

# Electroacupuncture attenuates learning and memory impairment via activation of $\alpha 7$ nAChR-mediated anti-inflammatory activity in focal cerebral ischemia/reperfusion injured rats

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**Abstract.** Studies have reported that electroacupuncture (EA) may reduce learning and memory impairment following cerebral ischemic injury. However, the precise mechanism of action remains unclear. In the present study, the attenuation of focal cerebral ischemia/reperfusion injury by EA in rats was investigated. EA at the Baihui (DU 20) and Shenting (DU 24) acupoints was demonstrated to significantly improve performance in the Morris water maze task, with shortened latency time and increased frequency of passing the platform. Molecular analysis revealed that EA activated the expression of  $\alpha 7$  nicotinic acetylcholine receptors ( $\alpha 7$ nAChR) in the hippocampus. In addition, EA led to a decreased expression of the microglia/macrophage marker Iba1 and the astrocyte marker glial fibrillary acidic protein in the hippocampus. EA treatment also led to decreased production of the inflammatory cytokines tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ . Treatment with methyllycaconitine, an  $\alpha 7$ nAChR antagonist, attenuated the improvement of learning and memory following EA treatment and the inhibitory effects of EA on glial cell activation and inflammatory cytokine production. In conclusion, the findings of the present study demonstrate that EA is able to improve learning and memory function following cerebral ischemic injury via activation of  $\alpha 7$ nAChR, which significantly decreases the neuroinflammatory response.

## Introduction

Stroke is one of the most common causes of mortality and a leading cause of disability worldwide (1). Survivors are typically afflicted by certain levels of functional impairment, including motor, sensory and cognitive dysfunction (2-4). Evidence suggests that up to 64% of patients exhibit a certain degree of cognitive impairment, with 20-30% of patients demonstrating dementia at 3 months following stroke (5-7). Learning and memory deficits are among the most common cognitive impairments and severely affect patients' daily activities and quality of life, which leads to them becoming a significant burden on their families and society (8,9).

Acupuncture is one of the most commonly used and important therapies in traditional Chinese medicine and has been applied clinically for over a millennium (10). Electroacupuncture (EA), a combination of electrical stimulation with acupuncture, has demonstrated efficacy in alleviating cognitive impairments and improving learning and memory in patients and animal models post-stroke (11-14). However, the exact mechanism of its benefits on impaired cognition remains unclear.

Neuroinflammation is a prime pathological factor in stroke, involving a number of complex cellular processes and activities, including astrocyte and microglial proliferation and the production of inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  (15,16). Previous animal studies suggested a mechanistic link between neuroinflammation and cognitive function following traumatic brain injury (TBI) and also in Alzheimer's disease (AD) models (17-19). In addition, hippocampal neuroinflammation is responsible for mediating cognitive dysfunction in the aging brain, in post-cerebral ischemia, and also in AD (20-22).

Cholinergic anti-inflammatory pathways are critical regulators of inflammation. These pathways primarily involve the interaction of vagus nerve cholinergic signaling with  $\alpha 7$  nicotinic acetylcholine receptors ( $\alpha 7$ nAChRs) on immune cells, which leads to inhibition of pro-inflammatory cytokine production, thereby preventing excessive inflammatory responses (23). The  $\alpha 7$ nAChR serves as an important

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signaling receptor in cholinergic anti-inflammatory pathways and is closely associated with learning and memory (24-26). Counteracting neuroinflammatory processes via cholinergic anti-inflammatory signaling prevents progressive tissue damage in the brain following stroke (27). EA is known to act in a similar manner by inhibiting neuroinflammation and preventing development of cognitive dysfunction in patients post-stroke (14,28). In addition, a previous study demonstrated that treatment with EA prior to ischemia/reperfusion (I/R) injury is neuroprotective because EA prevents the downregulation of  $\alpha 7$ nAChR in neurons within the ischemic penumbra (29). The present study aimed to elucidate whether EA ameliorates learning and memory through  $\alpha 7$ nAChR-mediated inhibition of neuroinflammation in a rat model of focal cerebral I/R injury.

