

Antidepressant, anti-inflammatory, and antioxidant effects of electroacupuncture through sonic hedgehog–signaling pathway in a rat model of poststroke depression

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Wa Cai^{1,2}
Wen Ma¹
Guan-Tao Wang¹
Yi-Jing Li¹
Wei-Dong Shen^{1,2}

¹Institute of Acupuncture and Anesthesia, Shanghai Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, People's Republic of China; ²Department of Acupuncture, Shanghai Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, People's Republic of China

Background: Poststroke depression (PSD) is the most frequent psychological sequela after stroke. Electroacupuncture (EA) treatment is effective for PSD. The study aimed at clarifying the mechanisms of EA's antidepressant effects in a PSD rat model.

Methods: We used middle cerebral artery occlusion to establish the rat model of PSD. Tests of sucrose preference and locomotor activity were performed to examine depressive-like behaviors. We measured malondialdehyde, GSH, SOD, IL6, IL1 β , TNF α , and 5HT with ELISA. The hippocampal Shh-signaling pathway was assessed by Western blot.

Results: EA significantly decreased sucrose preference and locomotor activities of PSD rats, reduced IL6, TNF α , increased GSH, and upregulated 5HT, and also slightly reduced IL1 β and malondialdehyde, all of which were measured with ELISA. The Shh-signaling pathway assessed by Western blotting was activated by EA. Those changes were inhibited by the Shh-pathway inhibitor cyclopamine.

Conclusion: EA effectively alleviated depressive-like behaviors in PSD by suppressing inflammation and oxidative stress through activation of the Shh-signaling pathway.

Keywords: electroacupuncture, poststroke depression, sonic hedgehog–signaling pathway, oxidative stress, inflammation, 5HT

Introduction

Poststroke depression (PSD) is the most frequent psychiatric consequence of stroke, with a prevalence of a third of stroke survivors.¹ PSD negatively affects stroke rehabilitation, cognitive function, and social activity, leading to increased mortality.² Research has shown that PSD is associated with lower levels of 5HT, one of the monoamines that can be significantly upregulated by antidepressants.³ Both oxidative stress and inflammation have been found to play a crucial role in the pathogenesis of PSD.⁴

Shh is a glycoprotein that is prominent in neurogenesis of neural stem cells. It binds to its receptor Ptc1 and suppresses the transducing protein Smo, which subsequently triggers the transcription factor Gli1 to promote neurogenesis, including proliferation, migration, and differentiation of cells.^{5,6} Since hippocampal neurogenesis is involved in protecting against and promoting recovery from depression,⁷ the hippocampus is important in the development of depressive-like behaviors of PSD.

Correspondence: Wei-Dong Shen
Shanghai Shuguang Hospital, 528
Zhangheng Road, Pudong Xinqu, Shanghai
201203, People's Republic of China
Tel +861 367 166 8310
Fax +86 212 025 6699
Email shenweidong_1018@163.com

Numerous studies have confirmed that electroacupuncture (EA) is an effective treatment for PSD.^{8–11} A recent meta-analysis indicated that EA caused fewer adverse events than antidepressants in treating PSD.¹² However, the mechanisms of EA treatment for PSD remain uncertain. The objective of this study was to find out whether the effectiveness of EA treatment for PSD is associated with the hippocampal Shh-signaling pathway.

Methods

Animals

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Shanghai University of Traditional Chinese Medicine and designed in accord with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (publication 8023, revised 1978). Experiments were conducted according to the National Institute of Health's Guide for the Care and Use of Laboratory Animals (publication 80–23), revised in 1996. The number of animals used and suffering caused was minimized in our experiments.

Male Sprague Dawley rats aged around 3 months were obtained from the Animal Center of Shanghai University of Traditional Chinese Medicine, Shanghai, China. Upon arrival, five rats were housed in each cage and acclimatized to a room with controlled temperature ($24\text{ }^{\circ}\text{C}\pm 2\text{ }^{\circ}\text{C}$), humidity ($60\%\pm 5\%$) and a natural 12 hr light/dark cycle. They were allowed to acclimatize to the environment for 7 days before experiments. All experimental rats (220–250 g) were allocated randomly into different treatment groups by an independent researcher. Rats in every group were mixed in behavioral tests during observation (9–11 am), and observers were blinded to group allocation. After

experiments had finished, all rats were killed by overdoses of chemical anesthetics.

