Electroacupuncture promotes neurological functional recovery via the retinoic acid signaling pathway in rats following cerebral ischemia-reperfusion injury

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Abstract. Neurogenesis is regulated by a number of signaling pathways, including the retinoic acid (RA) pathway, a key regulator of neurogenesis in the subventricular zone (SVZ) and hippocampus. Acupuncture has been used to treat neurological conditions and is known to potentially enhance cell proliferation in the neurogenic area (hippocampal dentate gyrus and the SVZ of the lateral ventricle walls) in pathological conditions, which is associated with improved brain function. However, whether or not the neuroprotective effects of electroacupuncture (EA) are mediated by the regulation of the RA signaling pathway remains to be determined. Using a transient middle cerebral artery occlusion model, in the present study we evaluated the effect of EA on the neurological functional recovery, infarction volume and investigated the underlying molecular mechanisms. Two hundred and sixteen SD rats were randomly divided into 3 groups: sham, model group (ischemic rats without EA stimulation) and EA group (ischemic rats with EA stimulation on ST36 and LI11). Behavioral deficits were detected with high-resolution digital analysis of 24-h home-cage video recordings. Infarct volume was determined by triphenyltetrazolium hydrochloride staining and the expression of RA mRNA and protein was measured using RT-PCR and western blotting, respectively. We found that EA decreased the infarct volume, promoted neurological functional recovery and increased the RA mRNA and protein expression, compared with the model group. Findings of this study suggest that promoting neurological functional recovery by modulating RA expression in the post-ischemic brain is one of the mechanisms by which EA can be effective in the treatment of ischemic stroke.

Introduction

Cerebral ischemia is considered a major cause of morbidity and mortality worldwide. Excitotoxicity is a major pathophysiological mechanism associated with stroke-induced inflammation and oxidative stress-associated disruption of the blood-brain barrier, resulting in enhancing neuronal cell death during ischemic stroke (1). Recombinant tissue plasminogen activator (t-PA) is the preferred treatment for acute ischemic stroke, however, due to a limited time window few patients are able to receive this therapy. Furthermore, t-PA treatment is likely to increase the risk of hemorrhage (2). Therefore, development of alternative therapies for ischemic stroke is important.

Mounting evidence indicates that potential neural stem/progenitor cells are present in various brain regions outside of the subventricular zone (SVZ) and subgranular zone of the hippocampal dentate gyrus (3,4). Stroke-induced neurogenesis has also been demonstrated in the adult human brain, even in patients of advanced age (5-7), indicating potential involvement of neural stem cells (8) and neurogenesis-regulating factors (9). Various factors regulate neurogenesis during development. One developmental molecule of particular interest in this regard is retinoic acid (RA), which is involved in the normal development of the central nervous system (10) and is also critical in the adult brain (11,12). RA expression and signaling continues in the postnatal and adult brain, stimulating neonatal SVZ and adult hippocampal neurogenesis (13-16). The hypothesis that RA is crucial in the transcriptional model after nerve injury is supported by indirect evidence regarding the regulation of trauma-related genes, by observations of retinoid receptors and binding proteins after nerve injury and by dorsal root ganglia, retinal and spinal cord explant cultures (17). Retinoid-binding proteins are expressed in the SVZ-olfactory bulb pathway and RA receptors persist in the olfactory bulb (18). Transplantation of neural cells obtained from RA-treated

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cynomolgus monkey embryonic stem cells successfully enhanced the motor function of hemiplegic mice with experimental brain injury (19).

Acupuncture has been used to treat neurologic conditions, and acupuncture reportedly enhances cell proliferation in the neurogenic area (hippocampal dentate gyrus and the SVZ of the lateral ventricle walls) in pathological conditions (20-22), which is associated with enhanced brain function (23,24). The LI11 (Quchi) and ST36 (Zusanli) acupoints have been selected for brain injury rehabilitation. Electroacupuncture (EA) at these acupoints has the potential to stimulate cell proliferation and reduce brain injury (20,21,25-27). However, the mechanisms underlying the effects of acupuncture are poorly understood. Thus, whether or not the neuroprotective effects of EA are mediated by regulation of the RA signaling pathway should be investigated.

