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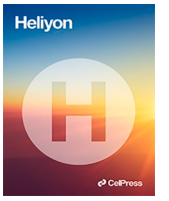

Research article

Electroacupuncture pretreatment enhances the calcium efflux activity of Na^+/Ca^{2+} exchanger to attenuate cerebral injury by PI3K/Akt-mediated NCX1 upregulation after focal cerebral ischaemia

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ABSTRACT

Electroacupuncture pretreatment is considered as an optimal strategy for inducing cerebral ischaemic tolerance. However, the underlying neuroprotective mechanism of this approach has never been explored from the perspective of calcium homeostasis. Intracellular calcium overload is a key inducer of cascade neuronal injury in the early stage after cerebral ischaemia attack and the Na^+/Ca^{2+} exchanger (NCX) is the main plasma membrane calcium extrusion pathway maintaining post-ischaemic calcium homeostasis. This study aims to investigate whether the regulation of NCX-mediated calcium transport contributes to the cerebroprotective effect of electroacupuncture pretreatment against ischaemic injury and to elucidate the underlying mechanisms involved in this process. Following five days of repeated electroacupuncture stimulation on Baihui (GV20), Neiguan (PC6), and Sanyinjiao (SP6) acupoints in rats, in vivo and in vitro models of cerebral ischaemia were induced through middle cerebral artery occlusion and oxygen/glucose deprivation (OGD), respectively. Firstly, we verified the neuroprotective effect of electroacupuncture pretreatment from the perspective of neurological score, infarct volume and neuronal apoptosis. Our findings from brain slice patch-clamp indicated that electroacupuncture pretreatment enhanced the Ca^{2+} efflux capacity of NCX after OGD. NCX1 expression in the ischaemic penumbra exhibited a consistent decline from 1 to 24 h in MCAO rats. Electroacupuncture pretreatment upregulated the expression of NCX1, especially at 24 h, and silencing NCX1 by short hairpin RNA (shRNA) administration reversed the protective effect of electroacupuncture pretreatment against cerebral ischaemic injury. Furthermore, we administered LY294002, a phosphatidylinositol 3 kinase (PI3K) inhibitor, prior to inducing ischaemia to

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investigate the upstream regulatory mechanism of electroacupuncture pretreatment on NCX1 expression. Electroacupuncture pretreatment activates PI3K/Akt pathway, leading to an increase in the expression of NCX1, which facilitates calcium extrusion and exerts a neuroprotective effect against cerebral ischaemia. These findings provided a novel insight into the prevention of ischemic stroke and other similar conditions characterized by brain ischaemia or hypoperfusion.

1. Introduction

Stroke is the leading cause of death and disability worldwide, and 87 % are ischaemic stroke [1]. The advancements in mechanical thrombectomy and pharmacological thrombolysis has brought new optimism to the field. Nevertheless, the effectiveness of these recanalization techniques is hindered by relatively narrow therapeutic time windows, various contraindications, and restricted accessibility [2,3]. Moreover, many promising neuroprotective medications for post-stroke treatment have failed to translate from animal experiments to clinical practice [4,5]. It is urgent to develop new options for stroke management. Therefore, exploring the best prevention method is listed as the first research priority to improve stroke outcomes [6].

Ischaemic preconditioning (IPC) has the translational potential to become a prophylactic treatment for ischaemic stroke [7,8]. The concept of IPC is using short periods of nonlethal ischaemia to induce endogenous protective action against a subsequent and more severe ischaemic insult [8]. Experimental studies have confirmed that IPC can effectively reduce cerebral ischaemia injury by activating multiple endogenous protective mechanisms [9]. Acupuncture, a prominent modality in traditional Chinese medicine for disease prevention and management, might be the most promising IPC method for stroke management due to its simplicity, availability and safety. Most of the pre-existing studies of acupuncture pretreatment for inducing cerebral ischemic tolerance adopted the electroacupuncture scheme. Because electroacupuncture, the combination of traditional acupuncture with modern acupoint stimulation methods, could provide quantifiable stimulation by applying an electrical current to the needles that are inserted into acupoints. Electroacupuncture pretreatment can reduce ischaemic brain injury and promote the recovery of neurological function by activating the endogenous cannabinoid system [10], inhibiting excitotoxicity [11], inhibiting inflammation [12], promoting mitochondrial biogenesis [13], regulating autophagy, decreasing oxidative stress and so on [14,15]. However, the underlying neuroprotective mechanism of electroacupuncture pretreatment has not been fully elucidated.

Calcium (Ca^{2+}) overload is the main factor underlying neuronal death in the early stage after cerebral ischaemia attack, and reducing the concentration of intracellular Ca^{2+} can play a neuroprotective role [16]. The Na^{+}/Ca^{2+} exchanger (NCX), a bidirectional membrane ion transporter, plays an essential role in maintaining intracellular Ca^{2+} homeostasis [17]. Under physiological conditions, NCX operates in the forward mode by extruding one Ca^{2+} against entering three Na^{+}. However, under certain pathological conditions, such as cerebral ischaemia, NCX might also work in the reverse mode, leading to Ca^{2+} overload and cell death [18]. Various isoforms (NCX1, NCX2, NCX3) of NCX have been identified in mammals [19], with NCX1 being the major subtype related to neuronal survival after ischaemic insults [20,21]. Inhibiting NCX1 expression through different methods can render the brain more vulnerable to ischaemic insult [22,23].

The purpose of this study was to investigate whether the regulation of NCX Ca^{2+} transportation contributes to the cerebroprotective effect of electroacupuncture pretreatment against ischaemic attack and to explore the underlying mechanism from the aspect of phosphatidylinositol 3 kinase (PI3K)/protein kinaseB (Akt) -mediated NCX1 expression.

2. Materials and methods

2.1. Animals

Healthy adult male Sprague-Dawley rats (200–230 g) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All animals were housed in a specific pathogen–free environment with regulated cycles of temperature and humidity, and a 12-h light-dark cycle.

2.2. Experimental protocols

2.2.1. Experiment I

To investigate the neuroprotective effect of electroacupuncture pretreatment, the rats aged 5–6 weeks were randomly divided into 3 groups: sham operation (Sham), middle cerebral artery occlusion (MCAO) and electroacupuncture + MCAO (EA + MCAO) (n = 10 for each group). As shown in Fig. 1A, rats in each group would be pretreated using electroacupuncture or only binding for 5 consecutive days prior to MCAO or sham-MCAO preparation. At 24 h after model preparation, the degree of neurological deficit was evaluated by neurobehavioural scoring, the cerebral infarction volume was measured by TTC staining, and neuronal apoptosis was detected by flow cytometry.

