








Original Research

The Role of the Hippocampal P2X7R/NLRP3 Signaling Pathway in Depression-Like Behavior Induced by the Interaction Between Chronic Stress and Time in a Rat Model of Stroke

Yi Zhang^{1,†}, Siyuan Wu^{1,2,3,†}, Wenjing Tang^{1,4}, Chen Yang^{1,5}, Yuqi Yin¹, Juan He⁶,
Xi Tao^{1,2,3,*}¹Department of Neurological Rehabilitation, Hunan Provincial People's Hospital, Hunan Normal University, 410016 Changsha, Hunan, China²Hunan Provincial Key Laboratory of Neurorestoratology, 410016 Changsha, Hunan, China³Clinical Research Center for Cerebrovascular Disease Rehabilitation in Hunan Province, 410016 Changsha, Hunan, China⁴Department of Rehabilitation, Rehabilitation Hospital of Hunan Province, 410003 Changsha, Hunan, China⁵Department of Emergency and Critical Care Medicine, Suzhou Municipal Hospital, Gusu School of Nanjing Medical University, 215001 Suzhou, Jiangsu, China⁶Department of Neurosurgery, Hunan Provincial People's Hospital, Hunan Normal University, 410016 Changsha, Hunan, China*Correspondence: taoxi567@hunnu.edu.cn (Xi Tao)

†These authors contributed equally.

Academic Editors: Domenico De Berardis and Bettina Platt

Submitted: 26 April 2025 Revised: 1 September 2025 Accepted: 25 September 2025 Published: 11 November 2025

Abstract

Background: Depression frequently manifests as a secondary affective disorder in individuals who have experienced a stroke. In laboratory rats subjected to stroke, prolonged exposure to chronic stress effectively replicates the physiological impairment and adverse environmental challenges encountered by stroke patients. Nevertheless, the complex mechanisms underlying these phenomena remain unclear. **Methods:** To elucidate the mechanisms underlying these impairments, we established a poststroke depression model by combining middle cerebral artery occlusion (MCAO) with 70 minutes of ischemia and chronic unpredictable mild stress (CUMS) exposure. Behavioral assessments, along with analyses of purinergic ligand-gated ion channel 7 receptor (P2X7R) and nucleotide-binding oligomerization domain, leucine-rich repeats, and pyrin domain-containing protein 3 (NLRP3)-associated inflammatory protein levels and peripheral blood inflammatory cytokine levels, were conducted at 1, 2 and 4 weeks post-MCAO, and the results were compared with those of rats subjected to stroke alone. **Results:** Depression-like behaviors were induced by CUMS exposure for three weeks. These changes were accompanied by significant increases in the protein levels of interleukin-1 β (IL-1 β), caspase-1, NLRP3 and Iba-1 in the hippocampus. Additionally, an increase in the fluorescence intensity of Iba-1, P2X7R, and NLRP3 in the Cornu Ammonis 1 (CA1) region was observed, along with dysregulation of plasma IL-6, IL-4, IL-10, and IL-1 β levels. Importantly, the interaction of CUMS exposure and time affected behavioral scores and the levels of IL-1 β . Notably, intraperitoneal administration of Brilliant blue G reversed depression-like behaviors and reduced the expression of NLRP3, caspase-1, IL-1 β and IL-18 in the affected hippocampus. **Conclusions:** These findings are consistent with the involvement of P2X7R/NLRP3 signaling in hippocampal impairment and inflammation/immune dysregulation in the context of depression-like behaviors induced by CUMS. In particular, behavioral scores may be affected by the interaction between CUMS exposure and time.

Keywords: stroke; depression; stress; P2X7 receptor; NLRP3 protein; microglia; hippocampus

1. Introduction

Poststroke depression (PSD) is an affective disorder that emerges in the aftermath of a stroke and is characterized primarily by diminished euphoria, reduced interest, low mood, feelings of worthlessness, and disrupted sleep patterns [1,2]. The prevalence of PSD varies, ranging from 23.30% to 36.0% among individuals recently affected by a stroke [2–4]. In more than 80% of related study, the presence of affective disorders at the time of the stroke correlated with the worsening of symptoms during subsequent follow-up [5]. Furthermore, PSD not only adversely affects quality of life and the ability of affected individuals to engage in daily activities but also increases the likelihood of recurrent strokes, morbidity, and mortality [1,4,5].

To identify the optimal strategies for modeling chronic stress exposure following stroke, we previously conducted a systematic review of rodent depression models and related assessment methods [6] and reported pertinent experimental findings [7]. According to the literature [8,9], 6–12 weeks of chronic unpredictable mild stress (CUMS) exposure is typically required for the induction of depression-like behavior in rats, whereas other reports have shown that only 4 weeks of CUMS exposure may be necessary [10,11]; however, the outcomes are contingent upon the particular protocol utilized. Ischemic stroke can induce depression-like phenotypes in rats; however, these phenotypes are typically brief in duration and tend to resolve within one week [6,12]. Therefore, a stroke-only model is not suitable



for long-term studies of PSD-like phenotypes. Combining stroke induction with CUMS exposure is a frequently used method for generating PSD models and often reduces the duration of CUMS exposure required to induce PSD symptoms [6]. Researchers believe that this phenomenon may be related to the fact that short-term stress exposure can exacerbate damage after stroke [13]. However, whether the duration of chronic stress or CUMS exposure determines whether depression-like behavior develops after stroke remains unknown. In particular, to date, no studies have reported whether depressive-like behavior in rats subjected to stroke is affected by the interaction between stress and time.

Purinergic ligand-gated ion channel 7 receptor (P2X7R) is an adenosine triphosphate (ATP)-dependent cationic channel protein expressed at varying levels on the membranes of neurons, glia, and endothelial cells [14]. Pathological stimuli such as hypoxia, inflammation, and hypoglycemia have been shown to trigger ATP release into the intercellular space [14,15]. The subsequent excessive accumulation of ATP induces the opening of the membrane receptor P2X7, leading to intracellular K^+ efflux and the activation of the nucleotide-binding oligomerization domain, leucine-rich repeats, and pyrin domain-containing protein 3 (NLRP3) inflammasome during neurovascular injury [14,16]. The NLRP3/caspase-1 pathway is a well-known molecular signaling cascade associated with pyroptosis [17,18]. In this pathway, interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) are released into the extracellular space following structural alterations in gasdermin D, amplifying local inflammation, cell swelling, rupture, and, ultimately, cell death [19]. The study has shown that in both rat and mouse ischemic brain models, the expression of *P2X7R* mRNA is significantly upregulated in the ischemic hemisphere, including the ischemic penumbra, core ischemic region, and adjacent nonischemic brain tissue [20]. Further study has shown that focal cortical ischemia in mice leads to the activation of P2X7R/NLRP3 signaling around lesions [21]. Moreover, intraventricular injection of a P2X7R-specific nanobody inhibits Caspase-1 activity and reduces IL-1 β expression, thus alleviating ischemic brain injury in mice [22]. Additionally, in generalized depression models, chronic stress increases the expression of P2X7R and NLRP3 in the hippocampus of rodents [15,23,24]. Further research has demonstrated that blocking P2X7R with brilliant blue G (BBG) significantly inhibits pyroptosis pathways, thereby reducing inflammation and depressive-like behaviors in rodents [25–27]. These findings suggest that the activation of the P2X7R/NLRP3 signaling pathway is closely associated with the onset of depression-like behaviors. However, to our knowledge, no studies have explored whether P2X7R/NLRP3 signaling mediates depression-like behaviors induced by chronic stress after ischemic stroke.

Previous studies have also highlighted the involvement of sensitized microglia in mediating central immune and inflammatory processes in response to both endoge-

nous and exogenous stressors [28,29]. Upon exposure to inflammatory factors, heat shock responses, or oxidative stress, resting microglia are activated, experience microenvironmental changes, and transform into phagocytic amoeboid cells [28–30]. Our previous work showed that CUMS promotes microglial sensitization in the ischemic corpus striatum and frontal lobe of rats subjected to stroke [7]. However, a comprehensive understanding of the dynamic changes in hippocampal microglia and the associated inflammatory responses is lacking. The aim of this study was to elucidate the potential mechanisms of PSD to address the limitations of prior studies.

2. Materials and Methods

2.1 Animals

Specific-pathogen-free male Sprague–Dawley rats were obtained from Hunan Slake Jingda Co., Ltd. [Changsha, Hunan, China, certificate No. SYXK (Xiang) 2020-0017]. The body weights of the rats ranged from 250 to 280 g. Throughout the experimental period, all the animals were housed at our institution's animal research center at a constant humidity and temperature with consistent lighting conditions, and they were acclimated to the environment for 1 week before the experiments. During this acclimatization period, the rats were provided unrestricted access to food and water.

2.2 Instruments and Reagents

A protein electrophoresis system (MINI-PROTEAN3; Bio-Rad, Hercules, CA, USA), Chemiluminescence detection system (1708370; Bio-Rad), Image Lab software (6.0.1; Bio-Rad), a high-speed refrigerated centrifuge (3.16R; Hengnuo, Changsha, Hunan, China), a fluorescence microscope (BA410T; Motic, Xiamen, Fujian, China), a filament (2636-A4; Xinon, Beijing, China), ImageJ software (V.2.0.0; NIH, Bethesda, MD, USA) and Microwave oven (M1-L202B; Midea, Foshan, Guangdong, China) were used in this study.

The main reagents used in this study were as follows. Radioimmunoprecipitation assay (RIPA) buffer (KGB5203; KeyGen, Nanjing, Jiangsu, China), BBG (B0770; Sigma-Aldrich, St. Louis, MO, USA) and 4% paraformaldehyde (G1101; Servicebio, Wuhan, Hubei, China) was used. Primary antibodies against P2X7R (PA5-77665, rabbit anti-rat, Thermo Fisher Scientific, Waltham, MA, USA), NLRP3 (ab263899, rabbit anti-rat, Abcam, Cambridge, MA, USA), Iba-1 (MA5-27726, mouse anti-rat, Thermo Fisher Scientific), caspase-1 (AF5418, rabbit anti-rat, Affinity, Nanjing, Jiangsu, China), IL-18 (DF6252, rabbit anti-rat, Affinity), IL-1 β (A20527, rabbit anti-rat, ABclonal), and GAPDH (AF7021, rabbit anti-rat, Affinity), as well as goat anti-mouse (SA00001-1) and goat anti-rabbit (SA00001-2) IgG (both from Proteintech, Chicago, IL, USA) were used. 4',6-Diamidino-2-phenylindole (DAPI) staining solution (AWC0293a; Abiwell, Changsha, Hunan,

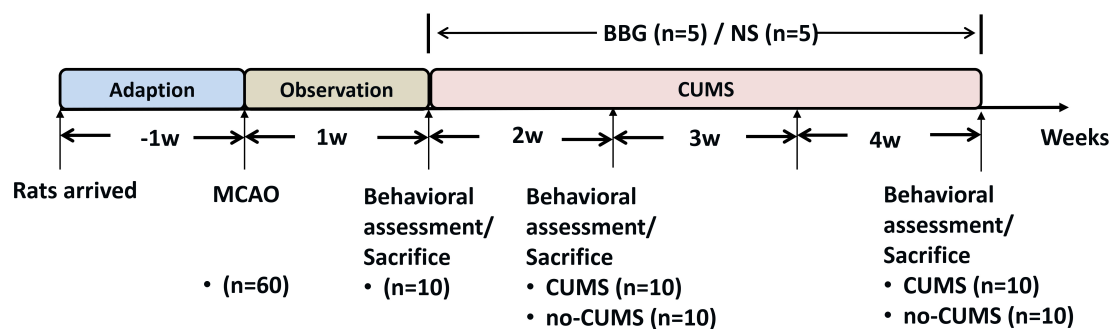


Fig. 1. Diagram of the experimental procedures. BBG, brilliant blue G; NS, normal saline; CUMS, chronic unpredictable mild stress; MCAO, middle cerebral artery occlusion.