To extend the clinical observations of the potential neuroprotective effect of EA and help to establish a scientific foundation for further research, the effect of EA on the infarct volume, neurological functional recovery, and the expression of RA mRNA and protein were examined in this study. EA was found to decrease the infarct volume, promote neurological functional recovery and regulate the expression of RA mRNA and protein. This finding suggests that promoting neurological functional recovery through modulating RA expression in the post-ischemic brain is one of the mechanisms by which EA can be effective in the treatment of ischemic stroke.

Materials and methods

Reagents. The Raldh1, Raldh2 and β-actin primers were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Raldh1 and Raldh2 antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies and the antibody against β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Any other chemicals used, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Animals. Healthy and pathogen-free male Sprague-Dawley rats (n=216), weighing 220-280 g, were purchased from SLAC Laboratory Animal Co., Ltd., Shanghai, China (Laboratory Animal Use Certificate no. SCXK(SH)2007000509570) and raised in a sterile environment. The care and use of the laboratory animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals, enforced by the Ministry of Science and Technology, China (2006) (28).

Animal model of transient middle cerebral artery occlusion. Animals were housed in a 12-h reverse light-dark cycle and were provided with food and water *ad libitum*. Rats were anesthetized with 10% chloral hydrate (3.5 ml/kg body weight). Body temperature was maintained using a 37°C water-recirculating pad. Transient middle cerebral artery occlusion (tMCAO) was produced for 120 min using the external carotid artery insertion method as described previously (29). Briefly, the left common carotid artery was exposed through a midline incision, and the internal and external carotid arteries were separated. A 3.0-mm nylon monofilament with a tip rounded by heat was placed in the external carotid artery and advanced through the internal carotid artery until resistance occurred. The monofilament was left in place for 120 min and then removed under anesthesia. Animals were observed until recovery from anesthesia. The sham group was treated in the same way, although tMCAO was not induced. This protocol produced infarcts involving the striatum and cortex, with a mortality rate of ~30%. The rats were subsequently randomized into 3 experimental groups: EA group (n=72), model group (n=72) and sham group (n=72). The rats were tested at 1, 7, 14 and 28 days subsequent to tMCAO. Eighteen rats were decapitated at each time point in each experimental group, after the neurological function was tested. Six rats were used for triphenyltetrazolium hydrochloride (TTC) staining, 6 rats were used for western blotting (Raldh1 and Raldh2 protein measurement), and the remaining 6 were used for Raldh1 and Raldh2 mRNA measurement.

Treatment. In the EA group, EA was applied to acupoints for hemiplegia. The acupoint prescription included ST36 (Zusanli, 5 mm below the head of fibula under the knee joint and 2 mm lateral to the anterior tibial tubercle) and LI11 (Quchi, in the depression lateral to the anterior aspect of the radius joint of the forelimb) at a depth of 5 mm into the skin with a stainless needle measuring 0.25x20 mm in length with a guide-tube for 20 min (Wujiang Shenli Medical & Health Material Co., Ltd., Wujiang, China). Electric stimulation was generated using an electrical stimulator (Huatuo SDZ-II; Suzhou Medical Appliance Factory Co., Ltd., China) for 20 min and the stimulation parameter exhibited disperse-dense waves of a frequency of 5/20 Hz (28.5 msec/15 msec pulse duration) and a current density of 2-4 mA. Model and sham groups did not undergo any EA, which was administered once a day for a period of 4 weeks.