## Materials and methods

**Animals.** A total of 65 male Sprague-Dawley (SD) rats (250-280 g, ages 10-12 weeks) were provided by Shanghai SLAC Laboratory Animal Co., Ltd. [Laboratory Animal Use Certificate no. SCXK (SH) 2012-0002] and housed under controlled conditions with a 12-h light/dark cycle,  $22 \pm 2^\circ\text{C}$  temperature and  $55 \pm 15\%$  humidity for at least 1 week prior to surgery and treatment. All animals were allowed *ad libitum* access to standard rodent food and water. All animal treatments and experiments were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (FUTCM; Fujian, China).

**Cerebral I/R injury model.** Transient focal cerebral ischemia was established via middle cerebral artery occlusion (MCAO) as previously described (30) in 55 rats. Briefly, rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (3 ml/kg body weight; catalogue no. 30037516; Changzhou Haituo Experimental Instrument Co., Ltd., Changzhou, China) diluted with 0.9% saline in a sterilized surgical site. Following a midline incision in the neck, the left common carotid artery (CCA), left external carotid artery (ECA) and the internal carotid artery (ICA) were carefully exposed and dissected. The left middle cerebral artery (MCA) was occluded by introducing an embolus through the ICA. To occlude the origin of the left MCA, a nylon filament ( $0.36 \pm 0.02$  tip diameter; Guangzhou Jialing Co. Ltd., Guangzhou, China) was inserted ( $\sim 20$  mm) from the ECA and ICA into the MCA. Reperfusion was accomplished by withdrawing the filament after 2 h of occlusion-mediated ischemia. The incision was sutured and sterilized. During and following surgery, the internal temperature of the animals was maintained at  $37^\circ\text{C}$  using a heating pad. For rats in the control group ( $n=10$ ), the arteries were similarly exposed, but not immobilized in a blinded manner as previously described (30). The neurological deficits of the rats were scored as follows: A score of 0 represented no neurological deficit, 1 indicated mild deficits (failure to fully extend the right forepaw), 2 (circling to the right) and 3 (falling to the right) indicated moderate deficits, and a score of 4 represented severe deficits (complete loss of walking ability). Rats

that received MCAO and a score of 0 or 4 were excluded from the experiment.

**Groups.** When the MCAO model was established, the rats were randomly assigned into four groups according to neurological deficit scores: i) MCAO group ( $n=13$ ); ii) MCAO + EA group (EA group,  $n=13$ ); iii) MCAO + EA + normal saline (EA + NS group,  $n=12$ ) and iv) MCAO + EA + methyllycaconitine group (EA + MLA group,  $n=12$ ). Control rats received surgery without artery ligation ( $n=10$ ). Therefore, there were five groups in total in the present study.

**EA treatment.** Rats were administered EA for 30 min daily for 7 days, starting 2 days after I/R surgery. The acupuncture needles (0.3 mm diameter) were inserted at a depth of 2-3 mm into the Baihui (DU 20) and Shenting (DU 24) acupoints, which are commonly used to treat post-stroke cognitive impairment in China (31). Electrical stimulation was then generated using the EA apparatus (Model G6805; Shanghai Medical Instrument Factory, Shanghai, China) with disperse-dense waves of a frequency of 2-10 Hz and an intensity of 2-4 mA. The rats in the control and MCAO groups remained in their cages without special intervention.

**Drug administration.** Methyllycaconitine (MLA; 5 mg/kg; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was diluted in 0.9% saline and administered intraperitoneally 30 min prior to each EA treatment (32). In the EA + NS group, 0.9% saline was injected, with a volume equivalent to that of the methyllycaconitine solution.

**Morris water maze.** Rats were subjected to the Morris water maze 3 days following surgery to assess spatial learning and memory as previously described (33). The water maze apparatus (Chinese Academy of Sciences, Beijing, China) consisted of a circular, black-painted pool (diameter, 120 cm; depth, 50 cm) filled with water tinted with black ink (depth, 30 cm; temperature,  $26 \pm 2^\circ\text{C}$ ). The tank was divided into four equal quadrants and a video camera attached to a computer was placed above the center of the tank to record the rats. A fixed, 6-cm platform was submerged 2 cm below the surface of the water. A number of visual cues were placed in each quadrant. During the first set of trials, each rat was placed in the water at four equidistant locations to the platform. When the rats arrived at the platform and remained on it for 3 sec they were considered to have found the platform. When the rats were unable to find the platform within 90 sec, they were placed on the platform for 10 sec and the time score was 90 sec. The latency time to find the submerged platform and the total swimming distance were recorded for 4 days.

