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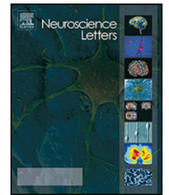

Granulocyte-colony stimulating factor (G-CSF) enhances recovery in mouse model of Parkinson's disease

G-CSF提升巴金森的恢復

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ABSTRACT

Introduction: Granulocyte-colony stimulating factor (G-CSF) is used routinely in clinical practice for the treatment of neutropenia and to increase generation of hematopoietic stem cells in bone marrow donors. A growing body of literature on the neurotrophic effects of G-CSF has led to clinical trials in stroke, Alzheimer's disease (AD) and Parkinson's disease (PD). *Objectives*: The primary objective of this study was to determine if G-CSF administration would rescue the nigro-striatal system and restore locomotor function after completion of a sub-acute course of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration (30 mg/kg i.p. for 5 days) in 12 month-old mice. A secondary aim was to determine if G-CSF affects the neuro-inflammatory response by modulating microglial activation in striatum and midbrain. *Results*: MPTP-treated mice were impaired on the rotometer test after the last dose of the toxicant and remained impaired until euthanasia. MPTP-treated mice that were given an 8-day regimen of G-CSF starting 2 days after the last dose of toxicant enhanced motor performance compared to the MPTP alone group. MPTP treatment depleted striatal DA (DA) levels; G-CSF given after MPTP resulted in a partial, significant repletion of DA levels. Total microglial burden in the striatum was increased significantly in MPTP-treated mice and was reduced after G-CSF rescue. *Conclusion*: G-CSF enhances recovery of DA nigro-striatal function from MPTP toxicity in part by modulating the microglial response to injury. The G-CSF receptor may provide a novel target for modifying the disease process in Parkinson's disease.

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The pathogenesis of Parkinson's disease (PD) involves a gradual demise of dopaminergic neurons of the midbrain, depletion of striatal dopamine (DA), accumulation of intracellular inclusions (Lewy bodies), and activation of microglia in the substantia nigra (SN) and striatum [2,13]. There is increasing evidence that neuroinflammatory responses drive neurodegenerative events [1,5,7,23]. In rodent models of PD, microglial activation precedes neurodegeneration in the SN, suggesting that the underlying pathogenesis involves a complex response in the nigrostriatal pathway, and that the immune system plays a significant role in the pathogenesis of PD [10].

Hematopoietic growth factors or cytokines such as granulocyte-colony stimulating factor (G-CSF) have been have been reported to modulate the immune system by down-regulating pro-inflammatory cytokines [8]. G-CSF is most commonly used to treat neutropenia, but its effects as an immune modulator and its direct actions on neurons and neural progenitors are gathering attention. G-CSF has been reported to directly stimulate proliferation and differentiation of neural stem/progenitor cells (NSC) and to turn on anti-apoptotic processes in neurons [11,19,20].

Administration of G-CSF has been increasingly studied in various animal models of neurologic disease including stroke, AD, PD and amyotrophic lateral sclerosis [3,14,17,19,21,22]. Two prior studies with G-CSF addressed its neuroprotective capacity against MPTP toxicity both *in vitro* and *in vivo* [3,14], but its capacity to repair an already injured nigro-striatal system has not been previously studied.

The primary objective of the present study was to determine whether G-CSF administration could replenish depleted striatal DA levels and reverse the locomotor deficit caused by a sub-acute course of MPTP in 12-month-old mice. A secondary aim was to

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determine if the restoration of striatal DA was related to the effects of G-CSF on microglial activity in the striatum and SN.

Male (C57BL/6J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were allowed to age to 12 months before administration of MPTP. Animals were randomly assigned to 3 groups ($n = 8$ per group). Group A = control mice (no MPTP and no G-CSF); Group B = daily MPTP injections (30 mg/kg i.p. for 5 days followed 2 days later by 8 days of vehicle injection; Group C = daily MPTP injections (30 mg/kg i.p. for 5 days followed 2 days later with 8 daily injections of G-CSF (250 μg/kg, s.c.). Group D (G-CSF control) = vehicle injections × 5 d followed by G-CSF × 8 d. In separate experiments, lower doses of G-CSF were administered (50 and 125 μg/kg) after completion of the MPTP injections. To determine if G-CSF alone had an impact on locomotor activity a group of 6 mice was given G-CSF alone for 8 d. Mice were housed and maintained in a specific pathogen free facility under a 12-h light/dark cycle, with *ad libitum* access to rodent chow and water. All experiments conformed to guidelines for the ethical use of animals as provided by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC #000434). The number of animals utilized was based on statistical power analysis and all means were taken to reduce suffering. The protocol was approved by the IACUC of the University of South Florida.

