Histol Histopathol (2025) 40: 919-933

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Aerobic exercise remodels gut microbiota to alleviate cerebral ischemia-reperfusion injury

Mingjin Zhu[1], Jiajie Zhu[2], Jiafei Pan[1], Rui Fu[2], Guoyuan Pan[1] and Jie Zhang[1]

[1]Department of Rehabilitation Medicine and [2]Department of Gastroenterology, Tongde Hospital of Zhejiang Province, Hanghzou, Zhejiang, PR China

Summary. Aerobic exercise exhibits a neuroprotective role against cerebral ischemia-reperfusion (I/R) injury, and the present study explored the underlying mechanisms. Adult Sprague-Dawley rats (n=87) were used in the study, and cerebral I/R injury in rats was modeled using middle cerebral artery occlusion and reperfusion (MCAOR), followed by interval aerobic exercise training at a moderate intensity. Colonization with gut microbiota from the trained rats was performed on MCAOR rats. Neurobehavioral assessments were performed. Cerebral infarction and neuronal damage were detected by tissue staining and molecular experiments. Gut microbiota composition was analyzed by 16S rRNA gene sequencing. Neuroinflammation was detected using an enzyme-linked immunosorbent assay. Aerobic exercise ameliorated neurological deficit, spontaneous locomotor activity, and spatial learning and memory impairment in MCAOR rats (p<0.001). Further, aerobic exercise decreased infarct volume, attenuated neuronal damage, increased SYN1 and PSD95 expression, as well as reduced neuroinflammation by upregulating IL-10 and downregulating IL-6, TNF-α, IL-17, and TGF-β in MCAOR rats (p<0.05). Aerobic exercise altered gut microbiota composition in MCAOR rats. Gut microbiota colonization in rats alleviated cerebral I/R injury by reducing neurological deficit scores, promoting spontaneous locomotor activity, decreasing infarct volume, elevating SYN1 and PSD95 expression, and improving neuroinflammation (p<0.05). In conclusion, aerobic exercise remodeled the gut microbiota in rats to attenuate cognitive dysfunction and neuroinflammation after cerebral I/R.

Key words: Cerebral ischemia-reperfusion, Gut-brain axis, Aerobic exercise, Gut microbiota, Neurological deficit

Corresponding Author: Jie Zhang, Department of Rehabilitation Medicine, Tongde Hospital of Zhejiang Province, 234 Gucui Road, Xihu District, Hanghzou, Zhejiang Province, 310012, China. e-mail: zhangjie198781@163.com

www.hh.um.es. DOI: 10.14670/HH-18-832

Introduction

The gut-brain axis (GBA) is a bidirectional communication network between microbiota, intestine, and the central nervous system (CNS), and is implicated in the regulation of host brain and behavior via different gut-brain signaling pathways (Cryan et al., 2019). Gut microbiota, an ecological community of symbiotic and pathogenic microorganisms, colonizes the surface of the human intestinal mucosa, and the mutualism of gut microbiota and its host is not only essential for intestinal health but also for the body as a whole (Rutsch et al., 2020). Recently, growing evidence has shown that dysbiosis of gut microbiota is linked to the pathological development of brain diseases including neurodegenerative disorders and acute CNS injuries (e.g., stroke) (Sampson et al., 2016; Pluta et al., 2021; Yuan et al., 2021).

Ischemic stroke (IS) accounts for approximately 85% of all stroke cases and is a leading cause of disability and death worldwide (Parr et al., 2017). The occlusion of the middle cerebral artery (MCA) is the main cause of IS, leading to local damage to the brain parenchyma and neurological deficits (Zhao et al., 2022). Despite the effect of the recanalization strategy on salvaging ischemic penumbra, the reperfusion of blood flow can induce secondary brain tissue damage (Zhang et al., 2022). Therefore, efforts have been dedicated to understanding the pathogenesis of cerebral ischemia-reperfusion (I/R) injury to seek novel therapies. Compared with health control, significant alterations in gut microbiota composition have been found in patients with IS (Bao et al., 2022). In addition, previous studies have reported that interventions related to the gut microbiome hold significant promise and potential for treating stroke (Cryan et al., 2020; Sorboni et al., 2022). A previous study reported that gut microbiota sampled from middle cerebral artery occlusion and reperfusion (MCAOR)-induced murine models resulted in changes in behavior, brain functional connectivity, neuronal plasticity, and neuroinflammation in germ-free healthy mice (Wang et al., 2021). Therefore, increasing interest has arisen regarding the

treatment of cerebral I/R injury through modulating gut microbiota composition (Li et al., 2023b).

Recently, several studies have documented the neuroprotective role of aerobic exercise training against cerebral I/R injury in animal models, however, its related mechanisms remain elusive (Liu et al., 2022; Luo et al., 2022). Exercise is thought to be one of the environmental factors influencing the ecological balance of gut microbiota (Gubert et al., 2020). In the study of metabolic diseases, exercise training has been found to induce compositional and functional changes in gut flora in patients, indicating its important potential in non-pharmacological therapies (Allen et al., 2018; Motiani et al., 2020; Quiroga et al., 2020). Taken together, we proposed a hypothesis that aerobic exercise may ameliorate cerebral I/R injury via remodeling gut microbiota composition.

Hence, the present study treated MCAOR rats with moderate-intensity interval training (MIIT), with nimodipine administration (a calcium channel blocker that can improve cerebral I/R injury (Tomassoni et al., 2008)) used as the positive control, to investigate whether exercise training affects the gut microbiota composition in rat models. Further, we implanted fecal samples from MIIT-treated MCAOR rats to (no exercise) MCAOR rats to investigate the aforementioned hypothesis.

Materials and methods

Animals and ethics statement

Adult Sprague-Dawley (SD) rats (n=87, male, weighing 290-310 g) were housed in a specific pathogen-free environment with food and drinking water *ad libitum*. All animal experiments in this study had been granted approval by the Ethics Committee of Zhejiang Baiyue Biotech Co., Ltd for Experimental Animals Welfare (ZJBYLA-IACUC-20230607), and experimental procedures were conducted based on the guidelines of the China Council on Animal Care and Use.

MCAOR experiment and grouping

MCAOR was carried out as described previously (Liu et al., 2021). Rats were anesthetized with 2% isoflurane (PHR2874, Sigma-Aldrich, St. Louis, MO, USA). After surgical exposure of neck vessels, a small incision was made on the internal carotid artery (ICA) and then, a silicone rubber-coated monofilament (602156PK5Re, Doccol, Redlands, CA, USA) was inserted into the ICA to occlude blood flow of the right MCA. After 90-min ischemic induction, the monofilament was gently withdrawn to allow cerebral reperfusion. The sham rats were subjected to the same surgical procedures except for the occlusion of blood flow.

All animal experiments had to be divided into two stages, and rats with or without surgery were grouped as follows:

Stage 1: rats were divided into the Control (normal rats), Sham, MCAOR, MCAOR-Ex (MCAOR rats given MIIT), and MCAOR-Nim groups (MCAOR rats administered nimodipine), with 12 rats in each group.

Stage 2: rats were randomly divided into the Control group (sham rats), MCAOR, and MCAOR+T groups (MCAOR rats colonized with gut microbiota), with 9 rats in each group.

All rats were euthanatized by cervical dislocation under deep anesthesia (150 mg/kg pentobarbital sodium, P3761, Sigma-Aldrich, USA) at the termination of the experiment. Brain and fecal samples were harvested and preserved as indicated for subsequent assays.

Exercise training

An animal treadmill apparatus (XR-PT-10B, Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China) was applied for exercise training in rats. Before surgery, all rats were subjected to adaptive aerobic training (speed: 7 m/min, time: 20 min/time/day) for 3 days. A 30-min MIIT (speed: 15 m/min) was performed in rats on day 2 after MCAOR, and the training lasted for 2 weeks with 5 days per week, during which the rats were stimulated with appropriate electric shocks (0.05 mA) to encourage locomotion. Nimodipine (N149, Sigma-Aldrich, USA) was prepared with 0.5% carboxymethylcellulose sodium (C91597, Acmec Biochemical, Shanghai, China), and rats in the MCAOR-Nim group (used as the positive control) were orally administered 50 mg/kg nimodipine once a day for 2 weeks (Liu et al., 2021).

16S rRNA gene sequencing

Rat fecal samples (190-230 mg) from the MCAOR-Ex group were collected after two weeks and stored in sterile cryopreservation tubes at -80°C. Total microbial DNA from the fecal samples was extracted using the EZNA Stool DNA Kit (D4015, Omega Bio-Tek, Norcross, GA, USA) and subsequently quantified by Qubit 2.0 fluorometer (Thermo Fisher, Waltham, MA, USA). Then, a polymerase chain reaction was carried out to amplify the V3-V4 region of the microbial 16S rRNA gene, as described previously (Li et al., 2020). Paired-end libraries were constructed using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA), and the libraries were sequenced in an Illumina MiSeq Benchtop Sequencer following the manufacturer's protocol. Sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software package, and operational taxonomic units (OTUs) were picked using the UPARSE pipeline and clustered with the similarity of sequences at ≥97%. The taxonomic information of microbiota was annotated by the GreenGene database (https://greengenes.secondgenome.com/).

Gut microbiota colonization

Fecal microbiota transplantation was performed for gut microbiota colonization according to a previous description with slight modifications (Wang et al., 2021). Fecal samples (200 mg) from rats in the MCAOR-Ex group were diluted with 2 mL of 5% sterile phosphate-buffered saline (PBS; C0221A, Beyotime, Shanghai, China). After 5-min vortex oscillation and centrifu-gation, the bacterial solution as transplantation material was obtained and orally administered to rats in the MCAOR+T group at a dose of 10 μL/g/day for two weeks.

