Research Article

Electroacupuncture improves learning and memory functions in a rat cerebral ischemia/reperfusion injury model through PI3K/Akt signaling pathway activation

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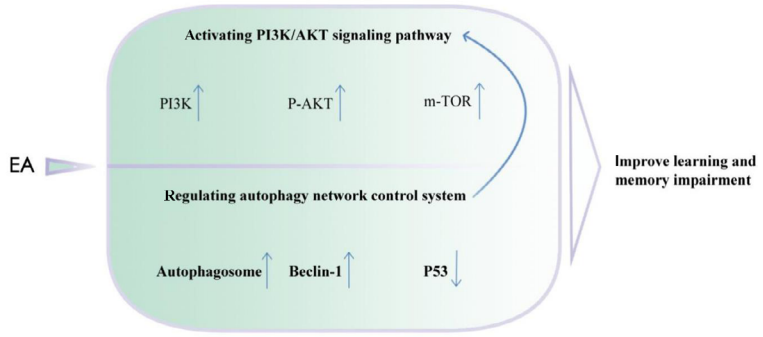
Graphical Abstract *Electroacupuncture (EA) at Baihui (GV20) and Shenting (GV24) alleviates learning and memory impairment after cerebral ischemia/reperfusion injury*



Abstract

Electroacupuncture has been widely used to treat cognitive impairment after cerebral ischemia, but the underlying mechanism has not yet been fully elucidated. Studies have shown that autophagy plays an important role in the formation and development of cognitive impairment, and the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays an important role in autophagy regulation. To investigate the role played by the PI3K/Akt signaling pathway in the electroacupuncture treatment of cerebral ischemia/reperfusion rat models, we first established a rat model of cerebral ischemia/reperfusion through the occlusion of the middle cerebral artery using the suture method. Starting at 2 hours after modeling, electroacupuncture was delivered at the *Shenting* (GV24) and *Baihui* (GV20) acupoints, with a dilatational wave (1–20 Hz frequency, 2 mA intensity, 6 V peak voltage), for 30 minutes/day over 8 consecutive days. Our results showed that electroacupuncture reduced the infarct volume in a rat model of cerebral ischemia/reperfusion injury, increased the mRNA expression levels of the PI3K/Akt signaling pathway-related factors *Beclin-1*, mammalian target of rapamycin (*mTOR*), and *PI3K*, increased the protein expression levels of phosphorylated Akt, Beclin-1, PI3K, and mTOR in the ischemic cerebral cortex, and simultaneously reduced *p53* mRNA and protein expression levels. In the Morris water maze test, the latency to find the hidden platform was significantly shortened among rats subjected to electroacupuncture stimulation compared with rats without electroacupuncture stimulation. In the spatial probe test, the number of times that a rat crossed the target quadrant was increased in rats subjected to electroacupuncture stimulation compared with rats without electroacupuncture stimulation. Electroacupuncture stimulation applied to the *Shenting* (GV24) and *Baihui* (GV20) acupoints activated the PI3K/Akt signaling pathway and improved rat learning and memory impairment. This study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China (approval No. 8150150901) on March 10, 2016.

Key Words: acupuncture; brain; central nervous system; factor; neurological function; pathways; protein; stroke

Chinese Library Classification No. R454.1; R741; R842.1

Introduction

Cognitive impairment and decline in patients with stroke during the acute and chronic phases, particularly the impairment of learning and memory, has a very high clinical incidence. Clinical studies have indicated that 25–30% of post-stroke impairment survivors suffer from immediate or delayed cognitive impairment, which markedly affects the rehabilitation programs necessary to improve physical ability

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and activities of daily living (Kalaria et al., 2016; Pantoni, 2017). Numerous recent studies have examined electroacupuncture (EA) treatment, which is based on acupuncture techniques from ancient China and combines traditional acupuncture with modern electrical stimulation. EA has dual therapeutic effects and represents a simple, convenient, and cost-effective treatment option that has been widely used to treat cognitive impairments following cerebral ischemia (Pollock et al., 2014; Liu et al., 2015; Han, 2019). However, the functional mechanisms that underlie the EA-mediated amelioration of post-stroke cognitive impairment have not yet been fully elucidated.

