Aerobic exercise reverses the NF-κB/NLRP3 inflammasome/5-HT pathway by upregulating irisin to alleviate post-stroke depression

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Background: Post-stroke depression (PSD) is one of the most common and serious sequelae of stroke. The pathogenesis of PSD involves both psychosocial and biological mechanisms, and aerobic exercise is a potential therapeutic target. We conducted an in-depth exploration of the protective mechanisms of aerobic exercise in a PSD mouse model.

Methods: In this study, C57BL/6 mice were used as the research objects, and a PSD mouse model was established by combining middle cerebral artery occlusion and chronic unpredictable mild stimulation. Real-time quantitative polymerase chain reaction, enzyme-linked immunosorbent assays, adeno-associated virus microinjection technology, co-immunoprecipitation, fluorescence in-situ hybridization, and western blotting were performed. A moderate-load treadmill exercise was used for aerobic exercise intervention. The moderate-intensity aerobic exercise training method adopted 0 slopes and treadmill adaptation training for 5 days. We verified the effects of aerobic exercise on the nuclear factor kappa B (NF-κB)/nucleotide-binding oligomerization domain--like receptor protein 3 (NLRP3) inflammasome/5-hydroxytryptamine (5-HT) pathway.

Results: Aerobic exercise effectively alleviated the neurological damage caused by PSD ($P<0.01$). The results from the PSD mouse model *in vivo* were consistent with those of the cell experiments. Moreover, overexpression of irisin improves depression-like behavior in PSD mice. We confirmed that aerobic exercise is involved in PSD through 5-HT, which inhibits NF-κB/NLRP3 inflammasome initiation through irisin and alleviates mitochondrial damage under stress by reducing calcium overload, thereby inhibiting NLRP3 inflammasome activation.

Conclusions: Aerobic exercise reversed the NF-κB/NLRP3 inflammasome/5-HT pathway by upregulating irisin expression to alleviate PSD.

Keywords: Post-stroke depression; aerobic exercise; irisin; nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3)

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Introduction

Post-stroke depression (PSD) is a syndrome that occurs after stroke and manifests as a series of depressive symptoms and corresponding physical symptoms (1). PSD is one of the most common and treatable complications after stroke. If the patient is not diagnosed and clinical intervention in time, it will affect the recovery of neurological function and self-care ability of patients after stroke (1). Epidemiological data showed that the combined incidence of PSD within 5 years after stroke was 31% (2). PSD can occur in the acute (<1 month), middle (1–6 months) and convalescent (>6 months) period after stroke, with incidence of 33%, 33% and 34%, respectively (2). A previous study has found that PSD is closely related to the poor prognosis of stroke, which can not only lead to prolonged hospital stay, neurological dysfunction, loss of independent living ability, and even lead to increased mortality and suicide rates (2).

The pathogenesis of PSD is complex, and any single mechanism cannot fully elucidate the occurrence and development of PSD (3-5). Based on the increased expression of inflammatory factors and the activation of inflammation-related pathways in PSD patients and animal models, a study has demonstrated that inflammatory mechanisms play an important role in the development of PSD (6). In recent years, studies have shown that step-by-step rehabilitation exercise training helps to reduce depressive symptoms and improves the quality of life of patients with PSD (6,7). We previously constructed a chronic unpredictable mild stress (CUMS) depression mouse model and observed that aerobic exercise alleviates depression in mice by inhibiting the nuclear factor kappa B (NF-κB) pathway and enhances immune and neuron repair functions (7).

The nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is a key inflammasome involved in multiple inflammatory responses, and its activation is associated with stress and depression (8,9). The components of the NLRP3 inflammasome and the effector product interleukin (IL)-1β in the peripheral blood mononuclear cells of patients with depression are significantly higher than those in normal people, and these abnormal indicators are gradually normalized after receiving antidepressant treatment (10). Kohman *et al.* (9) found that NLRP3 inflammasome aggregation existed in the brain of patients with neurodegenerative diseases mainly manifested by cognitive impairment. Niu *et al.* (8) confirmed that chronic social isolation may promote the release of inflammatory factors and inhibit synaptic plasticity by activating the NLRP3 inflammasome in hippocampal microglia, eventually leading to cognitive impairment in mice. NLRP3 inflammasome activation must progress through the following 2 stages: (I) inflammasome priming; and (II) inflammasome activation (11). The initiation of the NLRP3 inflammasome is required to complete the synthesis of the NLRP3 inflammasome protein, caspase-1 precursor protein, and IL-1β precursor protein, and the NF-κB pathway is a key signaling pathway involved in inflammasome initiation (12).

Studies have shown that physical exercise inhibits inflammation by inhibiting the NF-κB signaling pathway (13,14). The findings of such studies suggest that aerobic exercise may be involved in inhibiting the initiation of the NLRP3 inflammasome. Additionally, a previous study has found that chronic stress leads to the decline of learning and memory function and the damage of hippocampal neurons in mice (15). These studies suggest that the overactivation of NLRP3 inflammasome is an important pathophysiological mechanism of depression. However, it is not yet known whether the NLRP3 inflammasome is involved in the occurrence and development of PSD.

Irisin is a newly discovered muscle cytokine. A study has found that irisin is closely related to PSD and is a useful biomarker for predicting PSD (16). Scholars have noted that regular exercise training increases the level of irisin, improves cognitive function, and may be involved in the pathological

Highlight box

Key findings

- Aerobic exercise reversed the NF-κB/NLRP3 inflammasome/5-HT pathway by upregulating irisin expression to alleviate PSD.

What is known and what is new?

- Post-stroke depression (PSD) is a syndrome that occurs after stroke and manifests as a series of depressive symptoms and corresponding physical symptoms.
- we established a PSD model through middle cerebral artery occlusion (MCAO) combined with CUMS to examine the relationship and mechanism of aerobic exercise and the NF-κB/NLRP3 pathway in the occurrence of PSD. Further, we explored the role of irisin in PSD, and provided novel insights into the pathogenesis and treatment of PSD.

What is the implication, and what should change now?

- This study found that aerobic exercise reversed the NF-κB/NLRP3 inflammasome/5-HT pathway by upregulating irisin expression to alleviate PSD.

process of depression (17). As a neurotransmitter, 5-hydroxytryptamine (5-HT) is closely related to the occurrence of PSD (18). After a stroke, the 5-HT pathway in the brain is destroyed, which reduces the level of 5-HT in the body and reduces the vitality of the body, which leads to the occurrence of depression. Treatment with drugs, such as monoamine oxidase inhibitors and selective 5-HT reuptake inhibitors, has been shown to improve depressive symptoms by increasing 5-HT levels in the body (18).

We hypothesized that stroke increased the expression of inflammatory factors in the brain and participated in PSD by regulating NF-κB/NLRP3. Additionally, we conjectured that irisin affects these pathways and exerts antidepressant effects. To examine the above hypothesis, we established a PSD model through middle cerebral artery occlusion (MCAO) combined with CUMS to examine the relationship and mechanism of aerobic exercise and the NF-κB/NLRP3 pathway in the occurrence of PSD. Further, we explored the role of irisin in PSD, and provided novel insights into the pathogenesis and treatment of PSD. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5443/rc).

Methods

PSD mouse model (MCAO + CUMS)

A total of 72 C57BL/6 mice (8-month aged mice, female to male ratio: 1:1, body weight 22–25 g) were purchased from Shanghai Jisijie Laboratory Animal Co., Ltd. [production license number: SCXK (Shanghai) 2018-0004]. The animals were kept at the Laboratory Animal Center of The First Affiliated Hospital of Hunan Normal University [license number SYXK (Hunan) 2018-0049]. The mice were divided into the following 6 groups: the sham group, the PSD group, the aerobic exercise + PSD group, the AAV8-irisin-GFP + PSD group, the AAV8-control-GFP + PSD group, and the BAY 11-7082 + PSD group. There were 12 mice in each group (female to male ratio: 1:1). Animal experiments were performed under a project license (2019-S90) granted by ethics committee of The First Affiliated Hospital of Hunan Normal University, in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

PSD modeling was carried out using the aforementioned method with a slight modification (16). A PSD mouse model was established by combining MCAO and CUMS. Anesthesia was induced with 3% isoflurane and then maintained with 1.5% isoflurane. The external and internal carotid arteries were separated. The distal end of the external carotid artery, the proximal end of the common carotid artery, and the pterygopalatine artery were ligated. A nylon thread was inserted from the bifurcation of the common carotid artery and pushed forward to 18.1±0.5 cm, and the excess thread was sutured and fixed to the skin. Penicillin (100,000 U/piece) was administered daily for 3 consecutive days. Subsequently, each mouse was housed individually in a cage and received CUMS treatment for 35 days. The mice in the sham group underwent the same surgical procedure, but no nylon thread was inserted.

