● RESEARCH ARTICLE

Electroacupuncture-induced neuroprotection against focal cerebral ischemia in the rat is mediated by adenosine A1 receptors

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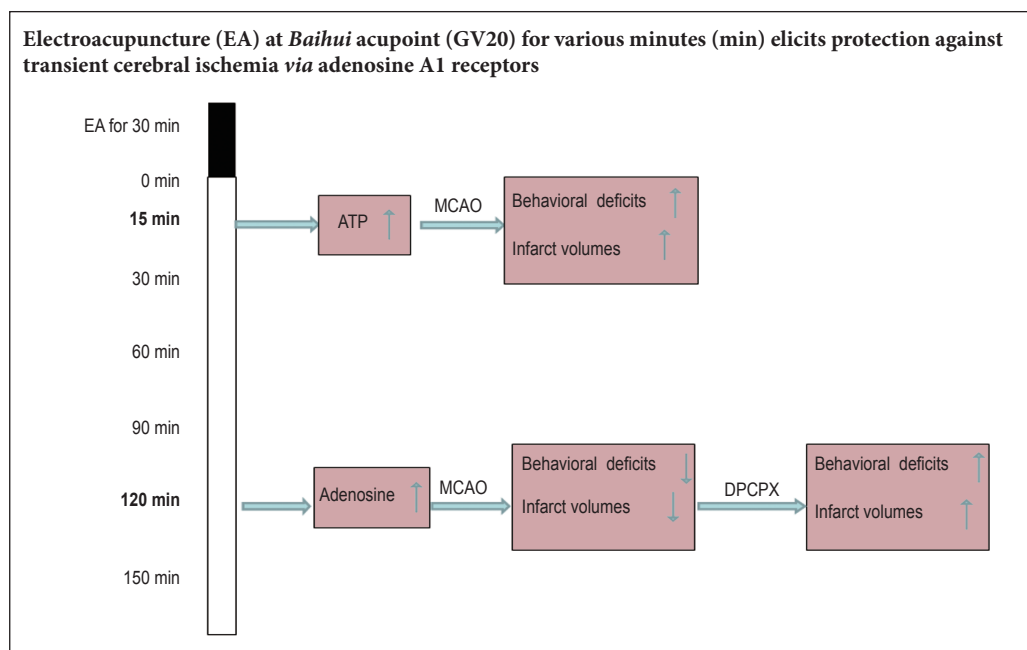2 Department of Neurology, Boston University School of Medicine, Boston, MA, USA

Graphical Abstract

Electroacupuncture (EA) at *Baihui* acupoint (GV20) for various minutes (min) elicits protection against transient cerebral ischemia *via* adenosine A1 receptors

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Abstract

The activation of adenosine A1 receptors is important for protecting against ischemic brain injury and pretreatment with electroacupuncture has been shown to mitigate ischemic brain insult. The aim of this study was to test whether the adenosine A1 receptor mediates electroacupuncture pretreatment-induced neuroprotection against ischemic brain injury. We first performed 30 minutes of electroacupuncture pretreatment at the *Baihui* acupoint (GV20), delivered with a current of 1 mA, a frequency of 2/15 Hz, and a depth of 1 mm. High-performance liquid chromatography found that adenosine triphosphate and adenosine levels peaked in the cerebral cortex at 15 minutes and 120 minutes after electroacupuncture pretreatment, respectively. We further examined the effect of 15 or 120 minutes electroacupuncture treatment on ischemic brain injury in a rat middle cerebral artery-occlusion model. We found that at 24 hours reperfusion, 120 minutes after electroacupuncture pretreatment, but not for 15 minutes, significantly reduced behavioral deficits and infarct volumes. Last, we demonstrated that the protective effect gained by 120 minutes after electroacupuncture treatment before ischemic injury was abolished by pretreatment with the A1-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (1 mg/kg, intraperitoneally). Our results suggest that pretreatment with electroacupuncture at the *Baihui* acupoint elicits protection against transient cerebral ischemia *via* action at adenosine A1 receptors.

Key Words: nerve regeneration; adenosine; adenosine triphosphate; adenosine A1 receptor; cerebral ischemia; electroacupuncture; pretreatment; 8-cyclopentyl-1,3-dipropylxanthine; high-performance liquid chromatography; neural regeneration

Introduction

Stroke is the second most common cause of morbidity and mortality worldwide (Donnan et al., 2008), and the majority of stroke cases (80%) are caused by cerebral ischemia (Donnan et al., 2008). Although agents such as tissue plasminogen activator and edaravone have been approved for treating cerebral ischemia (Lansberg et al., 2009), they do not reduce infarct volume or improve long-term neurological outcomes (Donnan et al., 2008). Thus, new and effective therapeutic strategies are urgently needed.