China) and IL-6 (E-EL-R0015c), IL-4 (E-EL-R0014c), IL-10 (GER0003) and IL-1 β (GER0002) kits (all from Servicebio) were also used.

In addition, there are some other auxiliary reagents and consumables, such as: 75% alcohol (67182260463; Annjet, Dezhou, Shandong, China), 0.9% physiological saline (XY4950; QiDuYaoYe, Zibo, Shandong, China), sodium pentobarbital (BC1040; Protein Biotechnology, Beijing, China), EDTA Buffer (CM12684; Proteintech), nonfat dry milk (GC310001; Servicebio), phosphate buffered saline with tween (PBST, G2157-1L; Servicebio, Wuhan, China), goat serum (50012-8615; Everygreen, Hangzhou, Zhejiang, China), sucrose water (XY4797; JinJianYaoYe, Changsha, Hunan, China), Pure water (G4701-500ML; Servicebio), antifuorescence quencher (G1401-5ML; Servicebio), phosphate buffered saline (PBS, BL302A; Biosharp, Hefei, Anhui, China), Xylene (N1-5L; Taikangyiliao, Wuhan, Hubei, China), Paraffin (C0175A-500g; Beyotime, Shanghai, China), and eppendorf (EP) tube (EP-150-M; Servicebio).

2.3 Animal Grouping

Following a one-week adaptation period, the rats were subjected to right middle cerebral artery occlusion (MCAO). The model rats were subsequently divided into three groups, the stroke ($n = 30$), stroke + CUMS ($n = 20$) and stroke + CUMS + intervention ($n = 10$) groups, utilizing random numbers generated by SPSS 24.0 software (IBM Corp., Chicago, IL, USA). To determine the impacts of CUMS exposure for different durations, the rats in the stroke + CUMS group were further divided into two subgroups and examined at 2 weeks ($n = 10$) or 4 weeks ($n = 10$) after MCAO. The stroke-only group was divided into three subgroups and studied at 1 week ($n = 10$), 2 weeks ($n = 10$), or 4 weeks ($n = 10$) after MCAO. Notably, the data collected from rats that had not undergone CUMS at one week after MCAO were used as common baseline data for the stroke + CUMS and stroke-only groups. Notably, two subsets of rats in the stroke + CUMS + intervention group were administered either normal saline (NS; $n = 5$) or BBG ($n = 5$) at the beginning of the CUMS protocol (Fig. 1).

2.4 Models

2.4.1 Middle Cerebral Artery Occlusion (MCAO)

MCAO was induced following the protocol outlined in our prior publication [7], with minor adjustments. Briefly, the rats were anesthetized by an intraperitoneal injection of 1% sodium pentobarbital (6 mL/kg) and placed in the supine position on a surgical platform, with their limbs secured. The hair on the right side of the neck was trimmed, and the skin was disinfected with choroidal iodine. The soft tissue was subsequently gently dissected along a longitudinal incision made in the neck skin. The experimenter meticulously ligated the starting point of the right external carotid artery and the proximal end of the common carotid artery. MCAO was induced by inserting a rounded, dull filament through a small oblique incision in the common carotid artery into the internal carotid artery. The ischemia duration was 70 minutes, and cerebral blood flow and rectal temperature were continuously monitored throughout the procedure. The rectal temperature was carefully monitored and maintained at 37 °C during the surgery. The rats that died during or after MCAO were categorized as failed models. In instances of failure, an equivalent number of additional rats were included to maintain a consistent number in the experimental cohort.

2.4.2 Chronic Unpredictable Mild Stress (CUMS)

PSD was induced by CUMS exposure in conjunction with isolation beginning one week after MCAO. The CUMS paradigm, a very popular and well-developed protocol, involves exposure to various stressors [7]: (1) circadian disruption for 36 hours, (2) water deprivation for 18 hours, (3) fasting for 20 hours, (4) exposure to a humid environment for 12 hours, (5) tilting of the cage at a 45° angle for 17 hours, (6) horizontal oscillation at a frequency of 90–100 cycles/min for 5 minutes, (7) noise stimulation at 80 dB for 10 minutes, (8) tail pinching for 1 minute, (9) forced swimming for 5 minutes in 4 °C water, and (10) space restriction for 2 hours. One of the first five “long” stimuli was randomly paired with one of the last five “short” stimuli daily. Stroke model rats were subjected to two of these stressors daily over a 3-week period.

2.5 Intraperitoneal Injection of Brilliant Blue G

A 7 mg/mL solution of BBG was prepared in sterile physiological saline and stored in the dark at 4 °C. This solution was injected into the peritoneal cavity of rats at a standard dose of 50 mg/kg. One week after MCAO, the rats in the stroke + CUMS + BBG group received daily injections of BBG solution beginning 30 minutes after the cessation of stress exposure. Physiological saline served as the control in the stroke + CUMS + NS group. The duration of drug injection was 3 weeks.

2.6 Neurological Deficit Score

As previously described [31], neurological deficit scores were assessed 12 hours after MCAO using the Longa method. A score of 0 represented walking in a straight line, indicating no neurological deficit, whereas a score of 4 represented an inability to walk or impairment of consciousness, indicating very severe neurological impairment and possible death. Therefore, rats with scores ranging from 1 to 3 were deemed successful models of ischemic stroke, whereas those with scores of 0 or 4 were excluded from the analysis.

After ~15% of the MCAO model rats were excluded based on the experimental requirements, a total of sixty rats were included in the statistical analysis.

2.7 Assessment of Body Weight and Behavior

Prior to testing, all the model rats were allowed to become familiar with and adapt to the testing environment. After each experiment, the equipment was thoroughly cleaned with 75% alcohol to eliminate any residual odor from the preceding animal. Behavioral assessments were consistently conducted at predetermined times to mitigate the potential impact of circadian rhythm variations.

2.7.1 Body Weight Measurements

Rat body weight measurements were conducted between 8:00 AM and 12:00 PM.

2.7.2 Open Field Test (OFT)

An uncovered box measuring 100 cm length × 100 cm width × 50 cm height in size was constructed. The box was divided into twenty-five 20 cm × 20 cm squares by drawing white lines on the bottom of the box. For the open field test (OFT), the model animals were gently placed in the central region of the box in a quiet room under low light conditions (approximately 15 lux), and their activity was meticulously observed for 10 minutes. During the test, behaviors, including the numbers of grid crossings and rearings, were quantified. The number of grid crossings was evaluated by determining the number of times a rat traversed a line on the bottom of the box. Similarly, the number of rearings was determined by evaluating the number of times a rat assumed an upright standing position [6].

2.7.3 Novelty-Suppressed Feeding Test (NSFT)

The novelty-suppressed feeding test (NSFT) was conducted in a different environment than the OFT. A new box with dimensions of 65 cm length × 35 cm width × 25 cm height was constructed as described in the literature, with slight modifications. Prior to the test, the rats were fasted for 16 hours. The formal test was divided into 2 stages. Three fresh biscuits were positioned on a white paper pad in the center of a sanitized cage, which served as the testing area. The rats were placed in a corner of the cage with their backs turned to the food. In the first stage, the time required for the rat to consume the first bite of food within 5 minutes (latency) was recorded. If the rat did not eat within 5 minutes, the latency was recorded as 5 minutes. In the second stage, after the first bite of food, the rat was immediately transferred to another identical cage. The food intake of the rats was measured within 10 minutes [6,32].

2.7.4 Sucrose Preference Test (SPT)

On the first day, two bottles of 1% sucrose water (200 mL each) were provided. On the second day, one bottle containing pure water and another containing 1% sucrose water were provided. Both water and food were withheld for 12 hours prior to the assessment. During the evaluation, 200 mL of pure water and 200 mL of 1% sucrose water were administered. The quantity of each liquid consumed by the rats over a 12-hour period was measured and analyzed. The sucrose preference index (SPI) was calculated using the following formula: $SPI = \frac{(\text{amount of 1\% sucrose water consumed})}{(\text{amount of 1\% sucrose water consumed} + \text{amount of pure water consumed})} \times 100\%$ [6].

All behavioral assessments were performed by two evaluators. First, rats with a suspected neurological deficit score of 0 or 4 after MCAO were excluded. Second, the behavior of the rats in the OFT, NSFT, and sucrose preference test (SPT) (in that order) was scored to minimize the influence of the previous tests on subsequent tests. These three tests took three days to complete. The average of the two scores for the OFT was analyzed. Body weight measurements, the NSFT, and the SPT were performed by one person, with another person supervising the procedure. All scorers were blinded to the experimental groups of the rats.

2.8 Preparation of Paraffin Sections and Immunofluorescence Staining

The rats (n = 5 per group) were anesthetized with 1% sodium pentobarbital at 1 week, 2 weeks, and 4 weeks after MCAO. Pentobarbital was administered by intraperitoneal injection into the lower left abdominal quadrant at a dose of 150 mg/kg. The animals were observed for 3–5 minutes. Death was confirmed by loss of consciousness, cessation of respiration and heartbeat, and absence of the pupillary light reflex. The cardiac apex was accessed via a venous indwelling needle, which was then inserted into the left ventricle to the ascending aorta. The right auricle was

subsequently cut, and 200 mL of normal saline was rapidly infused. Perfusion with 200 mL of 4% paraformaldehyde was terminated upon observing the stiffness of the tail or extremities. The brains were extracted and immersed in 4% paraformaldehyde overnight in a refrigerator set at 4 °C. Finally, the brain tissue was dehydrated and embedded in paraffin. The embedded tissue was subsequently systematically cut into 5 µm thick slices.

