

Original Article

Effects of electroacupuncture at Shenting and Baihui on cAMP-PKA-CREB signaling pathways in rats with cognitive dysfunction caused by cerebral ischemia reperfusion

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Abstract: Background: This study aimed to explore the effects of electroacupuncture at Shenting (Governor Vessel (GV) 24) and Baihui (GV 20) acupoints on rat behavior and the mechanisms of electroacupuncture on change of cAMP-PKA-CREB signaling pathways in the hippocampi. Methods: Sixty-three male Sprague-Dawley rats were randomly divided into the electroacupuncture group (E), model group (M), and sham-operation group (F), according to the random number table, with 21 rats in each group. Model rats with cognitive dysfunction caused by cerebral ischemia reperfusion were obtained by middle cerebral artery occlusion. Rats in group F were with artery separation. Model rats without electroacupuncture therapy were in group M. In group E, model rats were acupunctured at Shenting and Baihui acupoints for 1 minute. They were treated with dilatational waves by electronic acupunctoscope for 15 minutes at 12 hours, 24 hours, and 48 hours after the operation. Recovery of neurological impairment was evaluated by neurological scores. Expression of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and CREB in the hippocampi of model rats was detected by immunohistochemistry. Results: Neurological scores in group E began to decrease at the 12th hour, compared with groups M and F ($P<0.05$). cAMP, PKA, and CREB expression in the hippocampi of rats significantly decreased in group M, compared with group F ($P<0.05$), and significantly increased in group E, compared with group M ($P<0.05$), with significant differences. Conclusion: Electroacupuncture at Shenting and Baihui acupoints plays a neuroprotective role by stimulating cAMP-PKA-CREB signaling pathways in rats with cognitive dysfunction caused by cerebral ischemia reperfusion.

Keywords: Electroacupuncture, cognitive function, cAMP-PKA-CREB signaling pathways

Introduction

Strokes, also called cerebrovascular accidents, are a common and frequently-occurring disease in the elderly. Data issued by the American Heart Association showed that there are 0.61 million people with new-onset strokes every year and strokes have characteristics of high morbidity, high mortality, high disability, and high recurrence. Strokes have been the first prevalence factor in China since 2011 [1]. Most stroke patients are accompanied with atherosclerosis, hypertension, diabetes, coronary heart disease, obesity, and unhealthy habits, like smoking and drinking [2]. Nearly 46% of pa-

tients have cognitive dysfunction within a half-year after a stroke attack [3]. Patients with cognitive dysfunction have obviously reduced daily activities. They cannot return to society normally, bringing a heavy financial load to society and their families. The pathophysiological pathogenesis of strokes involves many harmful cascade reactions, such as excitotoxicity, oxidative stress, nitrative stress, inflammatory response, and apoptosis [4]. Some studies have demonstrated a connection between the pathogenesis and prognosis of strokes with cAMP-PKA-CREB signaling pathways. Activation of cAMP-PKA-CREB signaling pathways can reduce brain damage caused by ischemia [5, 6].

Acupuncture, especially electroacupuncture, has been clinically proven to be a feasible method of treating cognitive dysfunction caused by brain events [7]. Some research has indicated that electroacupuncture at acupoints can inhibit brain limbic structure atrophy of epileptics, improving patient cognitive function [8]. In China, Baihui and Shenting are usually used to clinically treat cognitive dysfunction caused by strokes [9, 10]. Electroacupuncture at Baihui and Shenting can inhibit apoptosis caused by cerebral ischemia reperfusion [11], with undiscovered internal mechanisms. In this study, model rats with cognitive dysfunction caused by focal cerebral ischemia reperfusion were obtained to investigate the therapeutic effects of electroacupuncture at Shenting (Governor Vessel (GV) 24) and Baihui (GV 20) acupoints on cognitive dysfunction caused by cerebral ischemia-reperfusion. This investigation was performed by stimulating cAMP-PKA-CREB signaling pathways, further researching the mechanisms of electroacupuncture in treating cognitive dysfunction caused by strokes.

