

Original Research

HDAC3 Contributes to Ischemic Stroke by Regulating Interferon Pathway

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Abstract

Background: The inflammation and immune response contribute to ischemic stroke pathology. Damaged brain cells release inflammatory substances to activate the immune system in the acute phase of stroke, including altering the interferon signaling pathway. However, the involvement of histone deacetylation in stroke remains unclear. **Methods:** To investigate whether histone deacetylation modulation could regulate the interferon signaling pathway and mediate the pathogenic changes after stroke, the middle cerebral artery occlusion (MCAO) mouse model was treated with histone deacetylase 3 (HDAC3) inhibitor and RGFP966. Additionally, a series of approaches, including middle cerebral artery occlusion (MCAO), real-time polymerase chain reaction (PCR), western blot, 2,3,5-triphenyltetrazolium chloride (TTC) staining, behavioral experiments, and confocal imaging were utilized. **Results:** It is observed that RGFP966 pretreatment could lead to better outcomes in the MCAO mouse model, including the decrease of infarction volumes, the amelioration of post-stroke anxiety-like behavior, and the relief of inflammatory responses. Furthermore, we found that RGFP966 could counteract the hyperactivation of the interferon signaling pathway and the excessive expression of Z-DNA Binding Protein 1 (ZBP1) in microglia. **Conclusions:** We demonstrated a novel mechanism that HDAC3 inhibition could ameliorate the pathological injury after ischemic stroke by downregulating the ZBP1/phosphorylated Interferon Regulatory Factor 3 (p-IRF3) pathway. Thus, these data provide a new promising target for therapies for ischemic stroke.

Keywords: ischemic stroke; histone deacetylase 3; Z-DNA binding protein 1; phosphorylated Interferon Regulatory Factor 3

1. Introduction

Stroke is a devastating cerebrovascular disease with high mortality and morbidity [1,2]. Ischemic stroke is commonly observed when arteries supplying blood to the brain become stenosed or occluded, resulting in local brain tissue hypoxia and ischemia, neuronal cell death, neuroinflammation, and secondary brain injury in hypoperfusion and ischemia/reperfusion [3–5]. Notably, epigenetic modifications, especially histone deacetylation, were found to be involved in the etiology of ischemia-related neuroinflammation.

HDACs, also known as histone deacetylases, are a class of essential epigenetic regulator enzymes. HDACs can remove the acetyl group from the target protein's lysine-amino group, thereby changing the activity of the protein or affecting gene transcription. Eighteen different HDACs have been discovered in eukaryotes, categorized into two groups based on their active mechanisms: classical HDACs and non-classical HDACs. Notably, the deacetylation ac-

tivity of classical HDACs (classes I, II, and IV) depends on the metal ion Zn^{2+} . At the same time, non-classical HDACs (Sirtuins, SIRT6) are dependent on the coenzyme nicotinamide adenine dinucleotide (NAD^+) [6]. Histone deacetylase 3 (HDAC3) can modulate histone acetylation or deacetylation and regulate cell survival, immunity, and inflammation [7]. Additionally, HDAC3 is crucial in maintaining neuronal plasticity, memory formation, and inflammatory response post-stroke. A robust expression of HDAC3 has been observed in neurons and glia after middle cerebral artery occlusion (MCAO) [8,9]. RGFP966, a selective inhibitor of HDAC3, induces neuroprotective effects in 3 days of transient focal brain ischemia, which was demonstrated by decreased infarct volume and improved neurological deficit scores [10]. Thus, these studies suggested selective inhibition of HDAC3 may benefit stroke injury. However, the regulating mechanism of HDAC3 on stroke injury remains to be elucidated.



Z-DNA Binding Protein 1 (ZBP1), one of the interferon-inducible nucleic acid sensors, plays a vital role in inflammatory cell death and innate immune responses [11]. A previous study has revealed that ZBP1 could recruit Interferon Regulatory Factor 3 (IRF3) in inflammatory lung disease in response to double-stranded DNA (dsDNA) [12]. Thus, the mechanism of ZBP1 recruiting IRF3 might provide novel insight for treating multiple myeloma [13]. Additionally, interferon regulatory factors are a family of transcription factors [14] involved in developing and differentiating distinct immune cells and responding to pathogen infection [15]. The IRF family includes 9 homogeneous isotypes with a common helix-turn-helix DNA binding motif. Notably, IRF-3 is a vital member of the IRF family, whose transcriptional activity is only influenced by post-translational alterations [14]. However, the role of the ZBP1/IRF3 pathway in ischemic stroke has not been investigated yet.

Here, we proposed that HDAC3 could regulate the expression/activity of ZBP1 and that the HDAC3-ZBP1-pIRF3 signaling pathway plays a vital role in ischemic brain injury. RGFP966, a specific suppressor of HDAC3, can decrease p-IRF3 and ZBP1 levels, inhibit proinflammatory cytokines expression, and relieve post-stroke anxious-like behavior. These effects indicate that targeting the HDAC3-ZBP1-pIRF3 signaling pathway might be a candidate therapeutic approach for ischemic stroke.

2. Methods

2.1 Animals and Drug Treatment

The Charles River Laboratory in Beijing provided the experimental mice. Age-matched male C57BL/6 wild-type mice were housed in an adaptive habitat with a 12-hour light/dark cycle and food and water accessibility. All animal procedures were carried out strictly per the National Institutes of Health's guidelines for the care and use of Laboratory Animals. They were followed by the Care Committee of the University of Science and Technology of China (USTC). RGFP966 (CSN17747, CSNpharm, Chicago, IL, USA) powder was dissolved in corn oil with 10% dimethylsulfoxide (DMSO), then RGFP966 solution (8 mg/kg) or Oil was delivered intraperitoneally twice before the ischemia-reperfusion injury according to our previous study [16].

2.2 Middle Cerebral Artery Occlusion Model (MCAO) and Neurological Function Assessment

Animals were intravenously anesthetized with 1% sodium pentobarbital at a 45 mg/kg dose. After the right common carotid artery and downstream external carotid artery (ECA) were exposed, a cut was made on the ECA, and a silicone-coated 6-0 monofilament suture (MSMC21B120PK50, RWD Life Science Corporation, Shenzhen, Guangdong, China) was inserted into the ECA and advanced into the internal carotid artery (ICA)

about 10 to 11 mm to block the initial segment of the middle cerebral artery (MCA). The artery was reperfusion by pulling out the suture after an occlusion over 90 minutes. The mice were kept warm through the operation with a heating pad with a consistent temperature of 37 °C for recovery. Additionally, sham control mice had undergone the same procedure as the MCAO group without the insertion of filament to occlude the MCA. Neurological function assessments from animals in each group were performed 24 h after reperfusion. A five-point scale was utilized for value: A score of 0 indicated no neurological deficit. Mouse with a score of 1 indicated mild focal neurological deficit, accompanied by failing to fully extend their forepaw. A score of 2 indicated moderate focal neurological deficit, with circling. A score of 3 indicated severe focal deficit, with slumping when walking. Mice with a score of 4 failed to walk independently and exhibited a depressed level of consciousness.

