSD-95, postsynaptic density protein 95; JAK2, Janus-activated kinase 2; STAT3, signal transducer and activator of transcription 3.

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Introduction

Stroke is ranked as the second leading cause of death after ischemic heart disease and the third commonest cause of disability globally.[1] Ischemic stroke accounts for approximately 80% of all strokes.[2] Increasing evidence has shown that ischemic stroke increases the risk of dementia or cognitive decline.[3,4] In fact, nearly 65% of stroke survivors are estimated to suffer from cognitive impairment.[5] According to report, the total annual expenditure of stroke and stroke-related injury is projected to increase to $240.67 billion in the United States by 2030, and a substantial proportion of that cost is due to cognitive impairment.[6]

Poststroke cognitive impairment is a multidomain impairment of cognitive ability particularly affecting learning and memory, language, and executive function.[7] Learning and memory problems are the most common form of cognitive impairment and can significantly impact rehabilitation of other cognitive functions. Thus, improvement of learning and memory benefits stroke rehabilitation. Studies have shown that hippocampal degeneration is central to memory loss in cognitive impairment.[8] Progressive decline of learning and memory is thought to result from synapse loss in the hippocampus[9] and is an important target for treatments aimed at improving cognitive deficits.[10]

Synaptophysin (SYN) is a 38-kDa calcium-binding glycoprotein located in the presynaptic membrane[11] that can be used as a specific marker of the presynaptic terminal.[12] SYN levels are an index of synaptic number and density.[13] Postsynaptic density protein-95 (PSD-95) is the most abundant protein of the PSD at glutamatergic synapses known for its role in regulation of synaptic plasticity.[14] Enhanced PSD-95 expression may be a marker of cognitive impairment.[15,16] Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) proteins may help drive the survival response of neurons following ischemia reperfusion injury, and this neuroprotective effect can be activated by many therapies, including acupuncture.[17] Previous studies have also shown the JAK2/STAT3 signaling pathway is associated with spatial learning and memory through synaptic plasticity in the hippocampal CA1 region.[18,19]

Acupuncture originated in ancient China has long been used as an alternative and complementary medicine for patients with stroke-related cognitive impairment to improve their quality of life and prevent further decline in cognitive function.[20] Recent clinical study demonstrated that electroacupuncture (EA) at points *Baihui* (GV 20; located in the median of the parietal bone and the line linking the 2 ears) and *Shenting* (GV 24; located in the median of frontalis) is an effective and safe strategy for treatment of cognitive dysfunction due to stroke.[20,21] The results of our previous study also showed that EA at GV 20 and GV 24 acupoints had significant neuroprotective effects against learning and memory impairment induced by cerebral ischemia-reperfusion injury.[22] Therefore, the hypothesis of current study was that the effects of EA at GV 20 and GV 24 on learning and memory impairment induced by ischemia-reperfusion injury plays via the JAK2/STAT3 signaling pathway associated with spatial learning and memory through synaptic plasticity in the hippocampal region.

Materials and Methods

Experimental Animals and Groups

Seventy-two 12-week-old SPF Male Sprague-Dawley rats (220-270 g) were provided by SLAC Laboratory Animal Co., Ltd. (SCXK 2012-003; Shanghai, China) and housed at the Fujian University of Traditional Chinese Medicine Laboratory Animal Center (Fuzhou, China). Rats were kept under a 12-hour light/12-hour dark cycle in a temperature-controlled room (22°C) and provided adequate water and food. All animal treatments were strictly in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (No.2016100). According to the method of random number table, all the rats were randomly divided into 4 groups (n = 18 each group): control group (sham operation control), middle cerebral artery occlusion and reperfusion (MCAO) group, GV 20 and GV 24 acupoint EA group (verum acupuncture, MCAO + VA), and nonacupoint EA group (control acupuncture, MCAO + CA). Cerebral ischemia-reperfusion injury was induced in MCAO, MCAO + VA, and MCAO + CA rats by and reperfusion (MCAO/R) according to previous studies.[23,24] Rats were anaesthetized using an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg). The left common carotid artery, the left external carotid artery, and internal carotid artery were carefully exposed by a midline neck incision. Approximately 18-22 mm of 4-0 nylon surgical thread was inserted into the left internal carotid artery internal carotid artery until the blunted distal end felt resistance to block the middle cerebral artery (MCA). The thread was removed to allow blood to the left MCA area following 90 minutes of occlusion. The control group rats underwent above procedure without the occlusion of the MCA as reported previously.[25,26]