Rats were subjected to the Morris water maze test with the platform removed 7 days following surgery. Rats were placed in the quadrant located diagonally from the target quadrant and allowed to swim for a maximum of 90 sec. The frequency of swimming across the former location of the platform in the target quadrant was recorded.

**Immunohistochemistry.** Rats were anesthetized with 10% chloral hydrate by intraperitoneal injection and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde

through the left ventricle. The brain was removed and fixed in 4% paraformaldehyde at 4°C for 24 h. Samples underwent dehydration with an ethanol gradient, 70, 80 and 90% for 1 h each and 100% ethanol for 20 min, 1 h and a final 20 min. Following washing with xylene, 20 min and 1 h followed by 20 min, the specimens were embedded in paraffin and cut into 5- $\mu$ m sections (RM2235 slice machine; Leica Microsystems GmbH, Wetzlar, Germany). Following deparaffinization, antigen retrieval was accomplished by immersing and boiling the sections in a Tris-EDTA Buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween-20, pH 9.0; for  $\alpha$ 7nAChR) or citrate buffer solution [10 mM Tris, 20% citrate, pH 6.0; for glial fibrillary acidic protein (GFAP) and microglial marker Iba1] in a microwave oven.

$\alpha$ 7nAChR, GFAP and Iba1 levels were analyzed using immunohistochemistry assay kits [diaminobenzidine (DAB) kit-0017; Maixin-Bio, Fujian, China] according to the manufacturer's protocol. Primary antibodies binding to  $\alpha$ 7nAChR (cat. no. ab24644; 1:100; Abcam, Cambridge, UK), GFAP (cat. no. 3670; 1:500; Cell Signaling Technologies, Inc., Danvers, MA, USA), and Iba1 (cat. no. NB100-1028; 1:250; Novus Biologicals, LLC, Littleton, CO, USA) were incubated with the sections at 4°C overnight and then the sections were incubated with secondary antibodies, provided in the DAB kit-0017, incubated at room temperature for 10 min. The positive cells were stained brown with DAB. Images were captured using a fluorescence microscope (DFC310 FX; Leica Microsystems GmbH) at x400 magnification. Positive cells were counted in four randomly selected microscopic fields using the Motic Med 6.0 CMIAS pathology image analysis system (Beihang Motic Inc., Beijing, China).

**Western blot analysis.** Hippocampal tissues were homogenized in non-denaturing lysis buffer (Beyotime Institute of Biotechnology Co., Ltd., Beijing, China; no. P0013B). Tissues were then ground on ice and incubated for 30 min prior to 10 min centrifugation at 1,465  $\times$  g at 4°C to separate the supernatant. Total protein in each sample was measured using the bicinchoninic acid (BCA) assay. A total of 50  $\mu$ g protein was separated on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked for 2 h with blocking buffer (P0023B; Beyotime Institute of Biotechnology Co., Ltd.) and then incubated with primary antibodies targeting  $\alpha$ 7nAChR (cat. no. ab24644; 1:10,000; Abcam), TNF- $\alpha$  (cat. no. 3707; 1:500; Cell Signaling Technologies, Inc.), IL-1 $\beta$  (1:200; sc-12742; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or GAPDH (cat. no. ab8245; 1:8,000; Abcam) at 4°C overnight. Following washing with TBS containing 0.05% Tween-20, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody (cat. no. 7076 for GAPDH; and cat. no. 7074 for  $\alpha$ 7nAChR, TNF- $\alpha$  and IL-1 $\beta$ ; 1:5,000; Cell Signaling Technologies, Inc.) for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (Beyotime Institute of Biotechnology, Co., Ltd.) and images were obtained and analyzed using a Bio-Image Analysis System, version 1.42q (Bio-Rad Laboratories, Hercules, CA, USA). The optical densities of the target protein were normalized against the GAPDH band and the analysis was replicated 3 times.

