

# Pretreatment With Electroacupuncture Induces Rapid Tolerance to Focal Cerebral Ischemia Through Regulation of Endocannabinoid System

Qiang Wang, MD, PhD; Ye Peng, MD; Shaoyang Chen, MD; Xingchun Gou, MD; Bo Hu, MD; Juan Du, MD; Yan Lu, MD, PhD; Lize Xiong, MD, PhD

**Background and Purpose**—Our previous study demonstrated that pretreatment with electroacupuncture (EA) induces rapid tolerance to focal cerebral ischemia. The present study was aimed to investigate the involvement of the endocannabinoid system in the early neuroprotection conferred by EA pretreatment in the animal model of focal cerebral ischemia.

**Methods**—Two hours after the end of EA pretreatment, focal cerebral ischemia was induced by middle cerebral artery occlusion for 120 minutes in male Sprague-Dawley rats or male C57BL/6 mice. The neurobehavioral scores, infarction volumes, and neuronal apoptosis were evaluated at 24 hours or 7 days after reperfusion in the presence or absence of AM251 (a selective cannabinoid receptor type 1 [CB1] receptor antagonist) or CB1 short interfering RNA. The expression of CB1 receptor and the content of endocannabinoids in the brains were also investigated.

**Results**—EA pretreatment reduced infarct size, improved neurological outcome, and inhibited neuronal apoptosis at 24 hours or 7 days after reperfusion. The beneficial effects were abolished by AM251. CB1 knockdown by CB1 short interfering RNA attenuated EA pretreatment-induced neuroprotection. EA pretreatment upregulated the neuronal expression of CB1 receptor in the rat brains and elevated the brain tissue content of the endocannabinoid 2-arachidonylglycerol and *N*-arachidonylethanolamine-anandamide. Pretreatment with 2-arachidonylglycerol and *N*-arachidonylethanolamine-anandamide also reduced infarct size and improved neurological outcome.

**Conclusion**—We conclude that pretreatment with EA increases the production of endocannabinoid 2-arachidonylglycerol and *N*-arachidonylethanolamine-anandamide, which elicits protective effects against transient cerebral ischemia through CB1 receptors. These results suggest a novel mechanism of EA pretreatment-induced rapid tolerance to focal cerebral ischemia. (*Stroke*. 2009;40:2157-2164.)

**Key Words:** cannabinoid ■ cerebral ischemia ■ electroacupuncture ■ pretreatment

Neurological damage, in particular stroke, accounts for approximately 40% of severely disabled adults. Although many advances have been made in the pharmacotherapy of stroke, clinical treatment of this debilitating disorder is inadequate. There is a huge unmet medical need for developing novel and rational strategies aimed at reducing impairments after stroke and other neurological conditions. Our previous study has demonstrated that pretreatment with electroacupuncture (EA) at the Baihui acupoint (GV 20) induces rapid tolerance to cerebral ischemic insult.<sup>1</sup> However, the underlying mechanism is not fully understood and more evidence is needed for EA pretreatment to be accepted clinically.

Studies on the pharmacology of cannabis were advanced considerably by the cloning of the centrally located cannabinoid receptor type 1 (CB1) receptors and the peripherally located cannabinoid CB2 receptors as well as the identifica-

tion of endogenous ligands.<sup>2-4</sup> Two endogenous ligands for the CB1 receptor have been proposed and termed endocannabinoids: *N*-arachidonylethanolamine-anandamide (AEA) and 2-arachidonylglycerol (2-AG). Immediately after the discovery of endogenous cannabinoid system, research activities had been focused on its protective effects against ischemic damage in the heart and brain. Administration of endocannabinoids such as 2-AG or AEA gives rise to neuroprotection in vivo models of closed head injury and acute cerebral infarction.<sup>5,6</sup> In addition, increased CB1 receptor expression in response to acute ischemic insult has been observed in the rat brain cortex.<sup>7</sup> Accordingly, cerebral ischemia has more severe effects in CB1 receptor knockout mice than wild-type mice.<sup>8</sup> These findings have prompted investigations into the role of the endocannabinoid system in neuroprotective effects.

Received November 17, 2008; final revision received January 23, 2009; accepted January 30, 2009.

From the Departments of Anesthesiology (Q.W., S.C., B.H., J.D., Y.L., L.X.) and Orthopedics (Y.P.), Xijing Hospital, Fourth Military Medical University, Xi'an, China; and the Cell Engineering Research Center & Department of Cell Biology (X.G.), State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi'an, China.

Q.W. and Y.P. contributed equally to this work.

Correspondence to Lize Xiong, MD, PhD, Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. E-mail lize.xiong@hotmail.com

© 2009 American Heart Association, Inc.

*Stroke* is available at <http://stroke.ahajournals.org>

DOI: 10.1161/STROKEAHA.108.541490

Several recent studies have reported the involvement of endocannabinoid system in the ischemic tolerance in brain. Repeated pretreatment with  $\Delta^9$ -tetrahydrocannabinol, the major active constituent of cannabis, led to tolerance to ischemic insult in mice subjected to 4 hours-middle cerebral artery occlusion (MCAO). The neuroprotective effect of  $\Delta^9$ -tetrahydrocannabinol was inhibited by SR141716, a CB1 receptor antagonist.<sup>9</sup> In another study, ischemic preconditioning downregulated the postischemic CB1 receptor in the gerbil hippocampus, indicating that the CB1 receptors may participate in the mechanisms of endogenous neuroprotection of ischemic preconditioning.<sup>10</sup> Based on these findings, we investigated whether the endocannabinoid system is involved in the mediation of the neuroprotective effect by EA pretreatment at the Baihui (GV 20) acupoint.

## Materials and Methods

### Experimental Protocols

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and was conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University (Xi'an, China). Male Sprague-Dawley rats (280 to 320 g) and male 10-week-old C57BL/6 mice (18 to 20 g) were provided by the Experimental Animal Center of the Fourth Military Medical University and housed under controlled conditions with a 12-hour light/dark cycle, a temperature at  $21 \pm 2^\circ\text{C}$ , and humidity at 60% to 70% for at least 1 week before drug treatment or surgery. The animals were allowed free access to standard rodent diet and tap water.

#### Experiment I

To assess the effect of AM251 on neuroprotection induced by EA pretreatment, rats were anesthetized with 1% sodium pentobarbital (SP, 40 mg/kg, intraperitoneally) at 2.5 hours before induction of focal cerebral ischemia and were randomly assigned to MCAO, EA+MCAO, AM251+MCAO, vehicle+EA, and AM251+EA groups. AM251, a CB1 receptor antagonist, was purchased from Tocris Bioscience and was dissolved in dimethyl sulfoxide and Tween-80 and then diluted with saline (dimethyl sulfoxide: Tween-80: saline=1:1:18) before use.