EA Treatment

After 24 hours of recovery from MCAO/R surgery, MCAO + VA group rats underwent EA stimulation for 30 minutes at GV 20 and GV 24 acupoints once a day for 14 days. All intervention processes were operated in a room which is freedom from interference. Rats were fixed by a special device (JKY/SGP-3, Xihuayi Science and Technology Ltd.,Beijing) during EA when they were awake. The acupuncture needles (diameter, 0.3 mm, Huatuo acupuncture

needle, Suzhou Medical Appliance Factory, Suzhou, China) were inserted at a depth of 2-3 mm into the points. EA stimulation was generated using a model G6805 EA device (Model G6805, Shanghai Huayi [Group] Company, Ltd., Shanghai, China) with condensation and rarefaction waves of 1-20 Hz and 0.2 mA intensity. Rats in the MCAO + CA group were administered EA stimulation at the bilateral costal region (below the costal region, 10 and 15 mm superior to the iliac crest)[27]; needles, stimulation parameters and EA apparatus were identical to that of the MCAO + VA group. Rats of the control group and the MCAO group were given no treatment. Manipulators were experienced and blinded to the grouping of the rats.

Neurological Assessment

Neurological deficit scores were assessed 2 hours after MCAO/R surgery, as well as 1, 7, and 14 days after EA intervention. Scoring rules were determined previously by Longa et al[28] as follows: score 0, no obvious neurological deficit symptoms; (1) failure to stretch the right forepaw completely; (2) circling to the right when walking; (3) falling to the right when walking; and (4) no spontaneous walking. In order to ensure the success of model and the survival rate of rats, rats which scored a 0 or 4 were excluded from the study according to previous reports[22,25]. The neurobehavioral test and histological scorings were performed in a blinded fashion.

Step-Down Passive Avoidance Test

The step-down apparatus (Xinruan Information Technology Co., Ltd., Shanghai, China) used in the present study was an experimental box (20 × 20 × 60 cm) with a bottom consisting of parallel stainless steel rods and a rubber platform placed on the center of the rods. All step-down testing equipment was located in a free-noise room. The experiment consisted of 2 parts as described below.

Training phase: Acquisition training was evaluated 2 hours after MCAO/R surgery and 13 days after EA intervention. Animals were placed in the step-down box 3 minutes before the test. During training, parallel stainless steel rods were electrified (36 V). The animals were initially placed on the rubber platform and received an electric shock by stepping off the platform onto the steel rods. The latency period from the time rats were placed on the rubber platform until they stepped down onto the steel rods was recorded.

Test phase: Step-down tests were conducted 24 hours after MCAO/R surgery and 14 days after EA intervention. The amount of time spent on the platform before stepping onto the rods was recorded as the latency. However, if the rats did not step off the rubber platform within 3 minutes of being placed there, the latency was recorded as 180 seconds.

Immunohistochemistry

Immunohistochemistry was performed according to previous studies.[29] Six rats from each experimental group were deeply anaesthetized with pentobarbital sodium (concentration 3%, 30 mg/kg per rat) by intraperitoneal injection. Their brains were harvested immediately and fixed in cold 4% paraformaldehyde, and then cut into 5-μm thick sections. Brain sections were incubated at 4°C overnight with rabbit anti-PSD-95 (1:400; D74D3, Cell Signaling Technology, Boston, MA) and rabbit anti-SYN (1:1000; ab32127, Abcam, Cambridge, UK) antibodies. The next day, avidin-biotin-peroxidase reagents were added, followed by staining with DAB (DAB kit-001, Maixin Technology Co., Ltd., Fuzhou, China). Images were captured using a Leica DM4-000B LED microscope (Leica, Wetzlar, Germany) at 400× magnification, and ImagePro Plus was used to analyze images. Positive cells were counted in 4 randomly selected microscopic fields.