Many studies have suggested that autophagy plays an important role in the formation and development of cognitive impairment, and the inhibition of cell apoptosis can reduce ischemia-reperfusion injury (Glatigny et al., 2019; Romeo et al., 2019; Mo et al., 2020). Many activating signaling pathways regulate autophagy and apoptosis during the post-stroke period, such as the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and nuclear factor-κB signaling pathways, which play key roles in the regulation of apoptosis (Wen et al., 2018; Huang et al., 2019). Therefore, we hypothesized that the regulation of the PI3K/Akt signaling pathway within the neural cell autophagy regulation network might represent a mechanism through which EA improves cognitive impairment after stroke. The aim of the present study was to investigate whether EA applied to the *Baihui* (GV20) and *Shenting* (GV24) acupoints improved learning and memory impairment following middle cerebral artery occlusion (MCAO) in rats via the PI3K/Akt signaling pathway.

Materials and Methods

Animals

A total of 36 specific-pathogen-free, male, Sprague-Dawley rats (aged 10–12 weeks, weighing 260 ± 20 g) were obtained from Beijing Vital River Laboratory Animal Technology, Beijing, China [license No. SCXK (Jing) 2016-0011]. All experiments were performed strictly in accordance with the International Ethical Guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996) and were approved by the Animal Ethics Committee of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China (approval No. 8150150901) on March 10, 2016. This study followed the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The rats were raised in a 22°C and 50% humidity atmosphere, under a 12 hour/12 hour light/dark cycle. For euthanasia, 2% pentobarbital sodium (40 mg/kg; intraperitoneal injection; Macklin, Shanghai, China) was used. The 36 rats were randomly divided into three groups: sham, MCAO, and MCAO + EA groups (n = 12 per group).

MCAO model establishment

The MCAO model was established using Longa's method (Longa et al., 1989). Based on the modified fornylon suture method, the left common carotid artery, left external carotid artery, and internal carotid artery were carefully exposed via a neck incision and dissected. Approximately 18–22 mm of nylon monofilament (Jialing-bio, Guangzhou, China), with a rounded tip, was inserted into the internal carotid artery and advanced until the origin of the middle cerebral artery was blocked. After 120 minutes of occlusion, the nylon monofilament was extracted to restore blood flow. For the sham group, the common carotid artery, internal carotid artery, and external carotid artery were simply separated. During and following surgery, the internal temperatures of the animals were maintained at 37°C using a heating pad. Neurological deficit scores were used to assess the success of MCAO.

EA treatment

Two hours after MCAO surgery, the rats in the MCAO + EA group received EA treatment at the *Baihui* (GV20, located in the center of the parietal bone) and *Shenting* (GV24, located in the anterior median line) acupoints using an EA apparatus (GM101; Huayi, Shanghai, China). The acupuncture needles (diameter 0.3 mm, Huatuo brand, Suzhou Medical Products Co., Ltd., Suzhou, China) were inserted at a depth of 2–3 mm into the GV20 and GV24 acupoints on the head, at a 45° angle. The stimulation parameters were as follows: dilatational wave frequency, 1–20 Hz (adjusted to the muscle twitch threshold); 6 V peak voltage; and 2 mA intensity for 30 minutes each day for 8 consecutive days.

Neurological deficit scores

At 2 hours after reperfusion and on the 8th day of EA treatment, the Longa Score Scale was used to evaluate neurological function (Longa et al., 1989): 0 indicated no neurological deficit, 1 indicated mild deficits (failure to fully extend the right forepaw), 2 indicated circling to the right, 3 indicated falling to the right, suggestive of moderate deficits, and 4 represented severe deficits (the complete loss of walking ability). Briefly, rats with scores between 1–3 indicated the successful modeling of MCAO and were included in subsequent experiments.

Morris water maze test

To evaluate learning and memory abilities, the rats were tested using the Morris water maze, as previously described (Feng et al., 2013; He et al., 2018; Wen et al., 2018), starting at the 4th day after surgery. The rats were trained from day 4 to day 8, and testing took place on day 9. The Morris water maze test consisted of two parts: the place navigation test and the spatial probe test. The water maze apparatus (Xin Ruan Information Technology, Shanghai, China) consists of a circular pool with a diameter of 150 cm and a height of 60 cm. A video camera (Canon, Tokyo, Japan) records and observes the activity of the rats. During the place navigation test, the rats were randomly assigned to one of four quadrants and were allowed to find a hidden platform. Then, the time taken for the rats to climb onto the platform was recorded, with an upper limit of 90 seconds. If the rat climbed the platform within 90 seconds and stayed for more than 3 seconds, the rat considered to have found the platform, and the time was recorded as the incubation period. If the rat was unable to locate the platform within 90 seconds, it was manually guided to the platform and allowed to familiarize itself with the platform location for 10 seconds. The incubation period was recorded as 90 seconds for rats that had to be guided to the platform. Training began at 10 a.m. each day, and rats were trained 4 times each day, with 10-minute intervals between sessions, for 5 consecutive days. The latency was recorded as the time necessary to reach the platform and was averaged over the 4 training sessions. To perform the spatial probe test, the hidden platform was removed on the sixth day, and the abilities of the rats to remember the hidden platform location were tested. The rats were allowed to swim freely for 90 seconds, and the number of times that the rats swam to the previous location of the platform during a 90-second trial was recorded.

