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Fasudil Enhances Therapeutic Efficacy of Neural Stem Cells in the Mouse Model of MPTP-Induced Parkinson's Disease

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Abstract Bone marrow-derived neural stem cells (NSCs) are ideal cells for cellular therapy because of their therapeutic potential for repairing and regenerating damaged neurons. However, the optimization of implanted cells and the improvement of microenvironment in the central nervous system (CNS) are still two critical elements for enhancing therapeutic effect. In the current study, we observed the combined therapeutic effect of NSCs with fasudil in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease (PD) mouse model and explored the possible cellular and molecular mechanisms. The results clearly show that combined treatment of NSCs with fasudil further improves motor capacity of PD mice, thus exerting double effect in treating MPTP-PD. The combined intervention more effectively protected dopaminergic (DA) neurons from loss in the substantia nigra pars compacta (SNpc), which may be associated with the increased number and survival of transplanted NSCs in the brain. Compared with the treatment of fasudil or NSCs alone, the combined intervention more effectively inhibited the activation and aggregation of microglia and astrocytes, displayed stronger anti-inflammatory and antioxidant effects, induced more neurotrophic factor NT-3, and affected the dynamic homeostasis of NMDA and AMPA receptors in MPTP-PD mice. Our study demonstrates that intranasal administration of NSCs, followed by fasudil administration, is a promising cell-based therapy for neuronal lesions.

Keywords Parkinson's disease (PD) · 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) · Rho kinase inhibitor fasudil · Bone marrow-neural stem cells

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Introduction

Parkinson's disease (PD) is the second most devastating neurodegenerative disease after Alzheimer's disease (AD) [1, 2]. It is estimated that, worldwide, more than eight million people could develop PD in the next decades [3]. PD is characterized by progressive loss of dopaminergic (DA) neurons, and at the time of clinical diagnosis, patients have already lost approximately 60 % of substantia nigra pars compacta (SNpc) neurons and 80 % of striatal dopamine neurons [4]. The degeneration of DA neurons in the substantia nigra (SN) and their projections to the striatum, leading to a severe loss of striatal dopamine, is the key pathology underlying motor deficits of PD. Current treatment can only control the symptoms but not halt the degenerative process of PD. Motor symptoms of PD can be successfully treated by dopaminergic drugs for several years but, over time, the drugs become less effective and are associated with side effects such

as involuntary movements (dyskinesias). An effective therapy for PD is thus of vital importance in medical practice and a challenge to researchers.

Neural stem cells (NSCs) exhibit stem cell properties, including self-renewal and production of a large number of progeny. In the past few decades, NSC transplantation has been suggested as a promising therapeutic strategy for various central nervous system (CNS) diseases, including degenerative disorders [5–10], stroke [11, 12], and spinal cord injury (SCI) [13, 14]. NSCs restore neuronal function via various mechanisms, including neurogenesis, angiogenesis, and synaptogenesis [11]. In addition, most preclinical studies have emphasized that NSCs enhanced self-repair systems rather than replacing lost cells by attenuating inflammation, participating in immunomodulation, enhancing autophagy, and normalizing the microenvironment [14]. Although preclinical data generated a great deal of enthusiasm, NSC therapy remains mainly at the experimental stages, its major limitation being instability of the therapeutic effect, which stems from a variety of factors. For example, the extracellular microenvironment surrounding transplanted cells affects the ultimate fate of these cells, such as migration, proliferation, differentiation, and cell–cell interactions [15]. Transplanted stem cells and microenvironments in the CNS are therefore two important factors that determine the clinical outcome of cell therapy.

It has been demonstrated that Rho kinase (ROCK) inhibitor, fasudil, may theoretically be an ideal candidate for optimizing cells and improving microenvironments in the CNS. First, ROCK inhibitors not only improve the in vitro growth and differentiation of stem cells but also improve their survival and engraftment during transplantation [16–18]. Combined treatment of SCI with fasudil and bone marrow stromal cell (BMSC) transplantation resulted in better locomotor recovery and axon regeneration [19, 20]. Second, studies in various animal models have shown that fasudil exhibited anti-inflammatory effects [21]. Fasudil can inhibit neutrophil infiltration in a cerebral model of infarction [22] and reduce synovial inflammation and production of proinflammatory cytokines in both mouse models and human cells isolated from rheumatoid arthritis (RA) patients [23]. In diabetes models, fasudil decreased monocyte adhesion to endothelial cells [24]. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, fasudil treatment resulted in decreased immune cell infiltration and tissue destruction, especially during disease onset [25–29].

To date, both preclinical and clinical studies have had mixed results for NSC therapy, and the discrepancy between expected and actual efficacy in several diseases has been discouraging. We consider that NSC therapy may now be at a critical stage for re-evaluation and re-consideration. In the present study, we attempt to explore whether the addition of fasudil can enhance therapeutic effect of NSCs by optimizing transplanted cells and improving the microenvironment within the CNS.

Materials and Methods

Animals

Sixty male C57BL/6 mice (26–30 g, 10–12 weeks) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All animal experiments in this study were carried out according to the guidelines for the care and use of laboratory animals and approved by the Ethics Committee of Shanxi Datong University, Datong, China. Mice were housed under pathogen-free conditions, received food and water ad libitum, and maintained in a reversed 12:12-h light/dark cycle in a temperature-controlled room (25 ± 2 °C) for 1 week prior to experimental manipulation.

Generation and Treatment of BM-NSCs

To generate bone marrow-derived neural stem cells (BM-NSCs), whole BM was harvested from the femurs of male green fluorescent protein (GFP) transgenic mice that constitutively express GFP (C57BL/6-Tg [ACTB-EGFP]) at 8 weeks of age, as in previously described protocols [30]. Briefly, cells were plated on poly-D-lysine/laminin (Sigma-Aldrich, St. Louis, MO)-coated 24-well plate and cultured in serum-free DMEM/F-12 (Invitrogen, Gaithersburg, MD) supplemented with 2 % B27, 20 ng/ml epidermal growth factor (EGF), and 20 ng/ml basic fibroblast growth factor (bFGF) along with antibiotics. Cells were plated at a density of 1×10^6 cells/well and medium changed every 4 days. After 2 weeks, a portion of individual cells proliferated to form distinct neurosphere. After 3–4 weeks, the neurospheres were collected, dissociated to single cells by Accutase (Thermo Fisher Scientific, Waltham, MA), and replated at 1.0×10^5 cells/ml for the next passage. These cells expressed NSC markers Nestin and Sox2, as determined by immunocytochemistry. To further confirm the NSC property of these cells in vitro, neurospheres at 5th–10th passages were dissociated into single cells and cultured in differentiation medium. After 10–14 days, BM-NSCs changed morphology and developed into neurons (NF-M$^+$), astrocytes (GFAP$^+$), and oligodendrocytes (GalC$^+$) as previously described [30]. Cells at 5th–10th passages were used. Single dissociated BM-NSCs were suspended in sterile PBS.

Experimental Design and MPTP-PD Model

Mice were divided into four groups, i.e., PBS-treated (PD), fasudil-treated (fasudil+PD), NSC-treated (NSCs+PD), and

fasudil+NSC-treated (fasudil+NSCs+PD) ($n = 15$ each group). MPTP (Sigma, USA) was dissolved in PBS. For the MPTP-PD model, mice were intraperitoneally injected with MPTP (15 mg/kg) four times at 2-h intervals for 1 day to induce Parkinsonism.

Administration of Fasudil and *BM-NSCs*

At 48 h after the final MPTP injection, mice were lightly anesthetized with diethylether, and two doses of 3 μl/hyaluronidase (total 100 U; Sigma-Aldrich Chemical Co.) in PBS were applied to each nostril, allowing the animal to sniff the solution into the upper nasal cavity. Subsequently, a total of 1.5×10^5 *BM-NSCs* in 12 μl PBS was administered to each nostril. Mice that received the same volume of PBS nasally served as untreated PD controls. At day 4 after MPTP injection, fasudil (from Tianjin Chase Sun Pharmaceutical Co., Ltd, China) was dissolved in PBS and injected intraperitoneally at 400 μg/mice every other day for six times.

