Electroacupuncture treatment enhances synaptic plasticity in middle cerebral artery occlusion mice via the miR-670-3p/HMGB1 axis

Jingyan Cheng[a], Guoyuan Pan[b], Zhen Wang[c], Haoran Chu[d,*], Yanpeng Pu[c,*]

[a]Department of Rehabilitation Treatment Center, the Second Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, China; [b]Department of Rehabilitation, Tongde Hospital of Zhejiang Province, Hangzhou, China; [c]Department of Encephalopathy, the Second Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, China; [d]Department of Spleen and Stomach Diseases, the Second Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, China

Abstract

Background: Electroacupuncture (EA) is a form of physical therapy rooted in traditional Chinese medicine, which has been widely used in clinical practice. This study aimed to explore the effect of EA treatment on synaptic plasticity in mice subjected to middle cerebral artery occlusion (MCAO) and to elucidate the associated molecular mechanisms.

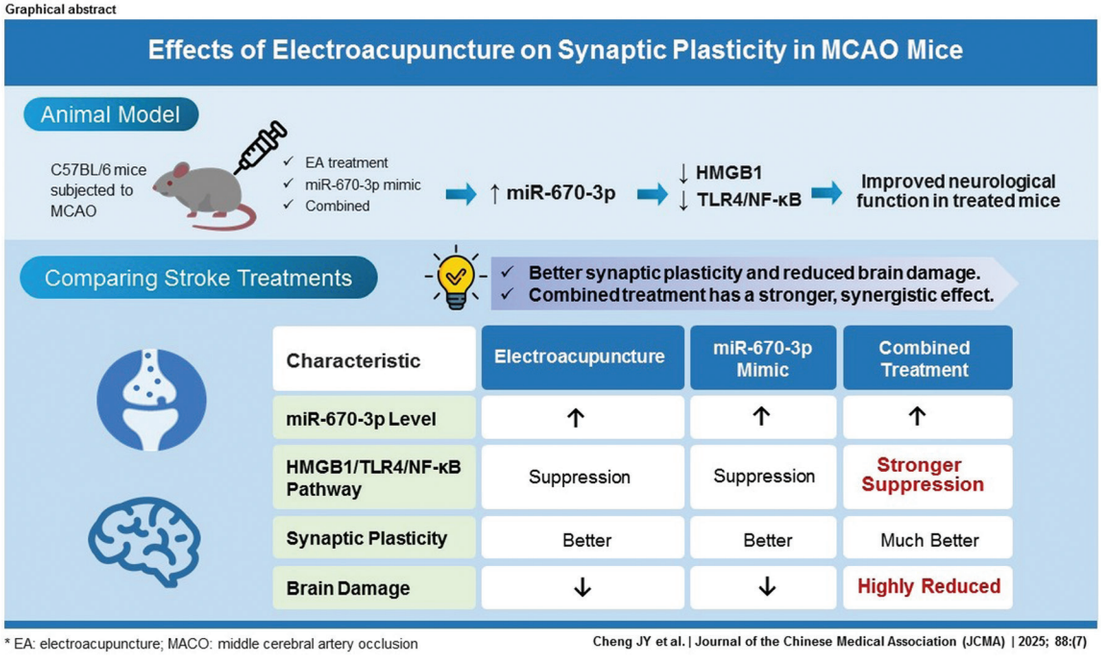
Methods: After MCAO modeling, C57BL/6 mice underwent EA treatment and/or miR-670-3p mimic injection, followed by assessment of neurological deficit by modified neurological severity score (mNSS) and cerebral infarction areas were evaluated via TTC staining. The changes of synaptic ultrastructure related parameters were observed using transmission electron microscopy (TEM). The expression levels of miR-670-3p, HMGB1, TLR4/NF-κB pathway-related proteins, and synapse-associated proteins (Synapsin I, PSD95, BDNF, and GAP43) were quantified by real-time quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The binding relationship between miR-670-3p and HMGB1 was assessed through dual-luciferase reporter assays and RNA pull-down assays.

Results: Mice that underwent EA treatment or miR-670-3p mimic injection exhibited increased miR-670-3p expression, reduced expression levels of HMGB1 and TLR4/NF-κB pathway-related proteins, improved neurological function, and enhanced synaptic plasticity. Furthermore, the combination of EA treatment and miR-670-3p mimic injection produced a synergistic effect, further amplifying these outcomes. Mechanistically, miR-670-3p was found to negatively regulate HMGB1.

Conclusion: EA treatment enhances synaptic plasticity in MCAO mice by promoting miR-670-3p expression to negatively regulate the HMGB1/TLR4/NF-κB pathway.

Keywords: Electroacupuncture; HMGB1; Middle cerebral artery occlusion

Graphical abstract



Lay Summary: Ischemic stroke often leads to lasting brain damage and impaired neurological function. This study investigated whether electroacupuncture, a traditional Chinese therapy that applies mild electrical currents to specific acupuncture points, could aid brain recovery after a stroke in mice. The researchers found that electroacupuncture promoted brain repair by increasing the activity of a small molecule called miR-670-3p. This molecule helped reduce inflammation-related proteins in the brain and enhanced the connections between neurons—a process known as synaptic plasticity. When electroacupuncture was combined with the direct injection of miR-670-3p, the recovery effects were even stronger. These results suggest that electroacupuncture may support brain healing after an ischemic stroke by reducing inflammation and improving neural communication.

1. INTRODUCTION

Ischemic stroke, the most common type of stroke, results from vascular occlusion and is a major cause of disability, causing brain infarction, cerebral tissue death, and focal neuronal damage.[1] Currently, there are two approved therapies for acute ischemic stroke: intravenous thrombolysis with tissue plasminogen activator (tPA) and mechanical thrombectomy, both of which aim to restore perfusion to the ischemic region.[2] Due to the limitations of the tPA treatment window and the risk of hemorrhagic transformation,[3] only a small proportion of patients receive adequate treatment. Endovascular therapy, such as stent retriever devices, has shown superior efficacy for managing acute ischemic stroke, particularly for large vessel occlusions in the middle cerebral artery and internal carotid artery, when administered within 6 hours of symptom onset.[4] Neuroprotection, a third strategy, targets the ischemic cascade to mitigate cellular damage.[5] Notably, synaptic plasticity plays crucial role in restoring neurological function following ischemic stroke.[6] Therefore, investigating the potential molecular mechanisms underlying synaptic plasticity holds promise for identifying effective therapeutic targets for ischemic stroke.

Electroacupuncture (EA), a safe and effective treatment that combines traditional Chinese acupuncture with modern electrical stimulation, is widely used in the treatment of ischemic stroke.[7] A growing number of studies have shown that EA treatment is protective in cerebral ischemic injury by mediating multiple mechanisms. For example, EA exerted a neuroprotective role in cerebral ischemia/reperfusion injury by reducing inflammation and cell pyroptosis.[8] Importantly, EA improved ischemic stroke by affecting microRNA (miRNA) expression and synaptic plasticity.[9] miRNAs exist in most eukaryotes and participate in transcriptional and post-transcriptional regulation,[10] of which have been used as one of the biomarkers for diagnosis and prognosis of ischemic stroke.[11] More importantly, miRNAs have been reported to affect synaptic function and plasticity by regulating gene expression.[12] In our preliminary study, we investigated various miRNAs modulated by EA treatment in ischemic stroke, and found that miR-670-3p was the most significantly regulated miRNA. MiR-670 was upregulated in transient middle cerebral artery occlusion (MCAO) and exacerbated neuronal apoptosis and neurological deficits through the Yap pathway.[13] In addition, EA could alleviate ischemic brain injury by inhibiting miR-34/Wnt/autophagy axis.[14] Based on these findings, we hypothesized that EA treatment might alleviate ischemic stroke by modulating miR-670-3p.

