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ORIGINAL RESEARCH

Nrf2 Deficiency Exacerbates Parkinson's Disease by Aggravating NLRP3 Inflammasome Activation in MPTP-Induced Mouse Models and LPS-Induced BV2 Cells

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Background: Parkinson's disease (PD) is a movement disorder characterized by the progressive loss of dopamine neurons. Microglia-mediated neuroinflammation drives disease progression and becomes a critical factor in neuronal degeneration. Recent studies have found that nuclear factor-erythroid 2-related-2 (Nrf2) expression levels are reduced during aging and neurodegenerative diseases, but its regulatory mechanism on microglia-induced neuroinflammation has not been fully elucidated.

Methods: In vivo, we used the intraperitoneal injection of the neurotoxic drug neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to establish an animal model of PD and, at the same time, administered Nrf2 inhibitors ML385 and dimethyl fumarate to regulate Nrf2 protein levels. In vitro, we used si-RNA to knock out the Nrf2 gene to intervene in BV2 cells and used lipopolysaccharide (LPS) to stimulate and induce the cell model.

Results: The study found that inhibition of Nrf2 expression aggravated the motor defects of PD mice, accompanied by a significant loss of dopaminergic neurons in the substantia nigra and striatum of the brain. In addition, after inhibition of Nrf2, the malondialdehyde (MDA) level in the substantia nigra of the midbrain of mice increased, and the levels of superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) decreased, accompanied by the proliferation of microglia and astrocytes. In addition, the activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, the assembly of apoptosis-associated speck-like protein containing a CARD (ASC) protein in microglia, and the release of downstream inflammatory factors caspase-1 and interleukin (IL)-1β, were aggravated. At the cellular level, it was found that knocking out the expression of Nrf2 would aggravate the activation of NLRP3 inflammasomes and the assembly of ASC in LPS-induced BV2 cells.

Conclusion: Inhibited Nrf2 activity can reduce the downstream antioxidant enzyme HO-1 and antioxidant levels, induce NLRP3 inflammasome activation and ASC protein assembly in microglia, and ultimately aggravate PD inflammatory response and dopamine neuron degeneration.

Keywords: Parkinson's disease, Nrf2, oxidative stress, NLRP3 inflammasome, microglia

Introduction

Parkinson's disease (PD) is an age-related multifactorial neurodegenerative disease, and its hallmark pathological is the severe loss of dopaminergic neurons in the substantia nigra and striatum, which leads to neurological impairment mainly characterized by tremors, bradykinesia, and movement disorders.[1,2] As the elderly survival rate increases, PD patients' absolute number and prevalence show a clear upward trend.[3] In addition, studies have confirmed that the etiology of PD is closely related to multiple factors such as immune disorders, neuroinflammation, oxidative stress, mitochondrial

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dysfunction, genetic background, and environment.[4–6] To date, no drug has been shown to have an apparent disease-modifying effect on PD.

The innate immune response is the first line of defense of the nervous system, which relies on specialized receptors to detect danger signals such as microbial infection and tissue damage, initiate the expression of antimicrobial molecules, and induce inflammatory genes.[7] The nuclear factor-erythroid 2-related-2 (Nrf2) is a pleiotropic transcription factor at the center of a complex regulatory network that modulates cellular defenses against toxic and oxidative damage by expressing genes involved in oxidative stress responses and drug detoxification.[8,9] In addition, Nrf2 is also an essential regulator of innate immunity, which can modulate immune responses by directly or indirectly interacting with one or more of the primary innate immune signaling components that maintain cellular homeostasis.[10] Many studies have found that increasing or decreasing Nrf2 activity by pharmacological and genetic manipulation is associated with many metabolic or inflammatory diseases.[11–13] Evidence from Parkinson's disease research has found that various natural compounds and herbs can promote Nrf2 activation to protect dopaminergic neurons and slow the progression of Parkinson's disease. For example, sulforaphane has been shown to exert neuroprotective effects in animal models of PD by increasing the activity of Nrf2 and its downstream antioxidant enzymes.[14,15] Dimethyl fumarate (DMF) is an antioxidant and anti-inflammatory drug that activates the Nrf2 pathway to enhance the natural antioxidant response in Parkinson's disease models.[16,17] DMF can promote autophagosome formation and inhibit cell apoptosis by eliminating the inhibitory effect of p53 on TIGAR.[18] In addition, studies have found that in a PD mouse model using an α-Syn Adeno-associated virus (AAV) vector, Nrf2 knockout mice showed dopaminergic neuron loss, dystrophic dendrites, protein aggregation, and increased neuroinflammation.[19] 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a commonly used treatment for inducing PD in mice, enhances microglial survival by activating the p21-Nrf2 pathway in this model and offers new therapeutic potential for neurodegeneration triggered by neuroinflammation.[20]

Neuroinflammation is an immune response caused by the malfunction of immune cells following stimulation or tissue damage to the central nervous system.[21] There is increasing evidence that excessive secretion of inflammatory factors is involved in the occurrence and development of PD, and the extent and duration of the inflammatory response ultimately determine the prognosis of PD patients.[22,23] Microglia and astrocytes, critical immune cell types in innate immunity, play an essential role in protecting neuronal survival and promoting tissue recovery.[24] Clinical studies have shown immune responses in the brains of PD patients, demonstrating an upregulation trend of immune-related proteins in microglia. For example, PET-MRI imaging showed that microglial activation preceded dopamine neuron loss in PD patients.[25] The NOD, LRR, and pyrin domain protein 3 (NLRP3) are components of the innate immune system that sense the intracellular environment and cellular stress responses—activated NLRP3 further associates with ASC and caspase-1 to form the inflammasome complex, leading to the maturation and secretion of proinflammatory cytokines IL-1β and IL-18.[26] The activation of NLRP3 inflammasome is strictly regulated in the nervous system, and its complex activation mechanism is affected by immune cells. NLRP3 inflammasome-mediated neuroinflammation in microglia plays a crucial role in the innate immune response in PD.[26] Mitochondrial dysfunction and NLRP3 activation are emerging as critical players in inducing and sustaining neuroinflammation.[27] For example, a study on PD found that the p38-TFEB signaling pathway affects NLRP3 degradation by inhibiting chaperone-mediated autophagy, promoting microglia activation, and promoting dopamine neuron death.[28] Carvacrol was able to regulate NLRP3 inflammasome via the Nrf2/HO-1 axis, modulate IL-18 in the rotenone-induced PD mouse model, alleviate neurotoxicity, reduce inflammatory cytokines, and improve rotenone-induced movement disorders.[29] It can be seen that regulating the activation of NLRP3 inflammasome in PD is the key to the body's resistance to the invasion of harmful substances and maintaining metabolic balance.

Nrf2 as a critical factor in anti-oxidative stress, can mediate oxidative stress and exert protective properties by widely controlling immune responses and immune inflammation by directly or indirectly interacting with important innate immune components, where its specific regulatory mechanism is still unclear. The novelty of this study is to explore the potential mechanism of Nrf2 as a transcriptional regulator of antioxidants in regulating immune cell-mediated neuroinflammation and its impact on the inflammatory signaling pathway NLRP3-ASC and the expression of inflammatory factors, providing new and valuable insights into alleviating neurodegeneration in PD.

Materials and Methods

Animals

Male C57BL/6 mice (6–8 weeks old) weighing 22–25g were purchased from Speifu Biotechnology Co., Ltd. (Beijing, China). Raising mice in a pathogen-free facility exposes them to an environment of 22–25 degrees Celsius, 50% to 60% humidity, a 12-hour light/12-hour dark cycle, and providing appropriate amounts of food and water. The animal experiments were approved by the Xinjiang Medical University Ethics Committee (approval number: IACUC-20230217-46), and all animal presentations were performed strictly with the Animal Care and Use Guide of Xinjiang Medical University. We used PASS software to estimate the number of animals in each group. Considering the chance of the experiment, 12 animals in each group were used to conduct the study to obtain statistically significant conclusions.

Reagents and Antibodies

Antibodies to the following proteins were used in this study: TH (1:1000; T2928, Sigma-Aldrich), NLRP3 (1:500, AG-20B-0014, AdipoGen), Iba1 (1:1000, 019–19741, Wako), Nrf2(1:500, ab62352, Abcam), HO-1 (1:500, AF5393, Affinity), GFAP (1:500, #3670, Cell Signaling Technology), ASC (1:400, #67824, Cell Signaling Technology), caspase-1 (1:1000, A21085- ABclone), IL-1β, 1:50,016,806-1-AP, Proteintech), actin (1:5000, 66,009-1-Ig, Proteintech), iNOS,1:1000, 18,985-1-AP, Proteintech. The fluorescently labeled secondary antibodies are goat anti-rabbit (1:1000, #4412, Cell Signaling Technology) and goat anti-mouse (1:1000, #4409, Cell Signaling Technology) Other reagents include: MPTP (M0896, Sigma-Aldrich, USA), ML385 (Mce, HY-100523), DMF (Mce, HY-17363).