**Statistical analysis.** The experimental results for each group are expressed as the mean  $\pm$  standard error of the mean. Statistical analysis was performed with one-way analysis of variance using the SPSS package for Windows (Version 18.0; SPSS, Inc., Chicago, IL, USA), homogeneity of variance with a LSD test, and heterogeneity of variance with Games-Howell (A) test.  $P < 0.05$  was considered to represent a statistically significant difference.

## Results

**EA reduces learning and memory impairment following focal cerebral ischemic injury.** Following modeling, the rats in the control group were of good health with no fatalities. In the model group, 3 rats died due to epilepsy. A further 3 mortalities were observed in the EA group, 2 in the EA + NS group and 2 in the EA + MLA group. Concerning the Morris water maze performance, as presented in Fig. 1, no significant differences were detected in swimming speed on days 3, 4, 5, 6 and 7 following MCAO among the five groups (Fig. 1A;  $P > 0.05$ ). This result indicates that the MCAO model did not affect rat motor function in the Morris water maze. Rats in the MCAO group demonstrated a longer latency time (Fig. 1B) to reach the hidden platform and passed the platform position fewer times (Fig. 1C and D) in the water maze tests than did those in the control group. In EA-treated rats, latency time was significantly reduced and frequency in passing the platform was increased compared with that in the MCAO group (Fig. 1B and C;  $P < 0.05$ ). However, rats treated with EA + MLA demonstrated prolonged latency with a decreased number of times crossing the platform compared with the EA + NS group.

**EA activates  $\alpha$ 7nAChR expression in the hippocampus.** As indicated in Fig. 2, immunohistochemical analysis revealed a significant reduction in the number of  $\alpha$ 7nAChR-positive cells in the CA1 region of the hippocampus in the MCAO group compared with the control group ( $P < 0.05$ ). However, EA reversed the  $\alpha$ 7nAChR reduction caused by I/R injury. Furthermore, MLA decreased  $\alpha$ 7nAChR expression levels compared with those in the EA + NS group.

**EA suppresses neuroinflammation via  $\alpha$ 7-dependent cholinergic pathways in the hippocampus.** Cerebral ischemic injury triggers a serious inflammatory response in the brain. As presented in Fig. 3, elevated expression of the astrocyte marker GFAP and microglia/macrophage marker Iba1 was detected in the hippocampus in the MCAO group compared with the control group. Western blot analysis of  $\alpha$ 7nAChR was consistent with the immunohistochemical analysis, and confirmed that the EA-induced increase in  $\alpha$ 7nAChR was attenuated by MLA (Fig. 4A and B). Furthermore, elevated expression of the key inflammatory factors TNF- $\alpha$  and IL-1 $\beta$  (Fig. 4A and C) was detected in the hippocampus in the MCAO group compared with the control group. In the EA treatment group, Iba1, GFAP and inflammatory factors were significantly reduced compared with those in the MCAO group (Figs. 3C and 4C;  $P < 0.05$ ). However, those inhibitions were markedly eradicated in the EA + MLA group compared with the EA + NS group.