Through bioinformatics analysis, miR-670-3p was found to target high mobility group box 1 (HMGB1). A compelling study revealed that HMGB1 was widely involved in the occurrence of ischemic stroke and the repair of nerve tissue after brain injury.[15] HMGB1 can affect cerebral ischemia through toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) pathway,[16] and the NF-κB pathway has also been shown to be an important pathway mediating neuroinflammation and synaptic plasticity.[17] Another study stated that miR-582-5p repressed apoptosis, inflammation, and oxidative stress by targeting HMGB1, thereby ameliorating neuronal damage.[18] Combining the above findings, we hypothesized that EA may regulate miR-670-3p expression to modulate the HMGB1/TLR4/NF-κB pathway, and ultimately improve ischemic stroke via synaptic plasticity. We further clarify the effect of EA pretreatment on ischemic stroke and explore its potential mechanism by establishing an MCAO model in C57BL/6 mice, to provide an experimental foundation for using EA as a treatment for ischemic stroke.

2. METHODS

2.1. Animals

C57BL/6 specific pathogen-free mice (male, 6-8 weeks) were purchased from Jiangxi Zhonghong Boyuan Biotechnology Co., Ltd. and housed in a controlled environment with free access to food and water. The room maintained a 12-hour light/dark cycle, a constant temperature of 25°C ± 2°C, and a relative humidity of 60% to 70%. All animal procedures were conducted in strict accordance with international animal welfare and ethical guidelines, with efforts made to minimize animal pain and distress. This study has been reviewed by the Experimental Ethics Committee, with the ethical approval number 2022120101.

2.2. Groups and MCAO modeling

The surgical procedures for the MCAO model were performed as previously described.[19,20] The mice were subdivided into eight groups: sham operation group (S), MCAO group (M), MCAO + EA group (EAM), MCAO + control mimic group (mimic NC), MCAO + miR-670-3p mimic group (miR-670-3p mimic), MCAO + miR-670-3p mimic + EA group (miR-670-3P mimic + EAM), MCAO + inhibitor NC + EA group (inhibitor NC + EAM), and MCAO+ miR-670-3p inhibitor + EA group (miR-670-3p inhibitor +EAM). All mice were acclimatized for 3 days before grouping. Twelve hours before modeling, the mice were weighed and their health status was assessed. Subsequently, bedding was replaced, and the mice were fasted but allowed free access to water. Surgical instruments were immersed in 75% medicinal alcohol 12 hours in advance and sterilized through high-pressure steam. MCAO mice underwent 2-hour ischemia and then 24-hour reperfusion. Mice in the S group were subjected to the same operation as in the MCAO group, such as skin incision, vessel separation and suture, except for insertion with thread into the internal carotid, and they were also bound

*Address correspondence. Dr. Haoran Chu, Department of Spleen and Stomach Diseases, the Second Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, Anhui 230061, China. E-mail: Chuhaoran62@163.com (H. Chu); Dr. Yanpeng Pu, Department of Encephalopathy, the Second Affiliated Hospital of Anhui University of Chinese Medicine, 300, Shouchun Road, Hefei 230061, China. E-mail address: puyanpeng1988@163.com (Y. Pu).

Author contributions: Dr. Jingyan Cheng, Dr. Guoyuan Pan, and Dr. Zhen Wang contributed equally to this research.

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2025): 88: 520-529.

Received March 7, 2024; accepted November 25, 2024.

doi: 10.1097/JCMA.0000000000001226

as mice in the EA group during EA treatment. Following 2 hours of ischemia, the mice were injected in the ipsilateral hemisphere of MCAO with 5 μL control mimics, inhibitor NC, miR-670-3p inhibitor or miR-670-3p mimic, followed by 24-hour reperfusion. Upon the completion of the operation, the neck of the mice was wrapped with new sterile gauze and the mice were housed in dry cages that were heated with a temperature-constant heating pad. An incandescent light was placed above the cages to maintain warmth. Vital signs and recovery time from anesthesia were recorded.

2.3. Intraventricular adenovirus injection

Mice in the mimic NC, miR-670-3p mimic, miR-670-3p mimic + EAM, inhibitor NC + EAM, and miR-670-3p inhibitor + EAM groups were anesthetized with 0.3% pentobarbital sodium and fixed on the brain stereotactic device. The injection site and method were as previously described.[20] Briefly, an adenovirus encoding miR-670-3p (5 μL) and a negative control adenovirus (5 μL) (from Zhonghong Boyuan Biological Technology Co., Ltd., Shanghai, China) were injected into the lateral ventricle of mice.

2.4. EA treatment

The acupoints including Baihui (GV20), Dazhui (GV14), and Shuigou (GV26) of mice were selected for EA pretreatment. Han's EA instrument (LH402A; Beijing Huawei Technologies Co., Ltd., Beijing, China) was connected to the acupuncture needles. Following 24 hours of MCAO modeling, mice in the EAM, inhibitor NC + EAM, miR-670-3p inhibitor + EAM, and miR-670-3P mimic + EAM groups underwent EA pretreatment with 15 Hz EA frequency and continuous wave. The mice remained calm and tolerated the procedure well, with the needle gently manipulated during treatment. The intervention was administered for 30 minutes daily over a period of three consecutive days.

2.5. Real-time quantitative polymerase chain reaction

MiRNA and total RNA were extracted from mouse brain tissue using Trizol reagent (CW0580S; CWBIO, Jiangsu, China). Next, the concentration and purity of miRNA and total RNA were determined, and total RNA electrophoresis and miRNA and RNA reverse transcription were performed. U6 and β-actin were selected as the internal controls. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the target genes. The primers are listed as follows: miR-670-3p (forward: 5'-GCGTTTCCTCATATCCATTCAG-3', reverse: 5'-AGTGCAGGGTCCGAGGTATT-3'), U6 (forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'), HMGB1 (forward: 5'-CGGAGAAACTTCAGACCGGA-3', reverse: 5'-TCCGGGTGCTTCTTCTTGTG-3'), and β-actin (forward: 5'-AGGGAAATCGTGCGTGAC-3', reverse: 5'-CATACCCAAGAAGGAAGGCT-3').

2.6. Western blot analysis

As previously described,[20] the total protein samples were extracted using RIPA lysis buffer (C1053; Beijing Pulilai Gene Technology Co., Ltd., Beijing, China), followed by protein quantification using the BCA kit (E-BC-K318-M; Elabscience, Wuhan, China). Next, the same amount of protein samples and markers were added to each well, followed by electrophoresis using a 10% SDS-PAGE separating gel and a 5% stacking gel. Afterward, the proteins were transferred to PVDF membranes (IPVH00010; Millipore, Bedford, MA). The membranes were blocked in 5% skim milk (P1622; Beijing Pulilai Gene Technology Co., Ltd.) for 2 hours and then probed with rabbit anti-PSD95 (20665-1-AP; Proteintech, Chicago, IL, 1:1000), rabbit anti-synapsin 1 (AF6201; Affinity, Jiangsu, China, 1:1000), rabbit anti-TLR4 (AF7017, Affinity, 1:1000), rabbit anti-NF-kB p65 (AF5006, Affinity, 1:1000), rabbit anti-p-NF-kB p65 (AF2006, Affinity, 1:1000), rabbit anti-BDNF (DF6387, Affinity, 1:1000), rabbit anti-GAP43 (DF7766, Affinity, 1:2000), and then HRP conjugated goat anti-rabbit IgG (H+L) (GB23303; Servicebio, Wuhan, China, 1:2000). Finally, the images were developed by automatic chemiluminescence image analysis system (Tanon-5200; Shanghai Tianneng Technology Co., Ltd., Shanghai, China). Data were analyzed by Image J software (ImageJ bundled with 64-bit Java 1.8.0_112; National Institutes of Health).

2.7. Infarction area assessment

After deep anesthesia, the mice were perfused with 0.9% normal saline through the heart. Mouse brain tissues were placed in a brain mold and cut into five consecutive coronal sections. The sections were immersed in 2% triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO) solution at 37°C for 30 minutes, followed by 24-hour fixation in 4% paraformaldehyde. Data were analyzed by Image J software (ImageJ bundled with 64-bit Java 1.8.0_112). The percentage of cerebral infarction area = total cerebral infarction area/total brain area × 100%.