MPTP Mice Models

MPTP was dissolved in 0.9% saline, and the drug concentration was 30 mg/kg. We randomly divided 60 mice into 4 groups: the sham group, the MPTP group, the MPTP+ML135 group, and the MPTP+DMF group. Mice in the MPTP group, MPTP+ML385 group, and MPTP+DMF group were injected intraperitoneally with MPTP (30 mg/kg) once a day for consecutive 5 -days, and the sham group was injected with an equal volume of normal saline.[30,31] Mice in the MPTP +ML385 group were injected intraperitoneally with ML385 (30 mg/kg) daily for consecutive 7 days. Mice in the MPTP +DMF group were orally administered DMF (50 mg/kg) daily for 7 days. Mice in each group were sacrificed after 21 days, and behavioral tests were conducted before execution, including an open field test, pole climbing test, and hanging test. Safety measures such as preparation and handling of MPTP comply with published guidelines.

Mouse Behavioral Tests

Open Field Test

We aimed to evaluate changes in spontaneous movement and exploratory behavior in mice. The Open field equipment (100 cm long, 100 cm wide, 50 cm high) is placed in a low-light, quiet environment. Before the test, the mice were placed alone in the middle of the equipment and walked for 5 minutes to adapt to the environment. The total site is divided into 16 areas; the sum of the distances of all 16 areas represents the “total distance”, and the sum of the distances of the four intermediate areas represents the “middle distance”. The distance the mice walked was measured by intelligent video tracking software, and the DigBehv animal behavior analysis software was used to analyze the distance and speed of the mice in the open field.

Pole Test

To evaluate the motor coordination ability of each group of mice, we placed the mice on the top of a pole 50 cm high and 1 cm in diameter. We recorded the time required for the mice to successfully descend from the top to the bottom of the pole.[32] Each mouse was placed at a specific time interval and tested three times per hour, with the average of the three tests taken.

Suspension Test

The muscle strength of the mouse’s limbs was measured by the suspension method. First, the mouse was placed on the lid of the mouse cage, and then the lid was turned upside down so that the mouse’s forelimbs could grasp the lid. The

suspension of the mouse's hind limbs was recorded and scored. When a mouse was inverted on the cage lid to when it fell, each mouse was measured three times, and the interval between the three measurements was at least 30 minutes.

Western Blotting

Proteins from mouse brain tissue and BV2 cells were extracted using a lysis buffer containing a protease inhibitor cocktail at 12,000 rpm. The supernatant was collected after 15 minutes, and the protein concentration was detected using a BCA kit. We separated the proteins using SDS-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to a PVDF membrane by electroporation. The membrane was blocked with 5% skim milk for 2 hours at room temperature on a shaker. The membrane was fully incubated with the corresponding primary antibody at 4°C overnight and washed three times with Tris-buffered saline containing Tween (TBST) the next day. The secondary antibody was incubated for 1 hour at room temperature and washed thrice with TBST. Finally, the data was detected using a chemical imaging system and processed with Image J.

Nissl Staining

Nissl staining is combined with nucleic acids in neural tissue and is used to evaluate the degree of neuronal damage. After behavioral testing, various mouse brain tissue samples were perfused and collected (n=3). When collecting the samples, avoid squeezing the tissue to avoid the illusion of neuronal contraction. Prepare frozen sections, drop tissue sections into Nissl staining solution, place in a 37°C incubator for staining for 30 minutes, wash twice with distilled water, differentiate with 95% ethanol for 5 seconds, dehydrate with graded alcohol, make it clear in xylene, seal with neutral gum for 10 minutes Observe and count under the microscope after the film.

Immunohistochemistry and Immunofluorescence

After the behavioral test, the mice were anesthetized with an intraperitoneal injection of 1% pentobarbital (n=3). The mice in each group were cardiac perfused, and their brains were removed for immunohistochemistry (IHC) and immunofluorescence (IF) staining. Brain slices were blocked with 3% catalase for 15 min, 0.3% TritonX-100 for 20 min, and goat serum was added dropwise for 30 min. The serum was discarded, and the primary antibody for TH (1:400) was added dropwise and incubated overnight at 4°C. The next day, the secondary antibody was incubated at 37°C for 1 hour, DAB staining was added dropwise, and the slides were transparently sealed, observed, and photographed under a microscope. The preparation process of immunofluorescence brain slices was consistent with the above. Add goat serum to the brain slices and incubate for 30 minutes for blocking. Add the primary antibody TH (1:400)-IBA1 (1:1000) mixture and keep it overnight at 4°C. Add fluorescent secondary antibody mixture dropwise and incubate at 37°C for 1h. Add an anti-fluorescence attenuation tablet containing DAPI, and take pictures under a laser confocal microscope. The results were calculated and analyzed using IPP6.0 image analysis software.

Measurement of Oxidative Stress Markers

Malondialdehyde (MDA) is a product of lipid peroxidation, and its content can indirectly reflect the tissue oxidative stress level. Superoxide dismutase (SOD) is a type of antioxidant enzyme whose function is to catalyze superoxide to generate hydrogen peroxide. Use according to the kit's instructions and the BCA protein analysis kit to detect the protein concentration after centrifugation. Detection was performed using MDA and SOD kits ((Jiangsu Jiancheng, Nanjing, China). The chemical colorimetric method was used to detect the MDA level and SOD activity in the substantia nigra of the brains of mice in each group. The thiobarbituric acid (TBA) method was used to determine MDA content, and the xanthine oxidase method was used to determine SOD activity. MDA levels were expressed in U/mgprot, and SOD activity was expressed in nmol/mgprot.

Cell Culture and Treatment

BV2 cells (Purchased from Procell Life Science&Technology Co., Ltd.)were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 12% fetal bovine serum (Gibco) and 1% penicillin-streptomycin at 37°C and saturated

humidity of 5% carbon dioxide. When BV2 cells grow to 60–70% confluence, refresh with a new medium containing PBS and incubate for 60 min. Then cells were stimulated with 500ng/mL PBS or LPS for 24 h.

Transfection of BV2 Cells with Nrf2-siRNA

Nrf2-siRNA (Purchased from GenePharma, shanghai) was used to knock down BV2 cells. Nrf2-siRNA consists of the following sequence: GGAAGCUGGAGAACAUUGUTT, ACAAAUGUUCUCCAGCUUCCTT. Scrambled-siRNA consists of the following sequence: UUCUUCGAACGUGUCACGUTT, ACGUGACACGUUCGGAGAATT. BV2 cells were transfected with Nrf2-siRNA or control siRNA for 48 h. RT-PCR and Western blot confirmed the knockdown efficiency of Nrf2-siRNA in BV2 cells.

Cell Viability Assay

The BV2 cell suspension was seeded into a 96-well plate at a cell number of 1×10^4 cells/well. After the cells had fully adhered to the wall, they were grouped into groups for plasmid transfection in the same manner as above, with 3 duplicate wells set in each group. After 24 hours of LPS intervention, the culture medium was removed, 10 ul of CCK-8 solution was added to each well, and the culture was continued for 2 hours. The absorbance (A) value at 450 nm was measured with a microplate reader. The absorbance data of each group were then compared with those of the control group (untreated cells), and all experiments were repeated three times.

Real-Time RT-PCR

Total RNA was extracted from BV2 cells using an RNA extraction kit (TIANGEN, Beijing, China). Total RNA was reverse transcribed into cDNA using the PrimeScrip RT Master Mix kit. The SYBR qPCR superMix Plus was used to perform qRT-PCR to evaluate the relative expression of mRNA. The reaction conditions were as follows: predenaturation at 94°C for 35 seconds, denaturation at 95°C for 5 seconds, annealing at 60°C for 34 seconds, 40 cycles. Data analysis used the $^{2-\Delta\Delta}$CT method, with GAPDH as the internal control. The primer sequences (5′-3′) are as follows: Nrf2 forward: GCTGGCTACCGCTGTTC, reverse: TGGAGAGGATGCTGAAAGAATC; GADPH forward: ACTCCACTCACGGCA AATTCAAC, reverse: ACACCAGTATCCACGACATAC.

Statistical Analysis

The experimental data were statistically analyzed using SPSS 27.0 statistical software. The comparison between two samples was performed using a *t*-test, and the comparison between multiple groups was performed using a one-way analysis of variance. The results were expressed as the mean±SD of at least three independent experiments. Statistical analysis used analysis of variance followed by Bonferroni post-correction for multiple comparisons. $P<0.05$ is considered statistically significant.

