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Post-MPTP Treatment with Granulocyte Colony-Stimulating Factor Improves Nigrostriatal Function in the Mouse Model of Parkinson's Disease

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Abstract The neuroprotective effects of granulocyte colony-stimulating factor (G-CSF) were reported in several neurological disease models, including Parkinson's disease (PD). In the present study, we investigated the therapeutic effect of G-CSF after the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD was established. G-CSF was subcutaneously administered into C57BL/6 mice that had undergone systemic MPTP injections. We found that G-CSF treatment markedly increased the number of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the G-CSF-treated group. Consistent with this finding, we found a significant increase in dopamine release under high K^+ stimulation in the striatum of the G-CSF-treated animals compared to the MPTP-exposed mice. Finally, we observed a persistent recovery of locomotor function in the G-CSF-treated animals. These results suggest the potential therapeutic value of G-CSF in treating PD. However, our bromodeoxyuridine labeling experiment failed to identify any newly generated dopaminergic neurons in SNpc. This might indicate an indirect effect of G-CSF on cell proliferation. The underlying mechanism of G-CSF is under further investigation.

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Keyword Parkinson's disease · Neurogenesis · G-CSF · Dopaminergic neurons · MPTP

Abbreviations

BrdU	Bromodeoxyuridine
DCX	Doublecortin
G-CSF	Granulocyte colony-stimulating factor
G-CSFR	G-CSF receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NSC	Neural stem cell
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
PFA	Paraformaldehyde
SGZ	Subgranular zone
SNpc	Substantia nigra pars compacta
SVZ	Subventricular zone
TH	Tyrosine hydroxylase

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease resulting from the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain. Normally, the dopaminergic neurons in the SNpc project to the striatum through the nigrostriatal pathway and release dopamine to maintain the balance of activity necessary for the control of involuntary movements. In PD, the profound loss of dopamine in the striatum causes tremor, rigidity, and slowness of movement. Currently, the most common therapeutic approach for PD is to restore striatal dopamine levels with drugs such as levodopa, dopamine agonists, and inhibitors of monoamine metabolism. Unfortunately, chronic treatment with these medications is

associated with serious side effects and fails to provide long-term control of the progression of the disease [1].

In addition, enormous efforts have also been made to search for novel neuroprotective drugs that can delay onset or slow down the progression of PD. Granulocyte colony-stimulating factor (G-CSF) is a growth factor known to stimulate the proliferation and survival of hematopoietic cells through the G-CSF receptor (G-CSFR) [2]. G-CSF can penetrate the blood brain barrier [3] and plays a prominent role in the CNS [4]. G-CSF and its receptor are expressed in neurons throughout the brain [3]. Increasing evidence from recent studies indicates that G-CSF is neuroprotective in vivo and in vitro [3, 5, 6]. For example, G-CSF protects against neurodegeneration in a number of neurological disease models such as PD [7, 8], Huntington's disease [9], and cerebral ischemia [10, 11].

More interestingly, G-CSF has been shown to be able to drive neurogenesis in vitro and in the rodent brain [3]. G-CSFRs are expressed by adult neural stem cells (NSCs) [3]. NSCs in the adult brain are found primarily in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus [12]. Several lines of evidence suggest that neurogenesis is impaired in PD because dopamine plays a neurotrophic role in the brain. Experimental depletion of dopamine in animal models resulted in decreased proliferation of NSCs in both SVZ and SGZ [13–15]. Analysis of postmortem human brains with PD also confirmed that there is a tremendous reduction in neurogenesis [13]. Recently, it has been proposed that the stimulation of neurogenesis by neurotrophic factors represents an important treatment strategy for PD and other neurodegenerative disorders [16, 17]. Peripheral infusion of G-CSF enhanced the recruitment of NSCs from the lateral ventricle wall into the ischemic area of the neocortex in rats [3]. Therefore, G-CSF may offer a novel regenerative therapy in treating neurodegenerative diseases. However, there have been no reports on the potential use of G-CSF to stimulate neuronal regeneration in a PD model.

In order to demonstrate this, it is first important to establish whether the post-symptomatic treatment with G-CSF is effective in PD. In this study, 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP) was used to generate the mouse model of PD. We then evaluated the ability of G-CSF to promote the regeneration of dopaminergic neurons in the SNpc and restore nigrostriatal function.

Experimental Procedures

Materials

MPTP-HCl, 5'-bromo-2'-deoxyuridine (BrdU) and mouse anti-BrdU were obtained from Sigma (St. Louis, MO, USA). Mouse anti-tyrosine hydroxylase (TH) was obtained from Chemicon (Temecula, CA, USA). Rabbit anti-TH and anti-proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG, Prolong®Gold antifade reagent with DAPI, and NeuroTrace green fluorescent Nissl stain were purchased from Invitrogen (Carlsbad, CA, USA). G-CSF was from Amgen (Thousand Oaks, CA, USA).

Animals

Male C57BL/6 mice (8–10 weeks of age; 22–26 g body weight) were purchased from Harlan (Indianapolis, Indiana, USA) and were maintained under temperature- and light-controlled conditions (20–23°C, 12-h-light/12-h-dark cycles) with continuous access to food and water. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida Atlantic University.