Experimental protocol

In order to find the exact efficacy of EA and its associations with the Shh-signaling pathway, 48 rats were allocated randomly into six groups ($n=8$ for each group): sham, PSD, EA + PSD, EA + PSD + cyclopamine (intraperitoneal administrations of 0.2 mg/kg/day), EA + PSD + vehicle (intraperitoneal administrations of 10 μL saline/day), and fluoxetine + PSD (intra-gastric administrations of 14.4 mg/kg/day). The sham group was the comparison PSD-model group for depressive behaviors. Fluoxetine was compared to EA in treatment effectiveness. The use of cyclopamine aimed at clarifying the role of the Shh-signaling pathway. The EA + PSD + vehicle group was set as the control group of the EA + PSD + cyclopamine group to reduce minor subjective errors.

ELISA was used on antioxidants GSH, SOD, and oxidative MDA to evaluate levels of oxidative stress. Inflammatory cytokines IL1 β , TNF α , and IL6 were measured with ELISA to assess inflammation. 5HT was also measured with ELISA to evaluate antidepressant effects. Shh, Smo, Ptch, and Gli1 were measured by Western blot to elucidate the role of the Shh-signaling pathway. Our study assessed depressive-like behavior through the sucrose-preference test (SPT) and locomotor activity (Figure 1).

PSD-model establishment

Transient focal cerebral ischemia was induced by 90 mins of left middle cerebral artery occlusion (MCAO), which was followed by reperfusion. The MCAO procedure was modified by a previous

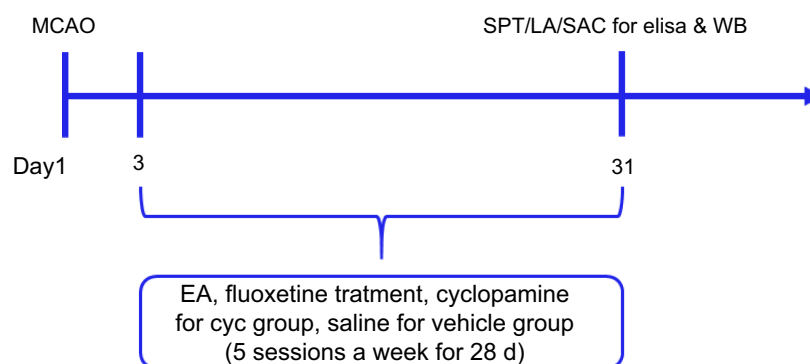


Figure 1 Protocol for the experiment. All other experimental procedures were done after the interventions. Rats were killed after the last test.

Abbreviations: SPT, sucrose-preference test; LA, locomotor activity; SAC, sacrifice; MCAO, middle cerebral artery occlusion; WB, Western blot.

method.¹³ Rats were anesthetized with 5% chloral hydrate. The left common carotid artery, external carotid artery, and internal carotid artery were exposed and isolated after a ventral neck incision. A monofilament (Sunbio Biotech, Beijing, China) was then introduced into the internal carotid artery and gently advanced to block the blood flow of the left middle cerebral artery for 90 mins. The wound was sutured shortly after completion of ischemia procedures. The same surgical procedures were done in the sham group, except that the left middle cerebral artery was not occluded. Rats were allowed 3 days' recovery before the start of any intervention.

Electroacupuncture treatment

The acupoints DU20 and DU24 were selected for EA treatment of PSD.¹⁴ Stainless-steel acupuncture needles (40×0.25 mm; Jiajian Medical Supplies Co Ltd, Wuxi, China) were inserted to a depth of about 3 mm using an electric current at a frequency of 2 Hz with an EA device (Hwato SDZ-II; Suzhou Medical Supplies, Suzhou, China) for about 30 mins per EA-treatment session.¹⁵ EA treatment lasted for a month (28 days), with 5 sessions a week.¹⁶

Behavior tests

The SPT and locomotor activity were assessed at baseline and on day 31 after treatment.