Result

Inhibition of Nrf2 Significantly Aggravates Motor Dysfunction in PD Model Mice

We used an intraperitoneal injection of an MPTP-induced mouse model of PD to observe dopamine neurons' degeneration process. To explore the impact of Nrf2 expression level on the pathological changes of PD, we used the Nrf2 inhibitor ML385 and the Nrf2 activator DMF to intervene in the PD animal model. The flowchart of experimental animal preparation is shown below (Figure 1A). The open field test, pole climbing test, and suspension test results confirmed that mice developed apparent movement disorders after intraperitoneal injection of MPTP. The results of behavioral experiments showed that the movement patterns of mice in the ML385 group were significantly different from those in the MPTP group. Compared with the MPTP group, the number of mice in the ML385 group entering the central area in the open field test was significantly reduced, the movement speed slowed, and the total movement distance was shortened (Figure 1B–D). Consistent with the above results, the time required for mice to climb the pole was prolonged, and the time for the forelimbs to hang and fall to the ground was shortened. The movement disorders of PD model mice were significantly improved when DMF was used (Figure 1E and F). In summary, inhibiting Nrf2 protein levels can aggravate

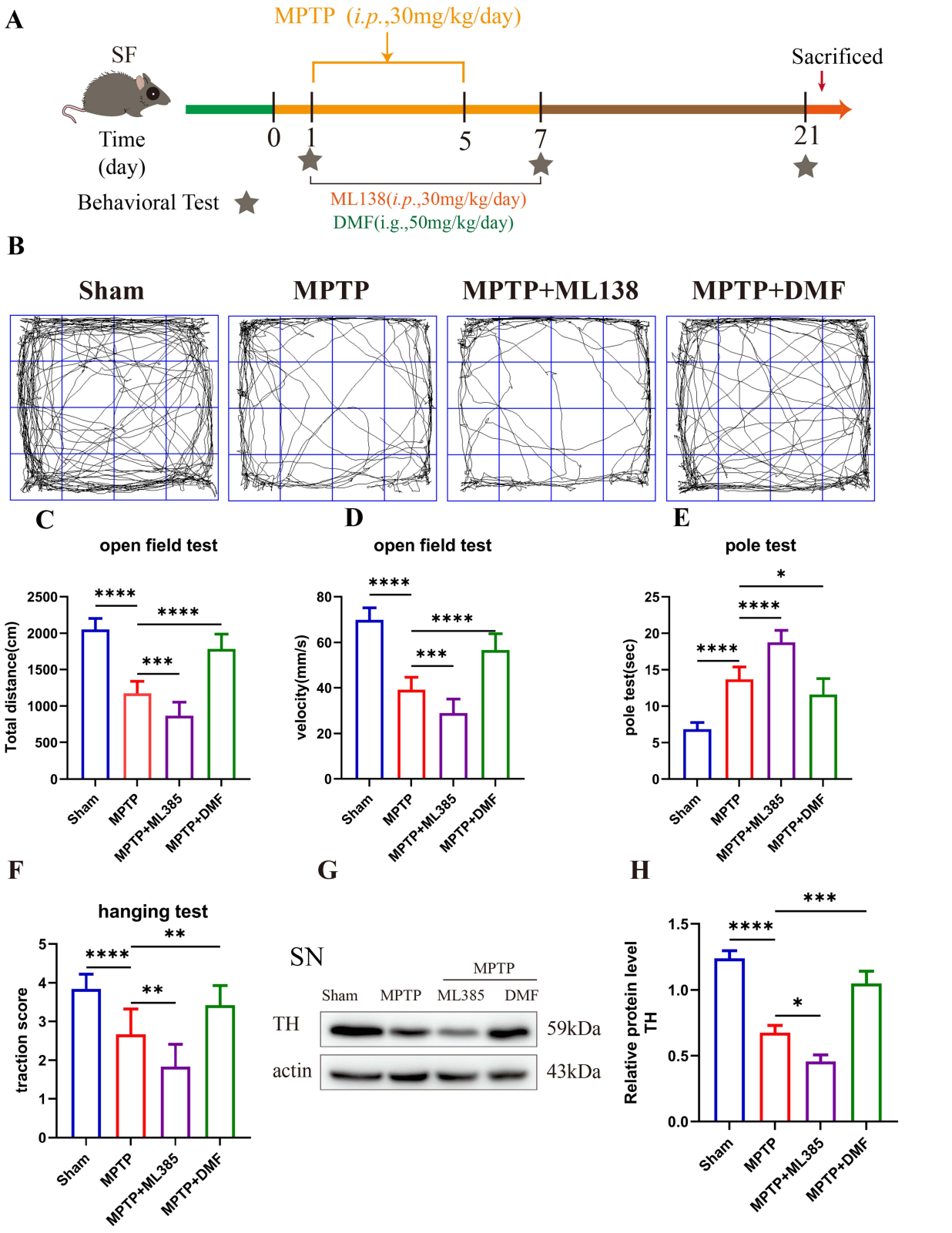

Figure 1 Flowchart of Parkinson's disease model mouse design and behavioral testing. (**A**) Experimental design flow chart. DMF (50 mg/kg) was intragastrically injected for 7 days, ml385 (30 mg/kg) was intraperitoneally injected for 7 days, and MPTP (30 mg/kg) was intraperitoneally injected for 5 days (n = 15 per group). (**B**) Open field test movement trajectory diagram. (**C**–**F**) Quantitative analysis of mouse behavioral results. (**G**) Western blotting of TH protein in the Substantia Nigra of mice. (**H**) Quantitative analysis of the expression level of TH. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

MPTP-induced PD mice's bradykinesia and balance disorders. Movement disorders improved after DMF treatment, confirming that regulating Nrf2 may be a potential intervention target for the treatment of PD.

Inhibiting Nrf2 Aggravates Dopamine Neuron Degeneration and Loss in PD Model Mice

To explore the role of Nrf2 activity in dopamine neurons in PD animal model mice, we used Western- blotting, and immunohistochemistry to detect the effects of changes in Nrf2 activity on the expression of dopamine neuron-related proteins and the morphology and number of neurons. Western blot results showed that compared with the sham operation group, the expression level of TH protein in the substantia nigra of the PD animal model group mice was reduced, and the expression of TH protein in mice after administration of ML385 was significantly decreased (Figure 1G and H). At the same time, we observed the results of immunohistochemistry and Nissl staining. We showed that compared with the sham operation group, the number of dopaminergic neurons in the substantia nigra of PD animal model mice was significantly reduced, the number of Nissl bodies was reduced, and the cell body morphology was smaller, confirming the existence of substantia nigra dopamine neuron cell damage (Figure 2A and B). Compared with the MPTP group, a large number of dopamine neurons were lost in the substantia nigra of the ML385-treated mice. The number of dopamine neurons decreased, and the remaining cells had fragmented cell bodies, light cytoplasm staining, and nuclear condensation (Figure 2C and D). Compared with the MPTP group, the loss of dopaminergic nerve fibers in the striatum of the ML385 group was more severe (Figure 3A and B). Moreover, Western blot analysis showed that the expression level of TH protein was significantly reduced, further confirming the above characteristics (Figure 3C and D). We demonstrate that administration of the Nrf2 inhibitor ML385 leads to more severe dopaminergic neuron loss in the striatum and substantia nigra. Treatment with DMF can improve dopamine synthesis in the brains of PD mice, maintain nigrostriatal function, and normalize motor functions. This result indicates that Nrf2 may play an essential role in the metabolic process of dopamine neurons in the striatum and substantia nigra. It even plays a crucial role in the occurrence and development of PD.

Inhibition of Nrf2 Enhances MPTP-Induced Microglia and Astrogliosis

The result of the study found that there was significant proliferation and activation of microglia and astrocytes in the substantia nigra and striatum of mice after MPTP injection, and the expression of dopamine neurons showed an opposite trend. We observed by immunofluorescence that the expression of microglial marker IBAI in the substantia nigra and striatum of ML385 group mice was different compared with that of MPTP group mice, with a significant increase in number and accompanied by changes in cell morphology (Figure 4A–D). In addition, the astrocyte surface marker GFAP showed a clear activation state, manifested by enlarged cell bodies, shorter branches, less complex structures, and a significant decrease in the expression of dopamine neurons (Figure 5A–D). After DMF treatment, the expression of GFAP and IBA1 was significantly reduced; microglia had more branches, longer processes, smaller cell bodies, and increased TH expression. As immune cells of the central nervous system, activation of microglia and astrocytes is crucial in the pathogenesis of neuroinflammation in PD. Targeted regulation of Nrf2 expression exerts specific effects on the activation and proliferation of microglia and astrocytes in MPTP-induced neuroinflammation.

