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Research Article

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Electroacupuncture at Shenting (GV24) and Baihui (GV20) Alleviates Cognitive Impairment in MCAO/R Rats by Inhibiting cGAS–STING Signaling Pathway–Associated Ferroptosis

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Abstract

Background: Post-stroke cognitive impairment (PSCI) is a serious complication of ischemic stroke (IS) and is associated with a substantially increased risk of mortality. However, effective interventions for PSCI remain limited. Clinical studies have shown that electroacupuncture (EA) can improve cognitive function after stroke, although the underlying mechanisms have not been systematically elucidated.

Objective: This study aimed to evaluate the effects of electroacupuncture at Shenting (GV24) and Baihui (GV20) on cognitive impairment in a rat model of middle cerebral artery occlusion/reperfusion (MCAO/R) and to investigate whether its neuroprotective effects are mediated through modulation of the cGAS-STING signaling pathway and inhibition of ferroptosis.

Methods: Neurological and behavioral assessments, histopathological examination, biochemical and molecular analyses, and pharmacological interventions were integrated in MCAO/R rats to investigate whether electroacupuncture alleviates ferroptosis by inhibiting the cGAS-STING signaling pathway, thereby improving post-stroke cognitive impairment.

Results: Compared with control rats, MCAO/R rats exhibited overall impairments in cognitive and neurological function, accompanied by enhanced hippocampal cGAS–STING-related signaling and abnormalities in ferroptosis-related indices. Electroacupuncture improved neurological and behavioral performance and reversed the aforementioned molecular alterations. The ferroptosis inhibitor ferrostatin-1 (Fer-1) partially mimicked the protective effects of electroacupuncture, whereas the STING agonist DMXAA attenuated electroacupuncture-associated improvements in cognitive function and ferroptosis-related alterations. The cGAS inhibitor RU.521 similarly improved cognitive function and ferroptosis-related indices; these protective effects of RU.521 were attenuated by co-administration of the ferroptosis inducer erastin.

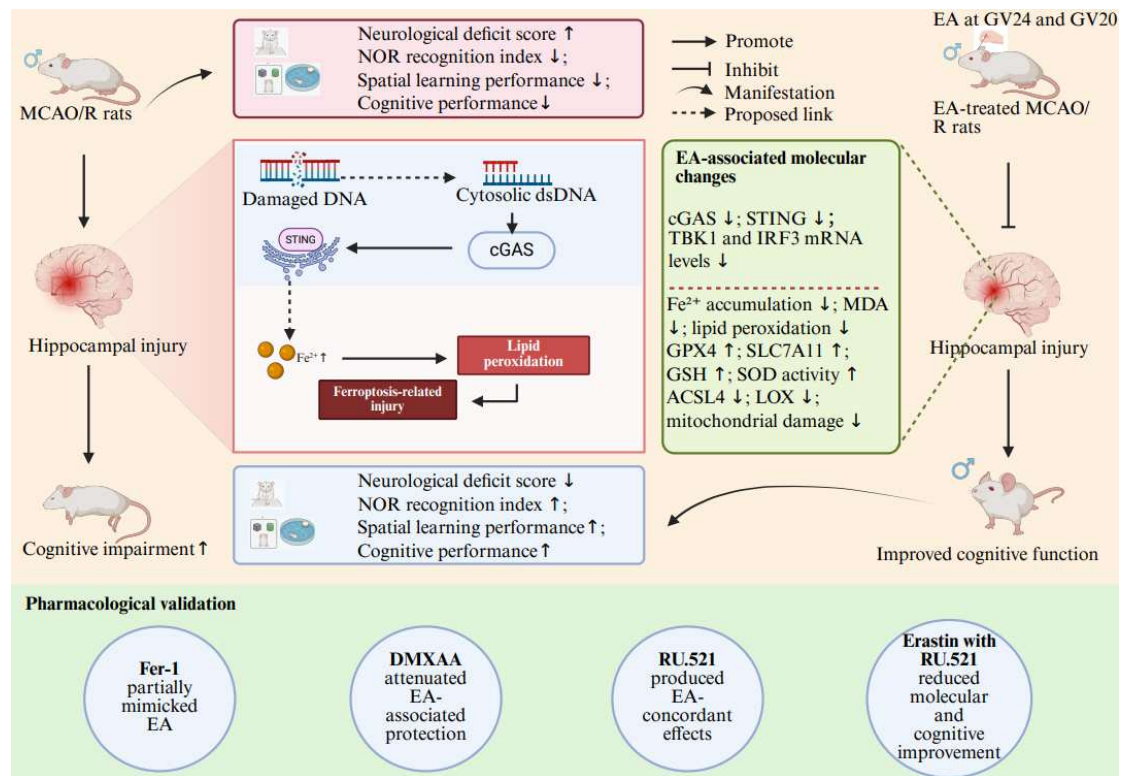
Conclusions: Electroacupuncture may improve cognitive function in MCAO/R rats by inhibiting the cGAS-STING signaling pathway, thereby attenuating ferroptosis.

Keywords: electroacupuncture; post-stroke cognitive impairment; middle cerebral artery occlusion/reperfusion; cGAS–STING signaling; ferroptosis.

Highlights

1. EA improved cognition and attenuated hippocampal injury after MCAO/R.
2. EA reduced cGAS–STING-related signaling and ferroptosis-related alterations.
3. Fer-1 partly reproduced the neurobehavioral and histological effects of EA.
4. DMXAA weakened EA effects, whereas RU.521 produced concordant molecular changes.
5. Erastin reduced RU.521-associated improvements despite sustained STING suppression.

56 **Graphical Abstract**



57

58 **1. Introduction**

59 Post-stroke cognitive impairment (PSCI) is a common and serious complication of
60 ischemic stroke (IS). Its clinical manifestations may involve multiple cognitive
61 domains, including attention, executive function, memory, and information-processing
62 speed, and are associated with an increased risk of recurrent stroke and mortality.
63 Despite continuing advances in reperfusion therapy, no specific treatment for PSCI has
64 yet achieved broad consensus. Acupuncture has accumulated a body of clinical
65 evidence for the treatment of PSCI. Animal studies have shown that electroacupuncture
66 at Shenting (GV24) and Baihui (GV20) improves learning and memory performance in
67 rats subjected to middle cerebral artery occlusion/reperfusion (MCAO/R); however, the
68 underlying molecular mechanisms have not been fully elucidated.

69 Previous studies suggest that ferroptosis may represent a potential pathological process
70 in PSCI. Ferroptosis is a regulated form of cell death driven by iron-dependent
71 phospholipid peroxidation and is jointly regulated by cellular iron homeostasis,

phospholipid metabolism, and antioxidant systems. Cerebral ischemia/reperfusion can disrupt these homeostatic processes, leading to iron accumulation, enhanced lipid peroxidation, and impaired antioxidant defenses. Further evidence indicates that the ferroptosis inhibitor ferrostatin-1 (Fer-1) can reduce cerebral infarction and neuronal injury. These findings suggest that ferroptosis may contribute to cognitive impairment associated with cerebral ischemia/reperfusion; however, its specific role in cognitive impairment after MCAO/R remains to be clarified.

The cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS-STING) pathway is a central signaling axis that senses cytosolic DNA and initiates innate immune responses. It may serve as an important upstream regulatory pathway in ferroptosis-related injury after cerebral ischemia/reperfusion. Under physiological conditions, nuclear and mitochondrial DNA are relatively segregated from the cytosol, and cGAS-STING signaling is tightly constrained to maintain appropriate innate immune homeostasis. Cerebral ischemia/reperfusion-induced nuclear and mitochondrial DNA damage and cytosolic leakage can aberrantly activate cGAS-STING signaling and its downstream TBK1-IRF3 and NF- κ B signaling pathways, concomitant with dysregulated iron metabolism, enhanced lipid peroxidation, and impaired GPX4-mediated antioxidant defense. Previous studies have shown that STING activation exacerbates lipid peroxidation and ferroptosis-related injury in ischemia/reperfusion and inflammatory neurological injury models. These findings suggest that STING may be a regulatory node linking innate immune activation to ferroptosis-related injury; however, its specific role in cognitive impairment after cerebral ischemia/reperfusion requires direct verification. Moreover, genetic deletion or pharmacological inhibition of cGAS-STING signaling has been shown to reduce iron accumulation, oxidative injury, and ferroptosis-related alterations, suggesting a potential functional link between this pathway and ferroptosis.

2. Materials and Methods

2.1. Animals

100 A total of 354 specific pathogen-free male Sprague-Dawley rats, aged 6-8 weeks and
101 weighing 250-280g, were purchased from Jinan Pengyue Experimental Animal
102 Breeding Co., Ltd. (Shandong, China). Rats were housed at the Animal Experimental
103 Center of Henan University of Chinese Medicine under controlled environmental
104 conditions (25 ± 2 °C; relative humidity, $50\% \pm 5\%$) with a 12-h light/12-h dark cycle
105 and ad libitum access to food and water. All rats were acclimatized for 7 days before
106 the start of the experiment. The experimental protocol was reviewed and approved by
107 the Ethics Committee for Laboratory Animals of Henan University of Chinese
108 Medicine (approval No. IACUC-202311033). All animal experiments were conducted
109 in accordance with relevant guidelines for the care and use of laboratory animals, and
110 the study was reported in accordance with the ARRIVE 2.0 guidelines. Sample sizes
111 for each experiment were determined with reference to previous studies using
112 comparable MCAO/R rat models and our preliminary experiments, with initial group
113 sizes expanded as appropriate to account for anticipated model-related mortality and
114 exclusions. No formal a priori power calculation was performed. Animals were
115 allocated to the experimental groups using a random number table. Model induction
116 and interventions were performed by investigators aware of group allocation, whereas
117 behavioral assessments and histological image acquisition and quantitative analyses
118 were conducted by investigators blinded to group allocation. For all outcomes,
119 *n* represents the number of independent animals; technical replicates were not
120 considered independent samples in the statistical analyses. Initial animal numbers,
121 mortality, exclusions, and final numbers included in the analyses are shown in Figure
122 1C. Experiment 1 evaluated the effects of EA on cognitive function and cGAS-STING-
123 related molecules in MCAO/R rats. Experiment 2 examined the effects of EA and Fer-
124 1 on ferroptosis-related indices and behavioral outcomes. Experiment 3 used DMXAA
125 to evaluate the effects of STING activation on EA-associated effects. Experiment 4
126 used RU.521 to determine whether cGAS inhibition could partially mimic the

molecular effects of EA. Experiment 5 combined RU.521 with erastin to examine the functional position of ferroptosis downstream of cGAS-related signaling.

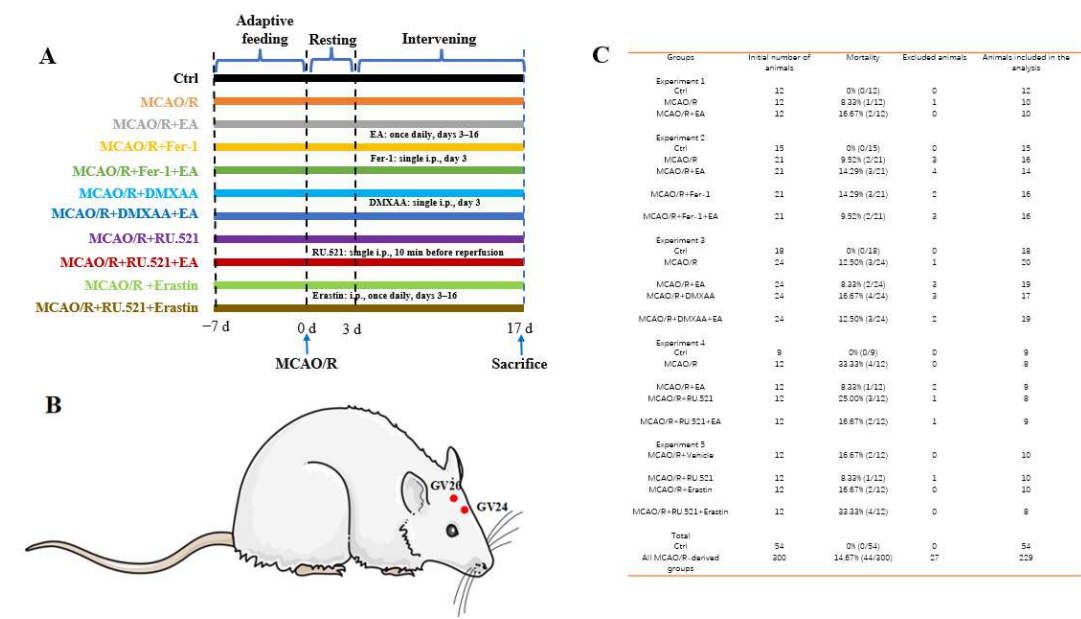


Figure 1. Experimental design, electroacupuncture protocol, acupoint locations, and animal allocation.

2.2. Establishment of the MCAO/R Model

The MCAO/R model was established using a modified intraluminal filament technique via the common carotid artery, with the surgical procedure and neurological function assessment based on the method described by Longa et al. Rats were anesthetized with 2% isoflurane by inhalation (RWD, Shenzhen, China). The left common carotid artery (CCA) was exposed and ligated proximally with 4-0 suture. A nylon monofilament (Beijing Cinontech, China) was then slowly introduced through the distal stump of the CCA into the internal carotid artery (ICA) until it occluded the origin of the middle cerebral artery (MCA). After 90 min of ischemia, the nylon monofilament was gently withdrawn to restore cerebral blood flow and initiate reperfusion. Body temperature was maintained at 37 °C throughout the surgical procedure using a heating pad. To prevent infection, gentamicin (25,000 U/kg) was administered by intraperitoneal injection once daily for 3 consecutive days after surgery. At 2h after reperfusion, neurological deficits were assessed using the Zea–Longa neurological deficit scoring

scale. Rats with scores of 1-3 were considered to have successful model establishment. Rats with Zea–Longa scores outside the range of 1-3 at 2h after reperfusion were excluded. Numbers of deaths and exclusions are presented in Figure 1C.