Materials and methods

Materials

Seventy-nine healthy adult male Sprague-Dawley rats with 250-300 g, provided by the Animal Experiment Center of Zhengzhou University in Henan Province (certification No.: SCXK (Henan) 2005-0001), were weighed and numbered. The modeling success rate was 80%. There were 63 rats participating in this study. Sixty-three rats, in accordance with the random number table, were separated into the electroacupuncture group (E), model group (M), and sham-operation group (F), with 21 rats in each group. Each group consisted of three subsets according to ischemia-reperfusion time: 12th hour subset, 24th hour subset, and 48th hour subset, with 7 rats in each subset. Experiment reagents used in this study were antimicrobial peptide cyclic adenosine monophosphate (cAMP) antibody and protein kinase A (PKA) (provided by Beijing Biosynthesis Biotechnology Co., LTD), CREB rabbit polyclonal antibody (provided by Wuhan Boster Biological Engineering Co., LTD), DAB reagent kit (provided by Beijing Zhongshan Biotechnology Co., LTD), and ready-to-use and no biotin-dependent immunohistochemical EliVision™

plus detection kit (provided by Fujian Maixin Biotechnology Co., LTD).

Methods

Modeling: The intraluminal thread was made by Zea Longa's method on the day before the surgery [12]. One end of the monofilament burned to a small smooth round head. A mark about 18mm at the end of the round head was made by a marker. The monofilament was immersed into poly-L-Lysine solution (0.1%) and dried in an oven at 60°C.

Cerebral ischemia reperfusion models were obtained by middle cerebral artery occlusion. First, rats were anesthetized by intravenous injections of 300 mg/kg chloral hydrate. Through midline incisions in the neck, the right common carotid artery was exposed. Heparin-treated and silicone-coated monofilament (diameter of 0.25 mm) was put into the internal carotid artery through the external carotid artery to block the blood supply of the middle cerebral artery. Core temperature was controlled at 37.0±0.5°C during ischemia. After blocking for 60 minutes, the monofilament was gently pulled out and the external carotid artery was closed by electrocoagulation. Right common carotid arteries of rats in group F were exposed and the external carotid artery was blocked by electrocoagulation, with no monofilament in the internal carotid artery.

Neurological scores

Neurological impairment was scored by Longa's method [12] at 3 hours after model establishment. Awakened animals after surgery were scored to select successful rat models. Successful models obtained 1-3 neurological scores by Longa's method, while failing models obtained 0 and 4 scores. Specific criteria were as follows (higher scores indicated more severe neurological impairment): a score of 0 indicated no neurologic deficit, a score of 1 (incompletely stretching contralateral forepaw) indicated a mild focal neurologic deficit, a score of 2 (circling to the right) indicated a moderate focal neurologic deficit, and a score of 3 (toppling and falling to the contralateral side) indicated a severe focal deficit. Rats with a score of 4 did not walk spontaneously and had a loss of consciousness.

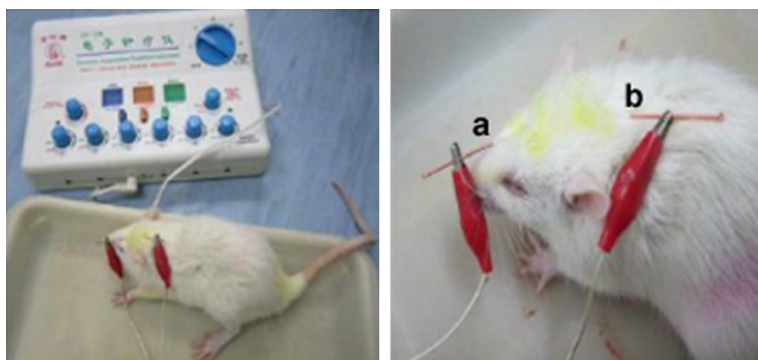


Figure 1. Diagram of electroacupuncture to Shenting and Baihui in rats. a: Shenting acupoint; b: Baihui acupoint.

Collection of samples

Rats were anesthetized by 0.35 mL/100g (10%) chloral hydrate at the 12th hour, 24th hour, and 48th hour after reperfusion of 3 hours of cerebral ischemia. Perfusion fixation was made by 4% paraformaldehyde. The heads were cut off and the brain tissue was taken out. External fixation on brain tissues was performed for 24 hours. Next, 2 mm coronary brain slices were cut. The third slice was embedded in paraffin and sectioned continuously.