Assessment of neurological deficit

The neurological deficit of rats in each group was assessed after corresponding interventions for 7 days using the modified neurological severity score (mNSS) test (Xie et al., 2019). The scoring system consists of motor, sensory, balance, and reflex functions of rats. The score was graded on a scale of 0 to 18 (0=normal score; 18=maximum score). A higher score indicated a more severe neurological deficit.

Behavioral tests

To evaluate the locomotor activity of rats, an uncovered box (100 cm × 100 cm × 50 cm, XR-XZ301, Shanghai Xinruan Information Technology Co., Ltd., China) was used for performing the open field test. Before each test, the box was sterilized with 75% alcohol (E99949, Acmec Biochemical, China) to eliminate any odors. The rats were individually placed into the center of the open field, which was divided into 16 districts, and they were allowed to explore the new environment for 15 min, with no disturbance throughout the test. The software SuperMaze (vision 2.0, Shanghai Xinruan Information Technology Co., Ltd., China) was applied to automatically record experimental data including total distance, central distance, time moving towards the central area, line crossing, and number of exits from the central area.

The Morris water maze (MWM) test was utilized to assess the spatial cognition of rats after corresponding interventions for 7 days. Briefly, the rats were placed in a circular pool in an MWM apparatus (XR-XM101, Shanghai Xinruan Information Technology Co., Ltd., China) equipped with video analysis systems. A platform (diameter 10 cm) was set up on the water's surface in the target zone. During the training in orientation navigation, when the rat found the platform within 90 s, the time was recorded as the latency to the platform. When the rat failed to find the platform within 90 s, it would be placed on the platform for 10 s. This training is lasted for 5 days. During the testing phase, elapsed time and distance before reaching the platform were recorded at 6th day. At 7th day, the platform was removed, and each rat in the pool was allowed to explore for 90 s. The number of platform crossings, as well as the time spent in the other three zones and the target zone, were digitally recorded.

Tissue staining

The infarct volume in rat brains was examined using 2,3,5-Triphenyltetrazolium chloride (TTC) staining. The frozen brain samples were cut into 5 μm sections. The sections were stained with TTC solution (17779, Sigma-Aldrich, USA) at 37°C for 30 min without light, rinsed with PBS, and fixed with 4% paraformaldehyde (PFD; P0099, Beyotime, China). After taking pictures, the infarction area was calculated using ImageJ Software (Vision 1.8.0, National Institutes of Health, Bethesda, MD, USA).

Fresh rat brains were fixed with 4% PFD and then assigned to dehydration and paraffin embedding. To examine pathological changes in the rat brains, 5-μm section samples were dewaxed and rehydrated, and then dyed with hematoxylin (E607317, Sangon Biotech, Shanghai, China) and counterstained with eosin (E607321, Sangon Biotech, China) at room temperature. The stained samples in five random fields (100× magnification) were captured using a light microscope (E600, Nikon, Tokyo, Japan).

The neuronal damage in rat brains was assayed using nissl staining. After dewaxing and rehydration, the coronal sections were stained with cresyl violet (G1430, Solarbio, Beijing, China) at 56°C for 1h, rinsed with deionized water, and developed with gradient ethanol. Finally, nissl-stained cells were observed by light microscopy (100× magnification).

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Rat brains were homogenized in Trizol (R1030, Applygen Technologies, Beijing, China) to extract total RNA, which was then reverse transcribed to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (04379012001, Roche, Basel, Switzerland). qRT-PCR was carried out using primers and SYBR Green Abstart PCR Mix (B110031, Sangon Biotech, China) in a LightCycler 480 real-time instrument (Roche, Switzerland) following the indicated thermo-cycling conditions. Relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH used as the normalization control. Primer sequences are listed as follows (5'-3'): synapsin I (SYN1; forward: GACAACCAACATGACTTCCAGG, reverse: TTCCA GTTCCCTGACACTGATG), postsynaptic density protein-95 (PSD95; forward: TTAGATCTCTGTG GGGCGGA, reverse: TGCCCACAAGCTCTTACCTG), and GAPDH (forward: CTCCTCGAAGTACCCTGT GC, reverse: CATGGTGCAGCGATGCTTTA).

Western blot

Rat brains were homogenized in phenylmethane-

sulfonyl fluoride-contained RIPA buffer (R0020, Solarbio, China) on ice to extract total protein. The lysed samples were subjected to a 5-min centrifugation (14,000×g) to collect the supernatant, which was subjected to protein quantification by the BCA Protein Assay Kit (T9300A, Takara, Beijing, China). Equal amounts of protein (40 μg) were separated via 10% SDS-PAGE gel under denaturing conditions, electrophoretically transferred onto polyvinylidene fluoride membranes, and treated with blocking buffer (P0252, Beyotime, China) at room temperature for 15 min. Primary antibodies were utilized to probe the separated blots on the membranes at 4°C overnight, followed by a 2h incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. At length, the blots were treated with chemiluminescence solution (P1000, Applygen Technologies, China) and analyzed in a Quantity One System image analyzer (Bio-Rad, Hercules, CA, USA). All antibodies were purchased from ABclonal (Wuhan, China) and are listed as follows: SYN1 (A17362, 74 kDa, 1:1000), PSD95 (A0131, 80 kDa, 1:2000), endogenous control

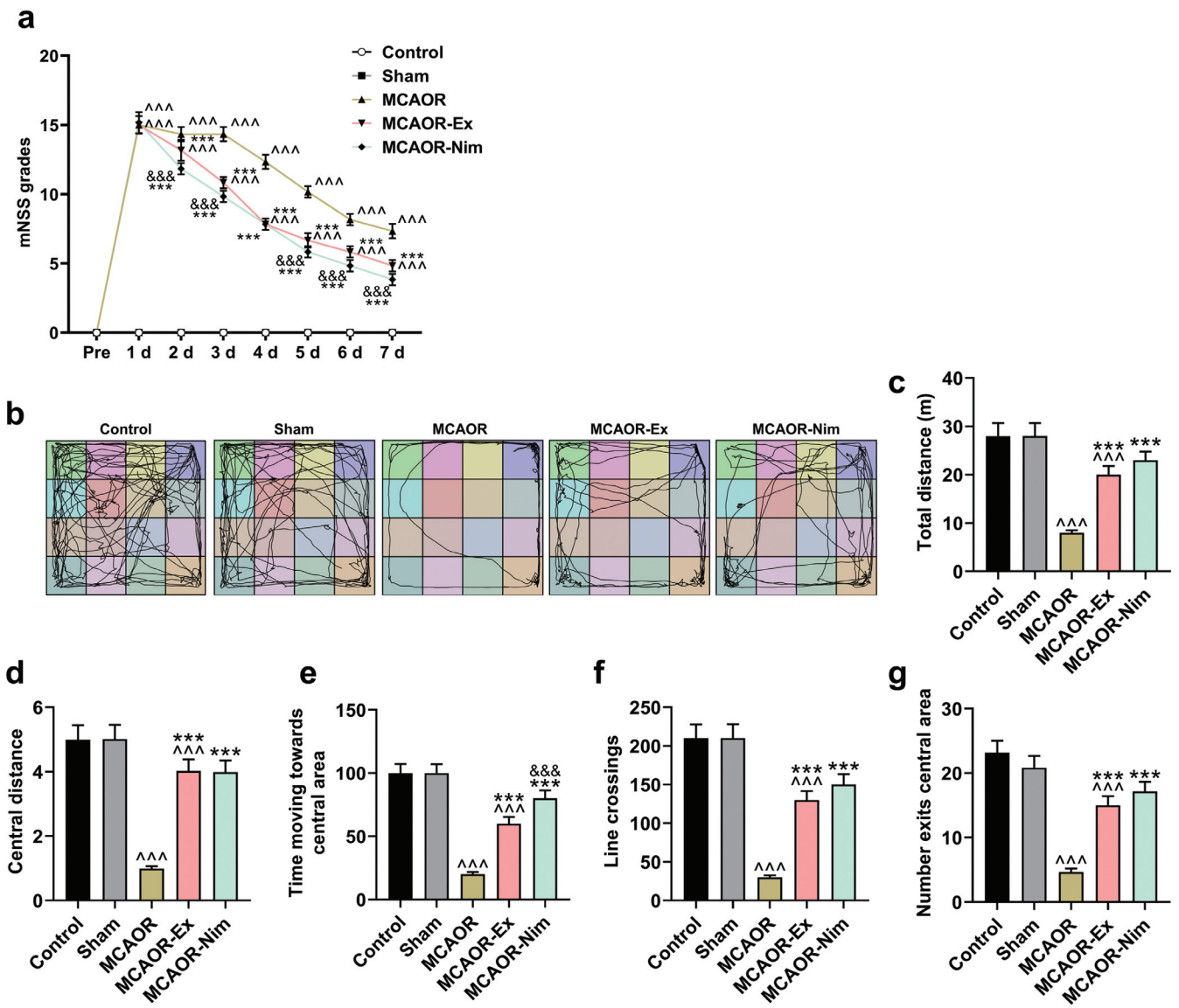


Fig. 1. Effects of aerobic exercise on neurological deficit and spontaneous locomotor activity in MCAOR rats. **a.** MCAOR rats were treated with or without interval aerobic exercise training or nimodipine for 2 weeks (MCAOR, MCAOR-Ex, and MCAOR-Nim groups). The rats that underwent sham operations were used as the Sham group, and normal rats were used as the Control group. The neurological deficit of rats in each group was assessed after corresponding interventions for 7 days by the mNSS test. **b-g.** The open field test was utilized to evaluate the locomotor activity of rats in each group. Data are shown as mean ± standard deviation. ^^^p<0.001, vs. Sham; ***p<0.001, vs. MCAOR; $^{\&\&\&}p$<0.001, vs. MCAOR-Ex. MCAOR, middle cerebral artery occlusion and reperfusion; mNSS, modified neurological severity score.

GAPDH (AC001, 36 kDa, 1:10000), and goat anti-rabbit IgG (AS014, 1:10000).

Enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin 6 (IL-6), IL-10, and tumor necrosis factor-alpha (TNF-α) in the infarcted area of rat brains were measured using commercial rat ELISA kits (BMS625/BMS629/KRC3011, Thermo Fisher, USA). IL-17 and transforming growth factor-beta (TGF-β) rat ELISA kits (PI548/PT878) were provided by Beyotime (China). The fresh samples were homogenized with pre-cooled PBS supplemented with protease inhibitor and centrifuged at 5,000×g for 10 min to obtain the supernatant. Then, 100 µL of diluted samples in each well of pre-coated ELISA plates were incubated with 100 µL biotinylated detection antibody for 1h and then HRP conjugate diluent for 30 min, in sequence. Following chromogenic treatment with the substrate, absorbance was measured at 450 nm using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland).

Statistical analysis

Data are presented as mean ± standard error of the mean from at least three independent experiments. Comparisons in multiple groups (Figs. 1A, 2B, 7A,C) were analyzed by two-way analysis of variance. The remaining muti-group comparisons were analyzed by a one-way analysis of variance, followed by the *post-hoc* analysis of Tukey's test. The Linear discriminant analysis effect size (LEfSe) statistical analysis was analyzed by the Galaxy Module online, based on $p<0.05$, and a logarithmic linear discriminant analysis (LDA) score was used for discriminant features. GraphPad Prism v8.0 (GraphPad Software Inc., San Diego, CA, USA) was used for calculating p values, where thresholds less than 0.05 were considered statistically significant.

Results

Aerobic exercise ameliorated neurological deficit and cognitive dysfunction in MCAOR rats

To confirm the neuroprotection of aerobic exercise against cerebral I/R injury, we trained rats with MIIT after MCAOR and used nimodipine administration as the positive control. The results of the mNSS test showed that scores after corresponding interventions for 7 days were decreased in the MCAOR-Ex group compared with the MCAOR group (Fig. 1A, $p<0.001$). According to the results of the open field test, MCAOR rats showed reduced spontaneous locomotor activity, as evidenced by decreased total distance, central distance, time to move towards the central area, line crossings, and number of exits from the central area (Fig. 1B-G, $p<0.001$). However, MIIT rescued these results in the

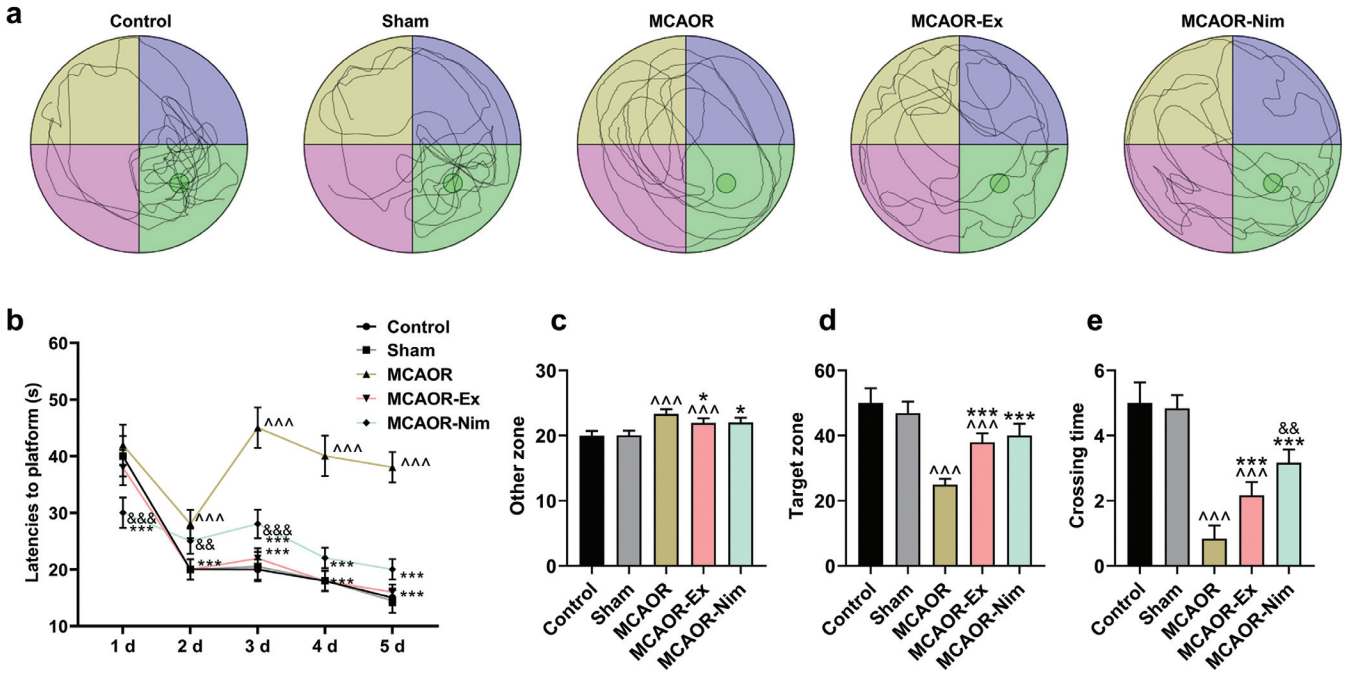


Fig. 2. The effect of aerobic exercise on the spatial learning and memory capacities of MCAOR rats. **a-e.** MCAOR rats were treated with or without interval aerobic exercise training or nimodipine for 2 weeks (MCAOR, MCAOR-Ex, and MCAOR-Nim groups). The rats that underwent sham operations were used as the Sham group, and normal rats were used as the Control group. The MWM test was utilized to assess the spatial cognition of rats in each group on days 1-5 after surgery. Data are shown as mean ± standard deviation. ^^^$p<0.001$, vs. Sham; *$p<0.05$, ***$p<0.001$, vs. MCAOR; $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ vs. MCAOR-Ex. MWM, Morris water maze.

MCAOR-Ex group (Fig. 1B-G, $p<0.001$). Compared with sham rats, the MWM test revealed spatial learning and memory impairment of rats at days 1-5 after MCAOR, as evidenced by longer escape latencies to the platform and time spent in other zones, as well as by shorter time spent in the target zone and decreased platform crossing time (Fig. 2A-E, $p<0.001$). However, MIIT alleviated spatial learning and memory impairment of MCAOR rats in the MCAOR-Ex group (Fig. 2A-E, $p<0.05$). Compared with the MCAOR group, nimodipine administration, a calcium channel antagonist used for treating cerebrovascular diseases, showed similar effects to MIIT intervention in MCAOR rats (Figs. 1A-2E, $p<0.05$). However, the levels of the open field test and MWM test-related indicators in the MCAOR-Ex group were lower than those in the sham group except for increased time spent in other zones (Figs. 1B-2E, $p<0.05$); therefore, the level of improvement in MCAOR-Ex rats based on behavioral indicators was in between MCAOR and Sham, and was similar to the positive control, except for time to move towards the central area and crossing time levels.

Aerobic exercise attenuated cerebral infarction, neuronal damage, and inflammation in MCAOR rats

Then, we performed TTC staining to explore the influence of aerobic exercise on cerebral infarction in rats after MCAOR. As illustrated in Figure 3A, we observed significant infarct areas in the coronal sections from the MCAOR group but fewer infarct areas were observed both in the MCAOR-Ex or MCAOR-Nim group (Fig. 3A-3B, $p<0.05$). The following hematoxylin and eosin staining as well as nissl staining demonstrated that there were many neurons in the hippocampus, with normal structure and neat arrangement in the control and sham groups, while the neurons showed obvious degeneration and necrosis in the MACOR group, and neurons shrank into a triangular shape, and the nuclei were concentrated and deeply stained (Fig. 3C-D). Also, MIIT and nimodipine administration attenuated MCAOR-induced neuronal necrosis and loss in rat