Sucrose-preference test

The SPT was conducted from 9 to 10 am to examine PSD-induced anhedonia. Rats were food- and water-deprived for 20 hrs prior to the test, followed by free access to one bottle with 2% sucrose solution (200 mL) and another bottle with water (200 mL) for 2 hrs. The sucrose-preference index was calculated:

$$\text{sucrose preference} = \frac{\text{sucrose intake (g)}}{\text{sucrose intake (g)} + \text{water intake (g)}}.$$
¹⁷

Locomotor activity

Locomotor activity was examined in a dark box (1×1 m chamber) with walls of 0.5 m height to assess rearing activity and general locomotor activity of rats. The floor of the chamber was divided into 25 equal square quadrants. Locomotor activity was measured when at least three paws were presented in a quadrant for 5 mins. Observer 2.0 software (Xinruan Information and Technology, Shanghai, China) was used to record the test.¹⁸

ELISA

Obtained from the abdominal aorta of anesthetized rats, blood was collected in anticoagulant-free tubes and quickly centrifuged to avoid glycolysis on day 31 after EA treatment. Serum samples were kept at -80 °C until assay. According to the manufacturer's instructions, IL6, TNF α , and IL1 β levels in serum were detected with ELISA kits (Xitang, Shanghai, China), and measurement of MDA (Beyotime, Haimen, China) and GSH and SOD (Qiaoyu, Shanghai, China) was performed. Serum levels of 5HT were measured with a sandwich-ELISA kit (R&D Systems).

Western blot

The brains of deeply anesthetized rats were promptly removed on day 31 after EA treatment. Ischemic hippocampi were isolated and frozen in liquid nitrogen. Total protein measurement for Shh, Gli1, Ptch1, and Smo in ischemic hippocampi (n=8 in each group) was performed in accordance with the manufacturer's instructions. With a BCA protein-assay reagent kit, concentrations of protein extracts were tested. Protein (50 μ g) were separated by SDS/PAGE and transferred to polyvinylidene difluoride membranes. Membranes loaded with proteins of interest were incubated with 5% nonfat dried milk in PBS at room temperature for 1 hr and then probed with anti-Ptch1 polyclonal antibody (1:500; Abcam), anti-Gli1 polyclonal antibody (1:500; Sigma-Aldrich), anti-Shh polyclonal antibody (1:1,000; Cell Signaling Technology) and anti-Smo monoclonal antibody (1:500; Santa Cruz Biotechnology) overnight at 4 °C, washed with 0.1% Tris-buffered saline on the second day, and then incubated with fluorescence-labeled goat antirabbit secondary antibodies for 1 hr at room temperature. Imaging densitometry (Li-Cor Biosciences) was used to analyze the relative density of each band. Changes in protein activation are presented as ratios of β -actin.

Statistical analysis

SPSS 21.0 for Windows (IBM, Armonk, NY, USA) was used for statistical analyses. All data are presented as means \pm SEM, and were analyzed with one-way ANOVA and paired *t*-tests. *P*<0.05 was considered statistically significant.

Results

EA treatment improved the depressive-like behaviors of PSD

Figure 2A shows that PSD rats had less sucrose preference than rats in the sham group (*P*<0.01). Both EA and fluoxetine

treatment significantly reversed low sucrose preference ($P<0.01$). PSD rats had significantly lower locomotor counts than sham-group rats ($P<0.001$, Figure 2B). Both EA and fluoxetine significantly increased locomotion counts ($P<0.001$). Sucrose preference and locomotor activity were assessed on day 31.

EA treatment alleviated oxidative stress and inflammation in PSD

Oxidative stress was measured through levels of GSH, SOD, and MDA on day 31 (Figure 3A–C). PSD rats had higher serum levels of MDA and lower levels of GSH than rats in

the sham group. Both EA and fluoxetine treatment slightly reduced MDA. Moreover, serum levels of GSH were elevated significantly by EA ($P<0.05$, Figure 3B). Serum levels of IL6, TNF α , and IL1 β increased significantly in PSD rats ($P<0.01$, Figure 3D–F). EA and fluoxetine alleviated inflammation in PSD via significantly decreasing serum levels of IL6 ($P<0.05$, Figure 3E) and TNF α ($P<0.001$, Figure 3F).

EA treatment regulated 5HT in PSD

As shown in Figure 4, serum levels of 5HT in PSD rats were significantly lower than sham rats ($P<0.01$, Figure 4). After treatment, 5HT levels in both the EA and fluoxetine groups were much higher than the PSD group ($P<0.01$, Figure 4).