#### Experiment II

To determine the regulatory effect of EA pretreatment on the endocannabinoid system before ischemia, rats were randomly divided into 3 groups: sham, SP, and EA groups.

#### Experiment III

To evaluate the neuroprotective effect of pretreatment with 2-AG and AEA, the rats were intraperitoneally injected with 5 mg/kg 2-AG, 10 mg/kg AEA, or 1 mL vehicle at 2 hours before MCAO. The 2-AG (No.1298) and AEA (No. 1339) were purchased from Tocris Bioscience and dissolved in anhydrous ethanol:emulphor:saline (1:1:18) before use. One milliliter per dosage was injected intraperitoneally. The dose and administration method of 2-AG and AEA in this experiment were chosen based on previous studies.<sup>5,6</sup>

#### Experiment IV

To further elucidate the role of the CB1 receptor in EA-induced early-phase neuroprotection, we first investigated the effectiveness of short interfering RNA (siRNA) *in vivo*; C57BL/6 mice were randomly divided into shams, siRNA, siRNA-c (control siRNA), siRNA+EA, and siRNA-c+EA groups. Then, we tested the effect of CB1 siRNA on neuroprotection induced by EA pretreatment; C57BL/6 mice were randomly divided into MCAO, EA+MCAO, siRNA+EA+MCAO, and siRNA-c+EA+MCAO groups.

Details of the experimental grouping and protocols are included in the Supplemental material, available online at <http://stroke.ahajournals.org>.

### Electroacupuncture Pretreatment

EA pretreatment was performed as described in our previous studies.<sup>1</sup> Briefly, animals were anesthetized with 40 mg/kg SP (intraperitoneally) and inhaled oxygen by face mask at a flow rate of 1 L/min. The acupoint "Baihui (GV 20)," which is located at the intersection of the sagittal midline and the line linking 2 rat ears, was stimulated with the intensity of 1 mA and frequency of 2/15 Hz for 30 minutes by using the G6805-2 EA Instrument (Model No.227033; Qingdao Xinsheng Ltd). The core temperature of all the rats was maintained (Spacelabs Medical Inc) at  $37.0 \pm 0.5^\circ\text{C}$  during EA pretreatment by surface heating or cooling. The right femoral artery was cannulated for continuous monitoring of blood pressure and for arterial blood sampling. Arterial blood gases and plasma glucose were measured at the onset of EA, 15 minutes after the onset of EA, and at the end of EA.

### Transfection of siRNA in the Mouse Brain

We performed *in vivo* siRNA transfection in C57BL/6 mice according to the method described by Luo et al.<sup>11</sup> Under anesthesia with SP (40 mg/kg), a stainless steel cannula was stereotaxically implanted in the unilateral cerebral ventricle. The stereotaxic coordinates were 0.4 mm posterior and 1.0 mm lateral to the bregma and 2.5 mm below the surface of the skull. In light of the manufacturer's instructions, 1  $\mu\text{L}$  of 10  $\mu\text{g}/\mu\text{L}$  CB1-siRNA (Santa Cruz Biotechnology, sc-39911) or control-siRNA (Santa Cruz Biotechnology, sc-37007) duplex stock solution was incubated with 10  $\mu\text{L}$  InvivoFectamine Reagent (Invitrogen Corporation; 1377-901) for 30 minutes at room temperature in an orbital shaker. Then the InvivoFectamine-siRNA mixture was diluted with 15 volumes (150  $\mu\text{L}$ ) of 5% glucose. Two microliters of the diluted mixture was stereotaxically delivered into the ipsilateral lateral ventricle. After recovering from anesthesia, mice were returned to their cages and given *ad libitum* access to food and water. The protein expression of CB1 receptor was evaluated 24 hours posttransfection using Western blot.

### Transient Focal Cerebral Ischemia

Focal cerebral ischemia was induced by MCAO in rats or mice using an intraluminal filament technique as described previously.<sup>1,12,13</sup> After 2 hours of MCAO, the filament was withdrawn, regional cerebral blood flow was restored to normal as monitored by laser Doppler flowmetry (PeriFlux 5000; Perimed AB), and wounds were sutured.

### Neurobehavioral Evaluation and Infarct Assessment

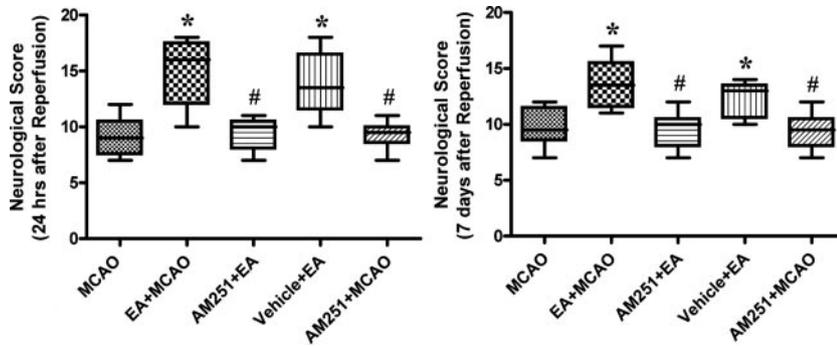
Twenty-four hours or 7 days after reperfusion, an 18-point scoring system reported by Garcia et al<sup>14</sup> with modifications was used for neurological assessment by a blinded observer. Then, animals ( $n=8$  for each group) were decapitated and 2-mm thick coronal sections from throughout the brain were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) to evaluate the infarct volume, as described previously.<sup>1,12,13</sup>

### TUNEL Staining

Samples from 5 groups ( $n=5$  for each group) in Experiment I were used for experiments. Twenty-four hours after reperfusion, neuronal apoptosis in the ischemic penumbra was assessed *in situ* by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining as described in our previous studies.<sup>12</sup> The TUNEL staining was quantitatively evaluated with the method described by Wang et al.<sup>15</sup> Briefly, 32 pixels of  $0.10 \text{ mm}^2$  were placed by light microscope with  $100\times$  magnification and then the total number of positively stained cells in these pixels was counted and expressed as cells per millimeter squared.