Two days after the final fasudil injection, mice were rapidly perfused with saline, followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were carefully removed and stored at −80 °C until use. One hundred sequential 10-μm-thick coronal sections (−2.80 to −3.88 mm posterior to bregma based on the paxinos atlas) were cut on freezing microtome using a Leica cryostat for immunohistochemistry.

Total proteins were extracted from the midbrain with 1 mM PMSF in 1 ml ice-cold RIPA buffer (Beyotime) and then added to EDTA-free protease inhibitor cocktail. Protein concentrations were determined using the bicinchoninic acid protein assay (Beyotime).

Behavioral Assessment

Open-Field Test

On days 14 and 15 after the MPTP intervention, mice were placed in the center of an open behavioral chamber to monitor general activity levels. Before recording, mice were allowed to freely explore the chamber for 15 min to get used to the space in order to reduce novelty-induced stress [31, 32]; their spontaneous activity was then analyzed for 30 min by an automated tracking system and distance traveled was recorded. The apparatus was washed out after each test in order to avoid interfering with the activities of other mice.

Traction Test

To test muscular relaxant activity, the traction test was performed as previously described [33, 34]. Animals grasped the taut metal wire with their forepaws and, when they were allowed to hang free, the time to bring their hind paws and tail up to the wire (time to BHPT) was recorded. The score to BHPT was graded according to the following criteria: 5 = able to do so within 5 s; 4 = within 10 s; 3 = within 20 s; 2 = within 40 s; and 1 = this did not occur or over 40 s. Then, the time of falling down from the wire was also recorded. The score was graded according to the following criteria: 5 = animals did not fall down from the wire; 4 = animals fell down from the wire, and the time is over 120 s; 3 = the time is over 60 s; 2 = over 20 s; and 1 = within 20 s. Finally, the time to climb to the platform on both sides was recorded. The criteria were follows: 5 = got to the platform within 40 s; 4 = within 60 s; 3 = within 100 s; 2 = within 130 s; and 1 = did not get to the platform, or the time was over 130 s. A cumulative clinical score for muscular relaxant activity was calculated for each mouse by adding these three scores [35]. A lower score was considered to be synonymous with bad coordination of the four paws and tail [33, 34].

Pole Test A pole test was performed to evaluate bradykinesia in the mice. The mouse was positioned head up at the top of a vertical rough-surfaced pole (10 mm diameter, 58 cm height). The time to turn and reach the floor was recorded. Two days before testing, each mouse was trained to descend the pole. On the test day, mice were allowed to practice five times and then tested three times, with each trial lasting for a maximum of 4 min [31].

Immunofluorescence Staining

Sections were permeabilized and blocked with 0.3 % Triton X-100/1 % bovine serum albumin in 0.01 M PBS for 1 h, then incubated overnight at 4 °C with primary antibodies directed against anti-tyrosine hydroxylase (1:1000, anti-TH, Millipore, Billericay, MA), anti-NF-kB-p65 (1:1000, Cell Signaling Technology, Danvers, MA), anti-CD11b (1:1000, eBioscience, San Diego, CA), anti-GFAP (1:500, DAKO, Z0334), and anti-NT-3 (1:1000, Abcam, USA). Sections were then washed with PBS and incubated with the corresponding fluorescein-labeled secondary antibodies with wavelengths of 555 or 405 μm for 2 h in the dark at room temperature. Finally, sections were cover-slipped with 50 % glycerinum and were counted under fluorescence microscope with Image-Pro Plus 6.0 software. As a negative control, additional sections were treated similarly, but the primary antibodies were omitted.

Western Blotting

Protein extracts (30 g) were separated by SDS-PAGE and transferred onto a PVDF membrane (Immobilon-P; Millipore). Membranes were then incubated overnight at 4 °C with primary antibodies (all at 1:1000) as follows: anti-CD11b, anti-Nrf2, anti-NMDAR1, anti-NMADR2, anti-AMPAR1, anti-AMPAR2, anti-NT-3 (all from Abcam, USA), anti-NF-kB-p65, anti-β-actin (both from Cell

Signaling Technology, Danvers, MA), anti-tyrosine hydroxylase (1:1000, anti-TH, Millipore, Billericay, MA), anti-GFAP (1:500, DAKO, Z0334), anti-ROCK II (1:1000, Cayman Chemicals Company, USA), and anti-HO-1 (1:1000, ABGENT). Bands were visualized by HRP-conjugated corresponding secondary antibodies (Thermo Scientific, Rockford, IL) and chemiluminescence (ECL) kit under ECL system (GE Healthcare Life Sciences, USA), followed by imaging and quantification of protein bands using Bio-Rad Quantity One 1-D software. β-actin was used as the internal control.

Statistical Analysis

All data are expressed as means ± standard error of measurement (SEM). Multiple comparisons were analyzed by one-way ANOVA. $P < 0.05$ was considered statistically significant. All statistical analyses and graphs were performed or generated with GraphPad Prism v5.0 (GraphPad Prism Software, Inc., San Diego, CA, USA).

Results

Combination of Fasudil and NSCs Improves Behavioral Scores of MPTP-PD

To validate the effect of fasudil and NSCs in PD, the model was induced in C57BL/6 mice by injection of MPTP, and the protective effect of fasudil and NSCs was examined by behavioral analysis of motor functions. On days 14 and 15 after the MPTP intervention, locomotor behavior of animals was examined using the open-field test. The total distance traveled (cm/30 min) by MPTP-PD mice under basal conditions was significantly lower than that of the fasudil-, NSC-, and fasudil+NSC-treated animals (Fig. 1a, $P < 0.05$–0.001). Importantly, the combined intervention of NSCs with fasudil further significantly ameliorated movement deficit when compared to fasudil (Fig. 1a, $P < 0.001$) or NSCs (Fig. 1a, $P < 0.001$) alone.

Using a traction test to monitor the muscular relaxant activity, we also observed that the group treated with NSCs plus

