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ORIGINAL RESEARCH

Downregulation of the HCN1 Channel Alleviates Anxiety- and Depression-Like Behaviors in Mice With Cerebral Ischemia–Reperfusion Injury by Suppressing the NLRP3 Inflammasome

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BACKGROUND: Post-stroke depression (PSD) is a prevalent neuropsychiatric complication of stroke. However, the mechanisms underlying PSD are still unclear. Here, we aimed to investigate the role of HCN1 (hyperpolarization-activated cyclic nucleotide-gated cation channel 1) in the pathogenesis of PSD and its underlying mechanisms.

METHODS: The PSD mice model was established by middle cerebral artery occlusion in vivo. Four weeks after middle cerebral artery occlusion, anxiety- and depression-like behaviors of mice were evaluated by various behavioral tests. HCN channels were downregulated by pharmacological inhibitor or neuron-specific adeno-associated virus. The oxygen–glucose deprivation/reoxygenation model in SY5Y cells was used to study the pathogenesis of PSD in vitro.

RESULTS: Mice exhibited anxiety- and depression-like behavior 4 weeks after middle cerebral artery occlusion, along with a significant increase in HCN1 protein expression in the ischemic hippocampus. Furthermore, the I_h current on neurons in the hippocampus was notably enhanced, whereas neuronal excitability was decreased in PSD mice. Treatment with HCN channel selective inhibitor ZD7288 protected SY5Y cells against oxygen–glucose deprivation/reoxygenation injury by suppressing K^+ efflux. Additionally, we observed a significant increase in protein expressions of NLRP3 (nucleotide-binding domain-like receptor protein 3) inflammasome pathway-related molecules in the ischemic hippocampus of PSD mice. Knockdown of HCN1 channels via virus injection into the hippocampus resulted in decreased protein expressions of NLRP3 inflammasome-related molecules and improvement in anxiety- and depression-like behaviors in PSD mice.

CONCLUSIONS: Downregulation of HCN1 channels has a beneficial effect on PSD by suppressing the NLRP3 inflammasome pathway, thus offering promise as a strategy for preventing and treating PSD.

Key Words: cerebral ischemia/reperfusion injury ■ depression ■ HCN channels ■ hippocampus ■ NLRP3 inflammasome

Stroke is recognized as one of the leading causes of death and disability worldwide, characterized by its high prevalence, mortality, and morbidity rates.[1] However, only a portion of patients with ischemic stroke can be treated with thrombolytic therapy or interventional therapy in the acute phase. Most

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RESEARCH PERSPECTIVE

What Is New?

- Using an experimental mouse model, we found that the HCN1 (hyperpolarization-activated cyclic nucleotide-gated cation channel 1) channel was significantly upregulated in the ipsilateral hippocampus of mice exhibiting anxiety- and depression-like behaviors induced by stroke.
- Pharmacological inhibition or genetic knockdown of the HCN1 channel in neurons could downregulate the NLRP3 (nucleotide-binding domain-like receptor protein 3) inflammasome pathway and improve anxiety- and depression-like behaviors in post-stroke depression mice.

What Question Should Be Addressed Next?

- This study unveils a novel mechanism of HCN1 channels contributing to post-stroke depression, and targeting HCN1 channels emerges as a potential strategy for improving depressive affective disorders in patients with stroke.

Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus
ASC	apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
DG	dentate gyrus
GSDMD	gasdermin D
HCN	hyperpolarization-activated cyclic nucleotide-gated cation channel
MCAO	middle cerebral artery occlusion
NLRP3	nucleotide-binding domain-like receptor protein 3
OGD/R	oxygen–glucose deprivation/reoxygenation
PSD	poststroke depression

survivors will experience neurological dysfunction such as cognitive impairment and affective disorders.[2] Poststroke depression (PSD) is a common neuropsychiatric complication after stroke, with an incidence in stroke survivors ranging from 30% to 60%.[3] The primary clinical manifestations of PSD include insomnia, anxiety, feelings of hopelessness, self-blame, social dysfunction, and even suicidal behavior. The presence of PSD in stroke survivors prolongs the recovery period for patients, reduces their quality of life, and places a heavy burden on both patients and society.[4] However, current pharmacological regimens for treating PSD using antidepressant drugs targeting neurotransmitters and nonpharmacological regimens such as psychotherapy are often unsatisfactory and may lead to undesirable side effects.[5] The pathophysiology of PSD remains largely unknown, hindering the development of novel therapeutic strategies. Therefore, it is crucial to explore the pathogenesis of PSD and identify specific therapeutic targets to promote rehabilitation for patients with PSD.

The HCN (hyperpolarization-activated cyclic nucleotide-gated cation channel) is a specialized type of voltage-gated ion channel that becomes activated during membrane potential hyperpolarization.[6] The HCN subfamily is primarily expressed in the heart and neurons, encoded by a family of 4 genes (*HCN*1–4), and they are responsible for generating the funny current (I_h), which exhibits mixed permeability to Na^+ and K^+.[7] The HCN channel plays a crucial role in regulating neuronal rhythm, transmitter release, synaptic plasticity, and other physiological processes. Its dysfunction is closely associated with the pathological mechanisms of various nervous system diseases.[8] Accumulating evidence suggests that the HCN channel is an important target for intervention in stroke treatment.[9] HCN inhibitors have been shown to suppress hyperautophagy induced by cerebral ischemia by promoting autophagosome-lysosome fusion, providing neuroprotection against hippocampal neuron damage in ischemic rats.[10] Furthermore, blockade of HCN channels has been found to improve cognitive dysfunction induced by cerebral ischemia in rats by reducing apoptosis of hippocampal neurons.[11] Our previous study also found that increased protein expression of the HCN1 channel in the amygdala was related to anxiety-like symptoms induced by chronic cerebral hypoperfusion in rats.[12] These studies collectively suggest that inhibiting HCN channels may have beneficial effects on brain injury caused by stroke. However, the role of HCN channels in the pathogenesis of poststroke depression remains unclear.

The activation of HCN channels has been shown to result in the outflow of intracellular K^+.[13] However, decreased intracellular K^+ concentration will trigger abnormal changes in cell function.[14,15] Recent studies have indicated that reduced intracellular K^+ concentration may activate the inflammatory pathway, such as the NLRP3 (nucleotide-binding domain-like receptor protein 3) inflammasome.[16] NLRP3 is a receptor protein that recognizes pathogenic and injury-related molecular patterns. It can form a complex with ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain) and pro-caspase-1 to generate activated caspase-1.[17] Activated caspase-1 acts as an effector agent by cleaving proteins and processing pro-IL (interleukin)-1β and pro-IL-18 into their mature forms IL-1β and IL-18, respectively, thereby leading to inflammatory responses and cell damage.[18] Accumulating evidence

suggests that inhibition of NLRP3-mediated inflammatory response can alleviate ischemic neuronal injury.[19] Exercise has been found to downregulate the expression of TLR4 (toll-like receptor 4)/NF-κB (nuclear factor kappa B)/NLRP3 protein in the hippocampus and improve PSD-like behavior in mice.[20] *Morinda officinalis* oligosaccharides have been shown to alleviate depression-like behavior in ischemic stroke rats by inhibiting the NLRP3 inflammasome and NF-κB pathway in the hippocampus.[21] Additionally, lithium has been found to inhibit the NLRP3 inflammasome by reducing reactive oxygen species production, thus improving depression-like behavior in stroke mice.[22] These studies suggest that the NLRP3 inflammasome plays an essential role in the pathogenesis of PSD. The NLRP3 inflammasome could be activated through several processes, including decreased intracellular K^+ concentration, intracellular Ca^{2+} accumulation, and reactive oxygen species production.[23] Given that opening HCN channels can cause K^+ efflux, it is hypothesized that HCN channels may regulate the activation of the NLRP3 inflammasome. However, there are no reports on the regulatory effect of HCN on NLRP3 inflammasome in the existing literature.

In the current study, we aimed to investigate the regulatory effect of HCN on the NLRP3 inflammasome and its role in the development of PSD. Using middle cerebral artery occlusion (MCAO) surgery in mice, which serves as a suitable model to study PSD induced by cerebral ischemia–reperfusion, we first examined anxiety- and depression-like behavior in mice after stroke. Additionally, we detected changes in the expression and function of the HCN channel and NLRP3 inflammasome pathway in the hippocampal region of PSD mice. Furthermore, we sought to elucidate the protective effect of HCN1 knockdown by virus injection on the anxiety and depression behavior of PSD mice and its underlying mechanisms. Considering the regulatory effect of the HCN channel on intracellular K^+ concentration, we hypothesize that downregulation of the HCN channel may protect against anxiety- and depression-like behavior induced by cerebral ischemia–reperfusion through inhibiting the NLRP3 inflammasome pathway. Overall, our study provides further insight into the mechanisms underlying PSD and suggests that inhibiting HCN channels may be a potential drug target for treating PSD.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

Male wild-type C57BL/6J mice (8–10 weeks, 20–25 g) were used in this study, and they were obtained from Vital River Laboratory Animal Technology Company (Beijing, China). Mice were housed 4 to 5 per cage under a 12-hour light/dark cycle with constant temperature and humidity, and had free access to food and water. All animal procedures were approved by the Animal Care and Use Ethics Committee in our university (ethical license number: JHDXLL2022-033) and guided through the Animal Research: Reporting of in vivo Experiments reporting guidelines.[24] Every effort was made to minimize both the suffering of animals and the number used.