### Double Immunofluorescence for CB1 Receptor and Neuronal Nuclei

At 120 minutes after the end of EA pretreatment, the rats ( $n=3$  for each group) in Experiment II were anesthetized and transcardially



**Figure 1.** Neurological scores 24 hours and 7 days after reperfusion in the rats with 120 minutes of MCAO. Pretreatment with EA significantly improved the neurological scores, whereas the CB1 receptor antagonist AM251 reversed the beneficial effect of EA pretreatment. \* $P < 0.01$  versus MCAO; # $P < 0.01$  versus EA+MCAO.

perfused with phosphate-buffered saline and 4% paraformaldehyde as described previously.<sup>12</sup> The 10- $\mu$ m thick coronal sections were incubated with the following primary antibodies: anti-CB1 receptor rabbit polyclonal antibody (Santa Cruz Biotechnology; 1:100 dilution) and antineuronal nuclei mouse monoclonal antibody (Chemicon International Inc; 1:1000 dilution) together for 12 hours at room temperature. After washing 3 times with phosphate-buffered saline, sections were next incubated with fluorescein isothiocyanate-labeled goat-antirabbit IgG (Molecular Probes Inc; 1:800 dilution) and Alexa-labeled goat-antimouse IgG (Sigma-Aldrich; 1:800 dilution) together for 1 hour at room temperature. Finally, sections were observed and images were captured using Olympus BX-60 fluorescence microscope (Olympus Corporation).

### Reverse Transcription–Polymerase Chain Reaction for Detection of CB1 mRNA

Animals were decapitated under deep anesthesia with 4% isoflurane in oxygen. The ipsilateral hemispheres were harvested for reverse transcription–polymerase chain reaction and Western blot analysis ( $n = 5$  for each group).

Total RNA was extracted from brain tissue using Trizol reagent (Invitrogen Corporation) according to the manufacturer's recommendations. The cDNA was synthesized using the Superscript First Strand Synthesis Kit (Promega Corporation) according to the manufacturer's instructions. The sequences of primers (CB1 and glyceraldehyde phosphate dehydrogenase) and PCR-reactive condition were described in previous study.<sup>16</sup> All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co Ltd (Shanghai, China).

### Western Blot Analysis of CB1 Protein

The hemispheres were homogenized in RIPA lysis buffer (Beyotime, Nantong, China) with  $1 \times$  Roche complete protease inhibitor cocktail and 1 mmol/L phenylmethylsulfonyl fluoride on ice. The Western blot was performed like our previous study.<sup>12</sup> The following primary antibodies were used in this study: anti-CB1 receptor rabbit polyclonal antibody (1:200 dilution) and antiglyceraldehyde phosphate dehydrogenase mouse monoclonal antibody (Santa Cruz Biotechnology; 1:1000 dilution). Appropriate secondary horseradish peroxidase-conjugated goat-antirabbit or goat-antimouse antibody (Pierce Biotechnology Inc; 1:5000 dilution) were used.

### Endocannabinoid Extraction and Liquid Chromatography/Mass Spectrometry

At 120 minutes after the end of EA pretreatment, the rats from 3 groups ( $n = 5$  for each group) in Experiment II were decapitated under deep anesthesia and the ipsilateral hemispheres were separated. Endocannabinoids were extracted from brains after EA pretreatment and endocannabinoid content was determined by isotope dilution and liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry as described by Patel et al.<sup>17</sup> Endocannabinoid contents were presented as nanograms per milliliter.

### Statistical Analysis

The software, SPSS 11.0 for Windows (SPSS Inc, Chicago, Ill), was used to conduct statistical analyses. All values, except for neurological scores, are presented as mean  $\pm$  SEM and were analyzed by one-way analysis of variance, and between-group differences were detected with post hoc Student-Newman-Keuls test. The neurological deficit scores were expressed as median (range) and were analyzed with Kruskal-Wallis test followed by the Mann-Whitney  $U$  test with Bonferroni correction. Values of  $P < 0.05$  were considered statistically significant.

## Results

### EA Pretreatment-Induced Improvement of Neurological Outcome Is Reversed by AM251

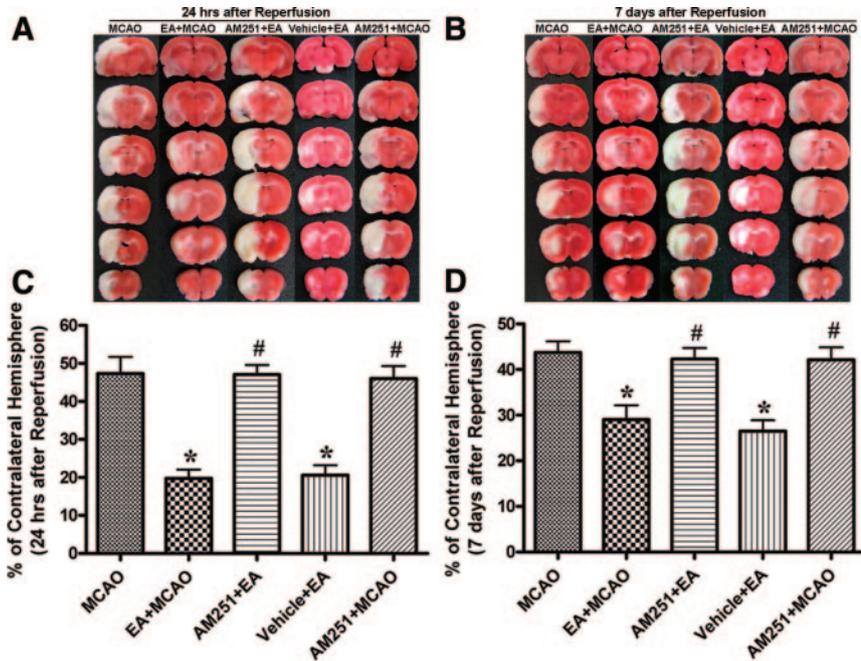
Twenty-four hours and 7 days after reperfusion, pretreatment with EA significantly improved the neurological scores compared with that of the MCAO group ( $P < 0.01$ ). The CB1 receptor antagonist AM251 had no effect on neurological scores when administered alone but reversed the beneficial effect of EA pretreatment given 30 minutes before the onset of EA pretreatment ( $P < 0.01$ , AM251+EA versus EA+MCAO). The neurological score of the vehicle+EA group was similar to that of the EA+MCAO group. There were no statistical differences in the neurological deficit scores among MCAO, AM251+MCAO, and AM251+EA groups (Figure 1).