2.8. Neurological deficit score

Two researchers independently assessed the mNSS scores of all mice on days 1 and 3 post-MCAO[20]

2.9. Transmission electron microscopy

The ultrastructural changes of synapses in the ischemic penumbra were observed by TEM as previously described.[20] Specifically, mice were anesthetized by inhalation of isoflurane and then euthanized. The ischemic penumbra was quickly excised and placed in 2.5% glutaraldehyde and the tissues were cut into small pieces of 1 × 1 × 1.5 mm. After that, the tissue pieces were fixed with 2.5% glutaraldehyde for 2 hours and then 1% osmic acid for 1 hour, followed by 2-hour 1% uranium acetate staining, dehydration with acetone, embedding in epoxy resin, and slicing with an ultramicrotome (RMC-PXL; RMC Boeckeler, Tucson, AZ). Finally, the images were captured under a transmission electron microscope (TEM) (H-7500; Hitachi, Tokyo, Japan).

2.10. Dual-luciferase reporter assay

The binding sites between miR-670-3p and HMGB1 were predicted using the TargetScan database (http://www.targetscan.org). According to the prediction results, wild and mutant sequences of binding sites (wt-HMGB1 and mut-HMGB1) were designed and synthesized. The wt-HMGB1 and mut-HMGB1 were inserted into the luciferase reporter gene vector (PGL3-Promoter), and the correctly-sequenced luciferase reporter plasmid was co-transfected into HEK239T cells (iCell Bioscience Inc, Shanghai, China) with miR-670-3p mimic or corresponding NC using lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). At 48 hours after transfection, firefly and Renilla luciferase activities were analyzed using a dual-luciferase reporter assay kit (D0010; Solaibao, Beijing, China).

2.11. RNA pull-down assay

The interaction between miR-670-3p and HMGB1 was verified using the RNA-protein pull-down kit (Thermo Scientific, MA). Briefly, HEK293T cells were cultured with biotinylated miR-670-3p-WT or miR-670-3p-Mut or NC (Gene Pharm, Shanghai, China). The enrichment of HMGB 1 in the biotin-coupled RNA

complexes was analyzed by quantitative reverse transcription PCR (qRT-PCR).

2.12. Statistical analysis

Data were all expressed in the form of mean ± SD. SPSS software (version 25.0, SPSS, Chicago, IL) and GraphPad Prism software were used for data analysis. Student's *t* test was used to compare the differences between the two groups and one-way analysis of variance was used for comparisons among multiple groups, with Tukey's post hoc test for pairwise comparisons. A p value of <0.05 was considered statistically significant.

3. RESULTS

3.1. EA treatment upregulates miR-670-3p expression in MCAO mice

First, we established MCAO mouse models and then treated MCAO mice with different treatments. Next, we detected miR-670-3p expression using real-time quantitative polymerase chain reaction (RT-qPCR) in the brain tissues of MCAO mice. As exhibited in Fig. 1 ($p < 0.05$), the M group had a lower expression of miR-670-3p than the S group. After EA treatment or miR-670-3p mimic injection, miR-670-3p expression was markedly increased in the brain tissues of MCAO mice, which was further increased following EA combined miR-670-3p mimic. Therefore, EA treatment effectively promoted miR-670-3p expression in MCAO mice.

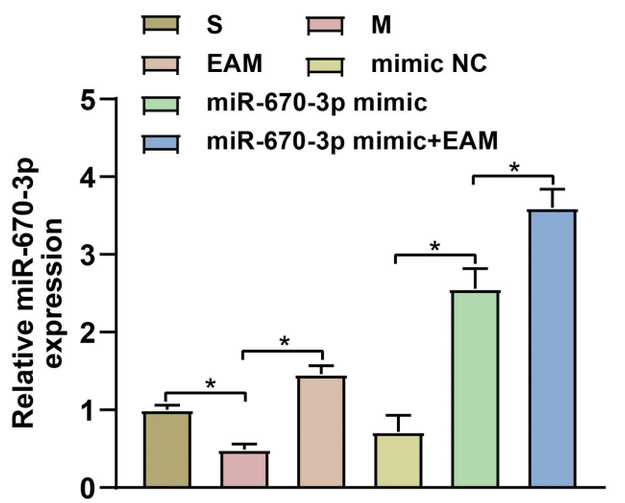


Fig. 1 EA treatment promotes miR-670-3p expression in MCAO mice. After MCAO modeling, the expression of miR-670-3p in the brain tissues of MCAO mice was measured by RT-qPCR. N = 6. Data were all expressed as mean ± SD, and one-way analysis of variance was used for comparisons among multiple groups with Tukey's post hoc test for pairwise comparisons. *$p < 0.05$. EA = electroacupuncture; M = MCAO; MCAO = middle cerebral artery occlusion; RT-qPCR = real-time quantitative polymerase chain reaction; S = sham operation.

3.2. EA treatment stimulates miR-670-3p expression to alleviate cerebral infarction area and improve neurological function in MCAO mice

To further investigate the role of miR-670-3p in MCAO mice, TTC staining was performed to assess the cerebral infarction area. In contrast to the S group, the cerebral infarction area was increased in the mice of the M group. EA treatment or miR-670-3p mimic injection reduced the cerebral infarction area, while the combined effects of EA treatment or miR-670-3p mimic injection on the cerebral infarction area were more significant (Fig. 2A, B, $p < 0.05$). The mNSS scores for evaluation of neurological function deficit revealed that, except for the S group, the other groups showed different degrees of neurological function injury. Specifically, the mNSS score in the M group was significantly increased, which was reversed by EA treatment or miR-670-3p mimic injection. Compared with the miR-670-3p mimic group, the miR-670-3p mimic + EAM group had a much lower mNSS score (Fig. 2C, $p < 0.05$). In conclusion, EA treatment reduced the cerebral infarction area and improved neurological function by upregulating miR-670-3p expression in MCAO mice.

3.3. EA treatment increases miR-670-3p expression to enhance synaptic plasticity in MCAO mice

Prior research unveiled that EA regulated the synaptic plasticity of hippocampal neurons by regulating miRNA expression, thus alleviating cognitive impairment.[21] Moreover, our previous study found that exercise could affect the synaptic plasticity of MCAO mice and improve the pathological changes of MCAO mice. Here, we evaluated the effect of EA on synaptic plasticity in MCAO mice by mediating miR-670-3p.

As displayed by western blot analysis, the protein expression of synaptic marker proteins (synapsin 1 and PSD95) was markedly reduced in the M group vs the S group, promoted by EA treatment or miR-670-3p mimic injection. Compared to the miR-670-3p mimic group, further increases of synapsin 1 and PSD95 were noted in the miR-670-3P mimic + EAM group (Fig. 3A, B, $p < 0.05$). As observed through TEM, in the M group, the presynaptic or postsynaptic membrane was blurred, the synaptic cleft width increased, and the postsynaptic density (PSD) thickness decreased. However, regular synaptic cleft, tight synaptic connections, decreased width of synaptic cleft, synaptic number density, and the density of synaptic connecting band surface, and increased PSD thickness were noticed after EA treatment or miR-670-3p mimic injection; moreover, the effect was more obvious after EA combined with miR-670-3p mimic (Fig. 3C–G, $p < 0.05$). A prior study illustrated that GAP43 and BDNF were closely related to the formation of synaptic plasticity.[22] Hence, we detected the expression of GAP43 and BDNF, and found that the expression levels of GAP43 and BDNF were consistent with those of synapsin 1 and PSD95 among all groups (Fig. 3H, I, $p < 0.05$). Taken together, these results suggest that EA treatment enhanced synaptic plasticity in MCAO mice by upregulating miR-670-3p expression.

3.4. Inhibiting miR-670-3p expression attenuates the improvement in cerebral infarction area and neurological function in mice induced by EA treatment

The function of miR-670-3p in MCAO was further verified by intracerebroventricular injection of miR-670-3p inhibitor adenovirus into MCAO mice. The results revealed that the cerebral infarction area was significantly larger, and the mNSS score was markedly higher in the miR-670-3p inhibitor + EA group compared to the inhibitor NC + EA group. However, these two indicators were insignificantly changed in the inhibitor NC + EAM group than in the EAM group (Fig. 4A–C). These results demonstrated that inhibiting miR-670-3p expression had a suppressive effect on EA treatment-induced improvement in cerebral infarction area and neurological function in mice.