Drugs and Antibodies

The HCN channel inhibitor ZD7288 was obtained from Sigma (St. Louis, MO). Antibodies against HCN1 (NBP1-22450) and HCN2 (NBP2-12895) were purchased from Novus (Cambridge, MA). Antibodies against NLRP3 (D4D8T) (15101), ASC (D2W8U) (67824), cleaved-gasdermin D (Asp276) (10137), cleaved-IL-1β (Asp117) (63124), and NeuN (D3S3I) (12943) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against cleaved-caspase-1 (22915-1-AP) and β-actin (66009-1-Ig) were purchased from Proteintech (Wuhan, China). Antibodies against Iba-1 (Ionized calcium binding adaptor molecule 1) (ab178846) and GFAP (glial fibrillary acidic protein) (ab7260) were purchased from Abcam (Waltham, MA). Alexa Fluor 488 conjugated anti-rabbit Ig (immunoglobulin) G (111–545-003) and Alexa Fluor 594 conjugated anti-mouse IgG (115–585-003) were bought from Jackson ImmunoResearch (West Grove, PA). HRP (horseradish peroxidase) conjugated anti-rabbit IgG (BA1055) and HRP conjugated anti-mouse IgG (BA1051) were acquired from Boster (Wuhan, China).

MCAO Surgery

Mice were randomly divided into a sham-operated group and MCAO group. MCAO surgery was conducted following the procedures outlined in our previous study.[25] Briefly, mice were anesthetized with 5% isoflurane in an induction chamber (RWD Life Technology, Shenzhen, China) and maintained using a nose cone. The core body temperature of mice was maintained at 37 °C using a heating plate. The left common carotid artery, external carotid artery, and internal carotid artery were carefully separated. A nylon monofilament (RWD Life Technology) was inserted through the internal carotid artery into the circle of Willis until encountering mild resistance to occlude the middle cerebral artery. The filament was withdrawn 90 minutes after occlusion for reperfusion. Cerebral blood flow was confirmed using a laser Doppler (Moor Instruments). Sham-operated mice underwent the same surgical procedure as MCAO mice but without nylon insertion.

2,3,5-Triphenyltetrazolium Chloride Staining

Brain infarction was assessed using 2,3,5-triphenyltetrazolium chloride (Sigma) staining at 24 hours and 4 weeks after reperfusion. Staining with 2,3,5-triphenyltetrazolium chloride was performed as previously described. Briefly, mice were anesthetized and euthanized by decapitation, and their brains were rapidly isolated on an ice pack. The brain tissue was then frozen at –20 °C for 5 minutes and sectioned horizontally into 2-mm-thick slices. Brain slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride at 37 °C in a 24-well plate for 30 minutes. Subsequently, the sections were arranged in the order they were cut and examined using a scanner.

Rotating-Rod Test

Briefly, mice were placed on the stationary bar of a rotarod apparatus (XR-6C, Shanghai Xinruan Information Technology, Shanghai, China) for 5 minutes for preadaptation. Subsequently, the rotating bar was set to a maximum rotation speed of 30 rpm during the test. The duration that each mouse remained on the rotating bar before falling was recorded, with a maximum cutoff time of 180 seconds. After each test, the rotarod was cleaned with alcohol and dried. The duration of time spent on the rotating rod was used as an assessment of motor balance ability.

Open Field Test

The open field test was performed as described in our previous study.[26] Briefly, the open field instrument was a plexiglass opening box (50×50×50 cm) with a tracking system XR-XZ301 (Shanghai Xinruan Information Technology). Mice were gently placed in the center of the brightly lit box. The movements of mice during 10 minutes were recorded using a computer-based image analyzer. The time spent in the central area served as an indicator of anxiety-like behaviors in mice. After behavioral testing, the equipment was cleaned with alcohol to remove any residual odors.

Three-Chamber Social Interaction Test

The sociability test was conducted using the 3-chamber social interaction apparatus (XR-XJ117; Shanghai Xinruan Information Technology). During the habituation phase, 2 small cages were placed inside the left and right chambers, and the test animal was allowed to freely explore the apparatus from the middle chamber for 10 minutes. In the test phase, a strange mouse was introduced in a small cage in the right chamber. The test mice were then allowed to freely explore the apparatus from the middle chamber for another 10 minutes. The time spent in each of the 3 chambers (either empty or containing a strange mouse) was measured by a computer-based image analyzer (Shanghai Xinruan Information Technology). The time spent in the right chamber containing a strange mouse was measured as the sociability of mice, which served as an indicator of depressive-like behavior.

Tail Suspension Test

The tail suspension test was conducted following previously reported methods.[27] Each mouse was suspended at a tail suspension bracket by wrapping a piece of tape 1 cm from the end of the tail. The behavior of the mice was recorded for 6 minutes by a tracking system XR-XQ203 (Shanghai Xinruan Information Technology). The first 2 minutes of mouse behavior was an adaptation period and was not analyzed. The immobility time of mice in the last 4 minutes was calculated to evaluate the desperation behavior.

Sucrose Preference Test

The sucrose preference test was performed as previously reported methods with minor modification.[28] In brief, mice were individually housed in cages and provided with 2 bottles of water for a 24-hour habituation period. Subsequently, 1 of the water bottles was replaced with a 1% sucrose solution. Twenty-four hours later, the positions of the 2 water bottles were switched, and the consumption of sucrose and water was observed for an additional 24 hours. The intake of both sucrose and water by mice was measured over the 48 hours. The sucrose preference index was calculated as (sucrose intake)/(sucrose intake+water intake).

Western Blotting

The day after the last behavioral experiment, animals were euthanized and collected for subsequent molecular biological analysis. This experiment was performed as described in our previous study.[29] Briefly, mice were anesthetized and euthanized by decapitation, and hippocampus tissues were rapidly isolated. The tissue was homogenized in a protein extraction buffer containing protease and phosphatase inhibitors. Protein concentration was measured using a protein assay kit (Boster, China). Subsequently, proteins were separated by 10% SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membranes (Merck Millipore). After blocking with 5% milk for 1 hour at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies: anti-HCN1 (1:1000), anti-HCN2 (1:1000), anti-NLRP3 (1:1000), anti-ASC (1:1000), anti-cleaved-gasdermin D (1:500), anti-cleaved-IL-1β (1:500), anti-cleaved-caspase-1 (1:500) and anti-β-actin (1:1000). Following incubation with HRP-conjugated secondary antibodies (1:3000), bands were treated

with chemiluminescent substrate (Thermo Fisher) and visualized by a chemiluminescence imaging system (ChemiDoc MP; Bio-Rad). Immunoreactive signals were quantified using National Institutes of Health ImageJ software.

Immunofluorescence Staining

Briefly, mice were anesthetized by isoflurane and then transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde fixative. The brain was subsequently dehydrated using sucrose solution and coronally sectioned at 10 μm using a freezing microtome (CM1860; Leica). After washing in phosphate buffer, the sections were incubated with 5% BSA at room temperature for 1 hour. The sections were incubated with the HCN1 (1:100) primary antibody overnight at 4 °C. After washing in phosphate buffer, sections were then transferred into the secondary antibodies Alexa Fluor 594 conjugate IgG (1:500) at room temperature for 1 hour. In addition, the sections were sequentially incubated with NeuN (1:300), Iba-1 (1:300), or GFAP (1:300) primary antibody overnight at 4 °C. After washing in phosphate buffer, sections were then transferred into the secondary antibodies Alexa Fluor 488 conjugate IgG (1:500) at room temperature for 1 hour. After bleaching with phosphate buffer, sections were analyzed using an Olympus BX51 FluoView microscope system (Olympus).

Electrophysiology Recordings

Electrophysiological recordings were conducted following previously described methods.[30] Briefly, mice were anesthetized with isoflurane, and their brains were rapidly removed and incubated in 4 °C modified artificial cerebrospinal fluid containing NaCl 119 mmol/L, KCl 2.5 mmol/L, NaH_2PO_4 1.25 mmol/L, $NaHCO_3$ 26 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgCl_2$ 1.3 mmol/L, and D-glucose 10 mmol/L saturated with a mixture of 95% O_2 and 5% CO_2. Coronal slices (300 μm thick) of the hippocampus were prepared using a Leica VT1200S vibratome and then incubated in artificial cerebrospinal fluidsolution. The hippocampal dentate gyrus (DG) cells were visualized using an upright microscope (BX51WI; Olympus) with infrared differential interference contrast optics. For characterization of HCN current (I_h), the slice was perfused with oxygen-saturated artificial cerebrospinal fluid at room temperature, and $BaCl_2$ (200 μmol/L) was added to the extracellular solution to block K^+ conductance in voltage clamp recordings. The I_h of hippocampus DG cells in the brain slice was recorded using micropipettes (5–8 MΩ) filled with an internal solution composed of K-gluconate 128 mmol/L, KCl 17.5 mmol/L, Na_2ATP 5 mmol/L, $MgCl_2$ 1 mmol/L, EGTA 0.2 mmol/L, and HEPES 10 mmol/L (pH 7.4).

Current steps (600 ms) were injected in increments of 20 pA from 0 to 40 pA to record AP (action potential) in the current clamp, and resting membrane potential was detected when the current injection was 0 pA. The I_h was recorded by a series of hyperpolarizing voltage steps (duration of 2.5 seconds) decreasing in increments of 10 mV from a holding potential of 50 mV to a final voltage of 130 mV and return to a fixed potential (–130 mV for 1.5 seconds) after the final voltage step. The maximal amplitude of I_h was determined from the –130 mV step. The input resistance was obtained from hyperpolarizing current injections of 5 pA into the neurons in the voltage clamp. The data were accepted only when the series resistance was <20 MΩ.

All drugs used in this electrophysiological recording were purchased from Sigma-Aldrich. Data acquisition was performed using a Multiclamp 700B patch clamp amplifier (Molecular Devices), sampled at 5 kHz and filtered at 2 kHz using a Digidata 1440A low-noise data acquisition system (Molecular Devices).