2.3 Western Blotting

The brain tissues were lysed in Radio-immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors. The lysate was homogenized using ultrasound and centrifuged at 13,000 g for 30 min. BCA Protein Assay (#23227, Thermo Fisher Scientific, Cambridge, UK) was applied to validate the protein concentration. After that, protein samples were dissolved in sodium dodecyl sulfate (SDS) buffer and divided into equal aliquots before being electrophoresed on an 8% to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The protein stripe was then transferred to polyvinylidene fluoride (PVDF) membranes, which were then incubated with primary antibodies cGAS (1:3000; #31659, Cell Signaling Technology, Danfoss, MA, USA), ZBP1 (1:3000; sc-271483, Santa Cruz Biotechnology, Dallas, TX, USA), p-IRF3 (1:3000; #79945, Cell Signaling Technology, Danfoss, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; ab8245, Abcam, Cambridge, UK) overnight at 4 °C after being blocked with 5% nonfat milk in 0.05% Tween TBS buffer for an hour. After 3 washes with PBS, PVDF membranes were incubated with Goat anti-Mouse HRP (1:10,000; Pierce, 1858413, Thermo Fisher Scientific Inc., Waltham, MA, USA) or Goat anti-Rabbit Horseradish Peroxidase (HRP) (1:10,000; Pierce, 1858415) secondary antibodies for 1 hour at room temperature. Furthermore, the ChemiDoc imaging system (#1708195, Bio-Rad, Hercules, CA, USA) was employed to detect the signals of the protein bands, and ImageJ (java8, LOCI, University of Wisconsin, Madison, WI, USA) was used to quantify the band intensity.

2.4 Quantitative Real-Time PCR

Total RNA from brain tissues was isolated using the TRIzol reagent according to the manufacturer's instructions (#15596018, Life Technologies, Carlsbad, CA, USA)

Table 1. Quantitative PCR (Q-PCR) primers information is displayed as follows.

Gene name	Primer (5'-3')
<i>IL-1β</i> (mouse)	F 5'-CCTTCCAGGATGAGGACATGA -3'. R 5'-TGAGTCACAGAGGATGGGCTC -3'.
<i>IL-6</i> (mouse)	F 5'-GAGGATACCACTCCCAACAGACC -3'. R 5'-AAGTGCATCATCGTTGTTTCATACA -3'.
<i>GAPDH</i> (mouse)	F 5'-CCCATCACCATTCTCCAGGAGC -3'. R 5'-CCAGTGAGCTTCCCGTTCAGC -3'.
<i>Myd88</i> (mouse)	F 5'-CACTCGCAGTTTGTGTTGATG -3'. R 5'-TCTGGAAGTCACATTCCTTGC -3'.
<i>IL33</i> (mouse)	F 5'-TCCAACCTCCAAGATTTCCTCCG -3'. R 5'-CATGCAGTAGACATGGCAGAA -3'.
<i>ZBP1</i> (mouse)	F 5'-TTGAGCACAGGAGACAATCTG -3'. R 5'-TTCAGGCGGTAAAGGACTTG -3'.
<i>ISG-15</i> (mouse)	F 5'-GGTGTCCGTGACTAACTCCAT -3'. R 5'-TGGAAAGGGTAAGACCGTCCT -3'.
<i>DDX58</i> (mouse)	F 5'-AGCCAAGGATGTCTCCGAGGAA -3'. R 5'-ACACTGAGCACGCTTTGTGGAC -3'.
<i>cGAS</i> (mouse)	F 5'-TTCCACGAGGAAATCCGCTGAG -3'. R 5'-CAGCAGGGCTTCCTGGTTTTC -3'.
<i>MAVS</i> (mouse)	F 5'-CTGCCAACACAATACCACCTGAG -3'. R 5'-TCTCTGGTCCAGAGTGCAAGCT -3'.
<i>MX1</i> (mouse)	F 5'-TCTGAGGAGAGCCAGACGAT -3'. R 5'-CTCAGGGTGTTCGATGAGGTC -3'.
<i>MDA5</i> (mouse)	F 5'-TGA TGC ACT ATT CCA AGA ACT AAC A -3'. R 5'- TCTGTGAGACGAGTTAGC CAA G -3'.
<i>TMEM173</i> (mouse)	F 5'-TGTGGCTGCTGATGCCATAC -3'. R 5'-CCAGCACGGTCGATGTTTTC -3'.

and reverse transcribed into complementary DNA (cDNA) by a cDNA Synthesis Kit (A5001, Promega, Madison, WI, USA). Then, SYBR qPCR Master Mix (4344463, Thermo Fisher, Danfoss, MA, USA) was adopted for Quantitative Real-time PCR (qRT-PCR) analysis from different groups (see Table 1). The expression of the gene was compared to either Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5 Immunofluorescence

Brain tissues were dehydrated with 20% sucrose and 30% sucrose successively after cardio-perfusion and fixation with 4% paraformaldehyde solution (PFA) for 8 hours. Using a cryostat, the optimal cutting temperature (OCT)-embedded brain tissue was cut into 16 μ m thick coronal sections (CM1950, Leica, Wetzlar, Germany). Subsequently, slides were permeabilized and blocked with 0.5% Triton X-100 (T8787, Sigma, St. Louis, MI, USA) and 3% Bovine Serum Albumin (A2153, Sigma, St. Louis, MIM, USA). Slides were incubated overnight at 4 °C with diluted primary antibodies against glial fibrillary acidic protein (GFAP) (1:2000; ab4674, Abcam, Cambridge, UK), Iba-1 (1:1000; PA5-27436, Invitrogen, Waltham, MA,

USA), ZBP1 (1:500; sc-271483, Santa Cruz Biotechnology, Dallas, TX, USA), NeuN (1:500; ABN91, Sigma, St. Louis, MI, USA) in 1% BSA. Brain samples were then stained with Alexa Fluo-conjugated secondary antibodies (including CY2 Goat anti-Chicken (A11039, Invitrogen, Waltham, MA, USA), CY3 Goat anti-Rabbit (A21429, Invitrogen, Waltham, MA, USA), CY5 Goat anti-Mouse (Z25008, Invitrogen, Waltham, MA, USA) for two hours at room temperature following three times washes with PBS, and the cell nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector labs H-1000, Burlingame, CA, USA) for 10 min. Images were captured with a Zeiss LSM880 microscope to visualize the fluorescence signals and qualified with Image J software (National Institutes of Health, <https://imagej.nih.gov/ij/>). For the analysis of microglia activation, unit about AnalyzeSkeleton was used for analyzing dendrite length. To analyze microglia soma size, a three-dimensional reconstruction of microglia was performed according to the 3D Script unit. The volume of microglia was then calculated. For the analysis of ZBP1 colocalization with other cells (Neuron (stained with NeuN), Astrocyte (stained with GFAP) and microglia/macrophage (stained with Iba-1)), Coloc 2 unit

in Image J was utilized. Respective colocalized signals in each cell were marked as an effective value to divide by the number of all cells per high power field (HPF). The percentage of ZBP1⁺ cells per view was then calculated in detail.