The tissue slices were dewaxed in xylene and subsequently hydrated in graded ethanol solutions. Antigen retrieval was performed by heating the slices in EDTA buffer in a microwave. After deparaffinization with xylene and rehydration in graded ethanol solutions, the slices were subjected to a second round of antigen retrieval with EDTA buffer in a microwave. The slices were subsequently covered completely with 10% goat serum at room temperature to block nonspecific binding. After one hour, the sections were incubated overnight at 4 °C with primary antibodies against P2X7R (1:50), NLRP3 (1:50), or Iba-1 (1:50). After primary antibody incubation, the slices were incubated with 50 µL of secondary antibody (1:100) in an opaque box for 1 hour at 37 °C. Excess antibody was removed, and the sections were thoroughly washed with PBS. Finally, the cell nuclei were incubated with DAPI working solution at 37 °C for 15 minutes. An antifluorescence quencher was applied, and the slices were mounted. Staining in the CA1 region was visualized under a fluorescence microscope and imaged in a dark environment. ImageJ software was used to quantify the fluorescence intensity per unit area in the CA1 region. The fluorescence intensities of P2X7R, NLRP3, and Iba-1 were calculated using DAPI as a reference and normalized.

2.9 Western Blotting

The remaining rats were euthanized with 1% sodium pentobarbital at 1 week, 2 weeks, and 4 weeks after MCAO. Affected hippocampal tissues preserved at -80 °C were fully lysed in RIPA lysis buffer, followed by ultrasonication on ice and centrifugation (12,000 r/min) for 15 minutes at 4 °C. The carefully collected supernatant was mixed with 5× SDS protein loading buffer and boiled for 5 minutes at 100 °C. After cooling, the sample was stored at -20 °C. Protein concentrations were determined through ultraviolet absorptiometry (A280). The proteins were subsequently separated via SDS-PAGE at different voltages and then transferred onto a PVDF membrane (BS-PVDF-45; Biosharp) at 4 °C at a constant voltage. The PVDF membrane was blocked with a PBS solution containing 5% nonfat dry milk. Afterward, the PVDF membrane was immersed in an aqueous mixture containing polyclonal anti-caspase-1 (1:1000), polyclonal anti-NLRP3 (1:1000), polyclonal anti-IL-18 (1:1000), polyclonal anti-IL-1β (1:1000), polyclonal anti-P2X7R (1:2000), or monoclonal anti-Iba-1 (1:3000) antibodies. After an overnight incubation at 4 °C, PBST was used to wash away the excess primary anti-

body. The membrane was then immersed in a diluent containing secondary antibody (1:3000) at room temperature for 1 hour. GAPDH (1:10,000) served as a reference protein for quantification, and Image Lab software was used to analyze the gray values of the bands.

2.10 Analysis of IL-6, IL-4, IL-10 and IL-1β Levels via Enzyme-Linked Immunosorbent Assays (ELISAs)

Following anesthesia (as described in Section 2.8), blood samples (approximately 1.5 mL) were drawn from the right retroorbital venous plexus of the rats. Samples (n = 5 per group) were collected at 2 and 4 weeks after MCAO and promptly subjected to centrifugation at 4 °C. The resulting plasma was aliquoted into EP tubes and stored at -80 °C. For analysis, the samples were diluted tenfold and processed in accordance with the manufacturer's instructions. The plasma levels of IL-6, IL-4, IL-10, and IL-1β were quantified. All the samples were concurrently analyzed by two experienced experimenters who were blinded to the specific grouping of the samples. Subsequently, standard curves were generated, and the concentration of each cytokine was calculated.

2.11 Statistical Methods

The data were analyzed using SPSS 24.0 software. In this study, all the data were tested with the Shapiro-Wilk test and met the criteria for a normal distribution. The data collected at 1 week after MCAO were used as the baseline data before chronic stress induction. The data collected at 2 weeks and 4 weeks after MCAO were standardized by calculating the values for 2 w/1 w (%) and 4 w/1 w (%) after MCAO, respectively. Differences in behavioral scores, the relative gray values of the protein bands and immunofluorescence intensity between the stroke group and the stroke + CUMS group were analyzed using two-way ANOVA. When an interaction between CUMS exposure and time was observed, simple effects analysis was performed, with comparisons between groups or between time points adjusted via Bonferroni correction. When no interaction between CUMS exposure and time was observed, main effects analysis with Bonferroni's post hoc correction was used. Plasma cytokine levels in the stroke group and the stroke + CUMS group were compared via an independent samples *t* test. Similarly, differences in behavioral scores and the relative gray values of protein bands in the stroke + CUMS + NS group and the stroke + CUMS + BBG group were analyzed via an independent samples *t* test. Statistical graphs were produced via GraphPad Prism 9.5.0 (Dotmatrix, San Diego, CA, USA). A *p* value < 0.05 was considered to indicate statistical significance.

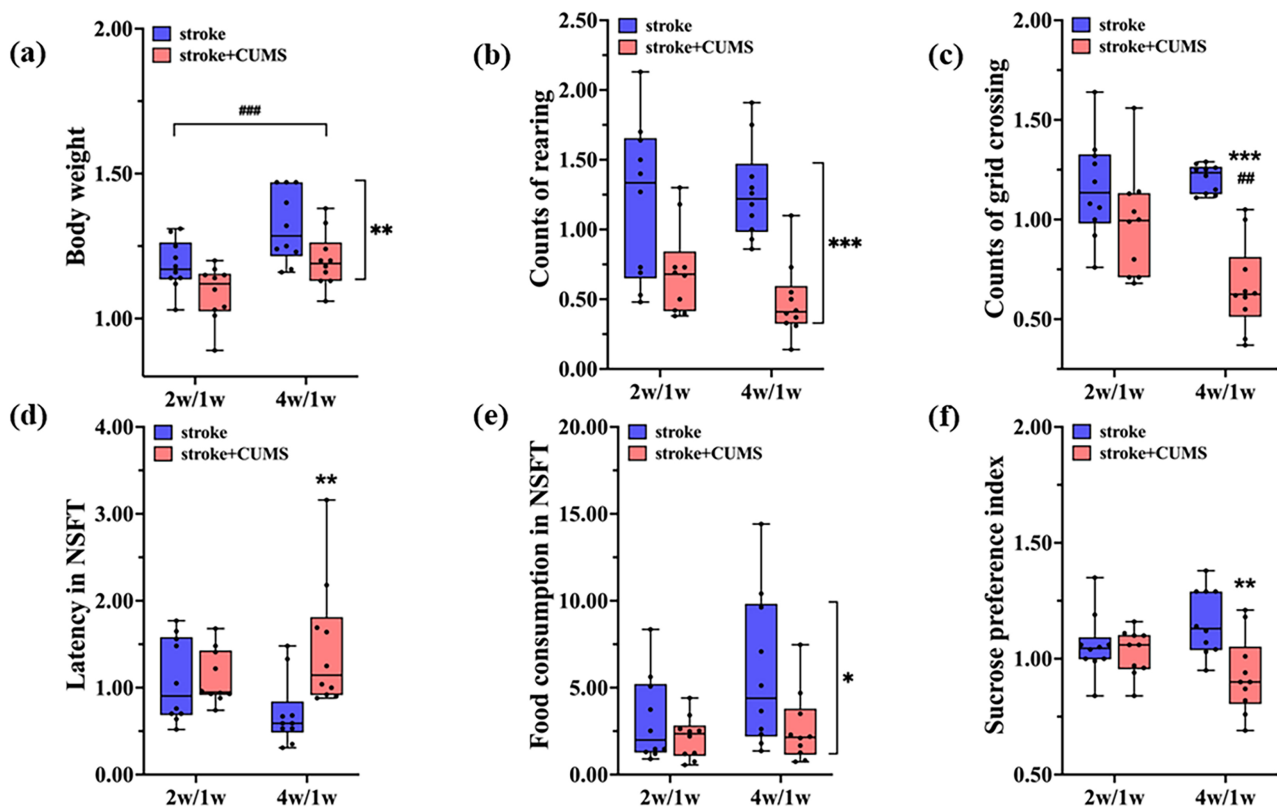


Fig. 2. Effects of CUMS and time on the body weight and behavior of rats subjected to stroke. Effects of CUMS and time on body weight (a) and performance in the OFT (b,c), NSFT (d,e) and SPT (f) in rats with stroke. The data are presented as the means \pm SDs ($n = 10$). In addition to body weight (a), the number of rearings in the OFT (b) and food consumption in the NSFT (e), there was an interaction effect between CUMS and time on the number of grid crossings in the OFT (c), latency in the NSFT (d), and SPI in the SPT (f). The stroke + CUMS group vs. the stroke group: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; 4 w/1 w post-MCAO vs. 2 w/1 w post-MCAO: ## $p < 0.01$, and ### $p < 0.001$. All comparisons were adjusted via Bonferroni's correction. OFT, open field test; NSFT, novelty-suppressed feeding test; SPI, sucrose preference index; SPT, sucrose preference test.

3. Results

3.1 Effects of CUMS and Time on the Body Weight and Behavior of Rats Subjected to Stroke

3.1.1 Body Weight

Body weight is a sensitive indicator of long-term mood disorders; however, conflicting results have been observed in clinical practice. In this study, no interaction effect between CUMS and time on body weight [$F_{(1, 36)} = 0.126, p = 0.724$] was detected. Main effect analysis revealed that CUMS had a significant effect on body weight [$F_{(1, 36)} = 10.889, p = 0.002$]. Specifically, the body weight of the stroke + CUMS group was lower than that of the stroke group ($t = 3.344, p = 0.002$). Main effect analysis also revealed that time had a significant effect on body weight [$F_{(1, 36)} = 14.757, p < 0.001$]. Body weight was significantly greater at 4 weeks after MCAO than at 2 weeks after MCAO ($t = 3.906, p < 0.001$) (Fig. 2a, **Supplementary Table 1**).

3.1.2 OFT

In this study, no interaction effect was detected between CUMS and time on the number of rearings [$F_{(1, 36)} = 1.256, p = 0.270$]. Main effect analysis revealed that CUMS had a significant effect on the number of rearings [$F_{(1, 36)} = 27.282, p < 0.001$]. Specifically, the number of rearings in the stroke + CUMS group was greater than that in the stroke group ($t = 5.236, p < 0.001$). Main effect analysis also revealed that time had no significant effect on the number of rearings [$F_{(1, 36)} = 0.390, p = 0.536$]. Therefore, there was no significant difference in the number of rearings between 2 and 4 weeks after MCAO.