Adeno-Associated Virus Microinjection

The day after the last behavioral experiment, animals were treated with adeno-associated virus (AAV) injections. PSD mice were randomly divided into 2 groups; 1 group of mice was given Hcn1 shRNA, and the other group mice were given control AAV injections. Hcn1 shRNA (rAAV-SYN-ZsGreen-Hcn1-shRNA) and control AAV injections (rAAV9-SYN-ZsGreen) were obtained from DesignGene biotechnology company (Shanghai, China). Briefly, mice were anesthetized with isoflurane using a gas anesthesia machine (RWD Life Technology) and then secured on a stereotaxic frame. A median incision was made on the mouse's head to expose the bregma. Subsequently, 1 μL of virus (1×10^{12} vg/mL) was injected into the left hippocampus (anteroposterior: –2.3 mm, mediolateral: 2.0 mm, dorsoventral: –2.0 mm) using a microinjection pump at a rate of 0.1 μL/min. The injector remained in place for 5 minutes to allow for AAV diffusion. Following surgery, mice were kept in an incubator until they regained consciousness and then transferred back to their home cage.

Cell Culture

The human neuroblastoma SY5Y cell line, BV2 mouse microglial cells, and human cervical cancer Hela cells were originally obtained from the Cell Bank (Shanghai, China). Cells were cultured in standard DMEM medium (Gibco) supplemented with 10% FBS (FS201-02; Transgene Biotech) and 1% penicillin–streptomycin. The cells were maintained at 37 °C in a cell incubator with 5% CO_2 and 95% air.

For the oxygen–glucose deprivation/reoxygenation (OGD/R) experiment, after removing the normal medium and washing with PBS, SY5Y cells were incubated with the glucose- and FBS-free DMEM, and then transferred to an anaerobic chamber

(Billups-Rothenberg, Del Mar, CA) containing 95% N_2 and 5% CO_2 for 5 hours. Following the OGD modeling period of 5 hours, SY5Y cells were reperfused with normal DMEM medium and environment for 1 hour. Cell viability was assessed using a cell counting kit-8 assay kit according to the manufacturer's instructions.

The HCN channel inhibitor ZD7288 was dissolved in dimethyl sulfoxide to a concentration of 100 mmol/L and then diluted to the required concentrations with DMEM. In the ZD7288 treatment group, SY5Y cells were treated with ZD7288 at the onset of oxygen and glucose deprivation treatment. In the control and OGD/R groups, cells were treated with the same concentration of dimethyl sulfoxide as a solvent control.

PBFI-AM (potassium-binding benzofuran isophthalate acetoxymethylester) Fluorescence Test

The intracellular concentration of K^+ was measured using the PBFI-AM (potassium-binding benzofuran isophthalate acetoxymethylester) fluorescence indicator (ab142804; Abcam). PBFI-AM was dissolved in dimethyl sulfoxide to a concentration of 5 mmol/L and then diluted to 2 μmol/L with normal saline. After removing the medium and washing with normal saline, cells were incubated with 2 μmol/L PBFI-AM for 40 minutes in the dark. Subsequently, cells were washed 3 times with normal saline. The excitation wavelength of the PBFI dye was 340 nm, whereas the emission wavelength was 500 nm. The fluorescence intensity of PBFI in cells was detected using a fluorescence microscope to determine the changes in intracellular K^+ concentration. Three visual fields in each cell culture dish were randomly selected and photographed under a ×400 fluorescence microscope (Olympus). Exposure time was kept constant for all conditions. The fluorescence intensity was quantified by ImageJ software.

Statistical Analysis

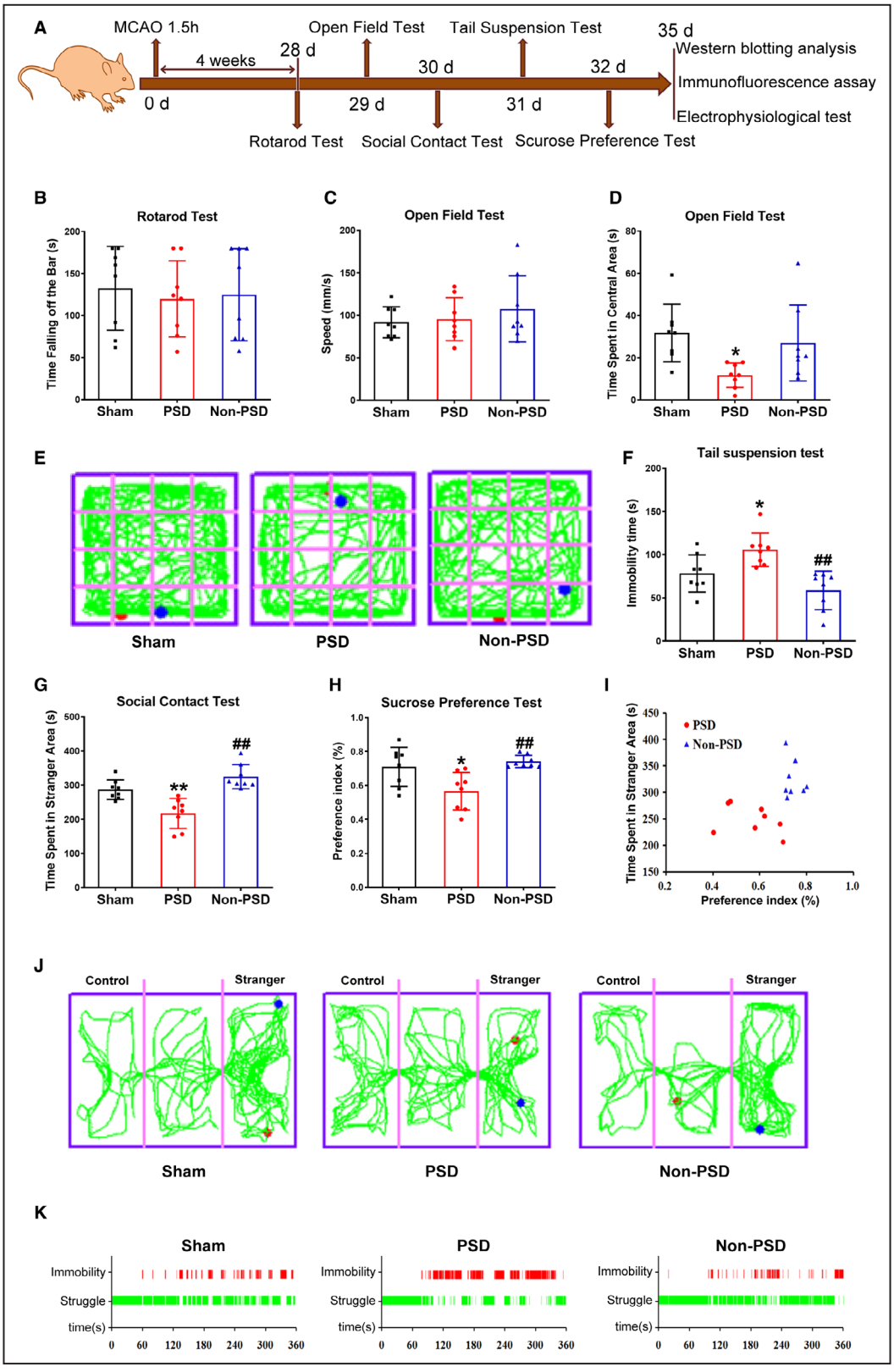
The data are presented as mean±SD (shown in Tables S1 and S4) and analyzed using GraphPad Prism software (version 8.0). Protein expression data were subjected to an unpaired Student *t* test, whereas other data were analyzed using 1-way ANOVA followed by Tukey multiple comparison test (equal variances assumed) or Games-Howell test (equal variances not assumed). $P<0.05$ was considered statistically significant.

RESULTS

Mice Exhibited Anxiety- and Depression-Like Behavior After Cerebral Ischemia–Reperfusion

The mouse model of focal cerebral ischemia–reperfusion was established by MCAO, which is the most widely used animal model of stroke.[31] The chronic period following stroke is often accompanied by psychiatric disorders, such as anxiety and depression.[32] Therefore, 4 weeks after MCAO, we conducted several behavioral tests to assess anxiety- and depression-like behaviors (Figure 1A). To evaluate the recovery of motor function after stroke, the rotarod test was initially performed in mice. We observed that the drop time of mice after stroke did not differ from that in the sham group, indicating that the motor function of mice had essentially recovered 4 weeks after cerebral ischemia (Figure 1B). Because the incidence of PSD in stroke survivors is approximately 30% to 60%, and not every survivor develops depressive behaviors, we differentiated between PSD mice and those who did not develop PSD after stroke using a sucrose preference test and social contact test. Mice after MCAO whose values were lower than the mean value of the sham operation group in both tests were categorized into the PSD group, whereas those whose values were not lower than the mean value of the sham group were placed into the non-PSD group. In the present study, about 46% of the stroke mice fell into the PSD group, and about 29% of the stroke mice fell into the non-PSD group. In the open field test, there were no significant differences in mean speed among groups, indicating that the motor function had basically recovered

Figure 1. Anxiety- and depression-like behavior in mice after cerebral ischemia–reperfusion.
A, Schematic diagram of the experimental timeline. **B**, The drop time of mice in the rotarod test indicated that the motor function of mice was basically recovered 4 weeks after cerebral ischemia (n=8). **C**, The mean speed of mice in each group did not show significant differences in open field test (n=8). **D**, Compared with the sham group, the time spent in the central area of PSD mice was significantly decreased in the open field test, indicating that mice exhibited anxiety-like behavior 4 weeks after MCAO. **E**, Representative graphs of mice trajectories in the open field test. The red dot was the starting point of the mouse, and the blue dot was the end point of the mouse. **F**, The immobility time of PSD mice was significantly increased in the tail suspension test (n=8). **G**, In the social contact test, time spent in the stranger area of PSD mice was significantly decreased, indicating that the sociability of PSD mice was impaired (n=8). **H**, The preference index in the sucrose preference test was significantly decreased in PSD mice, indicating that mice exhibited depression-like behavior 4 weeks after MCAO (n=8). **I**, The *x*–*y* plot of social contact vs sucrose preference. **J**, Representative graphs of mice trajectories in social contact test. The red dot was the starting point of the mouse, and the blue dot was the end point of the mouse. **K**, Representative graphs of mice trajectories in the tail suspension test. Data are presented as mean±SD, n=8 per group. **P*<0.05 and ***P*<0.01 vs the sham group, ##*P*<0.01 vs the PSD group. PSD indicates poststroke depression; and MCAO, middle cerebral artery occlusion.