2.6 Open Field Test

An open-field test was conducted to assess the anxiety-like behavior of the mice. It was carried out using smooth wooden equipment (50 cm width × 50 cm length × 40 cm height). Mice had a two-hour acclimatization period in the testing room. A video-tracking system (Video camera system, Smart 3.0, Panlab, Spain) was placed right above the center (20 × 20 cm) of the arena, and each mouse was allowed to roam freely for 10 minutes. Every mouse in the group was summarized as time spent (s) or cumulative distance (cm) in the center and total distance (cm) subsequently for further statistics.

2.7 Marble Burying Test

Shortly after the compounds or their corresponding vehicle were supplied, each mouse was placed individually in the open container after a two-hour acclimation phase. Fresh sawdust of 3 cm thickness was paved in a polycarbonate cage (26.5 cm length × 16 cm width × 14 cm height), with 20 glass marbles spaced randomly on the paved surface. The subject was left in the cage for about 30 minutes without interference. Then the number of marbles with more than half of their surface covered with sawdust was counted.

2.8 Elevated Plus Maze Test

The elevated plus maze was used to assess the anxiety-like behavior of the mice. The maze comprises two open and two closed arms with a 50 cm wall and a central zone. After a two-hour adaptation phase in the test room, the mice were placed in the maze's center facing the closed arm. The mice were allowed to explore the maze freely for five minutes. The video-tracking system (Video camera system, Smart 3.0, Panlab, Spain) recorded their motion traces. The duration and entries into the open arms and closed arms were reported. Every mouse in the group was summarized according to open arm entry (counts), open arm time (s), and open arm time (%) for further statistics.

2.9 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

To evaluate the change in the size of the ischemic zones, 2,3,5-Triphenyltetrazolium Chloride (TTC) staining (1% 2,3,5-triphenyltetrazolium chloride, Sigma-Aldrich Co., St. Louis, MO, USA) was applied. Brain tissues were cut into 1-mm thick coronal slices and moved slightly into a dish containing TTC dye for 30 minutes at 37 °C. TTC labels non-injured tissue, leaving the infarct area white. The stained slices were scanned and photographed for further Image J software analysis after being fixed in a 4% formaldehyde solution for two hours, as previously reported [17].

2.10 Statistical Analysis

Data were presented as the mean ± standard error of the mean (SEM) or standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA) and carried out with a *t*-test for two independent groups and one-way analysis of variance (ANOVA) when faced with multiple groups with a single variance. A value of *p* < 0.05 was considered statistically significant, and the statistical legends marked in the chart are explained as follows (* indicates *p* < 0.05; ** indicates *p* < 0.01; *** represents *p* < 0.001, # represents *p* < 0.0001).

3. Results

3.1 HDAC3 Inhibition can Significantly Attenuate Ischemic Brain Injury

We assessed the mice's infarct size and neurobehavioral prognosis from different groups using TTC staining and the neurological function score. As demonstrated, the average infarct size of mice with MCAO was about twice as large as that of mice pretreated with RGFP966 (Fig. 1C). In addition, RGFP966 significantly reduced motor impairment in mice following ischemia (Fig. 1D, * *p* < 0.05, MCAO-RGFP966 group vs. MCAO-Oil group).

3.2 HDAC3 Inhibition can Significantly Ameliorate Anxiety-Like Behavior in Mice after Ischemia Stroke

To assess the anxiety-like behavior of mice following infarction, a variety of behavioral tests were performed on three groups of mice. In the open field test, as shown in Fig. 2A–C, the anxiety-like behavior of the mice suffering from cerebral infarction is greatly reduced after RGFP966 treatment, as indicated by increased time spent in the central area. Similarly, administration of RGFP966 prior to MCAO significantly increased the number of entries and percentage of time spent in the open arm in the elevated plus maze test compared to the MCAO-Oil group, as demonstrated in Fig. 2D–F. Furthermore, the burying behavior in the RGFP966 group was greatly decreased compared to the vehicle group (Fig. 2G,H), suggesting that RGFP966 pretreatment can alleviate anxiety-like behavior in mice after an ischemic stroke attack.

3.3 ZBP1/p-IRF3 was Downregulated after HDAC3 Inhibition in MCAO

To investigate the mechanism of HDAC3 inhibition in alleviating ischemic brain damage, we obtained brain tissue from each group 3 days after surgery for quantitative PCR (qPCR) quantitative analysis of inflammation-related cytokines. Fig. 3A illustrates a member of the Toll-like receptor family in mice related to ischemia damage, the myeloid differentiation factor 88 (MYD88), and the inflammatory cytokines like interleukin-1β (IL-1β), interleukin-33 (IL-33), and interleukin-6 (IL-6), were dramatically up-regulated in MCAO-Oil group. Additionally, pretreatment with RGFP966 significantly decreased the expression of