There was an interaction effect between CUMS and time on the number of grid crossings [$F_{(1, 36)} = 6.857, p = 0.013$]. Simple effect analysis revealed that in the stroke + CUMS group [$F_{(1, 36)} = 10.494, p = 0.003$], the number of grid crossings at 4 weeks after MCAO was significantly lower than that at 2 weeks after MCAO ($t = 3.247, p = 0.003$). However, in the stroke group [$F_{(1, 36)} = 0.215, p = 0.646$], there was no obvious difference. Simple effect analysis revealed that at 4 weeks after MCAO [$F_{(1, 36)}$]

= 31.356, $p < 0.001$], the number of grid crossings in the stroke + CUMS group was lower than that in the stroke group ($t = 5.608$, $p < 0.001$). However, at 2 weeks after MCAO [$F_{(1, 36)} = 3.596$, $p = 0.066$], there was no obvious difference (Fig. 2b,c; **Supplementary Table 1**).

Performance in the OFT can reflect the locomotor activity of rats. These findings indicate that stroke model rats exhibit a gradual decrease in their ability to engage in physical activity as the duration of stress increases.

3.1.3 NSFT

Increased latency to feeding and little change in food intake in the NSFT are two important indicators of depressive-like behavior. There was an interaction effect between CUMS and time on the latency in the NSFT [$F_{(1, 36)} = 5.150$, $p = 0.029$]. Simple effect analysis revealed no significant differences in latency between 2 and 4 weeks after MCAO in the stroke group [$F_{(1, 36)} = 2.760$, $p = 0.105$] or the stroke + CUMS group [$F_{(1, 36)} = 2.396$, $p = 0.130$]. Simple effect analysis revealed that at 4 weeks after MCAO [$F_{(1, 36)} = 11.326$, $p = 0.002$], the latency of the stroke + CUMS group was longer than that of the stroke group ($t = 3.367$, $p = 0.002$), whereas at 2 weeks after MCAO [$F_{(1, 36)} = 0.024$, $p = 0.877$], there was no significant difference in latency between the two groups.

There was no interaction effect between CUMS and time on the amount of food consumed in the NSFT [$F_{(1, 36)} = 1.470$, $p = 0.233$]. Main effect analysis revealed that CUMS had a significant effect on the amount of food consumed [$F_{(1, 36)} = 5.596$, $p = 0.024$]. The amount of food consumed in the stroke + CUMS group was lower than that in the stroke group ($t = 2.366$, $p = 0.024$). Main effect analysis also revealed that time had no significant effect on the amount of food consumed [$F_{(1, 36)} = 3.280$, $p = 0.078$]. Therefore, there was no significant difference in food intake between 2 and 4 weeks after MCAO (Fig. 2d,e; **Supplementary Table 1**).

3.1.4 SPT

The sensation of sweet taste is associated with pleasure, and the SPT is used to determine whether a depressed animal is experiencing anhedonia. There was an interaction effect between CUMS and time on the SPI [$F_{(1, 36)} = 5.319$, $p = 0.027$]. Simple effect analysis revealed no significant difference in the SPI between 2 and 4 weeks after MCAO in the stroke group [$F_{(1, 36)} = 2.660$, $p = 0.112$] or the stroke + CUMS group [$F_{(1, 36)} = 2.659$, $p = 0.112$]. Simple effect analysis revealed that at 4 weeks after MCAO [$F_{(1, 36)} = 13.802$, $p = 0.001$], the SPI of the stroke + CUMS group was significantly lower than that of the stroke group ($t = 3.726$, $p = 0.001$). However, this effect was not obvious at 2 weeks after MCAO [$F_{(1, 36)} = 0.206$, $p = 0.653$] (Fig. 2f, **Supplementary Table 1**). These findings suggest the successful establishment of a chronic stress-induced depression model.

3.2 Chronic Stress Increases the Expression of Iba-1, P2X7R and Inflammation-Related Proteins in the Affected Hippocampus

There was no interaction effect between CUMS and time on the expression of Iba-1 [$F_{(1, 16)} = 3.716$, $p = 0.072$], caspase-1 [$F_{(1, 16)} = 0.007$, $p = 0.934$], IL-18 [$F_{(1, 16)} = 1.915$, $p = 0.185$], P2X7R [$F_{(1, 16)} = 0.402$, $p = 0.535$] or NLRP3 [$F_{(1, 16)} = 0.458$, $p = 0.508$]. Main effect analysis revealed that CUMS had a significant effect on Iba-1 [$F_{(1, 16)} = 37.824$, $p < 0.001$], caspase-1 [$F_{(1, 16)} = 14.115$, $p = 0.002$], and NLRP3 [$F_{(1, 16)} = 4.702$, $p = 0.046$] expression. Specifically, the expression of Iba-1 ($t = 6.136$, $p < 0.001$), caspase-1 ($t = 3.758$, $p = 0.002$), and NLRP3 ($t = 2.169$, $p = 0.046$) was significantly higher in the stroke + CUMS group than in the stroke group. However, the same results were not found for IL-18 [$F_{(1, 16)} = 2.408$, $p = 0.140$] or P2X7R [$F_{(1, 16)} = 1.455$, $p = 0.245$] expression (Fig. 3a–g; **Supplementary Table 2**).

Moreover, main effect analysis revealed that time significantly affected the expression of Iba-1 [$F_{(1, 16)} = 13.127$, $p = 0.002$], caspase-1 [$F_{(1, 16)} = 6.442$, $p = 0.022$], and IL-18 [$F_{(1, 16)} = 9.914$, $p = 0.006$] and NLRP3 [$F_{(1, 16)} = 5.194$, $p = 0.037$]. Iba-1 ($t = 3.610$, $p = 0.002$), caspase-1 ($t = 2.539$, $p = 0.022$), IL-18 ($t = 3.150$, $p = 0.006$) and NLRP3 ($t = 2.279$, $p = 0.037$) expression was increased at 4 weeks after MCAO compared with 2 weeks after MCAO. However, the same results were not obtained for P2X7R [$F_{(1, 16)} = 0.754$, $p = 0.398$] (Fig. 3a–g; **Supplementary Table 2**; The original western blot images for Fig. 3a and Fig. 3b are provided in the **Supplementary Figures**).

There was an interaction effect between CUMS and time on the expression of IL-1 β [$F_{(1, 16)} = 22.216$, $p < 0.001$]. Simple effect analysis revealed that the expression levels of IL-1 β in the stroke + CUMS group [$F_{(1, 16)} = 48.498$, $p < 0.001$] were significantly higher at 4 weeks after MCAO than at 2 weeks after MCAO ($t = 6.959$; $p < 0.001$). However, in the stroke group [$F_{(1, 16)} = 0.089$, $p = 0.769$], the difference in IL-1 β expression between the two time points was not obvious. Simple effect analysis also revealed that at 4 weeks after MCAO [$F_{(1, 16)} = 51.700$, $p < 0.001$], the expression of IL-1 β in the stroke + CUMS group was significantly higher than that in the stroke group ($t = 7.181$, $p < 0.001$). However, this difference was not obvious at 2 weeks after MCAO [$F_{(1, 16)} = 0.275$, $p = 0.607$] (Fig. 3h; **Supplementary Table 2**).

Ischemia can induce an inflammatory response in the hippocampus, and chronic stress can exacerbate structural damage, which is involved in mood disorders [33,34]. We observed that the levels of some inflammatory proteins increased with prolonged stress, which may have been at least partially related to increased Iba-1 expression or microglial activation. These results suggest that the levels of some inflammatory markers gradually increase over time, as expected, at least in part.

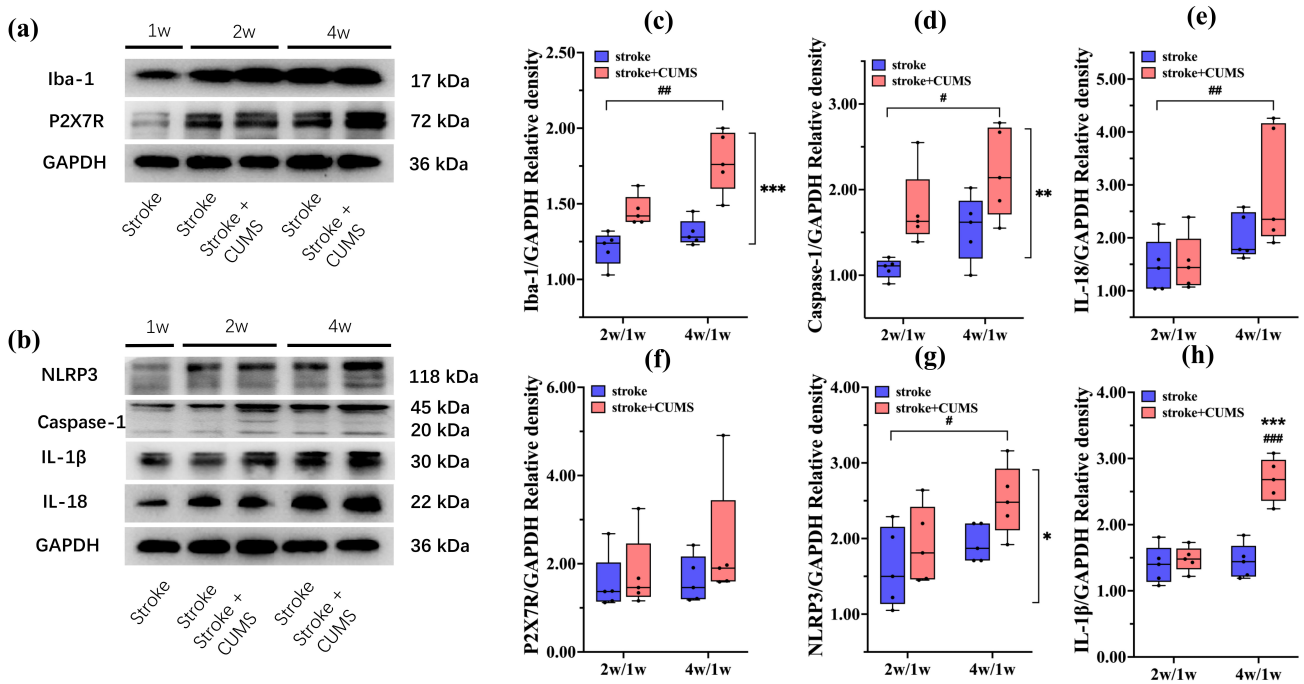


Fig. 3. Relative expression of Iba-1 and P2X7R/NLRP3 signaling pathway-related proteins in the affected hippocampus in the stroke and stroke + CUMS groups. Representative blots showing the levels of Iba-1 and proteins related to the P2X7R/NLRP3 signaling pathway (a,b). Graphs showing the relative protein expression levels of Iba-1 (c), caspase-1 (d), IL-18 (e), P2X7R (f), NLRP3 (g), and IL-1 β (h) are presented. The data are presented as the means \pm SDs ($n = 5$). Unlike for the expression of IL-1 β , there was no interaction effect between CUMS and time on the expression of Iba-1, P2X7R, NLRP3, caspase-1 or IL-18. The stroke + CUMS group vs. the stroke group: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; 4 w/1 w post-MCAO vs. 2 w/1 w post-MCAO: # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$. All comparisons were adjusted via Bonferroni's correction. P2X7R/NLRP3, Purinergic ligand-gated ion channel 7 receptor/nucleotide-binding oligomerization domain, leucine-rich repeats, and pyrin domain-containing protein 3; IL, interleukin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

3.3 Chronic Stress Increases P2X7R, NLRP3 and Iba-1 Expression in the CA1 Region on the Affected Side

The CA1 region, which is closely associated with the depressive phenotype, has structural and functional importance [35]. Our focus was on the distribution and fluorescence signals of three core proteins within this region. Sections were specifically stained for P2X7R (red fluorescence), NLRP3 (red fluorescence), Iba-1 (green fluorescence) and DAPI (blue fluorescence).