2.2.2. Experiment II

To observe the influence of electroacupuncture pretreatment on NCX activity using patch-clamp technology. The rat brain slice oxygen/glucose deprivation (OGD) method was used to simulate the rat cerebral infarction model in vitro to observe the changes in

neuronal NCX currents. The rats aged 3–4 weeks, with better brain slice activity [24], were randomly divided into 3 groups: Control, OGD, and EA + OGD (n = 4 for each group). Rats in EA + OGD group would be pretreated using electroacupuncture for 5 consecutive days prior to brain slice preparation.

2.2.3. Experiment III

To investigate the impact of electroacupuncture pretreatment on the regulation of NCX1 expression in ischaemic penumbra following model preparation at various time intervals (0, 1, 2, 24 h). The rats aged 5–6 weeks were randomly divided into 3 groups: sham, MCAO and EA + MCAO. Each group was subdivided into 0-h, 1-h, 2-h and 24-h subgroups (n = 7 for each subgroup). All pretreatment protocol were consistent with those in experiment I, and the brain tissue samples were extracted to measure expression level of NCX1 at different timepoints post cerebral ischaemia.

2.2.4. Experiment IV

To observe whether knockdown of NCX1 expression by short hairpin RNA (shRNA) would affect the therapeutic effects of electroacupuncture pretreatment. Rats aged 5–6 weeks were randomly divided into 4 groups: MCAO, EA + MCAO, NCX1 shRNA + EA + MCAO, and vehicle + EA + MCAO (n = 10 for each group). As Fig. 4A, rat in the NCX1 shRNA + EA + MCAO group and vehicle + EA + MCAO group would be injected with the NCX1 shRNA or its vehicle at the brain region prior to electroacupuncture pretreatment.

2.2.5. Experiment V

To explore whether electroacupuncture pretreatment plays a protective role in the brain by activating the PI3K/Akt-mediated NCX1 up-regulation using the PI3K inhibitor LY294002. The rats aged 5–6 weeks were randomly divided into 4 groups: MCAO, EA + MCAO, LY294002 + EA + MCAO, and vehicle + EA + MCAO (n = 14 for each group). Rat in the LY294002 + EA + MCAO group, and vehicle + EA + MCAO group would be intraperitoneally injected with the PI3K inhibitor LY294002 or its vehicle prior to electroacupuncture pretreatment.

2.3. Electroacupuncture pretreatment

The electroacupuncture pretreatment was performed as described previously [25]. Rat were restrained using homemade rat clothing during the pretreatment sessions. One-off sterile acupuncture needles (Hanyi, Beijing, China) of 0.25 × 13 mm was used to stimulate specific points. Baihui (GV20) was selected at the midpoint between the tips of the ears and punctured backwards at a depth of 2 mm. The bilateral Neiguan points (PC6) were selected 3 mm above the wrist joint on the medial side of the forelimb between the ulna and the radius and were vertically punctured to a depth of 1 mm. Bilateral Sanyinjiao (SP6) were selected 10 mm above the tip of the medial malleolus at the hind limbs and was punctured vertically to a depth of 5 mm. All points were stimulated at an intensity of 1 mA and a frequency of 2/15 Hz for 20 min per day five days in a row using an electroacupuncture therapeutic apparatus (HANS-100A, Nanjing Jisheng Medical Technology, China). The limbs and head would slightly tremor during electrical stimulation, which was considered a sign of Deqi. In the non-electroacupuncture group, rats were restrained using the homemade rat clothing for the same duration.

2.4. Animal model of focal cerebral ischaemia

Rats fasted and drank water freely for 12 h prior to undergoing surgery. After anesthesia induction with 4 % isoflurane (RWD Life science, Shenzhen, China) and maintenance with 2 % isoflurane, the right MCAO model was established in accordance with the method described by Longa et al. [26]. Briefly, the right common carotid artery, external carotid artery and internal carotid artery were exposed. A nylon filament suture (RWD Life science, Shenzhen, China) rounded by paraffin wax at the tip was advanced from the external carotid artery into the internal carotid artery. The internal carotid artery and common carotid artery were ligated to fix the thread bolt and the incision was sutured layer by layer after hemostasis and disinfection. The nylon suture remained in place in rats receiving MCAO until execute. In sham-MCAO rats, the same operation was performed except for the insertion suture: the right common carotid artery, external carotid artery and internal carotid artery were also exposed, but no nylon filament suture was inserted into artery before the incision was sutured layer by layer. Regional cerebral blood flow was monitored through a laser Doppler flow meter (DRT4; Hewlett-Packard, USA), and the adequacy of the model was confirmed by observing a significant reduction in regional cerebral blood flow to 30 % of baseline levels prior to the onset of ischaemia.

2.5. Neurological score

Twenty-four hours after MCAO, the neurological deficit was evaluated by an investigator unaware of group allocation according to the 18-point scoring system of Garcia et al. [27]. The neurological score ranged from 3(most severe neural deficit) to 18 (normal neural function) given to each rat with the summation of six distinct test: spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch.

2.6. Evaluation of the infarct volume

2,3,5-Triphenyltetrazolium chloride (TTC, Solarbio, Beijing, China) staining was used to determine the infarction volume 24 h after

MCAO. In brief, the rat was anaesthetized with 4 % isoflurane. The brain was rapidly removed and sectioned coronally into 2-mm-thick slices starting from the frontal pole. Slices were incubated in 2 % TTC at 37 °C for 30 min, after which the slices were removed and placed for imaging. TTC stains viable brain tissue deep red, while the infarcted tissue remains unstained. The areas of infarcted and total hemispheres of each section were quantified and analysed using ImageJ software. Brain infarct volume was expressed as a percentage relative to the total brain tissue volume.

2.7. Flow cytometry

Nerve cell apoptosis was detected using fluorescence-activated cell sorting with Annexin V-FITC/PI staining (Beamdiag, Changzhou, China). Briefly, nerve cell from the ischaemic penumbra were collected and resuspended in binding buffer at a cell concentration of approximately 1×10^6/ml after MCAO. An Annexin V-FITC Apoptosis Detection Kit (4A Biotech, Beijing, China) was used in accordance with the manufacturer's instructions. Annexin V-FITC (5 μl) and propidium iodide (10 μl) were added to each 100 μl of cell suspension. Following a 5-min reaction at room temperature in a light-protected setting, apoptotic cells were detected by fluorescence-activated cell sorting.