2,3,5-Triphenyltetrazolium chloride staining

2,3,5-Triphenyltetrazolium chloride staining was used to evaluate cerebral infarctions at the morphological level, as previously reported (Kakimoto et al., 2013). In brief, after the Morris water maze test, the rats were perfused with normal saline through the heart, and the brain was quickly removed and frozen at –20°C for 30 minutes. Brain tissues were cut into five 2-mm coronal slices and incubated immediately in 2% 2,3,5-triphenyltetrazolium chloride solution (Beijing Solarbio Science & Technology Co, Ltd., Beijing, China) at 37°C for 20

minutes, in the dark, and then fixed in 4% polyoxymethylene for 24 hours. Stained sections were photographed using a high-resolution digital camera (Canon, Tokyo, Japan). The percent cerebral infarct volume was calculated using Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, MD, USA) (Cheng et al., 2015).

Transmission electron microscopy

Transmission electron microscopy was used to observe changes in neuronal apoptosis and autophagy (Edwards et al., 2013). After anesthesia, the peri-ischemic region of the cortex was fixed with a mixture of 3% glutaraldehyde and 1.5% paraformaldehyde for 8 days at 4°C, followed by 1% osmium tetroxide in cacodylate buffer for 2 hours. Sections were washed with 0.1% phosphate buffer (pH 7.2) at room temperature. After washing with phosphate-buffered saline (PBS), the samples were gradient dehydrated with the 30–100% alcohol-acetone, embedded in an Epon-Araldite 618 mixture, for 2 hours at 37°C, and then sectioned into 80-nm, modular, ultrathin slices and observed with an electron microscope (JEM-1400; JEOL, Tokyo, Japan). The photos were obtained on an HP digital CCD camera (SIS4million Volex, Tokyo, Japan).

Western blot analysis

Western blot assay was used to qualitatively assess the related protein expression, as previously reported (Moriya et al., 2014). Total protein lysates were prepared from peri-ischemic regions of brain tissues. After total protein was collected and quantified, using a bicinchoninic acid assay (Brown et al., 1989), the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The samples were incubated overnight at 4°C with rabbit anti-Beclin-1 polyclonal antibody (1:1000; Cat# GTX55535; GeneTex, Irvine, CA, USA), rabbit anti-p53 polyclonal antibody (1:000; Cat# Ab26; Abcam, Cambridge, MA, USA), rabbit anti-PI3K polyclonal antibody (1:1000; Cat# GTX111173; GeneTex), rabbit anti-Akt polyclonal antibody (1:1000; Cat# Ab179463; GeneTex), rabbit anti-mTOR polyclonal antibody (1:1000; Cat# Ab134903; Abcam, Chicago, IL, USA) and rabbit anti-phospho-Akt (p-Akt) polyclonal antibody (1:1000; Cat# 13038T; CXT, Boston, MA, USA). Then, the samples were incubated with goat anti-rabbit IgG (1:5000; Cat# GTX213110-01; GeneTex) at 37°C for 1 hour. Bound secondary antibodies were visualized using an enhanced chemiluminescence kit (Bio-Rad, Philadelphia, PA, USA), and the protein expression levels were analyzed using Image-Pro Plus software. Protein expression levels were calculated as the optical density ratio between the target protein and β-actin (1:1000; PTGCN, Wuhan, China).