4 weeks after MCAO (Figure 1C). Moreover, time spent in the central area during open field testing was significantly decreased in PSD mice compared with that of the sham group mice (Figure 1D and 1E), suggesting that mice exhibited anxiety-like behavior 4 weeks after MCAO. In the tail suspension test, the immobility time was significantly increased in PSD mice compared with that of the sham group mice and non-PSD mice

(Figure 1F and 1K). Meanwhile, time spent in stranger areas during social contact testing and preference index during sucrose preference testing were both significantly decreased in PSD mice (Figure 1G through 1J). These results suggest that mice exhibit anxiety- and depression-like behavior 4 weeks after cerebral ischemia–reperfusion.

HCN1 Channel Was Upregulated in the Hippocampus of PSD Mice

The hippocampus is a well-known component of the brain's limbic system, playing a crucial role in various physiological functions and pathological processes such as memory and emotion.[33,34] In this study, we observed infarct damage in the hippocampus 24 hours after reperfusion using 2,3,5-triphenyltetrazolium chloride staining (Figure 2A). Furthermore, hippocampal atrophy was evident in both PSD mice and non-PSD mice 4 weeks after cerebral ischemia–reperfusion (Figure 2A). Given that HCN channels are implicated in the pathogenesis of numerous neurodegenerative diseases,[9] we investigated the expression and distribution of HCN channels in the ipsilateral hippocampus region of PSD mice. Compared with the sham group, there was a significant increase in HCN1 protein expression in the ipsilateral hippocampus of PSD mice, whereas HCN2 protein expression showed no significant change (Figure 2B through 2D). Full blot (uncropped) images of Western blots are shown in Figure S1. Additionally, we used immunofluorescence experiments to examine the cellular localization of HCN1 channel and found that HCN1 was predominantly expressed on neurons (Figure 2E). These results suggest that cerebral ischemia–reperfusion injury leads to an upregulation of HCN1 channels in the ipsilateral hippocampal neurons of mice, potentially contributing to anxiety- and depression-like behavior following stroke.

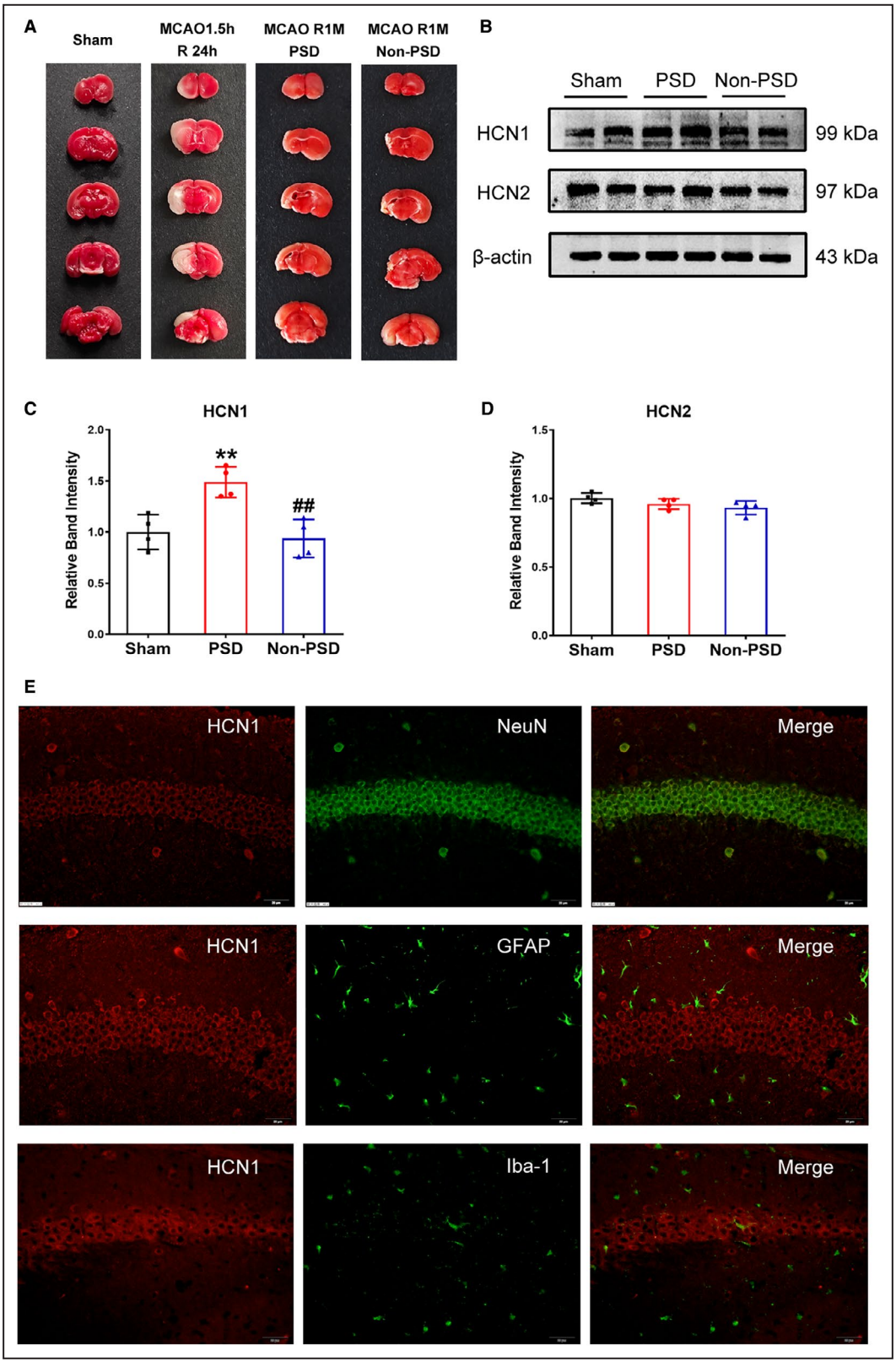
I_h Current Was Enhanced in Hippocampal Dentate Granule Neuron of PSD Mice

Because the protein expression of HCN1 channel was found to be increased in the hippocampus of PSD mice, we next investigated whether the function of HCN channel (I_h current) was altered in PSD mice. The I_h current was recorded using whole-cell patch-clamp recordings in the hippocampal DG neuron with typical voltage- and time-dependent activation (Figure 3A and 3B). Furthermore, we observed that I_h was abolished by perfusion with the selective HCN channel inhibitor ZD7288 (15 μmol/L) for 15 minutes (Figure 3C). As shown in Figure 3D through 3G, the maximal current of I_h in the hippocampus of PSD mice was significantly increased compared with that of sham mice and non-PSD mice. HCN channels are known to be versatile regulators of neuronal excitability in the nervous system.[35,36] Therefore, it is possible that the observed hyperpolarizing shift in I_h activation may affect the intrinsic excitability of hippocampal DG neuron. To investigate this possibility, we measured the resting membrane potentials of the hippocampal DG neuron in the current clamp when the current injection is 0 pA, and measured the input resistance in the voltage clamp. We observed a significant decrease in resting membrane potential and no significant decrease in input resistance in the PSD group compared with the sham group and non-PSD group (Figure 3H through 3L), indicating reduced neuronal excitability in the hippocampal DG neuron of PSD mice. HCN channels are a depolarizing conductance, so increased I_h and other potassium channels (eg, Eag-related gene K^+ channels, Kv 2.1 voltage-gated K^+ channel) may contribute to the hyperpolarized resting potential.[37] These findings suggest that the decreased neuronal excitability observed in the hippocampus of PSD mice may stem from the increased availability of HCN channels, which is potentially involved in the pathogenesis of PSD.

