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ORIGINAL ARTICLE

FTY720 Inhibits MPP$^+$-Induced Microglial Activation by Affecting NLRP3 Inflammasome Activation

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Abstract

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons and excessive microglial activation in the substantia nigra *pars compacta* (SNpc). In the present study, we aimed to demonstrate the therapeutic effectiveness of the potent sphingosine-1-phosphate receptor antagonist fingolimod (FTY720) in an animal model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and to identify the potential mechanisms underlying these therapeutic effects. C57BL/6J mice were orally administered FTY720 before subcutaneous injection of MPTP. Open-field and rotarod tests were performed to determine the therapeutic effect of FTY720. The damage to dopaminergic neurons and the production of monoamine neurotransmitters were assessed using immunohistochemistry, high-performance liquid chromatography, and flow cytometry. Immunofluorescence (CD68- positive) and enzyme-linked immunosorbent assay were used to analyze the activation of microglia, and the levels of activated signaling molecules were measured using Western blotting. Our findings indicated that FTY720 significantly attenuated MPTP-induced behavioral deficits, reduced the loss of dopaminergic neurons, and increased dopamine release. FTY720 directly inhibited MPTP-induced microglial activation in the SNpc, suppressed the production of interleukin (IL)-6, IL-1β, and tumor necrosis factor-α in BV-2 microglial cells treated with 1-methyl-4-phenylpyridinium (MPP$^+$), and subsequently decreased apoptosis in SH-SY5Y neuroblastoma cells. Moreover, in MPP$^+$-treated BV-2 cells and primary microglia, FTY720 treatment significantly attenuated the increases in the phosphorylation of PI3K/AKT/GSK-3β, reduced ROS generation and p65 activation, and also inhibited the activation of NLRP3 inflammasome and caspase-1. In conclusion, FTY720 may reduce PD progression by inhibiting NLRP3 inflammasome activation via its effects on ROS generation and p65 activation in microglia. These findings provide novel insights into the mechanisms underlying the therapeutic effects of FTY720, suggesting its potential as a novel therapeutic strategy against PD.

Keywords Parkinson's disease · Fingolimod (FTY720) · Reactive oxygen species (ROS) · Microglia · NLRP3 inflammasome

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Introduction

Parkinson's disease (PD) is among the most common neurodegenerative disorders of the motor system, affecting approximately 1% of the population older than 60 years of age (Martin et al. 2011). Although PD is characterized by motor abnormalities, such as muscle stiffness, tremor, and postural instability (Liu et al. 2015b), cognitive and behavioral symptoms occur in advanced stages of the disease (Papagno and Trojano 2018). The main neuropathological feature of PD is the selective loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) (Kinoshita et al. 2015). Such dopaminergic neurodegeneration is typically associated with an inflammatory component, manifested in part by the presence of activated microglia. Microglial activation leads to the production of pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which trigger the apoptosis of dopaminergic neurons in the SNpc (Block et al. 2007). The activation of NLRP3 inflammasome plays a significant role in mediating neuroinflammation (Coll et al. 2015). NLRP3 inflammasome activation requires two signals: signal 1 activates nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling, facilitating the transcription of pro-IL-1β and NLRP3; signal 2 forms the inflammasome complex, comprising NLRP3, apoptosis-associated speck-like protein containing a carboxy-terminal CARD, and caspase-1, which in turn cleaves pro-IL-1β to IL-1β (Latz et al. 2013). Some research groups have performed experiments to establish the link between mitochondrial impairment and inflammasome activation in microglial cells, which may help in identifying the mechanism underlying inflammation-driven neurodegenerative diseases (Sarkar et al. 2017).

Fingolimod (FTY720), a structural analog of sphingosine-1-phosphate (S1P), is phosphorylated in the body by sphingosine kinases, whereupon it becomes an antagonist for four of the five G protein-coupled S1P receptor subtypes (Brinkmann 2009). It was the first oral drug approved by the US Food and Drug Administration for the treatment of relapsing-remitting forms of multiple sclerosis (Kappos et al. 2010; Montalban et al. 2011). Additionally, numerous studies have elucidated the role of FTY720 in the treatment of various health conditions, such as intracerebral hemorrhage (Rolland et al. 2011; Rolland et al. 2013), ischemic stroke (Hasegawa et al. 2010; Wei et al. 2011), Huntington's disease (Di Pardo et al. 2014), Alzheimer's disease (Hemmati et al. 2013), epilepsy (Gao et al. 2012), and spinal cord injury (Norimatsu et al. 2012). As a lipophilic agent, FTY720 crosses the blood-brain barrier (BBB) and accumulates in the brain, directly targeting S1P receptor-expressing cells of the central nervous system, including neurons and microglia (Hunter et al. 2016). Emerging evidence has demonstrated that FTY720 protects against neural injury caused by mitochondrial dysfunction (Moon et al. 2013), ischemia-reperfusion injury (Shichita et al. 2009), and cytotoxicity (Zhao et al. 2017b). Recent studies reported that FTY720 significantly reduced motor deficits and attenuated the loss of dopaminergic neurons through direct neuroprotective effects in animal models of PD that received 6-hydroxydopamine (6-OHDA) or rotenone (Ren et al. 2017; Zhao et al. 2017a). FTY720 also attenuated locomotor deficiency via the activation of sphingosine kinase 1 and Akt kinase in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD animal model. However, whether FTY720 attenuates neuronal injury in MPTP-induced PD mouse model by inhibiting NLRP3 inflammasome activation during microglial inflammatory response remains unclear.

In the present study, we developed an in vivo MPTP-induced animal model of PD, and an in vitro 1-methyl-4-phenylpyridinium (MPP^+)-induced model of microglial activation to investigate the protective effects of FTY720 against PD, as well as the potential mechanisms underlying these effects. Our data demonstrate that FTY720 relieves motor dysfunction, attenuates the loss of dopaminergic neurons, and reduces inflammation in MPTP-induced animal model of PD. Furthermore, our findings indicate that FTY720 prevents the death of dopaminergic neurons by inhibiting NLRP3 inflammasome activation by reducing ROS generation and p65 phosphorylation in both primary microglia and BV-2 cells, suggesting its potential as a novel neuroprotective agent in the treatment of PD.

Methods

Experimental Animals

Male C57BL/6J mice (20–25 g; 8 weeks old) were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China). All mice were housed at room temperature (RT: 22 ± 3 °C) under a 12-h light/dark cycle and provided food and water ad libitum (standard conditions). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Drug Treatments

MPTP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline; FTY720 (Cayman, Ann Arbor, MI, USA) was dissolved in ethanol. Mice were randomly divided into four groups: a control group, an FTY720-treated group, an MPTP-induced PD group, and an MPTP + FTY720 group. Mice in MPTP-induced PD group received subcutaneous injections of MPTP (20 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) for 5 consecutive days. The control mice received equal volumes of saline. Mice in the MPTP + FTY720 group received oral FTY720 (2 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) 0.5 h before each MPTP injection. The FTY720-treated group received oral FTY720 (2 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) alone. Mice were sacrificed 7 days after the last MPTP injection.

Behavioral Analyses

Parkinsonian symptoms were evaluated 7 days after the final injection of MPTP or saline using the open-field test (OFT) and rotarod test. The OFT was performed to evaluate the speed and locomotion. Three days before testing, mice were placed in the behavioral testing laboratory to allow for adaptation to the environment. On the day of the experiment, they were allowed to move freely in an open box for 10 min, during which the walking distance, time, average speed (averaged over the entire arena), and distance of central regional activity were recorded using a behavior recording system (Clever Sys Inc., Reston, VA, USA) (Belichenko et al. 2009).

Motor coordination was assessed using the rotarod test. The rotarod apparatus consisted of a motor-control unit (DigBehv-010, Jiliang, Shanghai, China) and a rotating horizontal cylinder (diameter: 30 mm), which was divided into five rotating compartments. Automatic timers recorded the time (in seconds) for which the mice remained on the cylinder, which rotated at a speed that accelerated from 5 to 15 rpm. One day before the test, all animals were pre-exposed to the rotarod procedure three times.