Real-time quantitative reverse transcription-polymerase chain reaction

Brain tissue was taken out from rats and immediately placed in liquid nitrogen and stored at –20°C. To explore the impact of acupuncture on mRNA expression of PI3K/Akt signaling pathway-related proteins (PI3K, Akt, mTOR, Beclin-1, p53), reverse transcription-polymerase chain reaction (PCR) method was used. For RNA extraction, tissue samples were homogenized in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Purified RNA (1 μg) was reverse transcribed into complementary DNA, according to the manufacturer's instructions. The RNA quantity was initially determined using an ultraviolet spectrophotometer (Precision Scientific Instruments Co., Ltd., Shanghai, China), then dissolved in diethylpyrocarbonate-treated water, and the purity and concentration were assessed. Complementary DNA was used to determine the mRNA levels of PI3K, Akt, mTOR, p53, and Beclin-1 by PCR with Taq DNA polymerase (Fermentas, Amherst, NY, USA). The concentrations of samples were determined, and RNA was reverse transcribed into complementary DNA using a reverse transcription kit (Takara Bio Inc.). Real-time quantitative PCR was performed using a kit, according to the manufacturer's instructions (Vazyme Biotech, Ltd., Nanjing, China), and relative mRNA expression analyses were performed on the Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) (Moriya et al., 2014). The sequences of the primers used for amplification, which were provided by Invitrogen (Waltham, MA, USA), are shown in **Table 1**.

Statistical analysis

All data were analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). Data are presented as the mean ± standard deviation (SD). Differences among groups were determined using a one-way analysis of variance followed by the least significant difference when equal variances were assumed. Otherwise, a Dunnett's T3 test was conducted. $P < 0.05$ was considered significant.

Results

Quantitative analysis of experimental animals

No rats died in the sham group. In the MCAO group, three rats died due to excessive hemorrhage. An additional three rats died in the MCAO + EA group.

EA attenuates neurological deficits and infarct volumes in rats with focal cerebral ischemia/reperfusion injury

In the present study, neurological scores were used to evaluate whether EA applied to the GV20 and GV24 acupoints can improve neurological function after MCAO. Decreased neurological scores indicated improved neurological function. The rats in the sham group did not display any signs of neurological deficits. However, 4–8 days following MCAO, the neurological scores of the MCAO + EA group were significantly reduced compared with those for the MCAO group ($P < 0.05$; **Table 2**). The infarct volume was measured using 2,3,5-triphenyltetrazolium chloride staining. The cerebral infarct volumes in the MCAO and MCAO + EA groups were significantly larger than that in the sham group ($P < 0.05$), indicating that the MCAO model was successfully established. At 8 days after surgery, a significant decrease in the cerebral infarction volume was observed in the MCAO + EA group ($P < 0.05$, *vs*. MCAO group), indicating that EA provided significant therapeutic efficacy for the prevention of focal cerebral injury (**Figure 1**).

EA treatment inhibits cell apoptosis in the peri-ischemic cortices of rats with focal cerebral ischemia/reperfusion injury

Under the electron microscope, abundant mitochondria,

Table 1 | **Primer sequences**

Primer	Sequences (5′–3′)	Product size (bp)
PI3K	Forward: GAA CAG GGC AGC TTC AAT GC	110
	Reverse: CTC CTT CTG GGT CCG GAG TA	
Akt1	Forward: CCT GGA CTA CTT GCA CTC CG	56
	Reverse: CAC AGC CCG AAG TCC GTT AT	
mTOR	Forward: CTG ATG TCA TTT ATT GGC ACA AA	289
	Reverse: CAG GGA CTC AGA ACA CAA ATG C	
p53	Forward: TCA CTC CAG CTA CCC GAA GA	53
	Reverse: GTC AGG CCC CAC TTT CTT GA	
Beclin-1	Forward: CCC AGC CAG GAT GAT GTC TAC	56
	Reverse: AGT CTC CGG CTG AGG TTC TC	
β-Actin	Forward: CAC CCG AGT ACA ACC TTC	42
	Reverse: CCC ATA CCC ATC ACA CC	

Akt: Protein kinase B; mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol-3-kinase.