MPTP and G-CSF Administration

MPTP-HCl was dissolved in saline. G-CSF was diluted in 5% dextrose. The mice were divided into three groups (n=6 for each group): group I, saline control; group II, MPTP-exposed group; and group III, MPTP-exposed group followed by G-CSF treatment. Mice were intraperitoneally (i.p.) injected with MPTP at 30 mg/kg for five consecutive days. G-CSF treatment started 4 days after the last MPTP injection by subcutaneous injection at 250 μg/kg for seven consecutive days. For the saline control group, mice were injected with an equal volume of saline instead of MPTP followed by 5% dextrose instead of G-CSF. Animals were sacrificed 8 days after the last G-CSF injection.

BrdU Incorporation

Animals from different experimental groups (n=6) received injections of BrdU (Sigma, 50 mg/kg dissolved in PBS, i.p.) twice daily for 6 days beginning 24 h after the first G-CSF injection. BrdU-positive cells were detected using immunohistochemistry. To further check whether these new neurons also express TH, co-staining with anti-TH was also performed.

Measurement of Striatal Dopamine Release by In Vivo Microdialysis

Guide cannulae, 10 mm in length prepared from 22 gauge stainless steel tubing, were implanted in anesthetized mice.

Stereotaxic coordinates for the guide cannulae relative to bregma were AP +0.6 mm, ML ±1.8 mm, and DV −2.0 mm. An I-shaped, 2.5 mm microdialysis probe (cut-off 18 kDa) was inserted through the guide cannulae targeting the striatum (AP +0.6 mm, ML ±1.8 mm, and DV −4.5 mm) and then secured in place with dental cement. With unrestricted behavior in the testing chamber, the mouse was attached to a fluid swivel (Instech Inc., W. Lafayett, IN, USA). The probe inlet was infused overnight with artificial cerebrospinal fluid (containing 140 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.25 mM NaH_2PO_4, and 1.0 mM Na_2HPO_4, pH=7.4) at a flow rate of 0.5 μl/min before sample collection.

Samples were collected at intervals of 20 min and analyzed by HPLC. Dopamine was separated via a reversed-phase column packed with a TSK gel ODS-80^{TM} and measured using a voltammetric detector (HTEC-500; EICOM, Japan). The potential on the graphite electrode was set at +400 mV (relative to Ag/AgCl reference electrode). The mobile phase consisted of 0.1 M phosphate buffer (pH=6.0), 1% methanol, 500 mg/L sodium-1-octanesulfonate, and 50 mg/L EDTA. The amount of dopamine in the samples was calculated by measurement of the peak areas with comparison to the known amounts in the external standards using PowerChrom software (AD Instruments, Boston, MA, USA).

Immunofluorescence

Mice were transcardially perfused with 50 ml 0.9% saline followed by 50 ml 4% paraformaldehyde (PFA) in PBS 8 days after the last G-CSF injection. Brains were removed, post-fixed overnight in 4% PFA at 4°C, and incubated in 30% sucrose in PBS at 4°C until equilibrated. Brains were rapidly frozen in embedding matrix using dry ice/acetone bath. Coronal sections of 40 μm were cut using a microtome and processed as free-floating sections. Sections were first blocked in blocking buffer (10% normal goat serum, 1% BSA, 0.3% Triton X-100 in PBS) for 30 min at room temperature. Sections were then subsequently incubated with the primary antibodies, anti-TH (1:1,000) and anti-PCNA (1:500), for 48 h at 4°C. For fluorescent visualization, sections were incubated with the respective secondary antibody conjugated to either Alexa Fluor 488 (green) or Alexa Fluor 594 (red) (1:2,000). Between steps, sections were washed three times for 5 min in PBS. For BrdU staining, sections were first denatured in 2N HCl for 30 min at 37°C, washed extensively in 0.1 M borate buffer (pH=8.5) and rinsed well in PBS to bring pH back to 7.4. The sections were then exposed to mouse anti-BrdU (1:500). After immunostaining, the sections were mounted on silane-coated microscope slides (Fisher Scientific) with Prolong®Gold antifade reagent containing DAPI, a nuclear counterstain. Fluorescent signals were detected using an Olympus AX70 fluorescent microscope (Center Valley, PA, USA) or a BioRad Radiance 2100 confocal laser scanning system (Hercules, CA, USA).

Nissl Staining

Some mouse brain tissue sections were treated with Nissl stain. These sections were incubated first with anti-TH (1:1,000) antibodies followed by Alexa Fluor 594 (red) fluorescent secondary antibodies as described above. After being rinsed with PBS, tissue sections were incubated with NeuroTrace green fluorescent Nissl (1:100) in buffer (10% normal goat serum, 0.1 % Triton X-100 in PBS) for 30 min at room temperature. Sections were washed three times for 5 min with PBS and mounted on slides with Prolong®Gold antifade reagent.

Cell Counting

The number of immunopositive cell bodies were counted by an observer blinded to treatment history. Every fourth section throughout, the entire SNpc was counted using NIH Image J software. After defining the boundary of the SNpc at low magnification (×4 objective), TH-positive neurons in the section from the entire SNpc were counted at a higher magnification (×40 objective). To avoid double counting of neurons, immunopositive cells were counted only when their nuclei were optically visible. All double-labeling was confirmed by rotating the image along each axis to ensure that the signals were localized within the same cell rather than separate cells in close vicinity. In experiments with Nissl staining, only TH- and Nissl-positive cells in SNpc were counted. The intensity of TH-immunoreactivity in the striatum was calculated by measuring the relative optical density with NIH Image J using the adjacent cortex as the background.