Behavioral testing. Neurological function deficits were evaluated using the HomeCageScan system at 1, 7, 14 and 28 days after tMCAO. Each rat was placed in a separate cage identical to its home-cage, with fresh bedding, food and water. A video camera (Sony DCR-HC62) was positioned perpendicular to the long axis of the cage so that the field of view included the entire length of the cage. The rats were acclimatized to their new environment for 1 h and activity was recorded for 24 h (12-hour light/dark cycle) under standard fluorescent lighting. The rats were then returned to their home-cages. The full length of each video was analyzed using HomeCageScan (version 3.0; Clever Systems, Inc.) for the quantification of rat motor deficits, specifically quantifying behaviors including grooming, feeding, walking and rearing. The sum of the time spent in each behavior was calculated, yielding an index of total activity.

Measurement of infarct volume. Infarct volume was assessed at 1, 7, 14 and 28 days after tMCAO using TTC staining. Animals were sacrificed by 10% chloral hydrate overdose, brains were rapidly removed, sectioned coronally at 2-mm intervals from the frontal pole and immersed in TTC (2%) at 37°C for 20 min, followed by formaldehyde (4%) for 15 min. The hemispheric infarcted area in each section was calculated by subtracting the area of normal TTC-stained brain in the ipsilateral cortex from the contralateral area. The

Table I. PT-PCR primers.

Gene	Primer sequence	Annealing temperature (˚C)
Raldh1	Forward 5'-CTTCTTTGTCCAGCCCACAGTCT-3'	
	Reverse 5'-GTTCACCCAGTTCTCGTCCATTT-3'	55
Raldh2	Forward 5'-GTCGTCAACATTCTGCCAGGGTAT-3'	
	Reverse 5'-CTGCTCCACAGCGTAGTCCAAGTC-3'	56
β-actin	Forward 5'-ACTGGCATTGTGATGGACTC-3'	
	Reverse 5'-CAGCACTGTGTTGGCATAGA-3'	55

data were analyzed with a computer-based image analysis system (Adobe Photoshop 8.0). The sum of the areas of all the sections was calculated and then multiplied by 2 mm, yielding the volume.

RNA extraction and RT-PCR analysis. A total of 72 rats (n=6/group) were sacrificed by decapitation at 1, 7, 14 and 28 days after tMCAO. The ischemic zone of rat brain was rapidly removed and stored at -80˚C for subsequent analysis. Total RNA from brain tissue was isolated with TRIzol reagent (Invitrogen). Oligo (dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Raldh1 or Raldh2 by PCR with Taq DNA polymerase (Fermentas). β-actin was used as an internal model. The primers used in these reactions are listed in Table I. The amplified products were analyzed by 1.5% agarose gel electrophoresis. Optical density ratios for Raldh1, Raldh2 and β-actin were used for the semi-quantitative analyses.

Western blot analysis. The ischemic zone of rat brain was sectioned, and the corresponding samples were homogenized with a homogenizer in lysis buffer (NP-40 lysis buffer and 100 mM PMSF). The homogenates were centrifuged at 14,000 rpm for 30 min at 4˚C. The supernatant was collected and the total protein content was determined using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Equal amounts of protein (20 μg) were boiled in loading buffer and separated by 12% SDS-PAGE gel under reducing condition using 80 V for 1 h. The proteins were then electrophoretically transferred onto nitrocellulose membranes using the iBlot Western Detection Stack/iBlot Dry Blotting system (Invitrogen). Membranes were blocked for 30 min with agitation at RT in SuperBlock T20 (TBS) blocking buffer (Thermo Scientific, Rockford, IL, USA). Membranes were washed in TBS with 0.25% Tween-20 (TBST) and exposed to primary antibodies against Raldh1 or Raldh2 (1:1,000) overnight at 4˚C on a rocking platform or rotator. β-actin (1:1,000) was also measured as an internal control for protein loading. After membranes were washed in TBST, secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-goat) were added at 1:5,000 dilution for 1 h at room temperature and the membranes were washed again in TBST. The antibody-bound protein bands were then detected with ECL, and images
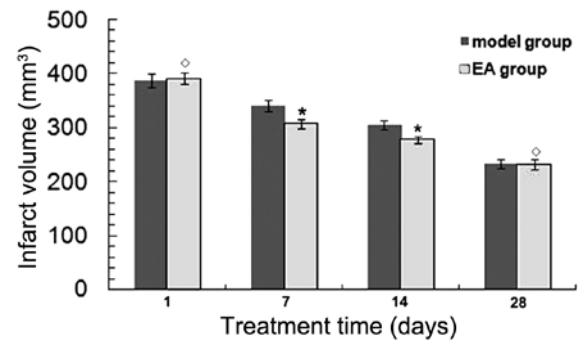