2.3. Electroacupuncture Intervention

Electroacupuncture was administered at Shenting (GV24) and Baihui (GV20). Acupoint localization and the stimulation protocol were based on previous studies of comparable MCAO/R rat models, with appropriate modifications. GV20 was located at the intersection of the dorsal midline of the head and the line connecting the tips of both ears. GV24 was located on the anterior midline of the head, anterior to the frontoparietal suture (Figure 1B). Sterile disposable acupuncture needles (0.25×25 mm) were inserted obliquely to a depth of approximately 3mm at GV24 and approximately 2mm at GV20. After needle insertion, the needle handles were connected to an electroacupuncture apparatus and stimulated with alternating sparse-dense waves at 2/10Hz. The stimulation parameters were determined on the basis of previous studies of GV20 and GV24 electroacupuncture in rats with focal cerebral ischemia/reperfusion and our preliminary protocol. EA treatment was initiated on day 3 after MCAO/R. The stimulation intensity was adjusted within a range of 1-3 mA, sufficient to induce slight rhythmic local tissue twitching without obvious struggling by the rats. Each session lasted 30 min and was administered once daily for 14 consecutive days.

2.4. Pharmacological Interventions

To evaluate the roles of ferroptosis and the cGAS-STING signaling pathway in the protective effects of EA and to further explore their potential upstream-downstream relationship, pharmacological interventions were performed using the ferroptosis inhibitor ferrostatin-1 (Fer-1), the STING agonist DMXAA, the cGAS inhibitor

RU.521, and the ferroptosis inducer erastin. The group assignments and intervention timelines for each experiment are shown in Figure 1A and C.

Fer-1 intervention: Fer-1 (GC10380; GlpBio, USA) was first dissolved in dimethyl sulfoxide (DMSO) to prepare a 10mmol/L stock solution. Before administration, PEG300, Tween 80, and normal saline were sequentially added to prepare a working solution containing 5% DMSO, 40% PEG300, 5% Tween 80, and 50% normal saline. The working solution was freshly prepared and thoroughly mixed before administration. Rats in the MCAO/R + Fer-1 group received a single intraperitoneal injection of Fer-1 (2.5 μ mol/kg; 5 mL/kg) on day 3 after MCAO/R surgery. Rats in the MCAO/R + Fer-1 + EA group received an intraperitoneal injection of Fer-1 at the same dose and volume on day 3 after surgery and then received EA for 14 consecutive days from the same day, according to the protocol described in Section 2.3. Corresponding control groups that did not receive Fer-1 in the Fer-1 experiment were intraperitoneally injected with an equivalent volume of vehicle at the same time point. The vehicle consisted of 5% DMSO, 40% PEG300, 5% Tween 80, and 50% normal saline.

DMXAA intervention: DMXAA (D5817; Merck, USA) was dissolved in sterile 5% sodium bicarbonate solution immediately before administration to prepare a 5 mg/mL working solution and was thoroughly mixed until clear. Rats in the MCAO/R + DMXAA group received a single intraperitoneal injection of DMXAA (25 mg/kg; 5 mL/kg) on day 3 after MCAO/R surgery. Rats in the MCAO/R + DMXAA + EA group received an intraperitoneal injection of DMXAA at the same dose and volume on day 3 after surgery and then received EA for 14 consecutive days from the same day, according to the protocol described in Section 2.3. Corresponding control groups that did not receive DMXAA in the DMXAA experiment were intraperitoneally injected with an equivalent volume of vehicle at the same time point. The vehicle was sterile 5% sodium bicarbonate solution.

cGAS inhibitor intervention: RU.521 (S6841; Selleck, USA) was first dissolved in anhydrous dimethyl sulfoxide (DMSO) to prepare a 50 mg/mL stock solution. Before

administration, PEG300, Tween 80, and sterile water for injection were sequentially added to prepare a working solution containing 2% DMSO, 40% PEG300, 5% Tween 80, and 53% sterile water for injection, with a final concentration of 1 mg/ml. The working solution was freshly prepared and thoroughly mixed before administration. Rats in the MCAO/R + RU.521 group received a single intraperitoneal injection of RU.521 (5 mg/kg; 5 mL/kg) at 80 min after the onset of ischemia, i.e., 10 min before filament withdrawal and restoration of cerebral blood flow for reperfusion. Rats in the MCAO/R + RU.521 + EA group received an intraperitoneal injection of RU.521 at the same dose and volume at the same time point and subsequently received EA for 14 consecutive days beginning on day 3 after MCAO/R, according to the protocol described in Section 2.3. Corresponding control groups that did not receive RU.521 in the RU.521 experiment received an intraperitoneal injection of an equivalent volume of vehicle at the same time point. The vehicle consisted of 2% DMSO, 40% PEG300, 5% Tween 80, and 53% sterile water for injection.

Erastin intervention: The ferroptosis inducer erastin (GC16630; GlpBio, USA) was first dissolved in dimethyl sulfoxide (DMSO) to prepare a 20mg/mL stock solution. Before administration, PEG300, Tween 80, and sterile water for injection were sequentially added to prepare a working solution containing 5% DMSO, 40% PEG300, 5% Tween 80, and 50% sterile water for injection, with a final concentration of 1 mg/mL. The working solution was freshly prepared and thoroughly mixed until clear before administration. Rats in the MCAO/R + Erastin group received intraperitoneal injections of erastin (10mg/kg) once daily for 14 consecutive days, beginning on day 3 after MCAO/R surgery. Rats in the MCAO/R + RU.521 + Erastin group received erastin according to the same regimen and were additionally administered a single intraperitoneal injection of RU.521 (5mg/kg) at 80 min after the onset of ischemia, i.e., 10 min before filament withdrawal and restoration of cerebral blood flow for reperfusion. Corresponding control groups that did not receive erastin received an intraperitoneal injection of an equivalent volume of vehicle at the corresponding time

points. The vehicle consisted of 5% DMSO, 40% PEG300, 5% Tween 80, and 50% sterile water for injection. Experiment 5 employed double-vehicle controls matched for administration timing and solvent composition. The MCAO/R + Vehicle group received RU.521 vehicle before reperfusion and erastin vehicle on postoperative days 3-16. The RU.521 group received RU.521 and erastin vehicle; the Erastin group received RU.521 vehicle and erastin; and the combined-treatment group received RU.521 and erastin. The compositions of the RU.521 vehicle and erastin vehicle were identical to those of the respective drug working solutions described above, and injection volumes were matched across groups at each corresponding time point.

2.5. Neurological Deficit Scoring and Infarct Volume Assessment

Neurological deficits were evaluated at 2 h after reperfusion and on days 7 and 14 after model establishment by investigators blinded to group allocation, using the Zea–Longa neurological deficit scoring scale. Scores ranged from 0 to 4, with higher scores indicating more severe neurological impairment. Rats with scores of 1-3 at 2 h after reperfusion were considered to have successful model establishment and were included in subsequent experiments. After completion of the 14-day intervention, brains were collected and serially sectioned into 2-mm-thick coronal slices. The brain slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC; G3005; Solarbio, China) solution at 37 °C for 15 min in the dark and were subsequently fixed overnight in 4% paraformaldehyde (BL539A; Biosharp, China). Viable brain tissue stained red, whereas unstained white areas were identified as infarcted regions. Stained brain slices were photographed, and the infarct area and total cross-sectional brain area of each slice were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The infarct areas and total brain cross-sectional areas were summed across slices. The percentage infarct volume was calculated as the summed infarct area divided by the summed total brain cross-sectional area. Because slice thickness was not completely

uniform across all brain sections, this area-based proportion was used to estimate relative infarct volume.

2.6. Novel Object Recognition Test

The novel object recognition (NOR) test was used to assess object recognition memory in rats. The experimental paradigm was based on the method established by Ennaceur and Delacour, with appropriate modifications. Testing was conducted in a separate behavioral laboratory under stable environmental conditions and during the same time period each day. The entire procedure was recorded using a camera mounted above the test arena and analyzed using an animal behavior video analysis system (XinRuan, Shanghai, China). Only animals that completed the test and met the prespecified inclusion criteria were included in the NOR analysis; the actual sample sizes for each experiment and group are presented in Figure 1C and the corresponding figure legends. The test comprised habituation, familiarization, and test phases. During habituation, each rat was placed in the center of the empty test arena and allowed to explore freely for 10 min. During the subsequent familiarization phase, two objects identical in appearance, material, and size were secured symmetrically on opposite sides of the arena. Each rat was then placed in the center of the arena and allowed to explore for 10 min. Twenty-four hours after familiarization, one of the familiar objects was replaced with a novel object of a different color and shape, and the rat was allowed to explore freely for 5 min. The left–right position of the novel object was counterbalanced within each group to minimize the influence of position preference. Object exploration was defined as the rat orienting its nose toward and actively approaching, sniffing, or touching an object. Sitting near, leaning against, or climbing on an object, as well as passing by without clear orientation toward the object, was not considered valid exploration. Investigators blinded to group allocation recorded the time spent exploring the novel and familiar objects during the test phase from the video recordings. The recognition index was calculated for each rat as the time spent exploring the novel

object divided by the combined time spent exploring the novel and familiar objects and ranged from 0 to 1. Statistical analyses were performed using individual rats as the experimental units. After each rat completed the relevant phase, the test arena and objects were thoroughly wiped with 75% ethanol. Testing of the next rat was initiated only after complete evaporation of the ethanol to minimize interference from residual olfactory cues.

2.7. Elevated Plus Maze Test

The elevated plus maze (EPM) test was used to assess anxiety-like behavior in rats. The maze consisted of two opposing open arms, two opposing enclosed arms, and a central platform. At the beginning of each test, a rat was placed on the central platform with its head facing an open arm and was allowed to explore freely for 10 min. A camera was positioned directly above the maze to record locomotor trajectories, time spent in each arm, and the number of arm entries using an animal behavior video analysis system (XinRuan, Shanghai, China). An entry was considered valid when all four paws of the rat entered the corresponding arm. The percentage of time spent in the open arms was defined as the time spent in the open arms divided by the combined time spent in the open and enclosed arms. The percentage of open-arm entries was defined as the number of entries into the open arms divided by the combined number of entries into the open and enclosed arms. Time spent on the central platform was not included in the time spent in either the open or enclosed arms. After each rat was tested, the maze was wiped with 75% ethanol. The next rat was tested only after complete evaporation of the ethanol to minimize the influence of residual olfactory cues. Behavioral data were analyzed by investigators blinded to group allocation.

2.8. Morris Water Maze Test

During days 9-14 of electroacupuncture treatment, the Morris water maze (MWM) test was used to assess spatial learning and memory in rats. The procedure was based on the

method established by Morris. The entire experiment was recorded by a camera system positioned above the pool, and swimming trajectories were analyzed using an animal behavior video analysis system (XinRuan, Shanghai, China). A 5-day acquisition training phase was conducted from days 9 to 13. Each day, rats were released from different starting points while facing the pool wall and were required to locate a hidden platform positioned below the water surface. Escape latency and swimming trajectories were recorded. On day 14, the platform was removed for a 90-s spatial probe trial, during which the number of crossings over the former platform location and swimming trajectories were recorded. The testing apparatus, platform location, and extramaze visual cues were kept constant throughout the experiment. Behavioral data were analyzed by investigators blinded to group allocation. After each trial, the rats were promptly dried and kept warm. The pool was cleaned after completion of the experiment.

2.9. Hematoxylin–Eosin and Prussian Blue Staining

After completion of the 14-day intervention, rats designated for histopathological examination were anesthetized and transcardially perfused. Following sequential perfusion with normal saline and 4% paraformaldehyde, brains were collected and post-fixed in 4% paraformaldehyde for 24 h. Brain tissues were then processed by routine dehydration, clearing, paraffin embedding, and preparation of coronal sections. Hematoxylin-eosin (H&E) staining (G1120; Solarbio, China) and Prussian blue staining (G1428; Solarbio, China) were performed according to the manufacturers' instructions. H&E staining was used to evaluate hippocampal tissue morphology and neuronal pathological alterations, whereas Prussian blue staining was used to assess iron deposition in the hippocampal region. Hippocampal areas were observed and imaged under identical imaging parameters using a light microscope (ECLIPSE Ts2; Nikon, Japan). Image acquisition and analysis were performed by investigators blinded to group allocation.

2.10. Transmission Electron Microscopy

After completion of the 14-day intervention, hippocampal tissues designated for transmission electron microscopy (TEM) were rapidly dissected, and tissue blocks of approximately 1 mm³ were prepared. The tissue blocks were immediately fixed in a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde. After thorough rinsing in 0.1 mol/L phosphate buffer (pH 7.4), the specimens were post-fixed in 1% osmium tetroxide for 90 min. They were then subjected sequentially to graded ethanol dehydration, epoxy resin infiltration, and embedding. Embedded tissues were sectioned into ultrathin sections of approximately 80 nm, mounted on copper grids, and double-stained with uranyl acetate and lead citrate. After the sections had dried, mitochondrial ultrastructural images of hippocampal neurons were obtained using a transmission electron microscope (HT7800; Hitachi, Japan). Morphological features including mitochondrial size, membrane integrity, cristae structure, and electron density were assessed. Image acquisition and morphological evaluation were performed by investigators blinded to group allocation.