Locomotor Activity

Locomotor activity was measured before animals were sacrificed for immunohistochemistry. Four individual rectangular open-field test chambers (25×25×24 cm) were placed in an isolated room for measuring locomotor activity. A camera, fixed to the ceiling above the open field, was connected to an automated video tracking system outside of the room (VIDEOTRACK NT4.0, View Point, Otterburn Park, Canada), allowing the experimenter to monitor and record the animal behavior. The total distance of movement covered by the animals from the four individual chambers was recorded at 5-min intervals for a total of 60 min.

Statistical Analysis

All data were expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Turkey's post

hoc test for dopaminergic neuron counts and optical density analysis. Two-way repeated measures ANOVA was used for in vivo microdialysis data and behavioral test. Scheffé's post hoc test was also used for multiple comparisons after two-way ANOVA analysis. A value of $P<0.05$ was considered to be statistically significant.

Results

Loss of Dopaminergic Neurons in the SNpc was Stable after MPTP Administration

In order to investigate whether G-CSF has a neuroregenerative effect, it is important verify that the degeneration of dopaminergic neurons is complete before G-CSF intervention to properly differentiate from the neuroprotective effect as reported [7, 8]. Therefore, we first generated the MPTP mice and examined the time-dependent degeneration of dopaminergic neurons. Dopaminergic neurons in SNpc were detected using an antibody against tyrosine hydroxylase (TH), a biochemical marker for dopaminergic neurons. Dopaminergic neurons were counted only when their nuclei were optically visible based on DAPI staining. As shown in Fig. 1a, a ~50% loss of dopaminergic neurons in SNpc was observed 4 days after the last MPTP injection compared to the saline control group (**$P<0.01$). The number of the lost dopaminergic neurons did not change significantly from 4 days to 22 days post-MPTP injection, although a slight increase was noted at 8 days and 15 days after MPTP injection (#$P<0.05$, compared to 4 days after the last MPTP injection, Fig. 1a). Therefore, we concluded that the dopaminergic neurons did not further degenerate in the time window that we examined (Fig. 1a). Optical density measurements of the TH-immunoreactive nerve fibers in the dorsal striatum also revealed a similar pattern (Fig. 1b, *$P<0.05$). The time-course and percentage loss of SNpc dopamine neurons were consistent with those reported by others using the MPTP mouse model [18]. Based on these observations, we decided to start G-CSF treatment 4 days after the last MPTP injection. Different dose of G-CSF ranging from 40–200 μg/kg has been reported in different studies depending on their purposes and delivery methods. Intravenous infusion of G-CSF at 50 μg/kg was used in a rat stroke model to study the neurogenesis [3]. Subcutaneous injection of 200 μg/kg G-CSF was used to study its