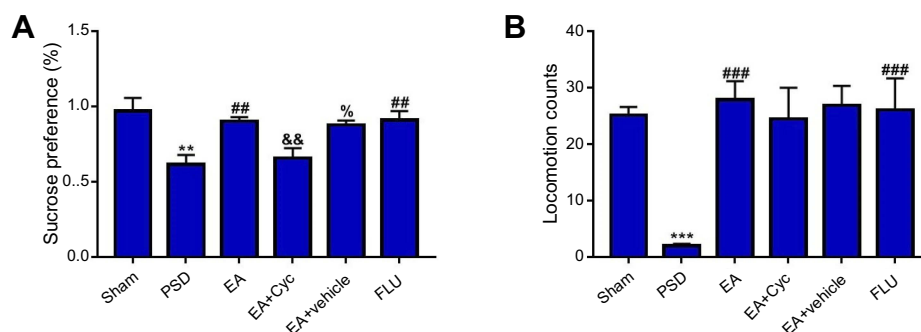


Figure 2 Improvement in depressive-like behaviors of PSD. (A) Effect of EA treatment on SPT; (B) changes in locomotion activity after EA treatment. Values are presented as means \pm SEM, with eight rats in each group. ** $P<0.01$ versus sham group; *** $P<0.001$ versus sham group; # $P<0.01$ versus PSD group; ### $P<0.001$ versus PSD group; && $P<0.01$ versus EA group; % $P<0.05$ versus EA + Cyc group.

Abbreviations: PSD, poststroke depression; EA, electroacupuncture; SPT, sucrose-preference test; Cyc, cyclopamine.

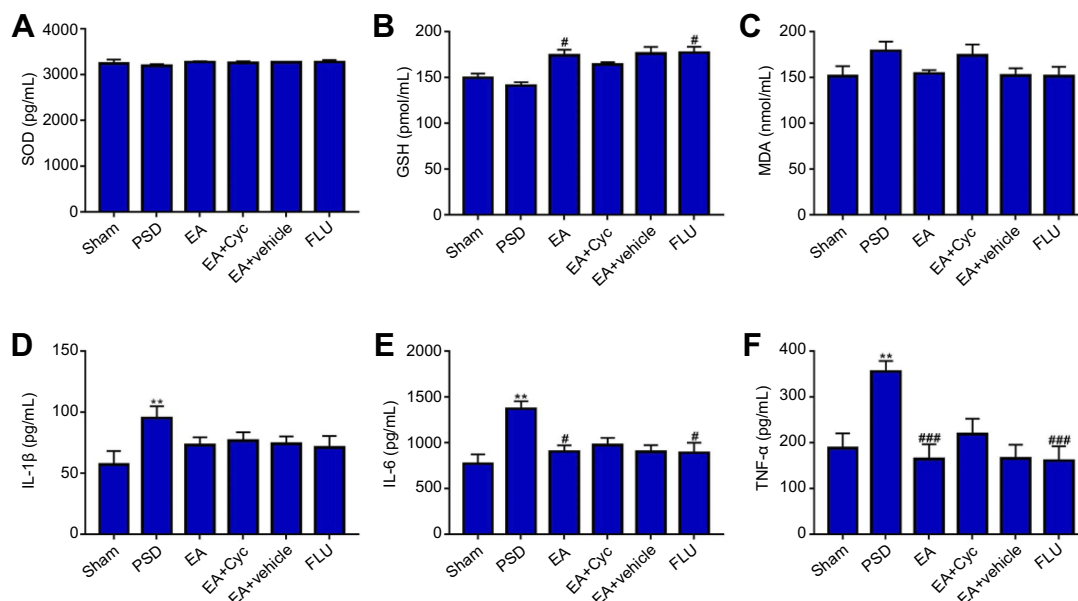


Figure 3 Alleviation of oxidative stress and inflammation. (A–F) Comparisons of SOD, GSH, MDA, IL6, TNF α , and IL1 β levels in sham, PSD, EA + PSD, EA + Cyc, EA + vehicle, and Flu groups by ELISA. Values are presented as means \pm SEM, with eight rats in each group. ** $P<0.01$ versus sham group; # $P<0.05$ versus PSD group; ### $P<0.001$ versus PSD group.