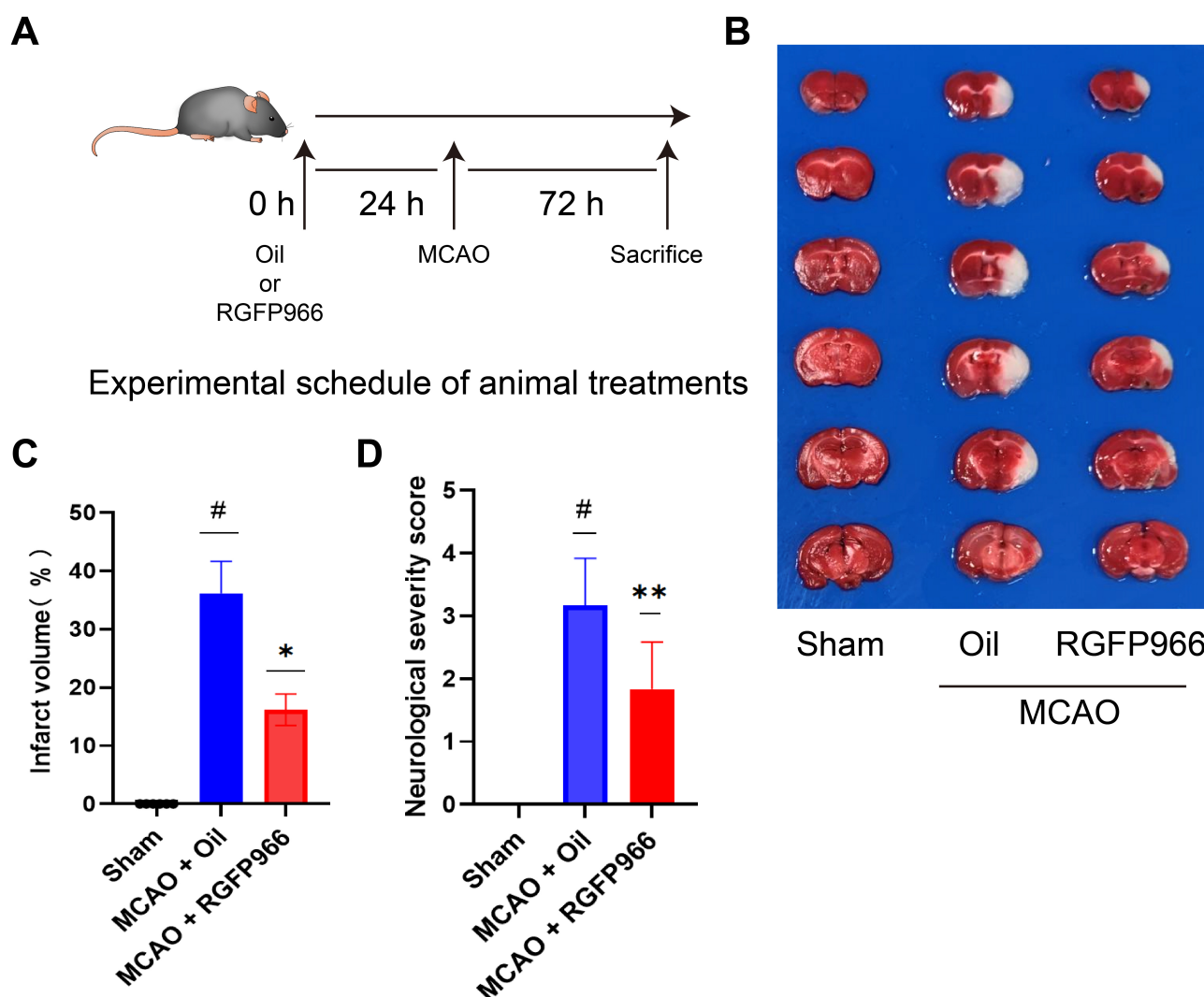


Fig. 1. HDAC3 inhibition can significantly attenuate ischemic brain injury. (A) Systematic scheme of the experiment. (B) An overall observation and TTC staining of the brains of the three groups: Sham, MCAO + Oil, and MCAO + RGFP966. (C) Statistical analysis of infarct volume of Sham, MCAO + Oil, and MCAO + RGFP966 (N = 6 per group). (D) Statistical analysis of neurological severity score of three groups (N = 6 per group). The above data were presented as the means $\pm$ SEM and analyzed by one-way ANOVA with Bonferroni post hoc test ($*p < 0.05$; $**p < 0.01$; $\#p < 0.0001$). HDAC3, histone deacetylase 3; TTC, 2,3,5-triphenyltetrazolium chloride; SEM, standard error of the mean; SD, standard deviation; ANOVA, analysis of variance; MCAO, middle cerebral artery occlusion.

these proinflammatory cytokines, suggesting that HDAC3 inhibitors could reduce ischemic brain damage by exerting anti-inflammatory effects [8]. Interestingly, the level of interferon pathway-related molecules was also found to be dramatically changed, especially DExD/H-box helicase 58 (DDX58) pattern recognition receptors, Melanoma differentiation-related gene 5 (*MDA5*), Interferon-Induced GTP-Binding Protein (Mx1), Mitochondrial antiviral signaling proteins (MAVS), and DNA sensor Cyclic GMP-AMP (cGAS).

Our results showed that ZBP1 was considerably up-regulated in the infarct periphery in the MCAO-Oil group compared to the Sham group (Fig. 4A), which aligned with the elevated ZBP1 mRNA expression in Fig. 3A. At the

same time, pre-treatment of RGFP966 substantially suppressed the expression of ZBP1 in the ischemic penumbra. Since activated ZBP1 has been shown to recruit TANK Binding Kinase 1 (TBK1) and IRF3 [18], we also detected the expression of IRF3. Notably, the level of p-IRF3 in mouse brain tissue was increased significantly after ischemia, while HDAC3 inhibition significantly decreased the p-IRF3 expression, as shown in Fig. 3B,C.

3.4 RGFP966 Treatment Inhibited the Over-Activation of ZBP1 in Microglia in the Ischemic Penumbra

There was obvious gliosis in the ischemic penumbra following ischemic brain injury (Fig. 4). As shown in Fig. 4C, microglia labeled with Iba-1 exhibited a more rounded morphology, and a decrease in both the length