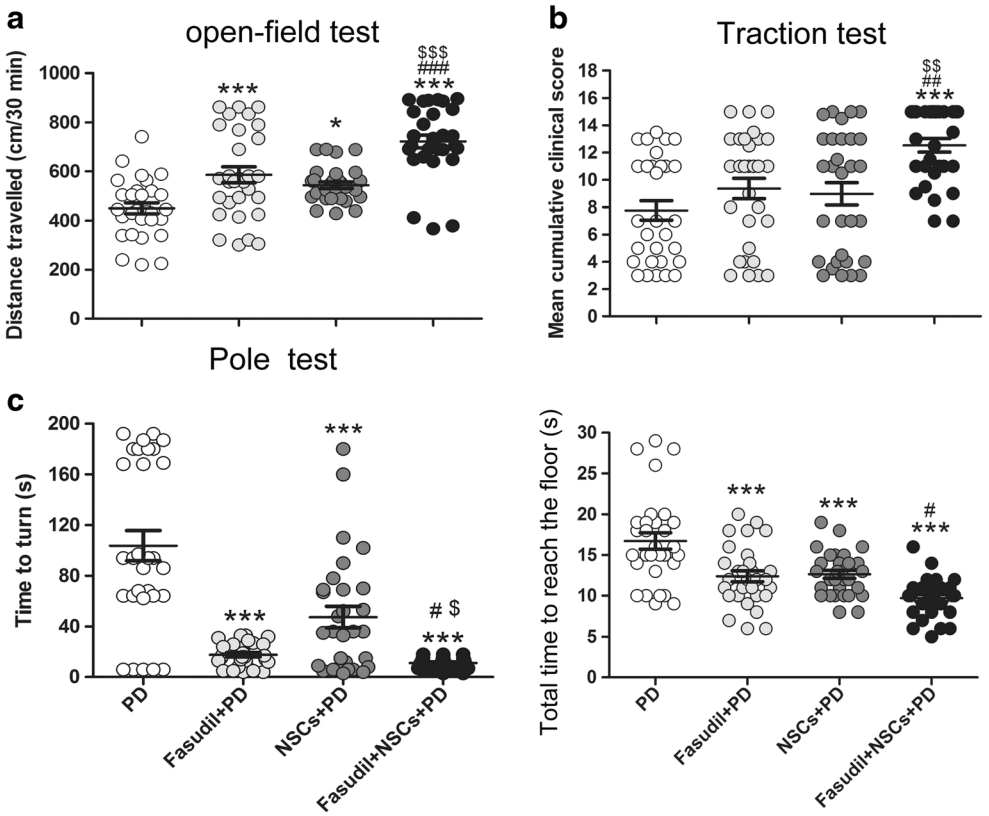

Fig. 1 Combination of fasudil and NSCs improved behavioral signs of MPTP-PD mice. NSCs were harvested from the femurs of male green fluorescent protein (GFP) transgenic mice and delivered into brain by intranasal route at day 2 after MPTP injection. Fasudil and PBS were intraperitoneally injected six times every other day, starting at day 4 after MPTP injection. **a** General locomotor activities (distance traveled for a period of 30 min, cm/30 min) were analyzed in an open-field test on days 14 and 15 after PD induction. **b** In a traction test, the muscular relaxant activity was graded and the mean cumulative clinical score recorded. **c** In the pole test, the time to turn and total time to reach the floor were recorded. Results are shown as mean ± SEM of 15 mice each group for two independent experiments, and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{*, \#, \$}P < 0.05$, $^{\#\#, \$\$}P < 0.01$, $^{***\#\#\#, \$\$\$}P < 0.001$

fasudil showed a significantly higher mean cumulative clinical score than the other groups (Fig. 1b, $P < 0.01$–0.001). However, fasudil or NSCs alone did not significantly improve muscular relaxant performance. The pole test, which is another sensitive method for determining nigrostriatal dysfunction, showed that with regard to bradykinesia of the MPTP-induced PD mice, the time to turn at the top (T-turn) and time to climb down (T-LA) increased to 103.73 ± 11.97 and 16.73 ± 1.01 s compared with the group treated with fasudil (17.63 ± 1.70 s, 12.40 ± 0.67 s, $P < 0.001$), NSCs (47.33 ± 8.39 s, 12.63 ± 0.49 s, $P < 0.001$), or with NSCs plus fasudil (11.17 ± 0.85 s, 9.70 ± 0.43 s, $P < 0.001$; all in Fig. 1c). Taken together, the combined intervention of NSCs with fasudil further significantly improved motor dysfunction compared with the treatment of fasudil alone (Fig. 1c, $P < 0.05$) or NSCs alone (Fig. 1c, $P < 0.05$).

Combination of Fasudil and NSCs Enhances the Protection of DA Neurons in MPTP-PD

The protective effects of NSCs plus fasudil on DA neurons were also investigated after MPTP-induced lesions had developed. The numbers of nigral TH-positive neurons were counted in the SN area. Administration of fasudil or NSCs alone reduced the loss of TH^+ DA neurons compared with control mice (Fig. 2, both $P < 0.001$), and the combination treatment of NSCs with fasudil further protected TH^+ DA neurons in the SNpc when compared with mice treated with NSCs or fasudil alone (Fig. 2, $P < 0.001$). There was no difference between mice treated with fasudil or NSCs alone in the loss of DA neurons. We further explored the levels of TH protein in the SN of brain by Western blot, and similar results were observed. These results are consistent with behavioral changes and

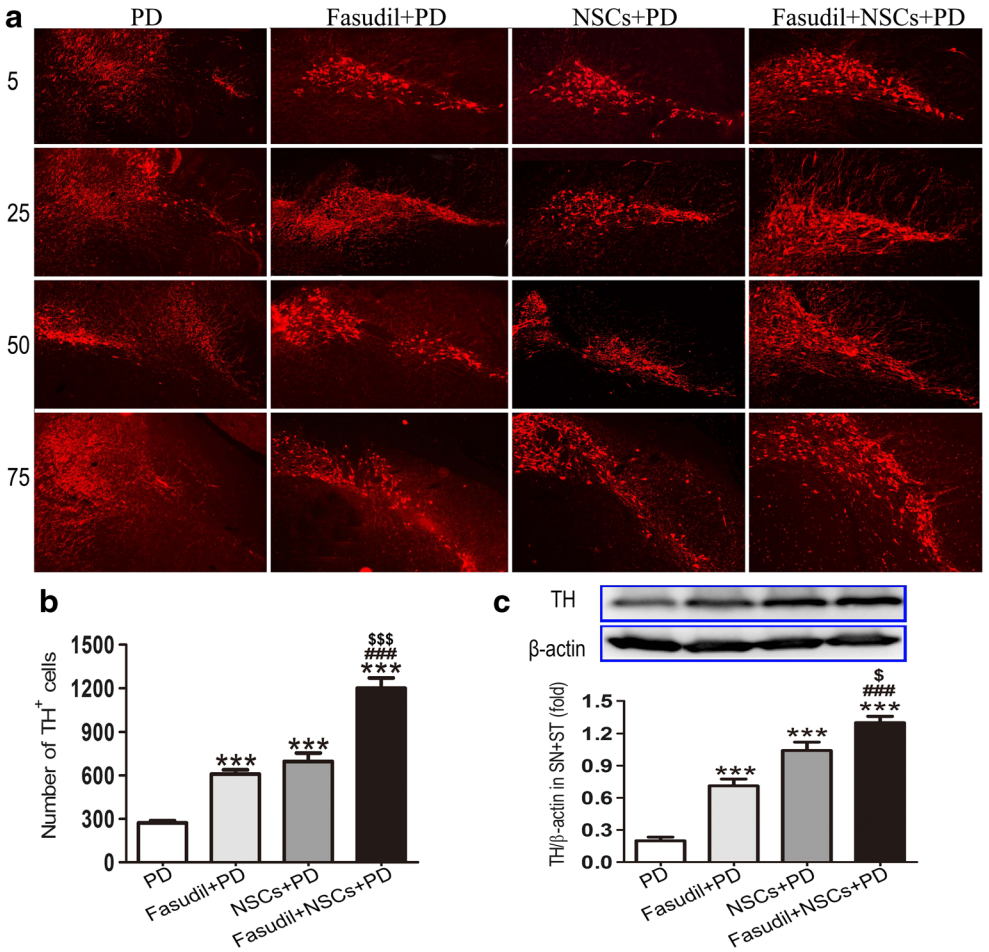

Fig. 2 Combination of fasudil and NSCs exhibits synergistic protection of DA neurons of MPTP-PD mice. Four consecutive brain sections (−2.80 to −3.88 mm posterior to bregma based on the paxinos atlas, the 5th, 25th, 50th, and 75th sections were chosen) were stained with anti-tyrosine hydroxylase (*TH*) antibody, followed by the corresponding fluorescein-labeled secondary antibodies. All slices were independently and blindly examined under a fluorescence light microscope by two investigators. **a** Immunohistochemistry of TH^+ DA neurons, **b** numbers of TH^+ cells in the SNpc, and **c** TH protein expression in SNpc (*SN*) and striatum (*ST*). Quantitative results are mean ± SEM ($n = 7$ in **a** and **b** and $n = 8$ in **c** each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{\$}P < 0.05$, ***, ###, $^{\$\$\$}P < 0.001$

indicate that combined intervention of NSCs with fasudil exerts a synergistic and superimposed effect in treating PD mice.