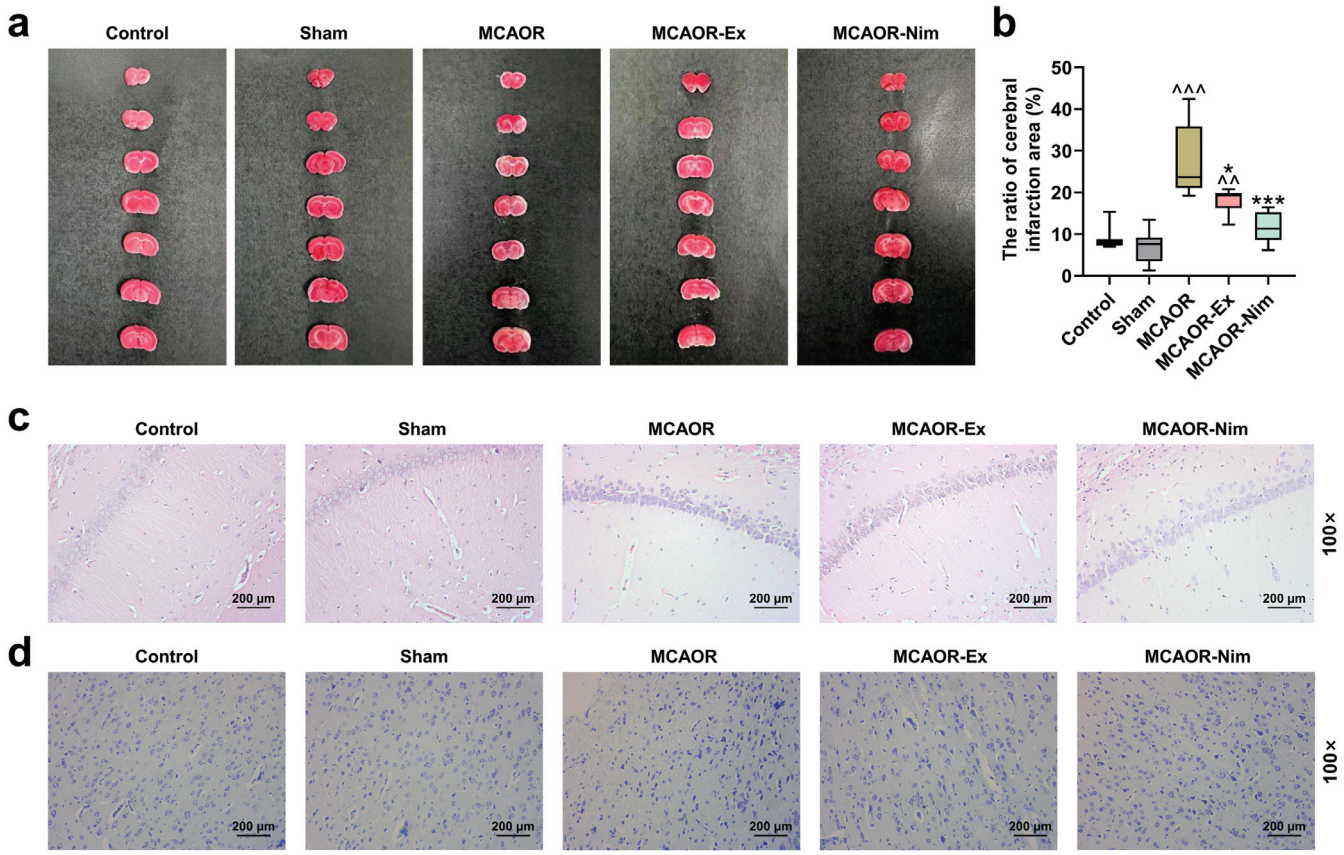


Fig. 3. Effects of aerobic exercise on cerebral infarction and neuronal damage in MCAOR rats. **a, b.** MCAOR rats were treated with or without interval aerobic exercise training or nimodipine for 2 weeks (MCAOR, MCAOR-Ex, and MCAOR-Nim groups). The rats that underwent sham operations were used as the Sham group, and normal rats were used as the Control group. The infarct volume in rat brains from each group was examined using TTC staining. **c.** The pathological changes in rat brains from each group were detected by hematoxylin and eosin staining. **d.** The neuronal damage in rat brains from each group was assayed using nissl staining. Data are shown as mean ± standard deviation. ^^$p<0.01$, ^^^$p<0.001$, vs. Sham; *$p<0.05$, ***$p<0.001$, vs. MCAOR. TTC, 2,3,5-Triphenyltetrazolium chloride.

brains (Fig. 3C-D). Additionally, lower expression of SYN1 and PSD95 were determined in the MCAOR group than in the Sham group (Fig. 4A-E, $p<0.001$). The MCAOR-Ex group exhibited decreased levels of SYN1 mRNA expression, PSD95 mRNA expression, and SYN1 protein expression in comparison with the Sham group (Fig. 4A-D, $p<0.05$), however, there was no significant difference in PSD95 protein expression between the two groups (Fig. 4E). More importantly, when compared to the MCAOR group, there was still increased expression of SYN1 and PSD95 in the MCAOR-Ex and MCAOR-Nim groups (Fig. 4A-E, $p<0.001$). Next, we detected the changes in neuronal inflammation in rats using an ELISA. As seen in Figure 4F-J, increased levels of IL-6, TNF-α, IL-17, and TGF-β as well as decreased levels of IL-10 were detected in the

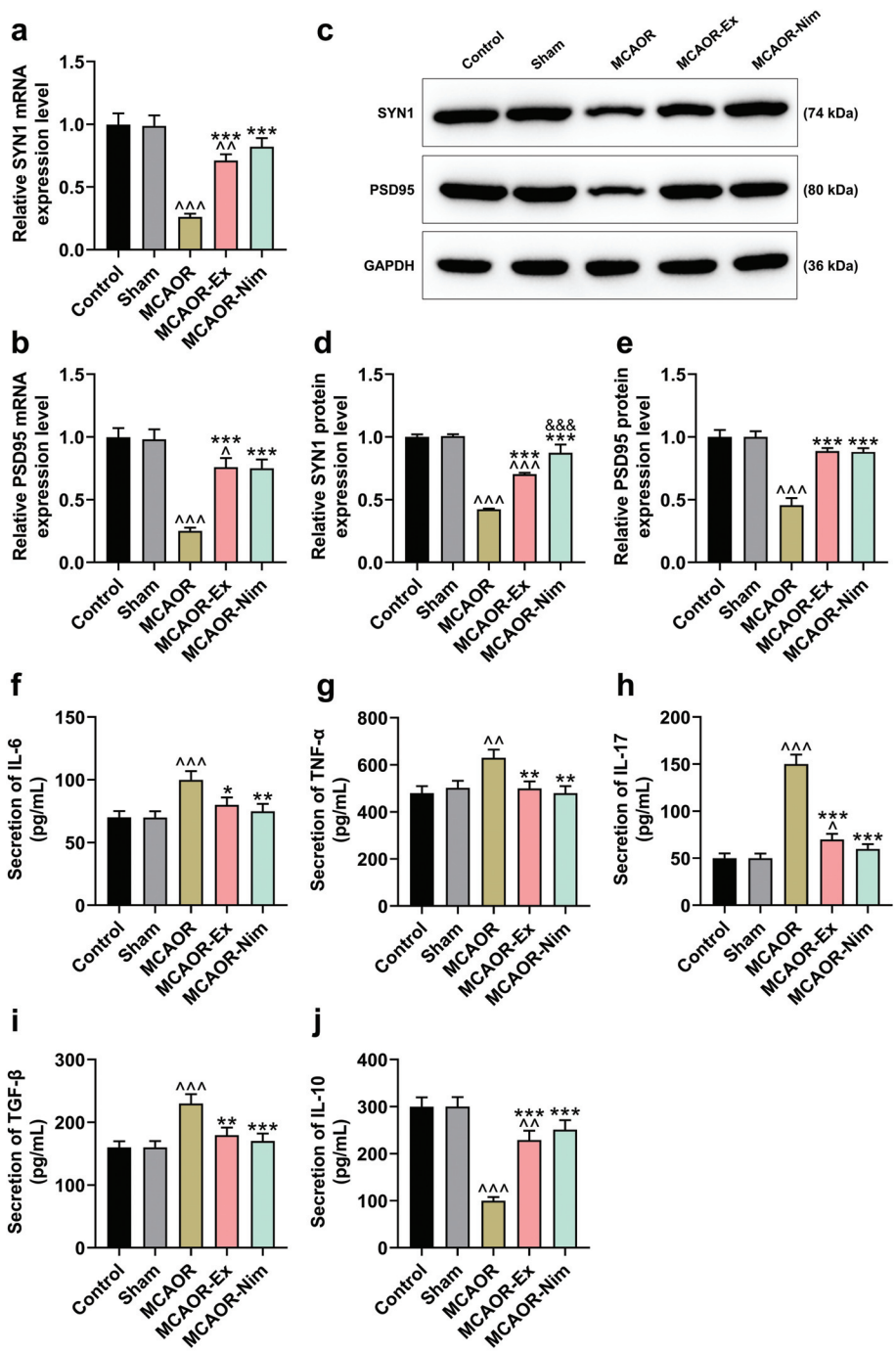


Fig. 4. Effects of aerobic exercise on SYN1 and PSD95 expression and neuroinflammation in MCAOR rats. **a. b.** MCAOR rats were treated with or without interval aerobic exercise training or nimodipine for 2 weeks (MCAOR, MCAOR-Ex, and MCAOR-Nim groups). The rats that underwent sham operations were used as the Sham group, and normal rats were used as the Control group. *SYN1* and *PSD95* mRNA expression in rat brains from each group were determined using qRT-PCR. **c, e.** SYN1 and PSD95 protein expression in rat brains from each group were determined using a western blot. **f-j.** Levels of IL-6, TNF-α, IL-17, TGF-β, and IL-10 in rat brains from each group were determined using ELISA. Data are shown as mean ± standard deviation. GAPDH was used as the endogenous control. ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$, vs. Sham; *$p<0.05$, **$p<0.01$, ***$p<0.001$, vs. MCAOR; &&&P<0.001, vs. MCAOR-Ex. SYN1, synapsin I; PSD95, postsynaptic density protein-95; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha; IL-17, interleukin 17; TGF-β, transforming growth factor-beta; IL-10, interleukin 10; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

MCAOR group compared with the Sham group (p<0.01). MIIT reduced IL-6, TNF-α, IL-17, and TGF-β levels and elevated IL-10 levels in the MCAOR-Ex group (Fig. 4F-J, p<0.05). The effects of MIIT on these inflammation-related cytokines were equivalent to nimodipine (Fig. 4F-J, p<0.01). In addition, the expression of the SYN1 protein in the MCAOR-Nim group was higher than that in the MCAOR-Ex group (Fig. 4D, p<0.001); the levels of other indicators in these two groups showed no significant difference (Fig. 4A-J).