Immunohistochemistry

Following anesthetization with 200 $mg \cdot kg^{-1}$ ketamine and 10 $mg \cdot kg^{-1}$ xylazine, mice were perfused with ice-cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. Brain tissues were isolated, post-fixed in 4% PFA at 4 °C for 24 h, and then transferred to 20% (*w*/*v*) and 30% (w/v) sucrose solutions for 3 days, following which they were embedded in optimal cutting temperature compound. The brains were then sliced into coronal sections (thickness: 25 μm) using a cryostat microtome (Leica CM1950, Nussloch, Germany). Following treatment with 3% H_2O_2 for 10 min, the sections were blocked with 5% bovine serum albumin (BSA) for 1 h. They were then incubated with a primary antibody against tyrosine hydroxylase (TH) (MAB318, Millipore, Billerica, MA, USA) at 4 °C overnight, washed with PBS, and incubated with the corresponding horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody for 1 h at RT. Thereafter, the color was developed using 3,3′-diaminobenzidine. The total number of TH-positive cells in the SNpc was counted under a microscope (Olympus BX51, Olympus, Tokyo, Japan) at 100× magnification.

Immunofluorescence

Frozen sections were used to determine the expression of CD68. Free-floating brain tissue sections were incubated with 5% BSA for 1 h, and then incubated with a primary antibody against CD68 (#ab125212, Abcam, MA, USA) at 4 °C overnight. Sections were then washed with PBS thrice, and incubated with fluorophore-labeled anti-rabbit secondary antibody for 1 h at RT. The nuclei were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a fluorescence microscope (Nikon Eclipse TE2000-S, Nikon, Tokyo, Japan) at 200× magnification.

High-Performance Liquid Chromatography

Brain homogenate sample preparation: Mice were sacrificed 7 days after the final injection of MPTP or saline, and the striatal tissues were quickly dissected on ice. The tissue samples were weighed and homogenized in a 10-fold volume of 0.1 M $HClO_4$ solution containing ethylenediaminetetracetic acid (EDTA, 0.1 mM). After homogenization, the tissue samples were centrifuged at 13,800×*g* for 25 min at 4 °C, and stored at −70 °C.

Monoamine neurotransmitter detection: Monoamine neurotransmitters, including dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (HIAA), were detected using the barium aluminosilicate high-performance liquid chromatography (HPLC) electrochemical detector system. This system consisted of an electrochemical detector, a chromatographic workstation (DA-5), a C18 reverse-phase column (2.2 μm, 120 Å, 2.1 × 100 mm, DIONEX, Sunnyvale, CA, USA), and a liquid-path transmission system. The working voltage was 500 mV.

The samples were injected into an autosampler at 4 °C (UltiMate 3000, ESA Biosciences, Inc., Chelmsford, MA, USA), eluted through a C18 column with a catecholamine-analysis mobile phase, and detected using an ESA Coulochem III electrochemical detector. The mobile phase consisted of 1.0 mM sodium octane, 50 mM NaH_2PO_4, 50 mM disodium citrate, 17 mM NaCl, 0.1 mM EDTA, and 4% acetonitrile (volume fraction), pH 4.0. The flow rate was 0.08 $ml \cdot min^{-1}$, with an injection volume of 10 μl. The content of monoamine neurotransmitters in the above samples was calculated using the external standard method.

Isolation of Primary Microglia

Primary microglia was isolated as previously described (Cao et al. 2018). The brains of newborn mice were harvested. After that, the cerebella, white matter, and leptomeninges were separated from the cerebral cortices. Then, the cerebral cortices were trypsinized for 5 min at 37 °C and filtrated through a 70-μm pore-size filter (Millipore, Billerica, MA, USA). Cells from six cerebra were seeded in a 75-cm^2 culture flask containing 15 ml of DMEM/F12 with 10% FBS and incubated in 5% CO_2 at 37 °C. After 24 h, the entire medium was replaced, and then half of the medium was replaced every 3–4 days. On days 13–14, microglia were isolated from the mixed glial culture by

shaking the flask at 200 rpm for 1 h at 37 °C. The microglia were centrifuged and seeded in 24-well plates for further stimulation. The purity of the isolated microglia was 90–95%.

Cell Line Culture

BV-2 microglial and SH-SY5Y neuroblastoma cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), at 37 °C in a 5% CO_2 incubator. BV-2 cells were pretreated with 0.5 μM FTY720 for 0.5 h, and then stimulated with 500 μM MPP^+ for 6 h for Western blotting, or for 12 h for enzyme-linked immunosorbent assay (ELISA). Cytotoxicity was measured using the Cell Counting Kit-8 method in accordance with the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Absorbance was measured at 450-nm wavelength.

Following stimulation with 500 μM MPP^+ for 9 h, with or without FTY720, BV-2 cells were washed thrice with PBS, and cultured in high-glucose DMEM containing 10% FBS for another 3 h. The supernatant was then collected to be used as a conditioned medium, and SH-SY5Y cells were cultured in the conditioned medium for 12 h.

Western Blotting

BV-2 cells were pretreated with 0.5 μM FTY720 for 0.5 h, and then stimulated with 500 μM MPP^+ for 6 h. The cells were then lysed on ice in radioimmunoprecipitation-assay lysis buffer (50 mM Tris, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.1% Na deoxycholate, and 1% Nonidet P-40, pH 7.2) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), for 30 min. The cells were then centrifuged at 13,800×*g* for 15 min at 4 °C. Samples containing equal amounts of protein were separated by 10% SDS-polyacrylamide gradient gel electrophoresis and were electroblotted onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% skim milk at RT for 2 h and incubated overnight at 4 °C with the following primary antibodies: anti-phospho-phosphoinositide 3-kinase (PI3K) (Tyr458/Tyr199; #4228, Cell Signaling Technology, Beverly, MA, USA), anti-PI3K (#4275, Cell Signaling Technology), anti-phospho-AKT (Ser473; #4060, Cell Signaling Technology), anti-AKT (#4691, Cell Signaling Technology), anti-phospho-glycogen synthase kinase-3 β (GSK-3β) (Ser 9; #5558, Cell Signaling Technology), anti-GSK-3β (#9315, Cell Signaling Technology), anti-phospho-p65 (Ser536; #3033, Cell Signaling Technology), anti-p65(#8242, Cell Signaling Technology), anti-NLRP3 (ab210491, Abcam), anti-Caspase-1 (06–503-1, Miliipore), and anti-GADPH (ab181602, Abcam). The membranes were then washed in PBS containing 0.05% Tween-20, and incubated with HRP-conjugated secondary antibody at RT for 2 h. Protein bands were visualized using chemiluminescence enhancement reagents (Perkin Elmer, Waltham, MA, USA).

ELISA for Cytokine Production

Seven days after MPTP injection, the mice were anesthetized with 200 $mg \cdot kg^{-1}$ ketamine and 10 $mg \cdot kg^{-1}$ xylazine, and transcardially perfused with ice-cold PBS (pH 7.4). The midbrain was isolated from the whole brain, homogenized in PBS, and centrifuged at 13,800×*g* for 15 min at 4 °C, following which the supernatant was collected. BV-2 cells were pretreated with 0.5 μM FTY720 for 0.5 h, and stimulated with 500 μM MPP^+ for 12 h, following which the supernatant was collected. Production of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α in vivo and in vitro was detected via ELISA (BD Biosciences, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Flow Cytometry

Following treatment with the conditioned medium from BV-2 cells, apoptosis of SH-SY5Y cells was investigated using a fluorescein isothiocyanate (FITC) Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences). Briefly, the cells were washed with ice-cold PBS, and then resuspended in 1× binding buffer at 1×10^6 $cells \cdot ml^{-1}$. The cell suspension was incubated in the dark with 1 $\mu l \cdot 100\ \mu l^{-1}$ of each FITC Annexin V and PI for 15 min at RT. The extent of apoptosis was analyzed within 1 h using a FACSCalibur flow cytometer (BD Biosciences).

MitoSOX™ Red Mitochondrial Superoxide Indicator for Live-Cell Imaging

The contents of one vial of MitoSOX™ mitochondrial superoxide indicator was dissolved in 13 μl of dimethylsulfoxide to make a 5 mM MitoSOX™ reagent stock solution, and further diluted in Hank's Balanced Salt Solution/Ca^{2+}/Mg^{2+} buffer to obtain a working solution with a 5-μM concentration of the reagent. The cells were incubated with the working solution at 37 °C for 10 min in the dark. Then the cells were washed three times with a warm buffer. After the nuclei were stained with DAPI, the cells were observed under a fluorescence microscope (Olympus TH4–200) at 10× magnification.

Statistical Analysis

The data are expressed as the mean ± standard error of the mean (SEM) of the indicated number of independent experiments. Statistical analysis was performed using Prism software (GraphPad Version 5, La Jolla, CA, USA). All data were analyzed using one- or two-way analyses of variance. Values of $p < 0.05$ were considered statistically significant.