Inhibition of HCN Channels Protected SY5Y Cells Against OGD/R Injury by Suppressing K^+ Efflux

The OGD/R model is the most widely known cell model of stroke, which is also used to study the pathogenesis of PSD in vitro.[38,39] Given that the upregulation of the HCN1 channel has been observed in PSD mice, we aimed to investigate whether inhibition of HCN channels using ZD7288, a selective HCN channel inhibitor, could protect against cell damage induced by OGD/R. As shown in Figure 4A, HCN1 channel was expressed in SY5Y cells. The viability of SY5Y cells was significantly reduced following OGD/R compared with the

Figure 2. Expression and distribution of HCN1 in the hippocampus of mice after cerebral ischemia–reperfusion.
A, TTC staining shows that hippocampus tissue was damaged and atrophic cerebral ischemia–reperfusion (n=3). White areas in the mouse brain slices represented ischemic infarction area. **B**, Representative bands of HCN1 and HCN2 in Western blotting analysis (n=4). **C**, Statistical results showed that the protein expression of HCN1 in the ipsilateral hippocampus of PSD mice was significantly increased (n=4). **D**, The protein expression of HCN2 in each group did not show significant differences. **E**, Double immunofluorescence staining showed that the HCN1 channel was predominantly colocalized with neurons, which was rarely colocalized with astrocytes or microglia. HCN1 (red), neuron marker NeuN (green), astrocyte marker GFAP (green), and microglia marker Iba-1 (green). Scale bar=20 μm. n=3 per group. $^{**}P<0.01$ vs sham group, $^{\#\#}P<0.01$ vs PSD group. HCN indicates hyperpolarization-activated cyclic nucleotide-gated cation channel; MCAO, middle cerebral artery occlusion; PSD, poststroke depression; NeuN (neuron-specific nuclear protein); Iba-1 (Ionized calcium binding adaptor molecule 1); GFAP (glial fibrillary acidic protein); and TTC, 2,3,5-triphenyltetrazolium chloride.



controls. Moreover, treatment with ZD7288 at concentrations ranging from 2.5 to 50 μmol/L markedly increased cell viability after OGD/R injury (Figure 4B).

Potassium is an essential ion present in high concentrations inside cells and plays a critical role in various physiological functions such as maintaining acid–base

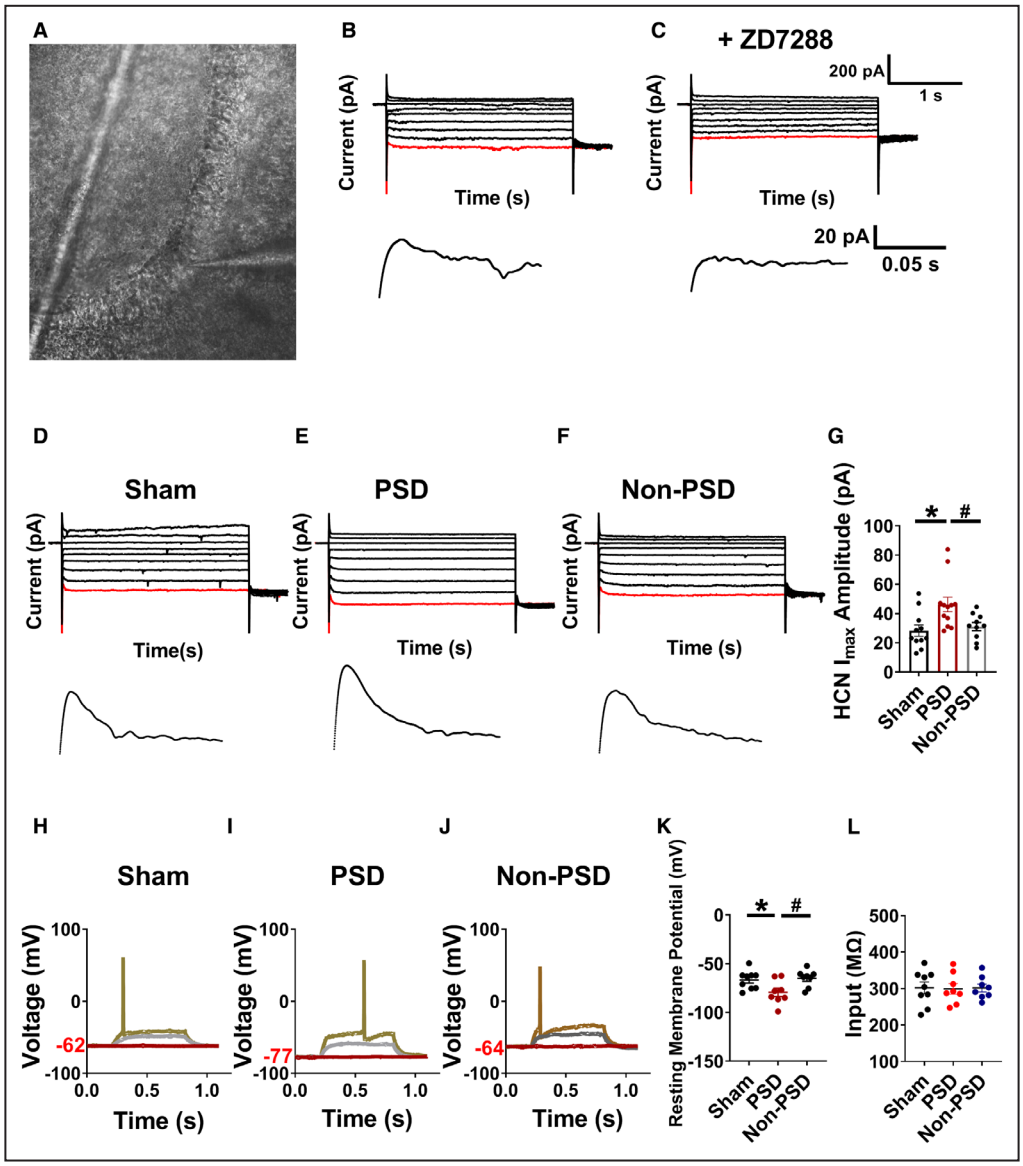


Figure 3. Alteration in I_h and neuronal excitability in hippocampal DG neurons of PSD mice.
A, Representative photograph of the recording setup by the whole-cell patch clamp in the mouse hippocampal DG neuron. **B**, Representative current of I_h with typical voltage- and time-dependent activation. **C**, I_h current was abolished by bathing with the selective HCN channel inhibitor ZD7288 (15 μmol/L) for 15 minutes. Representative current of I_h in hippocampal DG neurons of sham mice (**D**), PSD mice (**E**), or non-PSD mice (**F**). **G**, Statistical results of maximal current showed that I_h in the hippocampal DG of PSD mice (n=12) was significantly increased compared with the sham group (n=11) and non-PSD group (n=10). Representative graph of the resting membrane potentials in current clamp when the current injection is 0 pA in hippocampal DG neurons of sham mice (**H**), PSD mice (**I**), or non-PSD mice (**J**). **K**, Statistical results showed that resting membrane potential in the hippocampal DG of PSD mice (n=8) was significantly decreased compared with the sham group (n=9) and non-PSD group (n=8), indicating that the neuronal excitability was reduced in PSD mice. **L**, Statistical results showed that the input resistance in the hippocampal DG of PSD mice (n=8) showed no significant difference compared with the sham group (n=9) and non-PSD group (n=8). **P*<0.05 vs sham group, #*P*<0.05 vs PSD group. DG indicates dentate gyrus; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; and PSD, poststroke depression.

balance, osmotic pressure, membrane potential, and neuromuscular function.[40] Using a K^+ fluorescence probe PBFI-AM, we observed a significant decrease in intracellular K^+ concentration after OGD/R (Figure 4C and 4D). Furthermore, treatment with the HCN channel inhibitor ZD7288 ameliorated the reduction of intracellular K^+ concentration induced by OGD/R (Figure 4C and 4D). These results suggest that inhibition of the HCN channels alleviates cell damage after OGD/R by increasing intracellular K^+ concentration.

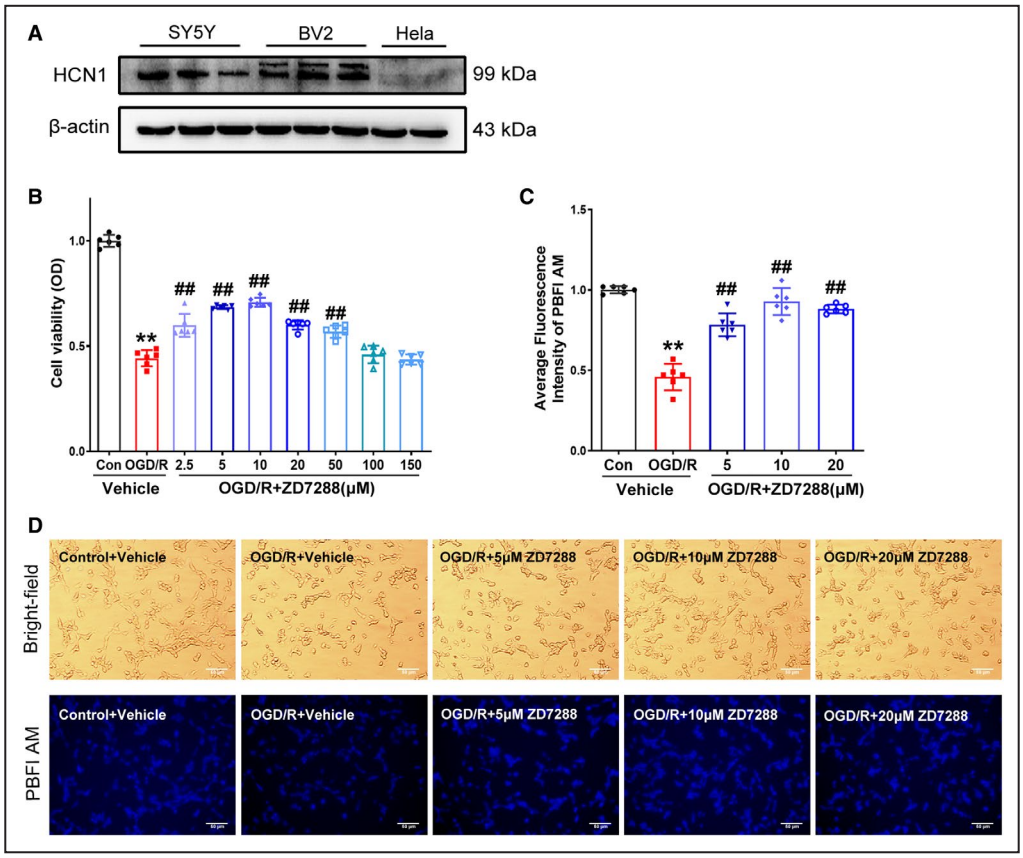


Figure 4. Inhibition of the HCN channel alleviates OGD/R-induced cell injury via increasing the intracellular K^+ concentration.