Transmission Electron Microscopy

Six rats that did not undergo immunohistochemistry from each experimental group were randomly selected for pathological synaptic ultrastructural experiment. The brain tissues were taken from the left ischemic hippocampus, cut into 1-mm^3 cubes, fixed in 1% paraformaldehyde with 1% lanthanum nitrate tracer for 24 hours, and fixed in 3% glutaraldehyde for another 24 hours. Then, samples were fixed with 1% osmium tetroxide for 2 hours and dehydrated in graded ethanol-1% lanthanum nitrate tracer solution and embedded in araldite. Ultrathin hippocampal CA1 slices (90-nm thick) were obtained and stained with lead citrate and uranyl acetate. A H-7650 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan) was used to observe ultrastructures, and images were captured with a digital CCD camera (SIS, 4 million voxels; Hewlett-Packard). Synapses of neuron cells were counted in 4 randomly selected microscopic fields.

Western Blotting

The levels of JAK2, STAT3, phosphorylated (p)-JAK2, and (p)-STAT3 expression were carried out by Western blot. The left hippocampal were separated quickly from rats that did not undergo immunohistochemistry or transmission electron microscopy (6 rats from each group). A total of protein (25 μg) obtained from the left cerebral hippocampal tissue of rats from each experimental group was loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluoride membrane. Blots were blocked with 5% skim milk for 2 hours, and then incubated with primary antibodies against JAK2 (1:2000; ab108586, Abcam), STAT3 (1:2000; ab68153, Abcam), p-JAK2 (1:2000; C8043, Cell Signaling Technology), p-STAT3 (1:200000; ab76315, Abcam), and β-actin (1:1000; HC201-01, TransGen Biotech, Beijing,

China) overnight at 4°C. The following day, blots were incubated with appropriate antirabbit or antimouse secondary antibodies (1:5000; Perkin-Elmer Life Sciences, Waltham, MA) for 1.5-2 hours (1.5 hours for JAK2, STAT3, and β-actin; 2 h for p-JAK2 and p-STAT3) with shaking. Proteins of interest were detected with enhanced chemiluminescence, and images were taken and examined with a ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA). Western blotting was repeated 3 times.

Statistical Analysis

Quantitative values shown represent the mean ± standard error of the mean. Statistical comparisons were made using a nonparametric test and 1-way analysis of variance between groups. A 2-tailed *P* value smaller than .05 was considered as statistically significant.

Results

Effect of EA on Neurological Deficits

Neurological deficit scores were evaluated as a measure of the neuroprotective function of EA. The results showed that rats in control group had no symptoms of neurological deficit, whereas MCAO, MCAO + VA, and MCAO + CA groups exhibited clear signs of cerebral injury. However, MCAO + VA rats showed significant improvement compared to MCAO rats ($P = .003$) and relative to MCAO + CA rats after 14 days ($P = .038$; Fig 1). These results suggest that EA at GV 20 and GV 24 has a therapeutic effect on cerebral ischemia-reperfusion injury.

Effect of EA on Step-Down Passive Avoidance

The step-down passive avoidance test was used to assess the therapeutic effect of EA on memory after MCAO/R surgery. Interestingly, the step-down latency was significantly shorter after the MCAO/R surgery than in control group (Fig 2). After 14 days, the step-down latency of MCAO rats was remarkably shorter than that of the control group ($P < .001$). On the other hand, the step-down latency of MCAO + VA rats was significantly prolonged relative to MCAO and MCAO + CA rats ($P = .012$ and $P = .022$ respectively; Fig 2). These data suggest that EA at GV 20 and GV 24 improves the memory of rats with MCAO/R injury, preventing an electric shock.

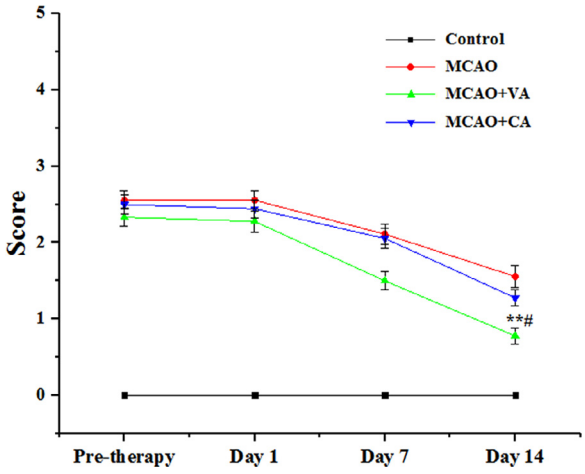

Figure 1. *Neurological deficit.* $^{\#}P < .05$, *MCAO + VA group versus MCAO + CA group;* $^{**}P < .01$, *the MCAO + VA group versus the MCAO group. Abbreviations: CA, control acupuncture; MCAO, middle cerebral artery occlusion; VA, verum acupuncture.*