3.5. Inhibiting miR-670-3p expression suppresses EA treatment-induced improvement in synaptic plasticity

The change in synaptic plasticity of MCAO mice was evaluated by western blot and the transmission electron scope.

The synapsin 1 and PSD95 protein levels were suppressed in mouse cerebral tissues of the miR-670-3p inhibitor + EAM group than in the inhibitor NC + EAM group (Fig. 5A, B, $p < 0.05$). Synaptic morphological change of the hippocampus was consistent with the synaptic marker proteins. Compared with the inhibitor NC + EAM group, the miR-670-3p inhibitor + EAM group showed blurry presynaptic or postsynaptic membrane, with elevated synaptic cleft and reduced PSD thickness (Fig. 5C, G, $p < 0.05$). Additionally, the levels of GAP43 and BDNF were downregulated in the miR-670-3p inhibitor + EA group compared to the inhibitor NC + EA group (Fig. 5H, I, $p < 0.05$). Accordingly, inhibiting miR-670-3p expression diminished the improvement of EA treatment in synaptic plasticity of MCAO mice.

3.6. miR-670-3p negatively targets HMGB1

Subsequently, we screened the downstream target genes of miR-670-3p through MiRTarBase and TargetScan databases, intersected the genes from the two databases, and found 20 intersecting genes (Fig. 6A). Of these 20 genes, HMGB1 has been reported to play an important role in brain injury.[23] Moreover, bioinformatics analysis showed binding sites between miR-670-3p and HMGB1 (Fig. 6B). Therefore, we hypothesized that HMGB1 may affect MCAO synaptic plasticity as a downstream target gene of miR-670-3p. To confirm our hypothesis, a dual-luciferase reporter assay was designed to assess the targeted binding relationship between miR-670-3p and HMGB1, which unveiled that miR-670-3p mimic prominently inhibited the luciferase activity of wild-type wt-HMGB1, while had no significant effect on the luciferase activity of mut-HMGB1 (Fig. 6C, $p < 0.05$). The interaction between miR-670-3p and HMGB1 was further verified by the RNA pull-down assay. In contrast to the NC group, cells transfected with biotinylated miR-670-3p-WT showed an increase in HMGB1 enrichment, while HMGB1 was not enriched in those transfected with biotinylated miR-670-3p-Mut (Fig. 6D, $p < 0.05$), which suggested the interaction between miR-670-3p and HMGB1. The above results indicated the binding relationship between miR-670-3p and HMGB1 in a targeted manner. Additionally, RT-qPCR and western blot analysis exhibited that the M group had higher expression of HMGB1 than the S group, and EA treatment or miR-670-3p mimic injection decreased HMGB1 expression. Compared with the miR-670-3p mimic group, HMGB1 expression was further reduced after EA combined with the miR-670-3p mimic (Fig. 6E, F, $p < 0.05$). Overall, miR-670-3p negatively targeted HMGB1 expression.

3.7. miR-670-3p negatively regulates HMGB1 to modulate TLR4/NF-κB pathway

Finally, we assessed the protein expression of the proteins associated with the TLR4/NF-κB pathway, and found that the expression of TLR4 and p-NF-κB p65 was consistent with that of HMGB1 (Fig. 7A, B, $p < 0.05$). Collectively, miR-670-3p negatively targeted HMGB1 to regulate the TLR4/NF-κB pathway, thereby improving synaptic plasticity in MCAO mice.

4. DISCUSSION

Currently, ischemic stroke remains one of the leading causes of death and long-term disability, presenting a significant challenge to both families and healthcare systems worldwide.[24] As evidenced by a large number of studies, the treatment of hyperbaric oxygen, lipopolysaccharide, or EA may assume a protective role in brain injury after ischemic stroke by modulating various pathological mechanisms.[25–27] It has been found that EA can positively regulate synaptic plasticity and is beneficial to the recovery of ischemic stroke.[28] Our research results also confirm this conclusion. Specifically, we observed that EA treatment enhanced synaptic plasticity by promoting miR-670-3p expression, which in turn downregulated the HMGB1/TLR4/NF-κB

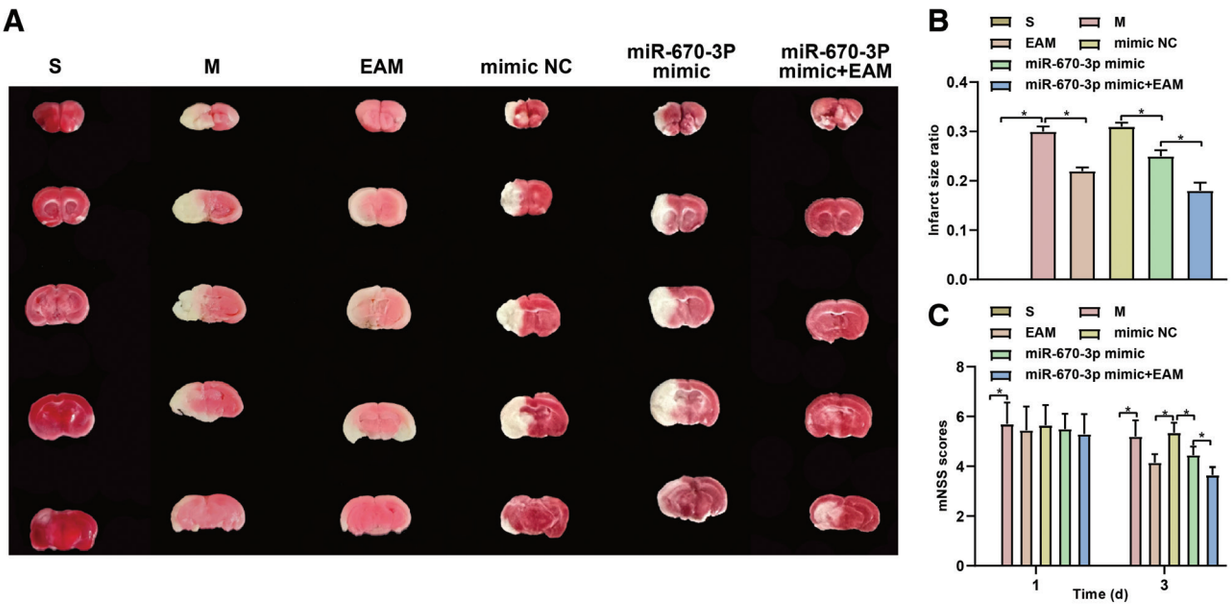


Fig. 2 EA treatment increases miR-670-3p expression to reduce cerebral infarction area and improve neurological function of MCAO mice. A-B, The cerebral infarction area of MCAO mice was observed through TTC staining. C, The mNSS scores were used to evaluate the neurological function deficit in MCAO mice. N = 6. Data were all expressed as mean ± SD, and one-way analysis of variance was used for comparisons among multiple groups with Tukey's post hoc test for pairwise comparisons. *$p < 0.05$. EA = electroacupuncture; M = MCAO; MCAO = middle cerebral artery occlusion; mNSS = modified neurological severity score; S = sham operation; TTC = triphenyl tetrazolium chloride.