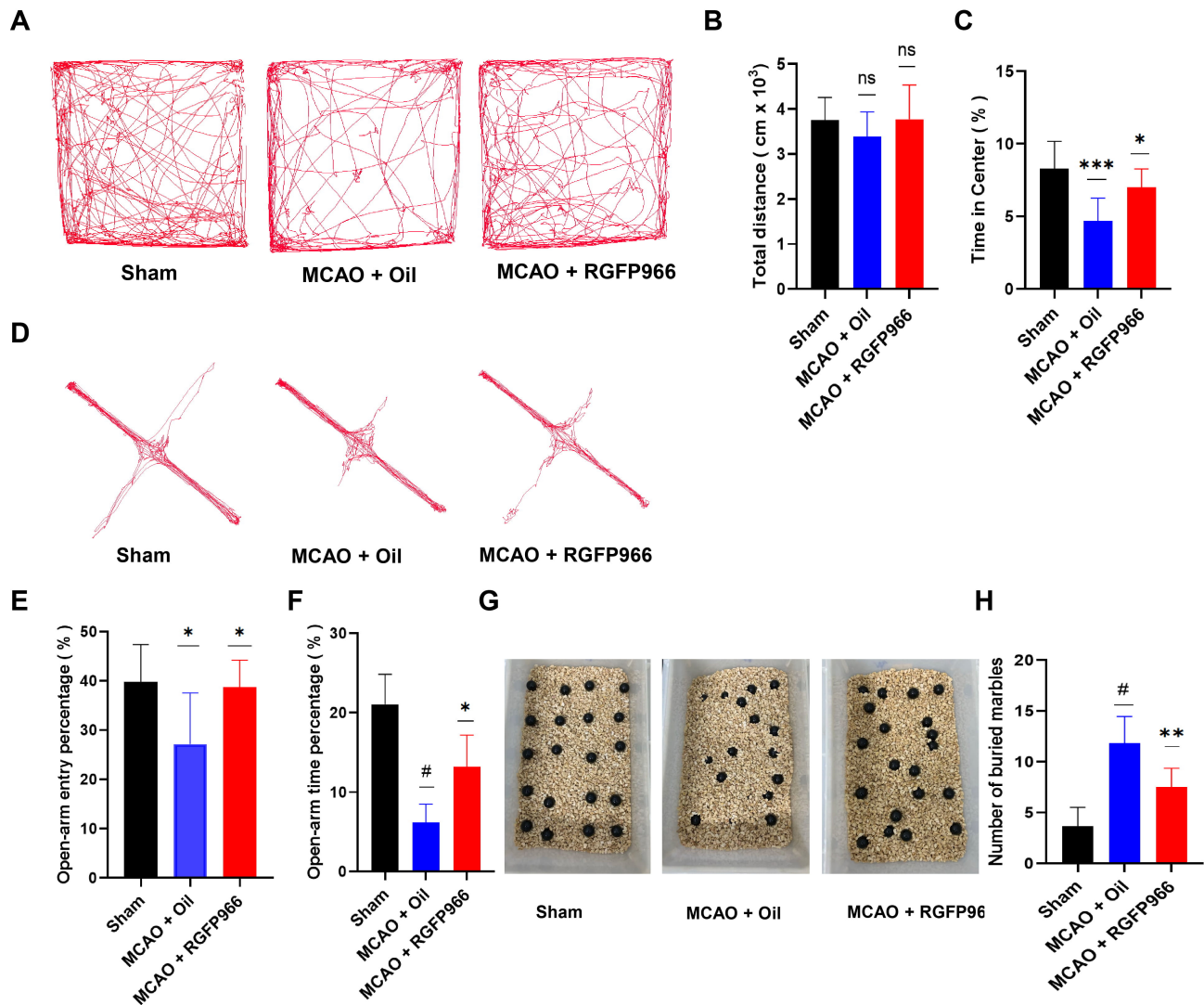


Fig. 2. HDAC3 inhibition can significantly reduce anxiety-like behavior in mice after stroke. (A) Typical tracks in the Open-Field Test (OFT) following model induction in mice with Sham, MCAO + Oil, and MCAO + RGFP966. (B) Statistical analysis of total distance traveled (N = 6 per group). (C) Statistical analysis of time in the center (%) (N = 6 per group). (D) Elevated plus-maze (EPM) represented in three groups of mice tracks. (E) Statistical analysis of the percentage of distance traveled in open-arm entry percentage (N = 6 per group). (F) Statistical analysis of the percentage of time spent in the center area (N = 6 per group). (G) Marbles buried test outcomes in Sham, MCAO + Oil, MCAO + RGFP966 group. (H) Statistical analysis of the number of buried marbles among three groups (N = 6 per group). The above data are presented as the means $\pm$ SEM and were analyzed by one-way ANOVA with Bonferroni post hoc test (ns, no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, # $p < 0.0001$).

of their branches after 72 hours of ischemia-reperfusion damage and reactive astrogliosis was also observed. Interestingly, the ZBP1-positive signal was also augmented, and ZBP1 was found predominantly colocalized with microglia. The percentage of ZBP1-positive microglia in the MCAO + Oil group was higher than in the Sham group ($p < 0.0001$, MCAO + RGFP966 group vs. MCAO + Oil group). However, the quantity of ZBP1-positive astrocytes was almost the same minor in all three groups, and neurons shared similar results (Fig. 4A,B).

4. Discussion

Ischemic stroke is closely linked to inflammation and immune reaction. Epigenetic modifications were recently found to play a significant role in the pathological damage caused by stroke, which was involved in regulating cellular damage, neuron inflammation, cognitive ability, and motor function [19].

HDACs are a set of contributing epigenetic enzymes that possess an essential function in regulating inflammation. Notably, previous studies have demonstrated that HDAC inhibitors have strong anti-inflammatory effects [8,20]. HDAC inhibitors manipulate the expres-