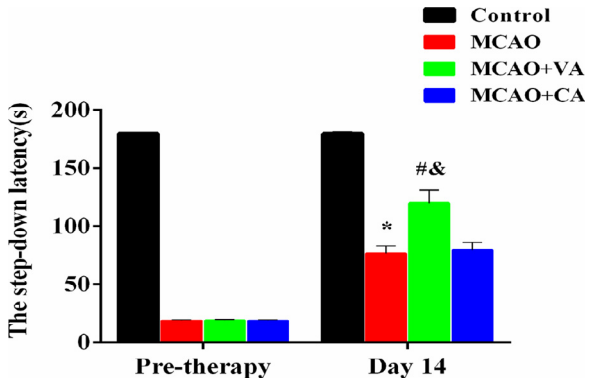

Figure 2. *Step-down passive avoidance text. The step-down latency before and after EA stimulation.* $^{*}P < .001$, *MCAO group versus control group;* $^{\#}P < .05$, *MCAO + VA group versus MCAO group;* $^{\&}P < .05$, *MCAO + VA group versus MCAO + CA group. Abbreviations: CA, control acupuncture; MCAO, middle cerebral artery occlusion; VA, verum acupuncture.*

Effect of EA on Levels of Hippocampal CA1 PSD-95 and SYN

The effect of EA on PSD-95 and SYN levels in the hippocampal CA1 region was assessed by immunohistochemistry. The number of PSD-95^{+} and SYN^{+} cells in the MCAO group was significantly lower than that in control group ($P = .003$ and $P = .048$, respectively; Fig 3A-D), whereas the number of PSD-95^{+} and SYN^{+} cells was significantly increased in the MCAO + VA group relative to MCAO and MCAO + CA group ($P = .030$, $P = .032$ for PSD-95^{+} and $P = .008$, $P = .013$ for SYN^{+}, respectively; Fig 3A-D). These results confirm that EA at GV 20 and GV 24 can increase the levels of PSD-95 and SYN in the hippocampal CA1 region after MCAO/R injury.

Effect of EA on the Number of Synapses and Ultrastructure

The effect of EA on ultrastructural morphology of hippocampal CA1 pyramidal neurons was assessed. In the MCAO group, the number of synapses was significantly lower than that in control group ($P < .001$; Fig 4A and C). In contrast, the number of synapses was significantly increased in the MCAO + VA group relative to both MCAO and MCAO + CA group (both $P < .001$; Fig 4A and C). The distribution of synaptic vesicles was abundant and closely