2.8. Western blot

Ischaemic penumbra tissues were extracted as described [28]. Protein concentrations were determined by the BCA protein assay (Solarbio, Beijing, China). Specific primary antibodies including anti-NCX1 (Abcam, Cambridge, UK), anti-Akt (CST, USA), anti-phosphorylated Akt (*p*-Akt) (CST, USA), and anti-β-actin (TransGen, China) were employed to detect the proteins of interest. Subsequently, a secondary HRP-conjugated goat anti-rabbit (1:5000, TransGen, China) or HRP-conjugated goat anti-mouse antibody (1:5000, TransGen, China) was used. The intensity of chemiluminescence was measured using ImageJ software.

2.9. Brain slice preparation and induction of in vitro ischaemia

After anaesthetization with isoflurane (RWD Life science, Shenzhen, China), the animals were euthanized by decapitation. Subsequently, brain tissues were quickly removed and immersed in slicing solution at 4 °C. Using a vibrating slicer, coronal slices were generated to prepare hippocampal coronal slices with a thickness of 400 μm. These brain slices were then incubated in saturated in artificial cerebrospinal fluid saturated with a gas mixture of 95 % O_2 and 5 % CO_2 at a room-temperature for over 1 h. The brain slices in the OGD group were exposed to a gas mixture of 95 % N_2 and 5 % CO_2 at room-temperature. Following this incubation period, the brain slices were transferred to the microscope perfusion tank for data recording. The flow rate of the artificial cerebrospinal fluid solution during perfusion was maintained at 2.0–2.5 mL/min. Throughout the experiment, a temperature control system was used to sustain a constant temperature of 31 ± 0.5 °C within the brain slices.

2.10. Electrophysiology

We recorded NCX currents with the patch-clamp whole-cell recording technique for neurons in hippocampal slices. The hard, thick-walled glass blank was made into a tip with a diameter of approximately 1 μm by using a programmable glass microelectrode drawing instrument for micrometre glass microelectrodes, and the resistance was 8–12MΩ after filling with electrode solution. Under an infrared phase contrast microscope, brain slices with a thickness of 200–300 μm were treated with a visual surface cleaning method. The glass microelectrode was driven by a microelectrode manipulator to approach the neurons. When the tip of the microelectrode just contacted the cell membrane, a slightly negative pressure was applied to form a high-resistance seal, with a seal resistance greater than 5 GΩ. The feedback resistance of the amplifier probe was 50 GΩ, and the filtering frequency of the low-pass filter was 1 kHz. Data acquisition was performed using recording software at a sampling frequency of 10 kHz. The cells were clamped at −40 mV, and ramp stimulation was given from 60 mV to 120 mV at a speed of 8.0 V/s, a duration of 2 s, and a frequency of 0.05 Hz. Ni ion can specifically block NCX [29,30], and could be used in the Patch clamp experiment to block the NCX current [31]. Following stabilization of the image, 25 mmol/L Ni^{2+} was added to the perfusion fluid to specifically block the NCX currents, and another I–V curve with significant attenuation was recorded. The Ni^{2+}-sensitive Na^+/Ca^{2+} exchange current was obtained by subtracting the two numbers. The data were analysed with pClampfit 10.6 software after 2-kHz low-pass filtering.

2.11. Intraventricular administration

The rats were anaesthetized and positioned on a stereotaxis apparatus, and a stainless-steel cannula was stereotaxically implanted in the unilateral cerebral ventricle using specific stereotaxic coordinates of 0.24 mm caudal, 3.3 mm lateral and 2.1 mm below the

surface of the skull. The shRNA for NCX1 and its scrambled RNA were supplied by Brain VTA (Wuhan, China). The sense and antisense sequences were as follows: 5′-GCAGATACAGAGGCAGAAACA-3′ and 5′-CCTAAGGTTAAGTCGCCCTCG-3′ respectively. The PI3K inhibitor LY294002 was purchased from Beyotime Biotechnology (Shanghai, China). The shRNA (1 μL, 80 nl/min) was administered 3 weeks before pretreatment induction, whereas LY294002 and the respective vehicle were injected 30 min before pretreatment.

2.12. Real time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Japan), according to the manufacturer's instructions. Transcript levels of indicated genes were quantified by quantitative RT-PCR on fluorescent quantitative PCR instrument (Bio-Rad, USA) with One Step SYBR®PrimeScript™RT-PCR Kit II. Relative expression was determined using GAPDH as an internal reference control. The primer sequences employed for this analysis are detailed below:

NCX1: 5′GCGCGTCGACCACAGTATTCCAAC3′ and 5′GGCCCCTTCGGAATCGTGGTCTG3';

GAPDH: 5′GCCCATCACCATCTTCCAGGAGCG 3′ and 5′GCAGAAGGGGCGGAGATGATGACC 3'.

2.13. Statistical analysis

The data were presented as the mean ± SD, and statistical analysis was conducted using SPSS 25.0 software. Statistical comparisons of different time points were made by one-way ANOVA. Data lacking a time sequence were compared using one-way ANOVA or the Kruskal–Wallis test, according to data distribution. Statistical significance was determined at a *P*-value less than 0.05.

3. Results

3.1. Electroacupuncture pretreatment induces tolerance against focal cerebral ischaemia

We evaluated the neuroprotective effect of electroacupuncture pretreatment through the evaluation of neurological function, infarct volume and neuronal apoptosis. Rats in the MCAO group exhibited neurological deficits 24 h after MCAO (Fig. 1C; 10.10 ± 1.29; $P < 0.01$ versus Sham). Conversely, rats subjected to electroacupuncture pretreatment displayed a significant improvement in the neurological function 24 h after MCAO (Fig. 1C; 14.00 ± 1.76; $P < 0.01$ versus MCAO). In addition, there was obvious ischaemic infarction in the MCAO and EA + MCAO groups. Analysis of the infarcts stained with TTC showed that the ratio of the infarcted tissue volume to the contralateral tissue 24 h after MCAO was 61.00 ± 9.69. Electroacupuncture pretreatment significantly reduced the infarct ratio (Fig. 1D and. E; 34.40 ± 8.73; $P < 0.01$ versus MCAO).

Nerve cell apoptosis within the ischaemic penumbra was detected 24 h after MCAO in each group. The MCAO group exhibited a notably elevated level of apoptosis compared to the Sham group (Fig. 1F; 12.73 ± 4.80; $P < 0.01$ versus Sham). Electroacupuncture pretreatment resulted in a significant decrease in the incidence of nerve cell apoptosis (Fig. 1F and. G; 3.88 ± 1.43; $P < 0.01$ versus MCAO).