We found no interaction effect between CUMS and time on the fluorescence intensity of Iba-1 [$F_{(1, 16)} = 0.789$, $p = 0.388$], NLRP3 [$F_{(1, 16)} = 1.440$; $p = 0.248$], or P2X7R [$F_{(1, 16)} = 1.967$, $p = 0.180$]. These findings are consistent with the results observed for the whole hippocampus. Main effect analysis revealed that CUMS had a significant effect on the fluorescence intensity of Iba-1 [$F_{(1, 16)} = 12.490$, $p = 0.003$], NLRP3 [$F_{(1, 16)} = 8.590$, $p = 0.010$] and P2X7R [$F_{(1, 16)} = 6.113$, $p = 0.025$]. Specifically, the fluorescence intensities of Iba-1 ($t = 3.532$, $p = 0.003$), NLRP3 ($t = 2.930$, $p = 0.010$) and P2X7R ($t = 2.471$, $p = 0.025$) were significantly higher in the stroke + CUMS group than in the stroke group (Fig. 4; Supplementary Table 3).

However, main effect analysis also revealed that time did not significantly affect the fluorescence intensity of Iba-1 [$F_{(1, 16)} = 3.332$, $p = 0.087$], NLRP3 [$F_{(1, 16)} = 4.312$, $p = 0.054$] or P2X7R [$F_{(1, 16)} = 1.616$, $p = 0.222$] (Fig. 4; Supplementary Table 3).

3.4 Chronic Stress Induces Pronounced Dysregulation of Cytokine Secretion Into the Peripheral Blood in Rats With Stroke

Systemic inflammatory responses occur in somatic lesions caused by chronic stress, indicating that peripheral inflammatory markers are potentially valuable for diagnosing or predicting mood disorders. Since the venous blood of rats could not be obtained or did not meet the requirements for effective analysis at 1 week after MCAO, we compared plasma levels of inflammatory factors between only 2 and 4 weeks after MCAO. Compared with the stroke group, the plasma levels of IL-4 ($t = 2.519$, $p = 0.036$) in the stroke + CUMS group were lower 4 weeks after MCAO, but the IL-10 levels ($t = 2.688$, $p = 0.028$) were increased. However, the plasma levels of IL-6 in the stroke + CUMS group were lower than those in the stroke group at both 2 ($t = 7.176$, $p < 0.001$) and 4 weeks ($t = 5.261$, $p < 0.01$) after MCAO.

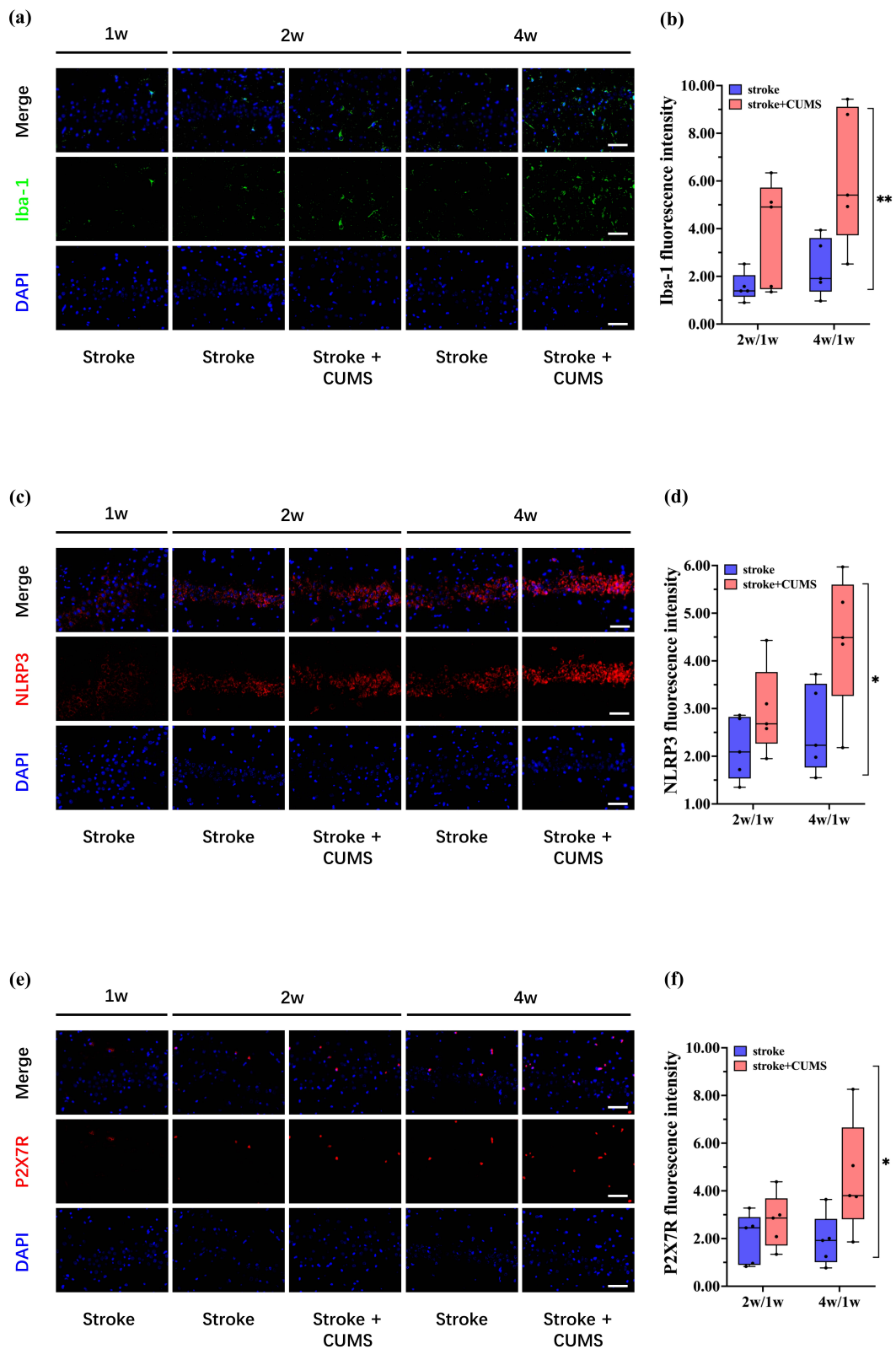


Fig. 4. Comparison of P2X7R, NLRP3 and Iba-1 staining in the affected CA1 region between the stroke and stroke + CUMS groups (400 \times). Images of immunofluorescence staining for P2X7R, NLRP3 and Iba-1 in the hippocampal CA1 region on the affected side. Quantitative analysis of the fluorescence intensities of Iba-1 (a,b), NLRP3 (c,d) and P2X7R (e,f) is shown. The data are presented as the means \pm SDs (n = 5). Scale bar = 100 μ m. There was no interaction effect between CUMS and time on the fluorescence intensity of Iba-1, P2X7R or NLRP3. The stroke + CUMS group vs. the stroke group: * p < 0.05, and ** p < 0.01. All comparisons were adjusted via Bonferroni's correction. DAPI, 4',6-Diamidino-2-phenylindole.

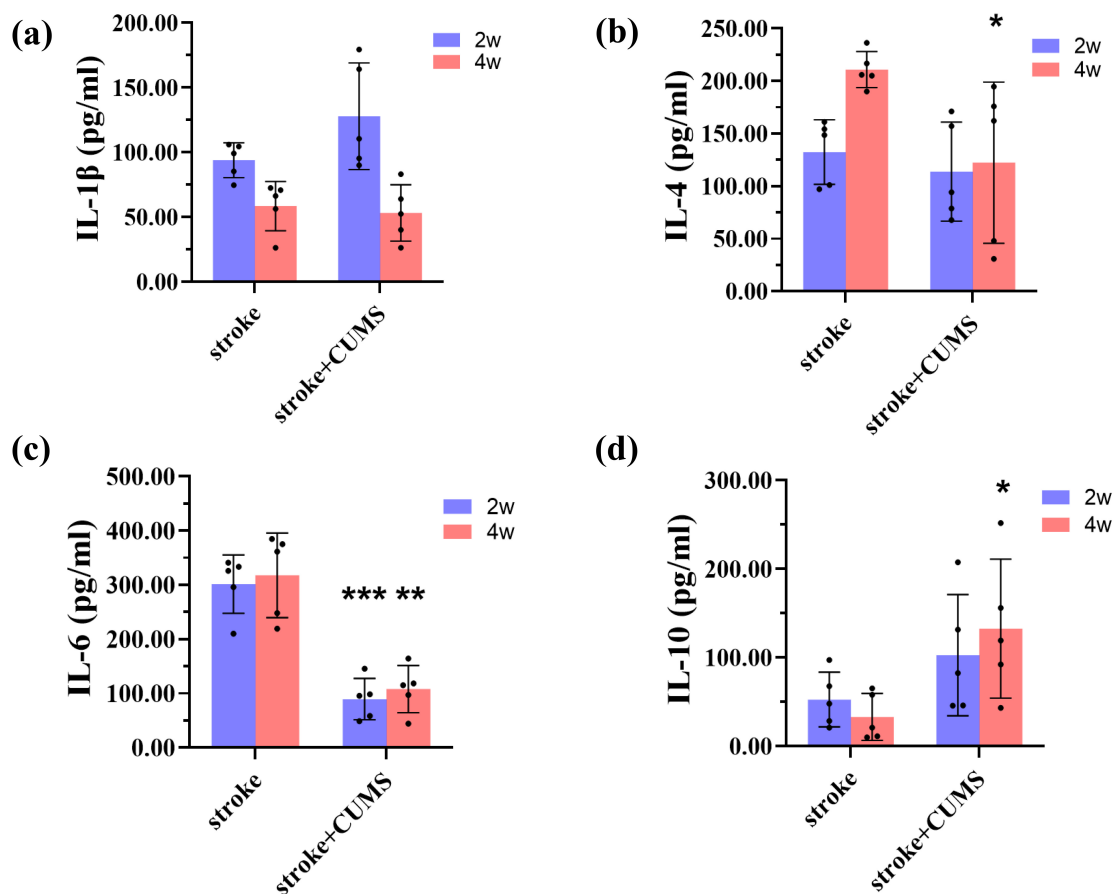


Fig. 5. Comparison of peripheral cytokine levels between the stroke and stroke + CUMS groups. (a–d) ELISA results for IL-1 β , IL-4, IL-6, and IL-10, respectively. The data are presented as the means \pm SDs ($n = 5$). Stroke + CUMS group vs. stroke group: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Interestingly, there was no significant difference in plasma IL-1 β levels between the two groups at 2 or 4 weeks after MCAO, unlike what was observed for hippocampal tissue (Fig. 5a–d).