Electroacupuncture (EA), a method in which traditional acupuncture is combined with modern electrotherapy, is widely used in the clinical management of several conditions, including stroke (Lu et al., 2010). Indeed, it has been found that 30 minutes of EA pretreatment at the *Baihui* acupoint (GV20) can induce cerebral ischemic tolerance (Wang et al., 2005, 2009).

Additionally, adenosine triphosphate (ATP), adenosine, and receptors for both are involved in the development of ischemic tolerance (Tozaki-Saitoh et al., 2011). Combined genetic and pharmacological approaches have demonstrated a critical role for the adenosine A1 receptor in mediating acupunctural analgesia (Goldman et al., 2010). Thus, we hypothesized that an extracellular increase in ATP or adenosine that was induced by EA treatment would act on the A1 receptor and afford neuroprotection against ischemic brain injury. To test this hypothesis, we used a rat model of middle cerebral arterial occlusion (MCAO) to evaluate whether increased extracellular levels of ATP or adenosine mediated ischemic tolerance induced by EA pretreatment.

Materials and Methods

Animals

Experimental protocols were approved by the Institutional Animal Experimental Ethics Committee at First Affiliated Hospital of Wenzhou Medical University in China (approval No. 12045) and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment. Two hundred and fifty-two male specific-pathogen-free Sprague-Dawley rats weighing 220 to 250 g and aged 3 months were purchased from the Animal Center at Wenzhou Medical University of China (approval No. SYXK (Zhe) 2010-0150). Rats were housed under controlled conditions with a 12-hour light-dark cycle and allowed free access to food and water. Fourteen of the rats were excluded from the final analysis because of anesthetic accidents ($n = 8$) and low body temperature ($n = 6$).

Experimental protocols

Experiment I: The first set of experiments was carried out to determine whether ATP or adenosine levels were upregulated in the rat cerebral cortex after EA pretreatment for 30 minutes. Rats were randomly assigned to a control group ($n = 8$), EA group ($n = 60$), and sham EA group ($n = 60$) (Wang et al., 2005). EA and sham EA groups were further divided into six subgroups based on the time point at which rats were sacrificed post-EA treatment: (0, 15, 30, 60, 120, or 150 minutes) ($n = 10$ per group). The presence of ATP and adenosine were then assessed at each time point using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (**Figure 1**).

Experiment II: Based on the results from Experiment I, Experiment II was carried out to assess how EA-induced changes in ATP and adenosine levels affect neuronal protection against cerebral ischemia. We selected two time points at which to induce transient focal cerebral ischemia. The first was at 15 minutes post-EA (post-EA15), when ATP levels had been shown to be significantly greatest (Exp. I results), and the second was at 120 minutes post-EA (post-EA120), when adenosine levels had been shown to be significantly the highest (Exp. I results). Thus, rats were randomly assigned to one of six groups: control, MCAO, MCAO post-EA15, MCAO post-sham EA15, MCAO post-EA120, MCAO post-sham EA120 ($n = 10$ per group). Neurological severity scores and infarct volumes (Wang et al., 2005) were assessed 24 hours after reperfusion to evaluate the neuroprotective effect of EA pretreatment and its relationship to ATP and adenosine levels (**Figure 1**).

Experiment III: Experiment III was carried out to assess the role of EA-induced adenosine in protecting the brain from cerebral ischemia. Briefly, the adenosine A1-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was intraperitoneally administered to rats 30 minutes before EA. Rats were then divided into MCAO, MCAO post-EA120, MCAO post-EA120 DPCPX, MCAO post-EA120 dimethyl sulfoxide (DMSO), and MCAO DPCPX groups ($n = 10$, per group). Neurological severity scores and infarct volumes were assessed 24 hours after reperfusion to evaluate the effect of DPCPX on the neuroprotective activity of EA-induced adenosine (**Figure 1**).