3.2. Electroacupuncture pretreatment promotes Ca^{2+} efflux through NCX in OGD-injured rat brain slices

In brain slices subjected to oxygen-glucose deprivation (OGD), alterations in NCX Ca^{2+} efflux capacity were examined using the patch-clamp technique. The results indicated a notable reduction in the Ni^{2+}-sensitive NCX current of Ca^{2+} efflux in brain slices following OGD. Conversely, electroacupuncture pretreatment significantly increased the NCX outward current of brain slices after OGD. These results suggested that electroacupuncture pretreatment might help preserve the Ca^{2+} efflux properties of NCX after OGD (Fig. 2A and. B).

3.3. Electroacupuncture pretreatment increases NCX1 expression at 24 h in ischaemic penumbra

The expression of NCX1 at various time points (0, 1, 2, and 24 h) post ischaemic attack in the ischaemic penumbra was measured using Western blot. The results indicated a gradual decrease in NCX1 levels, with a significant reduction at 1 h post ischaemia (Fig. 3A and. B; $P < 0.05$; versus Sham), reaching its lowest point at 24 h (Fig. 3B; $P < 0.01$; versus Sham) post ischaemia. Electroacupuncture pretreatment led to a significant increase in NCX1 expression at 24-h time point when compared to the MCAO model animals (Fig. 3B; 1.22 ± 0.26; $P < 0.01$; versus MCAO).

NCX1 shRNA abolishes the therapeutic effects of electroacupuncture pretreatment on brain infarction and neurological function after MCAO.

To demonstrate the role of NCX1 in neuroprotective effect elicited by electroacupuncture pretreatment, NCX1 shRNA was utilized to suppress the expression of NCX1. Western blot and PCR confirmed the effective suppression of NCX1expression by NCX1 shRNA (Figure in the Data Supplement). As shown in Fig. 4, compared with the MCAO group, the EA + MCAO group had a significantly improved neurological score (Fig. 4B; 12.70 ± 1.25; $P < 0.01$; versus MCAO) and decreased infarct volume (Fig. 4B; 29.00 ± 3.61; $P < 0.05$; versus MCAO). Additionally, knockdown of NCX1 by intracerebroventricular injection of NCX1-shRNA before MCAO significantly increased the infarct ratio (Fig. 4C and. D; 61.33 ± 7.64; $P < 0.01$ versus vehicle + EA + MCAO). Neurological function was exacerbated by NCX1-shRNA (Fig. 4B; 10.40 ± 2.01; $P < 0.01$ versus vehicle + EA + MCAO). These results suggested that NCX1 is a critical mediator of electroacupuncture pretreatment induced neuroprotection.