Inhibition of Nrf2 Exacerbates NLRP3 Inflammasome Activation in MPTP-Induced PD Mouse Model

Through immunofluorescence detection, we found that the expression of NLRP3 (green) in the substantia nigra of mice in the MPTP group increased, which was positively correlated with the expression of microglia (red)activation (Figure 6A and B), and the expression of GFAP (green) and ASC (red) was positively correlated. Related (Figure 6C and D). Western blotting detected the protein expression levels of NLRP3, ASC, IBA1, and GFAP in the substantia nigra area of the midbrain of mice after intraperitoneal injection of MPTP. Compared with the control group, the expression of NLRP3 and ASC proteins in the substantia nigra area of the midbrain of mice in the MPTP group was significantly increased (Figure 7A–C), and the expression of IBA1 and GFAP was increased (Figure 7D–G). This result suggests that the proliferation and activation of

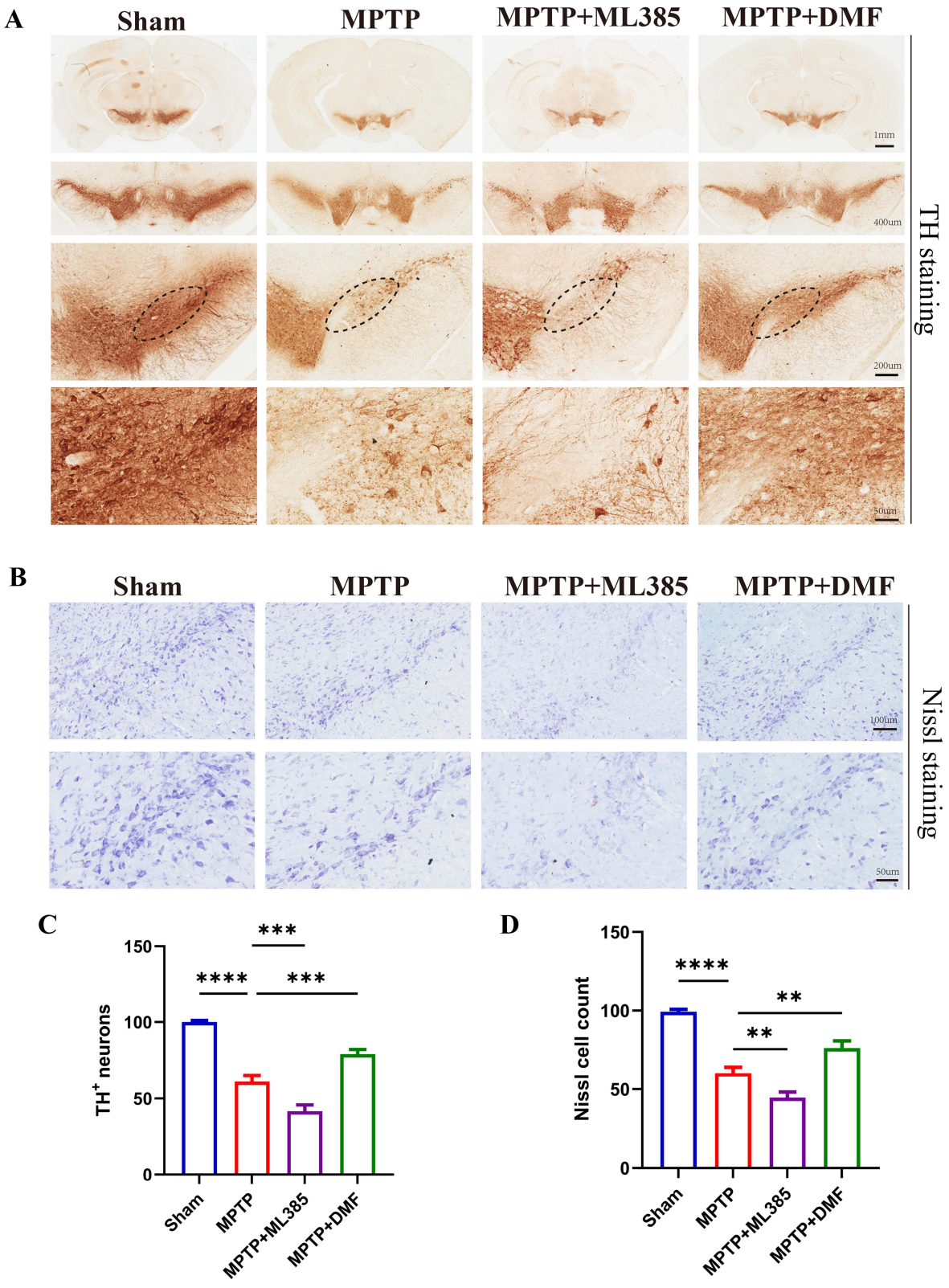

Figure 2 Immunohistochemical TH staining and Nissl staining of substantia nigra in each group of mice. (**A**) TH Immunohistochemical staining image of the substantia nigra area of mice (1×, 3×, 5×, 20×), n = 3 per group. (**B**) Nissl staining image of the substantia nigra area of mice (10×, 20×), n = 3 per group. (**C**) Quantitative analysis of the number of dopamine neuron cells in the substantia nigra area of mice. (**D**): Quantitative analysis of the number of Nissl-positive cells in the substantia nigra area of mice. Data were presented as mean ± SD, a one-way analysis of variance. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

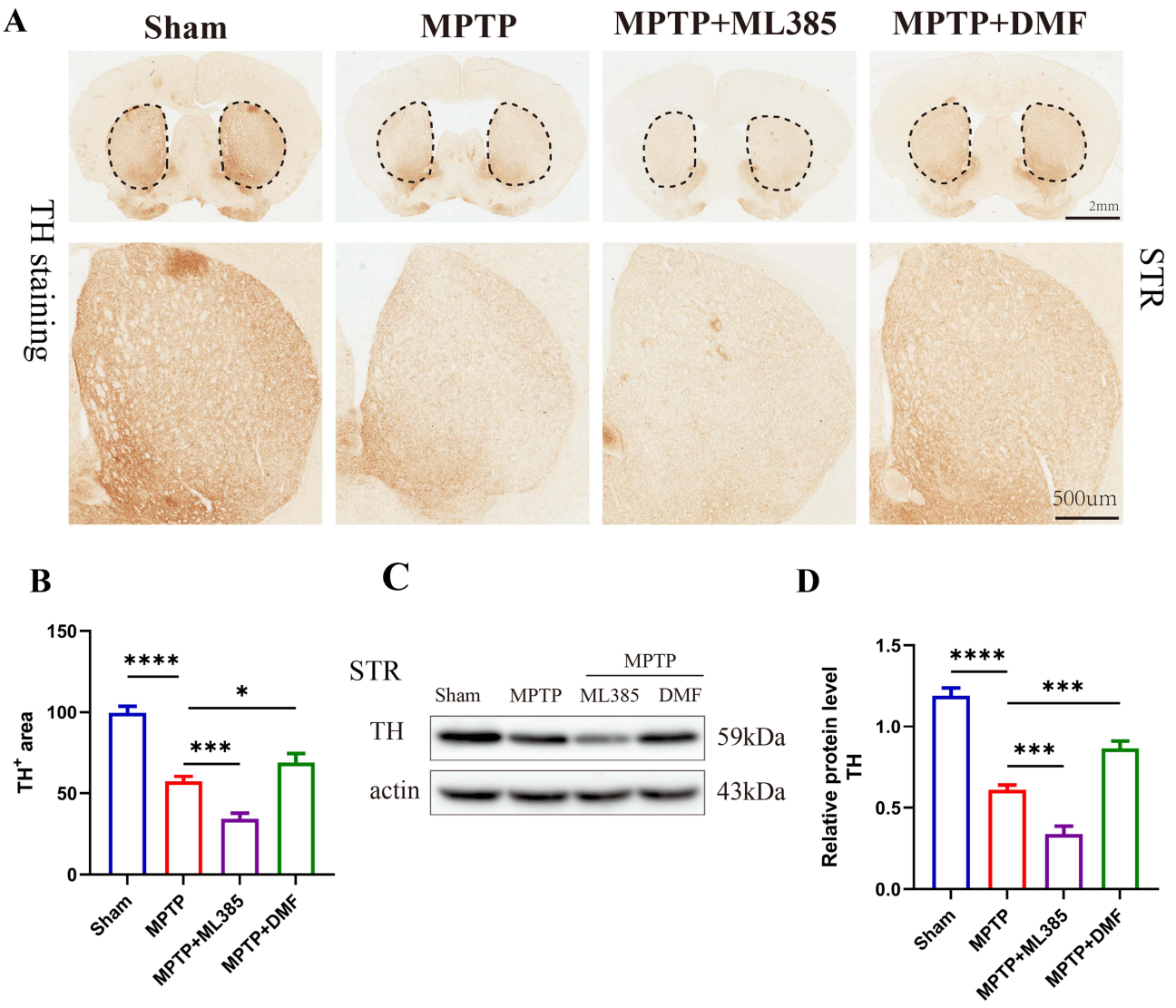

Figure 3 Immunohistochemical TH staining and Western blot of the striatum of each group of mice. (**A** and **B**) Immunohistochemical TH staining in the striatum and quantitative analysis of striatal axonal fibers of mice in each group, n = 3 per group. (**C** and **D**) A representative Western blot and quantitative analysis showed protein levels of TH in the striatum of each group of mice, n = 3 per group. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