### EA Pretreatment-Induced Reduction of Infarct Volume Is Abolished by AM251

The EA+MCAO group showed a smaller brain infarct volume compared with the MCAO group ( $P < 0.01$ ). The infarct volume of the AM251+EA group was similar to that of the MCAO group and was larger than that of the EA+MCAO group ( $P < 0.01$ ). The infarct volume of the vehicle+EA group was still significantly different from that of the MCAO group ( $P < 0.01$ ) and was similar to that of the EA+MCAO group. The result of the AM251+MCAO group was not significantly different from that of the MCAO group (Figure 2).

### EA Pretreatment-Induced Reduction of Neuronal Apoptosis Is Attenuated by AM251

No positive TUNEL staining (brown) was detected in the brain sections of sham animals 24 hours after reperfusion. However, a large number of TUNEL-positive cells in the ischemic penumbra of rat brain were seen in the MCAO, AM251+EA, and AM251+MCAO groups, whereas in contrast, only a small amount of TUNEL-positive cells in the EA+MCAO and vehicle+EA groups were observed (Figure 3A). The quantitative analysis of the number of TUNEL-positive cells and viable



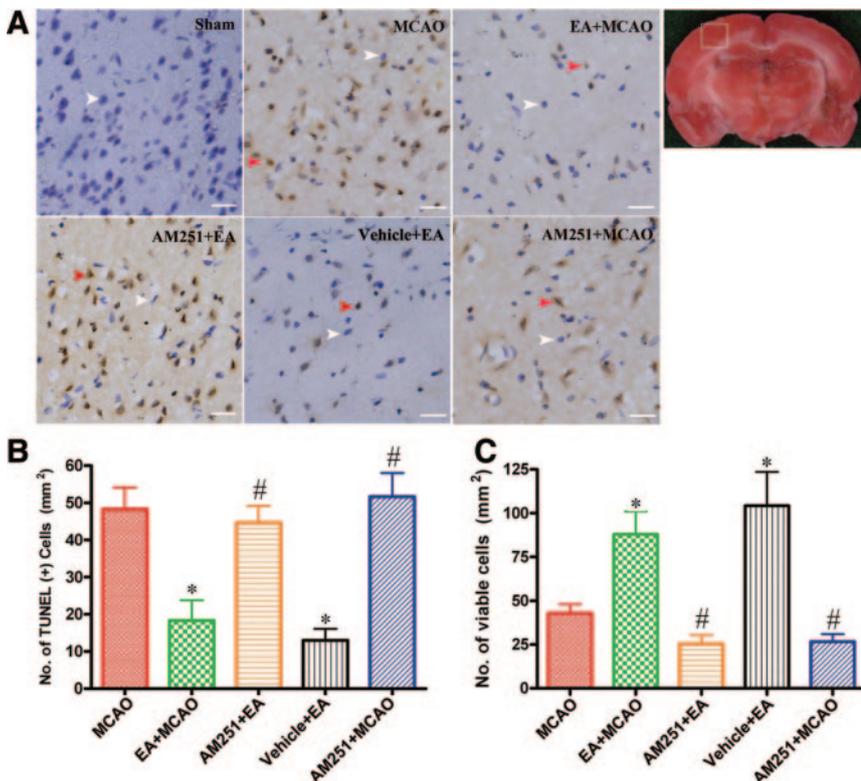
**Figure 2.** Infarct sizes at 24 hours and 7 days after reperfusion in the rats with 120 minutes of MCAO. A–B, Representative 2,3,5-triphenyltetrazolium chloride staining of the cerebral infarct in comparable sections of rat brain from 5 groups at 24 hours and 7 days after reperfusion. C–D, Quantification of infarct volume at 24 hours and 7 days after reperfusion, respectively. Pretreatment with EA significantly reduced the infarct volume, whereas the CB1 receptor antagonist AM251 abolished the protective effect of EA pretreatment. \* $P < 0.01$  versus MCAO; # $P < 0.01$  versus EA+MCAO.

cells in the ischemic penumbra of rats showed that the pretreatment with EA significantly reduced the number of TUNEL-positive cells at 24 hours after reperfusion compared with MCAO, AM251+EA, and AM251+MCAO groups. There was no difference among the MCAO, AM251+EA, and AM251+MCAO groups (Figure 3B). On the other hand, the number of viable cells (blue) in the ischemic penumbra was significantly increased in EA+MCAO and vehicle+EA groups in comparison to MCAO, AM251+EA, and

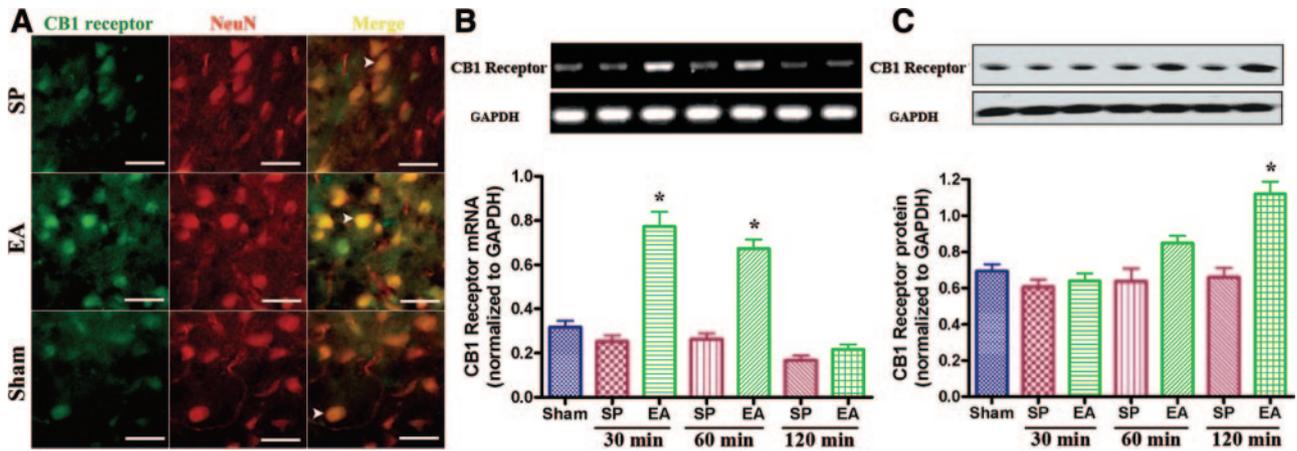
AM251+MCAO groups ( $P < 0.05$ ) as revealed by hematoxylin staining, but there was no difference among the latter 3 groups (Figure 3C).