Addition of Fasudil Contributes to the Survival of Transplanted NSCs in Brain

In order to better relieve and prevent disease with NSC treatment, it would be ideal if a greater number of transplanted cells could reach the lesion and survive there. In this study, the numbers of transplanted cells (GFP^+) were more highly elevated in the hippocampus and SN in mice treated with fasudil plus NSCs than in mice treated with NSCs alone (Fig. 3, $P < 0.01$ or $P < 0.001$). Thus, addition of fasudil likely increases the numbers or promotes the survival ratio of transplanted NSCs in the brain.

Combination of Fasudil and NSCs Inhibits Activation and Aggregation of Glia Around SNpc and Striatum

The distribution of microglia varies greatly throughout the brain during the course of PD, and the SN contains the highest density of microglia, contributing to neuronal death [36, 37]. Thus, the effect of single or combined treatment on microglia aggregation around SNpc and striatum was investigated in the present study. An increased CD11b expression and microglia aggregation were observed around SNpc and striatum of brain in MPTP-PD mice (Fig. 4a). Either fasudil or NSCs obviously inhibited expression of CD11b and aggregation of microglia when compared with MPTP-PD mice (Fig. 4b–d, both $P < 0.001$), but there was no significant difference between the two groups. The combined intervention of NSCs with fasudil further limited CD11b expression and microglia aggregation around SNpc and striatum compared with NSCs or fasudil alone ($P < 0.05$–0.01; Fig. 4b–d). These results suggested that reduced microglia activation and aggregation around SNpc and striatum may be a significant protection for nigrostriatal DA neurons in the MPTP-PD model.

In addition to microglia, astrocytes also play a critical role in mediating neuronal survival and function and in inducing neurodegeneration by secreting soluble toxic molecules [38]. In Fig. 5a, we showed that GFAP expression was correlated with that of p-NF-kB in the brain of MPTP-PD mice, indicating that astrocytes were activated. Similar to the results with microglia, either fasudil or NSCs obviously inhibited GFAP expression (Fig. 5b, c), while the combined intervention of NSCs with fasudil further suppressed GFAP expression when compared with fasudil alone (Fig. 5b, c, $P < 0.05$). GFAP expression from Western blot was consistent with the results derived from immunohistochemistry (Fig. 5d, $P < 0.01$).

Combination of Fasudil and NSCs Displays Stronger Anti-Inflammatory and Anti-Antioxidant Effects in MPTP-PD

Glial cells (microglia and astrocytes) are a double-edged sword, displaying a beneficial or destructive role when they

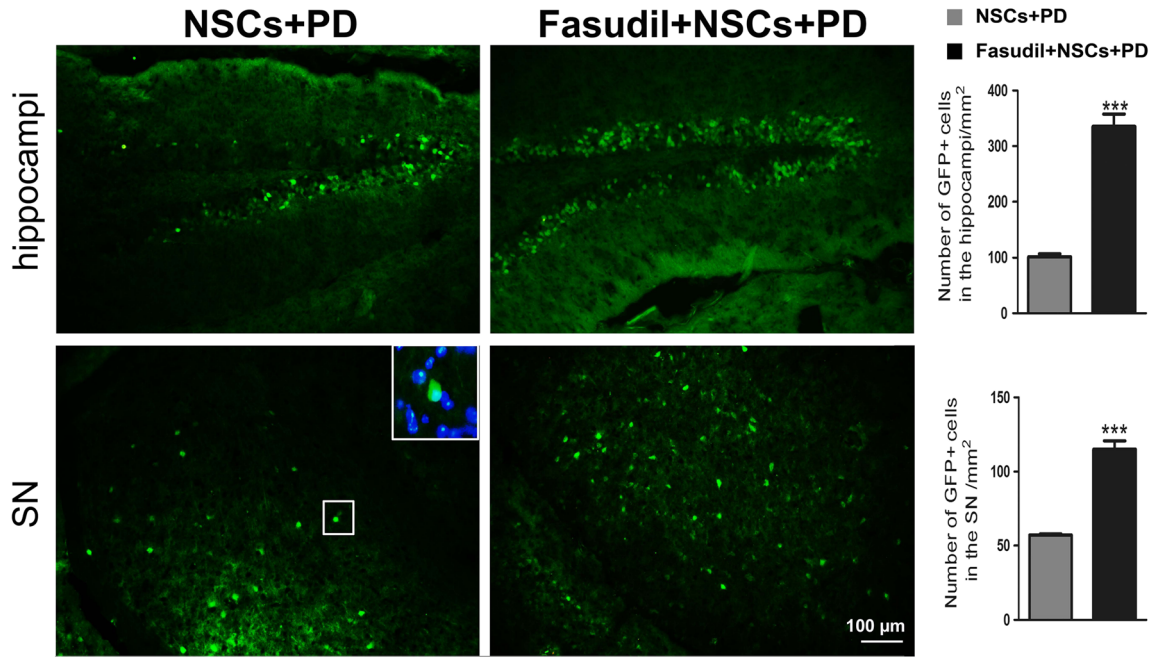

Fig. 3 Combination of fasudil and NSCs improves the survival of transplanted NSCs in brain. GFP^+ NSCs of brain sections were directly observed under a fluorescence microscope. The number of GFP^+ NSCs in the hippocampi and SN was averaged from five different levels 20 μm apart (bregma −2.80, −3.00, −3.20, −3.40, −3.60 mm). Quantitative results are mean ± SEM per square millimeter ($n = 7$ each group), and multiple comparisons were analyzed by one-way ANOVA. $^{***}P < 0.001$