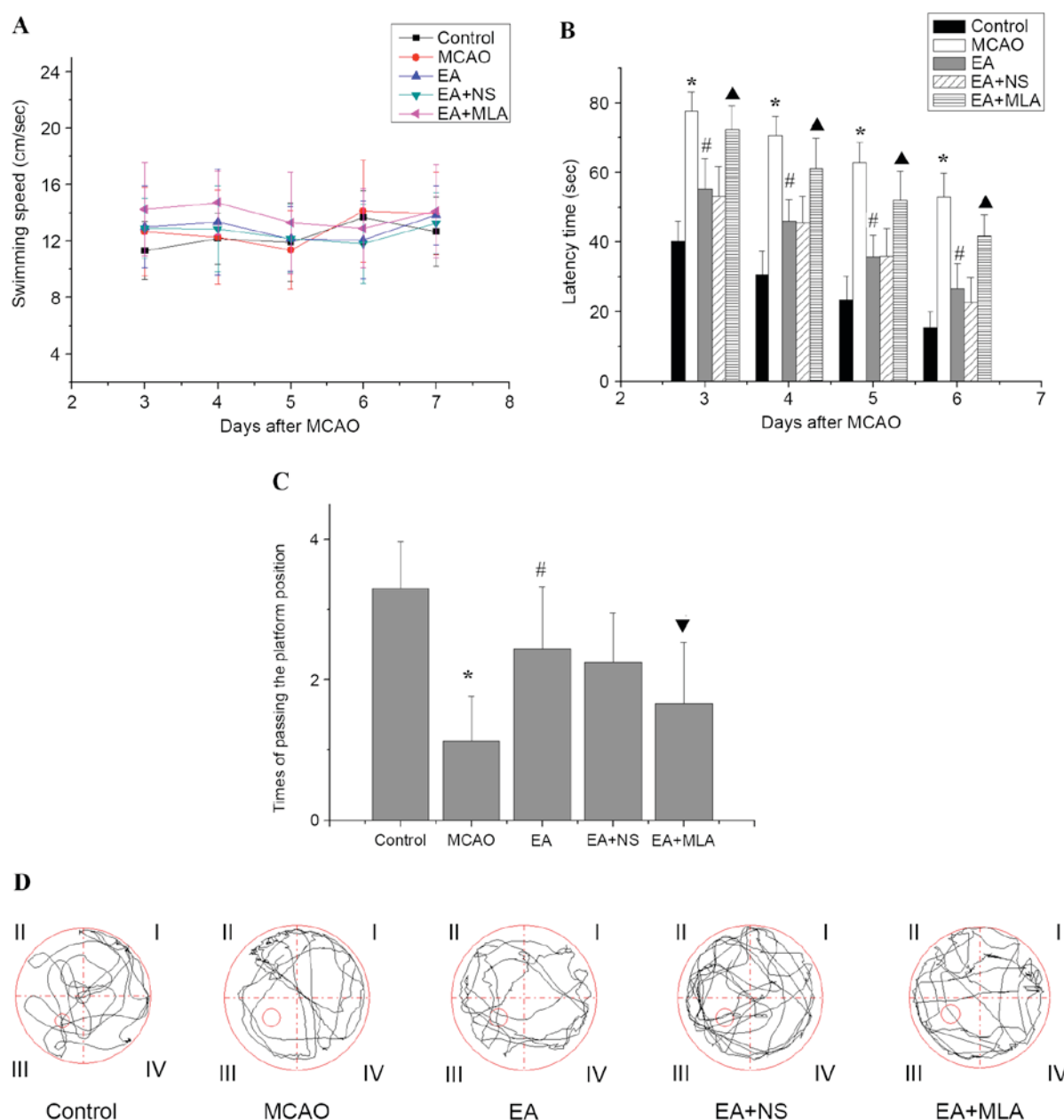


Figure 1. Effects of EA on the learning and memory of transient focal cerebral ischemic injured rats in the water maze. (A) No significant differences in swimming speed were observed among the control (n=10), MCAO (n=8), EA (n=9), EA + NS (n=9) and EA+ MLA (n=9) groups up to day 7. (B) The latency time to reach the hidden platform, (C) the number of times the rats passed the platform position and (D) typical traces during the test. \*P<0.01 vs. control, #P<0.01 vs. MCAO, ▲P<0.01 vs. EA + NS and ▼P<0.05 vs. EA + NS. Data are expressed as mean  $\pm$  standard error of the mean. EA, electroacupuncture; MCAO, middle cerebral artery occlusion; NS, normal saline; MLA, methyllycaconitine.

## Discussion

In the present study, EA was demonstrated to reduce learning and memory deficits via activation of  $\alpha 7$ nAChR-dependent anti-inflammatory pathways. In the Morris water maze assessment, the rats demonstrated a prolonged latency to find the hidden platform and decreased times of passing the platform position following MCAO treatment. In rats treated with EA, latency was shortened and the platform crossing time was increased. These results suggest that EA at the DU 20 and DU 24 acupoints improved learning and memory ability in cerebral ischemia-injured rats, which is consistent with previous studies (33,34).