Aerobic exercise modulated the composition of gut microbiota in MCAOR rats

To detect whether aerobic exercise influences the gut microbiota in MCAOR rats, we collected fecal samples from indicated rats and analyzed the microbial community structure using 16S rRNA gene sequencing; the results of PCA analysis are shown in Figure 5A,B. As shown in Figure 6A, rarefaction curves of observed species in all groups were gradually plateaued, indicating a reasonable amount of sequencing data. Similarly, saturated Shannon-Wiener curves and species accumulation curves were observed (Fig. 6B,C), reflecting the adequate amount of sequencing data and the great majority of microbial information. As demonstrated in Figure 6D, g-Vescimonas, g-Bacteroides and g-Blautia were common genera in the MCAOR group; g-Hominimerdicola, g-Ruminococcus, and g-Akkermansia were common genera in the MCAOR-Ex group; and g-Limosilactobacillus, g-Lactobacillus, and g-Neglecta were common genera in the MCAOR-Nim group. After analysis of microbial richness using alpha diversity indexes, including Sobs, Ace, Coverage, Shannon, Chao, and Simpson, it was revealed that the abundance of gut microbiota was decreased in MCAOR rats (Fig. 6E-J). Notably, the increased abundance and decreased diversity of gut microbiota were discerned in MCAOR rats after aerobic exercise training and nimodipine administration, which was related to the increased Sobs, Ace, Coverage, and Chao levels (for abundance) as well as the decreased Shannon level and increased Simpson level (for diversity) (Fig. 6E-J). Next, the structure and predominant gut microbiota from the MCAOR and MCAOR-Ex groups were displayed as a cladogram (Fig. 7A). According to the LEfSe analysis, a relatively increased abundance of p-Verrucomicrobia, p-Deferribacteres, p-Firmicutes, and g-Bacteroides was demonstrated in the MCAOR-Ex group at the phyla level (Fig. 7B). At the genera level, gut microbiota in MCAOR rats contained a higher abundance of g-Anaerotignum, g-Bradyrhizobium, g-Streptococcus, g-Barnesiella, and g-Blautia (Fig. 7B). Collectively, this indicated that aerobic exercise altered the gut microbiota in rats with cerebral I/R injury.

Aerobic exercise-remodeled gut microbiota exerted a neuroprotective effect on MCAOR rats

To investigate whether aerobic exercise attenuates cerebral I/R injury via altering the gut microbiota, we colonized gut microbiota from the MCAOR-Ex group in MCAOR rats through fecal implantation. Compared with the rats in the MCAOR group, gut microbiota-colonized rats had lower mNSS grades after corresponding interventions for 7 days (Fig. 8A, p<0.001), as well as better spatial learning and memory capacities (Fig. 8B-F, p<0.01). Based on the TTC staining, decreased cerebral infarct areas were discerned in MCAOR rats colonized with gut microbiota (Fig. 8G-H, p<0.001). As shown in Figure 9A-E, the colonization with gut microbiota elevated SYN1 and PSD95 expression in the brains of MCAOR rats (p<0.001). As expected, MCAOR-induced upregulation of IL-6, TNF-α, IL-17, and TGF-β as well as downregulation of IL-10 in rat brains were reversed after gut microbiota colonization (Fig. 9F-J, p<0.05).

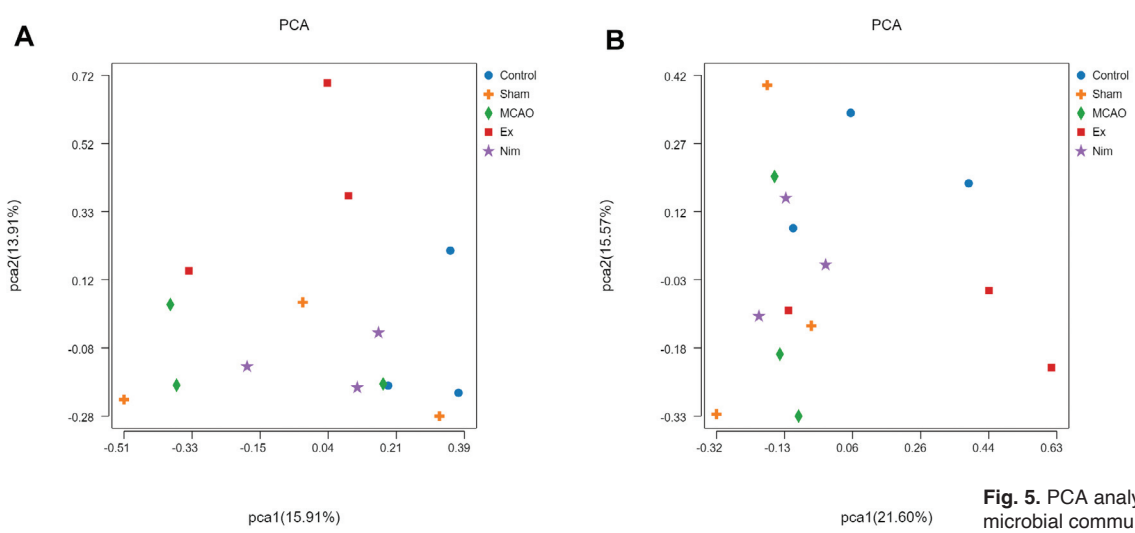


Fig. 5. PCA analysis by 16S rRNA of the microbial community of fecal samples.

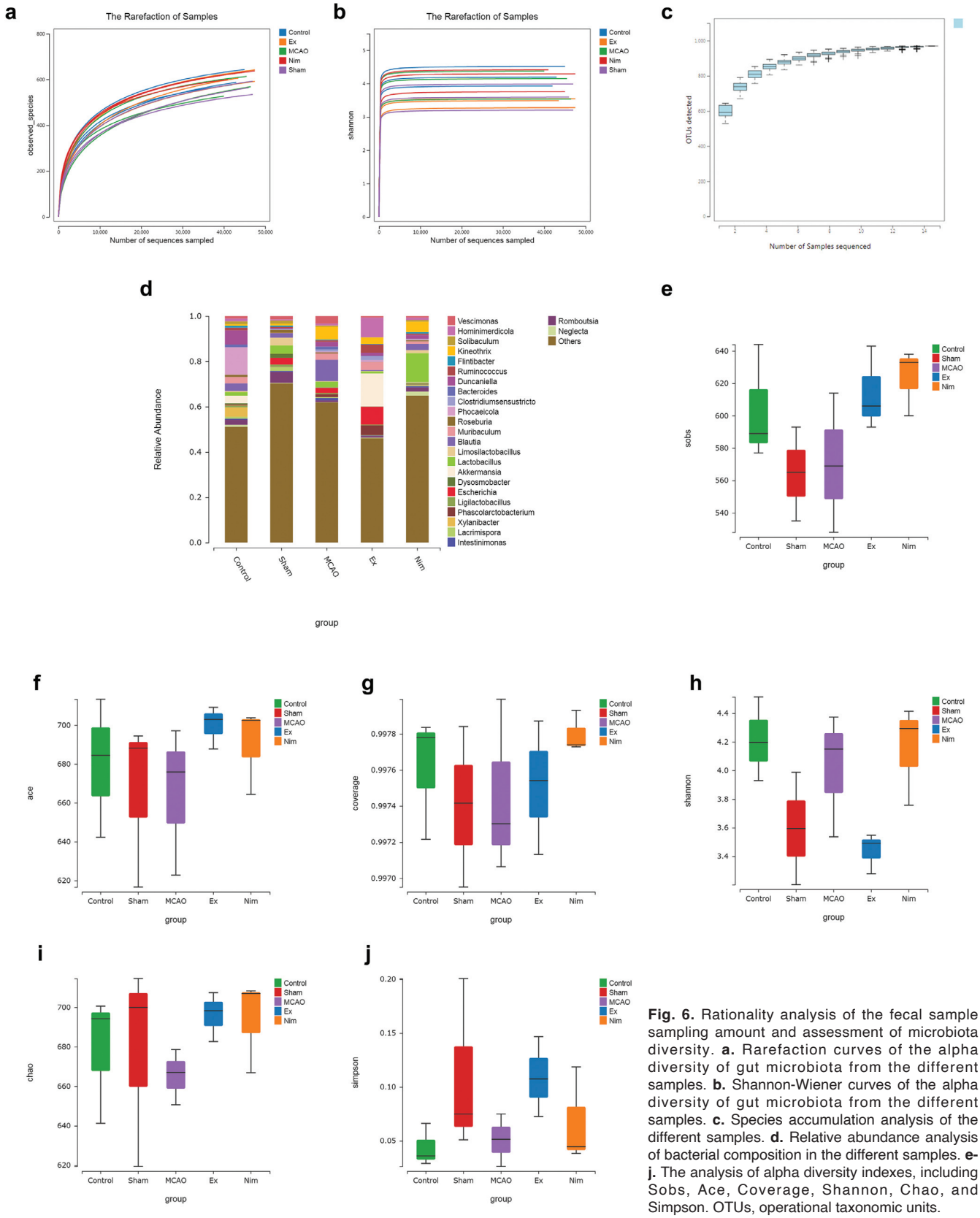
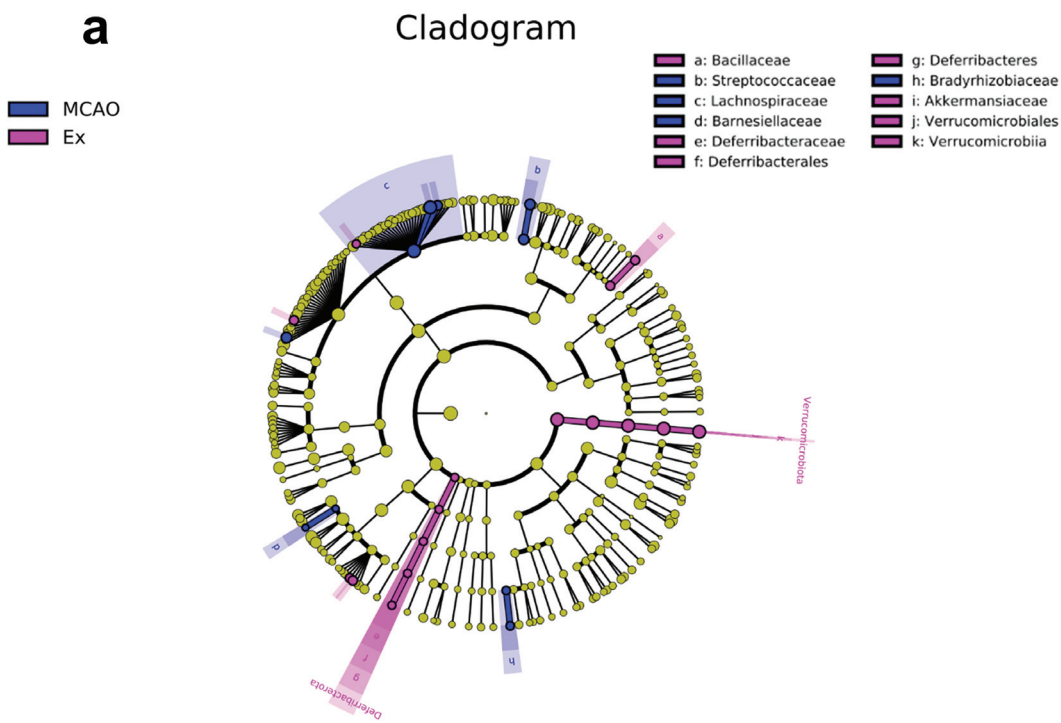


Fig. 6. Rationality analysis of the fecal sample sampling amount and assessment of microbiota diversity. **a.** Rarefaction curves of the alpha diversity of gut microbiota from the different samples. **b.** Shannon-Wiener curves of the alpha diversity of gut microbiota from the different samples. **c.** Species accumulation analysis of the different samples. **d.** Relative abundance analysis of bacterial composition in the different samples. **e-j.** The analysis of alpha diversity indexes, including Sobs, Ace, Coverage, Shannon, Chao, and Simpson. OTUs, operational taxonomic units.