### EA Pretreatment Upregulates the Expression of Neuronal CB1 Receptor

The expression and localization of CB1 receptor was examined by double immunofluorescence staining with neuronal nuclei (neuronal marker, red) and CB1 receptor (green). As



**Figure 3.** Neuronal apoptosis at 24 hours after reperfusion in the rats with 120 minutes of MCAO. A, Representative photomicrographs of TUNEL staining in the peri-infarct zone (region of interest identified in adjoining coronal section). The white arrowhead indicates viable cell and the red arrowhead indicates TUNEL-positive cell. Scale bars=50  $\mu\text{m}$ . B–C, Quantitative analysis of the number of TUNEL-positive cells and viable cells in the ischemic penumbra of rats in 5 groups. Pretreatment with EA significantly decreased the number of TUNEL-positive cells, whereas the CB1 receptor antagonist AM251 attenuated the reduction in the number of TUNEL-positive cells. \* $P < 0.01$  versus control; # $P < 0.01$  versus EA+MCAO.



**Figure 4.** Upregulation of neuronal CB1 receptor in the rat brain after EA pretreatment. A, Representative double immunofluorescence staining for CB1 receptor (green) and neuronal nuclei (neuronal marker, red) in brain sections. The CB1 receptors are increased and colocalized in the neurons (arrowheads) 2 hours after the end of EA pretreatment. Scale bars=20  $\mu$ m. B, The mRNA expression of CB1 receptor was analyzed by using reverse transcription–polymerase chain reaction. EA pretreatment significantly upregulated the mRNA expression of CB1 receptor at 30 and 60 minutes after the end of EA pretreatment. C, The protein expression of CB1 receptor was evaluated by using Western blot. Pretreatment with EA significantly upregulated the protein expression of CB1 receptor at 120 minutes after the end of EA pretreatment. \* $P$ <0.05 versus sham group.

shown in Figure 4A, in the sham and SP groups, little expression of CB1 receptor was observed throughout the hemisphere. However, the expression of CB1 receptor in the ipsilateral hemisphere was significantly increased after the EA pretreatment. Double immunofluorescence for CB1 receptor and neuronal nuclei demonstrated that the CB1 receptor was mainly localized in neurons in the ipsilateral hemisphere. Thus, the CB1 receptors were upregulated and colocalized with the neurons (arrowheads) 2 hours after the end of EA pretreatment in the ipsilateral hemisphere.

EA pretreatment produced an evident upregulation of CB1 receptor in the ipsilateral hemisphere as revealed by the presence of both mRNA (Figure 4B) and protein (Figure 4C). The expression of CB1 receptor mRNA significantly increased at 30 and 60 minutes after the end of EA pretreatment as compared with that in sham or SP groups (Figure 4B;  $P$ <0.05) but returned to normal level at 120 minutes after the end of EA pretreatment. The semiquantitative analysis of Western blot indicated that EA pretreatment significantly increased the expression of CB1 receptor protein compared with that in sham or SP groups at 120 minutes after the end of EA pretreatment (Figure 4C;  $P$ <0.05). However, there was no difference in CB1 receptor mRNA or protein expression between the sham and SP groups at 30, 60, and 120 minutes after the end of EA pretreatment.

### EA Pretreatment Increases the Content of Endocannabinoid in Brain Tissue

Pretreatment with EA increased the brain tissue content of the endocannabinoid 2-AG from  $5.20 \pm 0.71$  ng/mL to  $9.60 \pm 0.93$  ng/mL ( $P$ <0.01) and AEA from  $0.81 \pm 0.12$  ng/mL to  $2.32 \pm 0.23$  ng/mL ( $P$ <0.01). However, endocannabinoid contents in the sham and SP groups were not significantly different from each other (Figure 5A–B).

### Endocannabinoid Pretreatment Induces Neuroprotection

Twenty-four hours after reperfusion, pretreatment with 2-AG and AEA significantly improved the neurological scores and

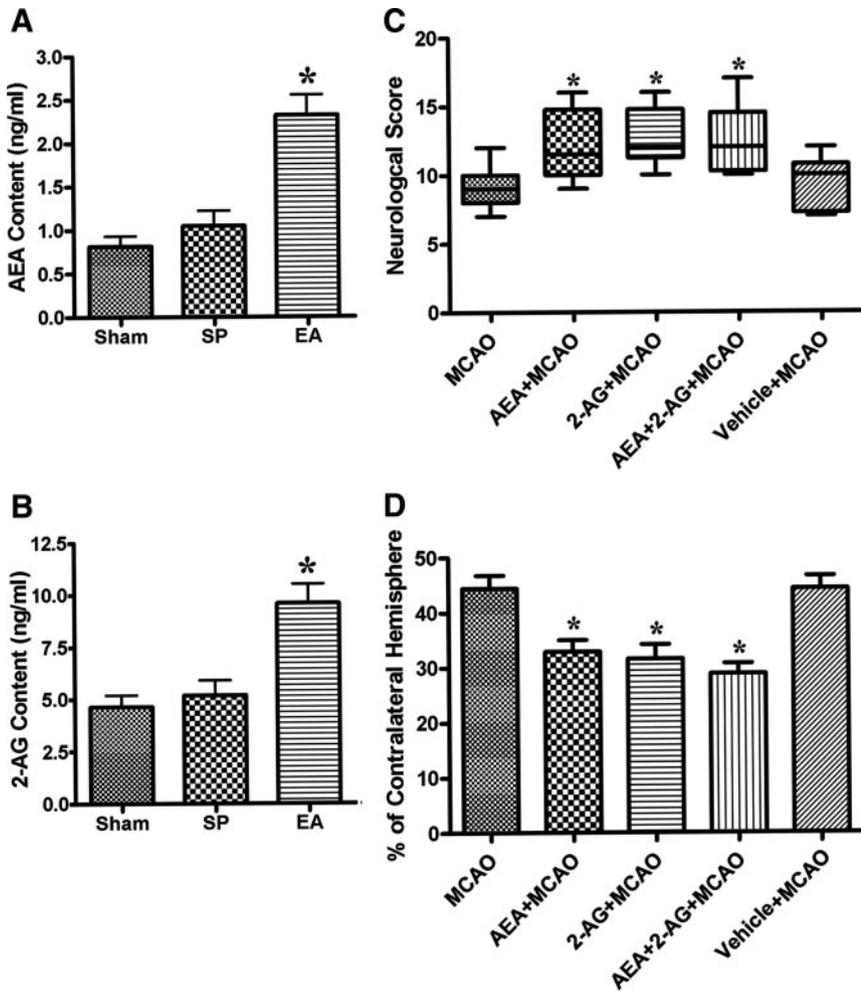
reduced the infarction volumes compared with those of the MCAO group ( $P$ <0.05). There were no statistical differences between MCAO and vehicle+MCAO groups (Figure 5C–D).