Group E

Shenting and Baihui acupoints were involved in this study. When the rats awoke, upward oblique insertion of 2 mm was performed in Shenting acupoints and forward oblique insertion of 2 mm was performed in Baihui acupoints (**Figure 1**). GM6805-2A electric acupuncture apparatus was used in a voltage peak of 6 V (rat ears shaking gently) and a dilatational wave of 10 Hz. Electroacupuncture was performed for 15 minutes at 12 hours, 24 hours, and 48 hours.

Acupoint locations referred to the *Experimental Acupuncture Science* (Appendix III: commonly used experimental animals (rats) acupuncture points) under Zhongren Li's general editorship [13]. Specific locations of Shenting and Baihui acupoints were as follows: Shenting, on anterior median line and the front of the border among frontal and parietal bones; Baihui, on anterior median line and the midpoint of a line between two Erjian acupoint (EX-HN 6).

Immunohistochemical detection of cAMP, PKA, and CREB in the hippocampi of rats in each group in each time period

Up, down, left, and right sides of the embedding block were trimmed in parallel surfaces with 2-3 mm paraffin around the tissue. The embedding block was cut into slices at a thickness of 5 μ m using a paraffin slicing machine. Slices were then unfolded by putting them into a stretching plate

with 45°C water. Afterward, the slices were taken out of the water and put in glass slides, baking for 3 hours in a 60°C in a dry oven. The dried slices were dewaxed in xylene (I) and xylene (II) for 10 minutes consecutively. Dewaxed slices were put into anhydrous alcohol for 10 minutes and into 95% alcohol, 80% alcohol, 70% alcohol, and distilled water for 5 minutes successively. The slices were incubated with 3% H₂O₂ for 10 minutes at room temperature to eliminate endogenous peroxidase activity and washed with distilled water for 10 minutes. After heating 0.01 mmol/L sodium citrate buffer (ph 6.0) up to about 95°C in water bath kettle, the slices were put in and heated for 15 minutes. After naturally cooling for 20-30 minutes at room temperature, the slices were washed with distilled water for 10 minutes. The slices were then digested with 37°C 0.1% trypsin for 30 minutes and washed with PBS (ph 7.4) for 10 minutes. After incubation with 5%-10% BSA for 20 minutes at room temperature, the serum was poured away and the slices were not washed. The slices were incubated with primary antibody of 1:100 dilutions in a 37°C wet box for 90 minutes and washed with PBS for 15 minutes. Next, the slices were incubated with secondary antibody labeled by biotin in a 37°C wet box for 30 minutes and washed with PBS for 15 minutes. The slices were then incubated with SABC (streptavidin biotin peroxidase complex) for 30 minutes in a 37°C wet box and washed with PBS for 20 minutes. The slices were then treated with DAB developer for 10 minutes. After thoroughly rinsing with distilled water, the slices were put into Harris hematoxylin for 4 minutes to stain cell

Table 1. Longa scores of rats at 12 hours, 24 hours, and 48 hours after ischemia-reperfusion in each group ($\bar{x} \pm s$)

Group	Postoperative instant score	12 h after reperfusion	24 h after reperfusion	48 h after reperfusion
F	0	0	0	0
M	2.9±0.71	2.8±0.63	2.5±0.32	2.3±0.65
E	2.8±0.95	2.7±0.86	2.1±0.56	1.3±0.21

Note: Comparing the postoperative instant scores in group E to that in group M, $P=0.745$. Comparing the scores at 12 hours, 24 hours, and 48 hours after reperfusion to postoperative instant scores, respectively: group M, $P=0.775$, $P=0.471$, $P=0.596$; group E, $P=0.009$, $P<0.001$, $P<0.001$. Comparing the scores at 12 hours, 24 hours, and 48 hours after reperfusion in group E to those in group M, respectively. All p values were less than 0.001 ($P<0.001$).

nucleus. Next, the slices were put into 70% alcohol, 80% alcohol, 95% alcohol, and anhydrous alcohol for 5 minutes successively to dehydrate. The slices were put into anhydrous alcohol, xylene, xylene (I), and xylene (II) for 10 minutes successively. The slices were taken out from xylene (III), wiping off the xylene by paper or cloth. A little drop of gum was then dripped in the center of the slices. Finally, the slices were sealed with cover glasses by tweezers. The brown reactant around nerve cell nucleus under a 400 times light microscope meant a positive expression. Image-Pro Plus system was used to detect and statistically analyze the percentage and coloration degree of positive cells.