The specific operation procedure of CUMS included day and night reversal, tail clipping for 3 min, swimming in 4 ℃ water for 5 min, horizontal oscillation at 160 Hz for 5 min, fasting for 1 d, water deprivation for 1 d, electric shocks for 2 min (30 V, electric shock for 5 s, at interval s of 5 s). The above stimuli were repeated 4 times to form 35 random stimuli, once per day. The mice in the sham group were fed routinely.

The aerobic exercise program for mice in the aerobic exercise + PSD group was as previously described (17). A moderate load treadmill exercise was used for the rehabilitation training intervention. The moderate-intensity aerobic exercise training method adopted a 0 slope, and the treadmill adaptive training was conducted for 5 days. The load intensity was equivalent to 60–70% maximal oxygen consumption (VO_2max), and the exercise was performed 6 times a week for a total of 12 weeks of training.

Depressive-like behavioral performance

Glucose consumption testing: The detection was performed according to the reference method (19). The mice were trained to drink glucose water before the experiment. After being deprived of water for 24 h, each mouse was given 20 mL of 1% sucrose water. After 1 h, the water bottle was removed, and the remaining volume was measured to obtain the consumption volume of glucose water (mL).

Open-field test (OFT) experiment: The detection was performed according to the reference method (20). A self-made open box without a lid with 64 equal black and white grids (10 cm × 10 cm) on the bottom was used. Each mouse was placed in the center square and timed for 3 min. The number of grids entered by all limbs when the mice climbed over the square observation area was used as the number of

horizontal movements. The number of vertical movements was taken as the number of times a mouse's forelimbs lifted off the ground or climbed the box wall and the hind limbs stood upright.

Morris water-maze test: The Morris water-maze experiment was carried out as described previously (19). The experiment used a stainless steel circular pool and a platform with a diameter of 100 cm and a water depth of 50 cm. First, clean water was poured into the pool, a little ink was then added to make the water black, and the water temperature was controlled at 22±2 ℃. The pool was divided into 2 areas. The near-platform area had a distance from the platform of <50 cm, and the far-platform area had a distance from the platform of >50 cm. The usual training time was set at 70 s as the maximum latency to escape to the platform, and if the time exceeded 70 s, the mice were directly pulled to the platform. During the experiment, each mouse was randomly placed in any quadrant, and the time to find the platform was taken as the latency period, and the path taken to the platform was taken as the total distance. The total route distance, the duration, the route near the platform, the route far from the platform, and the distance ratio of the near (near the platform) to far (far from the platform) of the mice were recorded.

Neurological deficit score

Neurological assessments were performed by investigators blinded to the experimental group. Neurological deficits were scored according to the Longa's scale (0–4 points) (18). Only mice with 1–3 points after 2 h of occlusion were considered successful models and included in the subsequent experiments.

Cell culture and primary microglia culture

On a clean bench, the BV-2 cells were transferred to a 25-mL culture flask and an appropriate amount of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin was added. The cells were cultured in a 37 ℃, 5% carbon dioxide incubator. After 8-10 h, the cells were completely adhered to the culture flask and washed twice with phosphate buffer solution (PBS). The medium was then replaced to remove any cells that had not adhered to the culture flask and debris, and the culture was continued.

The cerebral cortexes of the 1–3-day-old mice were cut to remove the meninges and cerebral blood vessels. The brain tissue was minced with scissors and trypsinized for 10 min at 37 ℃. After trypsin termination, the cells were filtered through a 100 μm cell strainer. After centrifugation at 1,500 rpm for 5 min, the supernatant was discarded. Cells were resuspended in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was changed after 3 days, and after 7 days, the medium was changed to medium containing 0.25 μg/mL of macrophage colony-stimulating factor to stimulate the proliferation of microglia. On days 12–14, the confluent mixed glial cells were shaken at 180 rpm for 3 h to isolate microglia.

AAV microinjection technology

Adeno-associated virus (AAV)-irisin-green fluorescent protein (GFP) and AAV8-control-GFP lentiviruses were purchased from Shanghai Hanheng Biological Company. The mice were intraperitoneally injected with 1% sodium pentobarbital, and after anesthesia, the mice were fixed on the brain stereotaxic apparatus. The hair on the head of each mouse was shaved, the mouse scalp was cut with a blade, the position of the front chimney point on the mouse head was confirmed, the coordinates of the front chimney point were read, and the corresponding coordinate values were recorded. The following locations in the left and right hippocampal regions of the mouse were confirmed: 2.0 mm behind the bony chimney point, 1.5 mm left and right, and 2.0 mm deep. Holes were drilled in the left and right hippocampal regions, and the needle was placed according to the coordinates. The needle reached the hippocampus on 1 side and was left for 10 min. The micro-syringe was slowly pushed to inject 1 μL of AAV (virus titer of 1×10^{9}) into the hippocampal area, and the injection speed was adjusted to 0.2 μL/min. After the injection of AAV, the needle was stopped at the original coordinates for 10 min, and the needle was slowly pulled out. The mouse scalp was sutured, and an ointment was applied to prevent infection. After the microinjection of AAV into the hippocampus, the mice were rested for 1 week before the subsequent experiments were conducted.

Immunofluorescence staining

The mouse hearts were perfused with 200 mL of PBS and then fixed with 200 mL 4% paraformaldehyde. After the removal of the mouse brains, the samples were incubated

Table 1 Primer sequences

Name	Forward/Sense (from 5' to 3')	Reverse/Antisense (from 5' to 3')
NLRP3	CCTTAAGCTGGAGCTGCTGT	TCACCTCTCGGCAGTGGATA
Caspase1	ACTGCTATGGACAAGGCACG	AGGGCAAAACTTGAGGGTCC
IL-1β	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
NEAT1	TTTGCCTAGGTTCCGTGCTT	GACCCAGGGGCAAGGTTTTA
Irisin	GGCAGGTGTTATAGCTCTCTTCT	ATATCTTGCTGCGGAGGAGAC
GAPDH	GGAGAGTGTTTCCTCGTCCC	ACTGTGCCGTTGAATTTGCC

NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; IL-1β, interleukin-1beta; NEATI, nuclear paraspeckle assembly transcript 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

at 4 ℃ overnight with 4% paraformaldehyde. Then, the brains were removed and dehydrated in 30% sucrose for 24 h. After the sedimentation was complete, the brain tissues were frozen at –80 ℃ and sliced at –20 ℃ the next day with a thickness of 30 μm. The 30-μm brain slices were washed with PBS for 5 min, incubated for 30 min at room temperature with 3% H_2O_2 (30% H_2O_2 + PBS =1:9), washed 3 times with PBS for 5 min each, and blocked with 0.1% Triton X-100 containing 10% goat serum for 60 min at room temperature. Primary antibody was added at a ratio of 1:1000, and the slices were placed on a shaker at 4 ℃ overnight. The brain slices were washed 5 times with PBS for 5 min each. The secondary antibody was biotinylated at 1:200 in PBS and incubated for 1 h at room temperature. The brain slices were re-washed 3 times with PBS for 5 min each, and incubated with avidin-biotin-HRP complex (ABC) reagent for 1 h at room temperature; the reagent was prepared 30 min in advance. The brain slices were re-washed 3 more times with PBS for 5 min each. The color of the 3,3'-diaminobenzidine (DAB) solution was left to develop for about 5 min.

RT-qPCR

For the real-time quantitative polymerase chain reaction (RT-qPCR), total ribonucleic acid (RNA) was extracted from the hippocampal tissue with TRIzol lysis buffer and reverse-transcribed into complementary deoxyribonucleic acid (cDNA). The cDNA and primers were amplified according to the steps of the reverse transcription kit. The primer sequences (forward and reverse) are listed in *Table 1*. The relative quantitative analysis of the messenger RNA (mRNA) was carried out using the $2^{-\Delta\Delta CT}$ method (12).

FISH

For the fluorescence *in-situ* hybridization (FISH), the brain sections or primary microglia cell slides were fixed with 4% paraformaldehyde (PFA) (enuclease) for 20 min at room temperature, and then rinsed twice with ribozyme-removing PBS for 5 mins each time. The wells were punched in PBS containing 0.25% Triton X-100 treated with de-nuclease for 15 min and washed twice with 2×saline sodium citrate (SSC) buffer for 15 min each time. The brain slices or primary microglia were prehybridized with prehybridization solution for 1 h at 37 ℃. The probe was dissolved in the hybridization solution at a concentration of 10 nM, and the probe was denatured in a water bath at 65 ℃ for 5 min. The slides were incubated with probe-dissolved hybridization solution and hybridized overnight at 37 ℃. The slides were rinsed 3 times with 2×SSC at 42 ℃ for 20 mins each time. The slides were rinsed twice with 0.2× SSC at 42 ℃ for 20 min each time. The slides were blocked with enzymatic PBS containing 1% BSA and 3% normal goat serum (NGS) for 1 h at room temperature. The slides were incubated with horseradish peroxidase-conjugated anti-digoxigenin antibody overnight at 4 ℃, and then rinsed 3 times with 1× tris-buffered saline (TBS) for 10 min each time. The slides were incubated with Cy5 labeling reagent for 10 min at room temperature. After rinsing twice with PBS, the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining mounting solution and stored at 4 ℃ in the dark.