2.11. Determination of Fe²⁺, GSH, SOD, and MDA

After completion of the respective 14-day interventions, hippocampal tissues designated for biochemical analyses were rapidly dissected. Appropriate amounts of tissue were homogenized on ice in pre-chilled homogenization medium prepared according to the instructions for each assay kit. Tissue homogenates were centrifuged at 4 °C for 15 min, and the supernatants were collected for subsequent analyses. Ferrous ion (Fe²⁺) content (E-BC-K773-M; Elabscience, China), reduced glutathione (GSH) content (E-BC-K030-M; Elabscience, China), superoxide dismutase (SOD) activity (E-BC-K020-M; Elabscience, China), and malondialdehyde (MDA) content (E-BC-K025-M; Elabscience, China) were determined according to the manufacturers' instructions. After reaction of the samples with the corresponding working reagents, absorbance was measured using a microplate reader at the wavelengths specified by the respective assay

kits. Fe²⁺, GSH, and MDA contents and SOD activity were calculated according to the procedures specified by each kit and normalized to tissue mass or total protein content in the supernatant. All samples were assayed under identical experimental conditions.

2.12. Western Blotting

Approximately 30 mg of rat hippocampal tissue was homogenized mechanically with three grinding beads (approximately 3mm in diameter) in 300 µL of pre-chilled radioimmunoprecipitation assay (RIPA) lysis buffer (R0010; Solarbio, China) supplemented with 3µL of phenylmethylsulfonyl fluoride (PMSF). Homogenates were lysed on ice for 30 min and then centrifuged at 12,000 × g for 15 min at 4 °C. The supernatants were collected, and total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (PC0020; Solarbio, China). The volume of each sample was adjusted to achieve the same protein concentration, after which 5× protein loading buffer (P1040; Solarbio, China) was added. Samples were heated at 100 °C for 5 min to ensure complete protein denaturation. Equal amounts of total protein were loaded, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes. Before transfer, PVDF membranes were activated in methanol for 3 min. Following transfer, membranes were blocked in blocking solution at room temperature for 2 h, washed three times with Tris-buffered saline containing Tween-20 (TBST) for 10 min each, and then incubated overnight at 4 °C with the corresponding primary antibodies.

The primary antibodies used were as follows: cGAS (1:1,000; YT7062; Immunoway), STING (1:500; 19851-1-AP; Proteintech), NCOA4 (1:1,000; YT0302; Immunoway), FTH1 (1:1,000; ab183781; Abcam), FTL (1:2,000; ab109373; Abcam), LC3 (1:2,000; ab192890; Abcam), Beclin-1 (1:1,000; AF5128; Affinity), p62 (1:10,000; ab109012; Abcam), GPX4 (1:2,000; ab125066; Abcam), SLC7A11 (1:2,000; ab175186; Abcam), ACSL4 (1:2,000; 22401-1-AP; Proteintech), LOX (1:5,000; ab174316; Abcam),

phospho-STING (p-STING; 1:1,000; AF7416; Affinity Biosciences), and GAPDH (1:5,000; 10494-1-AP; Proteintech) as the loading control. After primary antibody incubation, the membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBST; T1082; Solarbio, China) for 10 min each. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (SA00001-2; Proteintech) for 1 h at room temperature. Following three additional 10-min washes with TBST, protein bands were detected and imaged using enhanced chemiluminescence reagent (2311006; Solarbio, China). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Except for LC3 and p-STING, all target proteins were normalized to GAPDH from the corresponding sample. LC3 was quantified as the LC3-II/LC3-I band-intensity ratio, whereas p-STING was quantified as the p-STING-to-total-STING band-intensity ratio. Exposure conditions were maintained within the linear detection range. Samples from different experimental groups for the same target protein were compared under identical electrophoresis, transfer, signal-development, and image-analysis conditions.

2.13. Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from hippocampal tissues using the G3013 kit (Servicebio, China), and its concentration and purity were determined. Complementary DNA (cDNA) was synthesized by reverse transcription using SweScript All-in-One RT SuperMix (G3337; Servicebio, China). The mRNA expression levels of TBK1, IRF3, IL-1 β , IL-10, and TNF- α were quantified using a SYBR qRT-PCR kit (G3326; Servicebio, China), with GAPDH serving as the internal reference gene. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed in Table 1.

Table 1. Primer Sequences Used for Quantitative Real-Time Polymerase Chain Reaction

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
TBK1	5'-ACCAAGCTGCTGA GACTTTCCA-3'	5'-GATGAGTGCCTTC CTGATGTGC-3'
IRF3	5'-CAGGCCCTTTGTG GCAGATC-3'	5'-TTGTACTGGTCAG AGGTAAGGGAG-3'
IL-1 β	5'-TGTGACTCGTGGG ATGATGAC-3'	5'-CCACTTGTTGGCT TATGTTCTGTC-3'
IL-10	5'-ACAATAACTGCAC CCACTTCCC-3'	5'-CCAAGTAACCCTT AAAGTCCTGC-3'
TNF- α	5'-CCACCACGCTCTT CTGTCTACTG-3'	5'-TGGGCTACGGGCT TGTCAC-3'
GAPDH	5'-CTGGAGAAACCT GCCAAGTATG-3'	5'-GGTGGAAGAATG GGAGTTGCT-3'

414 2.14. Immunofluorescence Staining

415 After deparaffinization and rehydration, paraffin sections underwent heat-mediated
416 antigen retrieval in 1×EDTA buffer. Following antigen retrieval, the sections were
417 cooled to room temperature, blocked to reduce nonspecific binding, and incubated
418 overnight at 4 °C with primary antibodies. The primary antibodies used in this study
419 were STING (1:100; 066680-1-Ig; Proteintech), NCOA4 (1:100; YT0302;
420 Immunoway), FTH1 (1:100; sc-376594; Santa Cruz Biotechnology), and LC3 (1:500;
421 GTX127375; GeneTex). After primary antibody incubation, sections were washed three
422 times with phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) for 5–10 min
423 each. Sections were then incubated for 50 min at room temperature in the dark with
424 fluorophore-conjugated secondary antibodies: the secondary antibody corresponding to
425 rabbit primary antibodies (1:1,000; RGAR003; Proteintech) and the secondary antibody
426 corresponding to mouse primary antibodies (1:1,000; RGAM002; Proteintech).
427 Sections were subsequently counterstained with DAPI for 10 min at room temperature

in the dark and mounted with anti-fade mounting medium. Images were acquired using a fluorescence microscope (ECLIPSE Ts2R-FL; Nikon, Japan). The mean fluorescence intensity of each target protein in the hippocampal region was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.15. Statistical Analysis

Statistical analyses were performed using SPSS version 25.0. Data with a normal distribution are presented as the mean $\pm$ standard deviation (SD). For comparisons among multiple groups at a single time point, one-way analysis of variance (ANOVA) was used. When homogeneity of variance was satisfied, the least significant difference (LSD) test was applied for post hoc comparisons; when the assumption of homogeneity of variance was not met, Welch's ANOVA followed by Dunnett's T3 test was used. Zea-Longa scores were analyzed using the Kruskal-Wallis test. For the RU.521-Erastin combined-intervention experiment, two-way ANOVA was used to examine the main effects of RU.521 and erastin and their interaction, followed by Sidak-adjusted between-group comparisons. Escape latency in the Morris water maze (MWM) was analyzed using repeated-measures ANOVA, with training day as the within-subject factor and experimental group as the between-subject factor. In Experiment 5, RU.521 and erastin were additionally modeled as between-subject factors to examine their main effects and interaction. Greenhouse–Geisser correction was applied when necessary. Effect sizes are reported as partial eta squared (η_p^2). All tests were two-sided, and $P < 0.05$ was considered statistically significant. Individual animals were treated as the experimental units for all statistical analyses, and n denotes the number of independent animals.

3. Results

3.1. MCAO/R Rats Exhibited Cognitive Impairment and Aberrant Hippocampal Expression of cGAS-STING-Related Molecules, Which Were Attenuated by Electroacupuncture

To characterize changes in cognitive function, brain tissue injury, and hippocampal cGAS-STING-related molecules after MCAO/R and to evaluate the effects of EA, relevant outcomes were compared among the Ctrl, MCAO/R, and MCAO/R + EA groups. TTC staining showed that, compared with the Ctrl group, rats in the MCAO/R group exhibited distinct infarcted areas and significantly higher neurological deficit scores. Following EA intervention, infarct volume was reduced and neurological deficit scores were lower (Figure 2A-C). In the Morris water maze, MCAO/R rats showed prolonged escape latencies during acquisition training and fewer crossings over the former platform location during the spatial probe trial. EA shortened escape latencies and increased the number of crossings over the former platform location (Figure 2D-F), indicating that EA improved spatial learning and memory performance in MCAO/R rats. Western blotting showed that total hippocampal cGAS and STING protein expression was significantly increased in the MCAO/R group relative to the Ctrl group and was reduced following EA intervention (Figure 2G-I). RT-qPCR showed increased hippocampal mRNA expression of TBK1, IRF3, and the proinflammatory cytokines IL-1 β and TNF- α , together with reduced mRNA expression of the anti-inflammatory cytokine IL-10, in the MCAO/R group. EA intervention improved these transcriptional abnormalities to varying degrees (Figure 2J-N). In summary, MCAO/R rats exhibited brain tissue injury, neurological deficits, and impaired spatial learning and memory, accompanied by aberrant expression of hippocampal cGAS-STING-related proteins and inflammation-related genes. EA attenuated these functional and molecular alterations.

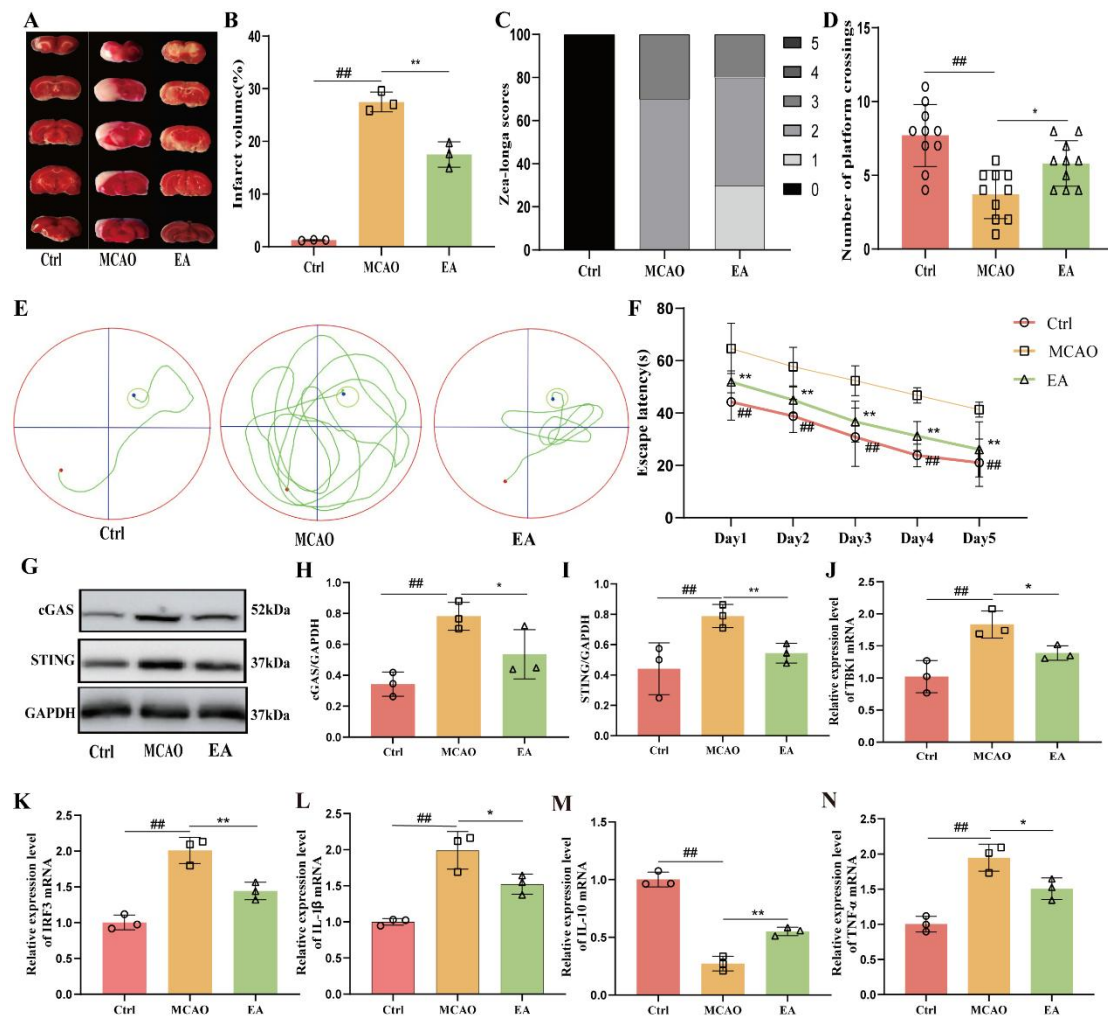


Figure 2. EA treatment reduces infarct volume, ameliorates neurological deficits, and attenuates cGAS–STING-related molecular alterations in MCAO/R rats.