Statistical analysis

SPSS 19.0 statistical analysis software was utilized to analyze data. Measurement data obeying a normal distribution are shown as $\bar{x} \pm s$. For non-normal distribution measurement data, median and interquartile ranges are used to show the central and discrete tendency. Measurement data were analyzed by variance analysis and enumeration data were analyzed by log rank sum test to compare differences among groups. Significant differences are indicated at $P<0.05$.

Results

Longa scores of ischemia reperfusion

Results indicated no neurological impairment occurring in group F, while there were different degrees of neurological impairment occurring in groups E and M. There were no significant differences between groups E and M ($P=0.745>0.05$). Scores indicated no significantly improved neurological function in group M, comparing scores at 12 hours, 24 hours, and 48 hours after reperfusion with postopera-

tive instant scores. There were no significant differences ($P=0.775$, 0.471 , $0.596>0.05$). Neurological function in group E was remarkably improved. There was a significant difference comparing the scores at 12 hours, 24 hours, and 48 hours after reperfusion with postoperative instant scores ($P=0.009$, $P<0.001$, $P<0.001$). Longa scores at 12 hours, 24 hours, and 48 hours after reperfusion in group E improved more than scores in group M, with significant differences ($P<0.01$) (Table 1).

CAMP-PKA-CREB pathway expression

Immunohistochemistry figures (Figure 2) showed different expression levels of CAMP, CREB, and PKA in three groups. Expression levels of CAMP, CREB, and PKA in group E were more closely related to levels in group F than in group M. Immunohistochemistry scores (IHS) of rats in each group (Tables 2-4) showed that IHS in group M was not significantly improved ($P>0.05$). There were significant differences in IHS at 12 hours, 24 hours, and 48 hours after reperfusion in group E ($P<0.05$). Scores in group E improved more than group M ($P<0.05$).

Discussion

Cognitive impairment after strokes has been recorded in some ancient Chinese literatures. It has been divided into mental diseases in Traditional Chinese Medicine, including dementia, melancholia, language disorder, and amnesia. Researchers have confirmed that acupuncture at specific acupoints could improve brain blood circulation, brain energy metabolism, and cognitive function [11, 14]. The governor vessel is an important pathway to deliver vital essence in the viscera, such as the heart and kidneys to the brain, filling encephalon and recovering primordial spirit. Baihui acupoint is a confluent acupoint of food-sanyang channel, foot-jueyin channel, and governor vessel, which