Western blot

The brain tissue was taken from mice, and the hippocampus was isolated. Total protein was extracted and quantified using the bicinchoninic acid (BCA) method. The samples

were loaded at 20 μg per well, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), membrane transfer, and blocking, respectively. The following primary antibodies were incubated overnight at 4 ℃: iNOS (1:5,000), NF-κB (1:3,000), NLRP3 (1:5,000), Caspase1 (1:500), IL-1β (1:500), and GAPDH (1:10,000). After washing each membrane 3 times with tris-buffered saline tween (TBST,) the samples were incubated with the secondary antibody at room temperature, and the membranes were re-washed 3 times. The expressions of inducible nitric oxide synthase (iNOS), NF-κB, nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), Caspase1, and interleukin-1beta (IL-1β) were compared to the GAPDH to obtain their relative expression levels.

CoIP

For the co-immunoprecipitation (CoIP) assays, the cells were lysed in immunoprecipitation buffer (Beyotime, Shanghai, China) with a cocktail of protease/phosphatase inhibitors (Beyotime, Shanghai, China). Next, the total proteins were incubated with anti-irisin or anti-immunoglobulin G overnight at 4 ℃. Subsequently, protein A/protein G-coated magnetic beads were used to capture protein complexes at 4 ℃ for 6 h. The immunoprecipitated proteins were examined by western blotting analysis after being washed in lysis buffer for 3 min.

ELISAs

For the enzyme-linked immunosorbent assays (ELISAs), the hippocampus of each mouse was collected, rinsed with 4 ℃ normal saline, and blotted dry with filter paper. The tissue was placed in a homogenizer, and after 9 times the volume of the normal saline was added for homogenization, the tissue was centrifuged at 3,000 rpm for 15 min, and the supernatant was stored at 4 ℃. The levels of irisin and 5-HT were measured according to the instructions of the ELISA kit (Wuhan Saipei Biotechnology Co., Ltd., irisin item no.: SP14895; 5-HT item no.: SP14055).

Statistical analysis

The mean ± standard error of the mean (SEM) are used to express the findings. The data were analyzed using GraphPad Prism 8.3. Comparisons between groups were performed using a 1-way analysis of variance and *t*-test. A P value <0.05 was considered statistically significant.

Results

Aerobic exercise improves neurological function and depressive-like behavioral performance in PSD mice

We examined the neurological changes in the mice (there were 12 mice per group; *Figure 1*). Aerobic exercise effectively alleviated the neurological damage caused by PSD (*Figure 1A*, P<0.01). The results of the glucose consumption testing are shown in Figure 1B. Compared to the sham group, the glucose water consumption of the mice in the PSD group was significantly reduced (P<0.05). Compared to the PSD group, aerobic exercise significantly increased the glucose water consumption of the PSD mice (P<0.05). The results of the OFT experiment are shown in *Figure 1C,1D*. Compared to the sham group, the horizontal and vertical movement counts of the mice in the PSD group were significantly reduced (P<0.01). Compared to the PSD group, aerobic exercise significantly increased the counts of the horizontal and vertical movements of the PSD mice (P<0.05). The results of the Morris water-maze test are shown in *Figure 1E*. Compared to the sham group, the total route distance (*Figure 1F*), the duration (*Figure 1G*), the route near the platform (*Figure 1H*), the route far from the platform (Figure 1I), and the distance ratio of the near (near the platform) to far (far from the platform) (*Figure 1J*) of the mice in the PSD group were significantly increased (P<0.05). Compared to the mice in the PSD group, aerobic exercise significantly reduced the total route distance, the duration, the route near the platform, the route far from the platform, and the distance ratio of the near (near the platform) to far (far from the platform) (*Figure 1E-1J*, P<0.05).

Aerobic exercise increases serum and hippocampal microglia levels of irisin

Studies have shown that aerobic exercise training increases the level of irisin, improves cognitive function (15-19), and participates in the pathological process of depression. However, the specific mechanism of irisin in PSD is still unclear. To investigate whether irisin participates in the effects of aerobic exercise on neurological function and depressive-like behavioral performance in PSD mice, we detected the irisin levels in serum and hippocampal microglia of the mice in each group by ELISAs and immunofluorescence staining, respectively. Compared

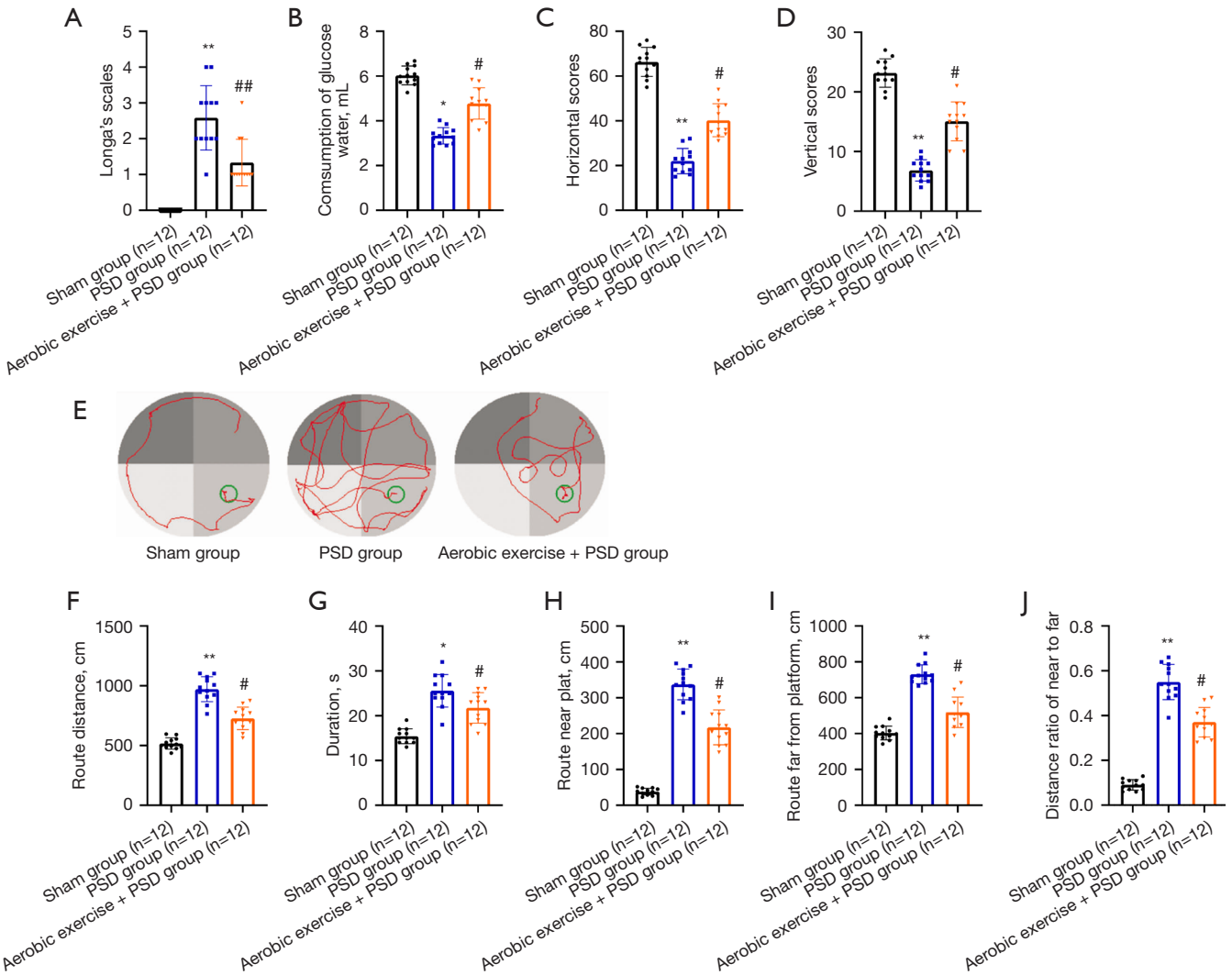

Figure 1 Aerobic exercise improves neurological function and depressive-like behavioral performance in PSD mice. (A) Longa's scale. (B) Glucose consumption testing. (C,D) OFT experiments. (E) Morris water-maze test. (F) Total route distance. (G) Duration. (H) Route near the platform. (I) Route far from the platform. (J) The distance ratio of the near (near the platform) to far (far from the platform). *, compared to the Sham group, P<0.05; **, compared to the Sham group, P<0.01; #, compared to the PSD group, P<0.05; ##, compared to the PSD, P<0.01. PSD, post-stroke depression; OFT, open-field test.

to the sham group, the serum irisin of the mice in the PSD group was significantly reduced (P<0.01, *Figure 2A*). Compared to the PSD group, aerobic exercise significantly increased the serum irisin level in the PSD mice (P<0.05, *Figure 2A*).