Preparation of the transient focal cerebral ischemia/reperfusion model

Two hours after EA pretreatment, rats were intraperitoneally anesthetized with 10% chloral hydrate at 350 mg/kg. Focal cerebral ischemia/reperfusion was induced *via* MCAO using an intraluminal filament (diameter 0.32 ± 0.02 mm, Beijing Shangdong Biotechnology Co., Ltd., China), as has been described elsewhere (Longa et al., 1989). In brief, an intraluminal filament was inserted from the external carotid artery into the left internal carotid artery to block the origin of the left middle cerebral artery. Regional cerebral blood flow was monitored using transcranial laser Doppler flowmetry (PeriFlux5000; Perimed AB, Sweden). MCAO was considered sufficient if the regional cerebral blood flow exhibited a sharp decrease down to 20% of baseline (preischemic) levels. Otherwise, the animal was excluded from analysis. The filament was withdrawn after 2 hours of MCAO, regional cerebral blood flow resumed, and the incision sites were sutured. During ischemia/reperfusion, the temperature of all rats was maintained at $37 \pm 0.2°C$ using a body-temperature blanket.

EA pretreatment

Rats were anesthetized with chloralic hydras (10%, 3 mL/kg, intraperitoneally) before EA pretreatment. According to

experimentally-based acupoint selection principles (Gao, 2008), the *Baihui* acupoint is located at the intersection of the line linking the two rat ear tips and the sagittal midline. To construct a circuit, we chose the *Baihui* acupoint as one electrode site and the left forelimb (the non-acupoint point) as the other. We used a HAN's EA Instrument (HANS, Beijing, China) to stimulate the *Baihui* acupoint for 30 minutes at a frequency of 2/15 Hz and an intensity of 1 mA. We inserted the acupuncture needle about 1 mm into the acupoint. The body temperature of all rats was maintained at 37.0 ± 0.2°C during EA pretreatment using a body temperature blanket until rats recovered from anesthesia. The physiological condition of the experimental rats was monitored using a blood gas analyzer (i-STAT, Abbot, IL, USA) at the beginning, middle, and end of EA pretreatment. As a control, one group of rats received sham EA pretreatment by inserting the acupuncture needle into the *Baihui* acupoint but not using any electrical stimulation. This sham EA procedure is widely used as a control for EA because it does not induce any effects of acupuncture (Wang et al., 2009; Han, 2011).

DPCPX administration

DPCPX (Sigma, New York, USA) was dissolved in DMSO (Sigma) at 1 mg/mL. DMSO (1 mL/kg) was intraperitoneally injected into rats 30 minutes before acupuncture in the MCAO post-EA120 DMSO group.

Detection of ATP and adenosine levels by HPLC

Rats were sacrificed at 0, 15, 30, 60, 120, or 150 minutes after EA treatment. Brain tissue was immediately extracted and dissected over an ice platform and stored at −80°C until HPLC analysis. To measure ATP and adenosine levels, frozen samples were homogenized with perchloric acid ($HClO_4$, 0.4 M, 10 mL/g) and centrifuged at 4,000 × *g* for 15 minutes. The supernatant was collected and neutralized with 4 M KOH and then re-centrifuged for 15 minutes. The resulting supernatant was collected and stored at −80°C until HPLC analysis.

ATP and adenosine levels in the cerebral cortex were determined by HPLC (Agilent 1100 system, Agilent Company, Santa Clara, CA, USA) using the reverse-phase column (ODS2-C18, 4.6 mm × 200 mm, 5 μm, Hypersil) to achieve chromatographic separation. For adenosine measurement, we employed a mobile phase that was 88 mM NaH_2PO_4 + 12 mM Na_2HPO_4:methanol = 85:15 (v/v, HPLC-grade water, pH 6.0). The flow rate was maintained at 1.0 mL/minutes and the detection wavelength was 260 nm. For ATP measurement, we followed the same procedure, but used a slightly different mobile phase (88 mM NaH_2PO_4 + 12 mM Na_2HPO_4: methanol = 95:5; Goldman et al., 2010). To establish calibration curves, ATP and adenosine five-point standards were obtained from Sigma and dissolved in ultrapure water (10, 5, 2.5, 1.25, and 0.625 ng/L) and the peak area of the five-point standards was recorded. Based on the five-point standards and peak area, we established calibration curves for adenosine (area = 71.6888 × amount + 20.571316,

r = 0.99776) and ATP (area = 34.05451 × amount + 6.13368, r = 0.99989).

Behavioral and infarct volume assessment

Samples and data were collected 24 hours after MCAO and reperfusion. Neurological severity scores were assessed *via* a single blind method using an established five-point scoring system (Longa et al., 1989): 0 = no deficit; 1 = forelimb weakness and torso turning to the ipsilateral side when held by the tail; 2 = circling to the affected side; 3 = unable to bear weight on the affected side; and 4 = no spontaneous locomotor activity or barrel rolling. At the end of behavioral testing, rats were once again anesthetized with chloralic hydras (Sigma) and decapitated. Brains were removed immediately over an ice platform and sliced into six 2-mm coronal sections using a brain matrix (68709; RWD Life Science Co., Ltd., Guangzhou, China). Brain sections were soaked in 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C. A digital camera (COOLPIX, L120; Nikon, Wuxi, China) connected to a computer was used to take photos of the stained brain sections. Unstained areas were considered infarct areas and were measured using image analysis software (Image-Pro Plus 6.0, Media Cybernetics, Singapore) by an investigator who was blind to the treatment group. The total infarct volume was presented as a proportion of the contralateral hemisphere volume.