3.2. Electroacupuncture Attenuated Ferroptosis- and Lipid Peroxidation-Related Alterations and Modulated Ferritinophagy-Related Molecular Expression in the Hippocampus of MCAO/R Rats

To determine whether the protective effects of EA were associated with attenuation of ferroptosis-related injury, we assessed indices related to iron load, lipid peroxidation, and antioxidant defense in hippocampal tissue. Prussian blue staining showed increased iron deposition in the hippocampal region of MCAO/R rats compared with the Ctrl group. In addition, hippocampal Fe²⁺ and MDA contents were increased, whereas GSH content and SOD activity were decreased (Figure 3L-P), indicating increased

489 hippocampal iron load and lipid peroxidation, together with impaired antioxidant
490 defense, after MCAO/R. Following EA intervention, hippocampal iron deposition and
491 Fe^{2+} content were reduced, MDA content was decreased, and GSH content and SOD
492 activity were increased. Fer-1 intervention induced changes in the same direction as
493 those observed with EA. Following combined Fer-1 and EA intervention, some indices
494 shifted further toward the levels observed in the Ctrl group (Figure 3L-P). These
495 findings indicate that MCAO/R was associated with ferroptosis-related biochemical
496 alterations in the hippocampus, including increased iron load and lipid peroxidation and
497 impaired antioxidant defense. EA attenuated these abnormalities, whereas Fer-1 exerted
498 regulatory effects in the same direction. Building on these findings, we further
499 examined changes in ferritin- and autophagy-related molecules. Western blotting
500 showed that, compared with the Ctrl group, the MCAO/R group exhibited increased
501 hippocampal expression of NCOA4 and Beclin-1 and an increased LC3-II/LC3-I ratio,
502 together with decreased expression of FTH1, FTL, and p62. EA or Fer-1 intervention
503 improved these abnormalities in protein expression to varying degrees (Figure 3A-H).
504 Immunofluorescence staining showed reduced FTH1 fluorescence intensity and
505 increased LC3 fluorescence intensity in the hippocampal CA1 region of the MCAO/R
506 group. Following EA or Fer-1 intervention, both fluorescence signals shifted toward the
507 levels observed in the Ctrl group (Figure 3I-K). The direction of changes in the
508 combined-intervention group was consistent with that in the single-intervention groups,
509 with further improvement observed for some indices. In summary, MCAO/R was
510 associated with ferroptosis-related alterations in the hippocampus, including increased
511 iron load and lipid peroxidation and impaired antioxidant defense. EA attenuated these
512 abnormalities, whereas Fer-1 exerted regulatory effects in the same direction. MCAO/R
513 was also accompanied by altered expression of NCOA4 and ferritin- and autophagy-
514 related molecules, and EA and Fer-1 interventions modulated these molecular
515 alterations to varying degrees.

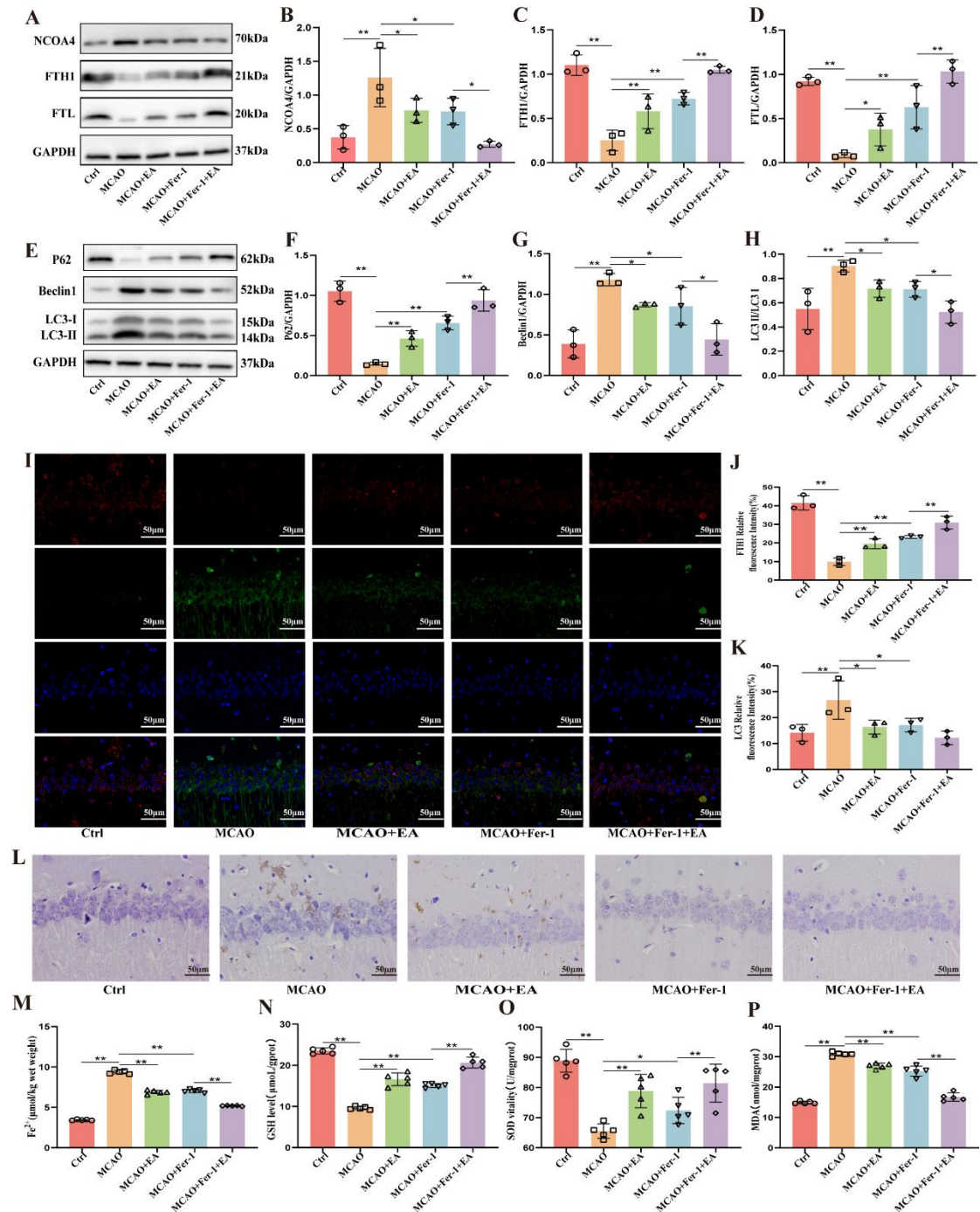


Figure 3. EA attenuates ferroptosis-related alterations and modulates ferritin- and autophagy-related proteins in the hippocampus of MCAO/R rats.

3.3. The Ferroptosis Inhibitor Fer-1 Partially Mimicked the Effects of Electroacupuncture on Cognitive Function in MCAO/R Rats

To investigate whether ferroptosis-related injury contributes to cognitive impairment after MCAO/R, we administered the ferroptosis inhibitor ferrostatin-1 (Fer-1) and

compared the effects of EA and Fer-1 administered alone or in combination. TTC staining showed that, compared with the Ctrl group, infarct volume was significantly increased in the MCAO/R group. Both EA and Fer-1 interventions markedly reduced infarct volume, and combined Fer-1 and EA intervention further reduced infarct volume (Figure 4A,B). Similarly, neurological deficit scores were significantly increased in the MCAO/R group but were reduced by EA or Fer-1 intervention, with a further reduction observed in the combined-intervention group (Figure 4C). In the novel object recognition test, compared with the Ctrl group, MCAO/R rats spent less time exploring the novel object, more time exploring the familiar object, and exhibited a lower recognition index. EA or Fer-1 intervention increased novel-object exploration time, decreased familiar-object exploration time, and increased the recognition index. These indices of object recognition memory were further improved following combined Fer-1 and EA intervention (Figure 4D-F). In the elevated plus maze test, the percentage of time spent in the open arms and the percentage of entries into the open arms were reduced in MCAO/R rats. Both measures increased after EA or Fer-1 intervention, with further improvement in the combined-intervention group (Figure 4G-I), indicating that both EA and Fer-1 alleviated anxiety-like behavior in MCAO/R rats. Hematoxylin-eosin staining showed disorganized cellular arrangement, widened intercellular spaces, and pyknotic, hyperchromatic nuclei in the hippocampal region of MCAO/R rats. These histopathological alterations were improved to varying degrees following EA or Fer-1 intervention, and hippocampal morphology was further improved in the combined-intervention group (Figure 4J). In summary, Fer-1 attenuated brain tissue injury and neurological deficits and improved object recognition memory and anxiety-like behavior in MCAO/R rats. The direction of its effects was consistent with that of EA.

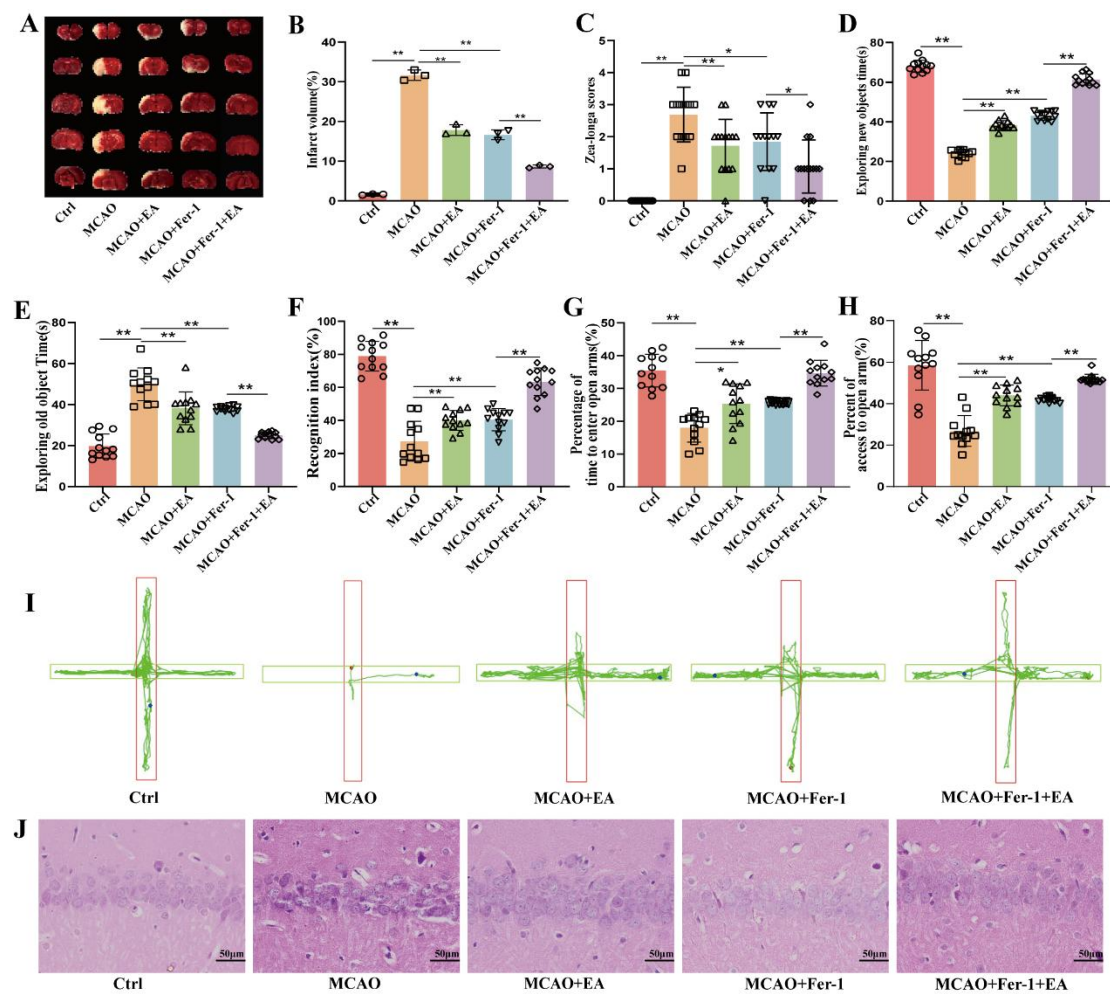


Figure 4. Ferrostatin-1 partially mimics the protective effects of EA against MCAO/R-induced neurological deficits and cognitive impairment.

3.4. STING Activation Attenuated the Protective Effects of Electroacupuncture on Cognitive Function in MCAO/R Rats

To evaluate the role of STING signaling in the protective effects of EA, the STING agonist DMXAA was administered to examine its effects on EA-associated improvements in brain tissue injury and cognitive behavior. TTC staining showed that, compared with the MCAO/R group, EA intervention significantly reduced infarct volume, whereas DMXAA intervention further increased infarct volume. Compared with the MCAO/R + EA group, combined DMXAA and EA intervention resulted in a markedly greater infarct volume, indicating that STING activation attenuated the EA-associated improvement in brain tissue injury (Figure 5A, B). In the elevated plus maze

test, the percentage of time spent in the open arms and the percentage of entries into the open arms were lower in the MCAO/R group than in the Ctrl group, and both measures increased following EA intervention. DMXAA intervention further decreased both measures, whereas these measures remained lower in the DMXAA+EA group than in the EA-alone group (Figure 5C, D), indicating that DMXAA attenuated the EA-associated improvement in anxiety-like behavior. In the novel object recognition test, compared with the MCAO/R group, EA intervention increased the time spent exploring the novel object, decreased the time spent exploring the familiar object, and increased the recognition index. DMXAA intervention decreased novel-object exploration time and the recognition index while increasing familiar-object exploration time. Compared with the MCAO/R+EA group, combined DMXAA and EA intervention decreased novel-object exploration time and the recognition index and increased familiar-object exploration time (Figure 5E-G), indicating that DMXAA attenuated the EA-associated improvement in object recognition memory. In the Morris water maze, EA intervention shortened escape latency during acquisition training and increased the number of crossings over the former platform location during the spatial probe trial. In contrast, DMXAA intervention prolonged escape latency and reduced the number of crossings over the former platform location. Compared with the MCAO/R + EA group, combined DMXAA and EA intervention prolonged escape latency and reduced the number of crossings over the former platform location. Swimming trajectories also showed reduced searching in the area where the platform had previously been located (Figure 5H-K), indicating that DMXAA attenuated the EA-associated improvement in spatial learning and memory. In summary, DMXAA intervention exacerbated brain tissue injury, cognitive behavioral abnormalities, and anxiety-like behavior in MCAO/R rats and attenuated the corresponding protective effects of EA. These findings indicate that STING activation may weaken the EA-associated improvement in cognitive function in MCAO/R rats.