Immunofluorescence staining was used to examine the co-localization of irisin with microglia in the hippocampus to determine the role of irisin in the pathogenesis of depression. We found that irisin was widely expressed in the hippocampus and co-localized with cells positive for the microglia-specific protein Iba-1 (*Figure 2B*). Compared to the sham group, the number of irisin-positive cells in the microglia in the hippocampus of the mice in the PSD group was significantly reduced (P<0.01, *Figure 2C*). Compared to the PSD group, aerobic exercise significantly increased the number of irisin-positive cells in microglia in the hippocampus of the PSD mice (P<0.05, *Figure 2C*). Notably, we found a good correlation between serum irisin and the number of irisin-positive microglia in the hippocampus (P<0.05, *Figure 2D-2F*). Our findings suggest

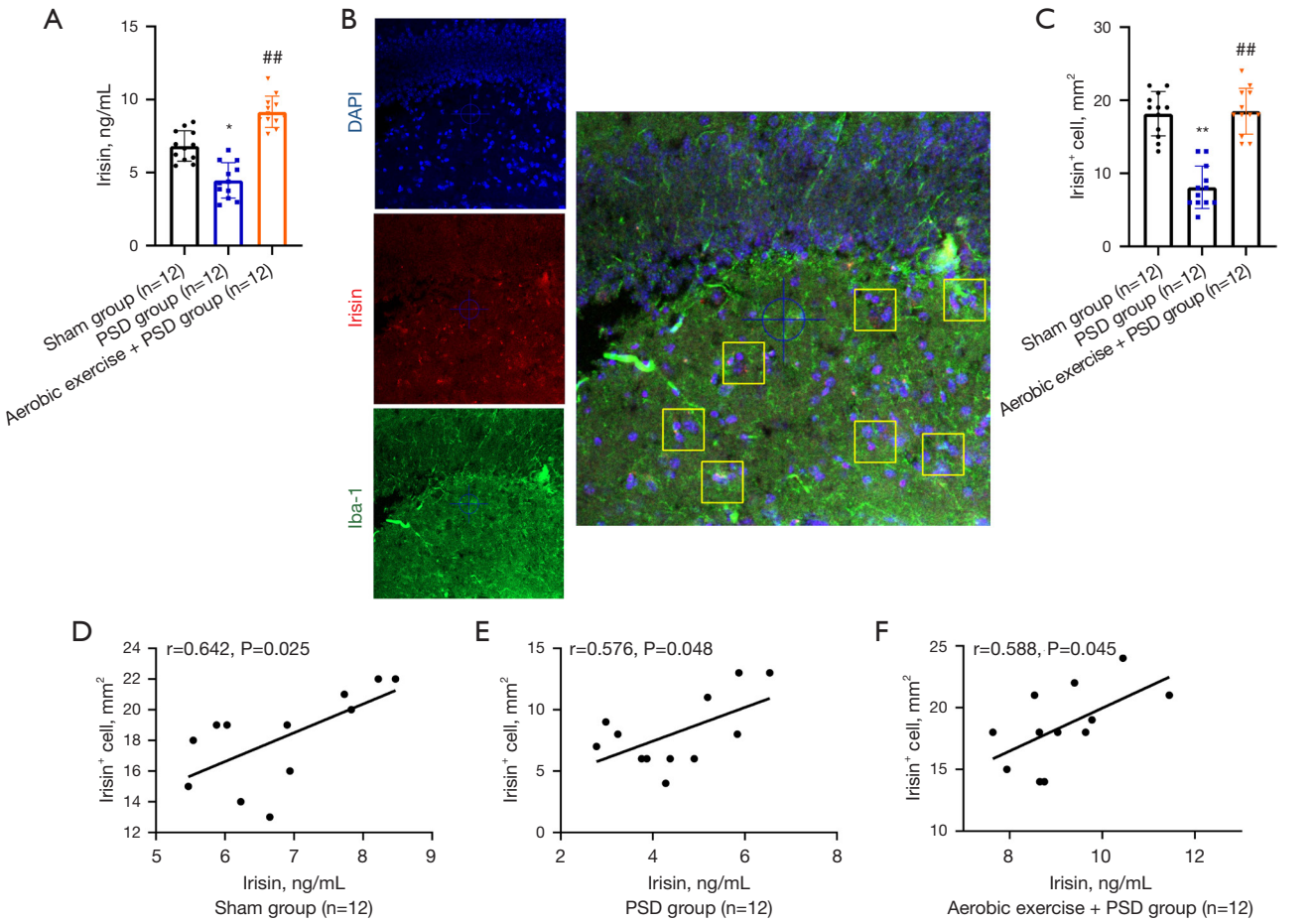

Figure 2 Aerobic exercise increases the serum and hippocampal microglia levels of irisin. (A) Compared to the sham group, the serum irisin of the mice of the PSD group was significantly reduced, while aerobic exercise significantly increased the serum irisin level in the PSD mice. (B) Irisin was widely expressed in the hippocampus and co-localized with cells positive for the microglia-specific protein Iba-1. Immunofluorescence staining (×400). Yellow boxes indicate co-location of Irisin and Iba-1. (C) Compared to the sham group, the number of irisin-positive cells in the microglia in the hippocampus of the mice in the PSD group was significantly reduced, while aerobic exercise significantly increased the number of irisin-positive cells in microglia in the hippocampus of the PSD mice. (D-F) Correlation between serum irisin and the number of hippocampal irisin-positive microglia in 3 groups of mice. *, compared to the Sham group, P<0.05; **, compared to the Sham group, P<0.01; ##, compared to the PSD, P<0.01. PSD, post-stroke depression; DAPI, 4',6-Diamidino-2'-phenylindole.

that aerobic exercise may be involved in the regulation of PSD by increasing irisin levels.

Further, we separately analyzed the correlation between the number of irisin-positive microglia in the hippocampus and neurological function and depression-like behavioral performance in the PSD model mice. We found that the number of irisin-positive microglia in the hippocampus of the PSD model mice was significantly positively correlated with neurological function and depression-like behavioral performance; that is, the higher the number of irisin-positive cells, the better the performance of neurological function and depression-like behavior (*Figure 3*).

Overexpression of irisin significantly improves depression-like behavior in PSD mice

To further verify the effect of irisin, we constructed irisin-overexpressing AAV8-irisin-GFP and transfected AAV8-irisin-GFP on BV-2 and primary microglia *in vitro*. RT-qPCR and immunofluorescence staining showed that