Statistical analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. With the exception of neurological scores, all values are presented as the mean ± SEM and were analyzed using a one-way analysis of variance followed by *post-hoc* least significant difference tests if the data met the assumption for the homogeneity of variance. Neurological scores are expressed as median (range) and were analyzed using the non-parametric Kruskal-Wallis *H* test followed by the Nemenyi test. $P < 0.05$ was considered statistically significant.

Results

ATP and adenosine levels increased only at 15 and 120 minutes, respectively, after EA treatment

EA treatment for 30 minutes resulted in greater cortical ATP levels at 15 minutes post-treatment compared with sham EA ($P = 0.009$) and control ($P = 0.001$) groups (**Figure 2**). In contrast, at 60 minutes post-treatment, rats in the EA group exhibited significantly lower ATP levels compared with the sham EA group ($P = 0.039$), but not with the control group ($P = 0.132$). Moreover, no differences among the groups were observed at 30, 90, 120, or 150 minutes post-EA treatment (all $P > 0.05$; **Figure 2**).

Adenosine levels in the EA group were greater than those of the sham and control groups. This result was present as a trend at 60 minutes and reached statistical significance at 120 minutes post-treatment (EA group *vs.* sham EA group, $P < 0.001$; EA group *vs.* control group, $P < 0.001$; **Figure 3**). No significant differences between groups were observed in adenosine levels at 0, 15, 30, 60, or 150 minutes after EA

treatment (all *P* > 0.05; **Figure 3**).

During EA treatment, we found no significant differences in physiological parameters, including arterial blood gases (PCO_2, PO_2, pH), body temperature, plasma glucose, hemoglobin, and lactic acid (data not shown).

EA pretreatment had no effect on neurobehavioral score or infarct volume of focal cerebral ischemia rats

Because ATP levels had risen significantly at 15 minutes and adenosine levels at 120 minutes post-EA treatment, we generated two MCAO models consistent with these time points. The goal was to confirm the presence of EA-mediated neuroprotection against ischemic brain injury and to investigate a potential association of this effect with greater ATP/adenosine levels in the cerebral cortex. Thus, at 15 and 120 minutes post-EA treatment, we examined the effect of EA on neurological scores and infarct volume. Again, during surgery, we monitored all physiological parameters and excluded outliers from further analysis.

As shown in **Table 1** and **Figure 4**, neurological scores and infarct volumes did not statistically differ among the MCAO, MCAO post-EA15, and MCAO post-sham EA15 groups (*P* > 0.05).

EA pretreatment was associated with reductions in both neurobehavioral deficits and infarct volumes

In contrast to MCAO 15 minutes after EA pretreatment, MCAO 120 minutes after EA, was associated with increased adenosine levels and resulted in significantly better neurobehavioral scores and reduced infarct volume after 24 hours. The neurobehavioral deficit scores for the EA group were significantly lower than those for the MCAO and MCAO post-sham EA120 groups (*P* < 0.001; **Table 2**). The infarct volumes in the EA group were also significantly smaller than those in the MCAO and MCAO post-sham EA120 groups (*P* < 0.001; **Figure 5**). Thus, EA treatment followed by MCA after 120 minutes partially and significantly improved neurobehavioral scores and infarct volume, and this was concurrent with increased adenosine levels in the cerebral cortex.

EA-mediated neuroprotection against ischemic brain injury was abolished by an A1 receptor antagonist

To further investigate the role of the adenosine-A1 receptor in mediating the benefits of EA, we examined how pretreatment with an A1-receptor antagonist (DPCPX) affected EA-mediated neuroprotection 24 hours after MCAO and reperfusion. We found that neurological deficit scores for the MCAO post-EA120 DPCPX group were significantly greater than those for the MCAO post-EA120 (*P* = 0.002) or MCAO post-EA120 DMSO (*P* = 0.003) groups, and were indistinguishable from those for the MCAO and MCAO DPCPX groups (**Table 3**). Similarly, infarct volumes were significantly greater in the MCAO post-EA120 DPCPX group compared with those in the MCAO post-EA120 group (*P* < 0.001) or the MCAO post-EA120 DMSO group (*P* < 0.001), and were comparable to those observed in the MCAO and MCAO + DPCPX groups (**Figure 6**).