Figure 1. Effect of EA treatment on the infarct volume in rats following tMCAO. After treatment with or without EA for the indicated time periods, the infarct volume changes were determined by TTC staining. Data shown are averages with SD (error bars) from 6 individual rats in each group. *P<0.05, **P<0.01 vs. the model group at 7 and 14 days. $^\circ$P>0.05 vs. the model groups at 1 and 28 days.

were captured using a Bio-Rad ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The ratio of gray scale values of the target protein to the internal control was used to measure the relative amount of Raldh1 and Raldh2.

Statistical analysis. Data are presented as the means ± SD for the indicated number of independently performed experiments. Comparisons between multiple groups were conducted with one-way ANOVA, whereas within each group data were analyzed with analysis of intraclass variance analysis. Differences between the 2 groups were assessed using the Student's t-test. P-values of <0.05 and <0.01 were considered as significant and highly significant differences, respectively.

Results

RA reduces infarct volume in rats following tMCAO. TTC staining was performed to determine the infarct volumes in brain sections at 1, 7, 14 and 28 days after tMCAO. The infarct volume was found to be decreased in a time-dependent manner in both the EA and model groups (P<0.05) (Fig. 1). At 7 and 14 days, the infarct volume was significantly decreased in the EA group (P<0.05) vs. the model group. No significant differences were detected in the infarct volume between the EA and model groups at 1 and 28 days after tMCAO (P>0.05), indicating that rats were able to recover naturally after tMCAO.
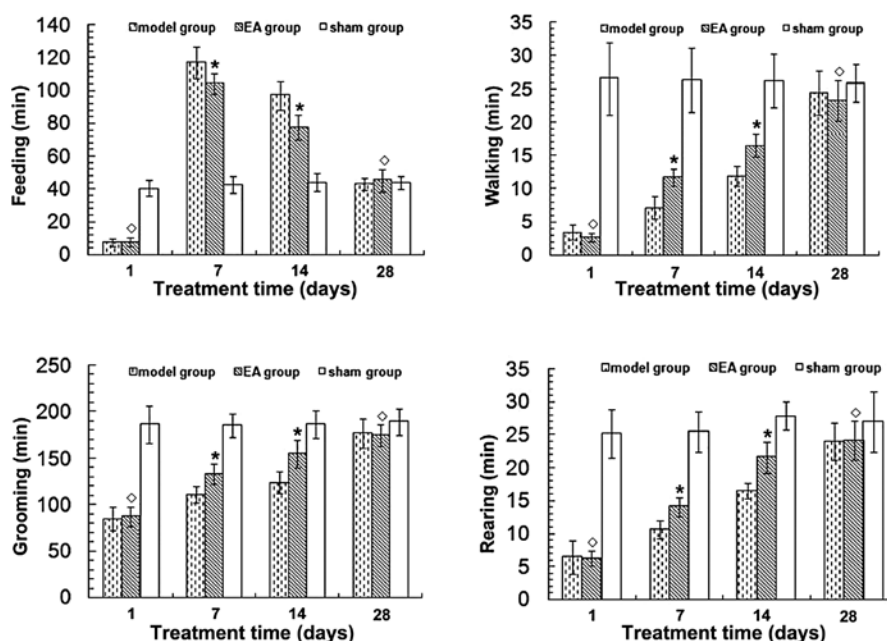
Figure 2. Effect of EA treatment on the neurological function in rats following tMCAO. After treatment with or without EA for the indicated time periods, the walking, rearing, feeding and grooming time changes were analyzed using a software program designed for rat home-cage behavioral assessment. Bar graphs represent the mean minutes of activity (sum of minutes spent walking, rearing, feeding and grooming, ±SD; 6 individual rats in each group). *P<0.05, **P<0.01, vs. the model group at 7 and 14 days. °P>0.05 vs. the model groups at 1 and 28 days.
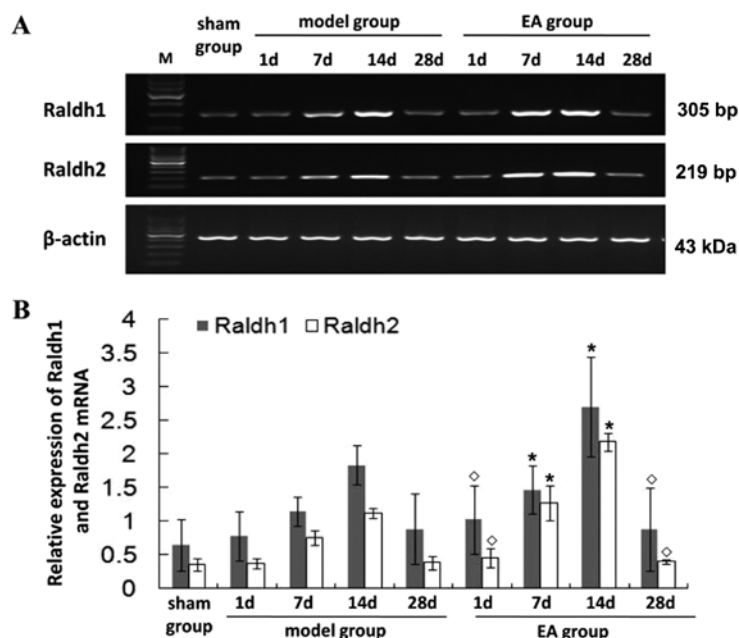