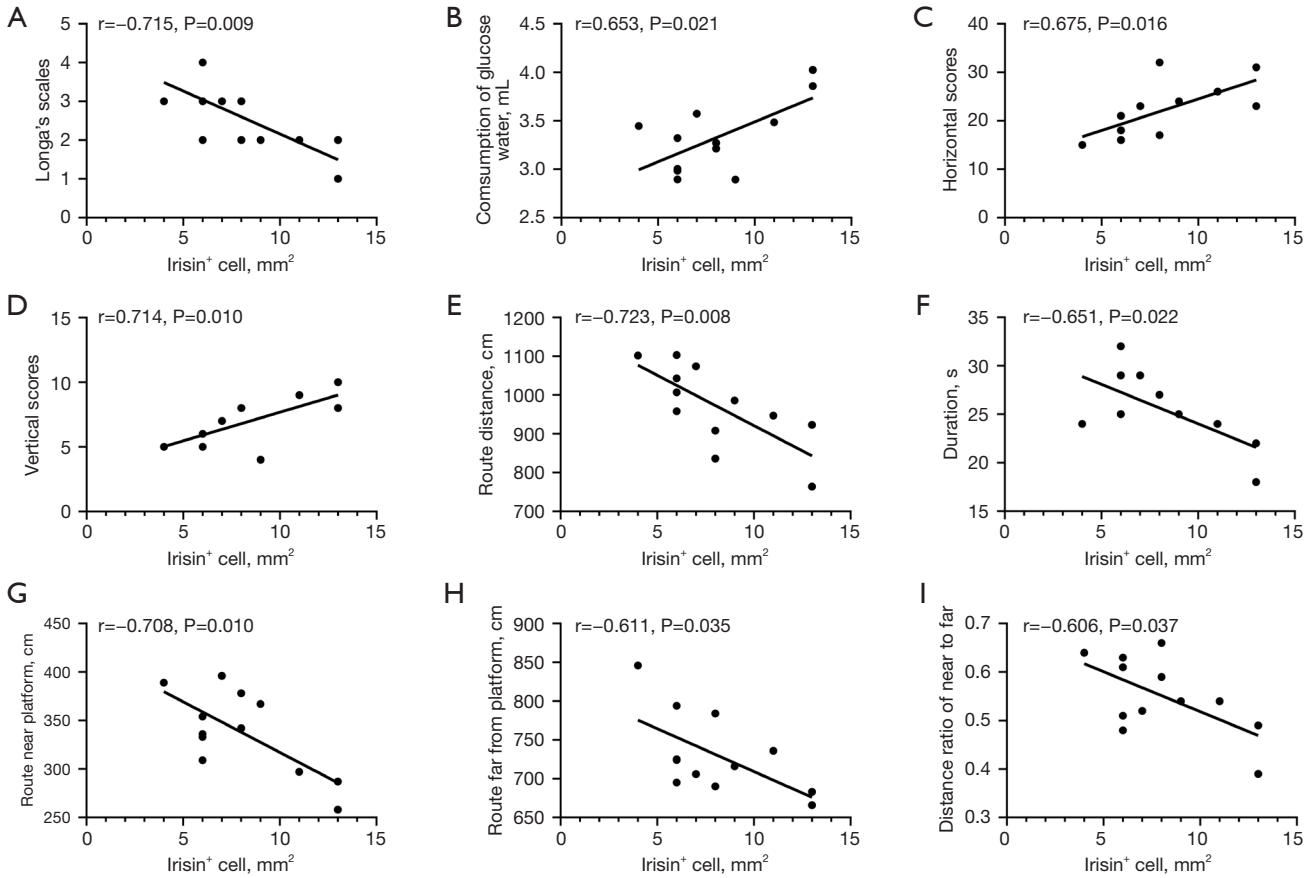

Figure 3 Correlation between the number of irisin-positive microglia in the hippocampus and neurological function and depression-like behavioral performance in the PSD model mice. (A) Correlation between the number of irisin-positive microglia and Longa's scale. (B) Correlation between the number of irisin-positive microglia and glucose water consumption. (C) Correlation between the number of irisin-positive microglia and horizontal movement. (D) Correlation between the number of irisin-positive microglia and vertical movement. (E) Correlation between the number of irisin-positive microglia and total route distance. (F) Correlation between the number of irisin-positive microglia and duration. (G) Correlation between the number of irisin-positive microglia and the route near the platform. (H) Correlation between the number of irisin-positive microglia and the route far from the platform. (I) Correlation between the number of irisin-positive microglia and the distance ratio of the near (near the platform) to far (far from the platform). PSD, post-stroke depression.

AAV8-irisin-GFP significantly upregulated the expression of irisin (*Figure 4A*,*4B*). To verify the efficacy of AAV8-irisin-GFP transduction *in vivo*, we microinjected AAV8-irisin-GFP into the mouse hippocampus. We found that GFP was widely expressed in the hippocampus and that there was a certain number of Iba-1-positive cells that co-localized with GFP (*Figure 4C*). Additionally, compared to the mice injected with AAV8-control-GFP, the RT-qPCR detection revealed that the expression of irisin was significantly increased in the mice injected with AAV8-irisin-GFP (*Figure 4D*).

We validated the effect of irisin *in vivo* by the brain microinjection of AAV8-control-GFP or AAV8-irisin-GFP into the hippocampus of the C57BL/6 mice. We examined the role of irisin in the pathogenesis of depression by monitoring neurological function and depressive-like behavioral performance in mice. A week after AAV microinjection, the mice were exposed to the MCAO+CUMS regimen for 5 weeks, and then underwent depressive-like behavioral testing (*Figure 5A*). We found that the overexpression of irisin significantly improved neurological function and depressive-like behavioral performance induced by MCAO + CUMS treatment compared to the AAV8-control-GFP (*Figure 5B-5J*). Our

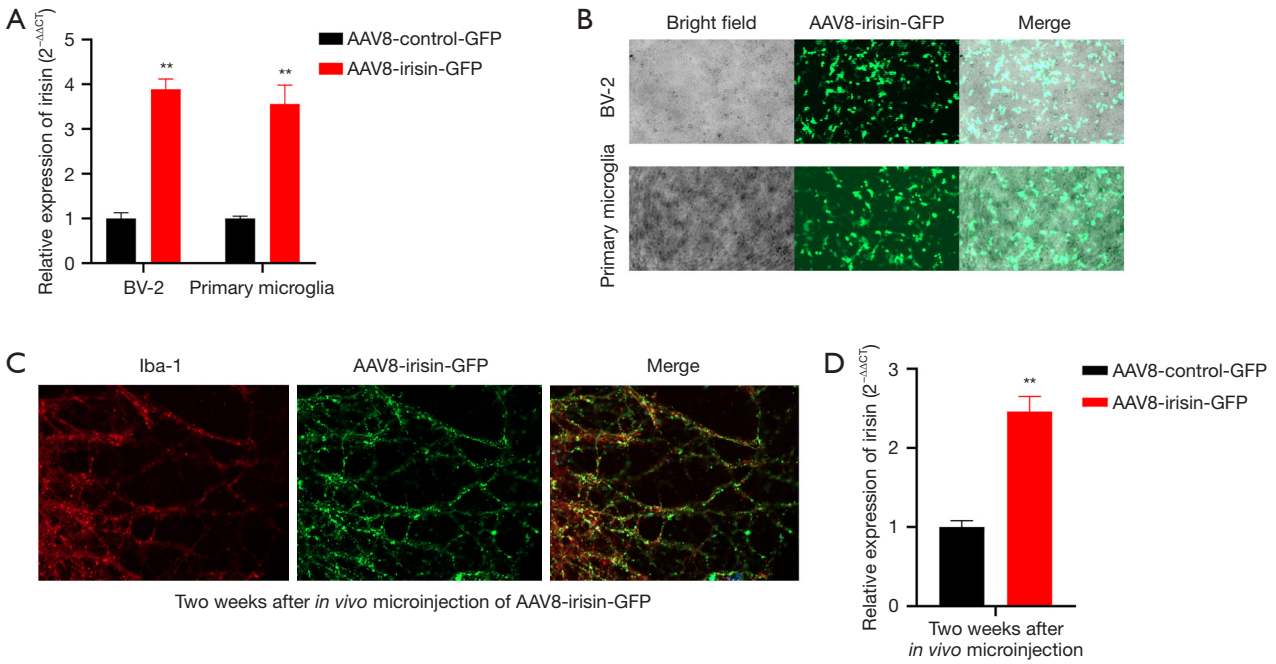

Figure 4 Construction and validation of AAV8-irisin-GFP overexpressing irisin. (A) RT-qPCR showed that AAV8-irisin-GFP significantly upregulated the expression of irisin. (B) Immunofluorescence staining showed that AAV8-irisin-green fluorescent protein significantly upregulated the expression of irisin. Immunofluorescence staining (×200). (C) AAV8-irisin-GFP was widely expressed in the hippocampus. Immunofluorescence staining (×200). (D) RT-qPCR detection showed that the expression of irisin was significantly increased in the mice injected with AAV8-irisin-GFP. **, compared to the AAV8-control-GFP group, P<0.01. RT-qPCR, real time-quantitative polymerase chain reaction.

results suggest that the overexpression of irisin improves depression-like behavior in PSD mice.

Overexpression of irisin in PSD model mice inhibits microglial activation in the hippocampus

The overexpression of irisin improves depression-like behavior in PSD mice. The relationship between irisin and neuroinflammation has been reported in the literature; however, the underlying mechanism required further exploration. We detected the expression levels of iNOS in the hippocampus of the mice treated with MCAO + CUMS after brain microinjection of AAV8-irisin-GFP. The expression of iNOS, a marker of microglial activation, was detected by Western blot. We found that compared to the AAV8-control-GFP, the overexpression of irisin significantly inhibited the increase in the expression of iNOS in the hippocampus of the PSD model mice (*Figure 6A,6B*).

Irisin interacts with NF-κB

To investigate the mechanisms of irisin, we first used subcellular fractionation location assays, which revealed a significant increase in irisin expression in the nucleus compared to the cytosol (*Figure 7A*), which suggests that irisin may play a key regulatory role on the transcriptional level by interacting with nucleus molecules or proteins. We then conducted a proteomic analysis to examine the irisin-associated protein complex in the BV2 cells. The protein complex of irisin was isolated, and mass spectrometry was then used to identify the proteins, and NF-κB was identified among the highly enriched proteins (*Figure 7B*). Further, the localization of irisin and NF-κB in the BV-2 and primary microglia cells was verified by. The confocal results indicated that irisin and NF-κB were mainly positioned in the nucleus of the BV-2 and primary microglia cells (*Figure 7C*). Additionally, the Co-IP analysis confirmed the interaction between irisin and NF-κB (*Figure 7D*).