3.5 BBG Mitigates Depression-Like Behavior While Suppressing Hippocampal Neuroinflammation in Rats With Stroke

To further investigate the role of the P2X7R/NLRP3 signaling pathway in PSD, we inhibited P2X7R on the cell membrane using BBG, and alterations in downstream inflammatory protein expression were assessed. At 4 weeks after MCAO, compared with the stroke + CUMS + NS group, the stroke + CUMS + BBG group presented increased numbers of rearings ($t = 4.706, p = 0.006$) and grid crossings ($t = 3.243, p = 0.012$) in the OFT, an elevated SPI ($t = 2.447, p = 0.040$), and a shortened latency in the NSFT ($t = 3.772, p = 0.005$), with no change in food intake in the NSFT ($t = 0.175, p = 0.866$) (Fig. 6a–d). In addition to reversing the changes in behavioral scores, BBG decreased the expression of IL-1 β ($t = 2.370, p = 0.045$), IL-18 ($t = 2.548, p = 0.034$), NLRP3 ($t = 3.492, p = 0.008$), and caspase-1 ($t = 3.223, p = 0.012$) in the affected hippocampus, whereas the expression of Iba-1 remained unaffected

($t = 1.215, p = 0.252$) (Fig. 6e,f. The original western blot images for Fig. 6e are provided in the **Supplementary Figures**).

4. Discussion

The novel insights from our study are as follows. (1) The hippocampal P2X7R/NLRP3-mediated inflammatory pathway is involved in chronic stress-induced depressive behavior in rats subjected to stroke. (2) A significant CUMS-by-time interaction effect on depression-like behavior was observed in rats subjected to stroke.

The CUMS paradigm is a protocol that is widely used in basic research to induce depression-like behavior in rats subjected to stroke [36]. Our investigation confirmed that 3 weeks of exposure to chronic stress induced depression-like behaviors in rats subjected to stroke. These behaviors included a reduced SPI, a prolonged latency and diminished food intake in the NSFT, and decreased locomotor activity in the OFT and were accompanied by a decline in body weight. These outcomes are consistent with findings from previous studies [7,37]. In this study, the OFT was conducted under dim lighting [38], and the reduction in locomotor activity may have been related to the systemic

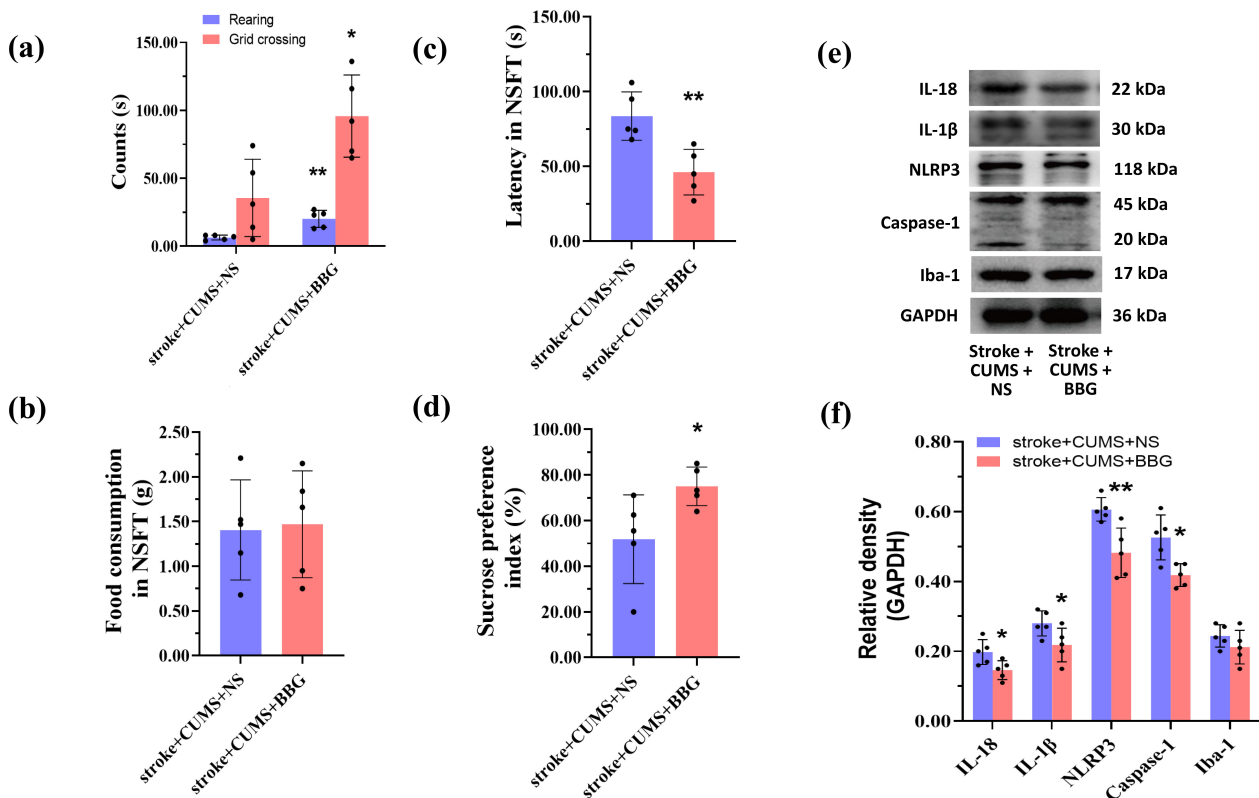


Fig. 6. BBG alleviates depression-like behaviors and mitigates hippocampal neuroinflammation in rats with stroke. Statistical analysis of the behavioral scores of rats subjected to stroke in the OFT (a), NSFT (b,c), and SPT (d). Western blot bands for inflammatory markers and statistical analysis of the relative gray values of the bands are displayed in (e) and (f), respectively. The data are presented as the means \pm SDs (n = 5). Stroke + CUMS + BBG group vs. stroke + CUMS + NS group: * $p < 0.05$ and ** $p < 0.01$.

inflammatory response induced by CUMS [39]. In contrast to previous studies, we assessed behavioral scores at three time points. Specifically, the score at one week after MCAO served as the baseline level before CUMS exposure. We report for the first time that behavioral scores and protein expression at two and four weeks after MCAO are affected by the interaction between CUMS and time. This study fills a gap in this field.

We analyzed the expression of P2X7R and various classic pyroptosis pathway-related proteins, including NLRP3, caspase-1, IL-1 β , and IL-18. The levels of these proteins were significantly increased after 3 weeks of CUMS compared with after stroke alone. In a focal cerebral ischemia mouse model, a notable increase in cortical P2X7R expression not only led to NLRP3 pathway sensitization but also triggered extensive neuronal apoptosis [21]. Similarly, in a general depression model, CUMS was shown to increase the levels of P2X7R and NLRP3 in the rodent hippocampus [23,24]. However, blockade of the membrane-anchored protein P2X7R has been shown to attenuate brain tissue damage induced by ischemic stroke or chronic stress [22,23]. Numerous studies of various disease states have confirmed the critical role of classic pyroptotic pathways mediated by the P2X7R/NLRP3 inflammasome in ischemic stroke and depression models [22–24]. In con-

trast to the models used in previous studies, our model involved both MCAO and CUMS exposure, allowing it to simulate the impact of additional stressors on secondary mood disorders in stroke survivors. We found that BBG inhibited NLRP3/caspase-1-related protein expression and reversed depressive-like behavior. This study revealed that chronic stress increases the risk of depression in rats subjected to stroke and that this effect is associated with the activation of P2X7R/NLRP3 signaling in the hippocampus on the affected side. Similarly, fluorescence staining for two key proteins, P2X7R and NLRP3, in the CA1 region revealed expression patterns generally consistent with those observed for hippocampus-related proteins. These findings further confirm the role of inflammation in the CA1 region in depressive-like behavior.

In addition to investigating the P2X7R/NLRP3 signaling pathway, we investigated hippocampal microglia and detected an increase in Iba-1 expression after 3 weeks of CUMS. Pathologically, a substantial increase in the number of microglia was observed in the CA1 region, and this alteration was predominantly evident in the group subjected to stroke and 3 weeks of CUMS exposure. As crucial immunocytes in the central nervous system, microglia play a critical role in the phagocytosis of cellular fragments and harmful metabolites, thereby mitigating the adverse effects

of intrinsic and extrinsic factors on neurons [40]. However, overactivation of microglia leads to the continuous production of cytotoxic products, such as reactive oxygen species and proinflammatory cytokines, exacerbating local damage within the microenvironment. This study highlights the pivotal involvement of hippocampal microglia in depressive-like behaviors induced by CUMS in rats subjected to stroke, which is consistent with findings from previous study [41].

In contrast to previous studies, in this study, behavioral scores and the expression of related proteins were investigated at different times before and after CUMS exposure. CUMS and time had interaction effects on performance in the OFT (number of grid crossings), NSFT (latency) and SPT (SPI). Thus, we conclude that fundamental indices of depressive behavior are not impacted solely by CUMS but are intricately linked to the duration of stress exposure. By reviewing the literature, we found that 4 to 12 weeks of chronic stress exposure is required to induce depressive-like behavior in stroke-free rats [8–11]. Notably, stroke model rats exhibited depressive-like behavior after CUMS exposure for a significantly shorter timeframe of 3 weeks. These findings may be related to the important role that different stress protocols play in this process. However, the results of the present study preliminarily verified the interaction between CUMS and the duration of stress exposure, resulting in the development of depressive-like behavior in stroke model rats over a relatively short period. Additionally, at the mechanistic level, our study reveals that CUMS and time have an interaction effect only on hippocampal IL-1 β levels. The expression of other proteins was minimally affected by the CUMS–time interaction, and correlation analysis of behavioral scores revealed no significant results (data not shown). These findings may be attributed to the small sample size, detection limitations, and substantial individual variability after surgery. Specifically, prolonged stress exposure correlated with increased inflammatory responses within the hippocampus. Nevertheless, within the broader context of the entire hippocampus and in the CA1 region, the expression of P2X7R was not affected by the CUMS–time interaction. This finding is similar to those of previous reports [42]. Additionally, these observations may be attributed to the primary biological role of P2X7R as a transmembrane protein (e.g., difficulty in desensitization) [43,44]. Concurrently, our two-way analysis suggested that IL-1 β may be associated with other upstream proteins involved in the development of depressive-like behavior in stroke model rats.