Table 2 | Effects of EA on the Longa Score Scale in rats with cerebral ischemia/reperfusion injury

	Longa Score Scale			
	0	1	2	3
Sham group (n = 12)				
After reperfusion	12	0	0	0
After EA treatment (at 8 days after surgery)	12	0	0	0
MCAO group (n = 9)				
After reperfusion	0	1	4	4
After EA treatment (at 8 days after surgery)	0	2	5	2†
MCAO + EA group (n = 9)				
After reperfusion	0	0	2	7
After EA treatment (at 8 days after surgery)	0	3	5	1$^{\dagger\#}$

A higher Longa Score Scale score correlates with more severe neurological dysfunction. Data are presented as the mean ± SD. Differences among groups were determined using a one-way analysis of variance followed by the least significant difference test. †P < 0.05, *vs*. after reperfusion; #P < 0.05, *vs*. MCAO group. EA: Electroacupuncture; MCAO: middle cerebral artery occlusion.

intact mitochondrial crests, ordered lysosomes, and complete cell nucleoli could be observed in the sham group. Large axons were observed, and chromatin was uniformly distributed. In the MCAO group, mitochondria were swollen, mitochondrial crests were destroyed, the lysosomes appeared disorganized, and the cells appeared structurally disordered in the peri-ischemic cortex, with euchromatin observed at the edge of the neuronal nuclei. The cells appeared to be smaller, and the electronic density was deeper in the MCAO group than in the sham group. All of these observations are consistent with early apoptotic signals. In the MCAO + EA group, the cell membrane was partially ruptured, and the mitochondria were slightly damaged. No changes in the rough endoplasmic reticulum and chromatin were observed in the MCAO + EA group. The cell nucleoli were complete, and chromatin was uniformly distributed (**Figure 2**).

EA affects gene and protein expression associated with the PI3K/Akt signaling pathway in the peri-ischemic cortices of rats with focal cerebral ischemia/reperfusion injury

The PCR results showed that compared with the sham group, the mRNA expression levels of *PI3K*, *p53*, *mTOR*, and *Beclin-1* were higher (P < 0.01), whereas the *PI3K*, *mTOR*, and *Beclin-1* mRNA expression were significantly higher in the MCAO + EA group than in the MCAO group (P < 0.05). In contrast, the mRNA expression of *p53* was lower in the MCAO + EA group than that in the MCAO group (P < 0.01). No significant difference in the mRNA expression level of *Akt* was observed between the MCAO and MCAO + EA groups (P > 0.05; **Figure 3**). Western blot assay results showed that compared with the MCAO group, the MCAO + EA group showed significantly increased levels of p-Akt, Beclin-1, PI3K, and mTOR in the peri-ischemic cortex (P < 0.05), whereas p53 was reduced (P < 0.05), and no change in Akt protein expression was observed (P > 0.05; **Figure 4**). These results suggest that EA modulated the activation of the PI3K/Akt signaling pathway.

EA reduces the learning and memory impairments observed in rats with focal cerebral ischemia/reperfusion injury

The Morris water maze test was used to assess the cognitive abilities of rats. During the place navigation test, rats in the MCAO group demonstrated a longer mean latency than the rats in the sham group. Compared with the latency in the MCAO group, the latency was significantly reduced in the MCAO + EA group (P < 0.05; **Table 3**). During the spatial probe test, the MCAO group crossed the target quadrant less frequently than the sham group (P < 0.05). However, compared with the MCAO group, the MCAO + EA group crossed the target quadrant more frequently (P < 0.01; **Figure 5**). These results indicate that EA treatment significantly improved the learning and memory abilities of the MCAO rats.

Discussion

Numerous studies have demonstrated that EA can significantly improve learning and memory impairments (Tao et al., 2010; Luo et al., 2013). However, the underlying mechanism of action for this effect has not been clarified.

Previous studies have shown that the regulatory mechanisms of the autophagy network system are primarily associated with changes in the PI3K and other signal transduction pathways and alterations in the expression level of the apoptosis-related genes such as caspsae-3 and p53 (Broughton et al., 2009; Luo et al., 2013). The PI3K signaling pathway plays a key role in the regulation of the autophagy network system. Shioda et al. (2008) also demonstrated that the PI3K signaling pathway plays an important role in the regulation of cognitive dysfunction associated with cerebral ischemia and hypoxia. The cell autophagy network is activated when autophagy inhibits cerebral cell apoptosis in the ischemic penumbra, degrading necrotic and denatured tissues. Therefore, autophagy activation could regulate apoptosis and promote cerebral cell proliferation post-stroke.