Results

FTY720 Relieves Motor Dysfunction in Mice with MPTP-Induced PD

Motor deficits were assessed using the OFT and rotarod test. After exposure to the novel environment in the OFT apparatus, the movement of individual mouse was recorded (Fig. 1a). Mice from MPTP-induced PD group engaged lesser in exploratory behavior than those from the control group, based on the total distance traveled (Fig. 1a, b). However, in mice treated with FTY720 plus MPTP, we observed a significant increase in the total distance traveled (Fig. 1b). Moreover, the speed of the mice pretreated with FTY720, averaged over the entire arena, was higher than those of the mice treated with MPTP alone (Fig. 1c).

During the rotarod test, MPTP-treated mice spent much less time on the cylinder (Fig. 1d) and traveled a much shorter distance (Fig. 1e) than saline-treated mice. Interestingly, FTY720 exerted beneficial effects in mice with MPTP-induced PD by prolonging the latency to fall (Fig. 1d), and increasing the total distance traveled (Fig. 1e). The motor function in both tests was not affected by FTY720 treatment alone (Fig. 1a–e); thus, the FTY720-treated group was excluded in the subsequent experiments. These results suggest that FTY720 ameliorates motor deficits in a mouse model of MPTP-induced PD.

FTY720 Prevents MPTP-Induced Loss of Dopaminergic Neurons and Modulates Striatal Levels of Biogenic Amines in PD Mice

The neural protection conferred by pretreatment with FTY720 was confirmed by counting the number of TH-positive neurons in the SNpc (Fig. 2a). Administration of MPTP led to a 53% decrease in the number of TH-positive cells in the SNpc relative to the levels observed in the control group (Fig. 2b). However, FTY720 intake significantly increased the number of TH-positive cells in the SNpc of MPTP-lesioned mice. The number of TH-positive cells was 0.58 times higher in the MPTP + FTY720 group than in the MPTP alone group. Thus, these findings suggest that FTY720 prevents the loss of dopaminergic neurons in the SNpc in a mouse model of MPTP-induced PD.

Because a significant reduction was observed in the loss of dopaminergic neurons in the MPTP + FTY720 group, we determined the levels of the monoamine neurotransmitters in the striatum. Striatal levels of DA and its major metabolites, DOPAC and HVA, as well as those of NE, 5-HT, and 5-HIAA were quantified using HPLC. The concentrations of DA (Fig. 2c), DOPAC (Fig. 2d), HVA (Fig. 2e), and 5-HT (Fig. 2f) in MPTP-treated mice were 84.47%, 81.32%, 43.77%, and 39.38% lower, respectively, than those in saline-treated mice. Pretreatment with FTY720 led to 164%, 87%, 59%, and 52% increase in the striatal levels of DA, DOPAC, HVA, and 5-HT, respectively, compared with the concentrations after treatment with MPTP alone (Fig. 2c–f). No significant differences in the levels of NE (Fig. 2g) or 5-HIAA (Fig. 2h) were observed. These results demonstrate that FTY720 diminished the reduction of DA and its metabolites levels in the striatum of mice with MPTP-induced PD.

FTY720 Attenuates Microglial Activation and Inflammatory Cytokine Production in the SNpc of Mice with MPTP-Induced PD

Since neuroinflammation plays a crucial role in PD pathology, we evaluated the effects of FTY720 on microglial activation and inflammatory cytokine production in MPTP-lesioned mice.

The behavioral tests and immunohistochemistry results showed that there was no difference between the FTY720 and control groups; therefore, the FTY720 group was excluded from the subsequent in vivo experiments. The brain sections were immunostained using an anti-CD68 (a marker for microglial activation) antibody. Activated microglia exhibited an amoeboid structure, with a large body and short processes. In the SNpc of saline-treated mice, microglia were only faintly immunoreactive for CD68. However, MPTP treatment produced a 490% increase in the number of CD68-positive microglia in this region, while pretreatment with FTY720 led to a 60.9% decrease of the MPTP-induced microglial activation (Fig. 3a, b).

In addition, we performed ELISA to determine the concentrations of the inflammatory cytokines TNF-α, IL-1β, and IL-6 in the midbrain. As shown in Fig. 3c–e, MPTP administration produced 307%, 302%, and 187% increases in TNF-α, IL-1β, and IL-6 production, respectively, relative to the levels after saline administration. Pretreatment with FTY720 significantly reduced the concentrations of TNF-α, IL-1β, and IL-6 in the midbrain by 43.9%, 52.2%, and 43.5%, respectively. Treatment with FTY720 alone did not affect the concentrations of the cytokines. These results indicate that FTY720 attenuates the inflammatory response to MPTP by inhibiting the activation of microglia and the subsequent production of TNF-α, IL-1β, and IL-6 in the midbrain.

FTY720 Reduces Apoptosis in SH-SY5Y Cells by Inhibiting Microglial Activation

BV-2 cells were treated with MPP^+ (500 μM) for 12 h, with and without FTY720. Our findings indicated that MPP^+ treatment increased the release of TNF-α (Fig. 4A), IL-1β (Fig. 4b), and IL-6 (Fig. 4c) by 282%, 356%, and 447%, respectively, relative to levels observed after the control saline treatment. However, FTY720 reduced the release of TNF-α, IL-1β, and IL-6 by 55.59%, 63.12%, and 54.72%, respectively, relative to the levels after MPP^+ treatment.

SH-SY5Y cells were treated with the conditioned medium collected from BV-2 cells treated with MPP^+ for