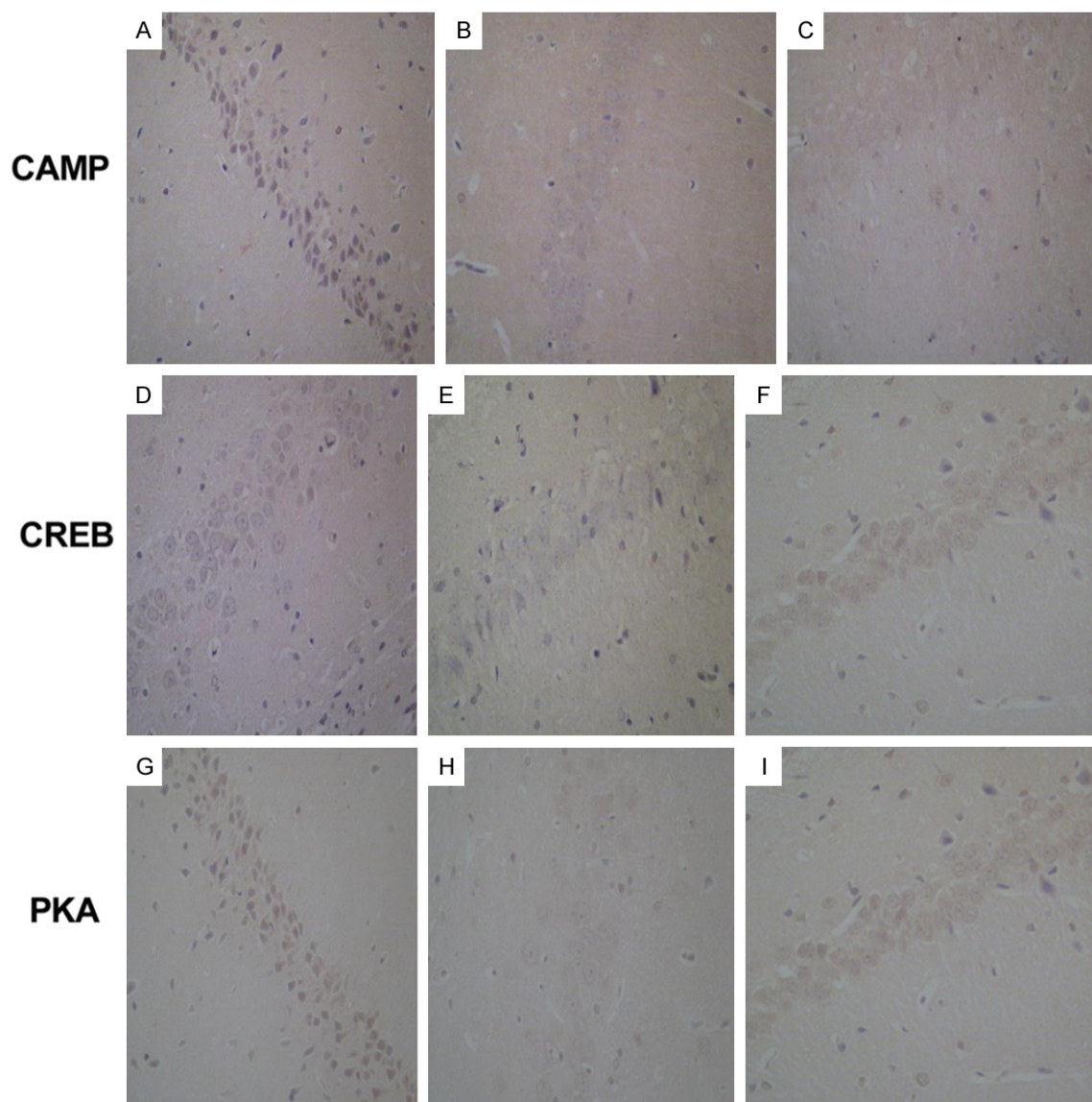


Figure 2. Expression of CAMP, CREB and PKA in the hippocampus of rats in each group. A-C: respectively are the expression of CAMP in groups F, M and E. D-F: respectively are expression of CREB in groups F, M and E. G-I: respectively, are the expression of PKA in groups F, M and E.

Table 2. Immunohistochemistry scores on CAMP in rat hippocampi of each group ($\bar{x} \pm s$)

	Group F	Group M	Group E	P
12 h	6.67±0.81	2.20±0.45	8.16±0.41	<0.001
24 h	6.43±0.76	2.71±0.34	8.97±0.49	<0.001
48 h	6.12±0.98	2.89±0.12	9.23±0.78	<0.001

Note: Compared CAMP at the three periods after reperfusion, group M: $P=0.518, 0.302, 0.893>0.05$; group E: $P<0.01, <0.01, 0.008<0.05$.

directly contacts the brain. Modern medical research has confirmed that acupuncture at Baihui acupoint has an effect on brain blood

circulation improvement, neuron repair, memory ability enhancement, and anti-depression. Acupuncture at Shenting and Baihui can improve the function of the brain, as Shenting and Baihui are perfectly located in the projection area of frontal lobe, temporal lobe, and parietal lobe relating to advanced thinking, memory, and spirit.