Discussion

In the current study, we evaluated whether EA pretreatment would increase ATP and adenosine levels in the rat cerebral cortex, and if so, whether increased adenosine or ATP participate in the neuroprotective effect associated with EA pretreatment. EA pretreatment resulted in significantly greater ATP levels (15 minutes after treatment) and adenosine levels (120 minutes after treatment) in the cerebral cortex compared with groups that did not receive treatment. Furthermore, EA pretreatment induced a significant reduction in infarct volume and improved neurobehavioral deficit scores at 120 minutes (but not 15 minutes), which was concurrent with the peak level of adenosine. Lastly, treatment with the adenosine A1 receptor antagonist, DPCPX, reversed the neuroprotective effect induced by EA pretreatment. These results suggest that EA pretreatment for 30 minutes triggers an increase in adenosine levels after 120 minutes, which alleviates cerebral ischemia/reperfusion injury in rats *via* adenosine-A1 receptors.

Clinical acupuncture has a long and wide ranging history; its use spanning from ancient China to modern western medicine. Western medical organizations have asserted that acupuncture is a useful and acceptable alternative or complementary treatment for various illnesses, including back pain, stroke, and asthma (No authors listed, 1998). In an effort to further explore these claims, Wang et al. found that EA could induce cerebral ischemic tolerance in a rat model of MCAO 120 minutes after EA pretreatment, and that this may involve the adenosine-A1 receptor (Wang et al., 2005). Here, we revealed that the protective effect observed 120 minutes after EA treatment was concurrent with the peak level of adenosine. Further, we found that that peak cortical ATP levels in the 15 minutes post-EA was not associated with a protective effect, and that pharmacologically blocking the A1 receptor with DPCPX abolished the beneficial effects of EA.

ATP is an important and ubiquitous purinergic nucleotide that plays a vital role in many physiological and pathophysiological conditions (Burnstock, 2011). ATP is released from damaged or normal neurons in response to hypoxia, mechanical deformation, and ischemia (Burnstock, 2009), and is catabolized to nucleotides (adenosine diphosphate, adenosine monophosphate, hypoxanthine nucleotides, adenosine, and inosine) by ectonucleotidases. In enzymatic pathways, ecto-5′-nucleotidase (CD73) and adenosine deaminase are rate-limiting enzymes; the former generates adenosine by hydrolyzing the 5′-phosphate from AMP, and the latter breaks down adenosine to inosine (Zimmermann, 2006). Adenosine, a metabolite of ATP, plays an important role in the brain under both physiological and pathophysiological conditions (Gomes et al., 2011). Elevated extracellular adenosine affords protection against neuronal damage in several *in vivo* and *in vitro* models of ischemia (Greene and Haas, 1991; von Lubitz, 2001). Deep brain stimulation, which has been widely used as a neurosurgical approach for treating tremor and other movement disorders, is associated with a significant increase in the release of ATP and the consequent accumulation of adenosine in thalamic tissue (von Lubitz, 2001). Acupuncture at the *Zusanli* (ST36) acupoint in mice
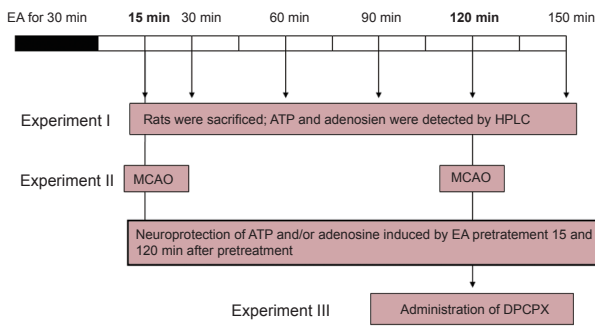
Figure 1 Experimental protocol for neuroprotection in ischemic rats induced by electroacupuncture *via* adenosine-A1 receptors.
EA: Electroacupuncture; min: minutes; ATP: adenosine triphosphate; HPLC: high-performance liquid chromatography; MCAO: middle cerebral artery occlusion; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine.

Table 1 Neurological scores among the different groups

Group	Neurological scores					Median (range)
	0	1	2	3	4	
Sham	8					0
MCAO		1	2	5	2	3(1–4)
MCAO post-EA15		2	2	4	2	3(1–4)
MCAO post-sham EA15		1	2	4	3	3(1–2)

Neurological scores are expressed as median (range) ($n = 10$ rats in each group) and were analyzed using the non-parametric Kruskal-Wallis H test followed by the Nemenyi test. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture.