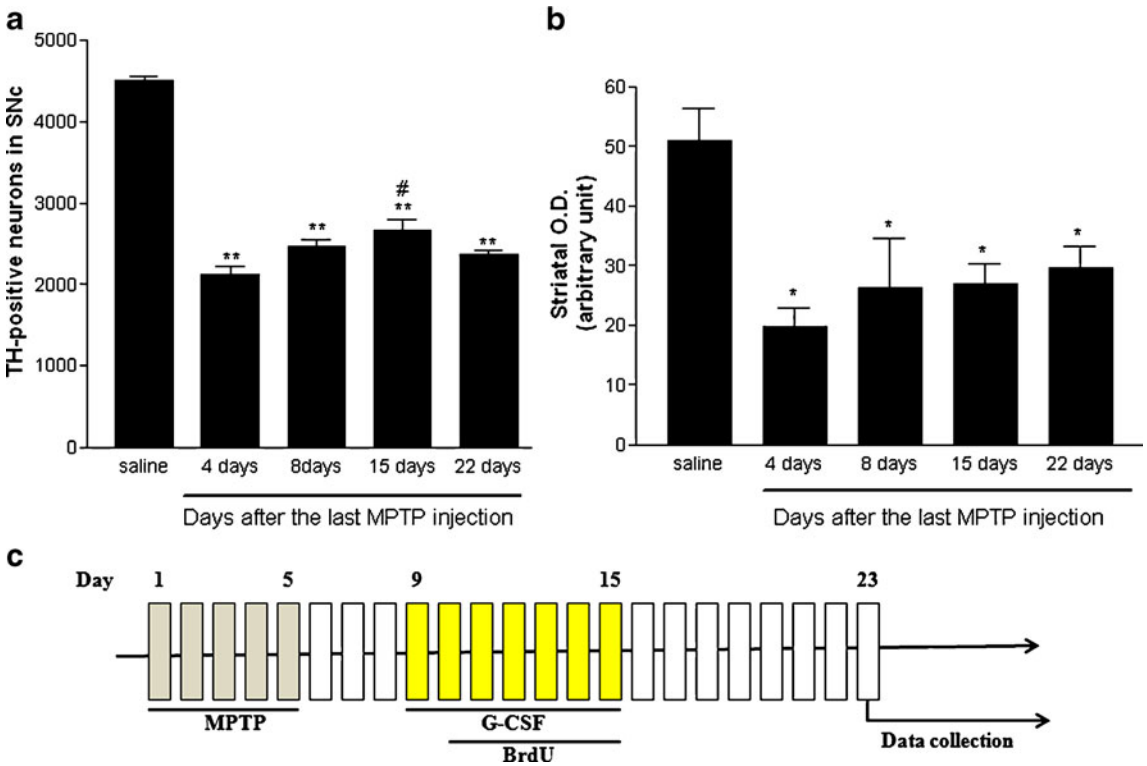

Fig. 1 Degeneration of dopaminergic neurons after MPTP administration. **a** Quantitative analysis of the number of the TH-positive neurons in SNpc after MPTP administration. Each error bar represents mean ± SEM. **$P<0.01$ vs. saline control group; #$P<0.05$ vs. 3 days after last MPTP injection group; one-way ANOVA followed by Turkey's post hoc test ($n=3$ mice per group). **b** Optical density analysis of the TH-immunoreactive nerve fibers in the striatum after MPTP administration. Each error bar represents mean ± SEM. *$P<0.05$ vs. saline control group; one-way ANOVA followed by Turkey's post hoc test; $n=3$ mice per group. **c** Experimental schedule of MPTP, G-CSF, and BrdU injections

neuroprotective effect in the MPTP mouse model of PD [7]. In this study, 250 μg/kg of G-CSF was subcutaneously injected into mice for seven consecutive days. Eight days after the last G-CSF injection, animals were analyzed using different approaches as described below. The experimental protocol is summarized in Fig. 1c.

G-CSF Treatment Promoted the Recovery of Dopaminergic Neurons after MPTP-Induced Neuronal Loss

MPTP caused a substantial loss of dopaminergic neurons in SNpc as well as degeneration of dopaminergic nerve fibers in the striatum. To investigate whether G-CSF has a therapeutic effect in the MPTP mouse model of PD, we first investigated whether G-CSF treatment could increase the number of dopaminergic neurons as stained by immunofluorescence. Representative images of dopaminergic neurons in SNpc of control (Fig. 2a), MPTP-treated (Fig. 2b), and G-CSF-treated mice (Fig. 2c) are shown. Every fourth section throughout the entire SNpc was then counted. As shown in Fig. 2d, the number of TH-immunoreactive cells in the SNpc of the control animal is 4,241±322. After MPTP treatment, the numbers reduced to 2,310±272. G-CSF treatment increased the number to 3,839±121. A statistical analysis revealed that the systemic MPTP treatment resulted in a reduction of TH-immunoreactive cells in the SNpc to an average 54% of the normal mice (Fig. 2d, $^{**}P<0.01$). G-CSF increased the number to 90% of the normal mice (Fig. 2d, $\#\#P<0.01$). In addition, we also counted the neuronal cells in SNpc by Nissl staining (Table 1). Consistent with our finding, G-CSF