In terms of pathophysiology, ischemic hypoxia injury after stroke leads to an increase in oxygen radical production, resulting in damaged protein fragmentation, cell division, and cell death. Mitochondria with abnormal appearances, such as those that are swollen or disorganization, are thought to be primary factors that induce apoptosis in the peri-ischemic region (Manzanero et al., 2013; Ritzenthaler et al., 2013), resulting in abnormal metabolism and development in neurons (Tyagi et al., 2010; Chan et al., 2015). Autophagy-related proteins include LC3, Beclin-1 (Tanida, 2011; Liu et al., 2016), p53, PI3K, Akt, and mTOR. Additionally, the PI3K/Akt signaling pathway, which has been associated with both apoptosis and autophagy, is essential for neuroprotection and cell-survival (Feng et al., 2013). The activation of the PI3K/Akt signaling pathway has a positive, protective effect on cell survival (Xue et al., 2014). PI3K, Akt, and mTOR proteins, which have been closely correlated with cell survival (Mullonkal and Toledo-Pereyra, 2007), are important downstream effectors of the PI3K/Akt signaling pathway (Mullonkal and Toledo-Pereyra, 2007; Xue et al., 2014). As a member of the apoptotic family, p53 participates in mitochondrial apoptotic and oxidative stress pathways. Previous studies have suggested a neuroprotective role for the PI3K/Akt signaling pathway in cerebral injury, resulting in the decreased number of apoptotic cells and smaller infarct volumes (Mullonkal and Toledo-Pereyra, 2007; Ishrat et al., 2012). Therefore, this pathway may represent a major target for the treatment of learning and memory impairments. However, this effect is temporary and must be sustained (Zhao et al., 2005). In the present study, we found that the protein and mRNA expression levels of PI3K, mTOR, and Beclin-1 were significantly increased in the MCAO + EA group compared with those in the MCAO group, whereas the p53 expression levels were reduced. These results suggested that autophagy was induced after ischemic injury and that EA application strengthened this effect. Therefore, EA appears to exert a neuroprotective effect via the activation of the PI3K/Akt signaling pathway.

EA has served as a mainstay treatment in traditional Chinese medicine for ischemic stroke and has demonstrated clinical efficacy. Recently, numerous studies have confirmed the neuroprotective mechanism of EA (Xie et al., 2013; Kim et al., 2014). EA at GV20 and GV24 of the *Du* meridian is used to treat cognitive impairment clinically (Zhao et al., 2009). To further explore the underlying mechanism associated with EA, we examined the effects of EA on the PI3K/Akt signaling pathway, which modulates apoptosis after ischemia-

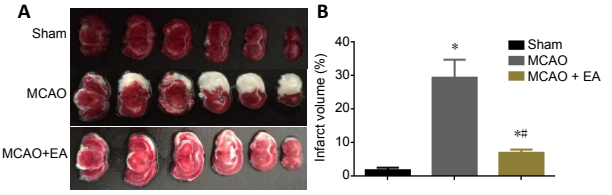

Figure 1 | Effects of EA on the infarct volumes of cerebral ischemia/reperfusion injury rats.
(A) The infarct areas were measured by 2,3,5-triphenyltetrazolium chloride staining. White color represents the ischemic area, and red shows the normal area. (B) Quantitative results of infarct volume. Data are presented as the mean ± SD (n = 12 in the sham group, 9 in MCAO and MCAO + EA groups, separately). *P < 0.05, *vs*. sham group; #P < 0.05, *vs*. MCAO group (one-way analysis of variance followed by the least significant difference test). EA: Electroacupuncture; MCAO: middle cerebral artery occlusion.

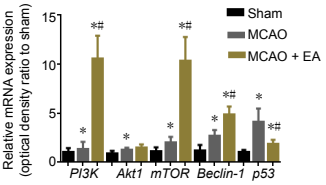

Figure 3 | Effects of EA on gene expression associated with the PI3K/Akt signaling pathway in the peri-ischemic cortices of rats with focal cerebral ischemia/reperfusion injury.
The mRNA expression levels were detected by real-time quantitative reverse transcription-polymerase chain reaction. Data are presented as the mean ± SD (n = 12 in the sham group, 9 in the MCAO and MCAP + EA groups, separately). *P < 0.05, *vs*. sham group; #P < 0.05, *vs*. MCAO group (one-way analysis of variance followed by the least significant difference test). Akt: Protein kinase B; EA: electroacupuncture; MCAO: middle cerebral artery occlusion; mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol-3-kinase.