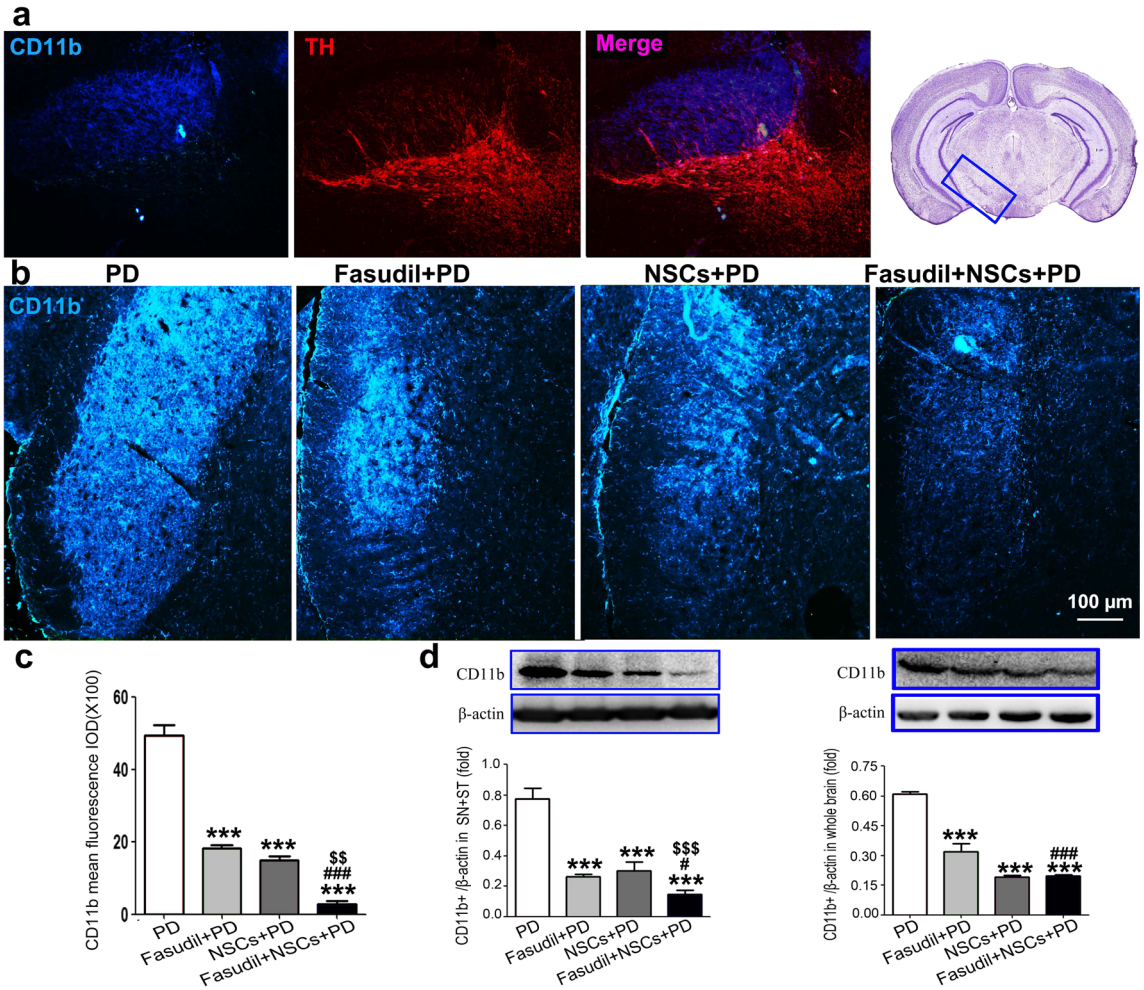

Fig. 4 Combination of fasudil and NSCs inhibits activation and aggregation of microglia around SNpc and striatum. Brain sections were stained with anti-CD11b and anti-tyrosine hydroxylase (*TH*), followed by the corresponding fluorescein-labeled secondary antibodies (wavelength of 405 μm for CD11b with *blue color* and 555 μm for TH with *red color*). All slices were independently and blindly examined under a fluorescence light microscope by two investigators. **a** Double immunostaining of CD11b and TH around SNpc of brain; **b** CD11b expression by immunohistochemistry in different groups; **c** fluorescence IOD of $CD11b^+$ microglia around SNpc in different groups; and **d** CD11b expression by Western blot in different groups. Quantitative results are mean ± SEM ($n = 7$ in **a**–**c** and $n = 8$ in **d** each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: Comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{\#}P < 0.05$, $^{\$\$}P < 0.01$, $^{***, ###, \$\$\$}P < 0.001$

are activated. Accumulating evidence suggests that the NF-kB signaling pathway contributes to inflammatory responses induced by glia [25, 28]. We determined the activity of NF-kB in SN and hippocampus by immunohistochemistry staining and Western blot. While MPTP-induced PD mice exhibited a high level of p-NF-kB/p65 expression, treatment of fasudil or NSCs alone effectively inhibited p-NF-kB/p65 expression in different regions of the brain compared with MPTP-PD mice (Fig. 6a, $P < 0.05$–0.01), and this effect was significantly enhanced by combined intervention of NSCs with fasudil (Fig. 6a, $P < 0.05$–0.001).

Further, fasudil inhibited ROCK II expression compared with that of MPTP-PD mice (Fig. 6b, $P < 0.001$), while the combined intervention of NSCs with fasudil enhanced the suppressive effects of NSCs on ROCK II (Fig. 6b, $P < 0.01$). Previous studies have indicated that ROCKII was activated in response to the myelin-associated inhibitor Nogo, which, in turn, enhanced ROCKII translocation to the cellular membrane of PC12 cells and the ROCKII activity in vitro [39]. Similarly, in the present study, we found that NSCs and fasudil alone significantly inhibited Nogo expression compared with untreated PD mice (Fig. 6b, $P < 0.001$), while combined treatment did not add to their individual effect on Nogo expression.

We also showed that treatment of fasudil, NSCs, or combined intervention induced expression of Nrf2 and Heme oxygenase isoform 1 (HO-1), while combined intervention showed a significantly higher expression of these molecules compared with fasudil (Fig. 6c, $P < 0.05$–0.001) or NSC treatment alone (Fig. 6c, $P < 0.05$). These results suggest that, in

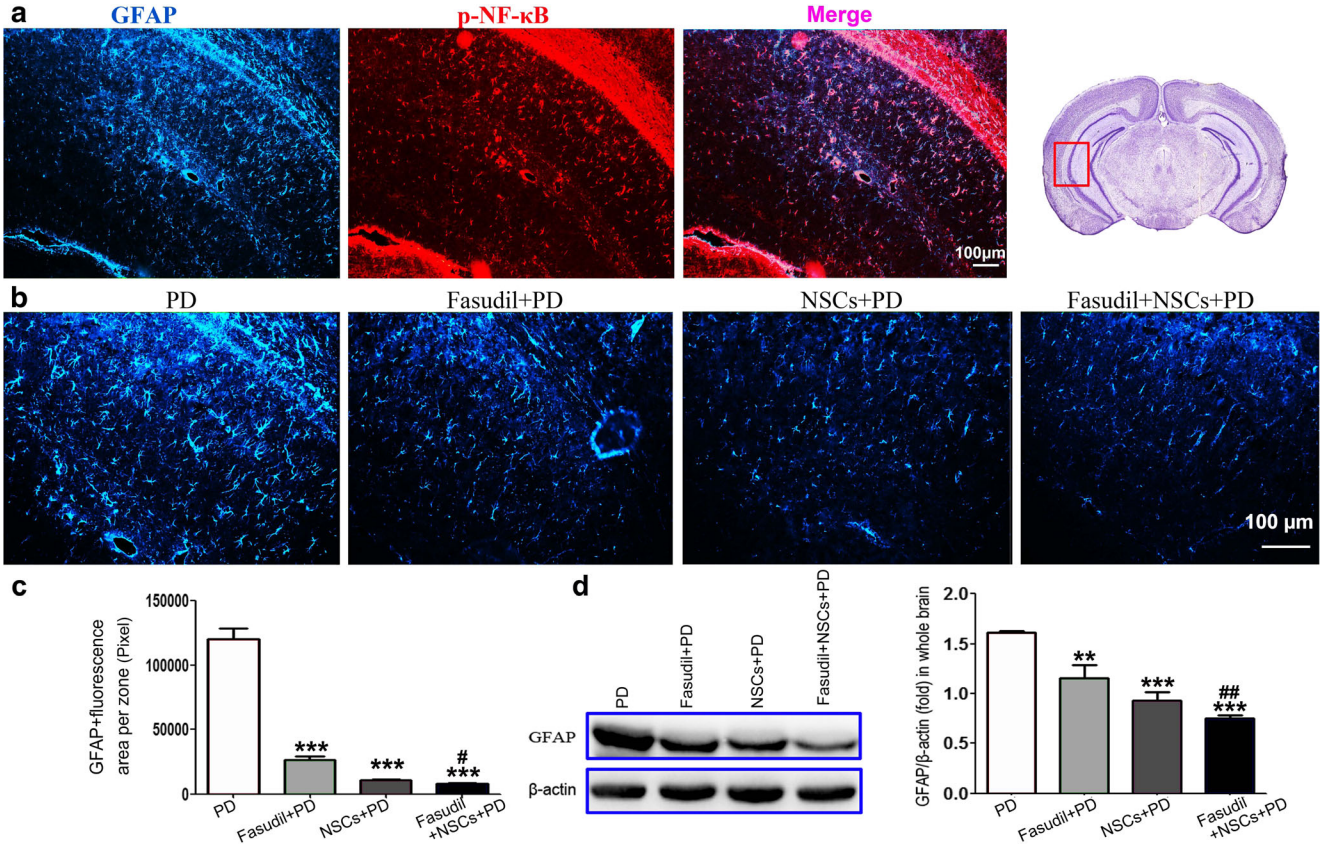

Fig. 5 Combination of fasudil and NSCs inhibits astrocyte activation. Brain sections were stained with anti-GFAP and anti-p-NF-kB antibodies, followed by the corresponding fluorescein-labeled secondary antibodies (wavelength of 405 μm for GFAP with *blue color* and 555 for p-NF-kB with *red color*). All slices were independently and blindly examined under a fluorescence light microscope by two investigators. **a** Double immunostaining of GFAP and p-NF-kB in the brain; **b** GFAP expression by immunohistochemistry in different groups; **c** the area of $GFAP^+$ astrocytes in different groups; and **d** GFAP expression by Western blot in different groups. Quantitative results are mean ± SEM ($n = 7$ in **a**–**c** and $n = 8$ in **d** each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{\#}P < 0.05$, **, $^{\#\#}P < 0.01$, $^{***}P < 0.001$

addition to an anti-inflammatory effect, ROCK inhibition may also have antioxidant effect.