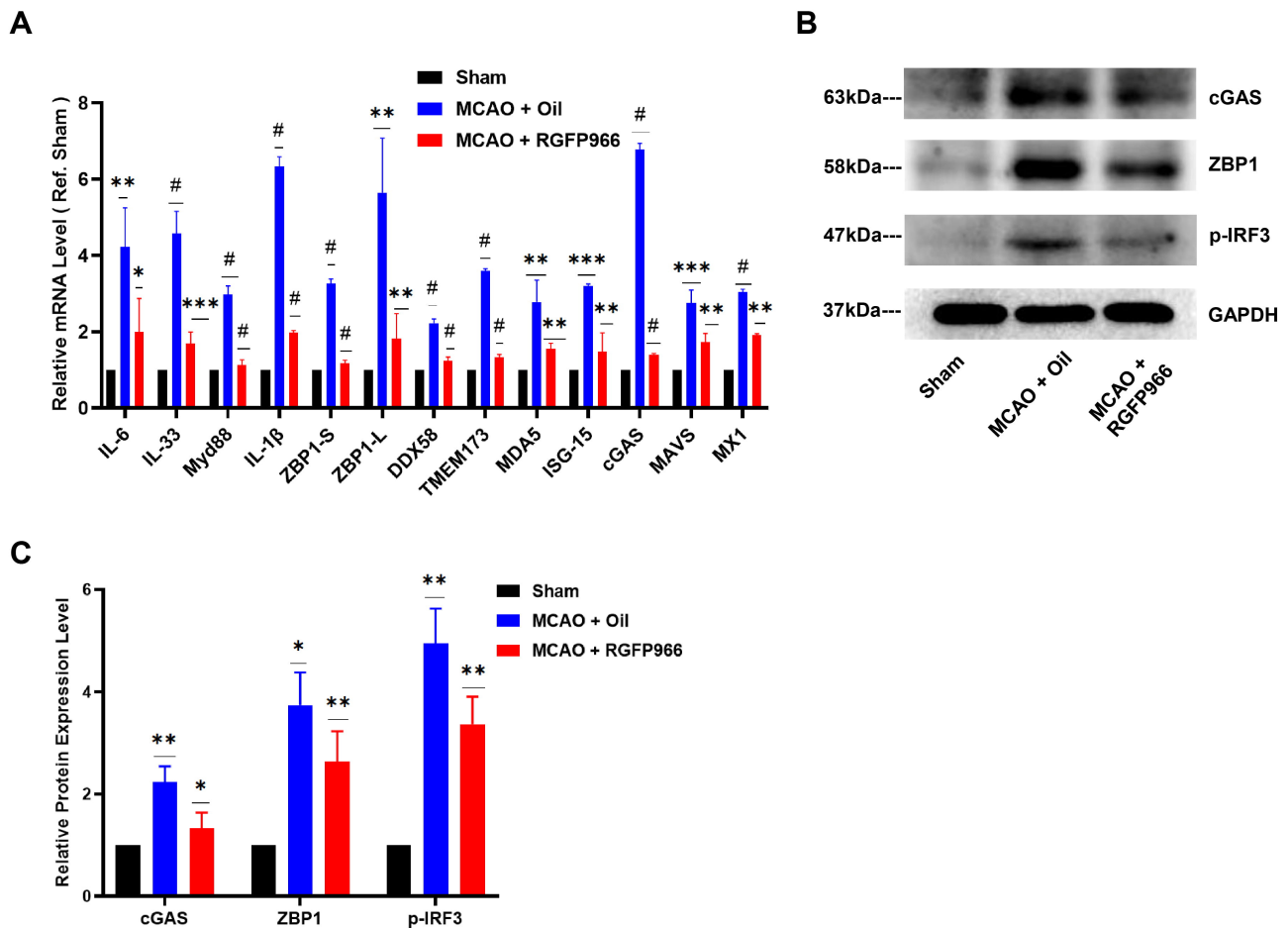


Fig. 3. ZBP1/p-IRF3 was downregulated after HDAC3 inhibition in MCAO. (A) RGFP966 pretreatment decreases the relative mRNA level of IL-6, IL-33, Myd88, IL-1 β , ZBP1-S, ZBP1-L, DDX58, TMEM173, MDA5, ISG-15, cGAS, MAVS and MX1 after MCAO (N = 3 per group). (B) RGFP966 pretreatment decreases the protein level of cGAS, pIRF3, and ZBP1 in the ischemic penumbra 72 h after reperfusion (N = 3 per group). (C) Statistical analysis of protein expression level. GAPDH was used as an internal control (N = 3 per group). The above data were presented as the means $\pm$ SEM and analyzed by one-way ANOVA with Bonferroni post hoc test (* p < 0.05; ** p < 0.01; *** p < 0.001, # p < 0.0001. N = 3 per group). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-33, interleukin-33; Myd88, myeloid differentiation factor 88; MDA5, Melanoma differentiation-related gene 5; cGAS, cyclic GMP-AMP synthase; MAVS, Mitochondrial antiviral signaling proteins.

sion patterns of downstream genes, protecting against ischemic stroke. Valproic acid, a non-selective HDAC inhibitor, has been demonstrated to drastically decrease cerebral infarction volume and enhance neurological behavioral scores in ischemic stroke [21]. Additionally, an investigation found that Trichostatin A enhanced behavioral score and decreased infarct volume in the MCAO model via the phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/AKT) pathway [22]. However, there are certain restrictions on the practical use of non-selective HDACs inhibition due to the adverse effects of activating other targets, such as the general toxicity of HDAC6 [23]. HDAC3 has tremendous potential for treating illness of the central nervous system due to its high expression in the brain and regulation of cerebral biological processes, notably learning and memorizing. This suggests that inhibiting HDAC3 ac-

tivity might be a way to reduce ischemia damage. It has also recently been established that selective inhibition of HDAC3 aids in managing type 1 and type 2 diabetes [24] and Huntington's disease in mice [25]. In our study, we discovered that RGFP966, a selective HDAC3 inhibitor, considerably reduced the extent of cerebral infarction and alleviated anxious behavior in MCAO mice, in line with the previous findings that HDAC3 inhibition enhances cognitive memory performance [26].

In recent years, innate immune receptors, cGAS and ZBP1, have become pivotal in activating the interferon pathway and releasing pro-inflammatory factors. Pathological changes were found in acute brain ischemia-reperfusion injury, including cell death, oxidative stress, and mitochondrial dysfunction. Due to their structural resemblance to viral DNA, these released dsDNA molecules (nuDNA,

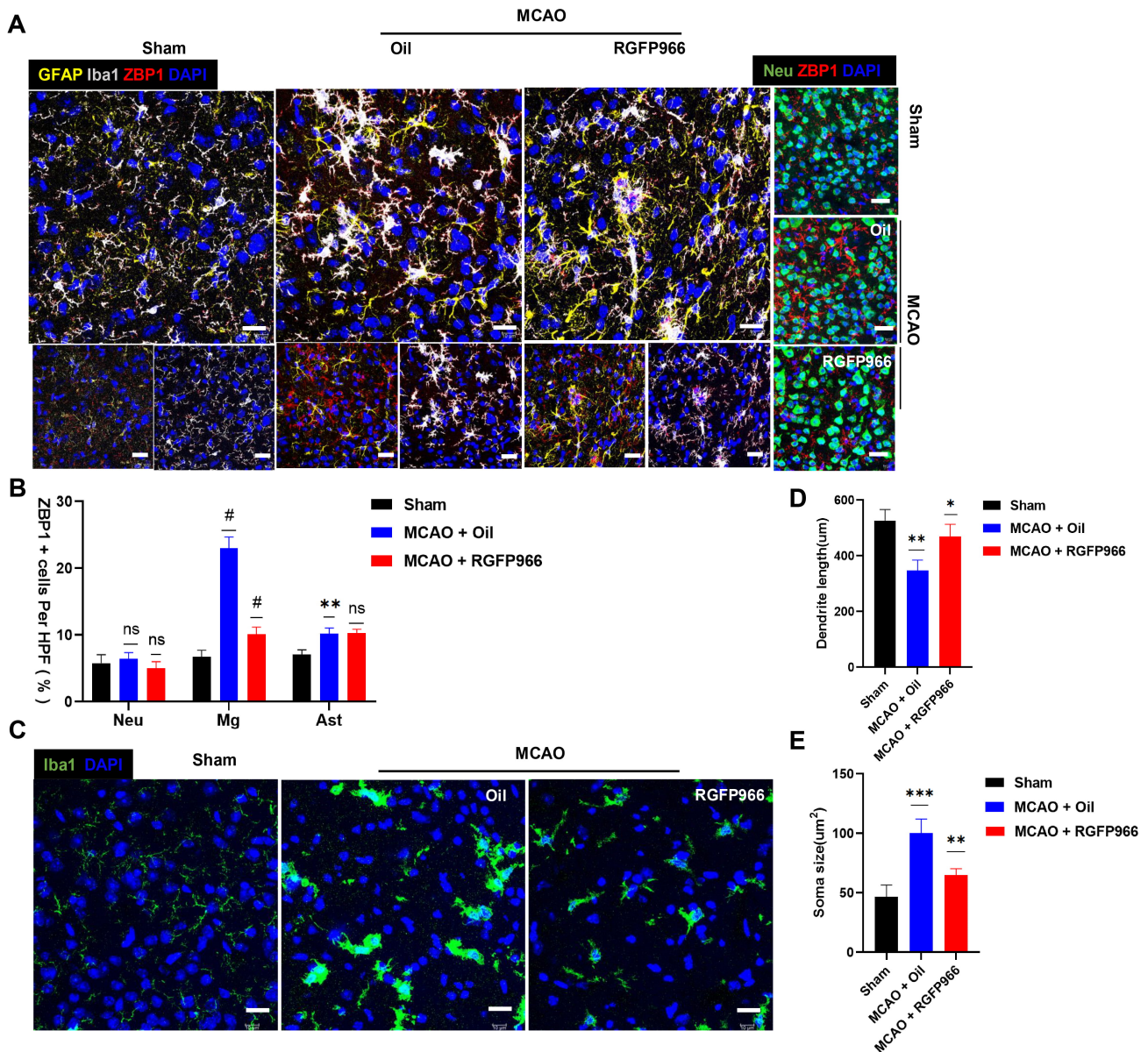


Fig. 4. RGFP966 treatment relieved over activation of ZBP1 in microglia in the ischemic penumbra. (A) Representative immunofluorescence images showing the morphological changes of microglia labeled with Iba-1 (white) and astrocyte labeled with GFAP (yellow), and neurons labeled with Neu (green), and colocalization of Iba-1 (white) and ZBP1 (red), and GFAP (yellow) and ZBP1 (red) in the ischemic penumbra 72 h after reperfusion, respectively. Scale bar = 10 μ m. (B) Percentages of ZBP1-positive cells in NeuN-positive, Iba-1-positive, GFAP-positive cells in the ischemic penumbra (Slices = 4 per group). (C) Histological analysis of microglia from Sham, MCAO + Oil, and MCAO + RGFP966 mice were performed by staining for Iba-1 (green) and DAPI (blue). Scale bar = 10 μ m. (D,E) Statistical analysis of microglial morphology (soma size and dendrite length) from three groups, respectively. ns, no significance; * p < 0.05; ** p < 0.01; *** p < 0.001, # p < 0.0001. Slices = 4 per group. ZBP1, Z-DNA Binding Protein 1; GFAP, Glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole.