A large hippocampal neuroinflammatory response was observed following MCAO, and EA reduced this inflammatory response as demonstrated by the reduction of Iba1 and GFAP expression and TNF- $\alpha$  and IL-1 $\beta$  production in the injured hippocampus. The histological evidence further suggests that  $\alpha 7$ nAChR mediates the reduction of neuroinflammation. It is known that  $\alpha 7$ nAChR is essential for the cholinergic anti-inflammatory response because loss of the  $\alpha 7$  nicotinic receptor subunit fails to inhibit cytokine synthesis (35). Studies indicate that two critical signaling pathways, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and janus kinase/signal transducer and activator of transcription (Jak/STAT), are required for the  $\alpha 7$ nAChR-dependent anti-inflammatory response. Activation



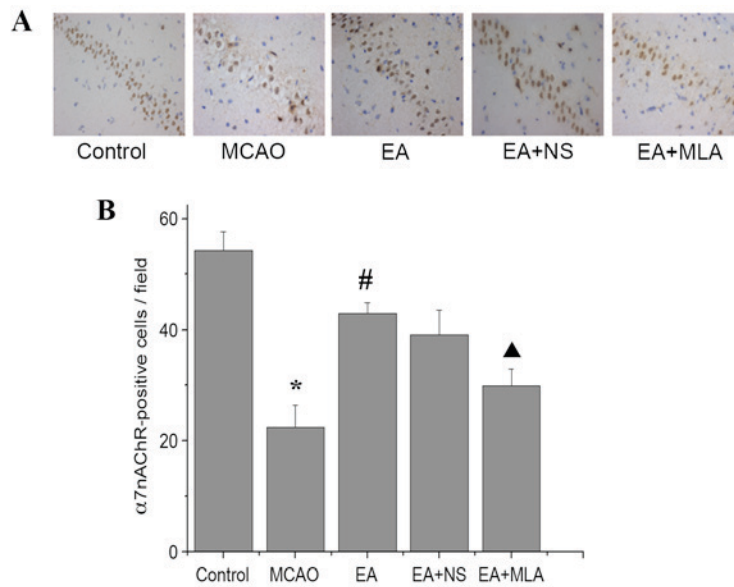


Figure 2. EA increases the expression of  $\alpha 7$ nAChR in the hippocampus. Distribution of  $\alpha 7$ nAChR-positive cells in the CA1 region of the hippocampus in the five groups stained with DAB (A) observed under a microscope (magnification, x400) and (B) quantified using an image analysis system. \* $P < 0.01$  vs. control, # $P < 0.01$  vs. MCAO and ▲ $P < 0.01$  vs. EA + NS. Data are expressed as mean  $\pm$  standard error of the mean (n=5). EA, electroacupuncture;  $\alpha 7$ nAChR,  $\alpha 7$  nicotinic acetylcholine receptor; MCAO, middle cerebral artery occlusion; NS, normal saline; MLA, methyllycaconitine; DAB, 3,3'-Diaminobenzidine.