Table 2 Neurological scores among the different groups

Group	Neurological scores					Median (range)
	0	1	2	3	4	
Sham	8					0
MCAO		1	2	5	2	3(1–4)
MCAO post-EA120[##]	2	5	2	1		1(0–3)
MCAO post-sham EA120[†††]			6	2	2	2(2–4)

Neurological scores are expressed as median (range) ($n = 10$ rats in each group) and were analyzed using the non-parametric Kruskal-Wallis H test followed by the Nemenyi test. ##$P < 0.001$, *vs.* MCAO; †††$P < 0.001$, *vs.* MCAO post-EA120 group. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture.

Table 3 Neurological scores among the different groups

Group	n	Neurological scores					Median (range)
		0	1	2	3	4	
MCAO	10		2	3	3	2	3(1–4)
MCAO post-EA120	10	2	5	2	1		1(0–3)
MCAO post-EA120 DPCPX[††]	10		1	4	3	2	2(2–4)
MCAO post-EA120 DMSO[§]	10	1	6	2	1		2(2–4)
MCAO DPCPX	10			4	4	2	2(2–4)

Neurological scores are expressed as median (range) ($n = 10$ rats in each group) and were analyzed using the non-parametric Kruskal-Wallis H test followed by the Nemenyi test. ††$P < 0.05$, *vs.* MCAO post-EA120 group; §§§$P < 0.05$, *vs.* MCAO post-EA120 DPCPX. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine; DMSO: dimethyl sulfoxide.
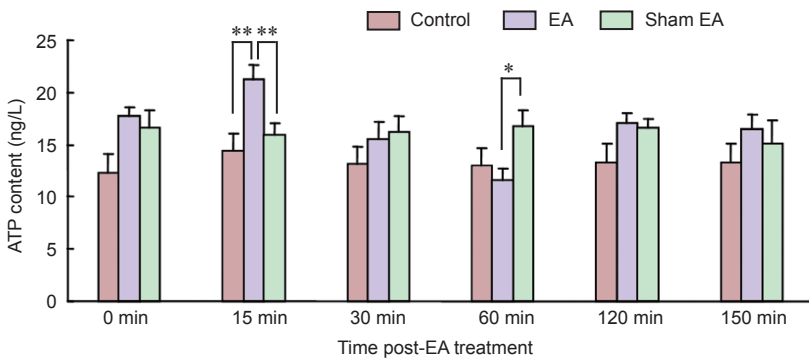

Figure 2 Extracellular ATP levels in the rat cerebral cortex increased by 15 min post-EA pretreatment, and returned to normal after 30 min.
Data are presented as the mean ± SEM ($n = 10$ rats in each group) and were analyzed using a one-way analysis of variance followed by a post-hoc least significant difference test. *$P < 0.05$, **$P < 0.01$. EA: Electroacupuncture; ATP: adenosine triphosphate; min: minutes.

has been found to trigger ATP release and its conversion into adenosine, which peaks locally at the acupuncture site (Goldman et al., 2010). Thus, acupuncture could trigger ATP and adenosine release in local tissues – at the *Zusanli* acupoint or in thalamic tissue. These results are consistent with the view that ATP is produced by cells in response to mechanical deformation and electrical currents (Burnstock, 2009). While these studies stress the local effect of EA treatment on adenosine signaling, local acupuncture at specific acupoints usually has a systemic effect, according to traditional Chinese theory (Yang et al., 2011). We found that EA treatment at the peripheral *Baihui* acupoint in rats markedly increased cortical ATP levels at 15 minutes post-treatment and resulted in a gradual increase in adenosine levels that peaked at 120 minutes post-treatment. Increased ATP and adenosine levels after EA treatment was detected by HPLC

of the tissue and therefore intracellular versus extracellular levels could not be distinguished. Additional studies, using microdialysis to assess extracellular levels of ATP and adenosine, will provide new insights into EA-induced adenosine signaling in the brain.