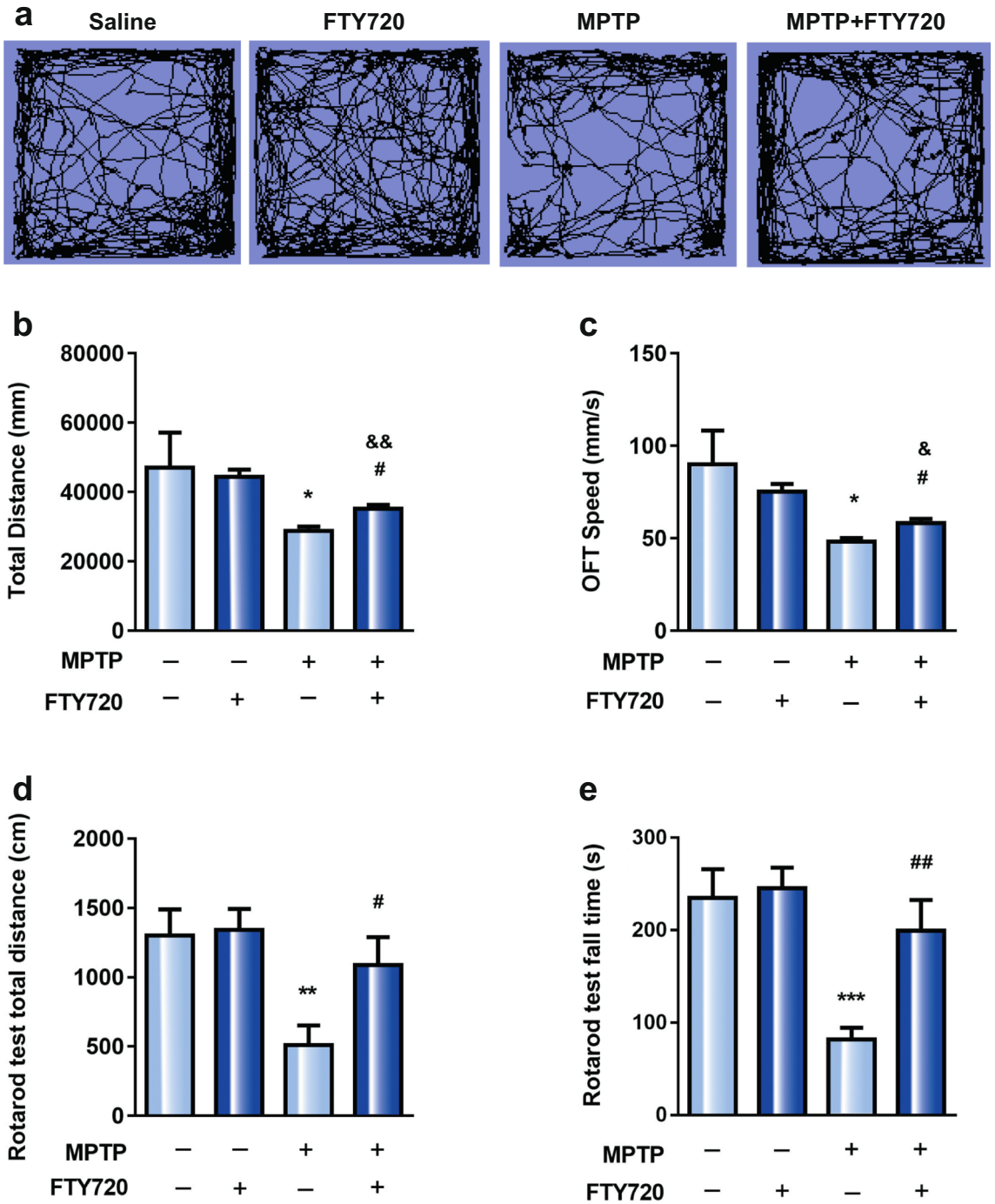


Fig. 1 FTY720 relieves motor deficits in MPTP-induced PD mice. Mice received subcutaneous injections of MPTP (20 mg·kg^{-1}·day^{-1}) for 5 consecutive days, with or without oral pretreatment with FTY720 (2 mg·kg^{-1}·day^{-1}) 0.5 h before each MPTP injection. The control group received equal amounts of saline. The FTY720 group received oral FTY720 alone. Behavioral analyses were performed 7 days after the last injection of MPTP. The motor behavior of the mice was evaluated for 10 min in an OFT and the movement of each individual mouse was recorded (**a**). Treatment with FTY720 significantly reversed the MPTP-induced decrease in the total distance traveled (**b**) and mean speed (**c**). Rotarod test results indicated that MPTP reduced the latency to fall (**d**) and total distance traveled (**e**); both effects were reversed by the treatment with FTY720 (**d** and **e**). Data are presented as the mean ± SEM, $n = 8$ mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group; # $p < 0.05$ and ## $p < 0.01$ vs. MPTP group; & $p < 0.05$ and && $p < 0.01$ vs. FTY720 group. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FTY720: fingolimod; OFT: open-field test

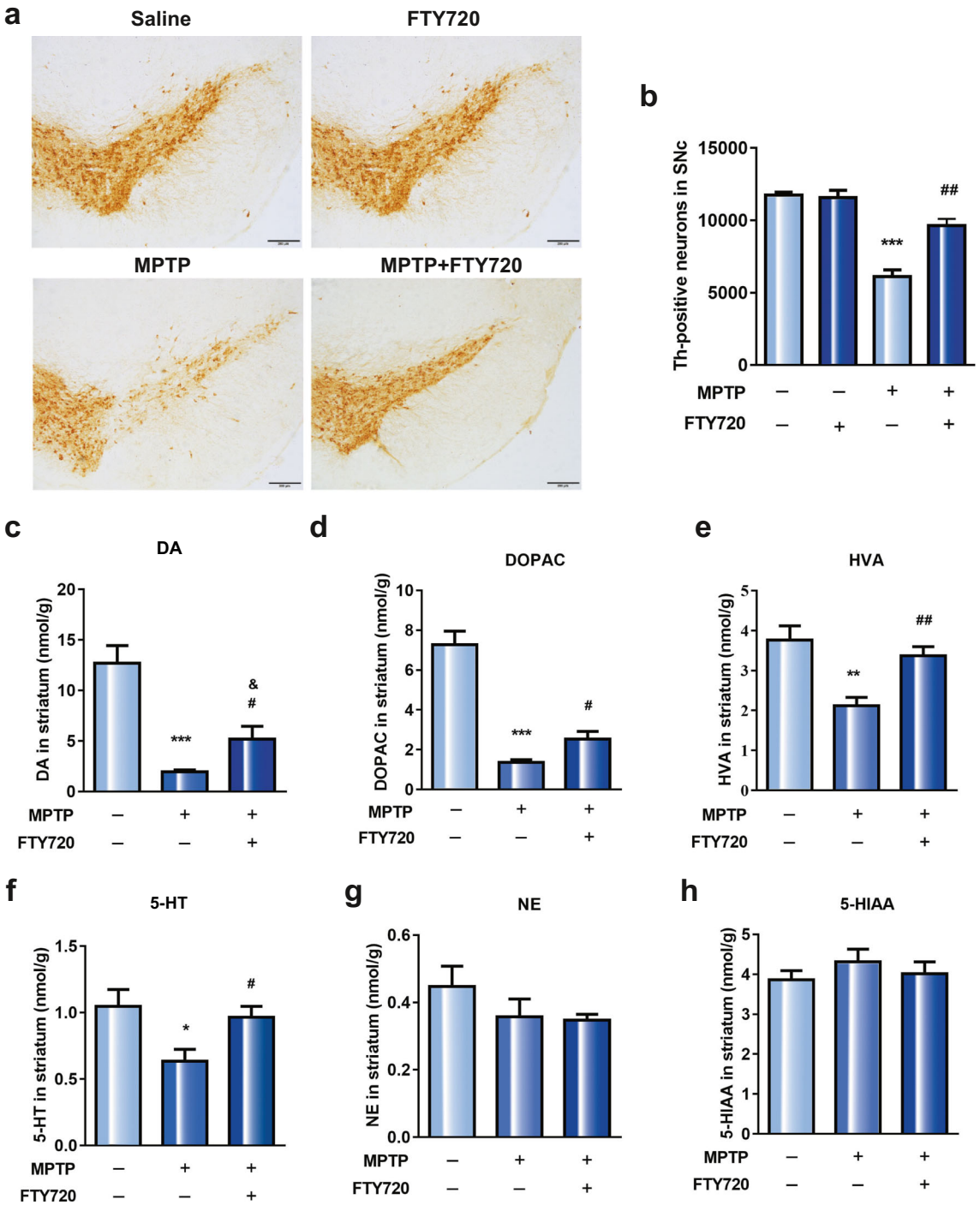


Fig. 2 FTY720 attenuates MPTP-induced loss of dopaminergic neurons in the SNpc and levels of biogenic amines. Mice received subcutaneous injections of MPTP (20 mg·kg^{-1}·day^{-1}) for 5 consecutive days, with or without oral pretreatment with FTY720 (2 mg·kg^{-1}·day^{-1}) 0.5 h before each MPTP injection. The control group received equal amounts of saline. Seven days after the last injection of MPTP, immunohistochemistry was performed to detect the number of TH-positive cells (brown) in the SNpc in each of the four groups (**a**). Scale bar = 200 μm. Treatment with FTY720 significantly attenuated MPTP-induced decrease in the stereological count of TH-positive cells in the SNpc (**b**). Data are presented as the mean ± SEM, $n = 5$ mice per group. The HPLC analysis revealed that FTY720 significantly increased the levels of DA (**c**), DOPAC (**d**), HVA (**e**), and 5-HT (F) 7 days after the last injection of MPTP, but had no effect on the levels of either NE (**g**) or 5-HIAA (**h**). Data are presented as the mean ± SEM, n = 8–10 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. MPTP group; && $p < 0.01$ vs. FTY720 group (**b**); & $p < 0.05$ and && $p < 0.01$ vs. the control group (**c** and **d**). SNpc: substantia nigra *pars compacta*; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FTY720: fingolimod; TH: tyrosine hydroxylase; HPLC: high-performance liquid chromatography; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; NE: norepinephrine; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindoleacetic acid