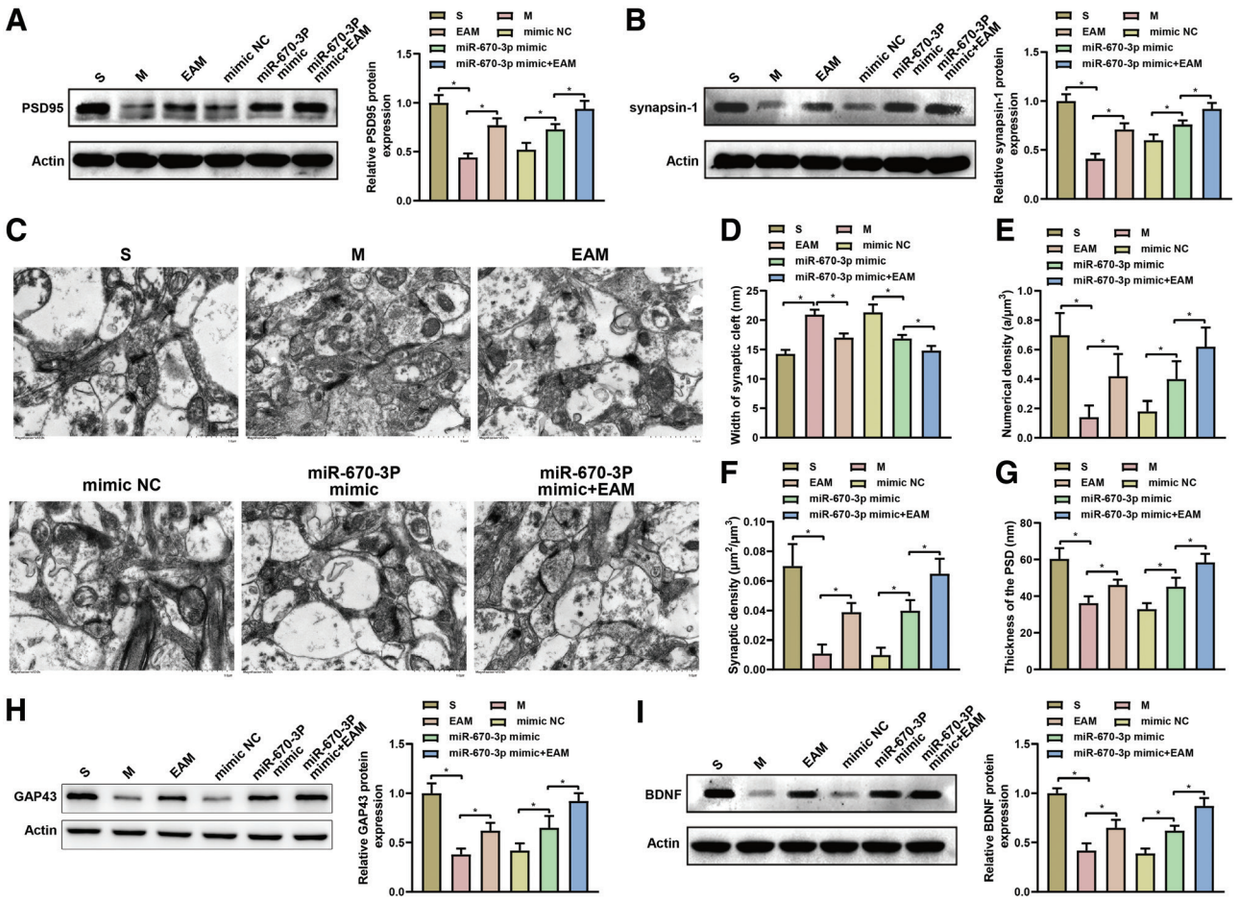


Fig. 3 EA treatment enhances synaptic plasticity in MCAO mice by increasing miR-670-3p expression. A-B, Western blot was used to measure the protein levels of PSD95 (A) and synapsin 1 (B). C-G, The synaptic structure (C), the width of synaptic cleft (D), synaptic number density (E), the density of synaptic connecting band surface (F), and synaptic PSD thickness (G) were observed by TEM. H-I, The protein expression of synaptic associated proteins GAP43 (H) and BDNF (I) in the brain tissues of MCAO mice were tested by western blot. N = 6. Data were all expressed as mean ± SD, and one-way analysis of variance was used for comparisons among multiple groups with Tukey's post hoc test for pairwise comparisons. *$p < 0.05$. EA = electroacupuncture; M = MCAO; MCAO = middle cerebral artery occlusion; PSD = postsynaptic density; S = sham operation; TEM = transmission electron microscopy.

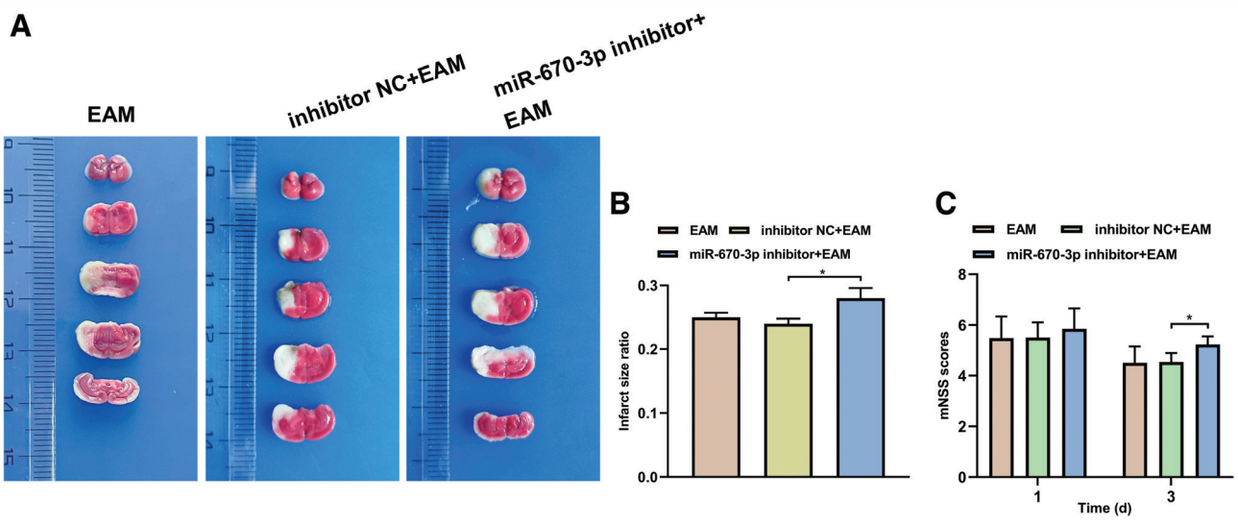


Fig. 4 miR-670-3p inhibition alleviates the therapeutic effect of EA treatment on cerebral infarction and neurological function. A-B, The cerebral infarction area of MCAO mice was observed through TTC staining. C, the neurological function deficit in MCAO mice was evaluated through mNSS. N = 6. Data were all expressed as mean ± SD. *$p < 0.05$. EA = electroacupuncture; MCAO = middle cerebral artery occlusion; mNSS = modified neurological severity score.