A number of studies have employed modern imaging technology, such as X-rays (Yan et al., 2009), computed tomography (Chen, 1992), and functional magnetic resonance imaging (Li et al., 2008), to investigate the mechanisms of action for acupuncture and have shown that stimulating peripheral acupoints can induce central nervous system activity. Our finding that EA treatment at the local (peripheral) *Baihui* acupoint can trigger an increase in adenosine and ATP levels in the cerebral cortex not only demonstrates the systemic effect of acupuncture in traditional Chinese theory, but also indicates that the effects of acupuncture may in part
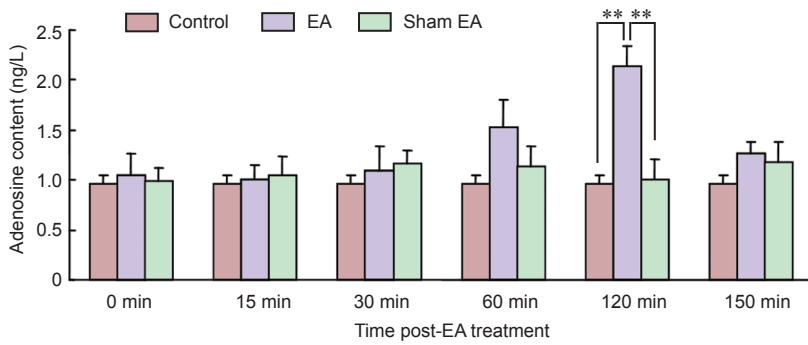
Figure 3 Extracellular adenosine content in the rat cerebral cortex was highest at 120 min post-EA pretreatment.
Data are presented as the mean ± SEM ($n = 10$ rats in each group) and were analyzed using a one-way analysis of variance followed by a *post-hoc* least significant difference test. **$P < 0.001$. EA: Electroacupuncture; min: minutes.
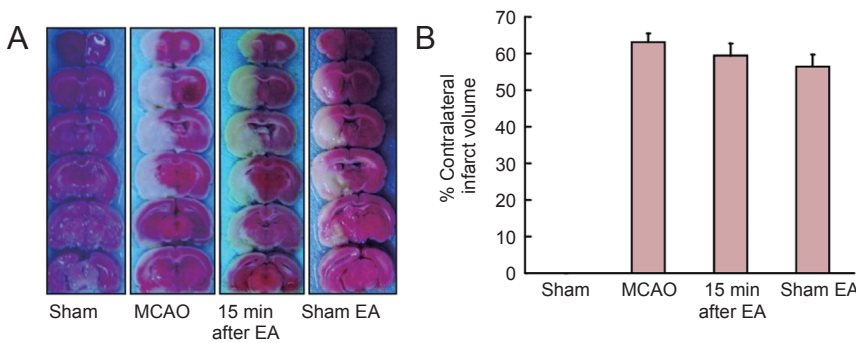

Figure 4 EA treatment before MCAO did not affect the rats' infarct volumes.
(A) 2,3,5-Triphenyltetrazolium chloride staining of the cerebral infarcts in rat-brain sections from the different groups 24 hours after reperfusion. (B) Quantitative analysis of the cerebral infarct volume in the different groups. Data are presented as the mean ± SEM ($n = 10$ rats in each group) and were analyzed using a one-way analysis of variance followed by *post-hoc* least significant difference tests. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture; min: minutes.
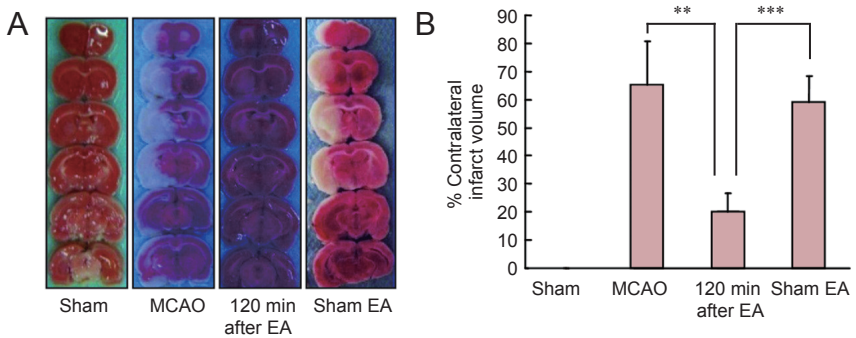

Figure 5 EA treatment prior to MCAO attenuated the infarct volumes.
(A) 2,3,5-Triphenyltetrazolium chloride staining of the cerebral infarcts in rat-brain sections from the different groups 24 hours after reperfusion. (B) Quantitative analysis of cerebral infarct volume in the different groups. Data are presented as the mean ± SEM ($n = 10$ rats in each group) and were analyzed using a one-way analysis of variance followed by *post-hoc* least significant difference tests. **$P < 0.01$, ***$P < 0.001$. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture; min: minutes.