Performance (latency to fall) on a rotating rod (Stoelting Co, Wood Dale, IL) was measured at baseline (Time 0), on the day after the last injection of MPTP (day 6) and again after last injection of G-CSF (day 12). The speed of rotation is gradually increased and the rodent's ability to remain on the rotating rod is recorded as duration (latency in seconds) before the mouse falls from the cylinder to the platform (9 cm below the rotating cylinder). Individual animals (outliers) that exhibited latency measurements at baseline that exceeded 2 SD from the mean were not included in final analyses, resulting in minimum of $n = 6$ per group. On day 15 after the last rotometer test was completed, animals were euthanized and brains were removed and bisected. One-half brain was dissected into striatum and ventral midbrain for analysis of DA and metabolites and the other half was immersion fixed and processed for immunohistochemistry.

MPTP (Sigma–Aldrich, St. Louis, MO) was handled using published operating procedures to protect health of animal care staff and researchers [15]. MPTP was administered at a dose of 30 mg/kg i.p. daily for 5 days. Recombinant human G-CSF (rhG-CSF, filgrastim; Neupogen®, Amgen, Inc., Thousand Oaks, CA) was administered daily for 8 days (50, 125 or 250 μg/kg, s.c.) starting 2 days after the last injection of MPTP. The highest dose of G-CSF was chosen based on results obtained in a recently published study from our laboratory in which a course of G-CSF administration improved cognitive performance and decreased amyloid burden in a mouse model of AD [17].

Measurement of striatal DA and metabolites was performed using a previously published method from our laboratory [4,18]. Briefly, striatal tissues samples were homogenized in 1 ml of perchloric acid (PCA, 0.05 M) containing 3,4-dihydrobenzylamine (DBA, 31 ng/ml) as an internal standard. Homogenates were centrifuged and supernatants were filtered through a 0.45 ml Acro LC 13 filter (Gelman Industries, Ann Arbor, MI). Amines were quantified by reverse-phase high performance liquid chromatography (HPLC). The ratio of the peak area produced by DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) to the peak height produced by DBA (internal standard) in the striatal samples was used to obtain the striatal levels from a calibration curve. This calibration curve was constructed by plotting the ratios of known amounts of DA, DOPAC, and HVA to DBA against the ratio of the peak heights produced by these known amounts to the peak area produced by DBA. Data was expressed as μg per g of wet weight.

After euthanasia and transcardial perfusion of the brain with 0.1 M phosphate-buffered saline (PBS), the brain was removed and bisected. The left hemisphere and entire midbrain was placed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight. Four half brains from each group of mice were routinely embedded in paraffin with 24-h processing and used for ionized calcium-binding adapter molecule 1 (Iba1, a marker of both resting and activated microglia) immunostaining to quantify microglia. Two half brains per group were transferred to graded sucrose solutions (10%, 20%, and finally 30%) for cryosectioning and immunostaining of tyrosine hydroxylase (TH) expressing DA neurons and fibers in midbrain and striatum. For paraffin sectioning, 5 coronal sections of midbrain with a 150-μm interval were cut at a thickness of 5-μm. Four sets of five sections were prepared for analysis of Iba1. Immunohistochemical staining was performed following the manufacturer's protocol using a Vectastain ABC *Elite* kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine reaction. The following antibodies were used for immunohistochemical staining: an Iba-1 polyclonal antibody (1:1000, Wako,