microglia and astrocytes are closely related to the NLRP3/ASC signaling pathway. Activation of the NLRP3 inflammasome leads to the antigen presentation response of immune cells and induces inflammation, which is ultimately positively correlated with PD progression. Compared with the MPTP group, immunofluorescence results showed that the activated expression of NLRP3/ASC in mice in the ML385 group was significantly enhanced and was positively correlated with IBA1 and GFAP. The Western blot results were consistent with the above, and the expression levels of NLRP3 and ASC proteins were also increased. After treatment with DMF, compared with the MPTP group, immunofluorescence showed reduced expression of NLRP3, ASC, IBA1, and GFAP. Western blot results showed that DMF could reduce the expression of NLRP3 and ASC proteins. It can be seen that the protein expression of NLRP3 and ASC is positively correlated with the activation of microglia and astrocytes, suggesting that the NLRP3/ASC signaling pathway may be involved in the pathogenesis of PD. When the expression of Nrf2 is reduced or missing, the NLRP3/ASC signaling pathway in PD is activated, further aggravating the proliferation and activation of microglia and astrocytes, promoting the irreversible development of the disease. These results indicate that Nrf2 may play an important role in the pathogenesis of PD by regulating the NLRP3/ASC signaling pathway.

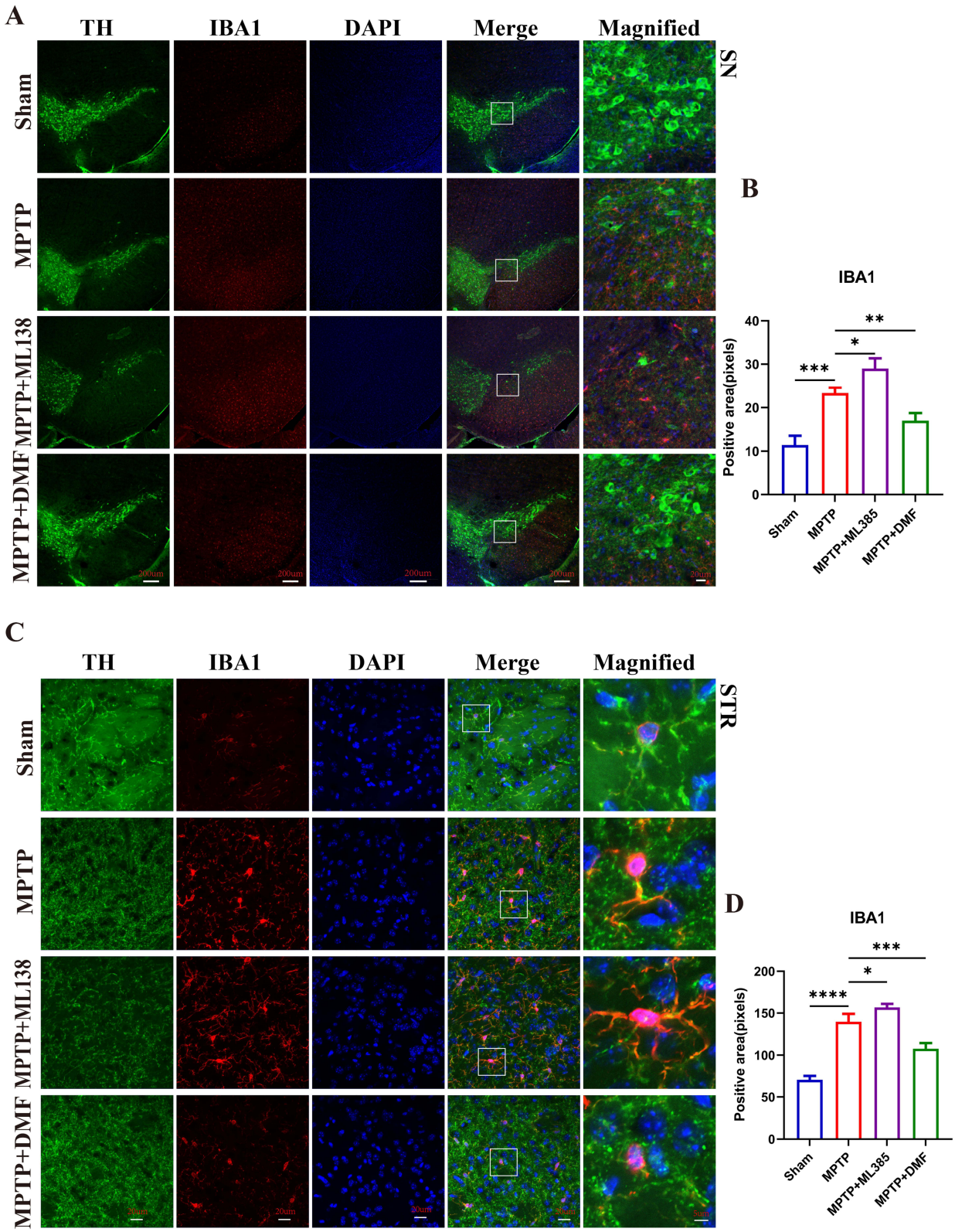

Figure 4 Microgliosis in the substantia nigra and striatum of mice. (**A** and **B**) Representative immunofluorescence image of TH (green) and IBA1 (red) labeled in the substantia nigra and quantitative analysis of the number of IBA1 positive areas, n = 3 per group, scale bar =200um. (**C** and **D**) Representative immunofluorescence image of TH (green) and IBA1 (red) labeled in the striatum and quantitative analysis of the number of IBA1 positive areas, scale bar =20um. The area outlined in the box is shown at higher magnification on the right. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