Abbreviations: MDA, malondialdehyde; PSD, poststroke depression; EA, electroacupuncture; Cyc, cyclopamine; Flu, fluoxetine.

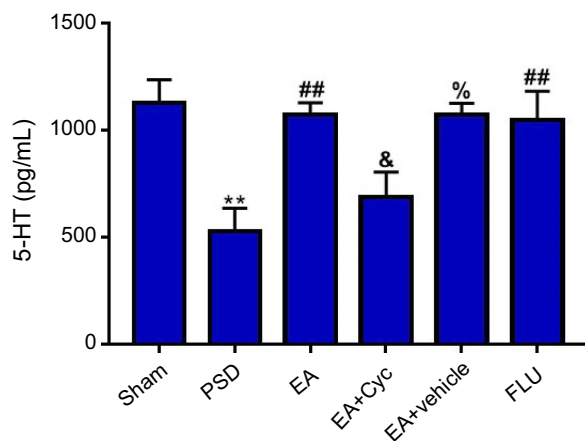


Figure 4 Changes in 5HT levels. Comparison of 5HT levels in sham, PSD, EA + PSD, EA + Cyc, EA + vehicle, and Flu groups by ELISA. Values are presented as means \pm SEM, with eight rats in each group. ** $P < 0.01$ versus sham group; ### $P < 0.05$ versus PSD group; & $P < 0.05$ versus EA group; % $P < 0.05$ versus EA + Cyc group. **Abbreviations:** PSD, poststroke depression; EA, electroacupuncture; Cyc, cyclopamine; Flu, fluoxetine.

EA treatment regulated Shh-signaling pathway in PSD

Expressions of Shh, Gli1, Smo, and Ptch1 proteins were measured through immunoblot analysis on day 31. Ratios of Shh, Gli1, Smo, and Ptch1 to β -actin in the PSD group were much lower than those in the sham group ($P < 0.05$ [Figure 5B and D], $P < 0.01$ [Figure 5C and E]). After EA and fluoxetine treatment, expression of all proteins had increased significantly ($P < 0.01$ [Figure 5C], $P < 0.05$

[Figure 5E]). The Gli1: β -actin ratio had also declined significantly after cyclopamine intervention ($P < 0.05$, Figure 5C). Shh, Gli1, Smo, and Ptch1 upregulation by EA was found to be inhibited by cyclopamine, an antagonist of the Shh-signaling pathway (Figure 5). Meanwhile, cyclopamine slightly reversed the upregulation of GSH and downregulation of MDA and IL6, TNF α , and IL1 β by EA (Figure 3). Compared to the EA group, 5HT in the EA + cyclopamine group was significantly decreased (Figure 4), with more serious depressive-like behaviors ($P < 0.01$, Figure 2A and B).

Discussion

PSD is the most frequent psychological sequela after stroke, and comes with neuronal injury, oxidative stress, and inflammation.^{18–20} The MCAO model is commonly used to induce ischemic brain injury in rats. Researchers have found that reduction in sucrose consumption and increase in immobility time in behavior tests are significant 2 weeks after MCAO surgery, indicating that the depressive-like behaviors are delayed effects of MCAO,²¹ which was also confirmed in our study. Although some results in our study, including behavior tests, Western blot, and ELISA, showed only a tendency toward alterations without statistical significance, which could be explained by some significant biochemical changes occurring prior to day 31, these tendencies were