### Knockdown of CB1 Receptor by siRNA Attenuates EA Pretreatment-Induced Neuroprotection

Twenty-four hours after intracerebroventricular administration of CB1 siRNA or siRNA-c, CB1 receptor protein in mouse brain was analyzed by Western blot (Figure 6A). The results indicated that the protein expression of CB1 receptor was downregulated by CB1 siRNA compared with either sham or siRNA-c ( $P$ <0.01). The upregulation of CB1 receptor by EA pretreatment was inhibited in CB1 siRNA-treated mice compared with siRNA-c-treated ones ( $P$ <0.01). Moreover, pretreatment with EA significantly reduced the infarction volumes at 24 hours after reperfusion ( $P$ <0.01, EA+MCAO versus MCAO). The CB1 knockdown by siRNA attenuated the protective effect of EA pretreatment ( $P$ <0.01, siRNA+EA+MCAO versus EA+MCAO), whereas siRNA-c had no effect on the neuroprotection of EA pretreatment (Figure 6B).

### Discussion

The present study demonstrated that the rapid tolerance to focal cerebral ischemia induced by EA pretreatment is attenuated by a specific CB1 receptor antagonist AM251, whereas the agent itself had no significant effect on neurological outcome, infarct volume, or neuronal apoptosis. Besides, pretreatment with EA at Baihui acupoint (GV 20) for 30 minutes increased the brain tissue content of the endocannabinoid 2-AG and AEA as well as upregulated the expression of neuronal CB1 receptor in the brain. Furthermore, pretreatment with 2-AG and AEA induced neuroprotection, and CB1 knockdown by CB1 siRNA in vivo attenuates EA pretreatment-induced reduction in infarction volumes. These results suggest that pretreatment with EA increases the production of the endocannabinoid 2-AG and AEA, which elicits protective effects against transient cerebral ischemia

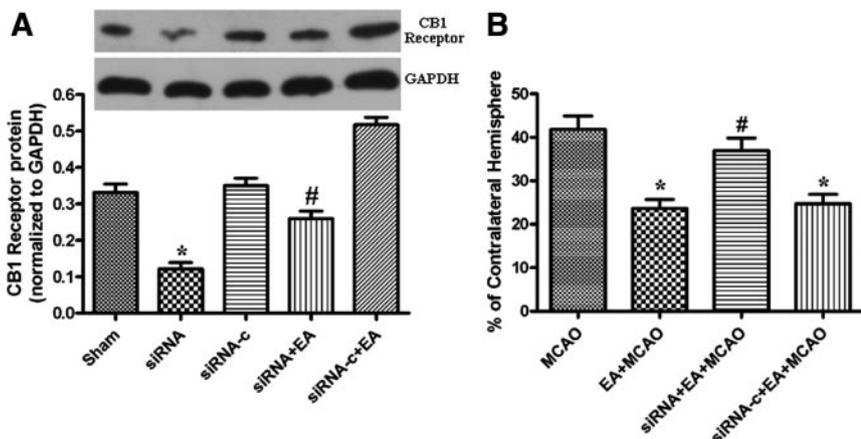


**Figure 5.** Endocannabinoid content in the rat brain tissue and neuroprotection of endocannabinoid pretreatment. A–B, The content of endocannabinoid in the rat brain was significantly increased in the EA group compared with that in the sham and SP groups. \* $P < 0.01$  versus sham group. C–D, Pretreatment with AEA and 2-AG significantly improved the neurological scores and reduced the infarction volumes compared with those of the MCAO group. \* $P < 0.05$  versus MCAO group.

through CB1 receptors, suggesting a new mechanism of EA pretreatment-induced rapid tolerance to focal cerebral ischemia.

The phenomenon of ischemic tolerance can be induced by pretreatment of sublethal stresses other than ischemia. The window of protection from preconditioning is bimodal. The first window of protection (rapid tolerance) lasting no longer than 3 hours appears immediately after the preconditioning maneuvers, after which protection wears off; the second window of protection (delayed tolerance) reap-

pears 24 hours after preconditioning, lasting 12 to 72 hours.<sup>18</sup> Our previous study demonstrated that similar to the rapid tolerance induced by ischemic preconditioning, 2 hours before lethal ischemic insult, EA pretreatment at Baihui acupoint (GV20) for 30 minutes could reduce infarct volumes and improve neurological deficit 24 hours after transient MCAO, indicating that the rapid tolerance to cerebral ischemic insult appears at 2 hours after the end of EA pretreatment.<sup>1</sup> In the present study, EA pretreatment induced rapid tolerance and exerted potent neuroprotection manifested fur-



**Figure 6.** Effect of CB1 siRNA on infarct volumes at 24 hours after reperfusion in the mice with 120 minutes of MCAO. A, The protein expression of CB1 receptor was analyzed by using Western blot. CB1 siRNA significantly downregulated the protein expression of CB1 receptor. \* $P < 0.01$  versus sham or siRNA-c; # $P < 0.01$  versus siRNA-c+EA. B, The infarct volumes were assessed by 2,3,5-triphenyltetrazolium chloride staining at 24 hours after reperfusion. Pretreatment with EA significantly reduced the infarct volume, whereas the CB1 siRNA attenuated the protective effect of EA pretreatment. \* $P < 0.01$  versus MCAO; # $P < 0.01$  versus EA+MCAO.

ther as significant reduction in infarct size and amelioration of neurological outcome lasting 7 days as well as inhibition of neuronal apoptosis after 24 hours reperfusion.

The involvement of the endocannabinoid system in the protective effect of preconditioning on ischemia in rat isolated hearts<sup>19</sup> as well as in rat hearts in vivo has been documented.<sup>20</sup> Recently, cannabinoids and CB1 receptor have been reported to participate in the mechanisms of endogenous neuroprotective effects of ischemic preconditioning in the brain.<sup>9,10</sup> However, little is known about the involvement of the endocannabinoid system in EA pretreatment in brains. We therefore investigated the role of the endocannabinoid system in the rapid ischemic tolerance induced by EA pretreatment. Our results showed that the neuroprotection induced by EA pretreatment were abolished by a selective antagonist of CB1 receptor AM251 given 30 minutes before the onset of EA pretreatment. While given alone, AM251 had no significant effect on ischemia-induced neurological outcome, infarct volume, or neuronal apoptosis. Similarly, administration of vehicle, the solvent of AM251, at 30 minutes before the onset of EA pretreatment did not alter the beneficial effects induced by EA pretreatment. These data indicated the absence of direct aggravating effects of AM251 or vehicle intervention in the experiments.