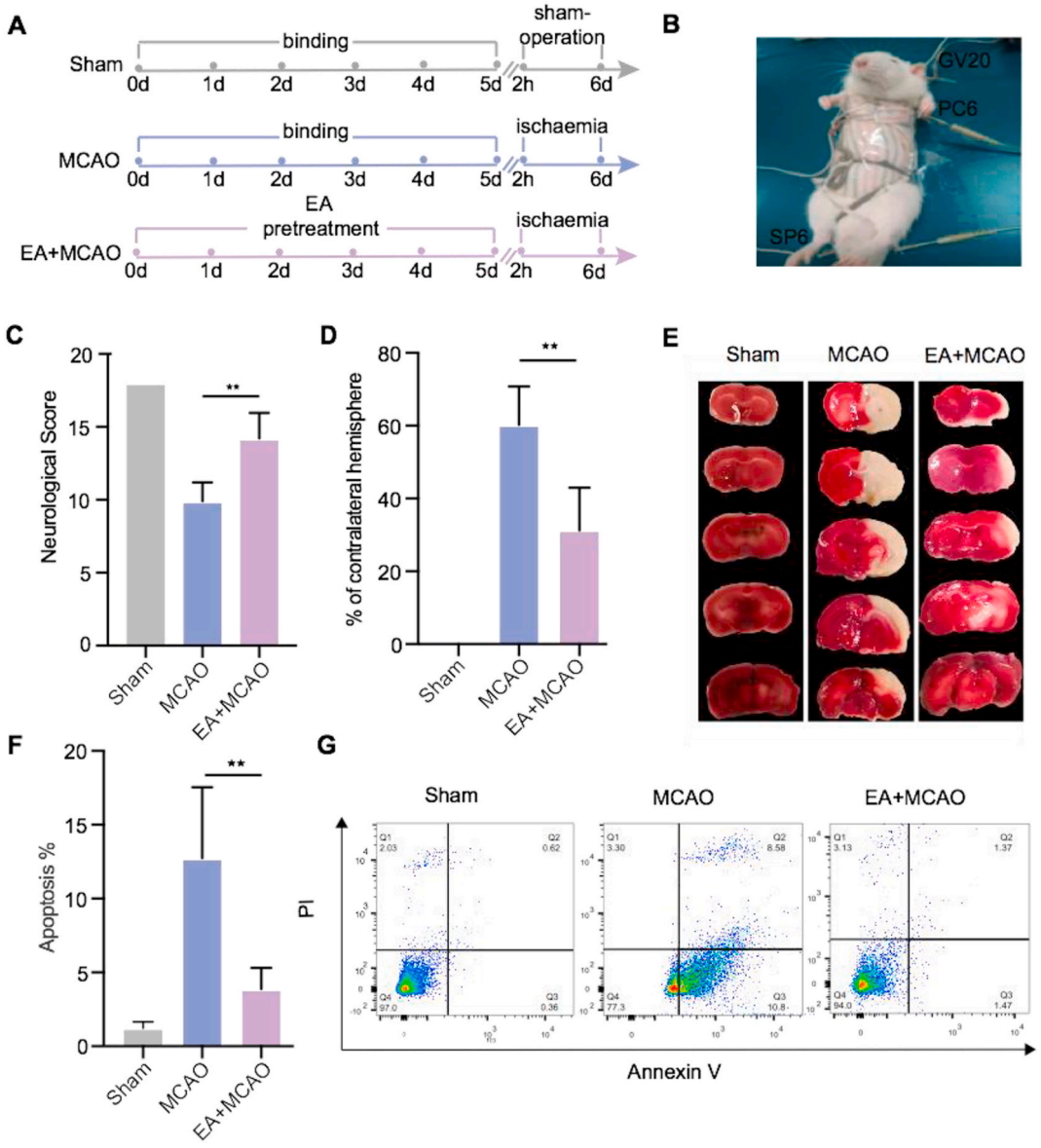

Fig. 1. Effect of electroacupuncture pretreatment on neurological deficit scores, infarction volume and the number of nerve cell apoptosis after MCAO. The neurological deficit was evaluated by the Garcia score (n = 10 per group), the infarct volume was detected by TTC staining of the brain slices (n = 5 per group), and the number of nerve cell apoptosis was detected by flow cytometry (n = 5 per group). **(A)** A schematic diagram illustrating the chronological events of experiments. The rats received 20 min electroacupuncture pretreatment once a day for five days prior to the ischaemic event, after which focal cerebral ischaemia was induced by MCAO. **(B)** Electroacupuncture stimulation. **(C)** Quantification of neurologic scores at 24 h after ischaemia. **(D)** Statistical analysis of the percentage of infarct volume was determined for each group. **(E)** Representative slice of TTC staining. The normal tissue was stained deep red and the infarct was stained white. Brain infarcts were quantified as percentage area of ischaemic hemisphere. **(F)** Quantitative analysis showed the number of nerve cell apoptosis in the ischaemic penumbra. **(G)** Representative flow cytometry showing the number of nerve cell apoptosis in each group at 24 h after ischaemia. Data are expressed as the means ± SD. $^{**}P < 0.01$ was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. PI3K inhibitor downregulates NCX1 and blocks the neuroprotective effect of electroacupuncture pretreatment

Previous studies have suggested that NCX1 is an additional target for the survival action of the PI3K/Akt pathway [32]. To elucidate the upstream molecular mechanisms through which EA pretreatment regulates NCX1, we administered an intraventricular injection of the PI3K inhibitor LY294002. Western blot analysis showed that the expression of Akt protein was similar between the groups, while LY294002 injection significantly reduced the level of (*p*-Akt) 24 h after MCAO (Fig. 5A and. B; 0.40 ± 0.93; $P < 0.01$ versus vehicle + EA + MCAO). As shown in Fig. 5, administration of LY294002 decreased the expression of NCX1 protein (Fig. 5B; 0.36 ± 0.17; $P < 0.01$ versus vehicle + EA + MCAO) and caused a significant impairment of neurological function (Fig. 5C; 11.89 ± 1.62; $P < 0.01$ versus vehicle + EA + MCAO). Furthermore, LY294002 treatment significantly affected the cerebral infarction volume (Fig. 5D and. E; 53.20 ± 5.07; $P < 0.05$ versus EA + MCAO) and nerve cell apoptosis rate (Fig. 5F and. G; 8.69 ± 4.08; $P < 0.05$ versus EA + MCAO). Thus, LY294002 treatment inhibited the upregulation of NCX1 and reversed the neuroprotective effect of electroacupuncture pretreatment.

4. Discussion

The concept of treatment prior to disease onset is a distinctive principle within traditional Chinese medicine (TCM), aimed at preventing the occurrence or progression of diseases by activating endogenous protective mechanisms and enhancing self-regulatory capability. This goal also applies to the understanding of IPC, which seeks to confer protection against severe or lethal ischaemia insult through the use of nonlethal ischaemia preconditioning [8]. According to the documentation in ancient traditional Chinese medicine texts, stroke prevention using acupuncture can be traced back to the Tang Dynasty (*Bei Ji Qian Jin Yao Fang*, also referred to as *Essential Prescriptions Worth a Thousand Gold for Emergencies* written by *Sun Simiao*). As the most widespread nonpharmaceutical therapy, acupuncture has been proven to have therapeutic value for post-stroke management [33]. Five major different mechanisms are involved in the beneficial effects of acupuncture or electroacupuncture on ischemic stroke rehabilitation [34]: promotion of neurogenesis and cell proliferation in the central nervous system; regulation of cerebral blood flow in the ischemic area; anti-apoptosis in the ischemic area; regulation of neurochemicals; and, improvement of impaired long-term potentiation and memory after stroke. Despite its proven benefits in post stroke management, acupuncture are not commonly utilized for stroke prevention. Nevertheless, it is posited acupuncture could serves as an optimal strategy to trigger endogenous protective mechanisms and enhance cerebral ischemic tolerance. A comprehensive understanding of the mechanisms through which preventive acupuncture mitigates cerebral ischemic injury may facilitate its broader clinical application among individuals at high risk of stroke occurrence, recurrence, or those undergoing cerebral hypoperfusion surgery.

Our previous experiment showed that 5 days of repeated electroacupuncture pretreatment on GV20, SP6, and PC6 could ameliorate neurological dysfunction, decrease infarct volume, and reduce apoptosis in an animal model of cerebral ischaemia/reperfusion [25]. This study further verifies that such acupuncture regimen could also elicit similar cerebral ischaemic tolerance in a model of permanent cerebral ischaemia. Such acupoints combination was selected to induce cerebral ischaemia tolerance by regulating the spirit and regulating the viscera from the perspective of the basic theory of TCM: GV20, located in the vertex, mainly regulate spirit and activate local collaterals for ischemic tolerance; SP6 could regulate meridian of liver, spleen and kidney; and the combination of PC6

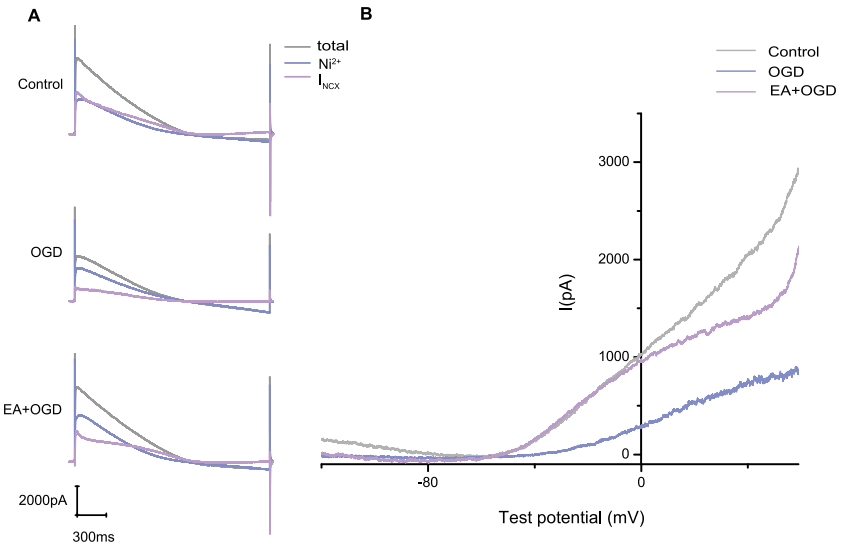

Fig. 2. Electroacupuncture Pretreatment Promotes Ca^{2+} Efflux through NCX in OGD-Injured Rat Brain Slices. (A) Representative patch-clamp I–V curve recording diagram of Control group, OGD group and EA + OGD group, respectively. **(B)** The comparison of Ni^{2+}-sensitive NCX current in each group (n = 4 per group).