Combination of Fasudil and NSCs Affects the Dynamic Homeostasis of NMDA and AMPA in MPTP-PD

By Western blot, we observed that fasudil treatment alone inhibited the expression of NMDAR1 and NMDAR2 ($P < 0.05$–0.001), while their expression was further inhibited by NSCs alone (both $P < 0.001$). Combined intervention did not further inhibit NMDAR1 and NMDAR2 expression when compared with NSC-treated PD mice (Fig. 7a). In contrast, treatment with NSCs, but not fasudil, induced both AMPAR1 and AMPAR2 expression compared with MPTP-PD ($P < 0.05$–0.001). Combined intervention effectively enhanced AMPAR1 and AMPAR2 expression compared with that of PD, fasudil, or NSC groups ($P < 0.05$–0.001). These results clearly show that combined intervention of NSCs with fasudil dramatically affects the dynamic homeostasis of NMDAR and AMPAR in MPTP-PD mice (Fig. 7b).

Combination of Fasudil and NSCs Enhances Neurotrophic Factors in MPTP-PD Mice

Given the important role of NT-3 in neuronal cell survival [40], we determined the effect of fasudil and/or NSCs on the induction of this neurotrophic factor. As shown in Fig. 8, treatment with fasudil or NSCs increased NT-3 expression compared with MPTP-PD mice ($P < 0.05$–0.001), while combined intervention further enhanced its expression when compared with that of other groups ($P < 0.01$–0.001). These results indicate that combined intervention of NSCs with fasudil synergistically upregulates NT-3 expression, which would be beneficial for neuron survival.

Discussion

Recently, NSC research has been one of the most exciting fields in neuroscience. NSC transplantation has been shown to effectively treat nervous system lesions. Its mechanisms of

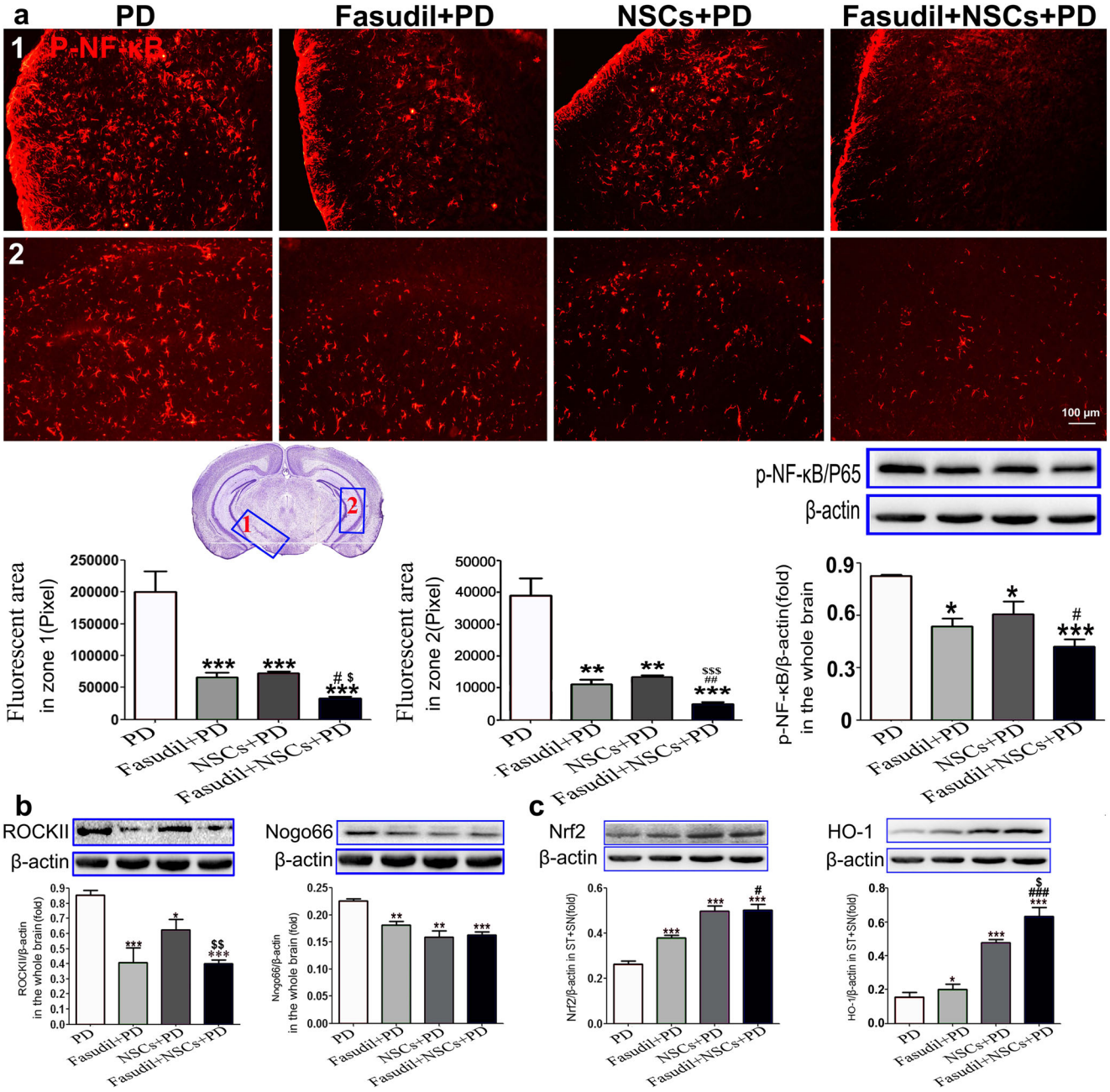

Fig. 6 Combination of fasudil and NSCs displays stronger anti-inflammatory and anti-antioxidant effects. Brain sections were stained with anti-p-NF-kB (*red*), followed by the corresponding fluorescein-labeled secondary antibodies (wavelength of 555 μm for p-NF-kB). All slices were independently and blindly examined under a fluorescence light microscope by two investigators. **a** p-NF-kB expression by immunohistochemistry in different regions of different groups; **b** ROCK and Nogo expression in brain by Western blot in different groups; and **c** Nrf2 and HO-1 expression by Western blot in different groups. Quantitative results are mean ± SEM ($n = 7$ in **a** and $n = 8$ in **b** and **c** each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{*}, ^{\#}, ^{\$}P < 0.05$, $^{**}, ^{\#\#}, ^{\$\$}P < 0.01$, $^{***}, ^{\#\#\#}, ^{\$\$\$}P < 0.001$

action are diverse, including neurogenesis, angiogenesis, synaptogenesis, secretion of neurotrophic factors and cytokines, scavenging of toxic molecules, and immunomodulation of inflammatory milieu [41, 42]. Among NSC sources, bone marrow- (BM-) and subventricular zone- (SVZ-) derived NSCs exhibit similar morphological properties, cellular markers, and the ability to differentiate, immunomodulation, and neurogenesis. However, given that SVZ-NSCs are hardly accessible for clinical application, BM-NSCs thus become a more attractive alternative for the treatment of neurological diseases. BM-NSCs have several advantages over other stem cells, e.g., they are free of ethical issues or possible allogenic