Moreover, the present immunofluorescence data showed that the CB1 receptors were increased and colocalized in the neurons 2 hours after the end of EA pretreatment, indicating that EA pretreatment upregulated the expression of neuronal CB1 receptor in the brain. Upregulation of neuronal CB1 receptor after EA pretreatment might be an important protective mechanism of tolerance against ischemia. Because CB1 receptors are expressed by neurons, vascular smooth muscle cells, and endothelial cells in the brain, it is plausible that activation of different "pools" of receptors could have opposite effects on neuronal survival during injury. There is evidence that the neuronal CB1 receptor is associated with a decrease in overall neuronal excitability, whereas activation of cerebrovascular CB1 receptors could be detrimental at times when the brain requires tight autoregulation of blood flow as occurs during ischemia.<sup>21,22</sup>

Several studies have shown that the CB1 receptor expression is increased in response to acute ischemic insult, and ischemia has more severe effects in CB1 knockout mice than wild-type mice after experimental brain ischemia, which suggested that upregulation of the CB1 receptor may be involved in preventing ischemic neuronal damage.<sup>7,8</sup> In the present study, we found that the mRNA expression of CB1 receptor increased after EA stimulus and reached peak 30 minutes after the end of EA pretreatment in the ipsilateral hemisphere. The CB1 mRNA in the anesthetic control hemisphere, however, remained unchanged after EA pretreatment. Interestingly, CB1 receptor proteins were upregulated 2 hours after the end of EA pretreatment. This finding is not in line with the well-recognized notion that rapid tolerance induction, in contrast to tolerance of the delayed type, does not depend on protein synthesis.<sup>23</sup> However, the fast protein expression of CB1 receptors is in accordance with the findings of a recent study,<sup>24</sup> which demonstrated that pretreatment with cannabidiol (50 mg/kg) and  $\Delta^9$ -tetrahydrocannabinol (1 mg/kg) enhanced the

expression level of CB1 receptor protein in the hippocampus and hypothalamus of rats 1 hour after the drugs were injected intraperitoneally. Although the mechanism underlying rapid protein expression of CB1 receptors induced by EA pretreatment in the brain needs further study, these results suggest that the EA-induced early-phase neuroprotection is mediated through a CB1 receptor-related pathway.

To strengthen the hypothesis that the neuroprotective effect of EA pretreatment is mediated through the CB1 receptor, the siRNA approach was used in this study. We demonstrated that the protein expression of CB1 receptor was downregulated by CB1 siRNA, and the upregulation of CB1 receptor by EA pretreatment was inhibited in CB1 siRNA-treated mice. Our results also showed that the neuroprotective effect induced by EA pretreatment was absent in animals treated with CB1 siRNA. The data further elucidate the role of the CB1 receptor-related pathway in EA-induced early-phase neuroprotection.

Another main hypothesis tested in this study is that the content of endocannabinoids in the brain would be modulated after pretreatment with EA. The present results showed that EA pretreatment produced an elevation of the content of the endocannabinoid 2-AG and EAE in brain tissue. It was reported that delayed preconditioning with the nitric oxide donor nitroglycerin increased the heart tissue content of the endocannabinoid 2-AG, whereas tissue levels of the endocannabinoid EAE remained unchanged.<sup>19</sup> This disagreement may be results from a different mode of preconditioning used or different tissue observed. The previous studies demonstrated that pretreatment with either 2-AG or EAE before the ischemia mimicked preconditioning inasmuch as it protected the ischemic insults in a similar fashion in rat isolated hearts.<sup>25</sup> Endogenous cannabinoids exert neuroprotective effects in animal models of various forms of acute neuronal injury such as cerebral ischemia, traumatic brain injury, and neurodegenerative diseases.<sup>5,6,26</sup> In the present study, pretreatment with 2-AG and AEA afforded neuroprotection manifested as the reduction of infarct sizes and improvement of neurological outcomes 24 hours after reperfusion followed by 120 minutes of focal ischemia. Thus, our finding suggests that pretreatment with EA increases the production of the endocannabinoid 2-AG and AEA, which elicit protective effects against transient cerebral ischemia.

According to the theory of Traditional Chinese Medicine, Baihui (GV 20) belongs to an acupoint of the DU series and has been commonly used for treating cerebrovascular diseases in China and other countries.<sup>27,28</sup> It has also been proved in experimental studies that acupuncture at the Baihui (GV 20) acupoint after ischemia could attenuate the cerebral ischemic injury in monkey and rat.<sup>29,30</sup> However, patients with stroke at the acute stage are usually too urgent to be treated by EA alone. Therefore, it would be beneficial to find a proper way to use EA in the prevention and alleviation of cerebral ischemic injury. Our preliminary results have shown that EA pretreatment produced a neuroprotective effect in patients, suggesting that pretreatment with acupuncture, ie, preventing the patients susceptible to stroke before they have an attack, may be a clinically applicable way to use EA to prevent and alleviate the disease.<sup>31</sup> The results of our previous

study showed that pretreatment with EA at Baihui acupoint (GV 20) protects the brain from injury by ischemia and reperfusion, whereas pretreatment with EA at nonacupoints does not produce evident neuroprotection. A detailed study on the cannabinoid signaling pathway and the link between EA and the cannabinoid system is needed to explore the precise mechanism of EA-induced neuroprotection.

### Summary

The present data indicated that pretreatment with EA may produce a neuroprotective effect through manipulation of the endocannabinoid system and/or its signaling pathway. These findings may provide a novel mechanism of pretreatment with EA-induced rapid tolerance to focal cerebral ischemia.

### Sources of Funding

This work was supported by the National Science Fund for Distinguished Young Scholars (Grant 30725039), the National Natural Science Foundation of China (Grant 30873326, 30572421), and the TCM Scientific Foundation of Shaanxi Provincial TCM Administrative Bureau (Grant 2008032).

### Disclosures

None.