(A) 2,3,5-Triphenyltetrazolium chloride staining of the cerebral infarcts in rat-brain sections from the different groups 24 hours after reperfusion. (B) Quantitative analysis of cerebral infarct volume in the different groups. Data are presented as the mean ± SEM ($n = 10$ rats in each group) and were analyzed using a one-way analysis of variance followed by *post-hoc* least significant difference tests. ***$P < 0.001$. MCAO: Middle cerebral artery occlusion; EA: electroacupuncture; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine. I: MCAO; II: 120 min after EA; III: 120 min after EA+DPCPX; IV: 120 min after EA+DMSO; V: MCAO+DPCPX; min: minutes.
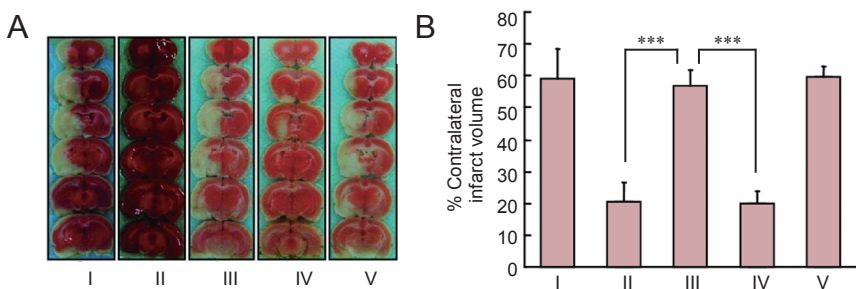
Figure 6 Pretreatment with the A1 receptor antagonist DPCPX reverses EA-mediated neuroprotection against MCAO-induced neurological deficits and infarct volume in rats.

be mediated by adenosine signaling. This is corroborated by the finding that neuronal stimulation can trigger ATP release from its terminals (Bekar et al., 2008; Goldman et al., 2010) and that ATP can be locally metabolized into adenosine by a series of ectonucleotidases such as CD39 and CD73 (Zimmermann, 2006). Further, our preliminary analysis also showed that adenosine deaminase and CD73 levels in the EA treatment group were greater than those in the control group

(data not shown). Thus, acupuncture at peripheral acupoints may trigger central nervous system activity that modulate levels of ATP and adenosine.

Another important finding of this study was that pharmacological blockade of A1 receptors by DPCPX abolished the neuroprotective effects observed 120 minutes post-EA pretreatment. This confirms previous results and provides independent support for the critical role of the A1 receptor

in mediating the protective effect of EA treatment against ischemic injury (Sun et al., 2005). Consistent with the theory that acupuncture has systemic effects, we were able to abolish the EA-induced protective benefits by systemic administration of an adenosine A1-receptor antagonist. Additional studies that administer A1-receptor antagonists through peripheral locations (such as near the *Baihui* acupoint) or through the central nervous system (such as intracerebroventricular injection) would reveal whether the effects are mediated by local or centralized mechanisms.

In terms of explaining the effect of acupuncture, the adenosine hypothesis has translational implications. For example, if this hypothesis is correct, then it implies that prolonging or diminishing the neuroprotective effect of EA treatment is possible by altering extracellular adenosine metabolism. Currently, there are several agents that have been approved for clinical use and are known to affect extracellular adenosine levels, including methotrexate, sulfasalazine, and salicylate (Morabito et al., 1998). Deoxycoformycin (pentostatin) is an antimicrobial nucleoside analogue that inhibits DNA synthesis and is approved by the FDA for the treatment of leukemia (Lamanna and Kay, 2009). Goldman et al. (2010) confirmed that deoxycoformycin promoted adenosine accumulation *in vivo* and enhanced analgesic effect of acupuncture. It would be interesting to evaluate whether deoxycoformycin can influence the effect of EA on ischemic brain injury. If any FDA-approved drugs exhibit a synergistic analgesic effect when combined with acupuncture, clinical trials can be designed to provide proof-of-principle data. Another example of the translational implications of our findings is that, caffeine–the most widely used psychoactive drug, regularly consumed by approximately 60–80% of the world's population–blocks adenosine A1 and A2A receptors in the brain (Chen et al., 2010). Thus, caffeine consumption may interfere with the effects of acupuncture.

In summary, neuroprotection *via* EA pretreatment against ischemic reperfusion injury at the *Baihui* acupoint in rats was related to peak levels of adenosine, but not ATP, in the cerebral cortex and was abolished by the pharmacological blockade of A1 receptors. Thus, EA treatment may exert its neuroprotective effect against ischemic brain injury by triggering extracellular adenosine to act on A1 receptors in the brain.

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Copyedited by Phillips A, Wysong S, Yu J, Li CH, Qiu Y, Song LP, Zhao M