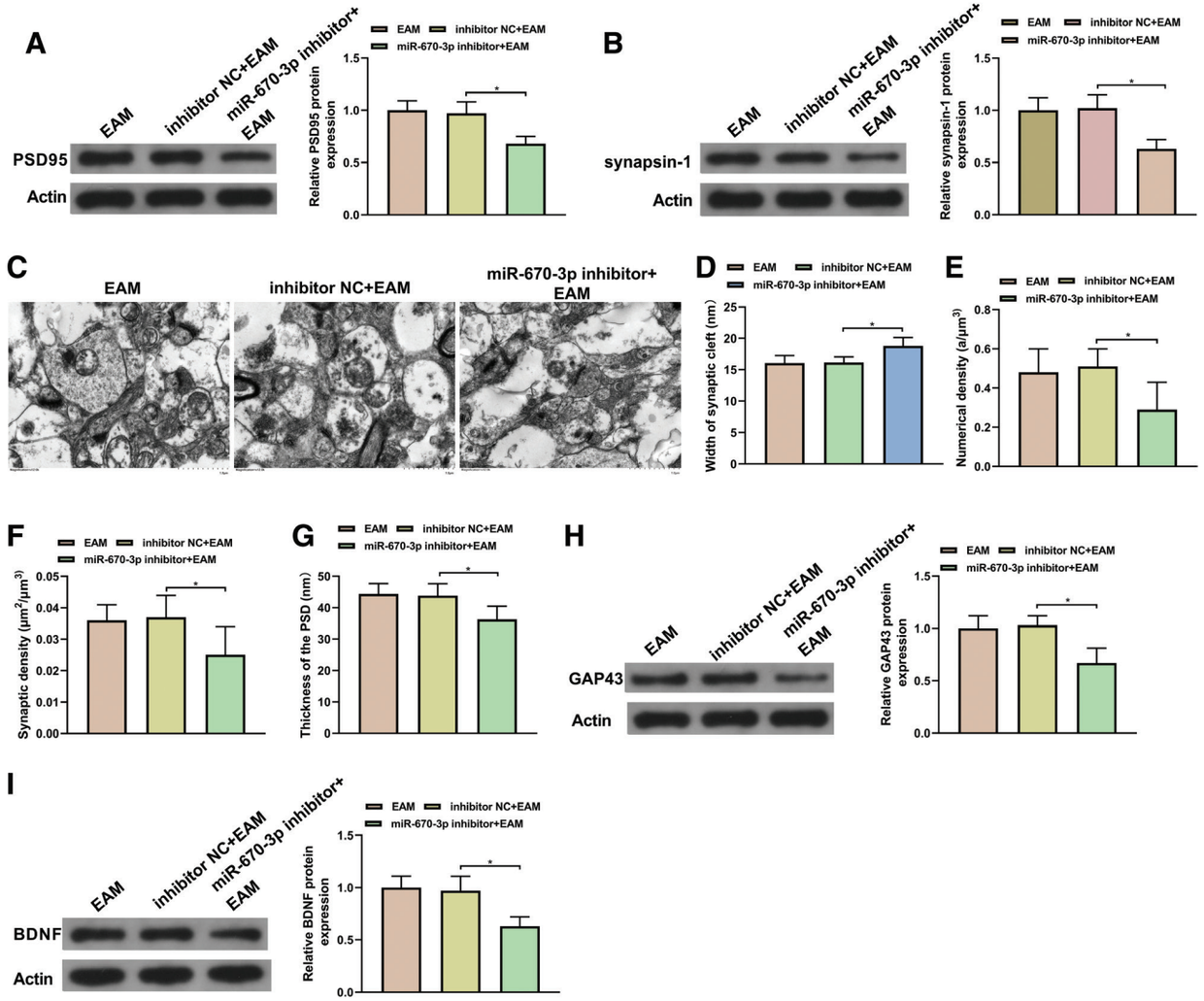


Fig. 5 Inhibiting miR-670-3p expression blunts the improvement of EA treatment in synaptic plasticity of MCAO mice. A-B, Western blot was used to measure the protein expression of PSD95 (A) and synapsin 1 (B). C-G, The synaptic structure (C), the width of synaptic cleft (D), synaptic number density (E), the density of synaptic connecting band surface (F), and synaptic PSD thickness (G) were observed by TEM. H-I, The protein expression of synaptic associated proteins GAP43 (H) and BDNF (I) in the brain tissues of MCAO mice was tested by western blot. N = 6. Data were all expressed as mean ± SD. *$p < 0.05$. EA = electroacupuncture; M = MCAO; MCAO = middle cerebral artery occlusion; PSD = postsynaptic density; S = sham operation; TEM = transmission electron microscopy.

signaling pathway, ultimately facilitating neurological recovery in MCAO mice.

In MCAO mice, miR-670-3p was detected to be lowly expressed, which could be upregulated by EA treatment. In diabetic mice, miR-670-3p has been determined as one of the top 10 upregulated circular RNA-bound miRNAs influenced by EA.[29] Further experiments displayed that EA-induced miR-670-3p upregulation alleviated cerebral infarction area and improved neurological function in MCAO mice. As a study by Zhao et al,[30] EA enhanced the recovery of neurobehavioral function in ischemic stroke through axonal regeneration mediated by miR-132 targeting SOX2. Another previous study reported that EA regulated hippocampal synaptic plasticity in ischemic stroke rats through LIMK1 function mediated by miR-134.[31] Consistently, our results showed that EA enhanced synaptic plasticity in MCAO mice via miR-670-3p upregulation. Fully understanding the specific mechanism of neuronal synaptic plasticity will help explore new therapeutic strategies for ischemic brain injury.[32] Synapsin 1 is an essential neuronal protein that plays a crucial role in synaptic communication and neuronal plasticity.[33] As a postsynaptic scaffolding protein, PSD95 plays essential roles in excitatory synaptic transmission and postsynaptic organization in the brain.[34] Additionally, BDNF is widely implicated in various brain disorders due to its role in regulating synaptic connections, synaptic structure, neurotransmitter release, and synaptic plasticity.[35] The continuous release of BDNF contributes to the recovery of ischemic stroke.[36] However, there is still no study directly reporting on the relationship between miR-670-3p and synaptic plasticity in ischemic stroke. In this study, we found that both EA and overexpression of miR-670-3p upregulated the expression of synapsin 1, PSD95, BDNF, and GAP43, demonstrating the synaptic regulatory function of EA and miR-670-3p. Consistently, inhibiting miR-670-3p attenuated the beneficial effects of EA treatment on cerebral infarction, neurological function and synaptic plasticity. From the above findings, it was concluded that EA can ameliorate ischemic stroke by regulating miRNA expression and synaptic plasticity. More importantly, our study

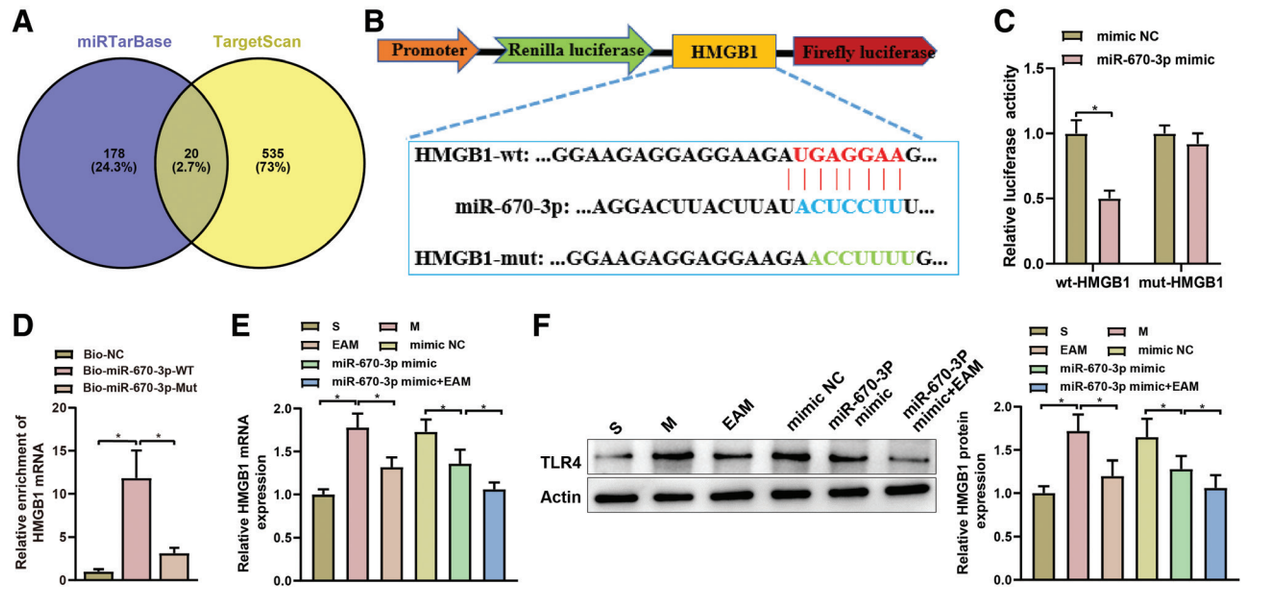


Fig. 6 HMGB1 is a downstream target gene of miR-670-3p. A, The downstream target genes of miR-670-3p were screened using MiRTarBase and TargetScan databases. B, The binding sites between miR-670-3p and HMGB1 were predicted through bioinformatics analysis. C, The binding relationship between miR-670-3p and HMGB1 was verified using a dual-luciferase reporter assay. D, The interaction between miR-670-3p and HMGB1 was verified by the RNA pull down assay. E-F, The expression of HMGB1 was measured by RT-qPCR (E) and western blot (F). N = 6 for animal experiments, and each cellular experiment was repeated three times. Data were all expressed as mean ± SD. Student's *t* test was used to compare the differences between the two groups and one-way analysis of variance was used for comparisons among multiple groups with Tukey's post hoc test for pairwise comparisons. *$p < 0.05$. EA = electroacupuncture; HMGB1 = high mobility group box B1; M = MCAO; MCAO = middle cerebral artery occlusion; RT-qPCR = real-time quantitative polymerase chain reaction; S = sham operation.

is the first to identify miR-670-3p as a potential therapeutic target for ischemic stroke, which represents a key highlight of our research. However, a previous study demonstrated that miR-670 was upregulated in transient MCAO and facilitated neuronal apoptosis and neurological deficits.[13] The difference in the role of miR-670-3p in MCAO between our study and the previous one may be caused by different occlusion durations. The previous study used a 1-hour occlusion, whereas our study utilized a 2-hour occlusion. The difference implies that miR-670-3p expression may be changing during the development of MCAO, but this hypothesis requires verification by extensive experiments.