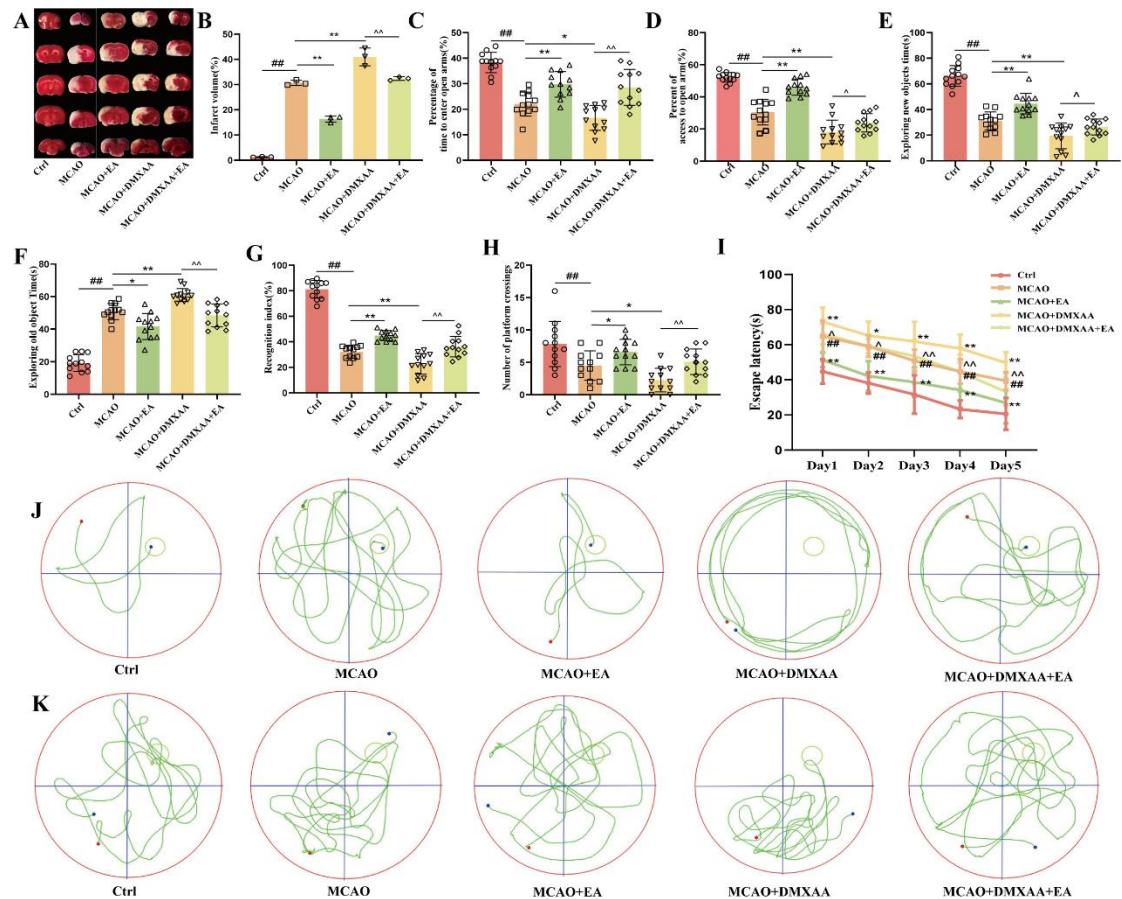


Figure 5. DMXAA attenuates the effects of EA on brain injury and behavioral deficits in MCAO/R rats.

3.5. STING Activation Attenuated EA-Associated Improvements in Ferroptosis-Related Injury and Was Accompanied by Changes in Ferritinophagy-Related Molecules

To further determine whether the attenuation of EA-associated cognitive protection by DMXAA was accompanied by ferroptosis-related alterations, we assessed hippocampal cGAS-STING-related proteins, ferroptosis-related molecules, biochemical indices, and tissue structure. Western blotting showed that, compared with the MCAO/R group, EA intervention reduced total hippocampal cGAS and STING protein expression, whereas DMXAA intervention further increased the expression of these proteins. Compared with the MCAO/R + EA group, combined DMXAA and EA intervention increased total cGAS and STING protein expression; however, expression levels remained lower than

those in the DMXAA-alone group (Figure 6A-C). Analysis of ferroptosis-related proteins showed that EA intervention increased GPX4 and SLC7A11 expression and reduced ACSL4 and LOX expression, whereas DMXAA intervention induced changes in the opposite direction. Compared with the MCAO/R+EA group, combined DMXAA and EA intervention decreased GPX4 and SLC7A11 expression and increased ACSL4 and LOX expression. However, compared with the DMXAA-alone group, these indices remained improved to varying degrees in the combined-intervention group (Figure 6O-S). Biochemical analyses showed that EA intervention increased hippocampal GSH content and SOD activity and reduced MDA content, whereas DMXAA intervention decreased GSH content and SOD activity and increased MDA content. In the DMXAA + EA group, these indices shifted in the direction of injury compared with the EA-alone group but remained improved relative to the DMXAA-alone group (Figure 6L-N).

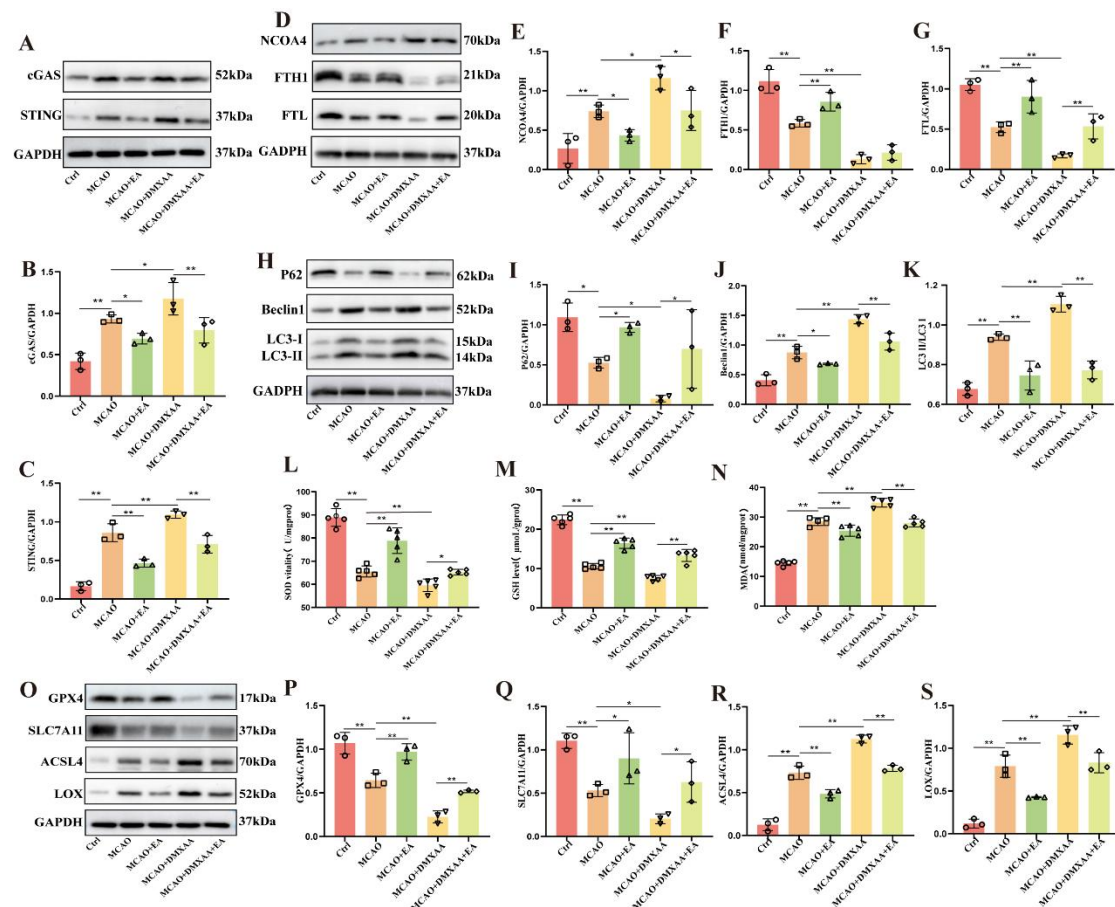


Figure 6. DMXAA attenuates the effects of EA on ferroptosis-related alterations and is accompanied by changes in ferritin- and autophagy-related proteins in MCAO/R rats.

Further analyses showed that DMXAA intervention increased hippocampal Fe^{2+} content and iron deposition and was accompanied by exacerbated hippocampal histopathological injury and mitochondrial ultrastructural abnormalities. EA intervention attenuated these alterations. However, following combined DMXAA and EA intervention, the EA-associated improvements in Fe^{2+} accumulation, iron deposition, hippocampal tissue morphology, and mitochondrial ultrastructural injury were attenuated (Figure 7B-E). These findings indicate that DMXAA intervention exacerbated abnormalities in iron load, lipid peroxidation, and anti-lipid peroxidation defense and attenuated the EA-associated improvement in ferroptosis-related injury after MCAO/R. We also examined changes in ferritin- and general autophagy-related molecules. Western blotting showed that, compared with the MCAO/R group, EA intervention decreased NCOA4 and Beclin-1 expression and the LC3-II/LC3-I ratio, while increasing FTH1, FTL, and p62 expression; DMXAA intervention induced changes in the opposite direction. Compared with the MCAO/R + EA group, combined DMXAA and EA intervention increased NCOA4 and Beclin-1 expression and the LC3-II/LC3-I ratio and decreased FTH1, FTL, and p62 expression. However, compared with the DMXAA-alone group, these indices remained improved to varying degrees in the combined-intervention group (Figure 6D-K). Immunofluorescence staining showed detectable STING and NCOA4 fluorescence signals in the hippocampal CA1 region in all groups, with partial spatial overlap between the two signals in merged images (Figure 7A). In summary, DMXAA intervention attenuated EA-associated improvements in hippocampal ferroptosis-related proteins, iron load, lipid peroxidation, antioxidant defense, and tissue structural injury, accompanied by changes in the expression of NCOA4 and ferritin- and general autophagy-related molecules.

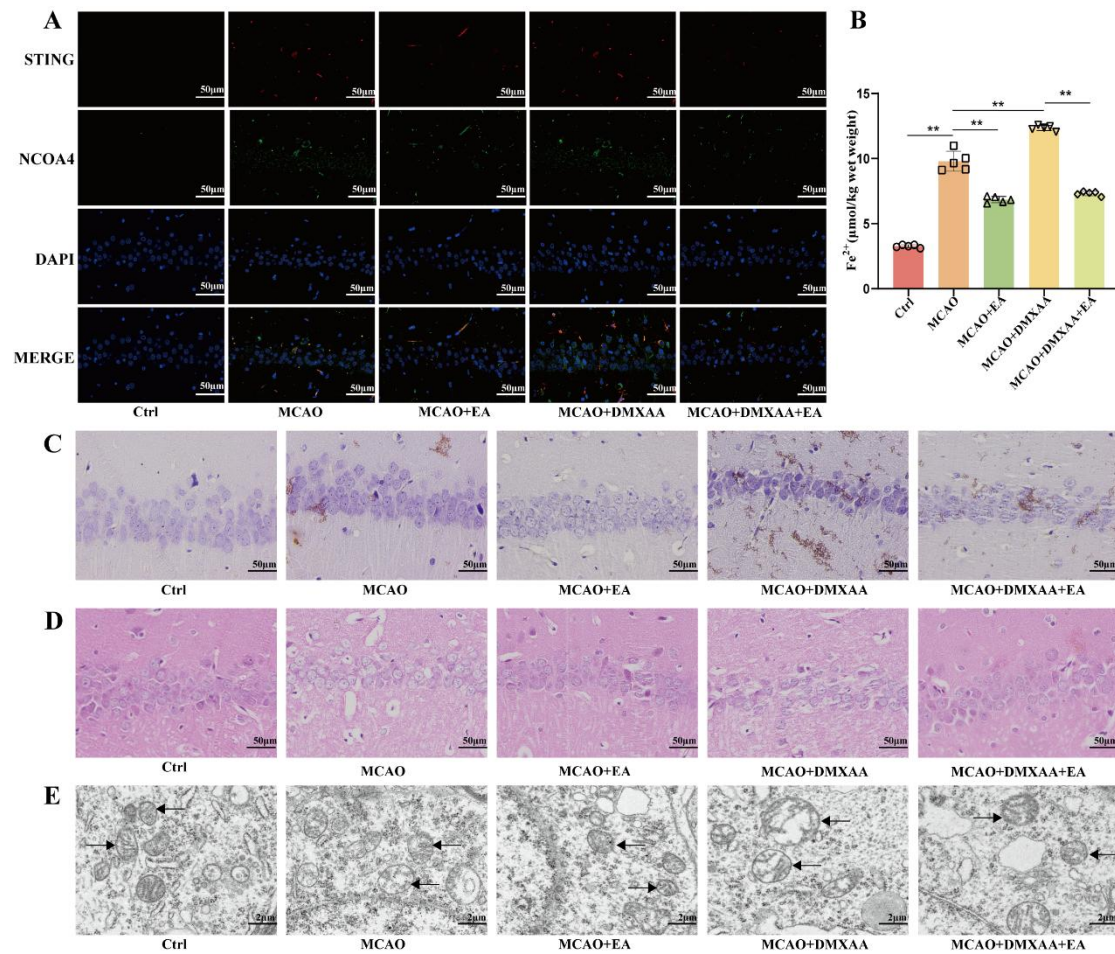


Figure 7. STING-NCOA4 co-localization, iron deposition, and mitochondrial damage reflect the reversal of EA's protective effects by DMXAA.