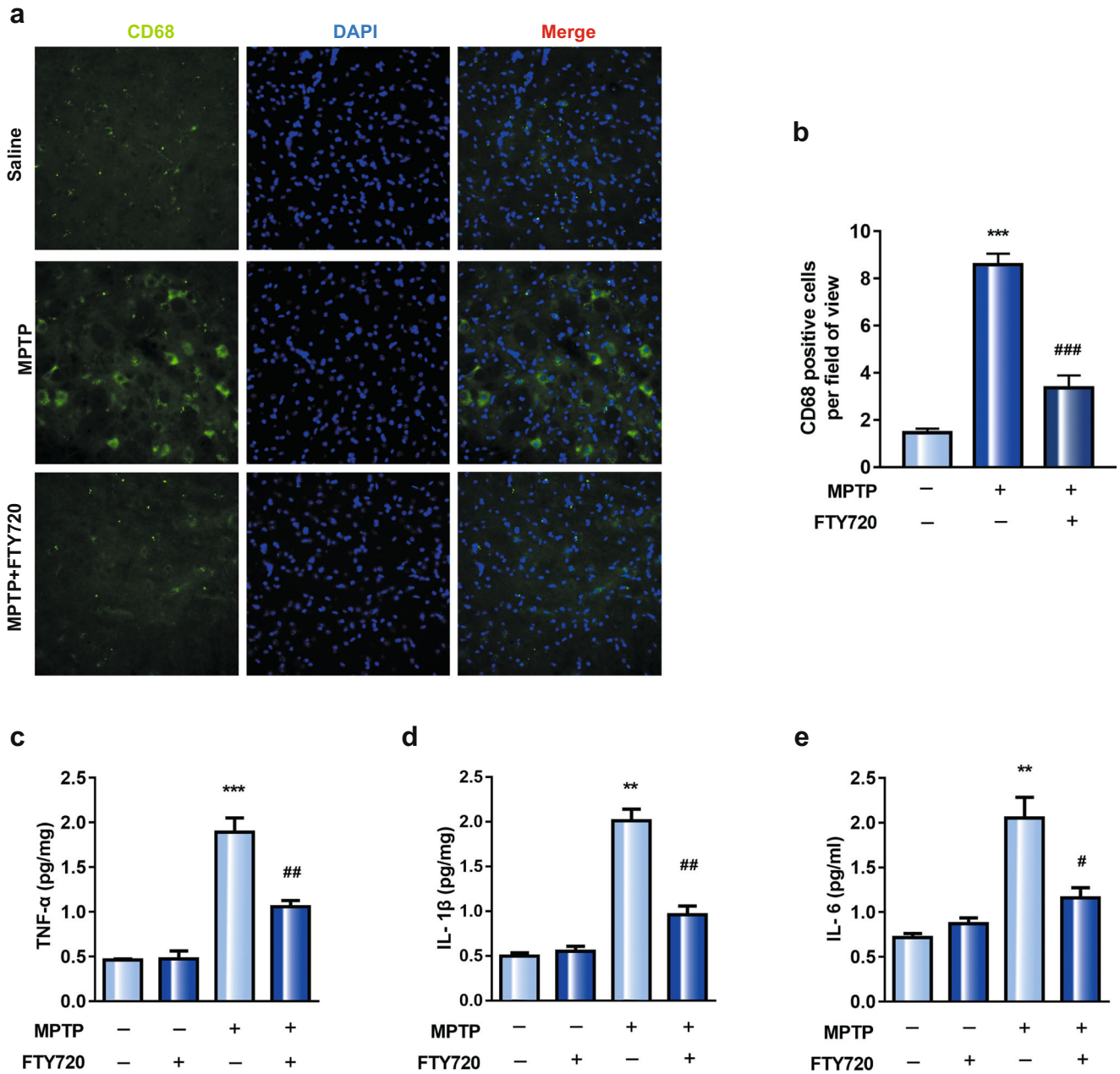


Fig. 3 FTY720 inhibits microglial activation and inflammatory cytokine production in the SNpc following treatment with MPTP. Mice received subcutaneous injections of MPTP (20 mg·kg^{-1}·day^{-1}) for 5 consecutive days, with or without oral pretreatment with FTY720 (2 mg·kg^{-1}·day^{-1}), 0.5 h before each MPTP injection. The control group received equal amounts of saline. Seven days after the last MPTP injection, brain sections were immunofluorescence-stained to detect microglial activation in the SNpc (CD68-positive, *green*, A). Nuclei were counterstained with DAPI (*blue*, **a**). Scale bar = 200 μm. Pre-treatment with FTY720 significantly reduced the number of CD68-positive cells in the SNpc following treatment with MPTP (**b**). Concentrations of TNF-α (**c**), IL-1β (**d**), and IL-6 (**e**) in the SNpc were also reduced by the treatment with FTY720. Data are presented as the mean ± SEM, $n = 6$ mice per group. ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. MPTP group. SNpc: substantia nigra *pars compacta*; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FTY720: fingolimod; IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α

12 h. Flow cytometry revealed increased apoptosis and necrosis of SH-SY5Y cells following treatment with the conditioned medium obtained from BV-2 cells treated with MPP^+ (Fig. 4d, e). The percentage of apoptotic and necrotic SH-SY5Y cells was significantly decreased when cells were incubated in the conditioned medium obtained from BV-2 cells treated with FTY720 + MPP^+ (Fig. 4d, e).

Collectively, our findings indicate that FTY720 treatment substantially reduces the release of TNF-α, IL-1β, and IL-6 from MPP^+-treated microglia, and protects SH-SY5Y cells against neurotoxicity by inhibiting microglial activation.

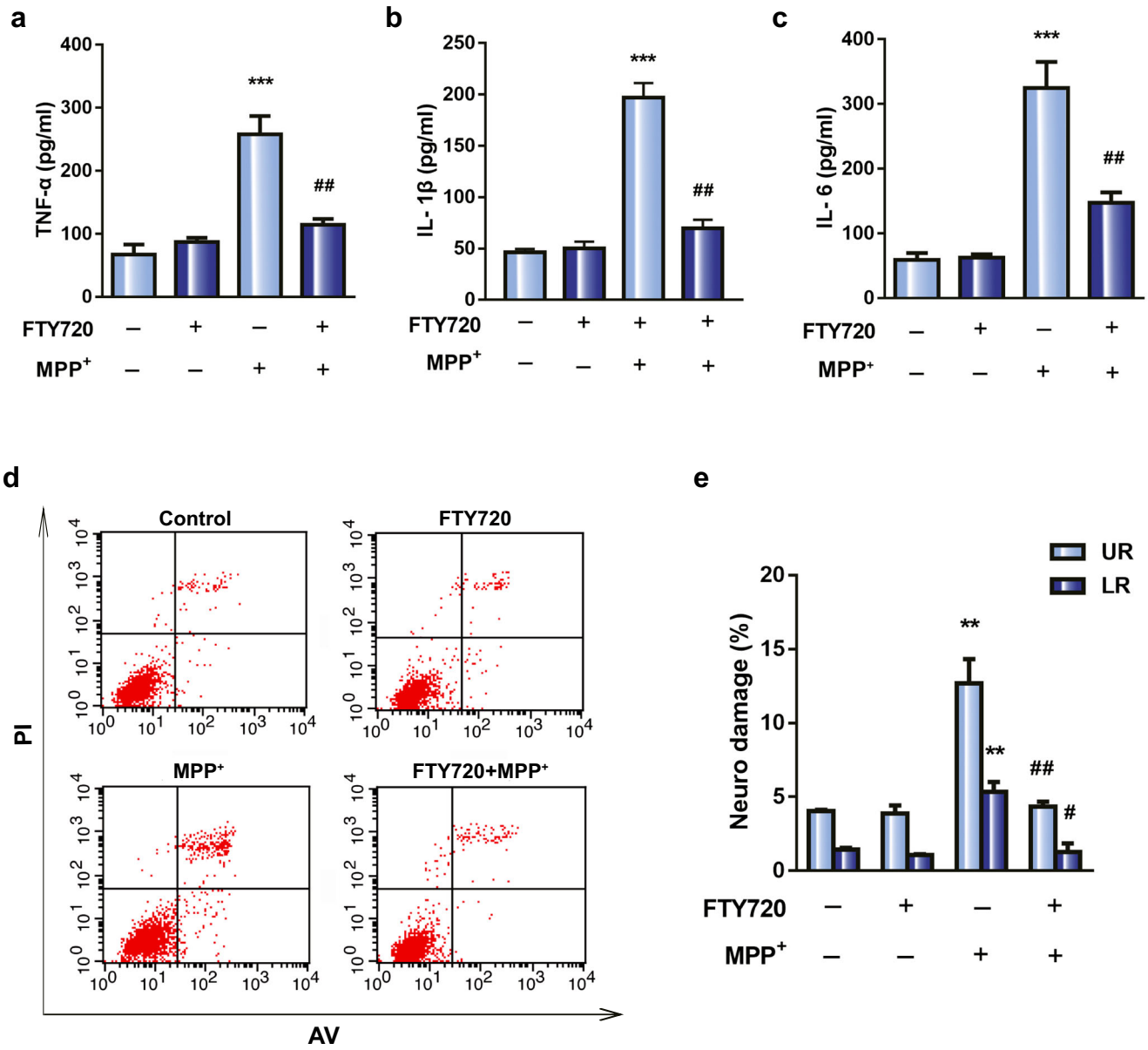


Fig. 4 FTY720 prevents apoptosis of SH-SY5Y cells by inhibiting MPP^+-induced activation of BV-2 cells. BV-2 cells were pretreated with 0.5 μM FTY720 for 0.5 h, and then treated with 500 μM MPP^+ for 12 h. The supernatants were then collected for ELISA. Treatment with MPP^+ significantly upregulated the concentrations of TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**), while pretreatment with FTY720 significantly attenuated these changes. After 12 h of stimulation with the conditioned medium collected from the supernatants of BV-2 cells, SH-SY5Y cells were analyzed for apoptosis (lower right quadrant) and necrosis (upper right quadrant) via flow cytometry (**d**). Conditioned medium from MPP^+-treated BV-2 cells significantly increased the apoptosis of SH-SY5Y cells, while that from MPP^+ + FTY720-treated BV-2 cells significantly reduced apoptosis in SH-SY5Y cells (**e**). Data are shown as the mean ± SEM, recorded across at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. MPP^+ group. MPP^+: 1-methyl-4-phenylpyridinium; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; FTY720: fingolimod; ELISA: enzyme-linked immunosorbent assay; UR: upper right; LR: lower right