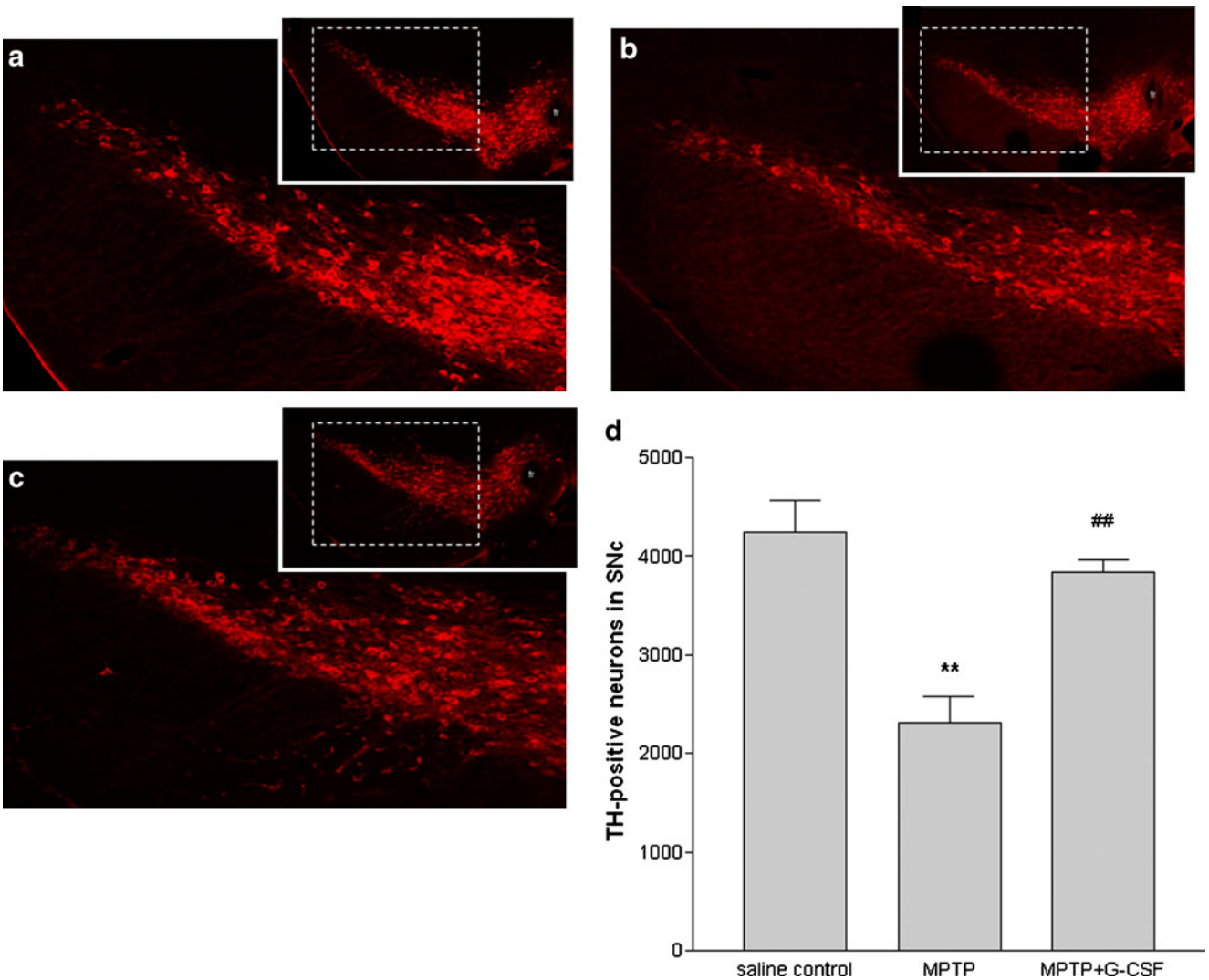

Fig. 2 Effect of G-CSF on the number of dopaminergic neurons in SNpc. Representative TH immunostaining in SNpc of the brain sections from mice injected with **a** saline, **b** MPTP alone, and **c** MPTP followed by G-CSF. *fr* indicates the position of fasciculus retroflexus. **d** Quantitative analysis of the number of the TH-positive neurons in SNpc. $^{**}P<0.01$ vs. saline control group; $\#\#P<0.01$ vs. MPTP group; one-way ANOVA followed by Turkey's post hoc test (n=4 mice per group)

Table 1 Number of $TH^+/Nissl^+$ neurons in SNpc in three different experimental groups

Group	$Nissl^+$ cells (percentage)	$TH^+/Nissl^+$ cells (percentage)
Saline control (n=4)	10,015±1,120 (100.0±11.2)	3,996±422 (100.0±10.6)
MPTP (n=4)	7,120±647 (71.1±3.5)*	7,120±647 (71.1±3.5)**
MPTP+G-CSF (n=4)	9,330±538 (93.2±5.4)$^{\#}$	3,197±210 (80.0±5.3)$^{\#}$

Values represent means ± SEM

*P<0.05; **P<0.01, compared to saline group; $^{\#}P$<0.05, compared to MPTP group

treatment increased the number of neuronal cells from 7,120±647 (MPTP group) to 9,330±538 (MPTP+G-CSF group, #P<0.05). Therefore, it is clear that post-MPTP treatment with G-CSF significantly increased the number of dopaminergic neurons after MPTP-induced neuronal loss.

We also examined TH-immunoreactive nerve fibers in the striatum (Fig. 3a–c). MPTP administration led to a ~62% loss of TH-immunoreactive fibers compared the saline control group (Fig. 3d, **P<0.01). G-CSF treatment increased the TH staining by ~20% compared to the MPTP mice (Fig. 2b). However, this effect failed to reach statistical significance (Fig. 2d, P=0.24). Mice treated with G-CSF alone did not exhibit a significant change in the number of TH-positive neurons in SNpc or TH immunostaining in the striatum (data not shown).

G-CSF Treatment Improved Striatal Dopamine Release

We next measured dopamine release in the striatum using in vivo microdialysis. As shown in Fig. 4a, the basal dopamine release in the saline control group was ~1.6±0.2 pg/sample in the dorsal striatum. Administration of MPTP produced a ~60% reduction in the basal dopamine release (0.67± 0.08 pg/sample, P=0.0031). A trend for improving the basal dopamine release impaired by MPTP administration was observed in the G-CSF-treated group, although the effect failed to reach statistical significance (1.22+0.56 pg/sample, Fig. 4a, P=0.3406). To further examine a functional change in dopamine release, dopamine release in response to high K^+ (100 mM) stimulation was measured. Two-way repeated measures ANOVA analysis of these data indicated a