### References

- Wang Q, Xiong L, Chen S, Liu Y, Zhu X. Rapid tolerance to focal cerebral ischemia in rats is induced by preconditioning with electroacupuncture: window of protection and the role of adenosine. *Neurosci Lett*. 2005;381:158–162.
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. 1990;346:561–564.
- Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 1993;365:61–65.
- Di Marzo V, Melck D, Bisogno T, De Petrocellis L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci*. 1998;21:521–528.
- Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, Shohami E. The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis*. 2006;22:257–264.
- Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, Fujiwara M. Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine<sub>1A</sub> receptor-dependent mechanism. *Stroke*. 2005;36:1077–1082.
- Jin KL, Mao XO, Goldsmith PC, Greenberg DA. CB1 cannabinoid receptor induction in experimental stroke. *Ann Neurol*. 2000;48:257–261.
- Parmentier-Batteur S, Jin K, Mao XO, Xie L, Greenberg DA. Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J Neurosci*. 2002;22:9771–9775.
- Hayakawa K, Mishima K, Nozako M, Ogata A, Hazekawa M, Liu AX, Fujioka M, Abe K, Hasebe N, Egashira N, Iwasaki K, Fujiwara M. Repeated treatment with cannabidiol but not Delta9-tetrahydrocannabinol has a neuroprotective effect without the development of tolerance. *Neuropharmacology*. 2007;52:1079–1087.
- Schomacher M, Müller HD, Sommer C. Short-term ischemia usually used for ischemic preconditioning down-regulates central cannabinoid receptors in the gerbil hippocampus. *Acta Neuropathol*. 2006;111:8–14.
- Luo J, Wang Y, Chen X, Chen H, Kintner DB, Shull GE, Philipson KD, Sun D. Increased tolerance to ischemic neuronal damage by knockdown of Na<sup>+</sup>-Ca<sup>2+</sup> exchanger isoform 1. *Ann N Y Acad Sci*. 2007;1099:292–305.
- Wang Q, Gou X, Xiong L, Jin W, Chen S, Hou L, Xu L. Trans-activator of transcription-mediated delivery of NEP1-40 protein into brain has a neuroprotective effect against focal cerebral ischemic injury via inhibition of neuronal apoptosis. *Anesthesiology*. 2008;108:1071–1080.
- Hata R, Mies G, Wiessner C, Fritze K, Hesselbarth D, Brinker G, Hossmann KA. A reproducible model of middle cerebral artery occlusion in mice: hemodynamic, biochemical, and magnetic resonance imaging. *J Cereb Blood Flow Metab*. 1998;18:367–375.
- Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats: statistical validation. *Stroke*. 1995;26:627–634.
- Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature*. 2002;417:941–944.
- Lépiciér P, Lagneux C, Sirois MG, Lamontagne D. Endothelial CB1-receptors limit infarct size through NO formation in rat isolated hearts. *Life Sci*. 2007;81:1373–1380.
- Patel S, Rademacher DJ, Hillard CJ. Differential regulation of the endocannabinoids anandamide and 2-arachidonylglycerol within the limbic forebrain by dopamine receptor activity. *J Pharmacol Exp Ther*. 2003;306:880–888.
- Pagliaro P, Gattullo D, Rastaldo R, Losano G. Ischemic preconditioning: from the first to the second window of protection. *Life Sci*. 2001;69:1–15.
- Wagner JA, Abesser M, Harvey-White J, Ertl G. 2-Arachidonylglycerol acting on CB1 cannabinoid receptors mediates delayed cardioprotection induced by nitric oxide in rat isolated hearts. *J Cardiovasc Pharmacol*. 2006;47:650–655.
- Hajrasouliha AR, Tavakoli S, Ghasemi M, Jabejdar-Maralani P, Sadeghipour H, Ebrahimi F, Dehpour AR. Endogenous cannabinoids contribute to remote ischemic preconditioning via cannabinoid CB2 receptors in the rat heart. *Eur J Pharmacol*. 2008;579:246–252.
- Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutierrez SO, van der Stelt M, Lopez-Rodriguez ML, Casanova E, Schutz G, Zieglerberger W, Di Marzo V, Behl C, Lutz B. CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science*. 2003;302:84–88.
- Wagner JA, Jarai Z, Batkai S, Kunos G. Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB(1) receptors. *Eur J Pharmacol*. 2001;423:203–210.
- Gidday JM. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci*. 2006;7:437–448.
- Hayakawa K, Mishima K, Hazekawa M, Sano K, Irie K, Orito K, Egawa T, Kitamura Y, Uchida N, Nishimura R, Egashira N, Iwasaki K, Fujiwara M. Cannabidiol potentiates pharmacological effects of Delta(9)-tetrahydrocannabinol via CB(1) receptor-dependent mechanism. *Brain Res*. 2008;1188:157–164.
- Bouchard JF, Lepicier P, Lamontagne D. Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart. *Life Sci*. 2003;72:1859–1870.
- Kreutz S, Koch M, Ghadban C, Korf HW, Dehghani F. Cannabinoids and neuronal damage: differential effects of THC, AEA and 2-AG on activated microglial cells and degenerating neurons in excitotoxically lesioned rat organotypic hippocampal slice cultures. *Exp Neurol*. 2007;203:246–257.
- Gosman-Hedstrom G, Claesson L, Klingenstierna U, Carlsson J, Olausson B, Frizell M, Fagerberg B, Blomstrand C. Effects of acupuncture treatment on daily life activities and quality of life: a controlled, prospective, and randomized study of acute stroke patients. *Stroke*. 1998;29:2100–2108.
- Mukherjee M, McPeak LK, Redford JB, Sun C, Liu W. The effect of electro-acupuncture on plasticity of the wrist joint in chronic stroke survivors. *Arch Phys Med Rehabil*. 2007;88:159–166.
- Gao H, Guo J, Zhao P, Cheng J. The neuroprotective effects of electroacupuncture on focal cerebral ischemia in monkey. *Acupunct Electrother Res*. 2002;27:45–57.
- Chuang CM, Hsieh CL, Li TC, Lin JG. Acupuncture stimulation at Baihui acupoint reduced cerebral infarct and increased dopamine levels in chronic cerebral hypoperfusion and ischemia-reperfusion injured Sprague-Dawley rats. *Am J Chin Med*. 2007;35:779–791.
- Xiong LZ. Neuroanesthesia and neuroprotection: where are we now? *Chin Med J (Engl)*. 2006;119:883–886.

**Pretreatment With Electroacupuncture Induces Rapid Tolerance to Focal Cerebral Ischemia Through Regulation of Endocannabinoid System**  
Qiang Wang, Ye Peng, Shaoyang Chen, Xingchun Gou, Bo Hu, Juan Du, Yan Lu and Lize Xiong

*Stroke*. 2009;40:2157-2164; originally published online April 16, 2009;  
doi: 10.1161/STROKEAHA.108.541490

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231  
Copyright © 2009 American Heart Association, Inc. All rights reserved.  
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://stroke.ahajournals.org/content/40/6/2157>

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

**Reprints:** Information about reprints can be found online at:  
<http://www.lww.com/reprints>

**Subscriptions:** Information about subscribing to *Stroke* is online at:  
<http://stroke.ahajournals.org/subscriptions/>