The PI3K/AKT/GSK-3β Pathway Is Implicated in the Effects of FTY720 on MPP^+-Treated BV-2 Cells and Primary Microglia

The PI3K/AKT signaling plays a crucial role in PD (Nakano et al. 2017) and lipopolysaccharide-induced microglial activation (Cianciulli et al. 2016; Dong et al. 2014). As a point of convergence of a large range of signaling pathways, GSK-3β has been recognized as an important regulator of inflammation (Jope et al. 2007; Martin et al. 2005); GSK-3β has been reported to be located in the mitochondria, where it is highly activated compared to its cytosolic form (Bijur and Jope 2003). In the present study, we measured the levels of PI3K/AKT/GSK-3β phosphorylation by Western

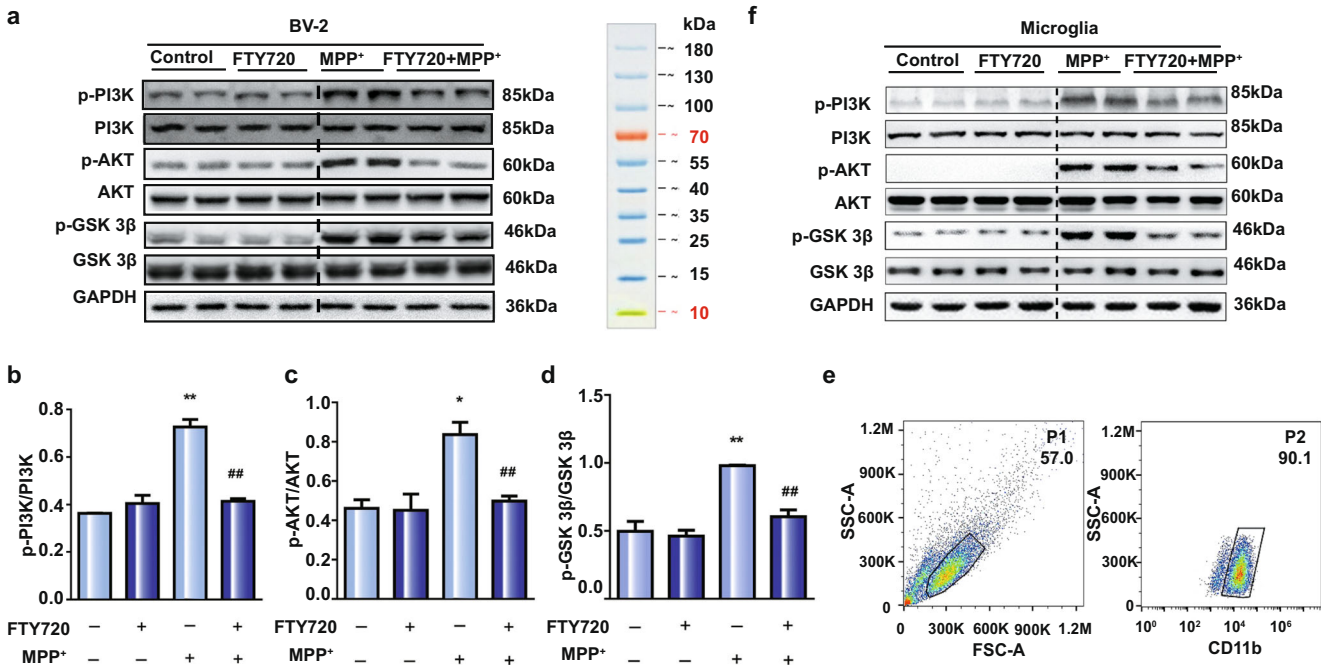


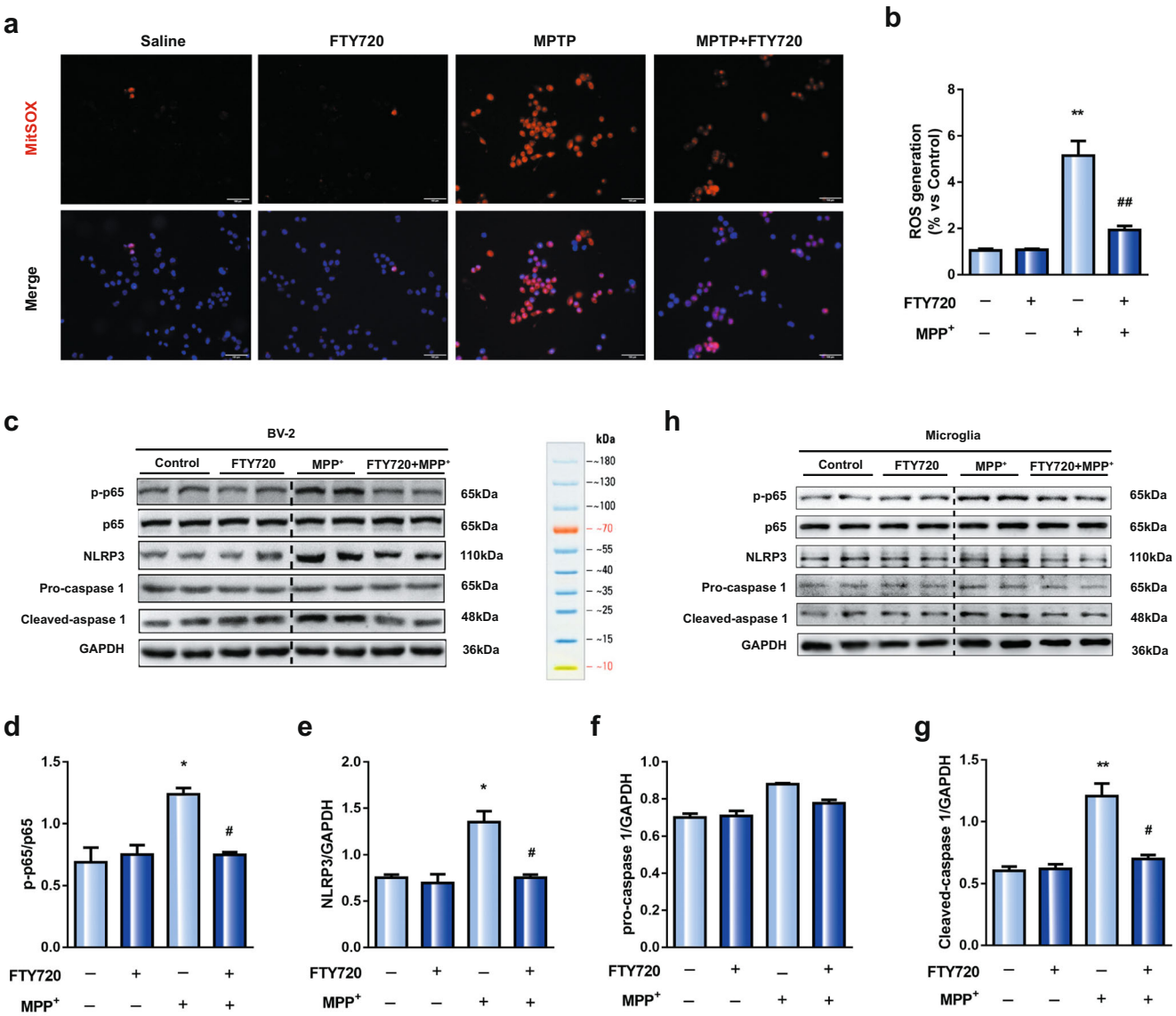
Fig. 5 FTY720 inhibits MPP$^+$-induced PI3K/AKT/GSK-3β phosphorylation. BV-2 cells and primary microglia were pretreated with 0.5 μM FTY720 for 0.5 h, and then stimulated with 500 μM MPP$^+$ for 6 h. Treatment with MPP$^+$ significantly increased the phosphorylation of PI3K (**a**, **b**), AKT (**a**, **c**), and GSK-3β (**a**, **d**) in BV-2 cells, and the effects were downregulated by pretreatment with FTY720. The results of the quantitative analysis were normalized by the corresponding total protein levels (**b**–**d**); FTY720 negatively regulates PI3K/AKT/GSK-3β signaling (**a**–**d**). Primary microglia was isolated from newborn mice with a purity of 90–95% (**e**). Same results were found in primary microglia (**f**). Data are shown as the mean ± SEM, recorded across at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. the control group; ## $p < 0.01$ vs. MPP$^+$ group. MPP$^+$: 1-methyl-4-phenylpyridinium; FTY720: fingolimod; PI3K: phosphoinositide 3-kinase; GSK-3β: glycogen synthase kinase-3 β

blotting. Treatment with MPP$^+$ alone significantly upregulated the phosphorylation levels of PI3K (Fig. 5a, b), AKT (Fig. 5a, c), GSK-3β (5A, D) in BV-2 cells, whereas pretreatment with FTY720 significantly attenuated these changes. We isolated primary microglia with a purity of 90–95% (Fig. 5e), and same results were observed (Fig. 5f). These results indicate that the inhibitory effect of FTY720 on MPP$^+$-treated BV-2 cells and primary microglia may be mediated by suppressing PI3K/AKT/GSK-3β signaling.