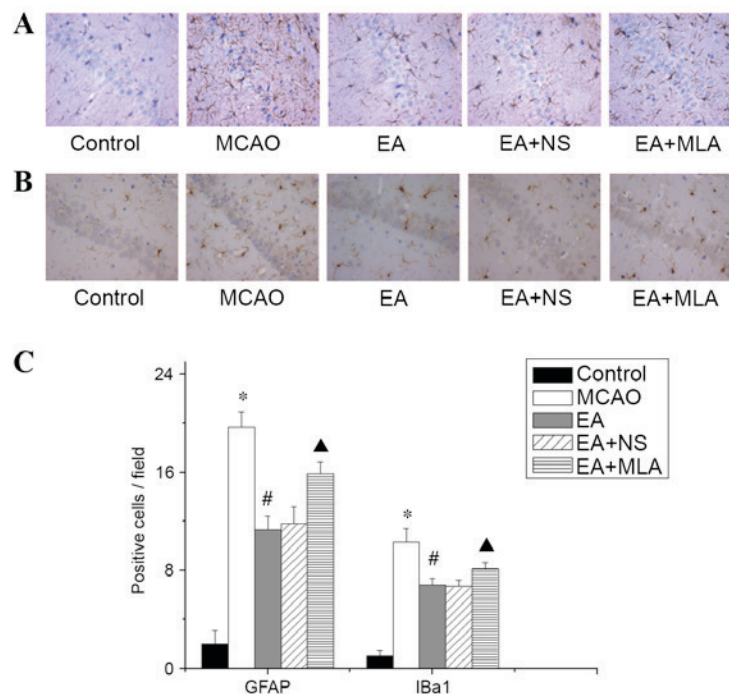


Figure 3. EA reduces the activity of microglia and astrocytes in the hippocampus. (A) GFAP-positive astrocytes and (B) Iba1-positive microglia in the CA1 region of the hippocampus in the five groups, stained with DAB. (C) Quantified data for the five groups. \* $P < 0.01$  vs. control, # $P < 0.01$  vs. MCAO and ▲ $P < 0.01$  vs. EA + NS. Data are expressed as mean  $\pm$  standard error of the mean (n=5). Magnification of x400. EA, electroacupuncture; GFAP, glial fibrillary acidic protein; Iba1, microglial marker; MCAO, middle cerebral artery occlusion; NS, normal saline; MLA, methyllycaconitine; DAB, 3,3'-Diaminobenzidine.

of the  $\alpha 7$ nAChR prevents Ik-B breakdown and promotes p65 nuclear translocation to suppress the transcription of inflammatory cytokines (36,37).  $\alpha 7$ nAChR may recruit the tyrosine kinase Jak2, and activate the transcription factor STAT3 to inhibit pro-inflammatory gene transcription (38). Previous research reports that EA may suppress NF- $\kappa$ B activation to induce an anti-inflammation response (39) and enhance STAT3 activation (40).

Global cerebral ischemia diminishes the capacity of the cholinergic anti-inflammatory pathway to control inflammation, but the application of an  $\alpha 7$ nAChR agonist protects against ischemia-induced cell death and inflammation in the hippocampus. This significantly decreases the mRNA expression of proinflammatory cytokines and reduces microglial activation, but not astrocyte activation (41,42). Conversely, treatment with an  $\alpha 7$ nAChR antagonist