Figure 3. Effect of EA treatment on the mRNA expression of Raldh1 and Raldh2 in rats following tMCAO. (A) After treatment with or without EA for the indicated time periods, the ischemic zone of rat brain was selected from each group, and the mRNA expression levels of Raldh1 and Raldh2 were determined by RT-PCR. β-actin was used as the internal control for RT-PCR. For each sample, the RT-PCR analysis was performed in triplicate. Data shown are representatives. (B) Densitometric analysis. *P<0.05, **P<0.01 vs. the model group at 7 and 14 days. °P>0.05 vs. the model groups at 1 and 28 days.

RA improves neurological function in rats following tMCAO. Cerebral cortex infarction caused neurological function deficits in rats, mainly manifested as right forelimb paralysis. To quantify this deficit, 24-h video recordings were analyzed using a software program designed for rat home-cage behavioral assessment. Neurological function was found to have improved in a time-dependent manner in the EA and model groups (P<0.05) (Fig. 2). Time spent walking, rearing and grooming was increased, while time spent feeding was reduced in the EA group 7 and 14 days after tMCAO (P<0.05) compared with the model group. No significant differences in neurological function were observed between the EA and model groups at 1 and 28 days after tMCAO (P>0.05). No neurological function deficits were evident in the sham group.
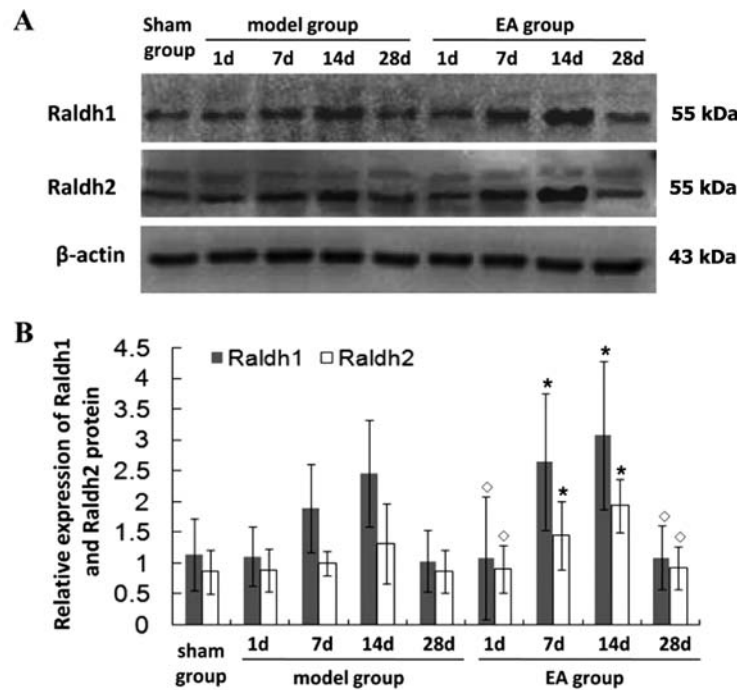
Figure 4. Effect of EA treatment on the protein expression of Raldh1 and Raldh2 in rats following tMCAO. (A) After treatment with or without EA, the ischemic zone of rat brain was selected from each group, and the protein expression levels of Raldh1 and Raldh2 were determined by western blotting. β-actin was used as the internal control for western blotting. For each sample, the western blot analysis was performed in triplicate. Data shown are representatives. (B) Densitometric analysis. *P<0.05, **P<0.01, vs. the model group at 7 and 14 days. °P>0.05 vs. the model groups at 1 and 28 days.