To explore the downstream genes of miR-670-3p, we used a database prediction method and found that HMGB1 was a target gene of miR-670-3p, which was further confirmed through functional assays. A forceful study showed that HMGB1 was increased in the plasma of stroke patients,[37] which aligns with our findings that HMGB1 expression was upregulated in the brain tissues of MCAO mice. Notably, Ye et al[38] pointed out that HMGB1 contributed to the pathogenesis of ischemic stroke via its presumed receptors, such as TLRs, matrix metalloproteinase enzymes, and receptors for advanced glycation end products. A recent study confirmed that the HMGB1-mediated TLR4/NF-κB pathway affected the progression of hepatic encephalopathy,[39] and the TLR4/NF-κB pathway was identified to be a vital pathway mediating neuroinflammation and synaptic plasticity.[17] In this study, our findings showed that miR-670-3p improved synaptic plasticity in MCAO mice by negatively targeting HMGB1 to regulate TLR4/NF-κB pathway. Interestingly, we also found an interesting phenomenon that EA combined with overexpression of miR-670-3p significantly reduced the area of cerebral infarction and alleviated neurological impairment than upregulation of miR-670-3p alone. In addition, the combination of EA and elevation of miR-670-3p could enhance synaptic plasticity. This synergistic effect may be attributed to the ability of EA treatment to upregulate endogenous miR-670-3p expression. However, given the complex biological functions of miR-670-3p, the precise mechanisms by which EA regulates its expression warrant further investigation.

According to the above discussion, we conclude that EA upregulates the expression of miR-670-3p and synaptic-related proteins; overexpression of miR-670-3P can enhance synaptic plasticity and protect the brain via the HMGB1/TLR4/NF-κB pathway. These findings suggest that miR-670-3p acts as a protective factor for ischemic stroke and serves as a key target for regulating synaptic plasticity.

ACKNOWLEDGMENTS

This study was funded by the Clinical Research Project of Anhui University of Traditional Chinese Medicine (grant no. 2021efylc20), the Scientific Research Project of Colleges and Universities in Anhui Province (grant nos. 2023AH050822 and 2023AH050829), and the Youth Project of Natural Science Foundation of Anhui Province (grant no. 2308085QH293).

REFERENCES

1. Zhao Y, Zhang X, Chen X, Wei Y. Neuronal injuries in cerebral infarction and ischemic stroke: from mechanisms to treatment (review). *Int J Mol Med* 2022;49:15.
2. Xiong Y, Wakhloo AK, Fisher M. Advances in acute ischemic stroke therapy. *Circ Res* 2022;130:1230–51.
3. Zhang S, Zhou Y, Li R, Chen Z, Fan X. Advanced drug delivery system against ischemic stroke. *J Control Release* 2022;344:173–201.
4. Jadhav AP, Desai SM, Jovin TG. Indications for mechanical thrombectomy for acute ischemic stroke: current guidelines and beyond. *Neurology* 2021;97:S126–36.

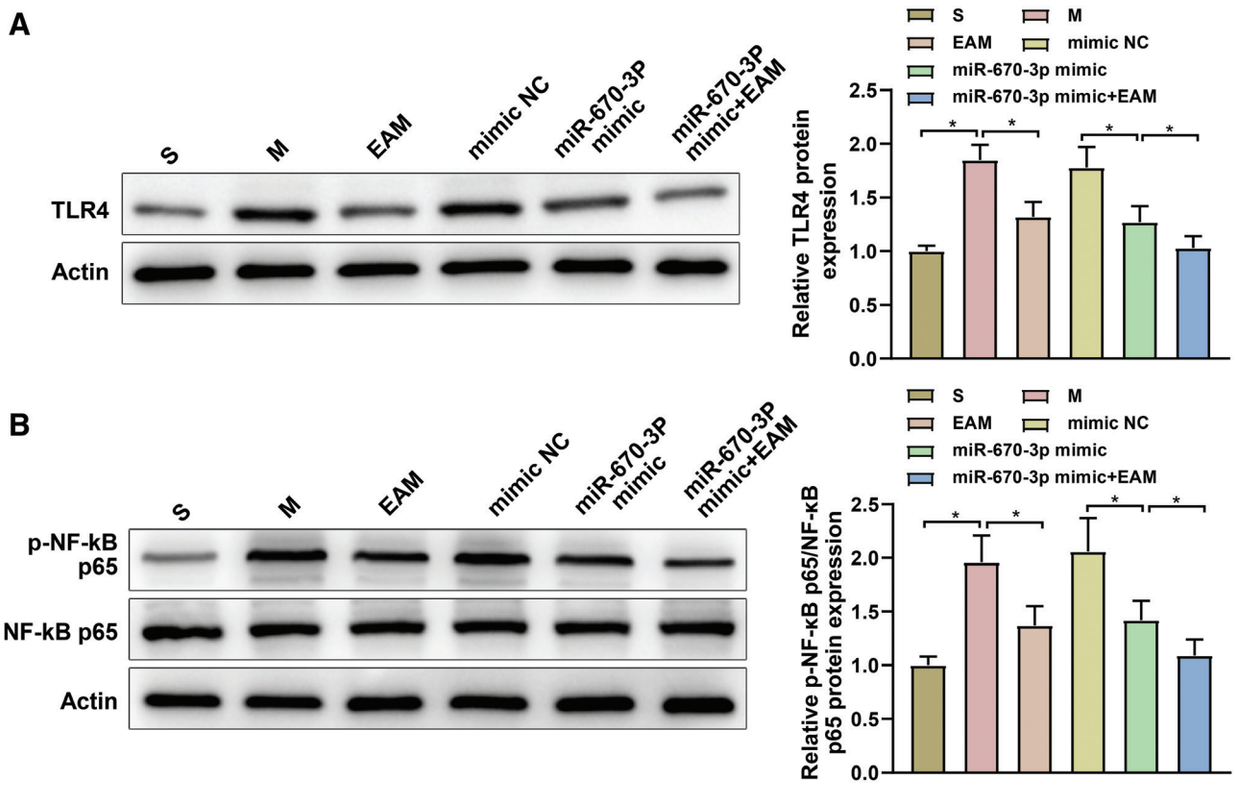


Fig. 7 miR-670-3p negatively targets HMGB1 and regulates the TLR4/NF-κB pathway. A-B, The protein expression of the TLR4/NF-κB pathway-related proteins TLR4 (A) and p-NF-κB p65 (B) was assessed by western blot. N = 6. Data were all expressed as mean ± SD, and one-way analysis of variance was used for comparisons among multiple groups with Tukey's post hoc test for pairwise comparisons. *$p < 0.05$. EA = electroacupuncture; HMGB1 = high mobility group box B1; M = MCAO; MCAO = middle cerebral artery occlusion; S = sham operation.