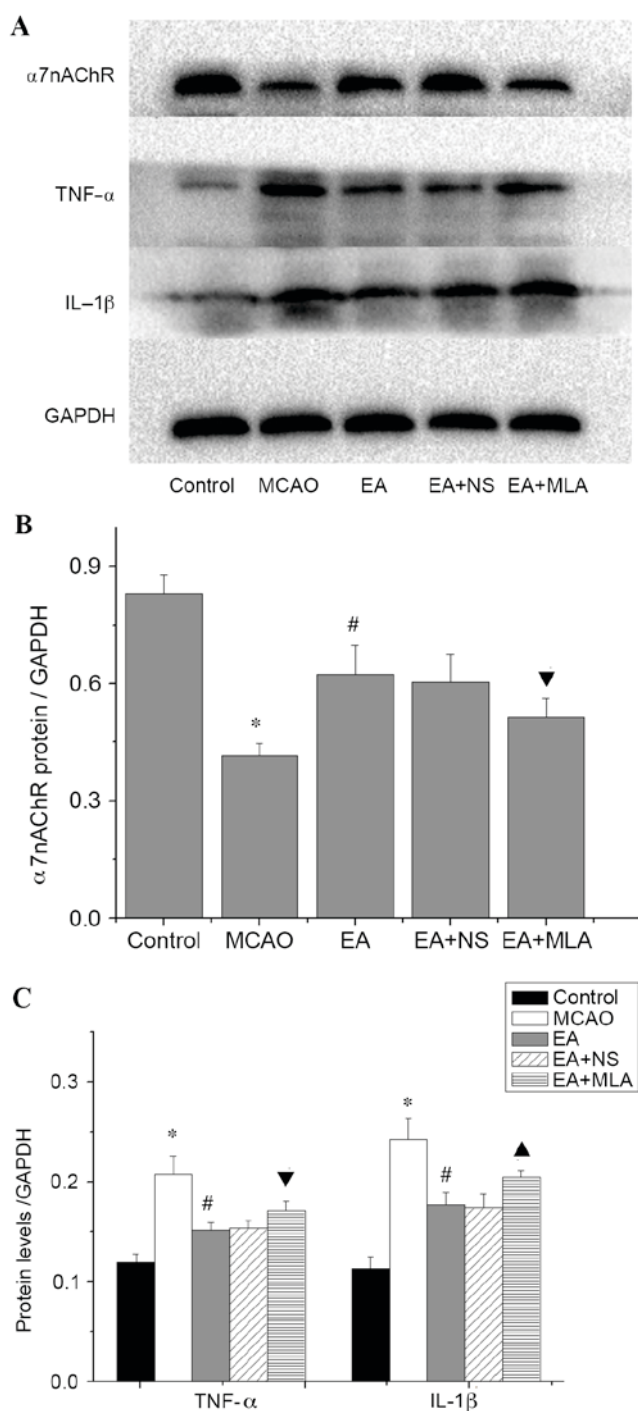


Figure 4. Western blot analysis of inflammatory protein expression in the hippocampus. (A) Western blot analysis demonstrating the concentrations of  $\alpha 7$ nAChR, TNF- $\alpha$ , IL-1 $\beta$  and GAPDH. (B) Quantification of the western blots for the levels of  $\alpha 7$ nAChR and (C) proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in the five groups. \* $P < 0.01$  vs. control, # $P < 0.01$  vs. MCAO, ▲ $P < 0.01$  vs. EA + NS and ▼ $P < 0.05$  vs. EA + NS. Data are expressed as mean  $\pm$  standard error of the mean ( $n = 5$ ).  $\alpha 7$ nAChR,  $\alpha 7$  nicotinic acetylcholine receptors; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCAO, middle cerebral artery occlusion; EA, electroacupuncture; NS, normal saline; MLA, methyllycaconitine.

following global cerebral ischemia worsened neuronal death and led to increased microglial activation (41,42). In a mouse model of Parkinson's disease, an  $\alpha 7$ nAChR agonist decreased the activation of astrocytes and microglia in the substantia nigra, and these protective effects were abolished

by administration of an  $\alpha 7$ nAChR-selective antagonist *in vitro* and *in vivo* (43). The current study indicates that the neuroprotective effects of EA are due to its actions as a  $\alpha 7$ nAChR agonist. Future studies may involve investigating an  $\alpha 7$ nAChR agonist in comparison with EA to validate this hypothesis.

Another potential mechanism for the action of EA-induced improvements in memory function involves effects on synaptic plasticity. Neuroinflammation has been confirmed to have a negative effect on learning and memory processes by blocking long-term potentiation (LTP) in the hippocampus *in vitro* and *in vivo* (44-46). Synaptic plasticity, neurotransmitter release and fast synaptic transmission are also modulated by the activation of neuronal  $\alpha 7$ nAChR (47,48). In addition, EA pretreatment has been demonstrated to protect the brain against transient cerebral ischemic injury via increased  $\alpha 7$ nAChR expression on neurons (29). The current study suggests that EA-mediated regulation of neuroinflammation may enhance LTP, and combined with EA regulation of  $\alpha 7$ nAChR expression on neurons, improve spatial learning and memory as a result.

In the present study, EA-induced expression of  $\alpha 7$ nAChR following temporary MCAO may serve the same role as an  $\alpha 7$ nAChR agonist in focal cerebral ischemia. Similarly, co-treatment with MLA, an  $\alpha 7$ nAChR antagonist, significantly affected the inhibitory effects of EA on glial activation and expression of inflammatory factors. In addition, MLA inhibited the improvement of spatial learning and memory following EA treatment. From these data, it may be inferred that EA activates an  $\alpha 7$ nAChR-dependent anti-inflammatory pathway to control neuroinflammation, which, in turn, leads to improvement in learning and memory outcomes after I/R brain injury.

In conclusion, the current study demonstrates that EA may reduce learning and memory impairment following cerebral I/R injury. The protective effects of EA appear to be mediated via the  $\alpha 7$ nAChR-mediated anti-inflammatory pathway. EA improves cognitive function by an  $\alpha 7$ nAChR-mediated mechanism that decreases neuroinflammation, as demonstrated by reduced glial activation and inflammatory cytokine production. However, A potential limitation of the present study is the lack of a positive control using an  $\alpha 7$ nAChR agonist group, which should be considered in future studies to compare the effects of EA and  $\alpha 7$ nAChR agonists.

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