FTY720 Inhibits MPP$^+$-Induced NLRP3 Inflammasome Activation by Affecting Mitochondrial Dysfunction and p65 Signaling in BV-2 Cells and Primary Microglia

A previous study indicated that GSK-3β significantly promotes reactive oxygen species (ROS) production by inhibiting NADH, which can be reversed by GSK-3β inhibitors in a PD model (King et al. 2008). To investigate whether FTY720 could reduce the production of ROS in MPP$^+$-induced BV-2 cells, cells were treated with MPP$^+$ (500 μM) for 6 h, with and without FTY720. The results showed that MPP$^+$ could cause generation of ROS in BV-2 cells. However, pretreatment with FTY720 reduced the production of ROS by 60.5% relative to the levels after MPP$^+$ treatment alone (Fig. 6a, b). It has been reported that inhibiting ROS production could attenuate the formation of NLRP3 inflammasome (Sarkar et al. 2017). Moreover, previous studies have reported that NLRP3 inflammasome expression may also be affected through the activation of NF-κB (Wen et al. 2018). To explore whether

Fig. 6 FTY720 inhibits MPP$^+$-induced activation of NLRP3 inflammasome by curbing ROS production and affecting the p65 signaling pathway. BV-2 cells and primary microglia were pretreated with 0.5 μM FTY720 for 0.5 h, and then stimulated with 500 μM MPP$^+$ for 6 h. Treatment with MPP$^+$ significantly increased the ROS production (**a**–**b**), phosphorylation of p65, activation of NLRP3 and caspase-1 in BV-2 cells (**c**–**g**) and primary microglia (**h**), while the effects were downregulated by pretreatment with FTY720. The results of the quantitative analysis in BV-2 cells were normalized by the corresponding total protein levels (**d**–**g**); FTY720 negatively regulated PI3K/AKT/GSK-3β and limits ROS production caused by mitochondrial functional disorders. Further, FTY720 negatively regulated the phosphorylation of p65 and the production of ROS caused by MPP$^+$; ROS and p65 further influenced NLRP3 and caspase-1 activation, and reduced the production of pro-inflammatory factors from microglia, thus decreasing the neuronal apoptosis and damage (**i**). Data are shown as the mean ± SEM, recorded across at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. MPP$^+$ group. MPP$^+$: 1-methyl-4-phenylpyridinium; FTY720: fingolimod; ROS: reactive oxygen species; PI3K: phosphoinositide 3-kinase; GSK-3β: glycogen synthase kinase-3 β; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β


a
Saline
FTY720
MPTP
MPTP+FTY720
MitSOX
Merge
b
ROS generation (% vs Control)
FTY720
MPP+
c
BV-2
Control
FTY720
MPP+
FTY720+MPP+
p-p65
p65
NLRP3
Pro-caspase 1
Cleaved-aspase 1
GAPDH
65kDa
110kDa
48kDa
36kDa
kDa
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e
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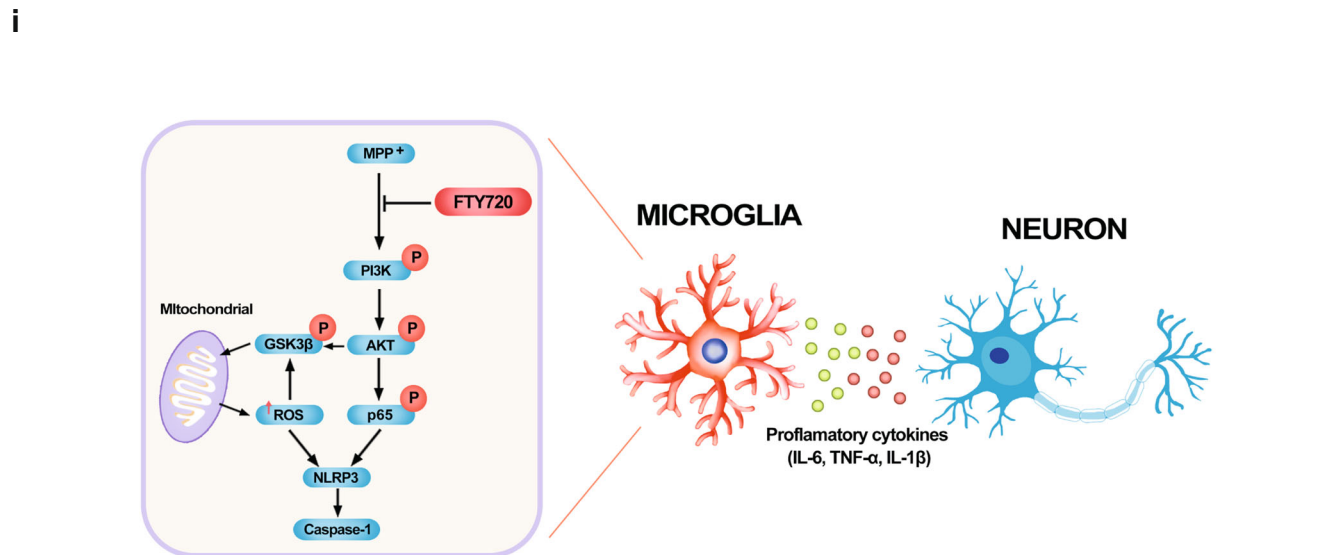
i
MPP+
FTY720
PI3K
Mitochondrial
GSK3β
AKT
ROS
p65
NLRP3
Caspase-1
MICROGLIA
NEURON
Proflamatory cytokines
(IL-6, TNF-α, IL-1β)

MPP^+-induced ROS production and p65 phosphorylation could affect the production of NLRP3 inflammasomes, BV-2 cells and primary microglial cells were exposed to MPP^+ for 6 h. Western blotting revealed that phosphorylation of p65 in BV-2 cells treated with FTY720 + MPP^+ was significantly lower than that in MPP^+-treated cells (Fig. 6c, d). Simultaneously, we found higher NLRP3 (Fig. 6c, e) and cleaved caspase-1 (Fig. 6c, g) levels in the MPP^+ group than in the control group, whereas pro-caspase-1 was not affected (Fig. 6c, f). However, in FTY720 pretreatment group, we observed that FTY720 reduced NLRP3 inflammasome and caspase-1 activation induced by MPP^+ (Fig. 6c–g). Same results were found in primary microglia (Fig. 6h). These findings indicate that, during MPP^+-induced microglial activation, FTY720 reduced ROS production by inhibiting the PI3K/AKT/GSK-3β signaling pathway, while at the same time reducing p65 phosphorylation, thus decreasing NLRP3 inflammasome activation through these two pathways (Fig. 6i).

Discussion

In the present study, we demonstrated that FTY720 relieves motor dysfunction in a mouse model of MPTP-induced PD by preventing the loss of dopaminergic neurons, and that this neuroprotective effect is indirectly mediated by inhibition of NLRP3 inflammasome activity via simultaneous influence over ROS generation and p65 signaling. Our study provides the first evidence that FTY720 protects dopaminergic neurons in animal models of PD by targeting NLRP3 inflammasome activation, supporting its role as a novel and effective therapeutic agent against PD.