Blockade of NF-κB attenuates NLRP3 inflammasome activation

Previous results have shown that aerobic exercise inhibits

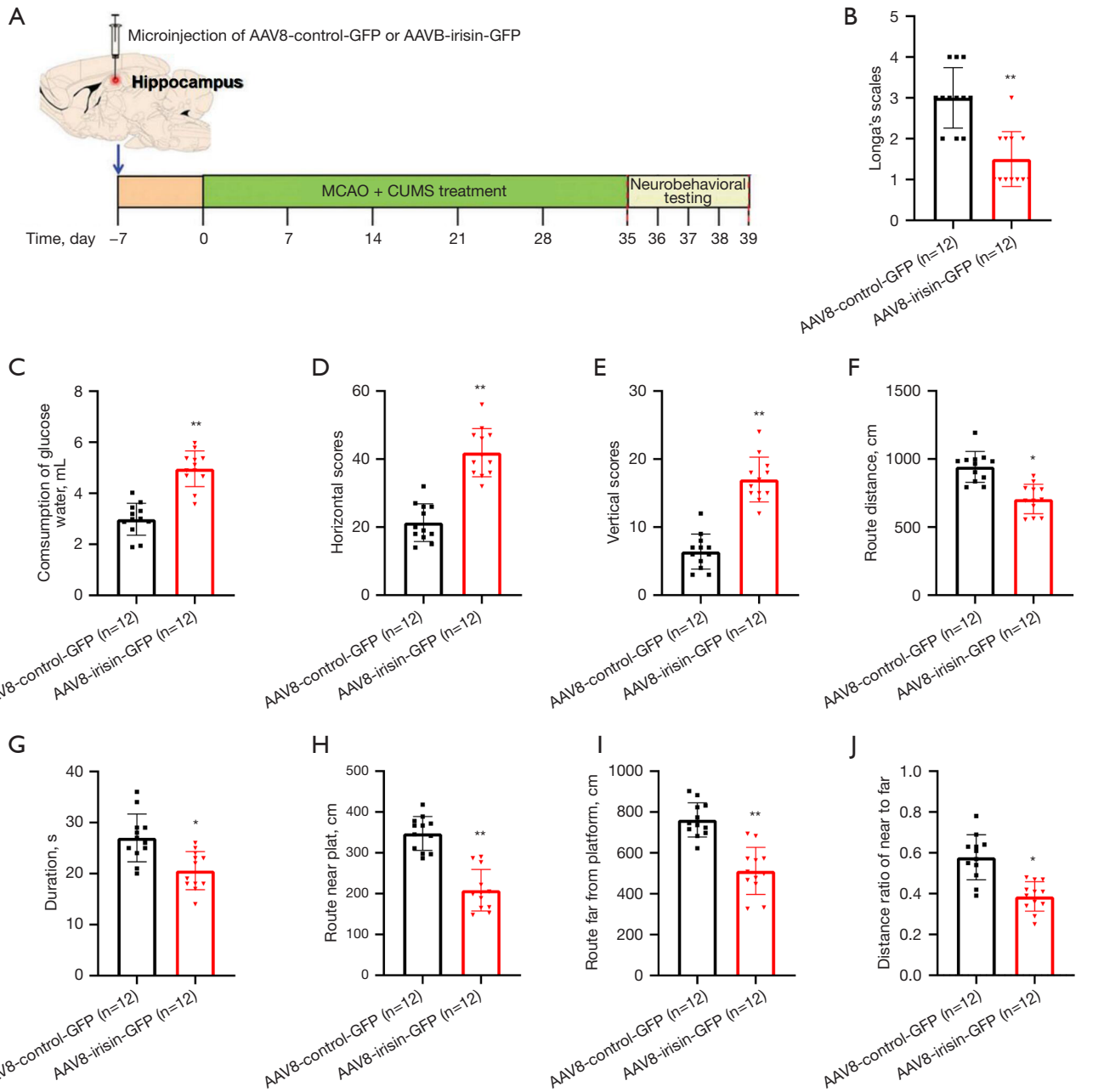

Figure 5 AAV8-irisin-GFP microinjection and validation. (A) Experimental design flow chart. (B) Longa's scale. (C) Glucose consumption testing. (D,E) OFT experiment. (F) Total route distance. (G) Duration. (H) Route near the platform. (I) Route far from the platform. (J) The distance ratio of the near (near the platform) to far (far from the platform). *, compared to the AAV8-control-GFP group, P<0.05; **, compared to the AAV8-control-GFP group, P<0.01. OFT, open-field test; MCAO, middle cerebral artery occlusion; CUMS, chronic unpredictable mild stress.

NF-κB by increasing irisin, and the accumulation of NF-κB is an important part of inflammatory activation (16,17). The NLRP3 inflammasome is a key inflammasome involved in multiple inflammatory responses, and its activation is associated with stress and depression (8,9). BAY 11-7082 is an NF-κB-targeted antagonist that inhibits NF-κB production and accumulation (18). In the present study, we examined changes in the NLRP3 inflammasome

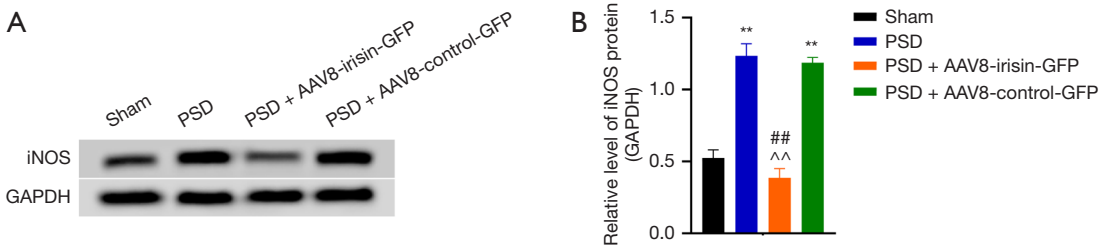

Figure 6 The overexpression of irisin in the PSD model mice inhibits microglial activation in the hippocampus. (A–B) Compared to the AAV8-control-GFP, the overexpression of irisin significantly inhibited the increase in the expression of iNOS in the hippocampus of the PSD model mice. **, compared to the Sham group, P<0.01; ##, compared to the PSD group, P<0.01; ^^, compared to the PSD+AAV8-control-GFP group, P<0.01. PSD, post-stroke depression; iNOS, inducible nitric oxide synthase; GFP, green fluorescent protein.

in the hippocampus of the PSD mice treated with BAY 11-7082 (3 mg/kg/day, subcutaneous injection for 28 days; *Figure 8A*). Compared to the PSD group, the mRNA (P<0.01, *Figure 8B*) and protein (P<0.01, *Figure 8C*) levels of NLRP3, Caspase1, and IL-1β in the hippocampus of the mice in the PSD group treated with BAY 11-7082 were significantly decreased.

Overexpression of irisin promotes 5-HT levels by reversing the NF-κB/NLRP3 inflammasome

As a neurotransmitter, 5-HT is closely related to the occurrence of PSD. After a stroke, the 5-HT pathway in the brain is disrupted, resulting in a decrease in 5-HT levels and activity in the body, which leads to depression. In this study, we further explored the effects and potential connections of aerobic exercise and/or the overexpression of irisin on 5-HT in the brain. Compared to the PSD group, the mice in the PSD + aerobic exercise group or PSD + irisin overexpression group or PSD+BAY 11-7082 group had significantly increased hippocampal irisin (P<0.05, Figure S1), 5-HT (P<0.05, *Figure 9A*), neurological function (*Figure 9B*) and depressive-like behavioral performance (*Figure 9C-9F*).

Discussion

PSD is considered a common psychiatric disorder after stroke; however, its pathogenesis is still unclear, and its treatment is also difficult (6). The activation of microglia is thought to be an important step leading to various neurological diseases, including depression (19). Classically activated M1-type microglia are pro-inflammatory and can cause neuronal damage; however, alternatively activated M2-type microglia are phagocytic, anti-inflammatory, and neurotrophic (19-21). After the body is stimulated by stress, the peripheral immune system induces the transport of cytokines to the central nervous system and stimulates the activation of microglia (20). Conversely, activated microglia secrete various pro-inflammatory cytokines and other inflammatory mediators that cause damage to neighboring neurons (17). Microglia regulate cytokine production and secretion and eliminate accumulated and invading pathogens, which are key to optimal immune function (22).