3.6. cGAS Inhibition Partially Mimicked the Regulatory Effects of Electroacupuncture on Ferroptosis- and Oxidative Stress-Related Molecules

To further determine whether pharmacological inhibition of cGAS could produce molecular regulatory effects similar to those of EA, we administered the cGAS inhibitor RU.521. Western blotting showed that, compared with the Ctrl group, total hippocampal cGAS and STING protein expression was increased in the MCAO/R group. Following EA or RU.521 intervention, expression of both proteins showed an overall downward trend, with a more pronounced decrease after combined RU.521 and EA intervention (Figure 8A-C). These findings indicate that RU.521 intervention was accompanied by changes in hippocampal cGAS-STING-related protein expression;

654 however, because only total cGAS and STING protein levels were measured, the extent
655 of pathway activation could not be directly determined. We subsequently assessed
656 ferroptosis-related proteins. Compared with the MCAO/R group, RU.521 intervention
657 increased hippocampal GPX4 and SLC7A11 expression and decreased ACSL4 and
658 LOX expression; the direction of these changes was broadly consistent with that
659 observed following EA intervention. After combined RU.521 and EA intervention,
660 these protein levels generally shifted further toward those observed in the Ctrl group
661 (Figure 8M-Q). These findings indicate that RU.521 partially mimicked the regulatory
662 effects of EA on ferroptosis-related molecular abnormalities after MCAO/R. In addition,
663 Western blotting showed that, compared with the MCAO/R group, RU.521 intervention
664 decreased NCOA4 and Beclin-1 expression and increased FTH1 and p62 expression.
665 Following combined EA and RU.521 intervention, these indices generally shifted
666 further toward those observed in the Ctrl group (Figure 8D-H). Immunofluorescence
667 staining showed increased NCOA4 and LC3 fluorescence intensities in the
668 hippocampal CA1 region of the MCAO/R group. Both fluorescence intensities were
669 reduced following EA or RU.521 intervention, with more pronounced reductions in the
670 combined-intervention group (Figure 8I-L). In summary, RU.521 intervention partially
671 mimicked the regulatory effects of EA on hippocampal ferroptosis-related proteins and
672 was accompanied by changes in cGAS-STING-related proteins and ferritin- and
673 general autophagy-related molecules.

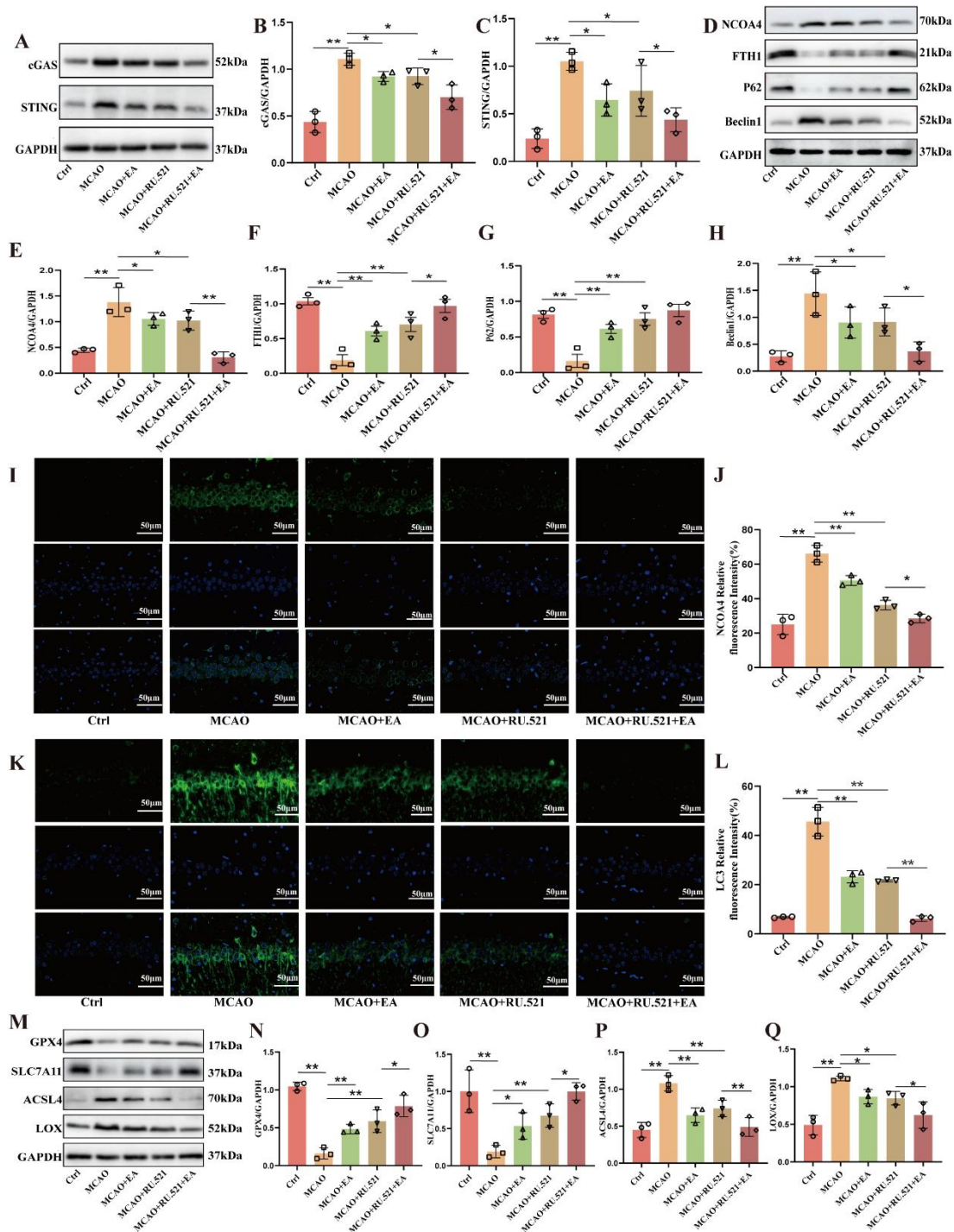


Figure 8. cGAS inhibitor RU.521 mimics the effects of EA on NCOA4-mediated ferritinophagy and ferroptosis in MCAO/R rats.

3.7. The Ferroptosis Inducer Erastin Attenuated RU.521-Associated Improvements in Cognitive Function in MCAO/R Rats

679 To determine whether ferroptosis may lie downstream of cGAS-STING signaling,
680 erastin was administered in the context of cGAS inhibition by RU.521. Western blotting
681 showed that, compared with the MCAO/R + Vehicle group, the MCAO/R + RU.521
682 group exhibited a lower hippocampal p-STING/STING ratio and higher GPX4
683 expression, whereas ACSL4 showed a downward trend. Compared with the MCAO/R
684 + RU.521 group, the MCAO/R + RU.521 + Erastin group maintained a low p-
685 STING/STING ratio, exhibited significantly reduced GPX4 expression, and showed an
686 upward trend in ACSL4 expression (Figure 9A-F). Two-way ANOVA showed a
687 significant main effect of erastin on ACSL4 expression ($F_{1,8} = 8.611$, $P = 0.019$,
688 $\eta_p^2 = 0.518$), whereas neither the main effect of RU.521 nor the RU.521 $\times$ erastin
689 interaction was significant (both $P > 0.05$). In the novel object recognition test, the
690 recognition index increased following RU.521 intervention but was significantly
691 reduced following combined administration of erastin. Total exploration time did not
692 differ significantly among groups (Figure 9I, J). In the Morris water maze, escape
693 latencies were comparable among groups on day 1. Over the training period, escape
694 latency decreased to a greater extent in the RU.521 group and to a lesser extent in the
695 Erastin group, whereas the reduction in the RU.521 + Erastin group was intermediate
696 between those observed in the two groups (Figure 9G, H). These findings indicate that
697 erastin exacerbated ferroptosis-related molecular abnormalities and attenuated the
698 RU.521-associated improvement in cognitive behavior in MCAO/R rats (Tables 2 and
699 3).

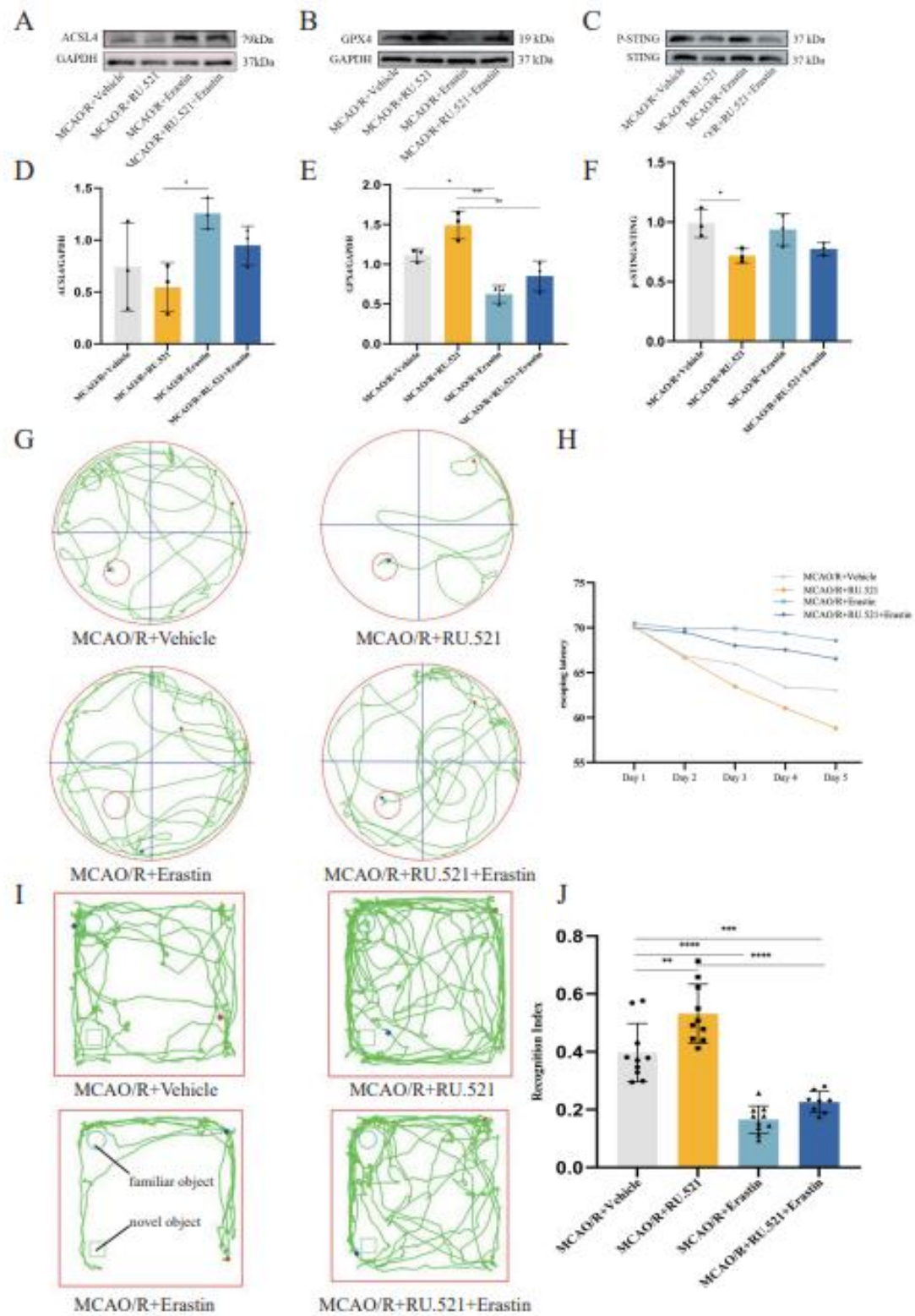


Figure 9. Erastin attenuates the molecular and cognitive effects of RU.521 in MCAO/R rats.