Behavioral scores of rats have been widely used in various experimental studies. Symptoms and signs caused by middle cerebral artery occlusion in rats can reflect the condition of brain injuries and recovery. They are

Table 3. Immunohistochemistry scores on PKA in rat hippocampi of each group ($\bar{x} \pm s$)

	Group F	Group M	Group E	P
12 h	5.27±2.06	1.90±1.14	4.13±0.41	<0.001
24 h	5.87±1.76	1.88±1.72	6.14±0.56	<0.001
48 h	6.11±1.36	1.79±1.75	6.97±0.76	<0.001

Note: Compared PKA at the three periods after reperfusion, group M: $P=0.893, 0.917, 0.499>0.05$; group E: $P<0.01$.

Table 4. Immunohistochemistry scores on CREB in rat hippocampi of each group ($\bar{x} \pm s$)

	Group F	Group M	Group E	P
12 h	4.50±1.05	1.40±0.43	4.77±0.65	<0.001
24 h	4.77±1.96	1.58±0.49	6.57±0.61	<0.001
48 h	4.99±0.96	1.66±0.67	6.54±0.98	<0.001

Note: Compared CREB at the three periods after reperfusion, group M: $P=0.995, 0.850, 0.884>0.05$; group E: $P<0.01$.

often used to judge efficacy in scientific research [15]. Middle cerebral artery occlusion model established by the suture method has been applied broadly. Its symptoms and signs can reflect the degree of brain injury and recovery, as marks of successful models [16]. Commonly used evaluation methods of nerve function defects are Bederson six scores [17] and Longa five scores. In this research, the simple and reliable Longa five-score method was used to evaluate a successful model and the curative effects of electroacupuncture on model rats.

In this research, neurological scores showed that instant scores after model establishment in groups M and E were significantly higher than scores in group F, demonstrating that the model was successful. In group E, neurological scores began to reduce at 12 hours after model establishment and there was a significant difference compared with group M. Results indicate that electroacupuncture at Shenting and Baihui could improve nerve function of model rats.

CAMP-PKA-CREB signal pathways are closely connected with learning and memory [18-20]. CAMP is the second messenger of intracellular signal transduction, which can stimulate PKA to phosphorylate target enzymes [21], express genes, and participate in the regulation of cell metabolism and cell growth and division. Some research has confirmed that the catalytic activ-

ity PKA plays a major role in long-term synaptic plasticity and long-term memory [22]. Regulation of cAMP is mainly realized by the combination of nucleotide cyclase and hydrolysis of phosphodiesterase4 (PDE-4). In Vecsey's research, memory loss model rats were made and treated with PDE-4. As a result, rat memories improved obviously [23, 24]. Some studies have shown that an increase of cAMP concentration in the brain can stimulate cAMP-PKA-CREB signaling pathways and further promote the formation of memory [25]. Damage to working memory and reference memory caused by scopolamine can be repaired by PDE-4 treatment [26]. In addition, electroacupuncture can partially restrain cAMP-PKA-CREB signaling pathways to alleviate pain [27].

CREB is the important component of various intracellular signal pathways in the nervous system and the transcription factor of cAMP change. It can regulate protein synthesis and genetic transcription [28]. Stimulation of cAMP-PKA pathways promotes the phosphorylation of CREB and activation of the transcription of downstream target genes [29]. Downstream effects include impact on the survival and growth of neurons, the plasticity of synaptic, and formation of long-term memory [30]. A related experimental research confirmed that reduction of CREB activity can control the formation of long-term memory and that increases of CREB counts or the enhancement of CREB activity can promote the formation of long-term memory [31]. CREB involves directly in the formation of spatial memory in a water maze [32] and the formation of long-term memory in early stage [33].

In the present study, detection results of cAMP, PKA, and CREB showed that positive cell expression of cAMP, PKA, and CREB in group E significantly increased, compared with groups F and M. This study demonstrated that electroacupuncture at Shenting and Baihui might promote recovery of neural function and improvement of spatial learning and memory ability in model rats through upregulation of cAMP-PKA-CREB signaling pathways.

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Disclosure of conflict of interest

None.

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