mtDNA) accumulate in the cytoplasm [27]. Additionally, these dsDNA molecules can be recognized by cGAS or ZBP1. The expression of cGAS and ZBP1 was found to be upregulated in the MCAO model. The cGAS enzyme catalyzes the production of cyclic guanosine monophosphate (cGAMP) [28,29]. Subsequently, the protein STING is recruited and continues to recruit the downstream TANK

Binding Kinase 1 (TBK1) in the forms of dimers [30], and IRF3 is subsequently recruited and phosphorylated, leading to the activation of cGAS-cGAMP-STING-IRF3 interferon signal pathway.

Besides acting as an interferon-stimulated gene (ISG), ZBP1 can also boost the gene expression of interferon (IFN) type I. ZBP1, also known as DAI or DLM-1, has

two Z α domains and is an immune sensor that binds both Z-DNA and Z-RNA [18]. It detects viral nucleic acids, mediates human defense against certain viruses, and regulates the programmed cell death process and inflammation [31–34]. ZBP1 can also be activated by host-derived nucleic acid receptors and damaged mtDNA [35]. In our study, the elevated expression of ZBP1 was detected three days after ischemia-reperfusion injury in the brain. The C-terminal domain of the activated ZBP1 could bind to and activate I κ B kinase TNFR-associated factor family member-associated NF- κ B activator-binding kinase (TBK1) [36]. In doing so, it activates the phosphorylation of IRF3, resulting in the transcriptional activation of ISGs and IFN- β . Likewise, ZBP1 can recruit IRF3 and TBK1 by itself [18]. Our data indicate that ZBP1 and other IFN-related factors were significantly upregulated after ischemia-reperfusion injury, and these alterations could be partly rescued by HDAC3 inhibitor. Furthermore, the RHIM domains of ZBP1 may mediate the interaction between toll-like receptor adapter molecule1 (TRIF) and receptor-interacting serine/threonine-protein kinase1 (RIPK1) [37], promoting NF- κ B nuclear translocation and inducing antiviral genes [38,39].

Microglia is one of the most significant immune cells in the central nervous system, participating in poststroke inflammation and neuronal injury regulation [40]. Microglia represents a hyperactivated state after an ischemic stroke. A previous study has shown that HDAC3 is mainly expressed in activated microglia in an ischemia mouse model [8]. Additionally, our finding indicated that the expression of ZBP1 is highly synchronized with the morphological changes of microglia. Notably, the microglial ZBP1 was significantly upregulated compared with neurons and astrocytes. Pre-administration of RGFP966 inhibited microglia activation and markedly decreased ZBP1 expression in microglia. Therefore, we speculated that lower ZBP1 expression in microglia induced by HDAC3 inhibitor might indicate a substantial protective effect.

The mechanism of HDAC3 is not thoroughly examined in this paper, for which we did not detect the enzyme activity or expression after utilizing an HDAC3 inhibitor. Additionally, it is unclear how HDAC3 may affect the long-term prognosis of stroke. Furthermore, we did not explore the direct roles of ZBP1 between HDAC3-mediated pathways. Theoretically, ZBP1 KO mice should be used because of non-candidates of ZBP1 specific inhibitors and non-reliable viruses to effectively knock down microglial ZBP1 in the brain. Thus, testing Z-RNA/Z-DNA and using ZBP1 gene knockout mice will provide more persuasive proof. Here, we provided evidence regarding the mechanism of inhibition of the ZBP1/p-IRF3 pathway involving HDAC3 to provide a more theoretical framework for the clinical transformation of HDAC3 inhibitors. However, we intend to further analyze the mechanism of HDAC3 and investigate the pertinent pathways.

5. Conclusions

We demonstrated HDAC3 inhibition could ameliorate the pathological changes after ischemic stroke by downregulating the hyperactivation of the ZBP1/p-IRF3 pathway, especially in microglia. Importantly, these findings provide a candidate therapeutic target for ischemia-reperfusion injury.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HZhu and SW designed the research study. JW, MY, and YC performed the research. YL provided help and advice on the experiments, such like instructing animal behavior experiments and helping immunofluorescence. HZhang, RT and WZ analyzed the data. 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

The protocol was approved by the Ethics Committee of First Affiliated Hospital of University of Science and Technology of China (approval number: 2022-N(A)-365).

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. *The Lancet. Neurology*. 2021; 20: 795–820.
- [2] Herpich F, Rincon F. Management of Acute Ischemic Stroke. *Critical Care Medicine*. 2020; 48: 1654–1663.
- [3] Jin L, Zhu Z, Hong L, Qian Z, Wang F, Mao Z. ROS-responsive 18 β -glycyrrhetic acid-conjugated polymeric nanoparticles mediate neuroprotection in ischemic stroke through HMGB1 inhi-