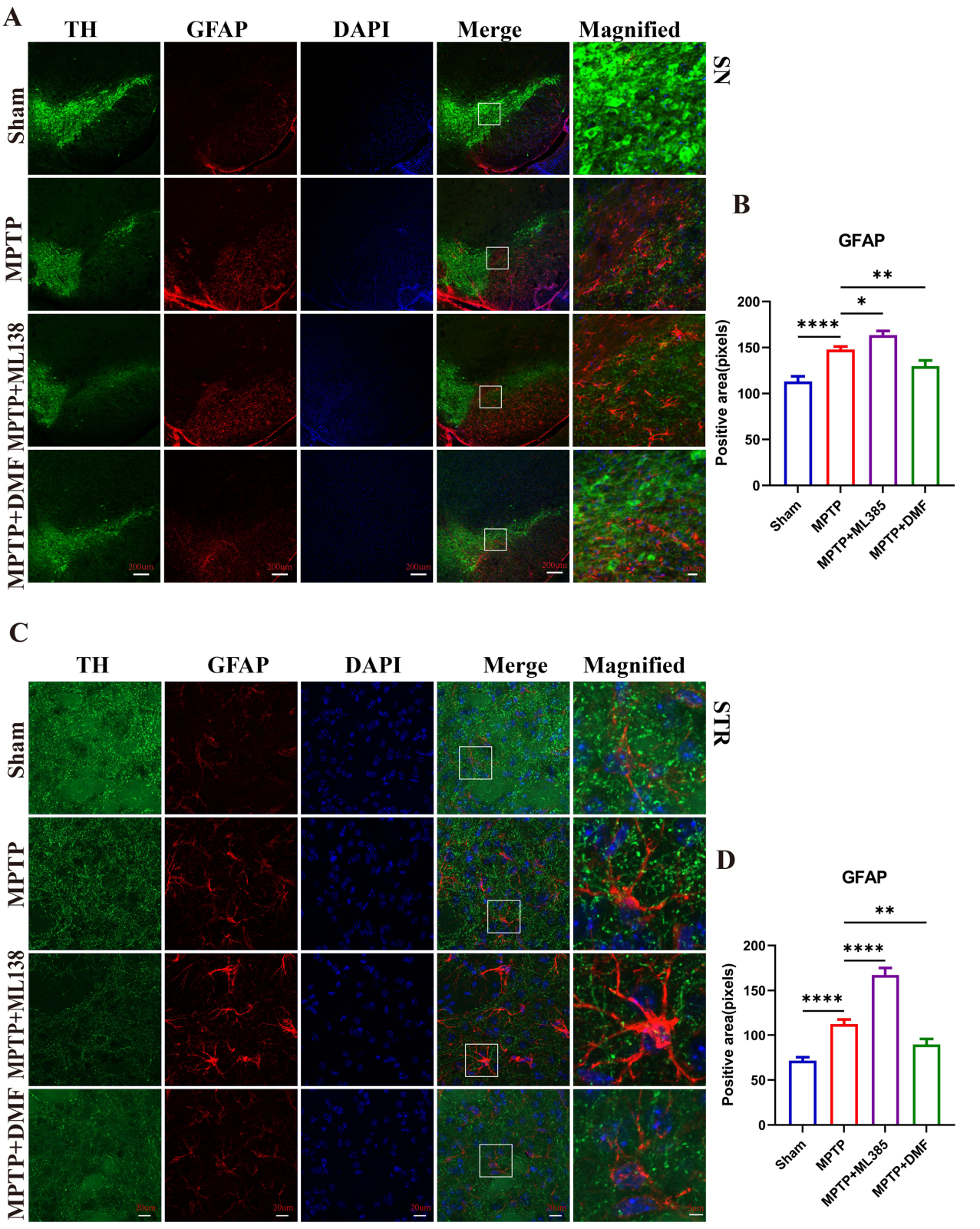

Figure 5 Astrogliosis in the substantia nigra and striatum of mice. (**A** and **B**) Representative immunofluorescence image of TH (green) and GFAP (red) labeled in the substantia nigra and quantitative analysis of the number of GFAP positive areas, n = 3 per group, scale bar =200um. (**C** and **D**) Representative immunofluorescence image of TH (green) and GFAP (red) labeled in the striatum and quantitative analysis of the number of GFAP positive areas, scale bar =20um. The area outlined in the box is shown at higher magnification on the right. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

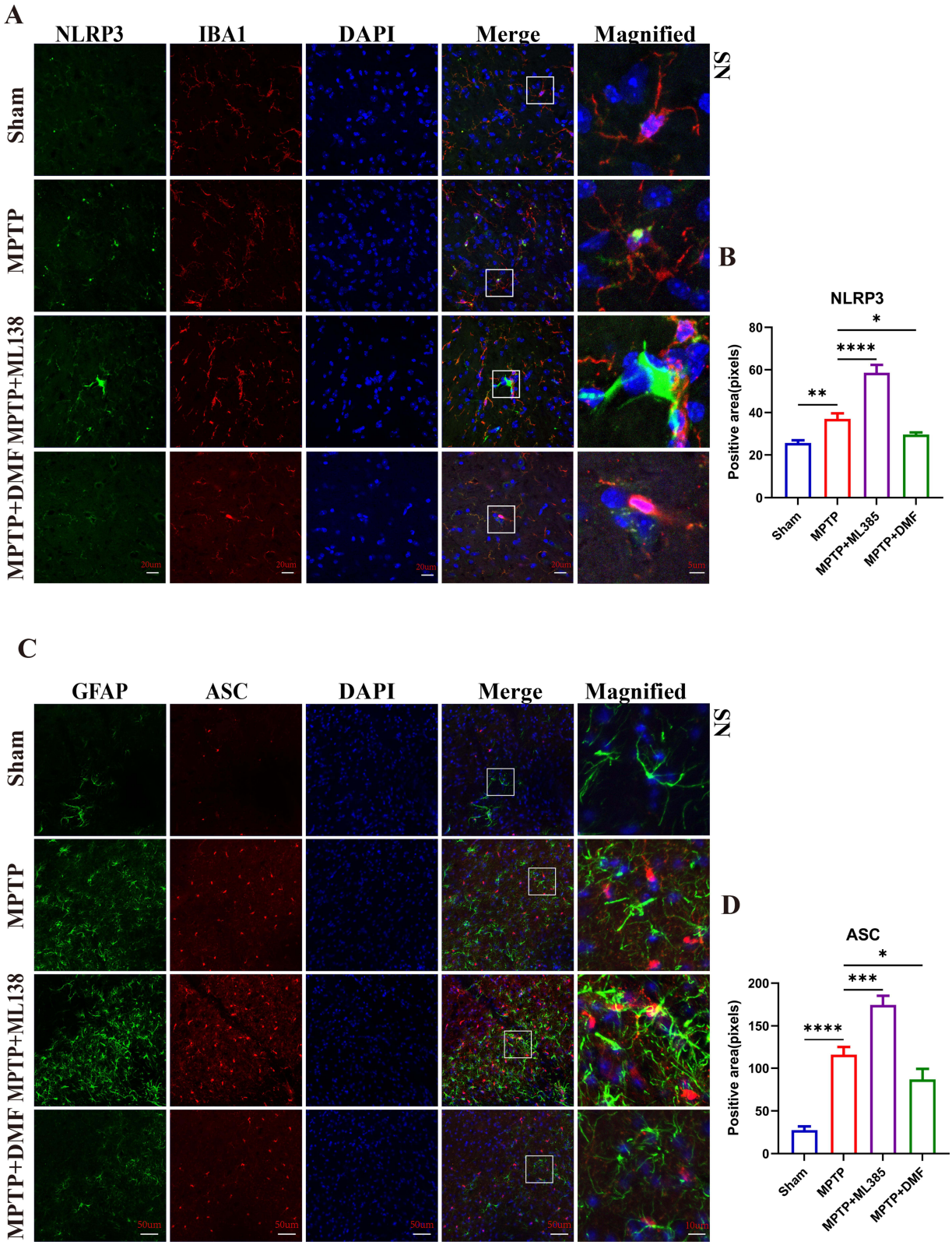

Figure 6 Activation of the NLRP3-ASC inflammasome in the substantia nigra of mice. (**A** and **B**) Representative immunofluorescence image of NLRP3 (green) and IBA1 (red) labeled in the substantia nigra and quantitative analysis of the number of NLRP3 positive areas, n = 3 per group, scale bar =20um. (**C** and **D**) Representative immunofluorescence image of GFAP (green) and ASC (red) labeled in the substantia nigra and quantitative analysis of the number of ASC positive areas, scale bar =50um. The area outlined in the box is shown at higher magnification on the right. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$, ****$p < 0.0001$.