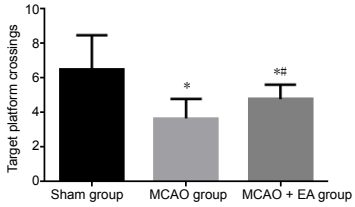

Figure 5 | Effects of EA on the number times the target quadrant was crossed during the spatial probe test in rats with focal cerebral ischemia/reperfusion injury.
Data are presented as the mean ± SD (n = 12 in the sham group, 9 in the MCAO and MCAO + EA groups, separately). *P < 0.05, *vs*. sham group; #P < 0.05, *vs*. MCAO group (one-way analysis of variance followed by the least significant difference test). EA: Electroacupuncture; MCAO: middle cerebral artery occlusion.

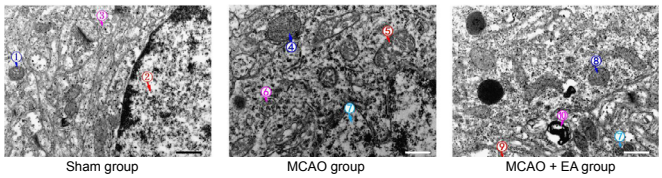

Figure 2 | Effects of EA on cell apoptosis in the brains of rats with focal cerebral ischemia/reperfusion injury.
Compared with the sham group, mitochondria were significantly destroyed and incomplete, and the distribution of chromatin and lysosomes were scattered, with cells appearing smaller and more electron-dense in the MCAO group, whereas autophagosomes significantly increased in the MCAO group. In the MCAO + EA group, the cell membrane was partly ruptured, the mitochondria were slightly damaged, and autophagosomes were significantly increased compared with the MCAO group. ①④⑧ indicate mitochondria; ②⑤⑨ indicate chromatin; ③⑥⑩ indicate lysosome; ⑦ indicates autophagosome. Scale bars: 1 μm. EA: Electroacupuncture; MCAO: middle cerebral artery occlusion.

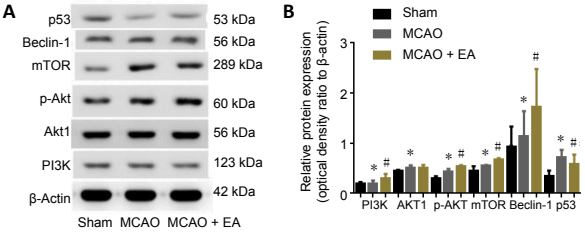

Figure 4 | Effects of EA on protein expression levels associated with the PI3K/Akt signaling pathway in the peri-ischemic cortices of rats with focal cerebral ischemia/reperfusion injury.
(A) Apoptosis-related proteins were detected by western blot analysis. (B) Quantification analysis of apoptosis-related proteins. Data are presented as the mean ± SD (n = 12 in the sham group, 9 in MCAO and MCAO + EA groups, separately). *P < 0.05, *vs*. sham group; #P < 0.05, *vs*. MCAO group (one-way analysis of variance followed by the least significant difference test). Akt: Protein kinase B; EA: electroacupuncture; MCAO: middle cerebral artery occlusion; mTOR: mammalian target of rapamycin; p-Akt: phospho-Akt; PI3K: phosphatidylinositol-3-kinase.

Table 3 | Effects of EA on the latency (s) during the place navigation test of rats with focal cerebral ischemia/reperfusion injury

Group	4th day	5th day	6th day	7th day	8th day
Sham	54.72±0.88	43.9±0.82	41.50±0.83	33.23±0.46	31.00±0.80
MCAO	66.02±0.90*	63.62±1.23*	62.91±0.64*	60.12±0.65*	59.18±0.69*
MCAO+EA	60.03±0.62*#	55.51±0.93*#	52.91±0.79*#	48.22±0.72*#	40.92±0.88*#

Data are presented as the mean ± SD (n = 12 in the sham group, 9 in the MCAO and MCAO + EA groups, separately). *P < 0.05, *vs*. sham group; #P < 0.05, *vs*. MCAO group (one-way analysis of variance followed by the least significant difference test). EA: Electroacupuncture; MCAO: middle cerebral artery occlusion.

reperfusion injury. In the present study, we only investigated the effects of EA on the brain regions directly affected by the ischemia-reperfusion injury, but did not explored the long-term neuroprotective and cognitive improvement effects of EA which was mediated by the anti-apoptotic and autophagy responses and the activation of the autophagy network system. In future studies, we will explore the relationship and include a sham acupuncture group, which would be useful to examine the cellular effects of acupuncture in the absence of injury. To further investigate the effects of EA in rats post-stroke, we will also perform further, in-depth explorations to determine whether the changes induced by EA are mediated by the proposed relationship between neuronal cell apoptosis and autophagy in future research.

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