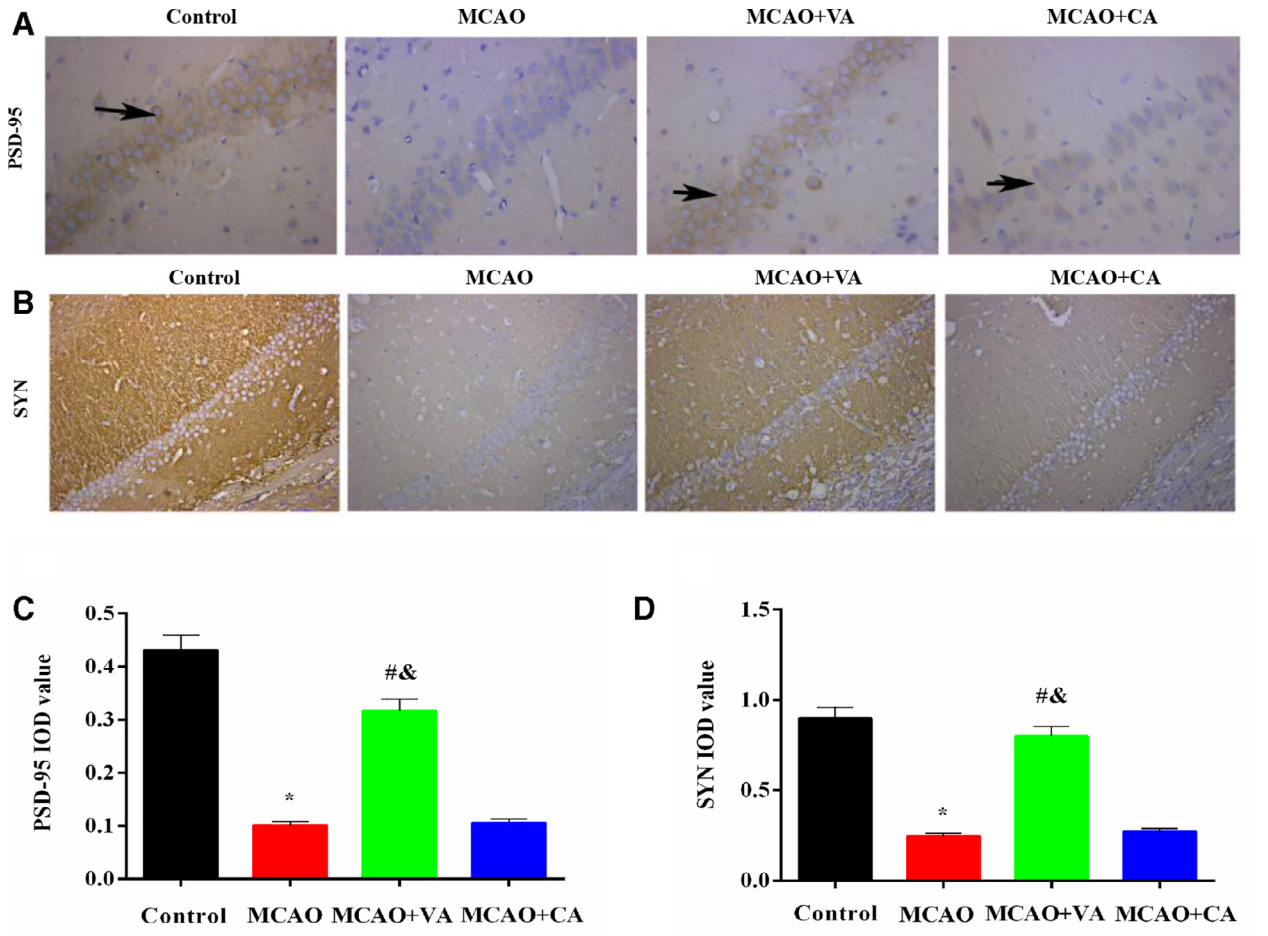

Figure 3. *Immunohistochemical detection of the PSD-95 and SYN expression in hippocampal CA1 region.* ***(A** and **B)** Immunohistochemistry of PSD-95-positive and SYN-positive cells at day 14 after EA intervention. (**C** and **D**) The positive cells of PSD-95 and SYN in MCAO group and MCAO + CA group presented lower density than that in MCAO + VA group and control group.* *P < .05, *MCAO group versus control group;* #P < .05, *MCAO + VA group versus MCAO group;* &P < .05, *MCAO + VA group versus MCAO + CA group. Abbreviations: CA, control acupuncture; MCAO, middle cerebral artery occlusion; PSD, postsynaptic density protein; VA, verum acupuncture.*

arranged in the control group, with a clearly visible synaptic structural profile (Fig 4B). However, fusion of synaptic space, loss of synaptic vesicles, incomplete synaptic structure, and swelling of the presynaptic terminal were observed in the MCAO and MCAO + CA groups; these changes were obviously improved in MCAO + VA group. Taken together, these results indicate that EA at GV 20 and GV 24 can improve the number and ultrastructure of synapses following MCAO/R injury.

Effect of EA on Levels and Phosphorylation of Hippocampal CA1 JAK2 and STAT3

To further explore the underlying molecular mechanism(s) of EA-induced improvements in synaptic plasticity, the levels and phosphorylation status of JAK2 and STAT3 expression in the hippocampal CA1 region were investigated by Western blot. As showed in Figures 5A and B, MCAO group p-JAK2 and p-STAT3 levels in the hippocampal CA1 region were significantly increased relative to control group (both $P < .001$). On the other hand, the levels of p-JAK2 and p-STAT3 were significantly decreased in MCAO + VA group compared to MCAO and MCAO + CA groups ($P < .001$, $P = .002$ for p-JAK2 and both $P < .001$ for p-STAT3, respectively; Fig 5A and B), indicating that EA intervention at GV 20 and GV 24 significantly affects the JAK2/STAT3 signaling pathway.