Despite emerging evidence that FTY720 is effective in reducing neurodegeneration and promoting reparative mechanisms in various neurological conditions (Tan et al. 2016), little is known regarding its mechanism of action or cellular targets in PD. Since the neurotransmitter DA directly affects animal behavior and is produced primarily by dopaminergic neurons (Volkow et al. 2017), the beneficial effect of FTY720 on motor dysfunction may be due to its protective effect on dopaminergic neurons. Previous studies have reported that FTY720 protects cortical neurons against excitotoxic death induced by N-methyl-D-aspartate (Di Menna et al. 2013), and prevents 6-hydroxydopamine-induced cytotoxicity and apoptosis in SH-SY5Y cells, suggesting that FTY720 exerts a direct effect on neurons (Ren et al. 2017). Additional studies have demonstrated that FTY720 promotes re-myelination in vitro by modulating microglial activation (Jackson et al. 2011) and induces peripheral nerve regeneration by enhancing the plasticity of Schwann cells (Heinen et al. 2015), demonstrating its indirect neuroprotective effects. In the present study, we observed the inhibition of MPP^+-induced mitochondrial superoxide generation and NLRP3 inflammasome activation in microglia following treatment with FTY720, suggesting that its neuroprotective effect is mediated indirectly. The pathogenesis of PD is associated with excessive mitochondrial dysfunction and microglial activation. A recent study has linked NLRP3 inflammasome activation to mitochondrial dysfunction in macrophages (Zhou et al. 2011); ROS-generating mitochondria could incite inflammation via NLRP3 inflammasome pathways, which can induce the microglia activity (van Horssen et al. 2017). Moreover, the NF-κB-activating stimuli transcriptionally enhance the expression of NLRP3 (Bauernfeind et al. 2009) and the activation of microglia. When microglia are activated, they produce large amounts of inflammatory cytokines, ultimately leading to the apoptosis of dopaminergic neurons (Phani et al. 2012). In animal models, glia oxidize MPTP to form MPP^+, which binds directly to dopaminergic neurons and activates glia to produce large amounts of pro-inflammatory cytokines (Liu et al. 2016). Both effects irreversibly switch neurons into the death program, apoptosis (Kempuraj et al. 2017). Therefore, microglial activation may precede the loss of dopaminergic neurons in PD, suggesting that the inhibition of microglial activity is more promising for the treatment of PD than the direct protection of dopaminergic neurons.

Mitochondrial superoxide generation and neuroinflammation play important roles in the degeneration of dopaminergic neurons in PD (Tansey and Goldberg 2010; Wu et al. 2003). Inflammasome activation in glia has been reported to contribute to Alzheimer's disease (Halle et al. 2008; Heneka et al. 2013). In the present study, we found that FTY720 could mitigate MPP^+-induced ROS production in BV-2 cells. Further, the phosphorylation of p65 by MPP^+ was also inhibited by FTY720 in both BV-2 cells and primary microglia. Previous experiments indicated that reducing ROS production can attenuate the activation of NLRP3 inflammasome; however, inhibiting the activation of NLRP3 inflammasome does not relieve mitochondrial damage conversely. It has been shown that mitochondrial dysfunction is the cause of the formation of inflammatory bodies (Sarkar et al. 2017). Therefore, FTY720 could reduce the neuroinflammation caused by microglial activation by inhibiting NLRP3 inflammasome activation upstream of mitochondrial damage and p65 phosphorylation. Besides caspase-1 activation and release of pro-inflammatory cytokines, inflammasome activation also leads to cell death in a process termed pyroptosis (Jamilloux et al. 2013). NLRP3 inflammasome-dependent pyroptosis was reported to be involved in age-dependent isoflurane-induced cognitive impairment (Yin et al. 2018), and NLRC4 inflammasome complex mediated pyroptosis in microglial cells under ischemic conditions (Poh et al. 2018). Although there's currently no published research on pyrotosis in the mechanism of PD, Deora V et al. reported that β-hydroxy butyrate could block all aspects of inflammasome activation and pyroptosis induced by ATP and MSU, which might have therapeutic benefits for PD (Deora et al. 2017).

The immunosuppressive effect of FTY720 has been widely reported, mainly because of its effectiveness in trapping T cells in the lymph nodes (Garris et al. 2014). Abnormal T cell-mediated immunity plays a fundamental role in the pathogenesis of PD (Chen et al. 2018). It's the major limitation of our current work that we didn't evaluate T cells counts in the blood or the brain. In the present study, we demonstrated that the anti-inflammatory effect of FTY720 in our PD model was mediated by the direct inhibition of MPP^+-induced microglial activity. Indeed, pathogenic activation of both murine and human astrocytes was suppressed by treatment with FTY720 (McManus et al. 2017; Wu et al. 2013). Additional studies have suggested that FTY720 promotes the neuroprotective effects of microglia (Noda et al. 2013). Due to its lipophilic nature, FTY720 can cross the BBB to exert direct effects on glia. Changes in microglial phenotypes are closely related to disease stages and severity; mastering the stage-specific switching of M1/M2 phenotypes within the appropriate time window may provide improved therapeutic benefits (Tang and Le 2016). However, in PD, it remains unclear whether FTY720 inhibits the inflammatory activation of microglia by modulating microglia/macrophage polarization. Future studies are warranted to verify this possible mechanism.

After docking to S1P receptors, FTY720-phosphate controls downstream signaling molecules, such as AKT, extracellular signal-regulated kinases, and STAT3 (Hou et al. 2016; Rutherford et al. 2013; Sekine et al. 2011). FTY720 also controls important inflammatory genes targets by modulating STAT1 and IRF8 levels at their promoter site (Das et al. 2017). In the present study, we demonstrated that pretreatment with FTY720 attenuated the phosphorylation of PI3K, AKT, and GSK-3β in BV-2 cells and primary microglia treated with MPP^+. A number of studies have reported that the release of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 from microglia was mediated by PI3K/AKT/GSK-3β signaling. Some chemical agents that pharmacologically inhibit PI3K/AKT/GSK-3β lead to reduced production of inflammatory cytokines (Zhang et al. 2018; Zassler et al. 2003; Kim et al. 2004). Based on these studies, we deduced that the inhibitory effect of FTY720 on the release of TNF-α, IL-1β, and IL-6 from microglia was mediated by PI3K/AKT/GSK-3β signaling. Protein phosphatase 2A (PP2A), a trimolecular complex, serves as a regulator of cell death and division, and can be activated by FTY720 (Cristobal et al. 2016). Studies have indicated that strategies involving PP2A activation may lead to the discovery of novel anti-inflammatories for chronic respiratory diseases as well as novel therapeutic strategies for cancer (Rahman et al. 2016; Velmurugan et al. 2018). A recent study has also revealed that the therapeutic potential of FTY720 for tumor suppression is associated with its ability to activate PP2A by targeting SET/I_2PP2A, an endogenous PP2A inhibitor (Liu et al. 2015a). In addition, PP2A is a physiologically relevant phosphatase for AKT (Ugi et al. 2004) and other signaling molecules. The inhibitory effect of FTY720 on PI3K/AKT/GSK-3β phosphorylation may also be mediated by the activation of PP2A or other phosphatases downstream of S1P receptors. However, further studies are required to investigate this possibility.

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Author Contributions DYS and HZ designed and supervised the study and contributed to the drafting of the manuscript. XS, KQZ, LH, LL, DYS, and HZ wrote the manuscript. SY and LJL conceptualized and performed the experiments and contributed to the drafting of the manuscript. XS performed the experiments of primary microglia. JH assisted with behavioral testing and cell culture. JH and LJL contributed to data analysis. All authors have read and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no competing interests.

Abbreviations *5-HIAA,* 5-hydroxyindoleacetic acid; *5-HT,* 5-hydroxytryptamine; *6-OHDA,* 6-hydroxydopamine; *BBB,* Blood-brain barrier; *DA,* Dopamine; *DOPAC,* 3,4-dihydroxy-phenylacetic acid; *ELISA,* Enzyme-linked immunosorbent assay; *FBS,* Fetal bovine serum; *FTY720,* Fingolimod; *HPLC,* High performance liquid chromatography; *HVA,* Homovanillic acid; *Iba-1,* Ionized-calcium–binding adapter molecule 1; *IL-6,* Interleukin-6; *MPP^+,* 1-methyl-4-phenylpyridinium; *MPTP,* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *NE,* Noradrenaline; *OFT,* Open-field test; *EDTA,* Ethylenediaminetetracetic acid; *PBS,* Phosphate-buffered saline; *PD,* Parkinson's disease; *PFA,* Paraformaldehyde; *PP2A,* Protein phosphatase 2A; *RT,* Room temperature; *S1P,* Sphingosine-1-phosphate; *SDS,* Sodium dodecyl sulfate; *SNpc,* Substantia nigra pars compacta; *TH,* Tyrosine hydroxylase; *TNF-α,* Tumor necrosis factor-α.

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