A, Representative bands of HCN1 protein expression in 3 cell types. **B**, Selective HCN channel inhibitor ZD7288 treatment (ranging from 2.5 to 50 μmol/L) could significantly enhance the SY5Y cell viability after OGD/R injury by CCK-8 detection (n=6). ZD7288 was dissolved in DMSO to a concentration of 100 mmol/L and then diluted to the required concentrations with DMEM. In the ZD7288 treatment group, cells were incubated with ZD7288 at the beginning of OGD treatment. **C**, The intracellular K^+ concentration was detected by a K^+ fluorescence probe PBFI-AM. ZD7288 treatment could ameliorate the reduction of intracellular K^+ concentration induced by OGD/R (n=6). **D**, Representative fluorescence images of K^+ probe PBFI-AM (blue) staining and bright-field photograph. Scale bar=50 μm. n=6 per group. $^{**}P<0.01$ vs control group, $^{\#\#}P<0.01$ vs OGD/R group. CCK-8 indicates cell counting kit-8; DMSO, dimethyl sulfoxide; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; OGD/R, oxygen–glucose deprivation/reoxygenation; and PBFI-AM (potassium-binding benzofuran isophthalate acetoxymethylester).

NLRP3 Signal Pathway Was Activated in the Ipsilateral Hippocampus of PSD Mice

Accumulating evidence suggests that the NLRP3 inflammasome pathway participates in brain damage following stroke, and can be activated by a decrease in intracellular K^+ concentration.[41,42] Therefore, we investigated the changes in expression of proteins related to the NLRP3 signaling pathway in the ipsilateral hippocampus of PSD mice. Compared with the sham group, the protein levels of NLRP3 and ASC were significantly increased in the ipsilateral hippocampus of PSD mice, whereas there was no significant change observed in non-PSD mice (Figure 5A through 5C). Additionally, we observed an increase in the protein levels of cleaved-caspase-1 and IL-1β, which are downstream factors of NLRP3 inflammasome, in the ipsilateral hippocampus of PSD mice (Figure 5D and 5E). These findings suggest that upregulation of the NLRP3 inflammasome pathway in the ipsilateral hippocampus may be involved in the pathogenesis of anxiety- and depression-like behavior after stroke.

Genetic Knockdown of the HCN1 Channel Resulted in an Improvement in Anxiety- and Depression-Like Behaviors of PSD Mice

To confirm the involvement of the HCN1 channel in anxiety- and depression-like behavior after stroke, we knocked down the HCN1 channel in hippocampal

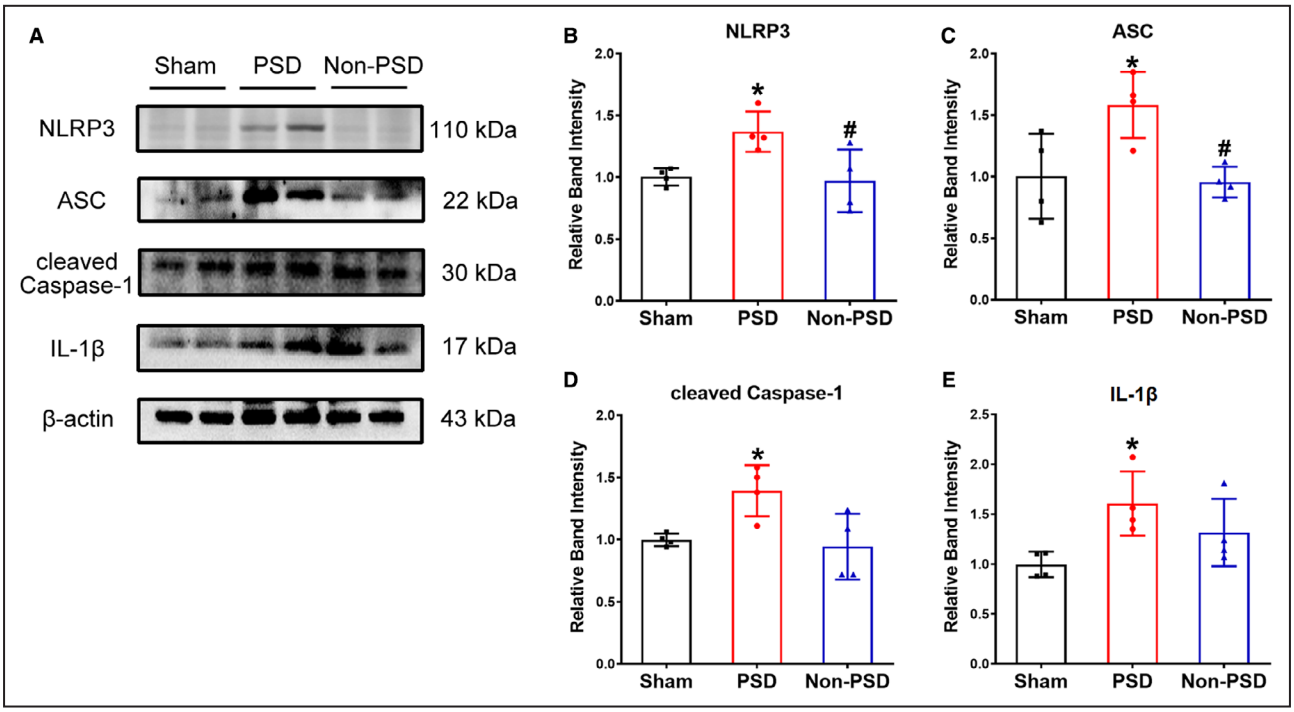


Figure 5. NLRP3 inflammasome pathway was increased in the hippocampus of PSD mice.
A, Representative band of NLRP3 inflammasome-related proteins. **B**, Statistical results show that the protein expression of NLRP3 was markedly increased in the ipsilateral hippocampus of PSD mice compared with the sham group, whereas it shows no significant change in the hippocampus of non-PSD mice (n=4). Meanwhile, the protein expressions of ASC (**C**), cleaved-caspase-1 (**D**), and IL-1β (**E**) were also observably increased in the ipsilateral hippocampus of PSD mice. n=4 per group. *P<0.05 vs sham group, #P<0.05 vs PSD group. ASC indicates apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; IL-1β, interleukin-1β; NLRP3, nucleotide-binding domain-like receptor protein 3; and PSD, poststroke depression.

neurons by microinjecting HCN1 shRNA (AAV-Hcn1) into the ipsilateral hippocampus (Figure 6A). As expected, the region of the hippocampus injected with the virus showed green fluorescence, whereas other brain regions showed no green fluorescence (Figure 6B). Consistent with the immunofluorescent results, Western blotting analysis demonstrated that the protein expression level of HCN1 in the hippocampus 4 weeks after AAV-Hcn1 microinjection was significantly reduced compared with scrambled AAV injection (Figure 6C and 6D). Meanwhile, there was no effect on the protein expression level of HCN2 in the hippocampus following AAV-Hcn1 microinjection (Figure 6E).

In an open field test, there were no significant differences in the mean speed among these groups, indicating that virus injection did not affect basic motor function in mice (Figure 7B). However, microinjection with AAV-Hcn1 into the ipsilateral hippocampus of PSD mice noticeably increased time spent in central areas compared with the PSD+AAV-Ctrl group, suggesting that knockdown of the HCN1 channel could improve the anxiety-like behavior of PSD mice (Figure 7C and 7F). Moreover, microinjection of AAV-Hcn1 tended to improve the preference index in a sucrose preference test compared with the PSD+AAV-Ctrl group, though this effect was not statistically significant (Figure 7D). Compared with the control AAV microinjection for PSD mice, AAV-Hcn1 microinjection also significantly increased time spent in stranger areas in a social contact test, indicating that knockdown of HCN1 channels could improve depression-like behavior of PSD mice (Figure 7E and 7G). Taken together, these results suggest that inhibition of HCN1 channels within hippocampal neurons could ameliorate anxiety- and depression-like behaviors seen within PSD disorder models.