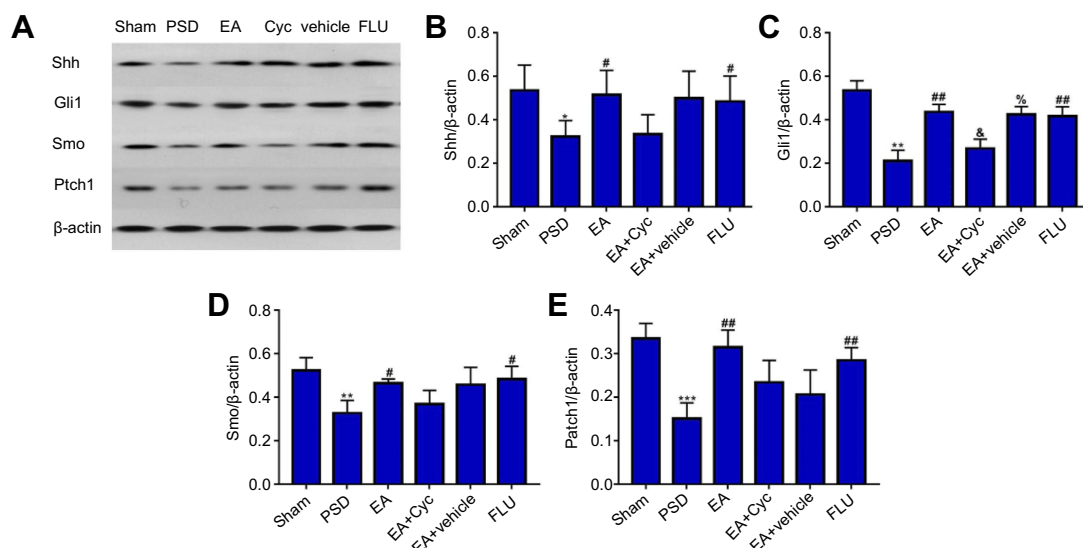


Figure 5 Regulation of Shh-signaling pathway. (A) Immunoblot analysis for expression of Shh, Gli1, Smo, Ptch1, and β -actin in sham, PSD, EA + PSD, EA + Cyc, EA + vehicle, and Flu groups. (B–E) Comparisons of Shh, Gli1, Smo, and Ptch1 levels in six groups by Western blot. Values are presented as means \pm SEM, with eight rats in each group. * $P < 0.05$ versus PSD group; ** $P < 0.01$ versus PSD group; *** $P < 0.001$ versus PSD group; # $P < 0.05$ versus PSD group; ## $P < 0.01$ versus PSD group; % $P < 0.05$ versus EA group; & $P < 0.05$ versus the EA + Cyc group.

Abbreviations: PSD, poststroke depression; EA, electroacupuncture; Cyc, cyclopamine; Flu, fluoxetine.

consistent with one another, as well as with findings of previous studies.

EA has been found to resist oxidation by reducing oxidants like reactive oxygen species (ROS), nitric oxide, inducible nitric oxide synthase, and COX2 in brain tissue after ischemic stroke²² and chronic unpredictable stress.²³ Inflammatory cytokines, including IL1 β , TNF α , and IL6 are reduced with EA in stroke survivors²⁴ and depression patients.²⁵ Significantly, our study had consistent findings: EA was found to resist oxidation effectively by reducing MDA and increasing GSH in brain tissue of PSD rats. IL1 β , TNF α , and IL6 were found to be reduced by EA. Improvements in depressive-like behavior tests (SPT and locomotor activity) and upregulation of 5HT levels verified the antidepressant effect of EA, accompanied by the anti-inflammatory and antioxidant effects of EA in PSD.

Most strokes are reportedly ischemic and lead to brain injury via oxygen and glucose,^{26,27} resulting in a loss of antioxidant defense and overproduction of ROS.²⁸ Depression is also related to increased oxidative stress and inhibition of antioxidant defense systems, causing neuronal damage.²⁹ Recent research has indicated that the Shh-signaling pathway is involved in cerebral ischemia and the underlying mechanisms associated with counteracting excessive ROS production.³⁰ It was found that Shh signaling reduced the detrimental effect of H₂O₂-like decreasing neuronal viability. Shh increased the activities of GSH-Px and attenuated the formation of MDA in H₂O₂-treated neurons. Shh plays a significant role in promoting neuritic outgrowth in H₂O₂-treated neurons in an in vitro model. Potential mechanisms of antioxidant effects by Shh may be associated with preventing mitochondrial dysfunction, inhibiting ROS release, and promoting the production of ATP.³¹ As the inhibitor of Smo in the Shh pathway, cyclopamine enhances the formation of MDA and reduces the activities of GSH-Px, Gli1, and Ptch1, which are critical effectors in the Shh pathway. A rat model of cerebral ischemia found that suppression of the Shh-signaling pathway aggravated ischemic injury.³²