4. Discussion

Post-stroke cognitive impairment (PSCI) is an important complication that affects long-term functional recovery in patients with ischemic stroke. Although electroacupuncture has shown some benefit for PSCI, its underlying mechanisms remain incompletely understood. In the present study, electroacupuncture improved cognitive impairment in MCAO/R rats and attenuated aberrant hippocampal cGAS-STING-related molecular changes and ferroptosis-related injury. Fer-1 partially mimicked the protective effects of electroacupuncture, whereas DMXAA attenuated electroacupuncture-associated improvements in cognitive function and ferroptosis-related injury, indicating that both ferroptosis and STING-related signaling participate in the effects of electroacupuncture. RU.521 induced regulatory effects in the same direction as electroacupuncture, whereas erastin attenuated the protective effects of RU.521, further supporting the possibility that ferroptosis represents a downstream component through which cGAS-STING-related signaling contributes to cognitive impairment after MCAO/R. Collectively, these findings support the possibility that electroacupuncture may improve cognitive function in MCAO/R rats by inhibiting cGAS-STING-related signaling and thereby attenuating downstream ferroptosis-related injury.

Ferroptosis may represent an important pathological component of cognitive impairment-related injury after MCAO/R. Previous studies have shown that cerebral ischemia/reperfusion disrupts cerebral iron homeostasis and antioxidant defenses, resulting in increased iron load, accumulation of lipid peroxidation products, and reduced GPX4-associated anti-lipid peroxidation capacity, thereby promoting ferroptosis-related brain tissue injury. Consistent with these findings, we observed increased hippocampal Fe^{2+} content and iron deposition, as well as increased ACSL4 and MDA levels, in MCAO/R rats, whereas SLC7A11, GPX4, GSH, and SOD levels were decreased. These changes indicate increased iron load and lipid peroxidation, accompanied by impaired anti-lipid peroxidation defense. ACSL4-mediated membrane phospholipid remodeling can increase cellular susceptibility to ferroptosis, whereas

731 disruption of the SLC7A11–GSH–GPX4 defense system limits the clearance of lipid
732 peroxides. The resulting oxidative imbalance may disrupt hippocampal tissue
733 homeostasis and affect cognitive function. EA intervention improved these ferroptosis-
734 related abnormalities to varying degrees, indicating that the cognitive protective effects
735 of EA were accompanied by attenuation of ferroptosis-related injury. Moreover, Fer-1
736 intervention reduced cerebral infarction and hippocampal tissue injury and improved
737 object recognition memory and neurological function, with effects broadly consistent
738 in direction with those of EA. The overall direction of these findings is consistent with
739 previous studies of cerebral ischemia/reperfusion. However, prior studies have largely
740 focused on acute cerebral infarction and short-term neurological deficits. The present
741 study further linked hippocampal ferroptosis-related alterations with cognitive
742 behavioral abnormalities and extended the evidence from concurrent changes in
743 molecular indices to pharmacological functional validation through Fer-1 intervention.
744 Therefore, ferroptosis-related injury may contribute to cognitive impairment after
745 MCAO/R and may represent a potential pathological component through which EA
746 exerts protective effects.

747 Building on these findings, we further examined whether cGAS-STING-related
748 signaling participates in the regulation of ferroptosis-related injury and cognitive
749 function by EA. The STING agonist DMXAA exacerbated hippocampal abnormalities
750 in iron load, lipid peroxidation, anti-lipid peroxidation defense, and mitochondrial
751 ultrastructure, and attenuated EA-associated improvements in these ferroptosis-related
752 alterations and cognitive behavioral abnormalities. This finding is broadly consistent
753 with previous evidence from cerebral ischemia/reperfusion studies: ischemic brain
754 injury can increase cytosolic double-stranded DNA and activate cGAS-related
755 inflammatory signaling, thereby promoting microglial activation, inflammatory
756 responses, and secondary brain injury. Previous studies have also shown that
757 electroacupuncture attenuates cerebral ischemia/reperfusion injury in association with
758 reduced expression of cGAS, STING, and downstream inflammation-related molecules.

759 Unlike previous studies, which have mainly focused on acute brain tissue injury and
760 inflammatory responses, the present study found that DMXAA, as a STING agonist,
761 simultaneously attenuated EA-associated improvements in ferroptosis-related injury
762 and cognitive behavioral abnormalities. These findings provide pharmacological
763 support for the involvement of STING-related signaling in the protective effects of EA.
764 However, because phosphorylation status across the STING–TBK1–IRF3 axis was not
765 systematically assessed in this experiment, the pathway-targeting effect of DMXAA
766 requires further verification.

767 In contrast to the effects of DMXAA, the cGAS inhibitor RU.521 induced molecular
768 regulatory effects in the same direction as those observed with EA. Following RU.521
769 intervention, hippocampal GPX4 and SLC7A11 expression increased, whereas ACSL4
770 and LOX expression decreased, suggesting that cGAS inhibition was accompanied by
771 improved anti-lipid peroxidation defense and reduced susceptibility to ferroptosis.
772 These findings are broadly consistent with those reported by Zhao et al. in the early
773 phase of cerebral ischemia/reperfusion in mice. In that study, the cGAS inhibitor
774 RU.521 attenuated NCOA4-mediated ferritinophagy, oxidative stress, and brain tissue
775 injury, whereas NCOA4 overexpression partially weakened these protective effects,
776 suggesting that cGAS-STING-related signaling may contribute to
777 ischemia/reperfusion-induced brain injury through regulation of NCOA4-mediated
778 ferritin turnover. Unlike that study, which focused on early brain tissue injury after
779 ischemia/reperfusion and NCOA4-related mechanisms, the present study primarily
780 examined ferroptosis-related alterations in the rat hippocampus and cognitive outcomes.
781 Although the two studies differed in animal species, observation periods, brain regions,
782 and outcome measures, the overall direction of their effects was consistent. Taken
783 together with the findings that DMXAA attenuated EA-associated effects and RU.521
784 partially mimicked EA-associated effects, pharmacological evidence in these two
785 opposing directions supports the involvement of cGAS-STING-related signaling in the
786 regulation of ferroptosis-related injury by EA after MCAO/R.

Building on these observations, combined RU.521 and erastin intervention provided further evidence regarding the potential hierarchical relationship between cGAS-STING-related signaling and ferroptosis. Previous studies suggest that inhibition of cGAS-STING-related signaling can attenuate NCOA4-mediated ferritinophagy, oxidative stress, and brain tissue injury after cerebral ischemia/reperfusion. NCOA4-mediated ferritinophagy and oxidative lipid injury may, in turn, increase susceptibility to ferroptosis by increasing the labile iron pool and promoting lipid peroxidation. However, whether ferroptosis constitutes a downstream component through which this signaling pathway affects cognitive function after MCAO/R has not been specifically verified. In the present study, under conditions in which RU.521 maintained a low p-STING/STING ratio, combined erastin intervention was accompanied by reduced GPX4 expression, increased ACSL4 expression, and poorer cognitive behavioral performance. These findings suggest that induction of ferroptosis-related injury can attenuate the molecular and behavioral protective effects associated with cGAS inhibition. This pharmacological dissociation between inhibition of upstream signaling and exacerbation of downstream injury indices provides supportive evidence that ferroptosis-related injury may lie downstream of cGAS-STING-related signaling and extends prior mechanistic observations on brain tissue injury to cognitive outcomes. However, it should be emphasized that the RU.521× erastin interaction did not reach statistical significance for the relevant outcomes; therefore, the present findings are insufficient to establish a strict causal hierarchical relationship between the two. Although this study provides relatively systematic evidence, through multi-node pharmacological interventions, that EA may modulate cGAS-STING-related signaling and ferroptosis in association with improved cognitive function after MCAO/R, several limitations should be acknowledged. First, behavioral assessments were primarily conducted at the end of the 14-day intervention period; therefore, the durability of the cognitive protective effects of EA remains unclear. In addition, swimming speed and

path length were not recorded during the Morris water maze test, and the potential influence of differences in motor function on escape latency cannot be fully excluded. Second, the early experiments primarily assessed total cGAS and STING protein expression and downstream gene expression. The p-STING/STING ratio was measured only in the combined RU.521-erastin intervention experiment; therefore, pathway activation status after EA and DMXAA interventions was not systematically evaluated. Third, mechanistic inferences were based primarily on pharmacological interventions and may have been affected by drug off-target effects and differences in intervention timing. Moreover, the RU.521 $\times$ erastin interaction did not reach statistical significance for some outcomes; thus, the available evidence is insufficient to establish a strict causal mediation relationship. Fourth, static autophagy-related protein measurements and fluorescence signal overlap cannot reflect ferritinophagic flux or establish its necessity in the effects of EA. Fifth, only male rats and a single MCAO/R model were used. Moreover, relevant measurements were primarily performed in whole hippocampal tissue, and the cell-specificity of the proposed mechanism remains unclear. These factors limit extrapolation of the findings to different biological contexts and to clinical populations with PSCI.

Future studies should undertake targeted validation along the central cGAS-STING-related signaling-ferroptosis-cognitive function axis. First, behavioral follow-up should be extended, and swimming speed and path length should be recorded concurrently in the Morris water maze to evaluate the persistence of EA-associated cognitive protection and control for potential confounding by motor function. Second, the phosphorylation status of the STING-TBK1-IRF3 axis should be systematically assessed. Genetic interventions targeting key molecules, including cGAS, STING, GPX4, and ACSL4, should also be incorporated to further verify the causal hierarchy between cGAS-STING-related signaling and ferroptosis. Third, cell-specific localization, conditional genetic manipulation, and direct assessment of lipid peroxidation could help identify the principal cell populations in which this mechanism occurs and clarify their

contribution to cognitive function. Finally, these findings should be replicated in animal models that include females, different age groups, and metabolic or cardiovascular risk factors. Clinical samples should also be used to examine the relationships among cGAS-STING activation, ferroptosis-related indices, and cognitive outcomes in PSCI, thereby enhancing the reproducibility and translational value of the findings.

Overall, the present study showed that electroacupuncture at GV24 and GV20 improved cognitive behavioral performance and attenuated brain tissue injury in MCAO/R rats. Multi-node pharmacological interventions further support the possibility that its protective effects are, at least in part, associated with modulation of cGAS-STING-related signaling and attenuation of downstream ferroptosis-related injury. The study also identified changes in ferritin- and general autophagy-related molecules; however, the available evidence remains insufficient to establish ferritinophagy as a necessary intermediate component of this proposed mechanistic pathway.

5. Conclusions

Although the cell-specificity and precise regulatory mechanisms underlying these effects require further elucidation, the present findings suggest that electroacupuncture at GV24 and GV20 may improve post-stroke cognitive and memory impairment by inhibiting hippocampal cGAS-STING-related signaling and attenuating downstream ferroptosis-related injury in MCAO/R rats. This study provides experimental evidence supporting electroacupuncture as an intervention for post-stroke cognitive impairment and identifies directions for further investigation of the roles of cGAS-STING signaling and ferroptosis in the cognitive protective effects of electroacupuncture.

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Author contributions

Jun Chen contributed to the conceptualization and design of the study, performed the
experiments, analyzed the data, and drafted the manuscript. Yamin Wang contributed
to the study design, performed the experiments, collected and analyzed the data, and
revised the manuscript. Yujie Zhang contributed to the establishment of the animal
model, pharmacological interventions, and data collection. Yaqi Luo performed the
animal experiments and behavioral assessments and collected the data. Kewen Ma
performed the biochemical and molecular experiments and analyzed the data. Xinbao
Tian performed the histological and imaging experiments and prepared the figures.
Zhaochun Wang contributed to data curation, statistical analysis, and validation of the
results. Tao Hu contributed to the methodology, interpretation of the results, and
revision of the manuscript. Jing Gao contributed to the conceptualization and
methodology of the study, supervised the research, interpreted the data, and critically
revised the manuscript. Xiaodong Feng contributed to the conceptualization of the study,
supervised and administered the project, acquired funding, and critically revised the
manuscript. All authors reviewed and approved the final manuscript and agreed to be
accountable for all aspects of the work.

Date availability

The datasets generated during the current study are available from the corresponding
author on reasonable request.

Ethics statement

All animal experiments complied with international animal experiments ethics and
requirements, and approved by the Ethics Committee of Laboratory Animals of the
Henan University of Chinese Medicine (IACUC-202311033).

895 **Conflicts of Interest**

896 All authors have no competing interests to declare.

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Table

Table 2. Effects of combined erastin intervention on ferroptosis-related protein expression and novel object recognition performance in RU.521-treated MCAO/R rats

Measure	Vehicle	RU.521	Erastin	RU.521+Erastin	Main effect of RU.521	Main effect of erastin	RU.521 × erastin interaction
p-STING/STING	0.990 ± 0.120	0.718 ± 0.058*	0.938 ± 0.132	0.775 ± 0.052	F(1,8) = 14.959 ; P = 0.005; η^2 =0.652	F(1,8) = 0.001 ; P = 0.970; η^2 <0.001	F(1,8)=0.940; P=0.361; η^2 =0.105
GPX4	1.112 ± 0.079	1.493 ± 0.177	0.624 ± 0.109*	0.853 ± 0.192†	F(1,8) = 12.944 ; P = 0.007; η^2 =0.618	F(1,8) = 44.261 ; P < 0.001; η^2 =0.847	F(1,8)=0.808; P=0.395; η^2 =0.092
ACSL4	0.742 ± 0.423	0.546 ± 0.237	1.256 ± 0.151	0.948 ± 0.186	F(1,8) = 2.613 ; P = 0.145; η^2 =0.246	F(1,8) = 8.611 ; P = 0.019; η^2 =0.518	F(1,8)=0.130; P=0.727; η^2 =0.016
NOR recognition index	0.397 ± 0.101	0.532 ± 0.102*	0.165 ± 0.048*	0.226 ± 0.037†	F(1,34) = 14.241 ; P < 0.001; η^2 =0.295	F(1,34)=108.086; P < 0.001; η^2 =0.761	F(1,34)=2.026; P=0.164; η^2 =0.056

Note: Data are presented as mean ± SD. For western blotting (WB) outcomes, $n = 3$ per group. For the NOR test, $n = 10$ in the Vehicle, RU.521, and Erastin groups and $n = 8$ in the RU.521 + Erastin group. Two-way analysis of variance based on Type III sums of squares was used, followed by Sidak-adjusted pairwise comparisons. * $P < 0.05$ versus the Vehicle group; † $P < 0.01$ versus the RU.521 group. η_p^2 , partial eta squared.