Discussion

Cognitive impairment significantly affects quality of life following a cerebral ischemic event.[30] In traditional Chinese medicine, acupuncture has been used to treat stroke for thousands of years. Previous study showed the clinical therapeutic effect of acupuncture on cognitive deficits caused by stroke.[31] Traditional Chinese medicine commonly uses GV 20 and GV 24 acupoints to treat cognitive deficits resulting from stroke.[21,32] However, the detailed mechanism(s) of this effect remain unclear. The current study used a rat model of MCAO/R which displays characteristics of cognitive dysfunction to investigate the mechanism(s) that EA at GV 20 and GV 24 acupoints improves learning and memory.

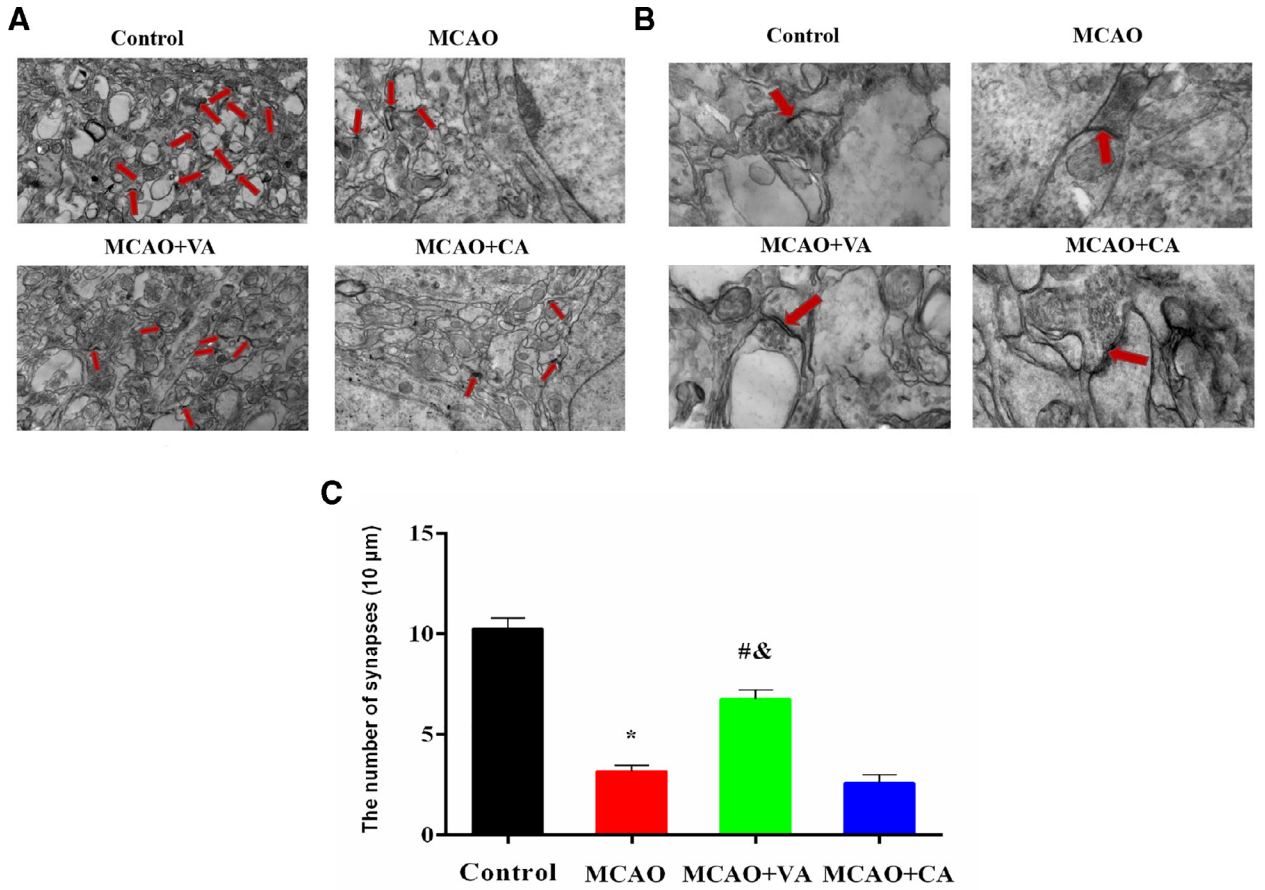

Figure 4. *Ultrastructure detection by transmission electron microscopy. The number of synapses in hippocampal CA1 region. (**A** and **B**) represent transmission electron micrographic images of synapses (arrows) in hippocampal CA1 region of each group (magnification, ×12000; ×80000). (**C**) Histogram shows the significant difference about the number of synapses.*$^{*}P < .001$, *MCAO group versus control group;* $^{\#}P < .001$, *MCAO + VA group versus MCAO group;* $^{\&}P < .001$, *MCAO + VA group versus MCAO + CA group. Abbreviations: CA, control acupuncture; MCAO, middle cerebral artery occlusion; VA, verum acupuncture.*

After 14 consecutive days of treatment, neurological deficit scores in the MCAO + VA group were significantly lower than those of MCAO and MCAO + CA groups, indicating an overall neuroprotective effect of EA. Next, estimation of learning and memory ability was done using the step-down passive avoidance test.[22] While step-down latency was shorter after MCAO/R injury in MCAO and MCAO + CA groups, EA treatment at GV 20 and GV 24 significantly prolonged step-down latency, suggesting improving behavioral performance, and therefore, learning and memory.