Many clinical studies have found that inflammation plays a crucial role in the pathogenesis of PSD.^{33–35} Notably, mutual interactions have been found between inflammation and oxidative stress. The Shh-signaling pathway can cope with inflammatory reactions,^{32,36,37} plays a part in ROS overproduction, and is regulated by brain injury-related inflammation.³⁸ Cyclopamine decreases Gli expression and reduces proliferation of astrocytes in the injured cortex.³⁸

Composed of astrocytes and capillary endothelial cells, blood–brain barrier (BBB) function is affected in stroke. Research has indicated that BBB dysfunction activates the Shh pathway during inflammation^{39–42} and that Shh released from astrocytes maintains the integrity of the BBB.⁴³ IL1 β was found to induce BBB disruption by downregulating Shh in astrocytes. Moreover, cross talk was found between the Wip1 and Shh pathways. Wip1 alleviated inflammation and BBB dysfunction via Shh signaling, which was increased by Wip1 overexpression and repressed by silencing of Wip1, indicating that Shh can interact with Wip1 to relieve inflammation and BBB disruption.⁴⁰

Researchers have reported that the antidepressant effects of fluoxetine were mediated by increased neurogenesis of the dentate gyrus (DG) of the adult hippocampus.⁴⁴ Impairment of DG neurogenesis might be one of the etiological factors of PSD. Cerebral ischemia triggered neurons generated to migrate from the subgranular zone into the granule-cell layer of the DG.^{45,46} Wang et al⁴⁷ found that ischemia hippocampal neurogenesis was inhibited in PSD rats, suggesting an interaction between depressive-like behaviors and ischemia-stimulated neurogenesis. Shh signaling can mediate the differentiation and proliferation of adult hippocampal neural progenitor cells (NPCs). If hippocampal NPCs are isolated from the hippocampus, Shh stimulates their proliferation,⁴⁸ whereas cyclopamine inhibits it. Harbored by adult hippocampus in the subgranular zone of the DG, NPCs can generate neurons that integrate into the DG and receive synaptic inputs from other hippocampal neurons.⁴⁹ Since deletion of Shh or Smo in nestin-expressing NPCs leads to reduced neurogenesis, Shh signaling is indispensable in hippocampal neurogenesis in adult rats.⁵⁰ Furthermore, due to the high prevalence of cognitive impairments in late-life depression, these were included in the diagnostic criteria of depressive-like disorders in the current *Diagnostic and Statistical Manual of Mental Disorders 5* classification.⁵¹ Studies have found that changes in the hippocampus were closely associated with cognitive impairments,^{52,53} which led to delayed neurological recovery time and negatively affected other depressive-like symptoms of stroke survivors, lowering the life quality of PSD patients.⁵⁴ Future research should be done to find associations between the hippocampal Shh-signaling pathway and cognitive impairments in PSD.

In our study, the significant upregulation of Shh, Gli1, Smo, and Ptch1 in rat hippocampi in the EA group and

fluoxetine group suggested that EA and fluoxetine activated the Shh-signaling pathway, while cyclopamine counteracted it. Additionally, anti-inflammatory and antioxidant effects of EA were inhibited by cyclopamine, consequently reversing the upregulation of 5HT by EA and aggravating depressive-like behaviors of PSD. Interestingly, cyclopamine significantly inhibited EA-mediated increases in sucrose preference, but no significant change was found in locomotor activity, which indicated that inhibiting the Shh-signaling pathway might have more association with anhedonia than with poor motivation. Our study found that the underlying mechanisms of EA's antidepressant, anti-inflammatory, and antioxidant effects on PSD were closely associated with activation of the Shh-signaling pathway.

Conclusion

This study aimed at clarifying potential mechanisms of EA treatment for PSD. We found that EA can effectively relieve depressive-like behaviors of PSD by suppressing inflammation and oxidative stress via activation of the hippocampal Shh-signaling pathway, suggesting that EA can be an effective treatment for PSD. Further research is needed to explore whether EA is associated with hippocampal neurogenesis mediated by Shh signaling.

Abbreviation list

EA, electroacupuncture; GSH, glutathione; LA, locomotor activity; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; NPCs, neural progenitor cells; PSD, poststroke depression; SPT, sucrose-preference test; WB, Western blot.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of Shanghai University of Traditional Chinese Medicine and performed according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (publication 8023, revised 1978).

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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