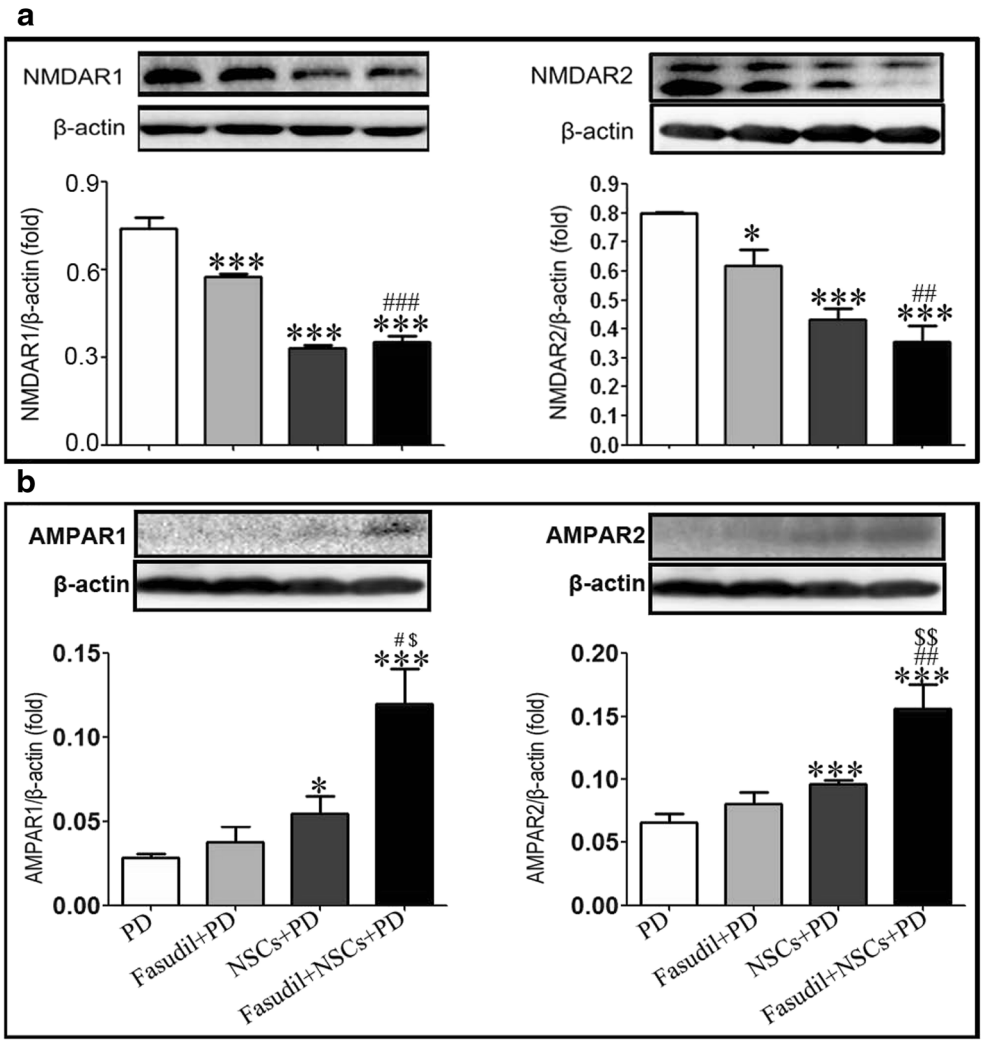

Fig. 7 Combination of fasudil and NSCs affects the dynamic homeostasis of NMDA and AMPA. NMDAR and AMPAR expression was measured by Western blot. **a** NMDAR1 and NMDAR2 expression in brain of different groups; **b** AMPAR1 and AMPAR2 expression in brain of different groups. Quantitative results are mean ± SEM ($n = 8$ each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{*, \#, \$}P < 0.05$, $^{\#\#, \$\$}P < 0.01$, $^{***, \#\#\#}P < 0.001$

rejection, i.e., they can be prepared from the patient's own BM cells, with minimal tumorigenicity [30, 43–45].

In stem cell therapies, a critical factor affecting neurogenesis is the cell delivery route. Current routes for administering stem cells to the brain include the system route (e.g., intravenous and intraperitoneal injections), direct CNS route (e.g., transcranial and intracerebroventricular injections), and intranasal (i.n.) delivery. Due to the special anatomical connection of the nasal route, i.n. administration provides an effective, noninvasive, and rapid route for delivering stem cells to the brain. Danielyan et al. demonstrated that i.n. delivery of stem cells improved the behavior of 6-hydroxydopamine (6-OHDA)-lesioned PD rats, increased the TH level, prevented dopamine loss, and reduced proinflammatory cytokine levels in lesion sites [46]. The highest number of stem cells migrated close to the α-synuclein aggregates and astrogliosis, suggesting the possible ability of stem cells to participate in clearance of α-synuclein [46–48]. NSCs can first be seen in the brain cortex 1 h after i.n. delivery; they then accumulate in the olfactory bulb, cortex, and spinal cord. These cells effectively induced functional recovery in CNS lesions by various mechanisms; interestingly, the immunomodulatory effect was CNS specific, without affecting immunological response in the periphery [49].

However, NSC transplantation alone is not sufficient for nerve regeneration and functional recovery, given that the majority of cells implanted into the CNS rarely survive. The microenvironment in the damaged CNS appears to be highly unfavorable for the survival of transplanted NSCs. To improve the efficacy of stem cells in target organs, combined intervention has been shown to improve graft efficiency and survival of stem cells and increase their therapeutic potential in the targeted organ compared with delivery of stem cells alone [50–52]. A variety of evidence indicates that injury to the CNS results in a strongly activated RhoA-ROCK pathway, making ROCK inhibition an attractive target for treatment of neurodegenerative diseases. Fasudil, a ROCK inhibitor, has received increasing attention in the treatment of neurological disease. A previous study showed that fasudil effectively ameliorated EAE development, possibly by blocking