Evidence shows that the mechanism by which aerobic exercise improves PSD is related to improvements in cerebral blood flow, the regulation of the expression of apoptotic genes, resistance to free radical damage, the reduction of the immune inflammatory response, the reduction of excitatory amino acid toxicity, the regulation of the expression of NF-κB, and the promotion of nerve cell repair (23,24). At present, studies suggest that the pathogenesis of PSD is closely related to the atrophy and necrosis of neurons in the hippocampus. Long-term stress stimuli can cause the functional and structural defects of neural networks in this region, and synaptic transmission disorders, leading to the occurrence of emotional and cognitive abnormalities in depression (25). There is increasing evidence that the hippocampal CA1 area is closely related to the stress response, and this area is considered an important brain area for the prevention and treatment of depression (26).

CUMS is the exposure of animals to mild and unpredictable external stimuli that induce behavioral changes similar to clinical depression, such as decreased exercise capacity, decreased sucrose intake, decreased learning and memory, and delayed responsiveness to stimuli. Thus, it is widely used to establish animal depression models (16).

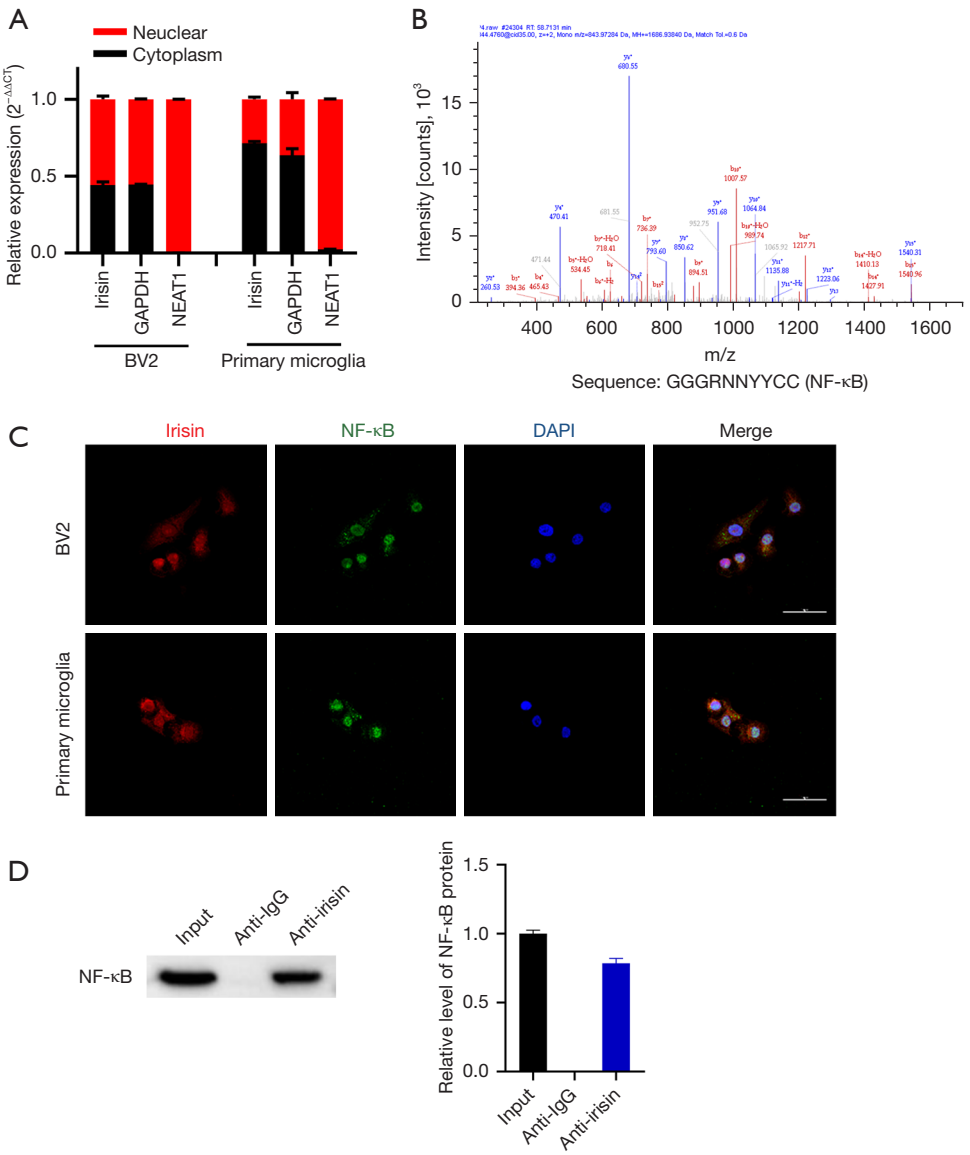

Figure 7 Irisin interacts with NF-κB. (A) A subcellular fractional localization analysis revealed that irisin expression was significantly increased in the nucleus compared to the cytosol. (B) The mass spectrum results indicated that irisin interacts with NF-κB. (C) A FISH experiment was conducted to verify the expression localization of irisin and NF-κB. FISH (×400). (D) CoIP assays verified the interaction between irisin and NF-κB. NF-κB, nuclear factor-kappaB; FISH, fluorescence in-situ hybridization; CoIP, co-immunoprecipitation; DAPI, 4',6-Diamidino-2'-phenylindole.

PSD is often accompanied by damage to neurons in the brain, resulting in a reduction or even loss of the ability to experience pleasure. The mouse model shows the inhibition of their sweet appetites, so glucose water consumption is often used as an important indicator to measure the degree of anhedonia (27). The OFT reflects an animal's exploration of a new environment and emotional changes. The horizontal activities mainly reflect an animal's emotional changes, while the vertical activities reflect an animal's desire to explore (20). As a classic behavioral evaluation method, the Morris water-maze test comprehensively evaluates animal learning, memory, and observation ability, and thus is widely used in the evaluation of neurological function in various neurological diseases (28).

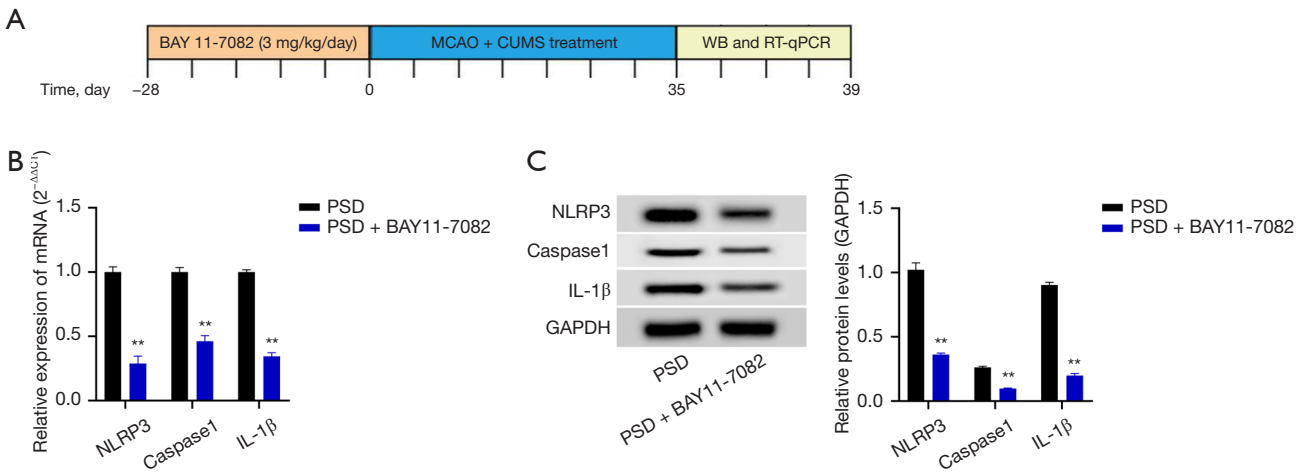

Figure 8 The blockade of NF-κB attenuates NLRP3 inflammasome activation. (A) Experimental design flow chart. (B) Compared to the PSD group, the mRNA levels of NLRP3, Caspase1, and IL-1β in the hippocampus of mice in the PSD group treated with BAY 11-7082 were significantly decreased. (C) Compared to the PSD group, the protein levels of NLRP3, caspase1, and IL-1β in the hippocampus of the mice in the PSD group treated with BAY 11-7082 were significantly decreased. **, compared to the PSD group, P<0.01. NF-κB, nuclear factor-kappaB; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; PSD, post-stroke depression; MCAO, middle cerebral artery occlusion; CUMS, chronic unpredictable mild stress; WB, western blot; RT-qPCR, real time-quantitative polymerase chain reaction.

In this experiment, we found that aerobic exercise significantly increased the glucose water consumption of PSD mice, increased the horizontal and vertical movement counts, and reduced the total route distance, duration, the route near the platform, the route far from the platform, and the distance ratio of the near (near the platform) to far (far from the platform). . These results suggest that aerobic exercise not only improves the emotional feedback of PSD mice to external environmental stimuli and enhances the animals' interest in spatial exploration, but also increases the learning and memory ability of depressed mice. In the Morris water-maze test, the trajectory distance in the far-platform area was not shortened, but the distance in the near-platform area was shortened, which suggests that aerobic exercise was more successful at restoring memory ability.