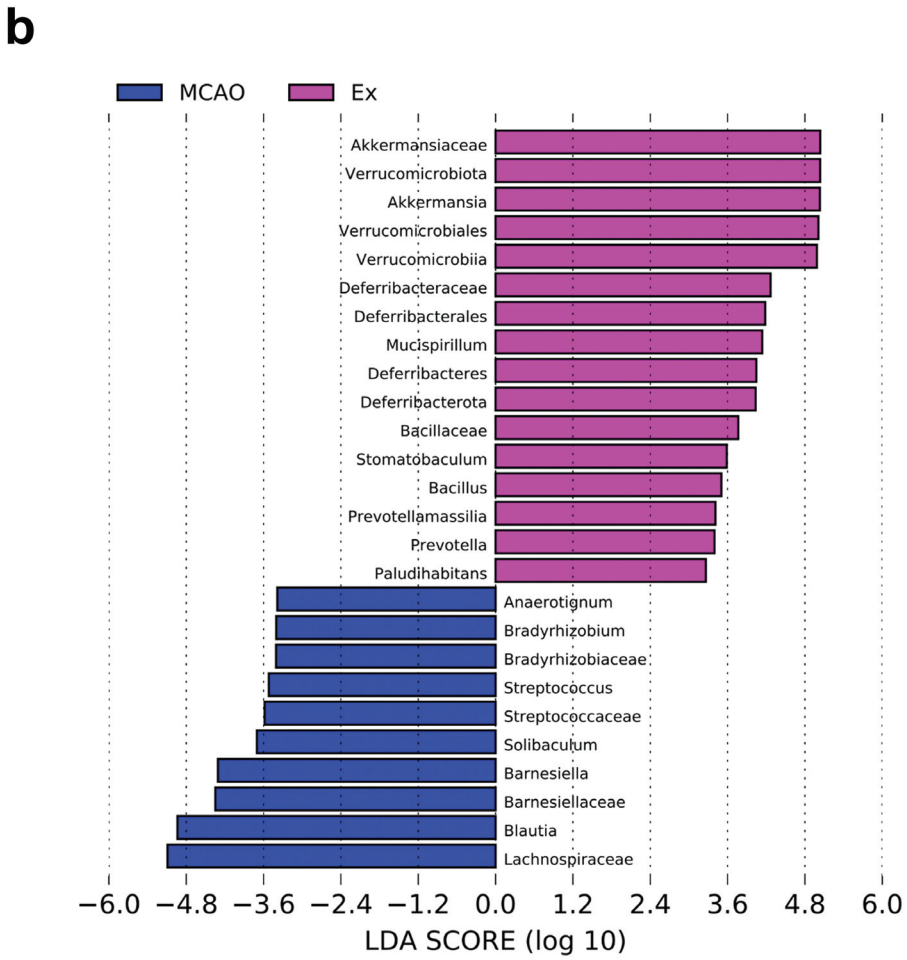


Fig. 7. Microbial analyses of rat fecal samples. **a.** Cladogram of the differential fecal bacteria in MCAOR rats received with or without interval aerobic exercise training. **b.** Comparisons of microbiota differences between MCAOR rats and exercise-intervened MCAOR rats using linear discriminant analysis effect size analysis. LDA, linear discriminant analysis.

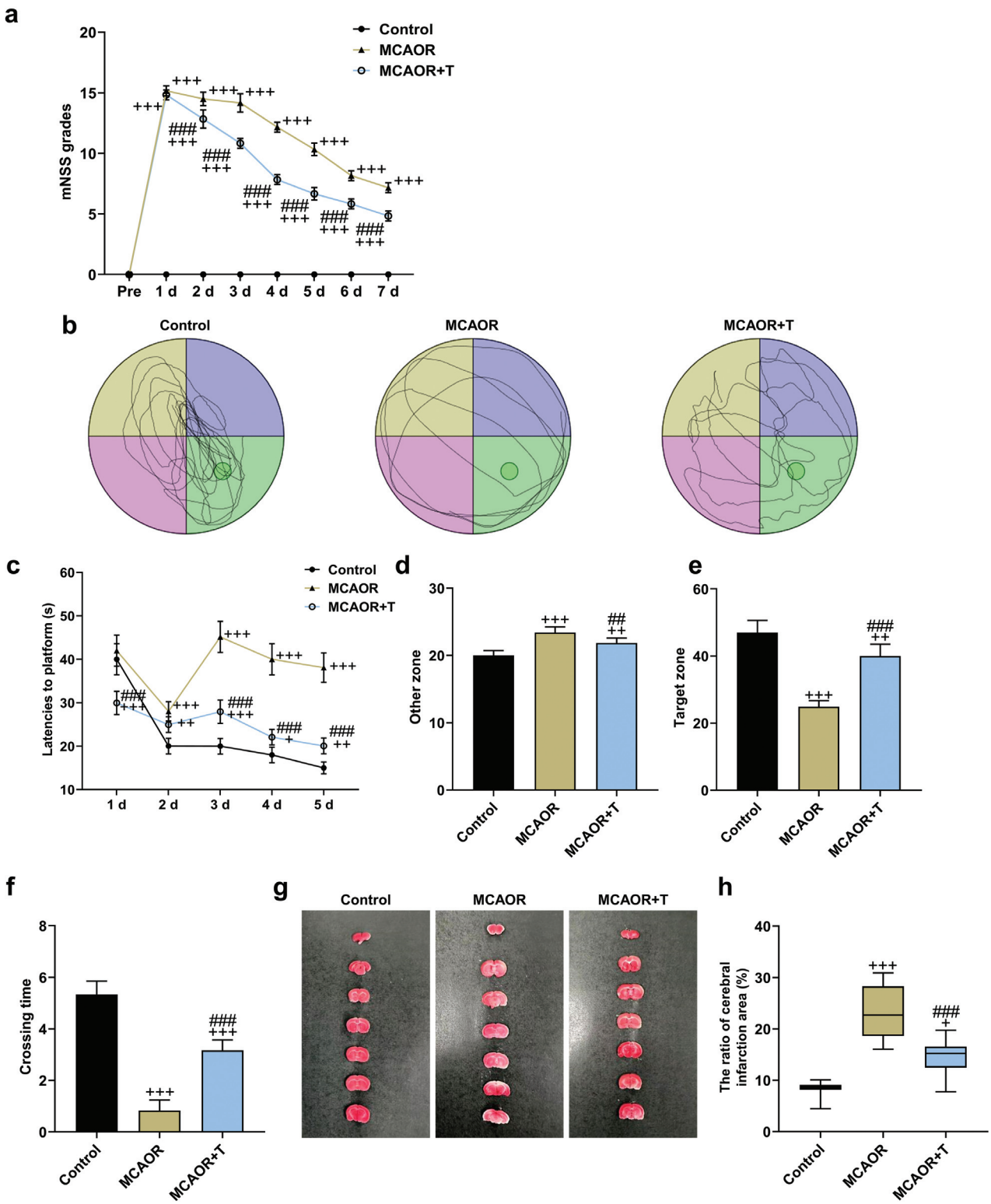


Fig. 8. Effects of aerobic exercise-remodeled gut microbiota on neurological deficit, spatial learning, and memory capacities, and cerebral infarction of MCAOR rats. **a.** MCAOR rats were colonized with or without gut microbiota from aerobic exercise-trained MCAOR rats (MCAOR and MCAOR+T groups). Sham rats were used as the Control group. The neurological deficit of rats in each group was assessed after corresponding interventions for 7 days by the mNSS test. **b-f.** An MWM test was utilized to assess the spatial cognition of rats in each group on days 1-5 after surgery. **g, h.** The infarct volume in rat brains from each group was examined using TTC staining. Data are shown as mean ± standard deviation. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, vs. Control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, vs. MCAOR.

Discussion

Studies have shown that microbes that colonize the gut are important players in influencing host health through crosstalk with various human organs, and the structural alterations in their activity and products are involved in the development and recovery of stroke (de Vos et al., 2022; Peh et al., 2022). Recently, modification of gut microbiota by diet and some natural compounds was reported to attenuate cerebral I/R injury *in vivo* through the mechanism of the GBA (Li et al., 2020; Pang et al., 2020). Different from previous studies, the present study demonstrated that MIIT intervention altered gut microbial communities in rats with cerebral I/R injury and thereby reduced cognitive dysfunction.