Inflammatory responses and impaired immune function constitute the pathophysiological underpinnings of stress-related injury. Many studies have confirmed the activation of the host immune system by both acute and chronic stressors [45–47]. Stress-related injuries primarily manifest as alterations in cellular and/or humoral immunity [47]. Adaptive changes in immune function, driven by the secretion of substantial amounts of inflammatory cytokines, are characteristic responses to stress [45]. In our investigation,

we determined the plasma levels of four cytokines known for their anti- or proinflammatory effects: IL-6, IL-4, IL-10, and IL-1 β . However, the findings did not reveal a straightforward increase in proinflammatory cytokine levels or a decrease in anti-inflammatory cytokine levels after chronic stress exposure. Notably, divergent trends were observed in the levels of IL-1 β in the periphery and the central nervous system. This paradoxical pattern of inflammatory cytokine expression aligns with extensive reports from previous studies [45,48,49]. For example, Khoury *et al.* [50] reported that chronic stress for 4 weeks did not increase IL-1 β levels and that lower IL-1 β levels were detected in the peripheral blood of stressed rats than in the peripheral blood of control rats. The inconsistency in IL-1 β expression between the peripheral and central systems may be related to differences in the central/peripheral microenvironments and immune regulation. In the central nervous system, microglia are the main immune regulators, although disruption of the blood–brain barrier increases the recruitment of peripheral neutrophils and macrophages [51,52]. In the peripheral blood, the complexity of humoral and cellular immune responses is much greater. This complexity includes whether the activation of central inflammasomes and blood-brain barrier disruption restricts the release of IL-1 β to the periphery, which requires further validation. The increase in IL-6 levels induced by acute stress is attributed primarily to adipocytes, indicating that IL-6 is an endocrine factor associated with cortisol and is involved in reducing tolerance to inflammatory responses [45]. In this study, we observed a discrepancy in peripheral IL-6 levels compared with those reported previously. This difference could be attributed to the negative feedback regulation induced by chronic stress or the stage of the stress process. Although varying degrees of elevated serum IL-10 levels and patterns of IL-10 expression have been observed in stress models [48,49], diminished levels of the anti-inflammatory factor IL-4 following stress are less commonly reported. The contradictory changes observed in the expression of the four inflammatory cytokines after exposure to CUMS compared with stroke alone in our study suggest that chronic stress adversely affects host immunity. Peripheral dysregulation of the inflammatory/immune response may play a pivotal role in mediating the development of depression-like behaviors induced by CUMS in rats with stroke. Finally, it should be noted that due to technical errors in blood collection at one week after MCAO, partial data loss occurred for this group, preventing the standardization of the peripheral inflammatory cytokine data.

Moreover, this study has the following limitations: (1) The duration of chronic stress was limited to either 1 or 3 weeks, and data from other time points were unavailable. (2) Only rats that were not exposed to CUMS served as controls. (3) Phenotypic alterations in microglia were not investigated. The potential infiltration of brain tissue by circulating macrophages, which also express Iba-1, could not be definitively ruled out. (4) The slightly insufficient sam-

ple size in some groups may have affected the statistical power. (5) Underlying mechanisms, such as hippocampal neuronal apoptosis, synaptic plasticity, blood-brain barrier disruption, and peripheral immune cell infiltration, were not validated. (6) Female rats were not included, as significant sex differences in depression susceptibility and stress tolerance have been observed. To gain deeper insight into the biological mechanisms underlying the development of chronic stress-induced PSD-like behavior, more reasonable classification methods or gene knockout could be used in future studies to observe phenotypic changes in microglia in the hippocampus or frontal cortex.

5. Conclusions

These findings are consistent with the involvement of P2X7R/NLRP3 signaling in the affected hippocampus and the dysregulation of inflammatory/immune responses in the manifestation of depression-like behaviors induced by chronic stress in rats subjected to stroke. In particular, a significant interaction effect of CUMS and time on depressive-like behavior was observed in stroke model rats.

Availability of Data and Materials

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

Author Contributions

XT, SW and WT conceived and designed the study. WT, SW, YZ and CY performed the study. YZ, YY, and JH performed data analysis, graphics, and interpretation. XT, YZ, YY and JH wrote the paper. XT, YZ, YY, JH and SW revised the paper for intellectual content. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This research received approval from the Animal Ethics Committee of the People's Hospital of Hunan Province, Hunan Normal University (Approval No. 202418). All procedures were conducted in accordance with the Regulations for the Administration of Laboratory Animals approved by the State Council of the People's Republic of China.

Acknowledgment

Not applicable.

Funding

This work was supported by the Hunan Provincial Natural Science Foundation (2023JJ30349) and Scientific Research Project of Hunan Education Department

(23A0078) to XT, and Scientific Research Project of Hunan Health Committee (202203102866) to JH.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/JIN40005>.

References

- [1] Towfighi A, Ovbiagele B, El Husseini N, Hackett ML, Jorge RE, Kissela BM, *et al.* Poststroke Depression: A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*. 2017; 48: e30–e43. <https://doi.org/10.1161/STR.0000000000000113>.
- [2] Yang F, Zhang P. Prevalence and Predictive factors of Post-Stroke Depression in Patients with Acute Cerebral Infarction. *Alpha Psychiatry*. 2024; 25: 592–597. <https://doi.org/10.5152/alphapsychiatry.2024.231381>.
- [3] Bi H, Wang M. Role of social support in poststroke depression: A meta-analysis. *Frontiers in Psychiatry*. 2022; 13: 924277. <https://doi.org/10.3389/fpsy.2022.924277>.
- [4] Robinson RG. Poststroke depression: prevalence, diagnosis, treatment, and disease progression. *Biological Psychiatry*. 2003; 54: 376–387. [https://doi.org/10.1016/s0006-3223\(03\)00423-2](https://doi.org/10.1016/s0006-3223(03)00423-2).
- [5] Robinson RG, Spalletta G. Poststroke depression: a review. *Canadian Journal of Psychiatry. Revue Canadienne De Psychiatrie*. 2010; 55: 341–349. <https://doi.org/10.1177/070674371005500602>.
- [6] Tao X, Yang W, Zhu S, Que R, Liu C, Fan T, *et al.* Models of poststroke depression and assessments of core depressive symptoms in rodents: How to choose? *Experimental Neurology*. 2019; 322: 113060. <https://doi.org/10.1016/j.expneurol.2019.113060>.
- [7] Tao X, Wu S, Tang W, Li L, Huang L, Mo D, *et al.* Alleviative effects of foraging exercise on depressive-like behaviors in chronic mild stress-induced ischemic rat model. *Brain Injury*. 2022; 36: 127–136. <https://doi.org/10.1080/02699052.2022.2034949>.
- [8] Wang X, Zhang F, Niu L, Yan J, Liu H, Wang D, *et al.* High-frequency repetitive transcranial magnetic stimulation improves depressive-like behaviors in CUMS-induced rats by modulating astrocyte GLT-1 to reduce glutamate toxicity. *Journal of Affective Disorders*. 2024; 348: 265–274. <https://doi.org/10.1016/j.jad.2023.12.068>.
- [9] Tan B, Jiang X, Chen L, Wang R, Wei H. Plasma exosomal miR-30a-5p inhibits osteogenic differentiation of bone marrow mesenchymal stem cells from a chronic unpredictable mild stress-induced depression rat model. *Molecular and Cellular Probes*. 2024; 75: 101957. <https://doi.org/10.1016/j.mcp.2024.101957>.
- [10] Han YX, Tao C, Gao XR, Wang LL, Jiang FH, Wang C, *et al.* BDNF-Related Imbalance of Copine 6 and Synaptic Plasticity Markers Couples With Depression-Like Behavior and Immune Activation in CUMS Rats. *Frontiers in Neuroscience*. 2018; 12: 731. <https://doi.org/10.3389/fnins.2018.00731>.
- [11] Liao D, Lv C, Cao L, Yao D, Wu Y, Long M, *et al.* Curcumin Attenuates Chronic Unpredictable Mild Stress-Induced Depressive-Like Behaviors via Restoring Changes in Oxidative Stress and the Activation of Nrf2 Signaling Pathway in Rats. *Oxidative Medicine and Cellular Longevity*. 2020; 2020: 9268083. <https://doi.org/10.1155/2020/9268083>.