5. Herpich F, Rincon F. Management of acute ischemic stroke. *Crit Care Med* 2020;48:1654–63.
6. Yepes M. Urokinase-type plasminogen activator is a modulator of synaptic plasticity in the central nervous system: implications for neurorepair in the ischemic brain. *Neural Regen Res* 2020;15:620–4.
7. Xing Y, Zhang M, Li WB, Dong F, Zhang F. Mechanisms involved in the neuroprotection of electroacupuncture therapy for ischemic stroke. *Front Neurosci* 2018;12:929.
8. Cai L, Yao ZY, Yang L, Xu XH, Luo M, Dong MM, et al. Mechanism of electroacupuncture against cerebral ischemia-reperfusion injury: reducing inflammatory response and cell pyroptosis by inhibiting NLRP3 and caspase-1. *Front Mol Neurosci* 2022;15:822088.
9. Dai Y, Wang S, Yang M, Zhuo P, Ding Y, Li X, et al. Electroacupuncture protective effects after cerebral ischemia are mediated through miR-219a inhibition. *Biol Res* 2023;56:36.
10. Tafrihi M, Hasheminasab E. MiRNAs: biology, biogenesis, their web-based tools, and databases. *Microrna* 2019;8:4–27.
11. Eyileten C, Wicik Z, De Rosa S, Mirowska-Guzel D, Soplinska A, Indolfi C, et al. MicroRNAs as diagnostic and prognostic biomarkers in ischemic stroke: a comprehensive review and bioinformatic analysis. *Cells* 2018;7:249.
12. Woods BJ, Van Vactor D. MiRNA: local guardians of presynaptic function in plasticity and disease. *RNA Biol* 2021;18:1014–24.
13. Yu SJ, Yu MJ, Bu ZQ, He PP, Feng J. MicroRNA-670 aggravates cerebral ischemia/reperfusion injury via the Yap pathway. *Neural Regen Res* 2021;16:1024–30.
14. Cao S, Yang Y, Yu Q, Shu S, Zhou S. Electroacupuncture alleviates ischaemic brain injury by regulating the miRNA-34/Wnt/autophagy axis. *Brain Res Bull* 2021;170:155–61.
15. Gao B, Wang S, Li J, Han N, Ge H, Zhang G, et al. HMGB1, angel or devil, in ischemic stroke. *Brain Behav* 2023;13:e2987.
16. Wang F, Ji S, Wang M, Liu L, Li Q, Jiang F, et al. HMGB1 promoted p-glycoprotein at the blood-brain barrier in MCAO rats via TLR4/NF-kB signaling pathway. *Eur J Pharmacol* 2020;880:173189.
17. Xu T, Liu J, Li XR, Yu Y, Luo X, Zheng X, et al. The mTOR/NF-kB pathway mediates neuroinflammation and synaptic plasticity in diabetic encephalopathy. *Mol Neurobiol* 2021;58:3848–62.
18. Yang G, Xue Z, Zhao Y. MiR-582-5p attenuates neonatal hypoxic-ischemic encephalopathy by targeting high mobility group box 1 (HMGB1) through inhibiting neuroinflammation and oxidative stress. *Curr Neurovasc Res* 2021;18:295–301.
19. Cheng J, Shen W, Jin L, Pan J, Zhou Y, Pan G, et al. Treadmill exercise promotes neurogenesis and myelin repair via upregulating WNT/β-catenin signaling pathways in the juvenile brain following focal cerebral ischemia/reperfusion. *Int J Mol Med* 2020;45:1447–63.
20. Shen W, Jin L, Zhu A, Lin Y, Pan G, Zhou S, et al. Treadmill exercise enhances synaptic plasticity in the ischemic penumbra of MCAO mice by inducing the expression of Camk2a via CYFIP1 upregulation. *Life Sci* 2021;270:119033.
21. Hao L, Wu Y, Xie J, Chen X. Electroacupuncture enhances cognitive deficits in a rat model of rapid eye movement sleep deprivation via targeting MiR-132. *Evid Based Complement Alternat Med* 2022;2022:7044208.
22. Lee YJ, Jeong YJ, Kang EJ, Kang BS, Lee SH, Kim YJ, et al. Gap-43 closely interacts with BDNF in hippocampal neurons and is associated with Alzheimer's disease progression. *Front Mol Neurosci* 2023;16:1150399.
23. Liu DD, Luo P, Gu L, Zhang Q, Gao P, Zhu Y, et al. Celastrol exerts a neuroprotective effect by directly binding to HMGB1 protein in cerebral ischemia-reperfusion. *J Neuroinflammation* 2021;18:174.
24. Chen H, Tian F. The residual risks associated with atherothrombosis of recurrent ischemic stroke (IS) after non-cardiogenic IS. *Curr Neurovasc Res* 2023;20:149–61.
25. Wang Z, Zhou Y, Yu Y, He K, Cheng LM. Lipopolysaccharide preconditioning increased the level of regulatory B cells in the spleen after acute ischaemia/reperfusion in mice. *Brain Res* 2018;1701:46–57.

26. Wu X, You J, Chen X, Zhou M, Ma H, Zhang T, et al. An overview of hyperbaric oxygen preconditioning against ischemic stroke. *Metab Brain Dis* 2023;38:855–72.
27. Zou J, Huang GF, Xia Q, Li X, Shi J, Sun N. Electroacupuncture promotes microglial M2 polarization in ischemic stroke via annexin A1. *Acupunct Med* 2022;40:258–67.
28. Xie G, Song C, Lin X, Yang M, Fan X, Liu W, et al. Electroacupuncture regulates hippocampal synaptic plasticity via inhibiting Janus-activated kinase 2/signal transducer and activator of transcription 3 signaling in cerebral ischemic rats. *J Stroke Cerebrovasc Dis* 2019;28:792–9.
29. Shou Y, Hu L, Zhang W, Gao Y, Xu P, Zhang B. Determination of electroacupuncture effects on circRNAs in plasma exosomes in diabetic mice: an RNA-sequencing approach. *Evid Based Complement Alternat Med* 2019;2019:7543049.
30. Zhao X, Bai F, Zhang E, Zhou D, Jiang T, Zhou H, et al. Electroacupuncture improves neurobehavioral function through targeting of SOX2-mediated axonal regeneration by MicroRNA-132 after ischemic stroke. *Front Mol Neurosci* 2018;11:471.
31. Liu W, Wu J, Huang J, Zhuo P, Lin Y, Wang L, et al. Electroacupuncture regulates hippocampal synaptic plasticity via miR-134-mediated LIMK1 function in rats with ischemic stroke. *Neural Plast* 2017;2017:9545646.
32. Xing Y, Bai Y. A review of exercise-induced neuroplasticity in ischemic stroke: pathology and mechanisms. *Mol Neurobiol* 2020;57:4218–31.
33. Bunger I, Makridis KL, Kreye J, Nikolaus M, Sedlin E, Ullrich T, et al. Maternal synapsin autoantibodies are associated with neurodevelopmental delay. *Front Immunol* 2023;14:1101087.
34. Fukata Y, Hirano Y, Miyazaki Y, Yokoi N, Fukata M. Trans-synaptic LGI1-ADAM22-MAGUK in AMPA and NMDA receptor regulation. *Neuropharmacology* 2021;194:108628.
35. Song M, Martinowich K, Lee FS. BDNF at the synapse: why location matters. *Mol Psychiatry* 2017;22:1370–5.
36. Wang C, Tian C, Cai D, Jiang H, Zhang W, Liu S, et al. BDNF-overexpressing MSCs delivered by hydrogel in acute ischemic stroke treatment. *Ann Transl Med* 2022;10:1393.
37. Denorme F, Portier I, Rustad JL, Cody MJ, de Araujo CV, Hoki C, et al. Neutrophil extracellular traps regulate ischemic stroke brain injury. *J Clin Invest* 2022;132:10.
38. Ye Y, Zeng Z, Jin T, Zhang H, Xiong X, Gu L. The role of high mobility group box 1 in ischemic stroke. *Front Cell Neurosci* 2019;13:127.
39. Essam RM, Saadawy MA, Gamal M, Abdelsalam RM, El-Sahar AE. Lactoferrinavertsneurologicalandbehavioralimpairmentsofthioacetamide-induced hepatic encephalopathy in rats via modulating HGMB1/TLR-4/MYD88/Nrf2 pathway. *Neuropharmacology* 2023;236:109575.