EA treatment regulates the expression of Raldh1 and Raldh2 after tMCAO. To explore the mechanism of the potential neuroprotective effect of EA, RT-PCR and western blot analysis were carried out to examine the mRNA and protein expression in the ischemic brain after tMCAO. In the model group, Raldh1 and Raldh2 mRNA was greatly increased at 7 days, peaked at 14 days and then gradually decreased (Fig. 3). Additionally, in the model group, higher Raldh1 and Raldh2 mRNA levels were found at 7 and 14 days (P<0.05) compared with the sham group. In the EA group, the Raldh1 and Raldh2 mRNA levels were higher than those in the model group at 7 and 14 days (P<0.05). No significant differences were detected in the Raldh1 and Raldh2 mRNA expression levels between the EA and model groups at 1 and 28 days after tMCAO (P>0.05), and the pattern of protein expression of Raldh1 and Raldh2 was similar to their respective mRNA levels (Fig. 4). These findings suggest that EA promotes neurological functional recovery in rats following tMCAO through the regulation of expression of the RA family.

Discussion

Neural stem cells from the adult forebrain SVZ give rise to olfactory bulb interneurons, while those in the hippocampal dentate gyrus generate new neurons in the granule cell layer (18). Brain insults such as seizure, stroke and trauma activate neural stem cells to proliferate more rapidly, migrate into injured regions and form new neurons and glia (30-36).

Retinoids, in particularly the active metabolite of vitamin A, RA, is known to stimulate neonatal SVZ and adult hippocampal neurogenesis (14-16). Retinol and its derivatives exert their biological actions via specific nuclear receptors (RARs and RXRs) that regulate gene transcription (37). RA receptors also interact with other nuclear receptors that possess neuroprotective properties, such as 1,25-dihydroxy-vitamin D (9) that is neuroprotective against stroke (38) and RXR receptors that form heterodimers with the vitamin D3 receptor (RXR-VDR). RXR is also able to form a dimer with thyroid hormone receptors, and thyroid hormone derivatives are protective against infarction (39). Thus, the neuroprotective effects of retinol and RA may be mediated, at least in part, by their formation of heterodimers with other nuclear receptors. In addition to its effect on nuclear receptors, retinol exerts acute effects on other potentially protective biological sites including calcium channels and gap junction channels, as well as having antioxidant properties. Calcium channel antagonists and antioxidants protect the brain from ischemia in animal models of stroke (40). RA also induced the expression of midkine, which is protective against brain ischemia (41).