- [12] Kato M, Iwata H, Okamoto M, Ishii T, Narita H. Focal cerebral ischemia-induced escape deficit in rats is ameliorated by a reversible inhibitor of monoamine oxidase-a: implications for a novel animal model of post-stroke depression. *Biological & Pharmaceutical Bulletin*. 2000; 23: 406–410. <https://doi.org/10.1248/bpb.23.406>.
- [13] Lowry CA, Jin AY. Improving the Social Relevance of Experimental Stroke Models: Social Isolation, Social Defeat Stress and Stroke Outcome in Animals and Humans. *Frontiers in Neurology*. 2020; 11: 427. <https://doi.org/10.3389/fneur.2020.00427>.
- [14] von Muecke-Heim IA, Ries C, Urbina L, Deussing JM. P2X7R antagonists in chronic stress-based depression models: a review. *European Archives of Psychiatry and Clinical Neuroscience*. 2021; 271: 1343–1358. <https://doi.org/10.1007/s00406-021-01306-3>.
- [15] Torres-Rodríguez O, Rivera-Escobales Y, Castillo-Ocampo Y, Velazquez B, Colón M, Porter JT. Purinergic P2X7 receptor-mediated inflammation precedes PTSD-related behaviors in rats. *Brain, Behavior, and Immunity*. 2023; 110: 107–118. <https://doi.org/10.1016/j.bbi.2023.02.015>.
- [16] Di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 Receptor in Infection and Inflammation. *Immunity*. 2017; 47: 15–31. <https://doi.org/10.1016/j.immuni.2017.06.020>.
- [17] Shi Y, Yang Y, Liu J, Zheng J. Avicularin Treatment Ameliorates Ischemic Stroke Damage by Regulating Microglia Polarization and its Exosomes via the NLRP3 Pathway. *Journal of Integrative Neuroscience*. 2024; 23: 196. <https://doi.org/10.31083/j.jin2311196>.
- [18] Zhu MJ, Zhang L, Wang CP. Copper Overload Promotes β -amyloid Induced NLRP3/Caspase-1/GSDMD-Mediated Pyroptosis in Alzheimer's Disease. *Journal of Integrative Neuroscience*. 2024; 23: 194. <https://doi.org/10.31083/j.jin2310194>.
- [19] Xu J, Núñez G. The NLRP3 inflammasome: activation and regulation. *Trends in Biochemical Sciences*. 2023; 48: 331–344. <https://doi.org/10.1016/j.tibs.2022.10.002>.
- [20] Challa SR, Levingston H, Fornal CA, Baker IM, Boston J, Shanthappa N, *et al.* Temporal mRNA Expression of Purinergic P2 Receptors in the Brain Following Cerebral Ischemia and Reperfusion: Similarities and Distinct Variations Between Rats and Mice. *International Journal of Molecular Sciences*. 2025; 26: 2379. <https://doi.org/10.3390/ijms26062379>.
- [21] Ye X, Shen T, Hu J, Zhang L, Zhang Y, Bao L, *et al.* Purinergic 2X7 receptor/NLRP3 pathway triggers neuronal apoptosis after ischemic stroke in the mouse. *Experimental Neurology*. 2017; 292: 46–55. <https://doi.org/10.1016/j.expneurol.2017.03.002>.
- [22] Wilmes M, Pinto Espinoza C, Ludewig P, Stabernack J, Liesz A, Nicke A, *et al.* Blocking P2X7 by intracerebroventricular injection of P2X7-specific nanobodies reduces stroke lesions. *Journal of Neuroinflammation*. 2022; 19: 256. <https://doi.org/10.1186/s12974-022-02601-z>.
- [23] Su WJ, Zhang T, Jiang CL, Wang W. Clemastine Alleviates Depressive-Like Behavior Through Reversing the Imbalance of Microglia-Related Pro-inflammatory State in Mouse Hippocampus. *Frontiers in Cellular Neuroscience*. 2018; 12: 412. <https://doi.org/10.3389/fncel.2018.00412>.
- [24] Zhang WY, Guo YJ, Han WX, Yang MQ, Wen LP, Wang KY, *et al.* Curcumin relieves depressive-like behaviors via inhibition of the NLRP3 inflammasome and kynurenine pathway in rats suffering from chronic unpredictable mild stress. *International Immunopharmacology*. 2019; 67: 138–144. <https://doi.org/10.1016/j.intimp.2018.12.012>.
- [25] Yue N, Huang H, Zhu X, Han Q, Wang Y, Li B, *et al.* Activation of P2X7 receptor and NLRP3 inflammasome assembly in hippocampal glial cells mediates chronic stress-induced depressive-like behaviors. *Journal of Neuroinflammation*. 2017; 14: 102. <https://doi.org/10.1186/s12974-017-0865-y>.
- [26] Wang C, Cui C, Xie X, Chen B, Feng L, Jiang P. Calcitriol attenuates lipopolysaccharide-induced neuroinflammation and depressive-like behaviors by suppressing the P2X7R/NLRP3/caspase-1 pathway. *Psychopharmacology*. 2024; 241: 1329–1343. <https://doi.org/10.1007/s00213-024-06565-1>.
- [27] Li S, Zhang Y, Wang Y, Zhang Z, Xin C, Wang Y, *et al.* Transcutaneous vagus nerve stimulation modulates depression-like phenotype induced by high-fat diet via P2X7R/NLRP3/IL-1 β in the prefrontal cortex. *CNS Neuroscience & Therapeutics*. 2024; 30: e14755. <https://doi.org/10.1111/cns.14755>.
- [28] Cao P, Chen C, Liu A, Shan Q, Zhu X, Jia C, *et al.* Early-life inflammation promotes depressive symptoms in adolescence via microglial engulfment of dendritic spines. *Neuron*. 2021; 109: 2573–2589.e9. <https://doi.org/10.1016/j.neuron.2021.06.012>.
- [29] Chen L, Zeng L, Li W, Li JS. The Activation of Hippocampal Microglial Cells and Their Role in the Regulation of Pain. *Journal of Integrative Neuroscience*. 2025; 24: 27730. <https://doi.org/10.31083/JIN27730>.
- [30] Zhang J, He H, Qiao Y, Zhou T, He H, Yi S, *et al.* Priming of microglia with IFN- γ impairs adult hippocampal neurogenesis and leads to depression-like behaviors and cognitive defects. *Glia*. 2020; 68: 2674–2692. <https://doi.org/10.1002/glia.23878>.
- [31] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989; 20: 84–91. <https://doi.org/10.1161/01.str.20.1.84>.
- [32] Vahid-Ansari F, Lagace DC, Albert PR. Persistent post-stroke depression in mice following unilateral medial prefrontal cortical stroke. *Translational Psychiatry*. 2016; 6: e863. <https://doi.org/10.1038/tp.2016.124>.
- [33] Zhang LM, Wu ZY, Liu JZ, Li Y, Lv JM, Wang LY, *et al.* Sub-anesthetic dose of S-ketamine improved cognitive dysfunction via the inhibition of hippocampal astrocytosis in a mouse model of post-stroke chronic stress. *Journal of Psychiatric Research*. 2023; 158: 1–14. <https://doi.org/10.1016/j.jpsychires.2022.12.010>.
- [34] Farajdokht F, Oghbaei F, Sadigh-Eteghad S, Majdi A, Aghsan SR, Farhoudi M, *et al.* Cerebrolysin® and Environmental Enrichment, Alone or in Combination, Ameliorate Anxiety- and Depressive-Like Behaviors in a Post-Ischemic Depression Model in Mice. *Journal of Stroke and Cerebrovascular Diseases*. 2022; 31: 106519. <https://doi.org/10.1016/j.jstrokecerebrovasdis.2022.106519>.
- [35] Kumari R, Verma V, Singaravel M. Simulated Chronic Jet Lag Affects the Structural and Functional Complexity of Hippocampal Neurons in Mice. *Neuroscience*. 2024; 543: 1–12. <https://doi.org/10.1016/j.neuroscience.2024.01.026>.
- [36] Tang C, Liu H, Zou H, Su M, Yin H, Sun M, *et al.* Dihydroartemisinin Protects Mice from CUMS-induced Depression-like Behaviors by Regulating Gut Microbes. *Neuroscience*. 2024; 547: 28–36. <https://doi.org/10.1016/j.neuroscience.2023.11.029>.
- [37] Wang SH, Zhang ZJ, Guo YJ, Teng GJ, Chen BA. Decreased expression of serotonin 1A receptor in the dentate gyrus in association with chronic mild stress: a rat model of post-stroke depression. *Psychiatry Research*. 2009; 170: 245–251. <https://doi.org/10.1016/j.psychres.2008.07.006>.
- [38] Neuwirth LS, Verrengia MT, Harikinish-Murrari ZI, Orens JE, Lopez OE. Under or Absent Reporting of Light Stimuli in Testing of Anxiety-Like Behaviors in Rodents: The Need for Standardization. *Frontiers in Molecular Neuroscience*. 2022; 15: 912146. <https://doi.org/10.3389/fnmol.2022.912146>.
- [39] Sun L, Ma S, Yu Y, Li X, Wei Q, Min L, *et al.* Transcutaneous auricular vagus nerve stimulation ameliorates adolescent depressive- and anxiety-like behaviors via hippocampus glycolysis and inflammation response. *CNS Neuroscience & Therapeutics*. 2024; 30: e14614. <https://doi.org/10.1111/cns.14614>.
- [40] Wu X, Wan T, Gao X, Fu M, Duan Y, Shen X, *et al.* Mi-

- croglia Pyroptosis: A Candidate Target for Neurological Diseases Treatment. *Frontiers in Neuroscience*. 2022; 16: 922331. <https://doi.org/10.3389/fnins.2022.922331>.
- [41] Wei L, Guo J, Yu X, Chen H, Du Y, Ji Z, *et al*. Role and characteristics of hippocampal region microglial activation in post-stroke depression. *Journal of Affective Disorders*. 2021; 291: 270–278. <https://doi.org/10.1016/j.jad.2021.05.022>.
- [42] Kongsui R, Beynon SB, Johnson SJ, Mayhew J, Kuter P, Nilsson M, *et al*. Chronic stress induces prolonged suppression of the P2X7 receptor within multiple regions of the hippocampus: a cumulative threshold spectra analysis. *Brain, Behavior, and Immunity*. 2014; 42: 69–80. <https://doi.org/10.1016/j.bbi.2014.05.017>.
- [43] Koshimizu T, Koshimizu M, Stojilkovic SS. Contributions of the C-terminal domain to the control of P2X receptor desensitization. *The Journal of Biological Chemistry*. 1999; 274: 37651–37657. <https://doi.org/10.1074/jbc.274.53.37651>.
- [44] Li Q, Luo X, Zeng W, Muallem S. Cell-specific behavior of P2X7 receptors in mouse parotid acinar and duct cells. *The Journal of Biological Chemistry*. 2003; 278: 47554–47561. <https://doi.org/10.1074/jbc.M308306200>.
- [45] Qing H, Desrouleaux R, Israni-Winger K, Mineur YS, Fogelman N, Zhang C, *et al*. Origin and Function of Stress-Induced IL-6 in Murine Models. *Cell*. 2020; 182: 372–387.e14. <https://doi.org/10.1016/j.cell.2020.05.054>.
- [46] Gu Y, Ye T, Tan P, Tong L, Ji J, Gu Y, *et al*. Tolerance-inducing effect and properties of innate immune stimulation on chronic stress-induced behavioral abnormalities in mice. *Brain, Behavior, and Immunity*. 2021; 91: 451–471. <https://doi.org/10.1016/j.bbi.2020.11.002>.
- [47] Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. *Immunologic Research*. 2014; 58: 193–210. <https://doi.org/10.1007/s12026-014-8517-0>.
- [48] Himmerich H, Fischer J, Bauer K, Kirkby KC, Sack U, Krügel U. Stress-induced cytokine changes in rats. *European Cytokine Network*. 2013; 24: 97–103. <https://doi.org/10.1684/ecn.2013.0338>.
- [49] Pertsov SS, Abramova AY, Chekhlov VV. Effect of Repeated Stress Exposures on the Blood Cytokine Profile in Rats with Different Behavioral Parameters. *Bulletin of Experimental Biology and Medicine*. 2022; 172: 397–401. <https://doi.org/10.1007/s10517-022-05401-w>.
- [50] Khoury RD, Prado RFD, Matos FDS, Meireles BRD, Cardoso FGDR, Oliveira LDD, *et al*. The influence of adrenergic blockade in rats with apical periodontitis under chronic stress conditions. *Archives of Oral Biology*. 2020; 110: 104590. <https://doi.org/10.1016/j.archoralbio.2019.104590>.
- [51] Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science*. 2005; 308: 1314–1318. <https://doi.org/10.1126/science.1110647>.
- [52] Lee J, Cho W, Yu JW, Hyun YM. NLRP3 activation induces BBB disruption and neutrophil infiltration via CXCR2 signaling in the mouse brain. *Journal of Neuroinflammation*. 2025; 22: 139. <https://doi.org/10.1186/s12974-025-03468-6>.