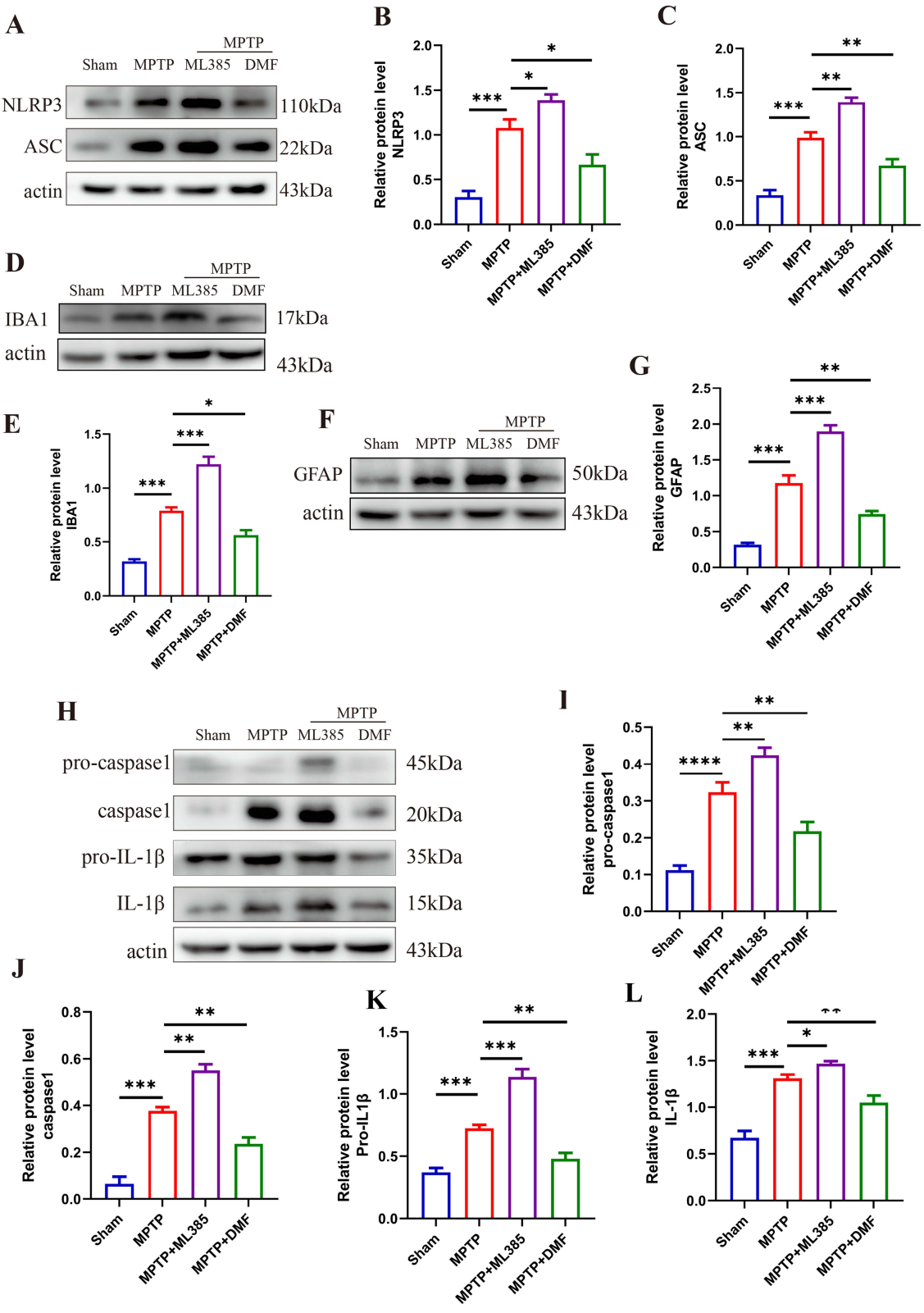

Figure 7 The release of NLRP3-ASC and inflammatory factors in the substantia nigra of mice. (**A**–**C**) The representative Western blotting of NLRP3-ASC-related proteins and quantitative analysis of the expression level, n = 3 per group. (**D**–**G**) The representative Western blotting of IBA1-GFAP-related proteins and quantitative analysis of the expression level. (**E**–**L**) The representative Western blotting of Caspase-1-IL-1β-related proteins and quantitative analysis of the expression level. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Inhibition of Nrf2 Regulates Oxidative Stress and Neuroinflammation in PD Model Mice

Western blotting detected the expression of inflammatory factors pro-caspase1, caspase1, pro-IL-1β, and IL-1β in the substantia nigra area. Compared with the MPTP group, the protein expression of caspase1 and IL-1β in mice in the ML385 group increased. The protein expression of caspase1 and IL-1β decreased after treatment with DMF (Figure 7H–L). At the same time, we also detected the protein expression levels of NRF2 and antioxidant enzyme HO-1. The results showed that compared with the MPTP group, the protein expression levels of NRF2 and HO-1 in the ML385 group were significantly reduced (Figure 8A–C). We detected oxidative stress markers in the substantia nigra of mice, and the results showed that compared with the MPTP group, the MDA values of mice in the ML385 group were significantly increased, and the SOD levels were decreased (Figure 8D–E). After treatment with DMF, the accumulation of MDA decreased, accompanied by an increase in the expression level of SOD. When ML385 is used to inhibit the expression of the Nrf2 pathway, it may activate NLRP3/ASC signaling-dependent caspase-1 maturation and IL-1β release in microglia and astrocytes by regulating the release of ROS during oxidative stress. Thereby accelerating dopaminergic neurodegeneration in PD mice. The above results confirm that Nrf2 can participate in the anti-inflammatory process and exert neuroprotective effects by regulating the protein expression of downstream antioxidant enzyme HO-1 and reducing the expression of oxidation products.

Nrf2 Gene Knockout in BV2 Cells Enhances LPS-Induced NLRP3 Inflammasome Activation

We used Nrf2-siRNA to knock down the gene and observed the activation of NLRP3 inflammasome and the assembly of ASC in LPS-stimulated BV2 microglia. We verified gene knockout efficiency by real-time fluorescence RT-PCR and Western blot (Figure 9A–D). It was observed under a fluorescence microscope that LPS stimulation caused the formation

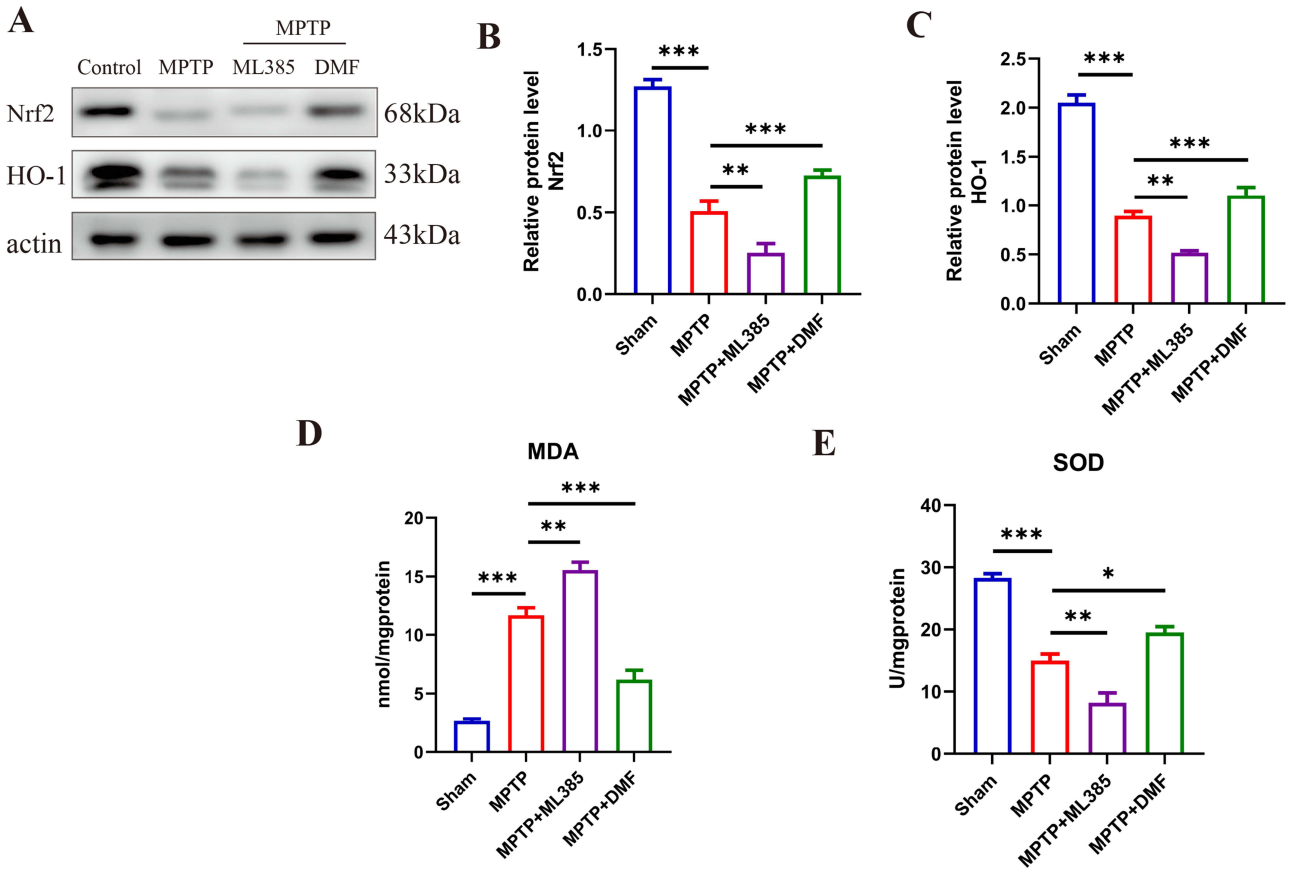

Figure 8 Nrf2/HO-1 protein and oxidative stress marker levels in the substantia nigra of mice. (**A**–**C**) The representative Western blotting of Nrf2/HO-1 related proteins and quantitative analysis of the expression level, n = 3 per group. (**D** and **E**) Quantitative analysis of SOD and MAD in the substantia nigra of mice. Data were presented as mean ± SD, a one-way analysis of variance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