Microglial lesions have been reported to lead to depression, and drugs that restore the function of these cells hold promise as fast-acting antidepressants. A previous study and magnetic resonance imaging techniques have shown that depression occurs when the shape and function of microglia changes in depressed animal models. Additionally, long-term exposure to chronic, unpredictable psychological stress can also lead to changes in the shape and function of microglia, which also contribute to depression (27). Neun was discovered in mouse brain nuclei by Depping *et al.* and is widely used as a marker for mature neurons in vertebrates (28).

We found that aerobic exercise significantly decreased microglial activation in PSD mice, which suggests that aerobic exercise protects nerve cell function. The present study also found that the neurite length and density of hippocampal neuronal precursor cells, which are the basis of neuronal regeneration, were reduced after PSD treatment. Thus, the inhibition of neuronal regeneration in PSD mice appears to be the fundamental reason for the decline of learning and memory performance in PSD mice. After aerobic exercise treatment in PSD mice, the neurite length and density of hippocampal neuron precursor cells increased, indicating that aerobic exercise promotes the regeneration of neurons in the hippocampus of PSD mice.

NLRP3 inflammasome initiation is required to complete the synthesis of the NLRP3 inflammasome protein, caspase-1 precursor protein, and IL-1β precursor protein. Among them, the NF-κB pathway is a key signaling pathway involved in the initiation of inflammasomes (11). A previous study has shown that irisin inhibits inflammation by inhibiting the NF-κB signaling pathway after physical exercise (29). It was suggested that aerobic exercise may

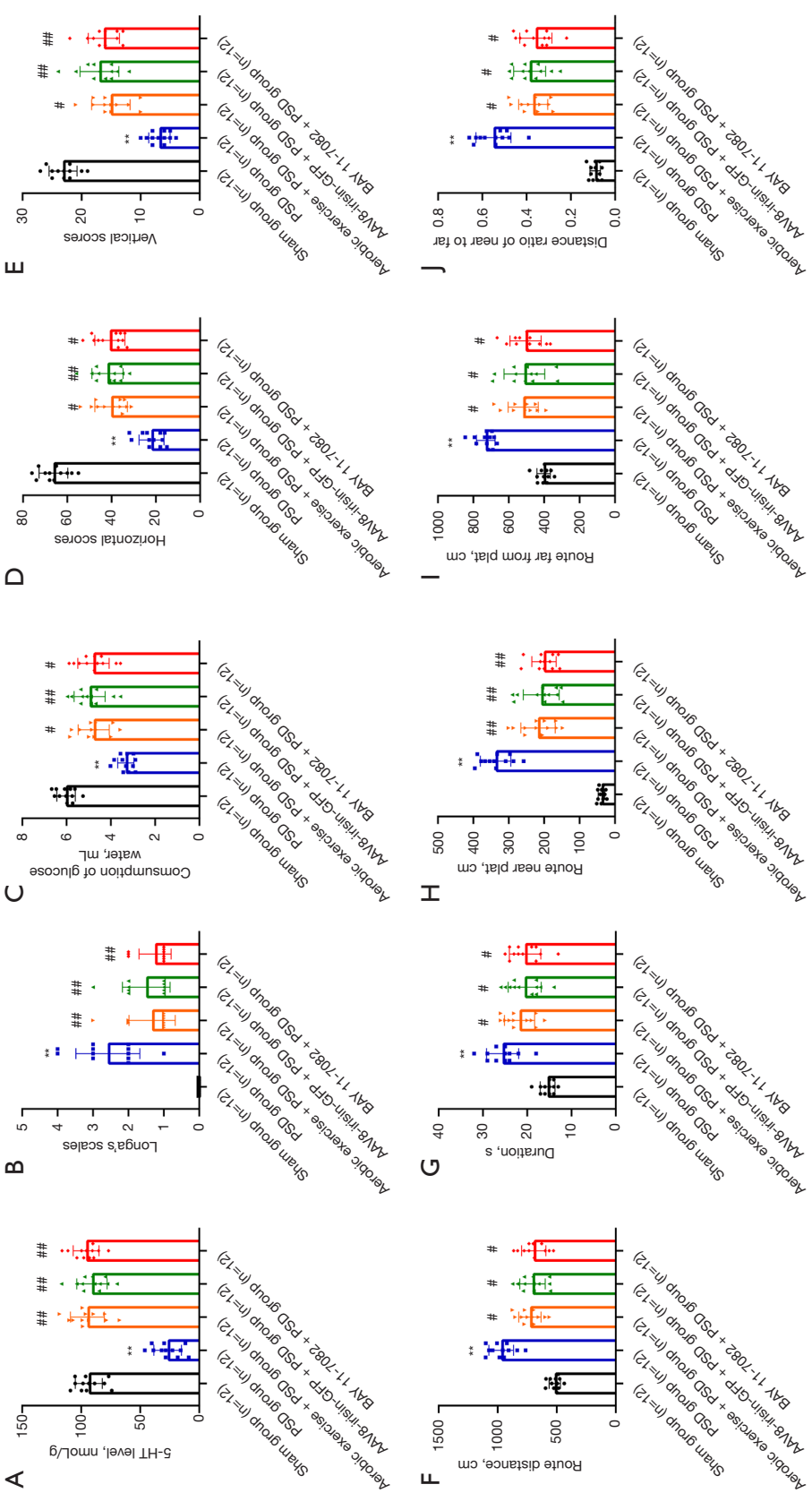

Figure 9 Overexpression of irisin promotes 5-HT levels by reversing the NF-κB/NLRP3 inflammasome. (A) 5-HT levels. (B) Longa's scale. (C) Glucose consumption testing. (D-E) OFT experiment. (F) Total route distance. (G) Duration. (H) Route near the platform. (I) Route far from the platform. (J) The distance ratio of the near (near the platform) to far (far from the platform). **, compared to the Sham group, P<0.01; #, compared to the PSD group, P<0.05; ##, compared to the PSD, P<0.01. 5-HT, 5-hydroxytryptamine; NF-κB, nuclear factor-kappaB; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; PSD, post-stroke depression; OFT, open-field test.

inhibit the activation of the NLRP3 inflammasome by upregulating irisin. NLRP3 inflammasome activation can be induced by a variety of stimuli.

Mitochondria are the energy factories in cells and the central link in the initiation or development of various diseases. Calcium overload caused by cellular Ca influx under stress has been shown to induce mitochondrial damage, increase ROS generation, and promote K^+ efflux (30-32), which suggests that mitochondrial damage may be the key to NLRP3 inflammasome activation. Studies have shown that irisin inhibits Ca^{2+} influx (33) and reduces mitochondrial damage (34), which suggests that aerobic exercise may inhibit the progression of PSD by inhibiting inflammasome activation. We found that aerobic exercise significantly reduced the expression of NLRP3, Caspase1, and IL-1β, which suggests that aerobic exercise plays a key role in inhibiting the activation of the NLRP3 inflammasome, which may be the mechanism underlying the anti-PSD effect.

Conclusions

In conclusion, this study found that aerobic exercise reversed the NF-κB/NLRP3 inflammasome/5-HT pathway by upregulating irisin expression to alleviate PSD.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5443/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5443/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5443/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (2019-S90) granted by committee of The First Affiliated Hospital of Hunan Normal University, in compliance with institutional guidelines for the care and use of animals.

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Supplementary

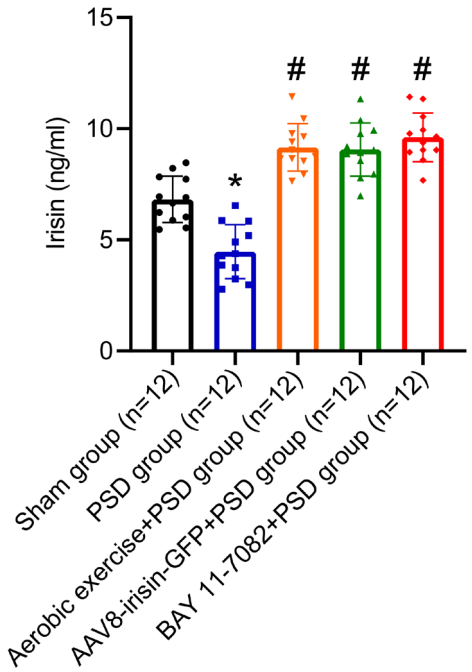

Figure S1 Hippocampal irisin levels in different groups. *, Compared to the Sham group, P<0.05; #, Compared to the PSD group, P<0.05.

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