Genetic Knockdown of HCN1 Channel Inhibited the NLRP3 Inflammasome Pathway in the Ipsilateral Hippocampus of PSD Mice

Because the activation of the HCN1 channel leads to the outflow of intracellular K^+, and decreased intracellular K^+ concentration may trigger the the NLRP3 inflammasome pathway, we next evaluated whether knockdown of the HCN1 channel improves anxiety- and depression-like behaviors in PSD mice through inhibiting the NLRP3 inflammasome pathway. As shown in Figure 7H through 7K, AAV-Hcn1 microinjection could significantly reduce the protein expression of HCN1, NLRP3, and the adaptor protein ASC in the ipsilateral hippocampus of PSD mice compared with AAV-Ctrl microinjection. Moreover,

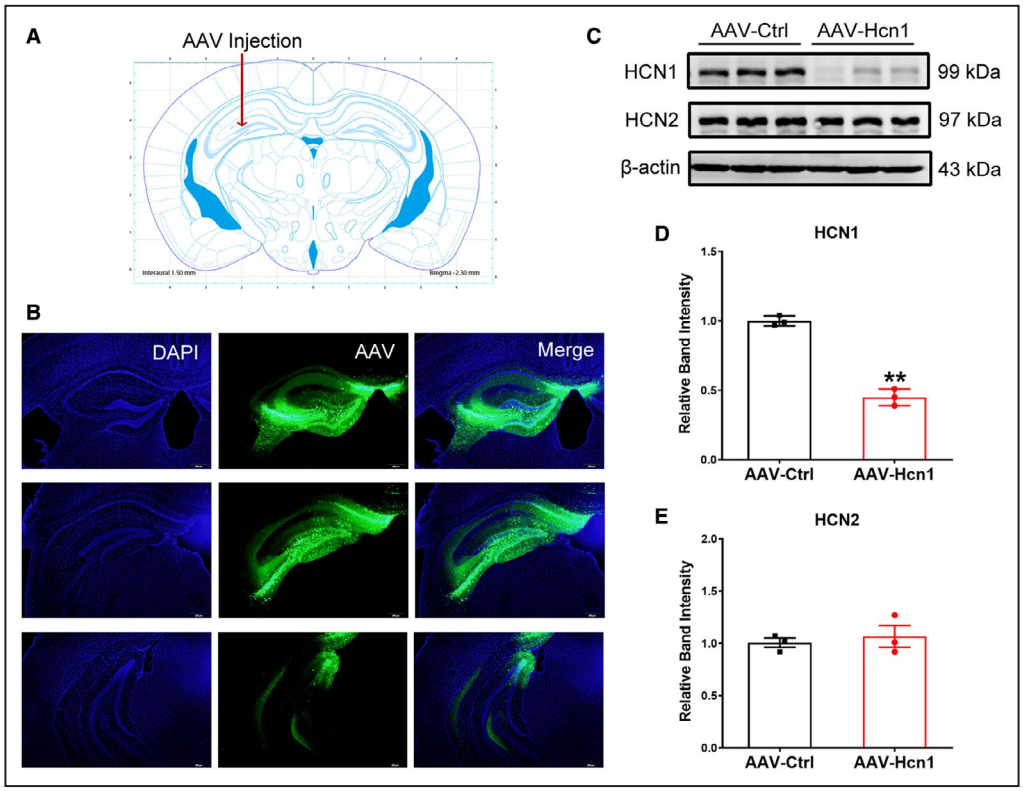


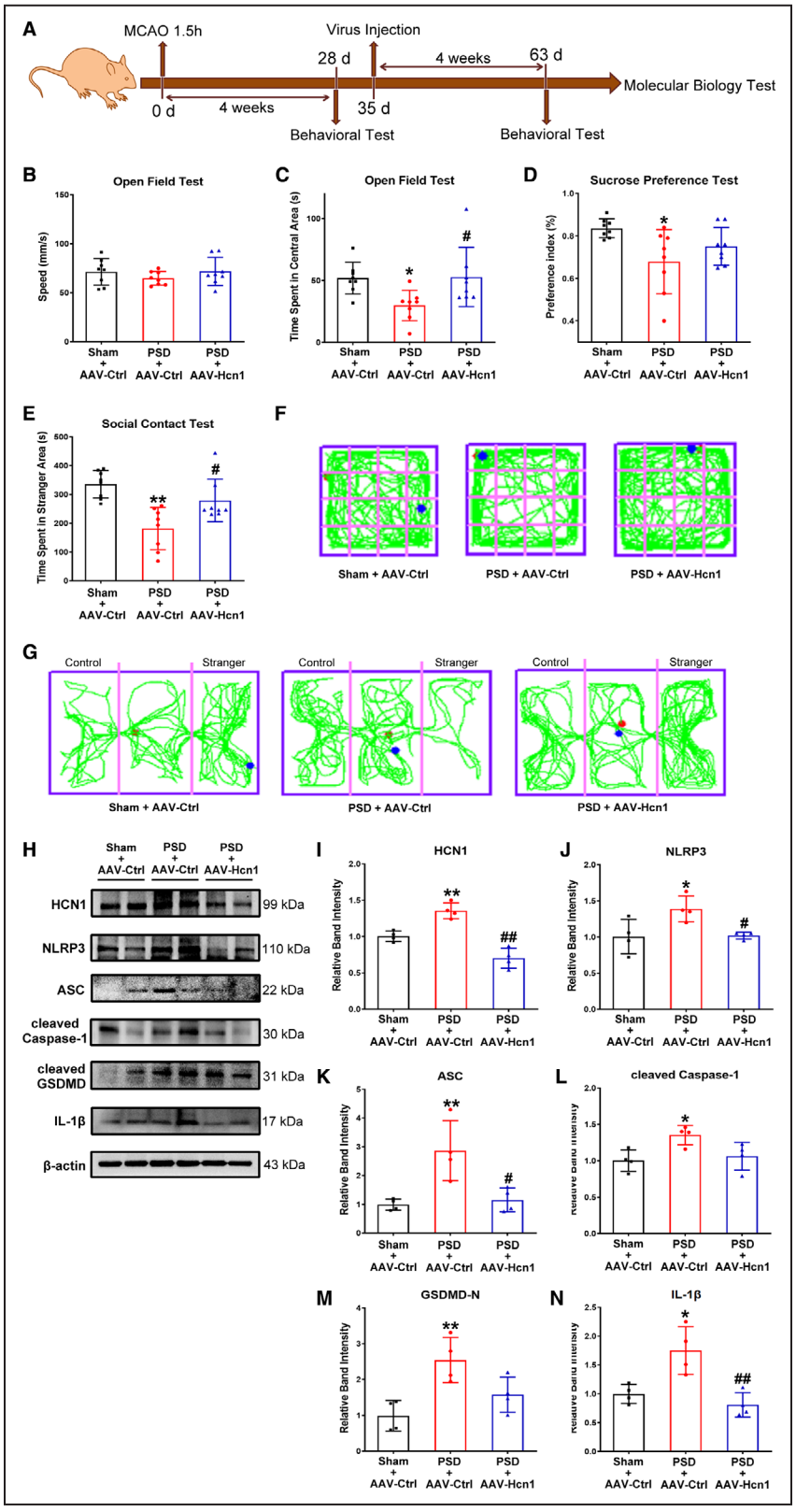
Figure 6. Targeted downregulation of HCN1 expression in the hippocampus by AAV microinjection. **A**, Schematic diagram of the AAV microinjection site in the hippocampus. The microinjection site in the left hippocampus of mice was AP: −2.3 mm, ML: 2.0 mm, DV: −2.0 mm. **B**, Representative fluorescence images of AAV (green) microinjection in the hippocampal slices (n=3). Four weeks after virus injection, the mice brains containing the hippocampus were sectioned coronally at 10 μm by a freezing microtome. Green fluorescence was observed on multiple hippocampal slices attached to the microinjection site. **C**, Representative blots of HCN1 and HCN2 proteins in the left hippocampus. **D**, Quantitative analysis showed that the protein expression level of HCN1 in the left hippocampus was markedly reduced 4 weeks after AAV-Hcn1 microinjection (n=3). **E**, The protein expression level of HCN2 in the hippocampus was not affected by AAV-Hcn1 microinjection. n=3 per group. $^{**}P<0.01$ vs AAV-Ctrl group. AAV indicates adeno-associated virus; AP, anteroposterior; DAPI, dihydrochloride; DV, dorsoventral; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; and ML, mediolateral.

microinjection of AAV-Hcn1 tended to decrease the protein expression of cleaved-caspase-1 and GSDMD (gasdermin D)-N in the ipsilateral hippocampus of PSD mice (Figure 7L and 7M). In addition, AAV-Hcn1 microinjection significantly attenuated the protein expression level of IL-1β in the hippocampus of PSD mice, which were downstream effectors of the NLRP3 inflammasome (Figure 7N). Overall, these results indicate that the knockdown of the HCN1 channel in the ipsilateral hippocampus may improve anxiety- and depression-like behaviors of PSD mice by downregulating the NLRP3 inflammasome pathway.

DISCUSSION

PSD is a psychiatric complication that occurs during the functional recovery of stroke survivors, significantly impacting the quality of life of patients.[43] However, current treatment options for PSD are unsatisfactory, and the pathological mechanism of PSD remains unclear.[44,45] In this study, we observed significant anxiety- and depression-like behaviors in PSD mice induced by cerebral ischemia–reperfusion. Furthermore, we found that the HCN1 channel and NLRP3 inflammasome pathway were significantly upregulated in the ipsilateral hippocampus of PSD mice. Treatment with the HCN channel selective inhibitor ZD7288 was able to alleviate OGD/R-induced cell damage by inhibiting K^+ efflux. Additionally, knockdown of the HCN1 channel in hippocampal neurons improved anxiety- and depression-like behaviors in PSD mice, possibly through inhibiting the NLRP3 inflammasome pathway.