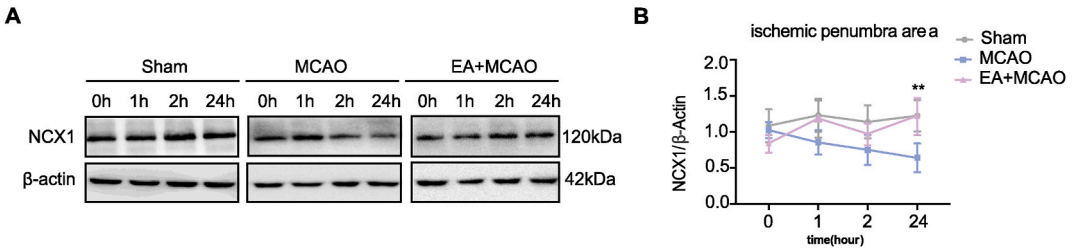

Fig. 3. Comparison of NCX1 expression in ischaemic penumbra of each group at different time points. The expression of NCX1 were detected by Western blot. **(A)** Western blot showing representative NCX1 protein expression at 0, 1, 2 and 24 h after ischaemia among the three groups. **(B)** The density of NCX1protein expression relative to β-actin protein expression at 0, 1, 2 and 24 h after ischaemia among the three groups (n = 4 per time point per group). Data are expressed as the means ± SD. $^{**}P < 0.01$ was considered significant. See uncropped versions of Western blot figures in the Supplementary materials.

and GV20 could promote holistic adaptation. Electroacupuncture is the combination of traditional acupuncture and modern method of acupoint stimulation. Most of the pre-existing studies of acupuncture pretreatment for inducing cerebral ischemic tolerance adopted the electroacupuncture scheme [10–13]. Those researchers choose similar acupoint stimulation method might because: electroacupuncture could induce similar effect as manual acupuncture, and it is easier to be standardized and repeated compared to manual acupuncture. Previous evidence showed that one session of electroacupuncture pretreatment could induce rapid tolerance to cerebral ischaemia within 2 h post pretreatment process [35], while repeated electroacupuncture could elicit both rapid and delayed tolerance to cerebral ischaemia [36]. In this study, 5 sessions of electroacupuncture pretreatment were chosen to extend the neural protective window observed in in vivo experiments.

Now that we have confirmed the neuroprotective effect of electroacupuncture preconditioning, the existing evidence of its underlying mechanism using animal model of MCAO should also be summarized: electroacupuncture pretreatment can induce cerebral ischaemic tolerance by activating the endogenous cannabinoid system [10], inhibiting excitotoxicity [11], inhibiting inflammation [12], promoting mitochondrial biogenesis [13], regulating autophagy, decreasing oxidative stress and so on [14,15]. The pathophysiological cascade involved in cerebral ischaemic injury is multifaceted. The existing evidence remains insufficient in elucidating the mechanisms by which electroacupuncture pretreatment induces cerebral ischaemic tolerance. Ischaemic attack triggers a cascade

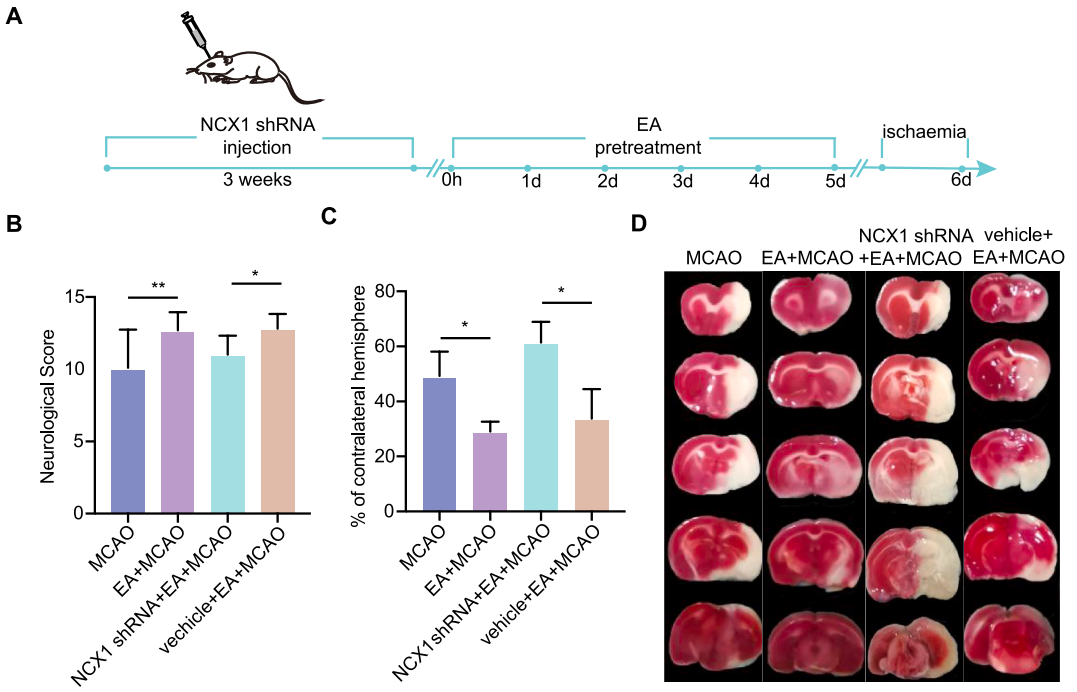

Fig. 4. Specific study on NCX1 response and neuroprotection effect of electroacupuncture pretreatment. (A) A schematic diagram of experimental flow. **(B)** Quantification of neurologic scores at 24 h after ischaemia (n = 10, per group). **(C)** Brain infarct volume presented as a percentage of the intact hemisphere (n = 4, per group). **(D)** Representative images of focal cerebral infarction staining by TTC in coronal brain slices (white, infarct tissue; red, noninfarct tissue). Data are presented as the means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$ was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of interconnected biochemical processes in response to inadequate energy and oxygen supply. Pretreatment intervention could influence the early-stage pathological process post ischaemic attack. This early-stage pathological progression is mainly characterized by the interplay between excessive extracellular glutamate and intracellular free Ca^{2+} overload, along with their subsequent downstream effects leading to cell death [37]. Previously, we confirmed that regulation of glutamate excitotoxicity is one protective pathway of electroacupuncture pretreatment against cerebral ischaemia [25]. However, whether the maintenance of calcium homeostasis is another key regulatory target of electroacupuncture pretreatment needs further exploration.