1 Table 3. Effects of Combined RU.521 and Erastin Intervention on Escape Latency in
2 the Morris Water Maze in MCAO/R Rats

Effect	F(df)	<i>P</i> value	partial η^2
Training day	F(4, 136)=44.146	<0.001	0.565
RU.521	F(1, 34)=0.532	0.471	0.015
Erastin	F(1, 34)=3.483	0.071	0.093
RU.521×Erastin	F(1, 34)=0.027	0.871	0.001
Training day×RU.521	F(4, 136)=3.758	0.006	0.100
Training day×Erastin	F(4, 136)=13.348	<0.001	0.282
Training day×RU.521×Erastin	F(4, 136)=0.510	0.728	0.015

3 **Note:** The Vehicle, RU.521, and Erastin groups each included $n = 10$ rats, and the
4 RU.521 + Erastin group included $n = 8$ rats. Escape latency was analyzed using
5 repeated-measures analysis of variance, with training day as the within-subject factor
6 and RU.521 and erastin as between-subject factors. Mauchly's test of sphericity
7 indicated that the assumption of sphericity was not violated ($W = 0.789$, $P = 0.568$);
8 therefore, no correction of degrees of freedom was applied. Effect sizes are reported as
9 partial eta squared (η_p^2).

10 Figure legends

11 **Figure 1. Experimental design, electroacupuncture protocol, acupoint locations,**
12 **and animal allocation.** (A) Schematic overview of the five independent experiments.
13 Experiment 1 evaluated the effects of electroacupuncture (EA) on neurological and
14 cognitive outcomes and cGAS–STING-related molecular changes in the Ctrl, MCAO/R,
15 and MCAO/R+EA groups. Experiment 2 examined whether the ferroptosis inhibitor
16 ferrostatin-1 (Fer-1) produced effects similar to those of EA in the Ctrl, MCAO/R,
17 MCAO/R+EA, MCAO/R+Fer-1, and MCAO/R+Fer-1+EA groups. Experiment 3
18 evaluated the effects of the STING agonist DMXAA on EA-associated behavioral and
19 molecular changes in the Ctrl, MCAO/R, MCAO/R+EA, MCAO/R+DMXAA, and
20 MCAO/R+DMXAA+EA groups. Experiment 4 examined the effects of the cGAS

inhibitor RU.521 alone or in combination with EA in the Ctrl, MCAO/R, MCAO/R+EA, MCAO/R+RU.521, and MCAO/R+RU.521+EA groups. Experiment 5 evaluated the effects of RU.521 and the ferroptosis inducer erastin using a 2×2 factorial design comprising the MCAO/R+Vehicle, MCAO/R+RU.521, MCAO/R+Erastin, and MCAO/R+RU.521+Erastin groups. MCAO/R surgery was performed on day 0. EA was initiated on day 3 after MCAO/R and administered once daily for 14 consecutive days. Fer-1 and DMXAA were administered once by intraperitoneal injection on day 3 after MCAO/R. RU.521 was administered once by intraperitoneal injection at 80 min after the onset of ischemia, 10 min before filament withdrawal and reperfusion. Erastin was administered intraperitoneally once daily from day 3 after MCAO/R for 14 consecutive days. (B) Schematic illustration of the EA acupoints. Baihui (GV20) was located on the dorsal midline at the intersection with the line connecting the tips of both ears. Shenting (GV24) was located on the anterior midline of the head, rostral to the frontoparietal junction. (C) Initial numbers, mortality, exclusions, and final numbers of animals included in the analysis for each group across the five experiments.

Figure 2. EA treatment reduces infarct volume, ameliorates neurological deficits, and attenuates cGAS–STING-related molecular alterations in MCAO/R rats. (A and B) Representative images of TTC-stained brain sections (A) and quantification of infarct volume (B) in Ctrl, MCAO/R, and MCAO/R+EA groups (n = 3 per group). (C) Neurological deficit scores of the three groups. (D) Number of platform crossings during the spatial probe trial of the Morris water maze test. (E) Representative swimming trajectories of rats in each group. (F) Escape latency to the hidden platform during the 5-day training trials of the Morris water maze test. (G–I) Representative western blot images (G) and quantification of cGAS (H) and STING (I) protein levels in the hippocampus (n = 3 per group). (J–N) Relative mRNA expression levels of TBK1 (J), IRF3 (K), IL-1 β (L), IL-10 (M), and TNF- α (N) in the hippocampus, measured by qRT-PCR (n = 3 per group). Data are presented as the mean $\pm$ SD. ##p < 0.01 vs. Ctrl group; *p < 0.05, **p < 0.01 vs. MCAO/R group.

Figure 3. EA attenuates ferroptosis-related alterations and modulates ferritin- and autophagy-related proteins in the hippocampus of MCAO/R rats. (A–D) Representative western blot images (A) and quantification of NCOA4 (B), FTH1 (C), and FTL (D) protein levels in hippocampal tissues from the Ctrl, MCAO/R, MCAO/R+EA, MCAO/R+Fer-1, and MCAO/R+Fer-1+EA groups (n = 3 per group). (E–H) Representative western blot images (E) and quantification of p62 (F), Beclin-1 (G), and the LC3-II/LC3-I ratio (H) in the hippocampus (n = 3 per group). (I–K) Representative immunofluorescence images of FTH1 (red), LC3 (green), and DAPI (blue) in the hippocampal CA1 region (I; scale bar = 50 μ m), with quantification of the relative fluorescence intensities of FTH1 (J) and LC3 (K) (n = 3 per group). (L) Representative Prussian blue staining images showing iron deposition in the hippocampal CA1 region (scale bar = 50 μ m). (M) Quantification of Fe²⁺ content in hippocampal tissue. (N–P) Quantification of GSH content (N), SOD activity (O), and MDA content (P) in hippocampal tissue (n = 5 per group). Data are presented as the mean $\pm$ SD. *p < 0.05 and **p < 0.01 for the comparisons indicated by the horizontal lines.

Figure 4. Ferrostatin-1 partially mimics the protective effects of EA against MCAO/R-induced neurological deficits and cognitive impairment. (A) Representative TTC-stained brain sections from the Ctrl, MCAO/R, MCAO/R+EA, MCAO/R+Fer-1, and MCAO/R+Fer-1+EA groups. (B) Quantification of infarct volume in each group (n = 3 per group). (C) Neurological deficit scores in the five groups (n = 12 per group). (D–F) Novel object recognition test showing the time spent exploring the novel object (D), the time spent exploring the familiar object (E), and the recognition index (F) (n = 12 per group). (G and H) Percentage of time spent in the open arms (G) and percentage of entries into the open arms (H) in the elevated plus maze test (n = 12 per group). (I) Representative movement trajectories in the elevated plus maze test. (J) Representative hematoxylin and eosin (H&E) staining images of the hippocampal CA1 region (scale bar = 50 μ m; n = 3 per group). Data are presented as

the mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ for the comparisons indicated by the horizontal lines.

Figure 5. DMXAA attenuates the effects of EA on brain injury and behavioral

deficits in MCAO/R rats. (A) Representative TTC-stained brain sections from Ctrl,

MCAO/R, MCAO/R+EA, MCAO/R+DMXAA, and MCAO/R+DMXAA+EA groups.

(B) Quantification of infarct volume in each group ($n = 3$ per group). (C and D)

Percentage of time spent in the open arms (C) and percentage of entries into the open

arms (D) in the elevated plus maze test ($n = 12$ per group). (E–G) Novel object

recognition test showing the time spent exploring the novel object (E), the time spent

exploring the familiar object (F), and the discrimination index (G) ($n = 12$ per group).

(H) Number of platform crossings during the spatial probe trial of the Morris water

maze test. (I) Escape latency to the hidden platform during the training trials of the

Morris water maze test. (J) Representative swimming trajectories during the training

phase of the Morris water maze test. (K) Representative tracking plots during the spatial

probe trial. Data are presented as the mean $\pm$ SD. ### $p < 0.01$ vs. Ctrl group; * $p < 0.05$,

** $p < 0.01$ vs. MCAO/R group; ^ $p < 0.05$, ^^ $p < 0.01$ vs. MCAO/R+DMXAA group.

Figure 6. DMXAA attenuates the effects of EA on ferroptosis-related alterations

and is accompanied by changes in ferritin- and autophagy-related proteins in

MCAO/R rats. (A–C) Representative western blot images (A) and quantification of

cGAS (B) and STING (C) protein levels in hippocampal tissues from the Ctrl,

MCAO/R, MCAO/R+EA, MCAO/R+DMXAA, and MCAO/R+DMXAA+EA groups

($n = 3$ per group). (D–G) Representative western blot images (D) and quantification of

NCOA4 (E), FTH1 (F), and FTL (G) protein levels in the hippocampus ($n = 3$ per

group). (H–K) Representative western blot images (H) and quantification of p62 (I),

Beclin-1 (J), and the LC3-II/LC3-I ratio (K) in the hippocampus ($n = 3$ per group). (L–

N) Quantification of SOD activity (L), GSH content (M), and MDA content (N) in

hippocampal tissue ($n = 5$ per group). (O–S) Representative western blot images (O)

and quantification of GPX4 (P), SLC7A11 (Q), ACSL4 (R), and LOX (S) protein levels

in the hippocampus (n = 3 per group). Data are presented as the mean $\pm$ SD. *p < 0.05 and **p < 0.01 for the comparisons indicated by the horizontal lines.

Figure 7. STING-NCOA4 co-localization, iron deposition, and mitochondrial damage reflect the reversal of EA's protective effects by DMXAA.

(A) Representative immunofluorescence images showing co-staining of STING (red), NCOA4 (green), and DAPI (blue) in the hippocampal CA1 region (400 $\times$). (B) Quantitative analysis of Fe²⁺ content in hippocampal tissue (n = 5 per group). (C and D) Representative Perls' staining images of iron deposition (C) and hematoxylin and eosin (H&E) staining images of hippocampal morphology (D) (400 $\times$). (E) Representative transmission electron microscopy images showing mitochondrial ultrastructural changes in the hippocampus. Data are presented as the mean $\pm$ SD. *p < 0.05, **p < 0.01 vs. MCAO/R+EA group.

Figure 8. cGAS inhibitor RU.521 mimics the effects of EA on NCOA4-mediated ferritinophagy and ferroptosis in MCAO/R rats.

(A–C) Representative western blot images of cGAS, STING, and GAPDH (A), and quantification of cGAS (B) and STING (C) protein levels in hippocampal tissues from the Ctrl, MCAO/R, MCAO/R+EA, MCAO/R+RU.521, and MCAO/R+RU.521+EA groups. (D–H) Representative western blot images of NCOA4, FTH1, p62, Beclin-1, and GAPDH (D), and quantification of NCOA4 (E), FTH1 (F), p62 (G), and Beclin-1 (H) protein levels. (I and J) Representative NCOA4 immunofluorescence images in the hippocampal CA1 region (I) and quantification of NCOA4 fluorescence intensity (J). (K and L) Representative LC3 immunofluorescence images in the hippocampal CA1 region (K) and quantification of LC3 fluorescence intensity (L). (M–Q) Representative western blot images of GPX4, SLC7A11, ACSL4, LOX, and GAPDH (M), and quantification of GPX4 (N), SLC7A11 (O), ACSL4 (P), and LOX (Q) protein levels. For western blot and immunofluorescence analyses, n=3 biologically independent animals per group. Data are presented as the mean $\pm$ SD. *P<0.05 and **P<0.01 for the comparisons indicated by the horizontal lines.

Figure 9. Erastin attenuates the molecular and cognitive effects of RU.521 in MCAO/R rats. (A–C) Representative western blot images of ACSL4 and GAPDH (A), GPX4 and GAPDH (B), and p-STING (Ser366) and total STING (C) in hippocampal tissues from the MCAO/R+Vehicle, MCAO/R+RU.521, MCAO/R+Erastin, and MCAO/R+RU.521+Erastin groups. (D–F) Quantification of ACSL4/GAPDH (D), GPX4/GAPDH (E), and p-STING (Ser366)/STING (F) ($n = 3$ per group). (G, H) Representative swimming trajectories (G) and escape latency during the 5-day acquisition phase of the Morris water maze test (H). (I, J) Representative movement trajectories in the novel object recognition test (I) and quantification of the recognition index (J). For the behavioral experiments, $n = 10$ in the MCAO/R+Vehicle, MCAO/R+RU.521, and MCAO/R+Erastin groups and $n = 8$ in the MCAO/R+RU.521+Erastin group. Quantitative data are presented as the mean $\pm$ SD. Western blot and novel object recognition data were analyzed by two-way ANOVA, with RU.521 and Erastin as fixed factors, followed by Sidak's multiple-comparisons test where appropriate. Escape latency was analyzed using a repeated-measures model. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ for the comparisons indicated by the horizontal lines in panels D–F and J.

Supplementary Files

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