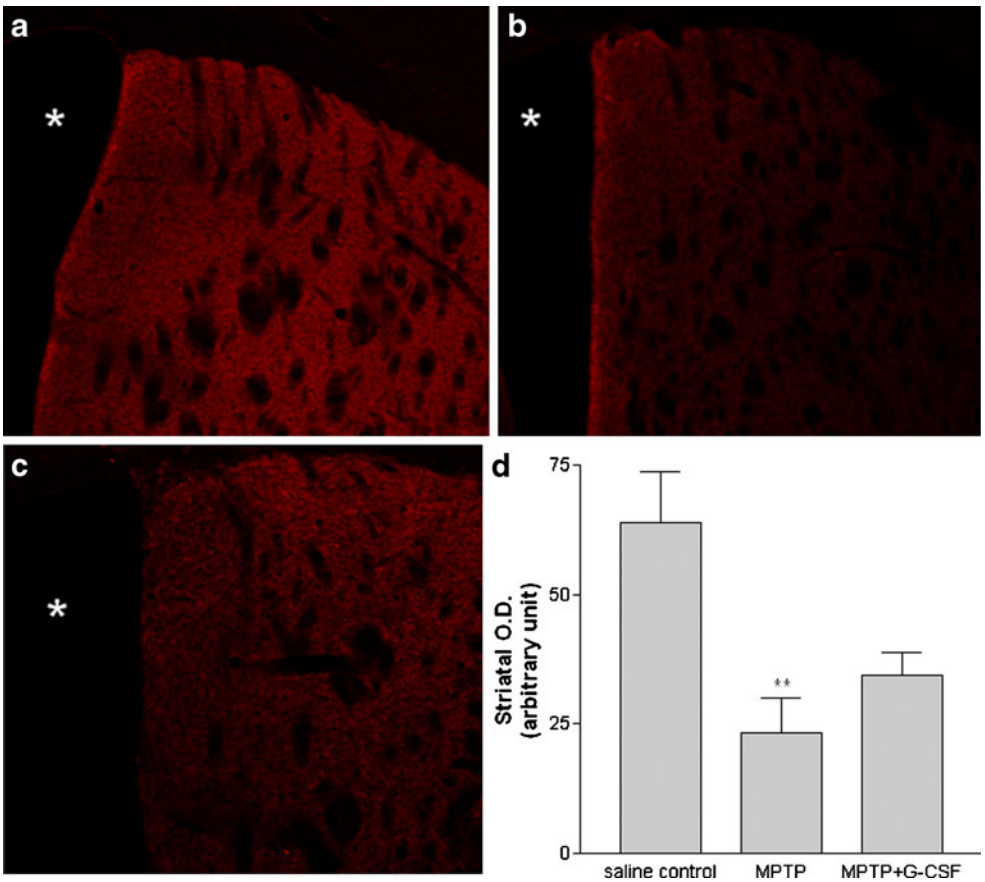

Fig. 3 Effect of G-CSF on dopaminergic nerve fibers in the striatum. Representative TH immunostaining in the striatum of the brain sections from mice injected with **a** saline, **b** MPTP alone, and **c** MPTP followed by G-CSF. *Asterisk* indicates the position of lateral ventricle. **d** Optical density analysis of the TH-immunoreactive nerve fibers in the striatum. Each error bar represents mean ± SEM. **P<0.01 vs. saline control group; ##P<0.01 vs. MPTP group; one-way ANOVA followed by Turkey's post hoc test (n=4 mice per group)

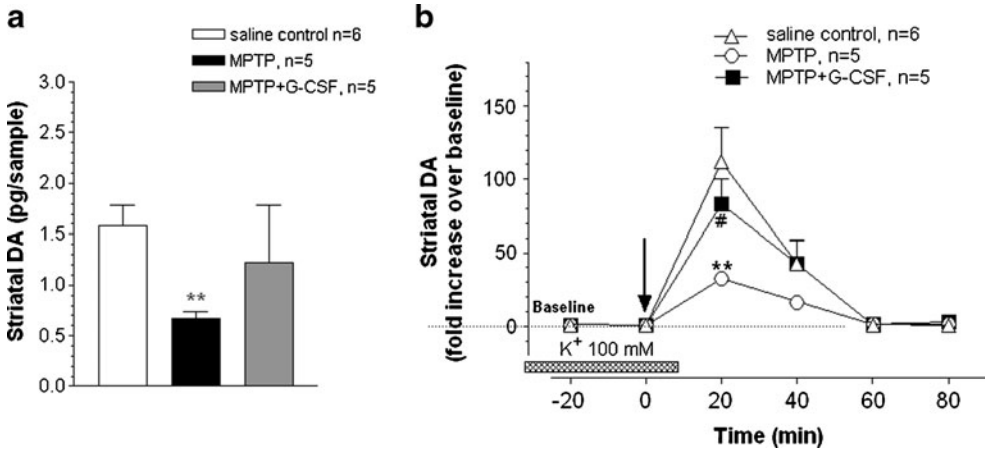

Fig. 4 Effect of G-CSF on striatal dopamine release in MPTP-treated mice. **a** Comparison of changes in basal dopamine release in mice treated with saline, MPTP alone, or MPTP followed by G-CSF treatment. Striatal dopamine levels were expressed as absolute values. $^{**}P<0.01$ MPTP vs. saline control analyzed by two-tailed unpaired Student *t* test. **b** Changes in dopamine release in response to local perfusion of K^+ (100 mM) into the striatum. Data were expressed as fold increases over their respective baselines. The baseline was calculated from the mean of two sequential effluxes before K^+ infusion. Data are expressed as mean ± SEM. *Horizontal bar* indicates the time window for K^+ perfusion. $^{**}P<0.01$ vs. saline control group and $\#P<0.05$ vs. MPTP group. Data were analyzed by the two-way repeated measures ANOVA followed by the post hoc Scheffé's test

significant treatment effect ($F(2, 13)=6.557$, $P=0.0107$) and time sampling ($F(1, 13)=9.132$, $P=0.0098$), without a significant interaction between treatment and sampling time ($F(2, 13)=1.273$, $P=0.3126$) (Fig. 4b). Specifically, a 110-fold increase in dopamine release over the baseline was observed at 20 min after K^+ perfusion in the saline-treated animals. This increase rapidly returned to the baseline after K^+ perfusion was terminated (Fig. 4b, empty triangle). In the MPTP-exposed animals, however, dopamine release in response to high K^+ was significantly smaller than that of the control animals ($F(1, 9)=15.763$, $P=0.0033$), with only a 32-fold increase over the baseline at 20 min after high K^+ perfusion (Fig. 4b, empty circle). Remarkably, G-CSF treatment significantly increased dopamine release compared to MPTP-exposed mice with an 83-fold increase observed over the baseline at 20 min after high K^+ perfusion ($F(1, 8)=8.305$, $P=0.0205$) (Fig. 4b, filled square).