When ischaemia happens, Ca^{2+} flows into the cytoplasm from the extracellular space through the plasma membrane or from intracellular Ca^{2+}-storage organelles, leading to an increase in cytosolic Ca^{2+} concentration [38]. Intracellular Ca^{2+} dyshomeostasis

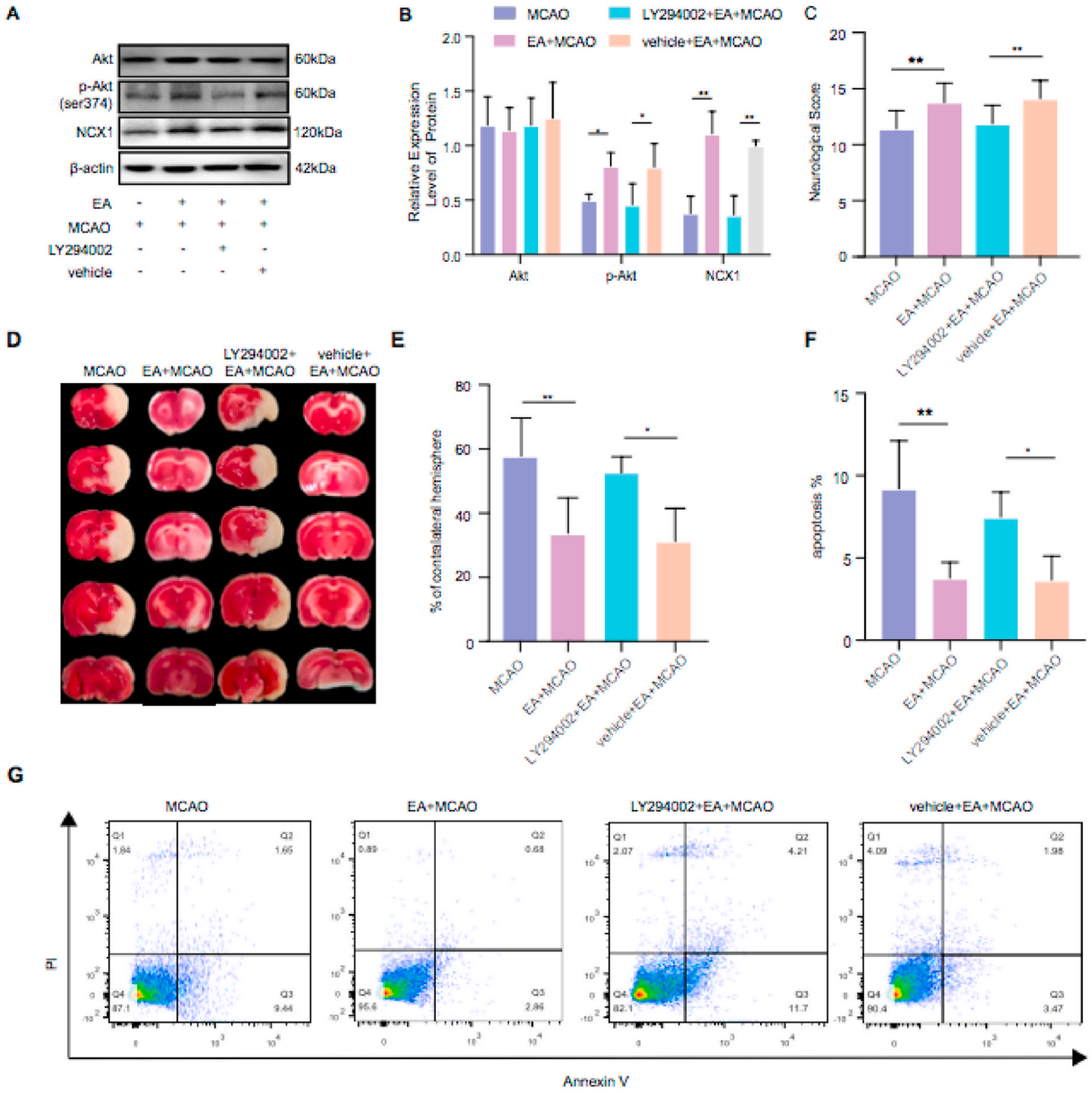

Fig. 5. PI3K Inhibitor Downregulates NCX1 and Blocks the Neuroprotective Effect of Electroacupuncture Pretreatment. (A) Representative pictures of Western blot bands showing the expression of Akt, *p*-Akt, NCX1, β-actin. **(B)** The density of Akt, *p*-Akt, NCX1 protein expression relative β-actin protein expression at 24 h after ischaemia (n = 3, per group). **(C)** Quantification of neurologic scores at 24 h after ischaemia (n = 9 per group). **(D)** Representative images of focal cerebral infarction staining by TTC in coronal brain slices (white, infarct tissue; red, noninfarct tissue). **(E)** Brain infarct volume presented as a percentage of the intact hemisphere (n = 5 per group). **(F)** Comparison of nerve cell apoptosis (n = 5 per group). **(G)** Representative flow cytometry showing the number of nerve cell apoptosis in each group at 24 h after ischaemia. Data are expressed as the means ± SD. $*P < 0.05$ vs MCAO; $**P < 0.01$ was considered significant. See uncropped versions of Western blot figures in the Supplementary materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

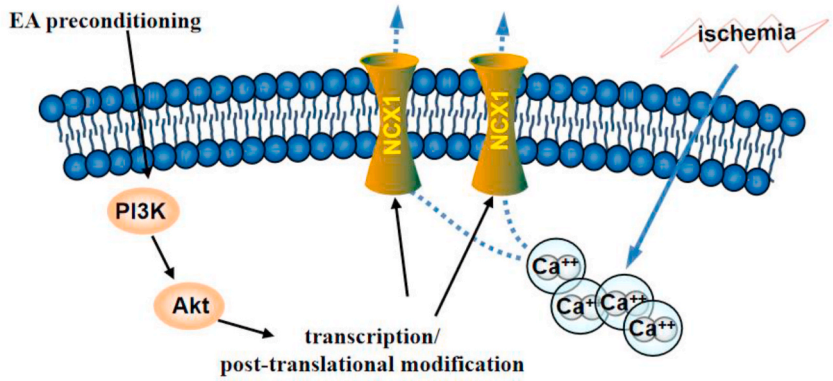

Fig. 6. Electroacupuncture pretreatment Enhances the calcium efflux activity of Na+/Ca2+ exchanger to Attenuate cerebral injury by PI3K/Akt-mediated NCX1 upregulation after focal cerebral ischaemia.