To further pinpoint the mechanism(s) that EA at GV 20 and GV 24 alleviates cognitive deficits following stroke, we also examined the levels and phosphorylation status of proteins implicated in learning and memory. A previous study reported that EA at appropriate acupoints improves synaptic plasticity after stroke. In the current study, we examined the levels of PSD-95 and SYN, which are known to be closely associated with synaptic plasticity.[33,34] Current results showed that MCAO/R injury (MCAO and MCAO + CA groups) significantly decreased the number of $PSD\text{-}95^{+}$ and SYN^{+} cells in the hippocampal CA1 region, while EA treatment at GV 20 and GV 24 ameliorated this effect. This result is consistent with previous studies and supports the positive impact of EA at appropriate acupoints on synaptic plasticity.

Previous studies in humans and animals have shown a decline in the number of synapses of CA1 pyramidal neurons in conjunction with cognitive impairment and suggested that hippocampal degeneration is central to memory loss in cognitive impairment.[8,35] Here, we also found a significant loss of hippocampal CA1 pyramidal cell synapses following the MCAO/R injuries (MCAO and MCAO + CA groups), indicating learning and memory deficits. However, this loss was reversed by EA treatment at GV 20 and GV 24. Synaptic ultrastructural characteristics are also associated with hippocampus-dependent learning and memory. Current results showed an abundance of closely arranged, yet clearly visible synapses in the control group that was not detected in rats subjected to MCAO/R. However, this damage was repaired by EA treatment at GV 20 and GV 24.

In addition, JAK2/STAT3 signaling has been shown to be associated with cognitive dysfunction after stroke.[36] Activation of this pathway can lead to spatial learning and memory impairment through inhibition of synaptic plasticity in