Accumulating evidence has supported that exercise training, as a non-pharmacological intervention, is effective in suppressing neuroinflammation and maintaining neurological function to protect the brain after cerebral I/R. For instance, the study of Xie et al. demonstrated that running exercise can facilitate dendritic cell growth and enhance synaptic plasticity in

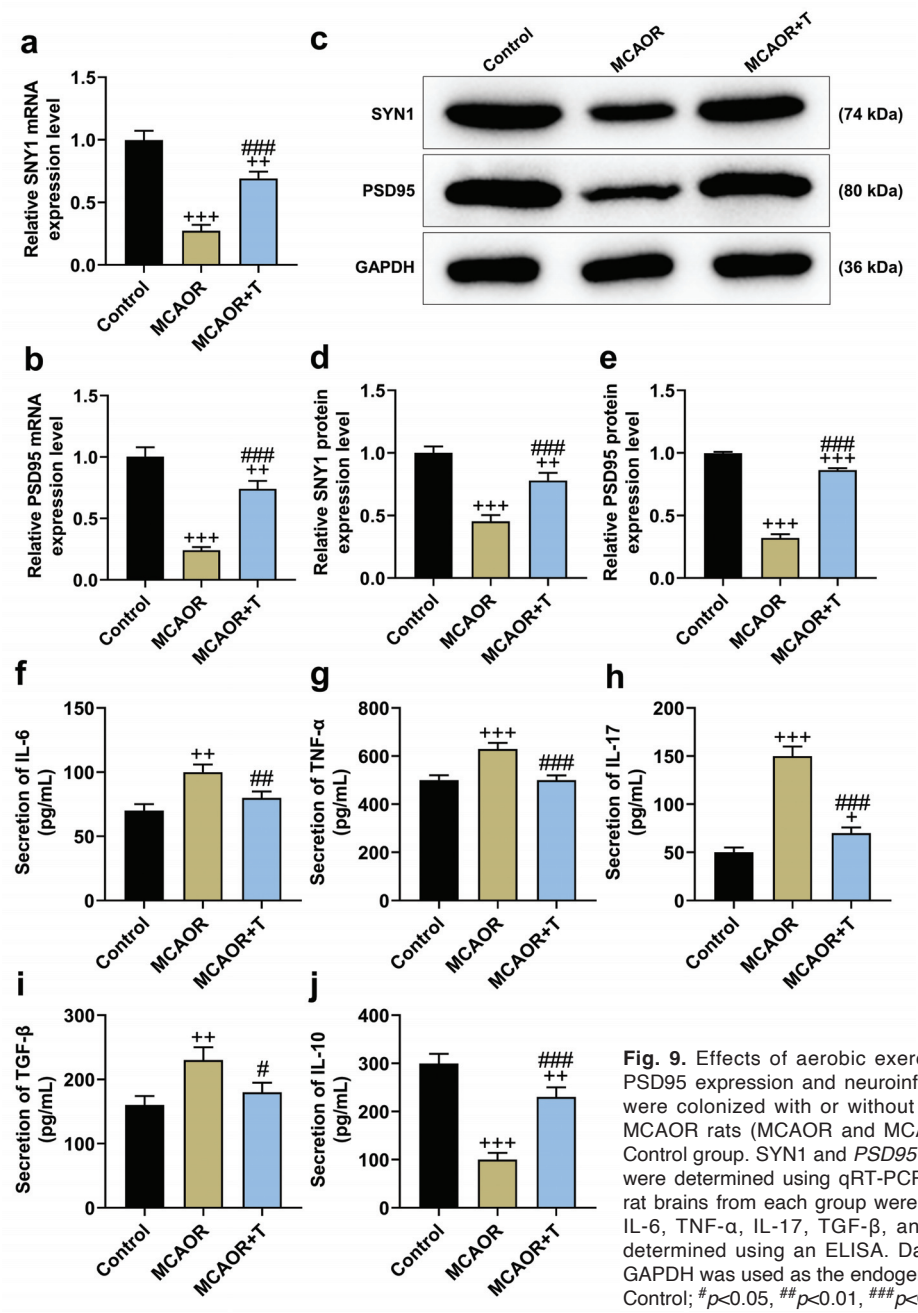


Fig. 9. Effects of aerobic exercise-remodeled gut microbiota on SYN1 and PSD95 expression and neuroinflammation in MCAOR rats. **a, b.** MCAOR rats were colonized with or without gut microbiota from aerobic exercise-trained MCAOR rats (MCAOR and MCAOR+T groups). Sham rats were used as the Control group. SYN1 and *PSD95* mRNA expression in rat brains from each group were determined using qRT-PCR. **c-e.** SYN1 and PSD95 protein expression in rat brains from each group were determined using a western blot. **f-j.** Levels of IL-6, TNF-α, IL-17, TGF-β, and IL-10 in rat brains from each group were determined using an ELISA. Data are shown as mean ± standard deviation. GAPDH was used as the endogenous control. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, vs. Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, vs. MCAOR.

the ischemic penumbra through regulation of the caveolin-1/VEGF signaling pathway, and thus reduce cerebral infarction volume and neurological defect in IS rat models (Xie et al., 2019). Compared with MCAOR rats, treadmill exercise-treated MCAOR rats presented better neurobehavioral outcomes largely due to the effect of treadmill exercise in inducing an anti-inflammatory phenotype in M2 microglia, thereby reducing inflammatory damage (Lu et al., 2021). Nimodipine is a calcium channel blocker used for protecting ischemic neurons and ameliorating cognitive impairment in the early phase of post-stroke patients (Li et al., 2023a). In this study, we found that nimodipine administration significantly reduced the neurological deficit, cerebral infarction volume, and hippocampal neuronal death, as well as enhanced autonomous motility, learning, and memory in MCAOR rats. Of note, MIIT after MCAOR yielded results consistent with nimodipine administration, indicating the improvement effect of MIIT on the neuron injury and cognitive impairment of MCAOR rats.

It is considered that neuroplasticity involving neurogenesis and synaptogenesis is critical for brain functional recovery after IS (Xing and Bai, 2020). In the brains of MCAOR rats, expression of SYN1, a key regulator of synaptic formation, and synaptic scaffold protein PSD95 were decreased (Wang et al., 2018). Based on western blot, this study confirmed that MIIT intervention upregulated SYN1 and PSD95 in MCAOR rats. Additionally, uncontrolled inflammation and immune response after reperfusion causally result in secondary brain injury (Liu et al., 2022). In this study, we found that MCAOR led to upregulation of pro-inflammatory cytokines (IL-6, TNF-α, and IL-17) and downregulation of anti-inflammatory cytokine IL-10 in rat brains; however, these situations were reversed by MIIT. These results may be related to the suppression of M1 microglia and the activation of M2 microglia (Liu et al., 2022), which may be a potential mechanism of MIIT in improving the cognitive dysfunction of MCAOR rats.

Exercise is considered a modulator of gut microbiota for alleviating neurodegenerative diseases, however, there is a lack of evidence as to whether the protective effect of this physical intervention post-stroke is mediated by the microbiota-GBA (Cryan et al., 2020; Gubert et al., 2020). Previously, a study demonstrated that MCAOR rats were characterized by a specific set of inflammation-related microbiota in the brain and peripheral organs including the gut, but these signatures were less observed in running exercise-trained stroke rats (Kingsbury et al., 2021). According to the results of 16S rRNA gene sequencing, the present study found that there were significant deviations in the abundance and diversity of gut microbial composition between the MCAOR and MCAOR-Ex groups. Clinically, the dysbiosis of gut microbiota in patients with stroke is mainly manifested in the alteration of the ratio of g-Bacteroides and p-Firmicutes (Chidambaram et al., 2022). Beneficial genera, such as g-Prevotella, g-Anaerotignum, g-Blautia, and g-Faecalibacterium, can produce short-chain fatty acids that can be absorbed into the circulation and cross the blood-brain barrier to promote stroke recovery (Hu et al., 2022). Moreover, g-Akkermansia belongs to the phyla of p-Verrucomicrobia and is considered a potential probiotic for the treatment of neuropsychiatric disorders given its positive effects on regulating immune and metabolic functions (Lei et al., 2023). In addition, it has been reported that the abundance of p-Firmicutes was decreased, whereas p-Proteobacteria and p-Deferribacteres were increased in rats with ischemic stroke (Wu et al., 2021), which was similar to our results. Based on the analysis of the LDA score, this study found that there were significant differences in species abundance at the level of p-Verrucomicrobia, g-Bacteroides, p-Deferribacteres, and p-Firmicutes between the MCAOR-Ex and MCAOR groups. In light of these findings, it is suggested that MIIT intervention post-stroke remodels microbial communities in the gut of rats. In addition, aerobic exercise training can lead to increased abundance and decreased diversity of gut microbiota in MCAOR rats, and the decrease in diversity may be related to the reshaping of gut microbiota through aerobic exercise, however, further exploration is needed to verify this hypothesis. Further, through transplantation of fecal microbiota from the MCAOR-Ex group, we found that impaired neurological function, brain damage, and neuroinflammation were attenuated in MCAOR rats.

In summary, our current findings support that aerobic exercise attenuates cerebral I/R injury-induced cognitive dysfunction by modifying the composition of the gut microbiota. Therefore, this study highlights that aerobic exercise can be used as a mechanical therapy for facilitating brain functional rehabilitation after IS based on an exercise-microbiota-GBA. However, whether the intestinal microbiota transplantation of MCAOR+T rats was truly successful has not been verified as yet, and further research is needed on the relationship between changes in gut microbiota and the studied genes (*SYN1* and *PSD95*) through rescue experiments, which will be supplemented in our future study.

Acknowledgements. Not applicable.

Funding. This work was supported by the Medical and Health Technology Planning Project of Zhejiang Province [NO: 2023RC139]; the Zhejiang Provincial Traditional Chinese Medicine Science and Technology Plan Project (NO: 2024031269&2024ZR049); and the State Administration of Traditional Chinese Medicine Science and Technology Department-Zhejiang Provincial Administration of Traditional Chinese Medicine Co-construction of Key Laboratory of Research on Prevention and Treatment for depression syndrome (GZY-ZJ-SY-2402).

Conflict of interest. The Authors declare that there is no conflict of interest.