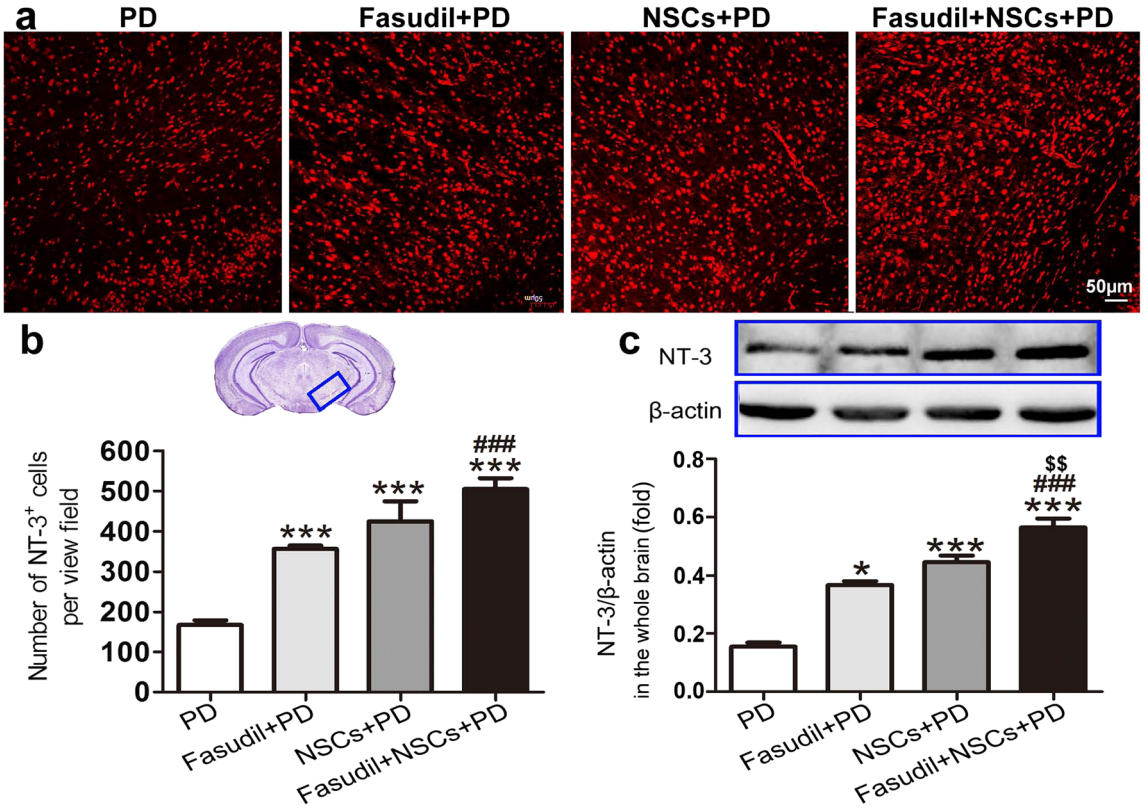

Fig. 8 Combination of fasudil and NSCs enhances NT-3 expression. Brain sections were stained with anti-NT-3 antibody (*red*), followed by the corresponding fluorescein-labeled secondary antibodies (wavelength of 555 μm for NT-3). All slices were independently and blindly examined under a fluorescence light microscope by two investigators. **a** NT-3 expression by immunohistochemistry. **b** Numbers of NT-3$^+$ cells in the different groups. **c** NT-3 expression in brain by Western blot in different groups. Quantitative results are mean ± SEM ($n = 7$ in **a** and **b** and $n = 8$ in **c** each group), and multiple comparisons were analyzed by one-way ANOVA. *Asterisk*: comparisons with the MPTP-PD group. *Number sign*: comparisons with the fasudil + PD group. *Dollar sign*: comparisons with the NSCs + PD group. $^{*}P < 0.05$, $^{\$\$}P < 0.01$, ***, $^{\#\#\#}P < 0.001$

inflammatory responses in the CNS and periphery [25, 26, 28, 29]. Fasudil also exerted protective effects for DA neurons and then motor deficits in the MPTP-PD model. Thus, the present study was designed to evaluate whether the combined intervention of BM-NSCs with fasudil further enhances functional recovery by i.n. route in mice subjected to MPTP.

While ROCK II expression was not inhibited by NSC treatment alone, it was significantly reduced in PD mice treated with NSCs plus fasudil. In neuron-microglia cultures, the ROCK inhibitor significantly reduced the MPP(+)-mediated loss of dopaminergic neurons. Inhibition of ROCK enhances survival of dopaminergic neurons and attenuates axonal loss in a mouse model of Parkinson's disease [53, 54]. However, treatment with ROCK inhibitor did not decrease the dopaminergic cell loss in primary neuron cultures without microglia [55], suggesting that the microglial response is crucial for the protective effects of ROCK inhibitors for dopaminergic neurons. The density of microglia in the healthy brain is remarkably higher in the SN compared with other midbrain areas and brain regions such as the hippocampus [56]. When mixed neuron-glia cultures derived from the hippocampus, cortex, or mesencephalon were treated with LPS, mesencephalic cultures became more sensitive to LPS compared with hippocampal and cortical cultures. The susceptibility of cortex, hippocampus, and DA neurons to LPS-induced toxicity varies, suggesting that the differential susceptibility of neurons is positively linked to the number of microglia in the various brain regions, and the highest proportion of microglia in the SN may facilitate degeneration of the nigrostriatal pathway in PD [36]. In the present study, we found that microglia were highly clustered around the striatum and substantia nigra. New reports have provided evidence that microglial phagocytosis might be a phenomenon for the induction of neuronal death [57] and promotion of apoptotic cell corpse degradation [58]. Activated microglia may play a critical role in inflammation-mediated dopaminergic neuronal death and provide the basis for further studies on the mechanisms of ATR-induced dopaminergic system toxicity [59, 60]. It has been reported that the inhibition of microglia activation decreased loss of nigral DA neurons [61], showing that, in addition to α-synuclein deposition, microglial activation is a prominent pathological feature in the SN of PD. Here, the administration of fasudil or NSCs alone reduced the CD11b$^+$ microglia around the striatum and substantia nigra, and the combined intervention of NSCs with

fasudil can further decline the presence of $CD11b^+$ microglia in these regions, thus contributing to the protection of DA neurons in nigral region.

Nrf2 is a transcription factor with a strong antioxidant effect, which protects neurons from ROS-induced damage, and targeting Nrf2 might, thus, be protective for DA neurons [62, 63]. HO-1 is crucial to inhibit oxidative stress via the catabolism of heme to carbon monoxide, bilirubin, and iron. Nigrostriatal Nrf2 antioxidant can protect dopaminergic neurons from MPP(+)-induced toxicity [64], while HO-1 activation might be neuroprotective [65]. In a PD mouse model, HO-1 significantly reduced MPTP-induced toxicity and dopaminergic neuronal death [66, 67]. Taken together, the Nrf2/HO-1 antioxidant pathway may offer neuroprotection for dopaminergic cells against oxidative stress [68]. In our study, Nrf2 and HO-1 expression, especially the latter, was obviously upregulated in PD mice treated with the combined NSC/fasudil intervention compared with those treated with NSCs or fasudil alone, suggesting that the addition of fasudil adds a synergistic effect to NSC therapy, possibly via activating the Nrf2/HO-1 antioxidant pathway.

NMDARs contribute to neural development, plasticity, and survival but are also linked with neurodegeneration [69]. NMDA receptors are distributed through broad areas of the brain, including the striatum. In this key structure of the basal ganglia, NMDA receptors are enriched in the medium spiny projection neurons and interneurons, in parallel with massive glutamatergic afferents from multiple forebrain sites, such as the cerebral cortex, hippocampus, amygdala, and thalamus [70]. In addition to the role of NMDA receptors in the regulation of normal striatal activities, malfunction of the receptors is linked to the pathogenesis of various neurological and neuropsychiatric disorders. Upregulation of NMDA receptors may increase intracellular Ca^{2+} with a subsequent increase in second messengers, which causes enhanced metabolic stress on mitochondria, excessive oxidative phosphorylation, increased production of reactive oxygen species, and finally neurodegeneration. Thus, NMDA receptor-mediated excitotoxicity has been suggested to be one of the possible causes of neuronal degeneration [71–73]. Reduced NMDARs by the combination of fasudil and NSCs may therefore be an important mechanism underlying their neuroprotective effects in our MPTP-PD model.

AMPARs are the major excitatory receptors of the brain and are fundamental to synaptic plasticity, memory, and cognition. Depletion of the AMPAR reserve pool impairs synaptic plasticity in a model of encephalopathy [74]. Paraquat inhibited postsynaptic AMPARs on dopaminergic neurons in SNpc, contributing to the pathogenesis of PD [75]. The numbers of cells positive for GluR1, a subunit of AMPA receptor, were significantly decreased in SNpc in the 6-OHDA-PD model [76]. These results show that a reduced AMPAR level is involved in the pathogenesis of PD and in experience-dependent spinal cord plasticity after injury and that it provides a pharmacologically targetable synaptic mechanism [77]. Our data demonstrate that combined intervention of NSCs with fasudil increased AMPAR1 and AMPAR2 expression. Collectively, these data delineate a novel signal cascade regulating AMPAR trafficking that may contribute to the molecular mechanisms that govern learning and cognition. We are trying to explore the cellular and molecular mechanisms of AMPA induction and to elucidate its role in governing learning and cognition.

In conclusion, our findings provide direct evidence that the addition of fasudil further enhances the therapeutic potential of NSC transplantation in MPTP-PD mice when compared with NSCs or fasudil alone. The synergistic and superimposed effect of NSCs combined with fasudil should lead to the following cellular and molecular changes: (1) reduction of glial cell aggregation around the striatum and SN; (2) inhibition of ROCK expression; (3) induction of Nrf2/HO-1 antioxidant pathway; and (4) regulation of NMDAR and AMPAR. These results indicate that intranasal NSC therapy, followed by fasudil administration, is a promising cell-based therapy for neuronal lesions, such as in PD.

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Compliance with Ethical Standards

Conflict of Interest None of the authors have any potential financial conflict of interest related to this manuscript.

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