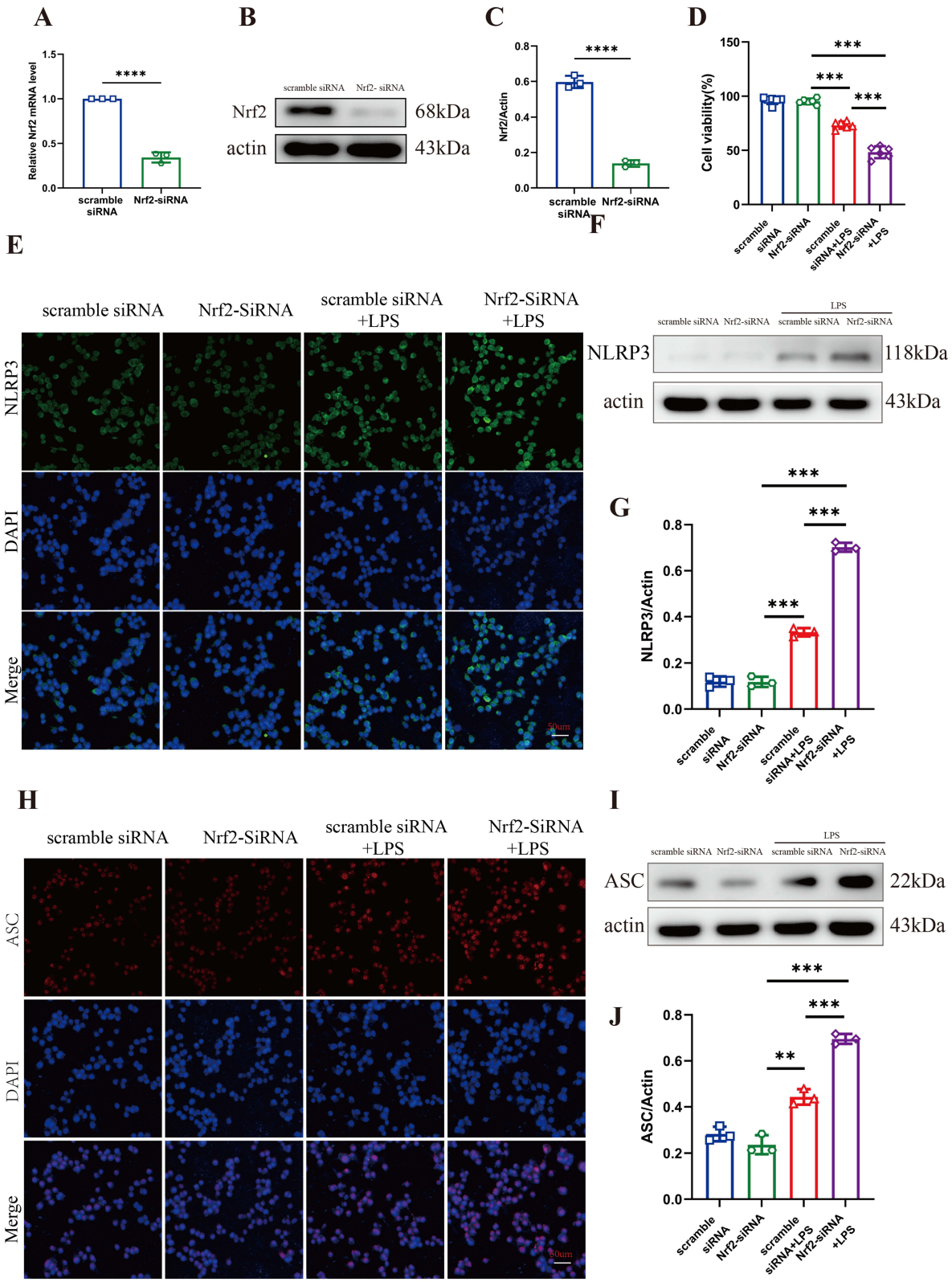

Figure 9 Nrf2 deficiency exacerbates NLRP3 inflammasome activation in BV2 cells. (**A**–**C**) RT-qPCR and Western blotting were used to detect the transfection efficiency of Nrf2-siRNA in BV2 cells. (**D**) Quantitative analysis of cell viability in each group. (**E**) Representative immunofluorescence of NLRP3 (green) labeled in the BV2 cell, scale bar is 50um. (**F** and **G**) The representative Western blotting of NLRP3-related proteins and quantitative analysis of the expression level (**H**) Representative immunofluorescence of ASC (red) labeled in the BV2 cell. (**I** and **J**) The representative Western blotting images of ASC-related protein and quantitative analysis of the expression level. Data were presented as mean ± SD, a one-way analysis of variance. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

of NLRP3 and ASC spots in BV2 cells, and these two spots were significantly enhanced after Nrf2-siRNA treatment (Figure 9E–G). Western blot analysis also showed that the protein levels of NLRP3 and ASC were further enhanced after inhibiting Nrf2 protein expression in BV2 microglia compared with disrupted siRNA-treated cells (Figure 9H–J). These results support that Nrf2-siRNA aggravates LPS-induced activation of the NLRP3 inflammasome.

Discuss

Neuroinflammation and oxidative stress are hallmarks of neurodegeneration and play an essential role in the development of PD.[33,34] Mitochondrial dysfunction is a critical immune checkpoint in neuroinflammation and neurodegeneration, and dysfunctional mitochondria aggregate and trigger innate immune signaling. As a transcription factor widely present in glial cells and neurons, Nrf2 can induce the expression of antioxidant genes to exert neuroprotective effects.[35] Studies have found that Nrf2 levels decrease with age, and irreversible decreases in Nrf2 protein expression have been observed in neurodegenerative diseases and aging.[36,37] Activation of Nrf2 can regulate the function of dopamine neurons, and many Nrf2 agonists have been shown to reverse behavioral and tissue abnormalities in PD animal models.[38] Data confirm that Nrf2 is involved in the occurrence and development of PD and is related to the progression of the disease.[39] In the early stages of PD, a-syn induces the production of a large amount of ROS in the body and upregulates the expression of Nrf2.[40] This may be related to the fact that Nrf2 regulates the expression of antioxidant genes, such as downstream antioxidant enzyme HO-1, to resist oxidative stress, thereby reducing ROS levels. As the disease progresses, Nrf2 levels begin to drop off a cliff. Studies have found that in a PD mouse model overexpressing a-syn, the loss of Nrf2 increased the degeneration of dopaminergic neurons and motor impairment in PD mice.[19] Overexpression of Nrf2 prevents a-syn aggregation and inhibits dopamine neuron degeneration.[41] We investigated the effects of Nrf2 on dopamine neuron neurodegeneration through multiple molecular signaling pathways mediated by immune responses following microglia and astrocyte activation.

Glial activation and subsequent immune response in the central nervous system are hallmarks of neuroinflammation in Parkinson's disease.[42] Excessive activation of microglia and the release of downstream pro-inflammatory cytokines accelerate disease progression.[43] The NLRP3 inflammasome, a key mediator of the innate immune system's response to harmful pathogens (cell debris and aggregated proteins), has emerged as a critical mechanism driving neuroinflammation in PD.[44,45] Clinical studies have found that the accumulation of NLRP3 inflammasome can be observed in dopaminergic neurons of PD patients and is highly expressed in microglia.[46] The activated NLRP3 inflammasome regulates the release of downstream inflammatory factors, thereby aggravating mitochondrial damage and promoting the irreversible loss of dopaminergic neurons, which is positively correlated with the progression of the disease.[47,48] In addition, studies have found that in animal models of PD neurotoxicity induced by MPTP, rotenone, and paraquat, there is the activation of the NLRP3 inflammasome and triggers caspase-1-mediated release of IL-1β and IL-18, driving dopamine neurons—the pathological process of gradual loss.[49] Studies have shown that small molecule NLRP3 inhibitors can inhibit the activation of NLRP3 inflammasome, effectively alleviate movement disorders in PD animal models, improve nigrostriatal dopaminergic degeneration, and prevent the formation and accumulation of a-syn aggregates. Therefore, neuroinflammation mediated by NLRP3 activation has become a key factor driving dopamine neuron loss in PD, making it a potential target for disease treatment.

Neurons in the central nervous system have high oxygen consumption rates and relatively low antioxidant enzyme activities, making them more susceptible to the harmful effects of oxidative stress than other cells.[50] Oxidative stress is a dangerous event that can generate large amounts of reactive oxygen species or cause insufficient antioxidant defense systems to trigger or enhance inflammatory responses.[51,52] The study found that when the antioxidant function in the body is weakened, MPTP-induced dopaminergic neurodegeneration and neuroinflammation will be aggravated.[53] Studies have shown that oxidative stress is essential for activating the NLRP3 inflammasome, enhancing inflammatory responses, and aggravating neuronal damage in the central nervous system.[54,55] To date, there are no disease-modifying drugs for PD, and recent gene therapies have not shown promising anti-neurodegenerative effects.[42,56] Therefore, the development of neuroprotective agents to arrest, delay, or halt the progression of PD remains a top priority.

Conclusion

Our data confirm previous observations that NLRP3 inflammasome activation is critical for amplifying the inflammatory response, leading to the death of substantia nigra DA neurons after MPTP treatment. Targeted activation of Nrf2 protein expression can improve motor function, reduce the loss of dopaminergic neurons, and inhibit gliosis and neuroinflammation. The mechanism may be increasing the expression of downstream antioxidant enzyme HO-1 and inhibiting the NLRP3 inflammasome of activation. It can be seen that Nrf2/HO-1 is involved in the regulation of neuroinflammation in the pathological process of PD and is a potential new target for the treatment of PD. It follows that oxidative stress and neuroinflammation play an integral role in the pathophysiology of PD. Increasing the expression of Nrf2/HO-1 to inhibit the assembly and activation of NLRP3 to play a regulatory role is expected to become a new target for maintaining brain function metabolism and improving brain function.

Ethics Statement

This animal study was reviewed and approved by the Institutional Animal Care and Utilization Committee of Xinjiang Medical University.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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