Compared with general depression, the manifestations and causes of human PSD are more complex, so the ideal animal model for PSD is still under investigation.[46] MCAO is a classical animal model of cerebral ischemia–reperfusion and has been proposed



for establishing PSD models.[47,48] Recent studies have shown that combining MCAO with depression models, such as chronic restraint stress or chronic mild stress, can lead to more obvious depressive-like behaviors after cerebral ischemia.[49,50] Although this combined animal model was effective in inducing depression representations, the causative factor of this combined model was not only stroke alone, which may be different from

Figure 7. Knockdown of the HCN1 channel improved anxiety- and depression-like behaviors of PSD mice though inhibiting NLRP3 inflammasome pathway.
A, Schematic diagram for the experimental timeline. **B**, The mean speed of mice in each group did not show significant differences in the open field test (n=8). **C**, Compared with the PSD+AAV-Ctrl group, time spent in the central area was significantly enhanced by microinjection with AAV-Hcn1 in the ipsilateral hippocampus of PSD mice (n=8). **D**, Microinjection of AAV-Hcn1 into the ipsilateral hippocampus of PSD mice tended to improve the preference index in the sucrose preference test compared with the PSD group (n=8). **E**, In the social contact test, the time spent in the stranger area was markedly increased after AAV-Hcn1 microinjection in PSD mice. **F**, Representative graphs of mice trajectories in the open field test (n=8). The red dot was the starting point of the mouse, and the blue dot was the end point of the mouse. **G**, Representative graphs of mice trajectories in the social contact test. The red dot was the starting point of the mouse, and the blue dot was the end point of the mouse. **H**, Representative bands of HCN1 and NLRP3 inflammasome-related proteins in the ipsilateral hippocampus. Compared with the PSD+AAV-Ctrl group, the protein expressions of HCN1 (**I**), NLRP3 (**J**), and ASC (**K**) in the ipsilateral hippocampus were significantly decreased by AAV-Hcn1 microinjection in PSD mice (n=4). Meanwhile, AAV-Hcn1 microinjection tended to decrease the protein expressions of cleaved-caspase-1 (**L**) and GSDMD-N (**M**) in the ipsilateral hippocampus of PSD mice (n=4). **N**, The protein expression of IL-1β in the ipsilateral hippocampus was significantly decreased by AAV-Hcn1 microinjection in PSD mice (n=4). $^{*}P<0.05$ and $^{**}P<0.01$ vs sham + AAV-Ctrl group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs PSD+AAV-Ctrl group. ASC indicates apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; AAV, adeno-associated virus; GSDMD, gasdermin D; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; IL-1β, interleukin-1β; MCAO, middle cerebral artery occlusion; NLRP3, nucleotide-binding domain-like receptor protein 3; and PSD, poststroke depression.

the cause in patients with clinical PSD. In some literature, only MCAO surgery was used to establish a PSD animal model.[51,52] Therefore, we first examined the anxiety and depression behaviors of MCAO mice using open field tests, sucrose preference tests, and social contact tests 4 weeks after cerebral ischemia–reperfusion. We then distinguished PSD mice from those that did not develop PSD based on their performance in the sucrose preference and social contact experiments. Compared with the mean value of sham-operated mice, MCAO mice that showed a tendency toward depression in both sucrose preference and social contact experiments were divided into the PSD group, whereas mice that did not show a tendency toward depression in either experiment were placed into the non-PSD group. As expected, mice in the PSD group exhibited anxiety- and depression-like behaviors 4 weeks after cerebral ischemia–reperfusion (Figure 1). We next prepared to investigate the pathogenesis of PSD mice.

HCN channels and their I_h current play a crucial role in various physiological processes such as rhythmic regulation of neurons, transmitter release, and synaptic plasticity.[53] Abnormal expression or dysfunction of HCN channels is closely associated with a variety of nervous system diseases.[54] Several studies have indicated that inhibiting HCN channels can improve brain damage caused by stroke.[10,11] Furthermore, recent studies have demonstrated the involvement of HCN channels in the pathogenesis of affective disorders.[55,56] For instance, ketamine has been shown to exhibit a fast-acting antidepressant effect by inhibiting presynaptic HCN1 channels,[57] whereas knockdown of HCN1 could increase the excitability of pyramidal neurons in the hippocampal CA1 (cornu ammonis 1) region and produce antidepressant-like behaviors.[58] Additionally, depression-like behaviors induced by chronic unpredictable stress have been linked to increased expression of HCN1 protein in the hippocampus; however, inhibitors of the HCN channels were able to improve these depression-like behaviors.[59] Collectively, these studies suggest that HCN channels may be involved in the pathogenesis of PSD. In our present study, we observed a significant increase in the protein expression of the HCN1 channel and the I_h current in the ipsilateral hippocampus of PSD mice (Figures 2C and 3G). Moreover, pharmacological inhibition of the HCN channels using ZD7288 showed neuroprotective effects on SY5Y cells against OGD/R injury (Figure 4B). Additionally, genetic knockdown of the HCN1 channel in hippocampal neurons via microinjecting AAV-Hcn1 remarkably improved anxiety- and depression-like behaviors in PSD mice (Figure 7A through 7G). Our findings suggest that inhibiting the HCN1 channel in hippocampal neurons has beneficial effects on anxiety- and depression-like behaviors induced by cerebral ischemia–reperfusion, which may offer a promising strategy for preventing and treating PSD.

The activation of HCN channels has been shown to result in the efflux of intracellular K^+, which in turn may trigger the NLRP3 inflammasome pathway.[60,61] The NLRP3 inflammasome is a multimolecular complex consisting of the NLRP3 protein, adaptor protein ASC, and effector protein pro-caspase-1, and it plays a crucial role in regulating innate immunity and inflammatory response.[62] Activation of the NLRP3 inflammasome can lead to the production of activated caspase-1, which then cleaves proteins GSDMD and pro-IL-1β into GSDMD-N and IL-1β, respectively. This process mediates cell dysfunction and ultimately results in cell death.[63,64] Accumulating evidence suggests that activation of the NLRP3 inflammasome is closely associated with the pathogenesis of various neurological diseases such as cerebral ischemia and depressive behaviors.[65–68] Aerobic exercise has been found to reverse the NF-κB/NLRP3/5-HT (5-hydroxytryptamine) pathway by upregulating irisin expression, thereby alleviating depressive-like behavior induced by combining MCAO and chronic unpredictable mild stimulation in mice.[69] Additionally, treatment with CysLT2R (cysteinyl leukotriene receptor

2) antagonist HAMI3379 has been shown to improve PSD in gerbils by inhibiting NLRP3 inflammasome/pyroptosis signaling,[70] whereas stellate ganglion block could ameliorate anxiety- and depression-like behaviors following thalamic hemorrhage via suppressing the HIF-1α (hypoxia-inducible factor-1α)/NLRP3 inflammatory pathway.[71] In this study, we observed similar results showing increased protein expression of the NLRP3 inflammasome pathway, including NLRP3, ASC, cleaved caspase-1, and IL-1β, in the ipsilateral hippocampus of PSD mice (Figure 5A through 5E).

The NLRP3 inflammasome can be activated through several processes, including a decrease in intracellular K^+ concentration, accumulation of intracellular Ca^{2+}, and production of reactive oxygen species.[72,73] Given that upregulation of HCN1 could lead to a decrease in intracellular K^+, we hypothesized that HCN channels may regulate the NLRP3 inflammasome pathway by influencing the intracellular K^+ concentration, thereby participating in the pathogenesis of PSD. In our vitro experiments, we observed that treatment with the HCN channel inhibitor ZD7288 could mitigate cell damage after OGD/R by increasing the intracellular K^+ concentration. Although ZD7288 is regarded as a fairly specific HCN inhibitor, potential off-target effects have been reported, such as its inhibitory effect on calcium channels.[74,75] Therefore, we next inhibited the expression of HCN1 at gene level to investigate the role of HCN1 in the pathogenesis of PSD. We found that genetic knockdown of the HCN1 channel via microinjection of HCN1 shRNA significantly decreased the protein expression of NLRP3 inflammasome pathway in the ipsilateral hippocampus of PSD mice (Figure 7H through 7N). These findings suggest that knockdown of the HCN1 channel can downregulate the NLRP3 inflammasome pathway, potentially contributing to the neuroprotective effect of inhibiting HCN1 channels on anxiety- and depression-like behaviors in PSD mice.

Some limitations need to be considered in this study. This study is mainly focused on changes occurring in male animals; however, male and female individuals have different susceptibility to depression.[76] Therefore, the role of HCN1 channels in PSD behavior in female animals warrants further study. Second, we only observed changes in the ischemic ipsilateral hippocampus and did not study changes in the contralateral brain region, which should be considered in future studies. Moreover, the hippocampus is formed by several subregions with different functions, such as CA1 (Cornu Ammonis 1), CA3 (Cornu Ammonis 3), and DG. Previous studies have reported that the CA1 region of the hippocampus is more sensitive to ischemia.[77] This study is mainly focused on changes occurring in the whole hippocampus but not in the hippocampal subregions, which should be considered in future studies. Moreover, we preliminarily found that inhibition of the HCN1 channel affected the intracellular potassium concentration and the protein expression of NLRP3 inflammasome pathway. Accumulating evidence suggests that NLRP3 activation correlates with K^+ efflux, Na^+ influx, production of reactive oxygen species, mitochondrial damage, and lysosomal damage.[78] It is not clear whether changes in intracellular potassium concentration are necessary for HCN to regulate the NLRP3 pathway. The role of HCN channels in the regulation of the NLRP3 pathway and its mechanism warrants further study.

In conclusion, this study demonstrates the role of the HCN1 channel in the development of PSD for the first time. Pharmacological inhibition or genetic knockdown of the HCN1 channel in neurons provided beneficial effects on the improvement of anxiety- and depression-like behaviors in mice induced by cerebral ischemia–reperfusion. More importantly, we revealed that the underlying mechanism of the neuroprotective effect of inhibiting HCN1 channels may be due to the downregulation of the NLRP3 inflammasome pathway. Overall, targeting the HCN1 channel activity in the hippocampus, especially its neuronal subcellular localization, may serve as a potential strategy for treating stroke-induced depressive behavior.

ARTICLE INFORMATION

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Author contributions: All authors have contributed significantly to this study. Mei Z., X.J.S., and W.L. conducted the study design. Most of the in vivo experiments were completed by Mei Z., X.T., and C.L. K.L. and Y.S. performed most of the in vitro experiments. Y.Y., Y.G., and Meiling Z. contributed data collection and data analysis. Mei Z. and W.L. wrote the main article. B.S., Y.X., and X.S. revised and edited the article. All authors read and approved the final article.

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Disclosure

None.

Supplemental Material

Tables S1–S4
Figure S1

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