RA is synthesized from retinol in a two-step pathway involving oxidation first to retinaldehyde, then to RA. The former step is predominantly catalyzed by retinol dehydrogenases, the latter step by retinaldehyde dehydrogenases (Raldhs), which are encoded by Raldh1, Raldh2 and Raldh3 expressed in non-overlapping patterns. Raldh3 is expressed only in the eye and facial region/ventral retina and the ganglionic eminence of the telencephalon. Later in development, this enzyme can be detected by zymography in a variety of sites, including the liver and adult skin. Raldh1 is present in only the dorsal retina, ventral mesencephalon and projections from here to the corpus striatum and the otic vesicle regions (data not shown). Although Raldh2 is absent from most of the brain proper, it is strongly expressed in the leptomeninges that

surround it (42). In the present study, we found that the Raldh1 and Raldh2 mRNA levels of the EA and model groups were greatly increased at 7 days, peaked at 14 days and gradually decreased at 28 days after tMCAO. A marked increase was observed in the Raldh1 and Raldh2 mRNA levels at 7 and 14 days in the EA group (P<0.05) compared with the model group. Expression of the Raldh1 and Raldh2 protein was similar to that of Raldh1 and Raldh2 mRNA.

Behavior is known to be the ultimate and most complex output of the brain. To quantify neurological function deficit, 24-h video recordings were conducted and digitally analyzed. The assessment of locomotor deficits using this method has been established in models of Huntington's and prion disease (43), with a similar approach having been described recently in a rat ICH model (44). Compared with traditional methods of behavioral assessment in stroke models, this digital approach has a number of benefits (45-47). First, it requires minimal investigator participation, reducing labor costs and the potential for bias. Second, it facilitates the analysis of night-time behavior. The validity of daytime testing of nocturnal animals in stroke models has never been established and is questionable given the behavioral inhibition and cognitive disturbances observed in rats during light phase testing. Third, this digital approach measures behaviors that are directly relevant to the animal's functional status. Most importantly, however, it eliminates the variability inherent in measuring brief episodes of behavior in animals stressed by environmental variation and human interference.

In this study, we found that time spent walking, rearing and grooming was markedly increased, whereas time spent feeding was reduced with locomotor activity improvement in the EA group at 7 and 14 days after tMCAO, compared with that in the model group (P<0.05). The changes we found in the neurological function were concomitant with improvements in RA mRNA and protein expression. These results therefore suggest that the administration of EA is likely to promote neurological functional recovery and regulate the expression of RA mRNA and protein. To the best of our knowledge, this is the first study to report that EA regulates RA expression.

It was also demonstrated that there was no significant difference in the infarct volume, neurological function, expression of RA mRNA and protein between the EA and model groups at 28 days after tMCAO (P>0.05), indicating that rats were able to recover naturally after tMCAO. Spontaneous recovery may be due to neurite regeneration and synapse remodeling, as well as a reduction in brain edema. A series of plasticity responses after tMCAO, including the release of neurotrophic factors, synthesis of neuron-specific proteins and rise of synaptic excitation, is involved in the process of functional recovery (48). Additionally, our study examined the correlation between neurogenesis and the use of young rats in behavioral recovery. The findings of this study are in contrast with the reality that the elderly population is most often affected by strokes and these people, unlike young healthy rodents, also have a multiplicity of other disease co-morbidities, such as hypertension and diabetes that along with increased age, are likely to limit or alter the neurogenic response post-stroke. Indeed, aged animals exhibit stroke-induced neurogenesis, however, their response is 'muted' compared with young animals (49,50). Although the spontaneous recovery of neurological function deficit may occur

after tMCAO, the administration of EA significantly promoted neural function recovery and decreased infarct volume. Raldh1 and Raldh2 mRNA and protein expression was increased by EA treatment following tMCAO compared to the model group.

In conclusion, administration of EA decreased the infarct volume, promoted neurological functional recovery and demonstrated that this neuroprotective effect is attributed, at least in part, to the expression of RA mRNA and protein. These findings likely provide an experimental basis for the treatment of ischemic stroke by electroacupuncture.

Acknowledgements

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