G-CSF Treatment Decreased Locomotor Hyperactivity Observed in MPTP-Exposed Mice

To assess the impact of these anatomical changes on the behavior of the animals, we assayed the effect of G-CSF on locomotor dysfunction induced by MPTP administration. MPTP-injected mice showed significantly increased motor activity compared to the saline control group (Fig. 5, $n=9$). After G-CSF treatment, the distance traveled by MPTP mice was significantly lowered when compared to MPTP-exposed mice alone ($F(1, 96)=9.06$, $P=0.0033$) (Fig. 5, $n=9$).

BrdU Incorporation Did Not Reveal the Co-localization of TH and BrdU in SN

Since G-CSF treatment was started after the MPTP-induced degeneration of dopaminergic neurons in the SNpc was

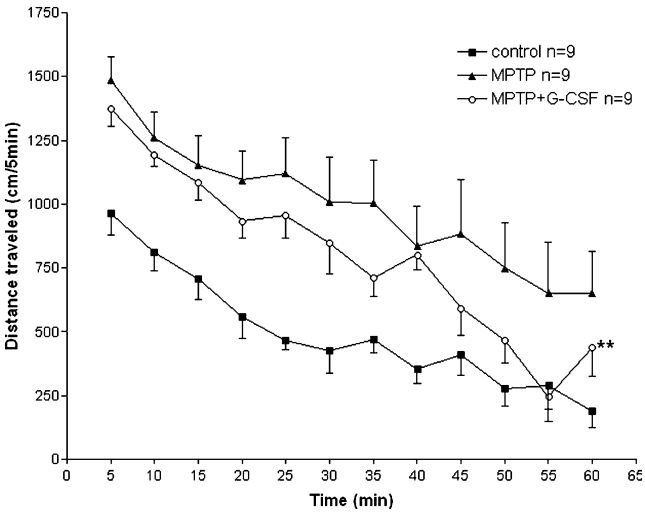

Fig. 5 Effect of G-CSF on locomotor hyperactivity observed in MPTP-treated animals. The distance traveled was collected in 5-min intervals for a total of 60 min. Compared to the saline control group, MPTP-exposed mice showed significantly increased motor activity compared to the control group ($F(1, 96)=67.98$, $P<0.0001$). G-CSF-treated mice displayed decreased locomotor activity compared to MPTP-exposed mice ($F(1, 96)=9.06$, $P=0.0033$, $^{**}P<0.01$). Data were analyzed by the two-way repeated measures ANOVA, $n=9$ mice per group

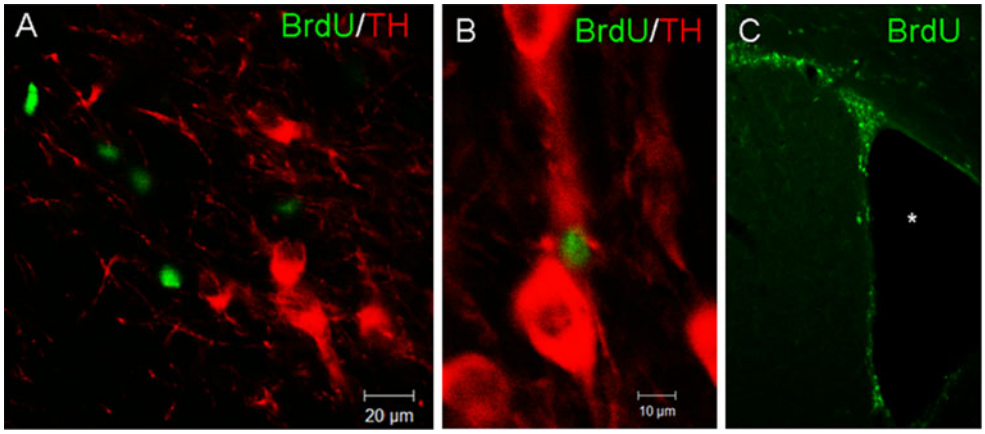

Fig. 6 Demonstration of neurogenesis in the mouse SN by BrdU incorporation. BrdU was administered twice daily for 6 days beginning 24 h after last G-CSF injection (50 mg/kg, i.p.) for five consecutive days. Representative images from the G-CSF-treated group are shown. **a** Laser confocal image of the distribution of BrdU (*green*) and TH (*red*) immunoreactive cells in the SN. **b** Confocal image of a BrdU-labeled cell (*green*) adjacent to a TH-positive cell (*red*). **c** BrdU labeling in the mouse SVZ. *Asterisk* indicates the lateral ventricle

complete, we reasoned that the increase of dopaminergic neurons after G-CSF treatment could be due to the enhanced neurogenesis in the SN. To demonstrate this, we studied the neurogenesis by BrdU incorporation. BrdU was administered twice daily for 6 days beginning 24 h after last G-CSF injection (50 mg/kg, i.p., Fig. 1c). To our surprise, we did not observe any significant difference in the number of BrdU-labeled cells in the SNpc among the three groups with a slight increase in the MPTP and G-CSF-treated groups (data not shown). More importantly, we did not find any BrdU/TH co-expressing cells in the SNpc from the G-CSF treated group (Fig. 6a). The closest example to co-localization in the SNpc is illustrated in Fig. 6b. To verify the BrdU labeling methodology, we also examined the mouse SVZ which is known to contain many BrdU-positive cells (Fig. 6c). We have also tried stem cell markers at different stages, such as progenitor cell nuclear antigen (PCNA) and doublecortin staining. Similar results were obtained (data not shown). Taken together, our data suggest that G-CSF may not have a direct role in activating dopaminergic neurogenesis in the SN.