due to intracellular free Ca^{2+} overload is directly related to increased free radical production and mitochondrial permeability and subsequent cell death [39]. Promoting Ca^{2+} efflux has the potential to promote neuronal survival and reduce cerebral ischaemic injury [40]. Ca^{2+} ATPase and NCX are responsible for exporting Ca^{2+} from the cell [41,42], with NCX being the main plasma membrane Ca^{2+} extrusion pathway to maintain Ca^{2+} homeostasis due to its high transport capacity [18]. Our results also show that the brain slice NCX current of Ca^{2} efflux decreased significantly after OGD. Activation of NCX maintains ionic homeostasis in the peri-infarct area by facilitating Ca^{2+} efflux [43], while its deactivation of NCX may aggravate ischaemic brain injury by dysregulating Ca^{2+} homeostasis [20,22,44]. Therefore, we propose that the neuroprotective action of electroacupuncture pretreatment might also contribute to the regulation of NCX. Our results of brain slice electrophysiology provide evidence for this hypothesis: the NCX outward current of brain slices pretreated with electroacupuncture showed no significant decrease after OGD, suggesting that electroacupuncture pretreatment can preserve the overall Ca^{2+} efflux property of NCX and regulate the Ca^{2+} homeostasis after oxygen glucose deprivation.

Among the three isoforms of the NCX protein family, NCX1 is the dominant exchanger gene expressed in the brain, and it plays a major role in the neuroprotective effect exerted by NCX during ischaemic injury [20,21,45]. The change in NCX1 expression can reflect the change in the ability of cells to control intracellular Ca^{2+} concentration [46], and inhibition of NCX1 expression can aggravate cerebral ischaemic injury [23,47]. During an ischaemic attack, NCX1 expression is decreased, leading to decreased efficacy in Ca^{2+} efflux [20,38]. Our study shows that NCX1 expression in the ischaemic penumbra constantly decreased from 1 h to 24 h after ischaemia. The downregulation of NCX1 protein may be attribute to the cleavage of NCX1 protein by proteolytic enzymes activated under ischaemia, such as caspases and calpain [48]. Upregulation of NCX1 expression contributes to the cerebral protective effect of various forms of cerebral ischaemic preconditioning [49,50]. Our findings showed that NCX1 plays a crucial role in the cerebral protection conferred by electroacupuncture pretreatment. Electroacupuncture pretreatment upregulated the expression of NCX1 protein, especially at 24 h, and NCX1 silencing by shRNA administration reversed the protective effects of electroacupuncture pretreatment in reducing cerebral ischaemic injury. Electroacupuncture pretreatment might enhance Ca^{2+} efflux efficiency and reduce the Ca^{2+} efflux concentration by upregulating NCX1 expression.

Our study set the 0h time point to clarify whether the modulation of NCX1 protein expression through electroacupuncture pretreatment occurs prior to ischemic preconditioning or in response to ischemic stress. And the results showed that there was no upregulation of NCX1 protein expression before the onset of ischaemia, even with electroacupuncture pretreatment. Electroacupuncture pretreatment induces ischemic tolerance, and alters the brain's responses to ischemic attack. Therefore, the predominant regulatory mechanism of EA pretreatment that induces ischemic tolerance might only become evident following the onset of an ischaemic event. In our investigation, specific timepoints of 1 h, 2 h and 24 h were established to clarify the commencement and duration of electroacupuncture pretreatment on the regulation of NCX1. The findings reveal that the impact of electroacupuncture pretreatment on the regulation of NCX1 protein level initiates as early as 1 h after the ischemic event and persists for a minimum of 24 h thereafter.

After establishing the role of NCX1 in the neuroprotective effects of electroacupuncture preconditioning, we further explored its upstream mechanism. Previous studies have indicated a significant association between NCX1 and *p*-Akt. In Akt-positive mutants, NCX1 serves as a pro-survival target, as its increased expression aids in neuronal survival under conditions of chemical hypoxia [32]. The upregulation of NCX1 expression in the ischemic temporoparietal cortex following pretreatment is facilitated by *p*-Akt [47]. Therefore, we proposed that the PI3K/AKT pathway, an significant regulatory pathway governing cell migration, proliferation, differentiation, and apoptosis [51], and a crucial target for ischemic stroke treatment [52], might also contribute to regulation of NCX1 induced by electroacupuncture pretreatment. Our results validated such hypothesis by demonstrating that electroacupuncture pretreatment could promote the phosphorylation of Akt and that injection of the PI3K inhibitor LY294002 prior to cerebral ischaemia could block the upregulation of NCX1 expression and thereby prevent the neuroprotective effect of electroacupuncture pretreatment.

As shown in Fig. 6, this study demonstrates that neuroprotection by electroacupuncture pretreatment might enhance the Ca^{2+} efflux activity of NCX by upregulating NCX1 via the PI3K/Akt upregulation. From the aspect of mechanism exploration, our finding firstly illustrates that electroacupuncture pretreatment induces cerebral ischaemic tolerance by maintain calcium homeostasis. From

the aspect of clinical application, our work might promote clinical translation of electroacupuncture for the patients at high risk of stroke, or those arranged for brain surgery where similar ischaemic or hypoperfusion condition would occur.

Ethics statement

All experimental protocols were approved by the Animal Care and Use Committee of Tianjin University of Traditional Chinese Medicine (TCM-LAEC2019019).

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Data availability statement

All data generated during this study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Wenhua Ning: Writing – original draft, Investigation, Funding acquisition, Data curation. **Li Li:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Ruiqi Wang:** Visualization, Investigation, Funding acquisition, Data curation. **Baoyu Zhang:** Methodology, Investigation. **Sha Yang:** Resources, Project administration. **Lili Zhang:** Software, Methodology. **Xiaonong Fan:** Resources, Project administration, Methodology. **Yan Shen:** Methodology, Formal analysis. **Yanan Zhang:** Formal analysis. **Mengxiong Zhao:** Software, Methodology. **Yang Wang:** Methodology. **Peizhe Liang:** Methodology. **Shu Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Wordvice AI in order to improve language. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33265.

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