References

Allen J.M., Mailing L.J., Niemiro G.M., Moore R., Cook M.D., White B.A., Holscher H.D. and Woods J.A. (2018). Exercise alters gut

microbiota composition and function in lean and obese humans. Med. Sci. Sports Exerc. 50, 747-757.

Bao Z., Zhang Z., Zhou G., Zhang A., Shao A. and Zhou F. (2022). Novel mechanisms and therapeutic targets for ischemic stroke: A focus on gut microbiota. Front. Cell. Neurosci. 16, 871720.

Chidambaram S.B., Rathipriya A.G., Mahalakshmi A.M., Sharma S., Hediyal T.A., Ray B., Sunanda T., Rungratanawanich W., Kashyap R.S., Qoronfleh M.W., Essa M.M., Song B.J. and Monaghan T.M. (2022). The influence of gut dysbiosis in the pathogenesis and management of ischemic stroke. Cells 11, 1239.

Cryan J.F., O'Riordan K.J., Cowan C.S.M., Sandhu K.V., Bastiaanssen T.F.S., Boehme M., Codagnone M.G., Cussotto S., Fulling C., Golubeva A.V., Guzzetta K.E., Jaggar M., Long-Smith C.M., Lyte J.M., Martin J.A., Molinero-Perez A., Moloney G., Morelli E., Morillas E., O'Connor R., Cruz-Pereira J.S., Peterson V.L., Rea K., Ritz N.L., Sherwin E., Spichak S., Teichman E.M., van de Wouw M., Ventura-Silva A.P., Wallace-Fitzsimons S.E., Hyland N., Clarke G. and Dinan T.G. (2019). The microbiota-gut-brain axis. Physiol. Rev. 99, 1877-2013.

Cryan J.F., O'Riordan K.J., Sandhu K., Peterson V. and Dinan T.G. (2020). The gut microbiome in neurological disorders. Lancet Neurol. 19, 179-194.

de Vos W.M., Tilg H., Van Hul M. and Cani P.D. (2022). Gut microbiome and health: Mechanistic insights. Gut 71, 1020-1032.

Gubert C., Kong G., Renoir T. and Hannan A.J. (2020). Exercise, diet and stress as modulators of gut microbiota: Implications for neurodegenerative diseases. Neurobiol. Dis. 134, 104621.

Hu W., Kong X., Wang H., Li Y. and Luo Y. (2022). Ischemic stroke and intestinal flora: An insight into brain-gut axis. Eur. J. Med. Res. 27, 73.

Kingsbury C., Shear A., Heyck M., Sadanandan N., Zhang H., Gonzales-Portillo B., Cozene B., Sheyner M., Navarro-Torres L., García-Sánchez J., Lee J.Y. and Borlongan C.V. (2021). Inflammation-relevant microbiome signature of the stroke brain, gut, spleen, and thymus and the impact of exercise. J. Cereb. Blood Flow Metab. 41, 3200-3212.

Lei W., Cheng Y., Gao J., Liu X., Shao L., Kong Q., Zheng N., Ling Z. and Hu W. (2023). *Akkermansia muciniphila* in neuropsychiatric disorders: Friend or foe? Front. Cell. Infect. Microbiol. 13, 1224155.

Li Q., Guo Y., Yu X., Liu W. and Zhou L. (2020). Protective mechanism of rhubarb anthraquinone glycosides in rats with cerebral ischaemia-reperfusion injury: Interactions between medicine and intestinal flora. Chin. Med. 15, 60.

Li Y., Cui R., Liu S., Qin Z., Sun W., Cheng Y. and Liu Q. (2023a). The efficacy and safety of post-stroke cognitive impairment therapies: An umbrella review. Front. Pharmacol. 14, 1207075.

Li H.J., Li D.Q., Zhang Y.L., Ding X.F., Gao H.T., Zhu Y., Liu J., Zhang L.X., Chen J., Chen G. and Yu Y. (2023b). Modulation of gut microbiota alleviates cerebral ischemia/reperfusion injury in rats by inhibiting M1 polarization of microglia. Front. Pharmacol. 14, 1123387.

Liu H., Zhang Z., Zang C., Wang L., Yang H., Sheng C., Shang J., Zhao Z., Yuan F., Yu Y., Yao X., Bao X. and Zhang D. (2021). GJ-4 ameliorates memory impairment in focal cerebral ischemia/reperfusion of rats via inhibiting JAK2/STAT1-mediated neuroinflammation. J. Ethnopharmacol. 267, 113491.

Liu M.X., Luo L., Fu J.H., He J.Y., Chen M.Y., He Z.J. and Jia J. (2022). Exercise-induced neuroprotection against cerebral ischemia/reperfusion injury is mediated via alleviating inflammasome-induced pyroptosis. Exp. Neurol. 349, 113952.

Lu J., Wang J., Yu L., Cui R., Zhang Y., Ding H. and Yan G. (2021). Treadmill exercise attenuates cerebral ischemia-reperfusion injury by promoting activation of M2 microglia via upregulation of interleukin-4. Front. Cardiovasc. Med. 8, 735485.

Luo F., Zhu M., Lv K., Sun D., Yang G. and Pan G. (2022). Treadmill training attenuates pyroptosis in rats with cerebral ischemia/reperfusion injury. Iran. J. Basic Med. Sci. 25, 1215-1221.

Motiani K.K., Collado M.C., Eskelinen J.J., Virtanen K.A., Löyttyniemi E., Salminen S., Nuutila P., Kalliokoski K.K. and Hannukainen J.C. (2020). Exercise training modulates gut microbiota profile and improves endotoxemia. Med. Sci. Sports Exerc. 52, 94-104.

Pang S.Q., Luo Z.T., Wang C.C., Hong X.P., Zhou J., Chen F., Ge L., Li X., Dai Y.L., Wu Y.L. and Zhang J.H. (2020). Effects of *Dioscorea polystachya* 'yam gruel' on the cognitive function of diabetic rats with focal cerebral ischemia-reperfusion injury via the gut-brain axis. J. Integr. Neurosci. 19, 273-283.

Parr E., Ferdinand P. and Roffe C. (2017). Management of acute stroke in the older person. Geriatrics 2, 27.

Peh A., O'Donnell J.A., Broughton B.R.S. and Marques F.Z. (2022). Gut microbiota and their metabolites in stroke: A double-edged sword. Stroke 53, 1788-1801.

Pluta R., Januszewski S. and Czuczwar S.J. (2021). The role of gut microbiota in an ischemic stroke. Int. J. Mol. Sci. 22, 915.

Quiroga R., Nistal E., Estébanez B., Porras D., Juárez-Fernández M., Martínez-Flórez S., García-Mediavilla M.V., de Paz J.A., González-Gallego J., Sánchez-Campos S. and Cuevas M.J. (2020). Exercise training modulates the gut microbiota profile and impairs inflammatory signaling pathways in obese children. Exp. Mol. Med. 52, 1048-1061.

Rutsch A., Kantsjö J.B. and Ronchi F. (2020). The gut-brain axis: How microbiota and host inflammasome influence brain physiology and pathology. Front. Immunol. 11, 604179.

Sampson T.R., Debelius J.W., Thron T., Janssen S., Shastri G.G., Ilhan Z.E., Challis C., Schretter C.E., Rocha S., Gradinaru V., Chesselet M.F., Keshavarzian A., Shannon K.M., Krajmalnik-Brown R., Wittung-Stafshede P., Knight R. and Mazmanian S.K. (2016). Gut microbiota regulate motor deficits and neuroinflammation in a model of parkinson's disease. Cell 167, 1469-1480.e1412.

Sorboni S.G., Moghaddam H.S., Jafarzadeh-Esfehani R. and Soleimanpour S. (2022). A comprehensive review on the role of the gut microbiome in human neurological disorders. Clin. Microbiol. Rev. 35, e0033820.

Tomassoni D., Lanari A., Silvestrelli G., Traini E. and Amenta F. (2008). Nimodipine and its use in cerebrovascular disease: Evidence from recent preclinical and controlled clinical studies. Clin. Exp. Hypertens. 30, 744-766.

Wang Y., Zhang Z., Zhang L., Yang H. and Shen Z. (2018). RLIPostC protects against cerebral ischemia through improved synaptogenesis in rats. Brain Inj. 32, 1429-1436.

Wang H., Ren S., Lv H. and Cao L. (2021). Gut microbiota from mice with cerebral ischemia-reperfusion injury affects the brain in healthy mice. Aging 13, 10058-10074.

Wu W., Sun Y., Luo N., Cheng C., Jiang C., Yu Q., Cheng S. and Ge J. (2021). Integrated 16s rRNA gene sequencing and LC-MS analysis revealed the interplay between gut microbiota and plasma metabolites in rats with ischemic stroke. J. Mol. Neurosci. 71, 2095-2106.

Xie Q., Cheng J., Pan G., Wu S., Hu Q., Jiang H., Wang Y., Xiong J.,

Pang Q. and Chen X. (2019). Treadmill exercise ameliorates focal cerebral ischemia/reperfusion-induced neurological deficit by promoting dendritic modification and synaptic plasticity via upregulating caveolin-1/VEGF signaling pathways. Exp. Neurol. 313, 60-78.

Xing Y. and Bai Y. (2020). A review of exercise-induced neuroplasticity in ischemic stroke: Pathology and mechanisms. Mol. Neurobiol. 57, 4218-4231.

Yuan B., Lu X.J. and Wu Q. (2021). Gut microbiota and acute central nervous system injury: A new target for therapeutic intervention. Front. Immunol. 12, 800796.

Zhang Q., Jia M., Wang Y., Wang Q. and Wu J. (2022). Cell death mechanisms in cerebral ischemia-reperfusion injury. Neurochem. Res. 47, 3525-3542.

Zhao Y., Zhang X., Chen X. and Wei Y. (2022). Neuronal injuries in cerebral infarction and ischemic stroke: From mechanisms to treatment (Review). Int. J. Mol. Med. 49, 15.

Accepted October 14, 2024