Discussion

The goal of this study was to evaluate the therapeutic potential of G-CSF in treating PD using the MPTP mouse model of PD. Following the MPTP-induced neurotoxicity, we found that G-CSF treatment increased the number of dopaminergic neurons in the SNpc. In addition, G-CSF treatment increased dopamine release under high K^+ stimulation. More importantly, G-CSF treatment reversed the locomotor hyperactivity induced by MPTP.

We have yet to establish the source of the increased TH-immunoreactive neurons observed in G-CSF-treated animals. It is possible that G-CSF could prevent the further degeneration of dopaminergic neurons by MPTP as reported [7, 8, 19]. However, it is unlikely in our studies because the MPTP-induced degeneration of dopaminergic neurons was complete and stable before G-CSF was administered (Fig. 1). Another possibility is that G-CSF could stimulate the generation of new dopaminergic neurons in the MPTP mouse model. However, recent reports on dopaminergic neurogenesis in adult mammalian SN remain very controversial. Dopaminergic neurogenesis in SN has been demonstrated in adult mice and was found to be enhanced in MPTP-lesioned mice [20, 21]. On the other hand, Frielingsdorf et al. found no evidence for dopaminergic neurogenesis in SN using similar methodological procedures as reported by Zhao et al. [21]. Nonetheless, the consistent finding among all the reports is that actively dividing neuron progenitor cells do present in SN as evidenced by BrdU labeling [20–22]. Instead of differentiating into dopaminergic neurons, these cells either remain as uncommitted [23] and give rise to new mature glial cells [24].

Although we could also demonstrate low level of neurogenesis in SN by BrdU incorporation in our present study, we failed to detect any co-labeling of TH and BrdU in SN from the G-CSF treated group (Fig. 5). Therefore, G-CSF is unlikely to play a direct role in stimulating the proliferation of neuron progenitor cells in our system. It has been suggested that local neuron proliferation occurs at a low level in SN of healthy mice, and this process greatly increased after MPTP lesion [21, 23, 25]. Robust cell proliferation, indicated by a 5-fold increase in BrdU incorporation, was observed in SN 3 days after MPTP treatment. However, most of the BrdU-positive cells remained as uncommitted progenitors since they did not express neuronal or glial markers [23, 25]. This raises an interesting possibility that G-CSF could induce these

uncommitted progenitors to differentiate into dopaminergic neurons in our study instead of promoting NSC proliferation. It is therefore important to mention the difference between their regimen of BrdU administration and ours. In the report by Mao et al., animals were given a single dose of BrdU (100 mg/kg, i.p.) 1 h after an acute injection of saline or MPTP (25 mg/kg, s.c.) for five consecutive days. In our present study, BrdU was administered on the fifth day after the last MPTP injection for 6 days (Fig. 1c). It is possible that our regimen of BrdU administration missed the critical period to label the dramatically increased uncommitted progenitor cells induced by MPTP as reported [23, 25]. It also explains why we could not detect BrdU/TH co-labeling in our study. This possibility needs to be tested by following the reported regimen of BrdU administration [23]. Indeed, G-CSF receptors are present on adult neural stem cells and G-CSF drives neural progenitor differentiation in vitro [3].

We did not detect a significant increase in the basal dopamine release after G-CSF treatment as measured by in vivo microdialysis. However, a significant increase in high K^+-evoked dopamine release was observed in the striatum after G-CSF treatment, indicating a functional recovery of the dopaminergic terminals. This may be the result of the sprouting of dopamine-releasing axon terminals from newly generated dopaminergic neurons. Alternatively, G-CSF could enhance the recovery of damaged striatal nerve terminals. Extensive astrogenesis occurs in the striatum after MPTP exposure [23, 25]. These new cells participate in reactive astrogliosis, a process thought to repair damaged axon terminals [26]. G-CSF-induced neurogenesis in the striatum may also play an important role in restoring dopamine levels. Progenitor cells in the SVZ are recruited to the rodent striatum after administration of growth factors such as transforming growth factor α [27] and brain-derived neurotrophic factor [28]. These cells appear to be able to differentiate into functioning dopaminergic neurons [27]. Thus, newly formed dopamine neurons may have contributed to the increased dopamine release as we observed.

We also found that chronic MPTP administration resulted in a significant increase in locomotor activity, and G-CSF appeared to partially reverse this effect. Historically, the behavioral analyses of mice exposed to MPTP have generated conflicting results [29]. Although most studies reported a decrease in locomotor activity after MPTP exposure, we and others [29, 30] found that MPTP-exposed mice were hyperactive compared to the control mice. A direct comparison of the locomotor activity results from different studies is not possible since there are variable factors such as animal age, gender, weight, MPTP dose, and administration schedule [29]. Based on the current understanding of the role of dopamine in the striatum and the clinical presentation of PD, MPTP-induced depletion of midbrain dopamine is expected to decrease locomotor activity. However, a more severe dopamine deficiency in the prefrontal cortex relative to the dorsal striatum may account for the MPTP-induced hyperactivity reported here [31]. In our study, G-CSF treatment appeared to reverse the MPTP-induced hyperactivity, which indicates an increase in dopaminergic function. Further studies on the mechanisms of its action should provide a better understanding of the therapeutic potential of G-CSF in treating PD.

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