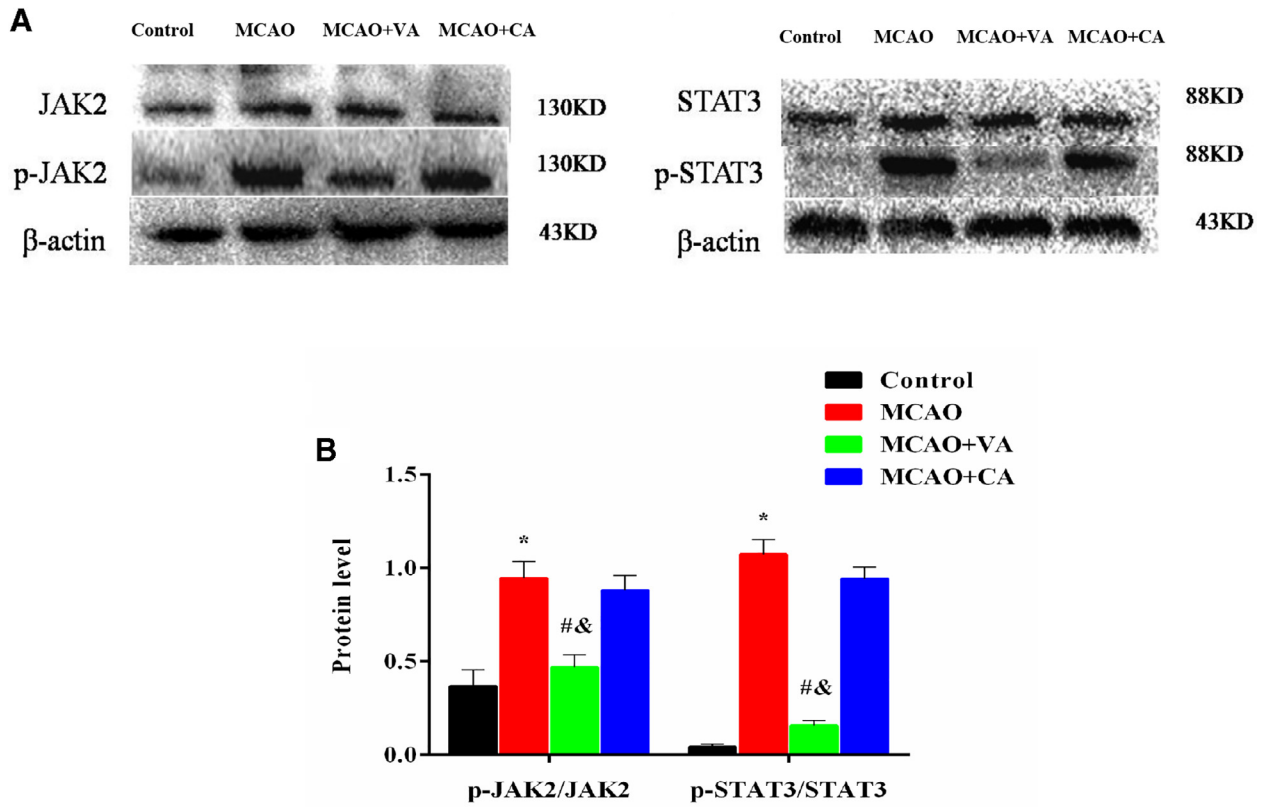

Figure 5. *Protein expression of JAK2-STAT3 in hippocampal CA1 region.(**A**) Expression of JAK2-STAT3 and phosphorylated JAK2-STAT3 was evaluated using western blotting. (**B**) Histogram shows significant difference about the p-JAK2 and p-STAT3.*P < .001, MCAO group versus control group; #P < .001, MCAO + VA group versus MCAO group; &P < .01, MCAO + VA group versus MCAO + CA group. Abbreviations: CA, control acupuncture; JAK2, Janus-activated kinase 2; MCAO, middle cerebral artery occlusion; STAT3, signal transducer and activator of transcription 3;VA, verum acupuncture.*

the hippocampal CA1 region.[18,19] The present study showed the levels of JAK2 and STAT3, as their phosphorylated counterparts, were significantly increased due to MCAO/R (MCAO and MCAO + CA groups). However, EA treatment at GV 20 and GV 24 inhibited the level of phosphorylation of both proteins in the hippocampal CA1 region, indicating that EA treatment at appropriate acupoints can significantly inhibit the level of JAK2/STAT3 signaling in the hippocampal CA1 region after MCAO/R injury.

Overall, the results of the present study indicate that short-term (14 days) EA treatment at GV 20 and GV 24 acupoints can ameliorate cognitive impairment induced by MCAO/R in rats. The mechanism that EA elicits its neuroprotective effect and consequently improves learning and memory is associated with greater synaptic number, plasticity, and ultrastructure (PSD-95 and SYN upregulation) congruent with inhibition of JAK2/STAT3 signaling in the hippocampal CA1 region. These findings likely provide an experimental basis for the treatment of cognitive dysfunction after ischemic stroke by EA. However, this experiment did not use inhibitors to block this pathway for validation. We will try to use JAK2-STAT3 signal pathway inhibitor to block this pathway to verify the results of this experiment in the future. Meanwhile, the effect of long-term EA treatment on cognitive impairment induced by MCAO/R in rats will be the another focus of our future research.

Competing Interests

All authors declared that they have no competing financial interests.

Authors' Contributions

JH and LDC conceived and designed the study; GLX, CMS, and XML performed the experiments and edited manuscript; MGY performed the EA intervention; XF and WLL supervised the research programme; JT, JH, and LDC revised the manuscript. All authors read and approved the manuscript.

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