- bition and microglia polarization regulation. *Bioactive Materials*. 2022; 19: 38–49.
- [4] Maida CD, Norrito RL, Daidone M, Tuttolomondo A, Pinto A. Neuroinflammatory Mechanisms in Ischemic Stroke: Focus on Cardioembolic Stroke, Background, and Therapeutic Approaches. *International Journal of Molecular Sciences*. 2020; 21: 6454.
 - [5] Miyamoto O, Auer RN. Hypoxia, hyperoxia, ischemia, and brain necrosis. *Neurology*. 2000; 54: 362–371.
 - [6] Milazzo G, Mercatelli D, Di Muzio G, Triboli L, De Rosa P, Perini G, *et al.* Histone Deacetylases (HDACs): Evolution, Specificity, Role in Transcriptional Complexes, and Pharmacological Actionability. *Genes*. 2020; 11: 556.
 - [7] Liao Y, Cheng J, Kong X, Li S, Li X, Zhang M, *et al.* HDAC3 inhibition ameliorates ischemia/reperfusion-induced brain injury by regulating the microglial cGAS-STING pathway. *Theranostics*. 2020; 10: 9644–9662.
 - [8] Zhang MJ, Zhao QC, Xia MX, Chen J, Chen YT, Cao X, *et al.* The HDAC3 inhibitor RGFP966 ameliorated ischemic brain damage by downregulating the AIM2 inflammasome. *FASEB Journal*. 2020; 34: 648–662.
 - [9] Zhou X, Qiao B. Inhibition of HDAC3 and ATXN3 by miR-25 prevents neuronal loss and ameliorates neurological recovery in cerebral stroke experimental rats. *Journal of Physiology and Biochemistry*. 2022; 78: 139–149.
 - [10] Matheson R, Chida K, Lu H, Clendaniel V, Fisher M, Thomas A, *et al.* Neuroprotective Effects of Selective Inhibition of Histone Deacetylase 3 in Experimental Stroke. *Translational Stroke Research*. 2020; 11: 1052–1063.
 - [11] Hao Y, Yang B, Yang J, Shi X, Yang X, Zhang D, *et al.* ZBP1: A Powerful Innate Immune Sensor and Double-Edged Sword in Host Immunity. *International Journal of Molecular Sciences*. 2022; 23: 10224.
 - [12] Szczesny B, Marcatti M, Ahmad A, Montalbano M, Bruny nszki A, Bibli SI, *et al.* Mitochondrial DNA damage and subsequent activation of Z-DNA binding protein 1 links oxidative stress to inflammation in epithelial cells. *Scientific Reports*. 2018; 8: 914.
 - [13] Ponnusamy K, Tzoni MM, Begum M, Robinson ME, Caputo VS, Katsarou A, *et al.* The innate sensor ZBP1-IRF3 axis regulates cell proliferation in multiple myeloma. *Haematologica*. 2022; 107: 721–732.
 - [14] Yoneyama M, Suhara W, Fujita T. Control of IRF-3 activation by phosphorylation. *Journal of Interferon & Cytokine Research*. 2002; 22: 73–76.
 - [15] Jefferies CA. Regulating IRFs in IFN Driven Disease. *Frontiers in Immunology*. 2019; 10: 325.
 - [16] Zhu H, Guo Y, Huang A, Shen H, Chen Y, Song J, *et al.* HDAC3-Regulated PGE2 Production by Microglia Induces Phobic Anxiety Susceptibility After Stroke and Pointedly Exploiting a Signal-Targeted Gamma Visual Stimulation New Therapy. *Frontiers in Immunology*. 2022; 13: 845678.
 - [17] Zong P, Feng J, Yue Z, Li Y, Wu G, Sun B, *et al.* Functional coupling of TRPM2 and extrasynaptic NMDARs exacerbates excitotoxicity in ischemic brain injury. *Neuron*. 2022; 110: 1944–1958.e8.
 - [18] Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature*. 2007; 448: 501–505.
 - [19] Hwang JY, Aromolaran KA, Zukin RS. Epigenetic mechanisms in stroke and epilepsy. *Neuropsychopharmacology*. 2013; 38: 167–182.
 - [20] Ghiboub M, Zhao J, Li Yim AYE, Schilderink R, Verseijden C, van Hamersveld PHP, *et al.* HDAC3 Mediates the Inflammatory Response and LPS Tolerance in Human Monocytes and Macrophages. *Frontiers in Immunology*. 2020; 11: 550769.
 - [21] Xuan A, Long D, Li J, Ji W, Hong L, Zhang M, *et al.* Neuroprotective effects of valproic acid following transient global ischemia in rats. *Life Sciences*. 2012; 90: 463–468.
 - [22] Ma XH, Gao Q, Jia Z, Zhang ZW. Neuroprotective capabilities of TSA against cerebral ischemia/reperfusion injury via PI3K/Akt signaling pathway in rats. *The International Journal of Neuroscience*. 2015; 125: 140–146.
 - [23] Malvaez M, McQuown SC, Rogge GA, Astarabadi M, Jacques V, Carreiro S, *et al.* HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110: 2647–2652.
 - [24] Zhao B, Yuan Q, Hou JB, Xia ZY, Zhan LY, Li M, *et al.* Inhibition of HDAC3 Ameliorates Cerebral Ischemia Reperfusion Injury in Diabetic Mice In Vivo and In Vitro. *Journal of Diabetes Research*. 2019; 2019: 8520856.
 - [25] Suelves N, Kirkham-McCarthy L, Lahue RS, Gin s S. A selective inhibitor of histone deacetylase 3 prevents cognitive deficits and suppresses striatal CAG repeat expansions in Huntington’s disease mice. *Scientific Reports*. 2017; 7: 6082.
 - [26] McQuown SC, Barrett RM, Matheos DP, Post RJ, Rogge GA, Alenghat T, *et al.* HDAC3 is a critical negative regulator of long-term memory formation. *The Journal of Neuroscience*. 2011; 31: 764–774.
 - [27] West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, *et al.* Mitochondrial DNA stress primes the antiviral innate immune response. *Nature*. 2015; 520: 553–557.
 - [28] Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science*. 2013; 339: 786–791.
 - [29] Kato K, Omura H, Ishitani R, Nureki O. Cyclic GMP-AMP as an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. *Annual Review of Biochemistry*. 2017; 86: 541–566.
 - [30] Shang G, Zhang C, Chen ZJ, Bai XC, Zhang X. Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. *Nature*. 2019; 567: 389–393.
 - [31] Ha SC, Kim D, Hwang HY, Rich A, Kim YG, Kim KK. The crystal structure of the second Z-DNA binding domain of human DAI (ZBP1) in complex with Z-DNA reveals an unusual binding mode to Z-DNA. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105: 20671–20676.
 - [32] Maelfait J, Liverpool L, Bridgeman A, Ragan KB, Upton JW, Rehwinkel J. Sensing of viral and endogenous RNA by ZBP1/DAI induces necroptosis. *The EMBO Journal*. 2017; 36: 2529–2543.
 - [33] Thapa RJ, Ingram JP, Ragan KB, Nogusa S, Boyd DF, Benitez AA, *et al.* DAI Senses Influenza A Virus Genomic RNA and Activates RIPK3-Dependent Cell Death. *Cell Host & Microbe*. 2016; 20: 674–681.
 - [34] Kuriakose T, Kanneganti TD. ZBP1: Innate Sensor Regulating Cell Death and Inflammation. *Trends in Immunology*. 2018; 39: 123–134.
 - [35] Chen D, Tong J, Yang L, Wei L, Stolz DB, Yu J, *et al.* PUMA amplifies necroptosis signaling by activating cytosolic DNA sensors. *Proceedings of the National Academy of Sciences of the United States of America*. 2018; 115: 3930–3935.
 - [36] Wang Z, Choi MK, Ban T, Yanai H, Negishi H, Lu Y, *et al.* Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105: 5477–5482.
 - [37] Kaiser WJ, Offermann MK. Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *Journal of Immunology*.

2005; 174: 4942–4952.

- [38] Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, *et al.* Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *Journal of Immunology*. 2003; 171: 4304–4310.
- [39] Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*. 1996; 4: 387–396.
- [40] Zhang W, Tian T, Gong SX, Huang WQ, Zhou QY, Wang AP, *et al.* Microglia-associated neuroinflammation is a potential therapeutic target for ischemic stroke. *Neural Regeneration Research*. 2021; 16: 6–11.