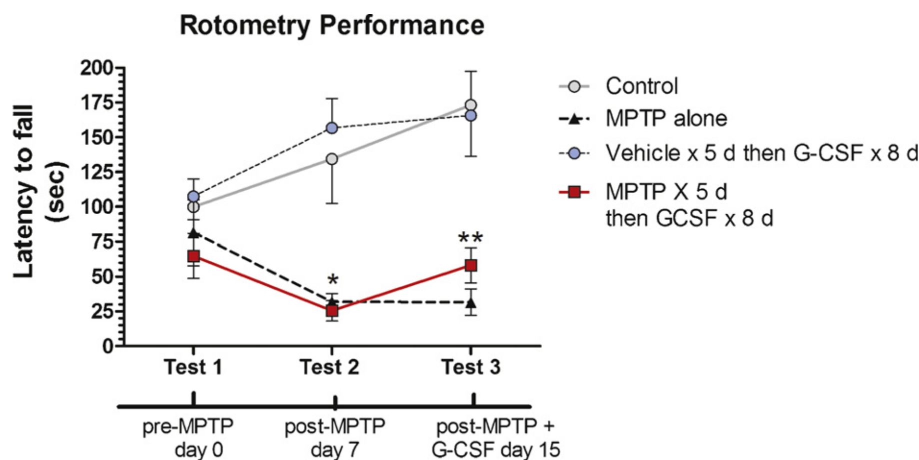


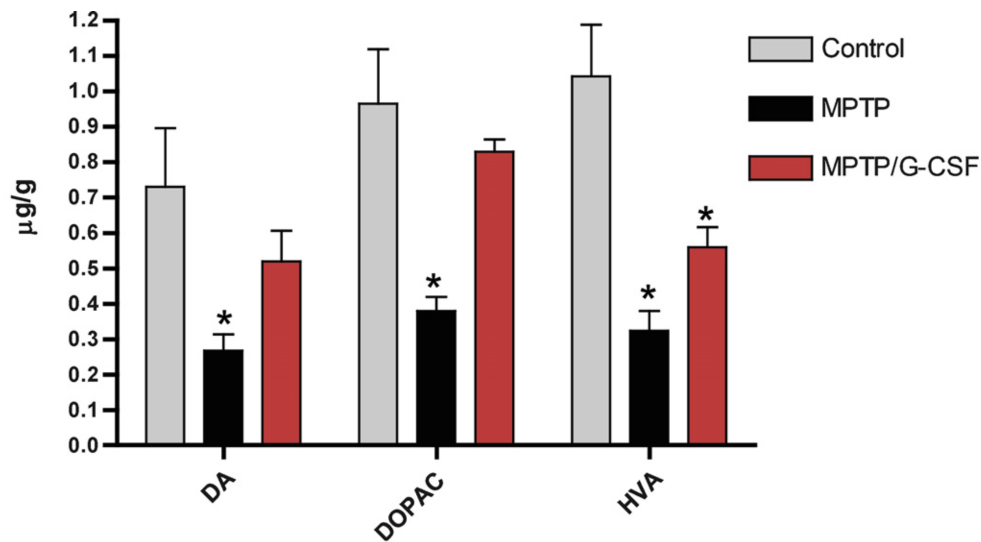
Fig. 1. Performance on rotating rod (latency to fall) at three time points for 3 groups of mice. Two-way ANOVA revealed that the experimental treatment accounted for 24.8% of total variance ($p < 0.001$) and time accounted for approximately 2.65% of total variance ($p = 0.26$). One-way ANOVA of Test 2 followed by Newman–Keuls multiple comparison test revealed that both the MPTP group and the MPTP/G-CSF group differed significantly from the control groups ($^*p < 0.01$). One-way ANOVA of Test 3 followed by Newman–Keuls multiple comparison test revealed that the two control groups were not significantly different from each other and the MPTP/G-CSF group differed significantly from the MPTP alone group ($^{**}p = 0.05$).

Osaka, Japan), and rabbit TH polyclonal antibody (1:500, Millepore, Billerica, MA).

Images were acquired as digitized tagged-image format files to retain maximum resolution using an Olympus BX60 microscope with an attached digital camera system (DP-70, Olympus, Tokyo Japan). Images of 5 sections (each 5-μm thick and 150-μm apart) were captured from serially sectioned striatum and midbrain and a threshold optical density was obtained that discriminated staining from background. Each anatomic region of interest was manually edited to eliminate artifacts. For Iba1 (microgliosis) burden analysis, data are reported as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels). Bias was eliminated by analyzing each entire region of interest represented by the sampling of 5 sections per region. Each analysis was done by a single examiner blinded to sample identities.

Behavioral performance was statistically evaluated to determine group differences based on treatment with G-CSF compared to placebo. The rotometery data were analyzed using both two-way repeated measure analysis of variance (ANOVA) and one-way ANOVA. After one-way ANOVA analysis, *post hoc* differences between groups (planned comparisons) were corrected with Newman–Keuls multiple comparison test. For analysis of striatal DA levels and immunostaining of Iba1, one-way ANOVA followed by Bonferroni's multiple comparison test was performed. All group data are presented as mean ± SEM. Group and all comparisons were considered significant at $P < 0.05$.

C57/BL6J mice of 12 months of age were randomly assigned to 4 groups of mice; those that received vehicle throughout the study (control); a group that received MPTP alone and no G-CSF; a group that received MPTP followed by 8 injections of G-CSF (MPTP/G-CSF) and a G-CSF vehicle group that only received 8d of G-CSF. Locomotor function on the rotometer was measured at 3 time points (Fig. 1). Test 1 was given before MPTP dosing (day 0). Test 2 was done 2 days after the last dose of MPTP and Test 3 after last dose of G-CSF was given (day 15). The two control groups of mice that did not receive MPTP improved their performance over the 3 test periods. The MPTP alone group showed a significant decrease in latency to fall 2 days after the last dose of MPTP (Test 2) and on day



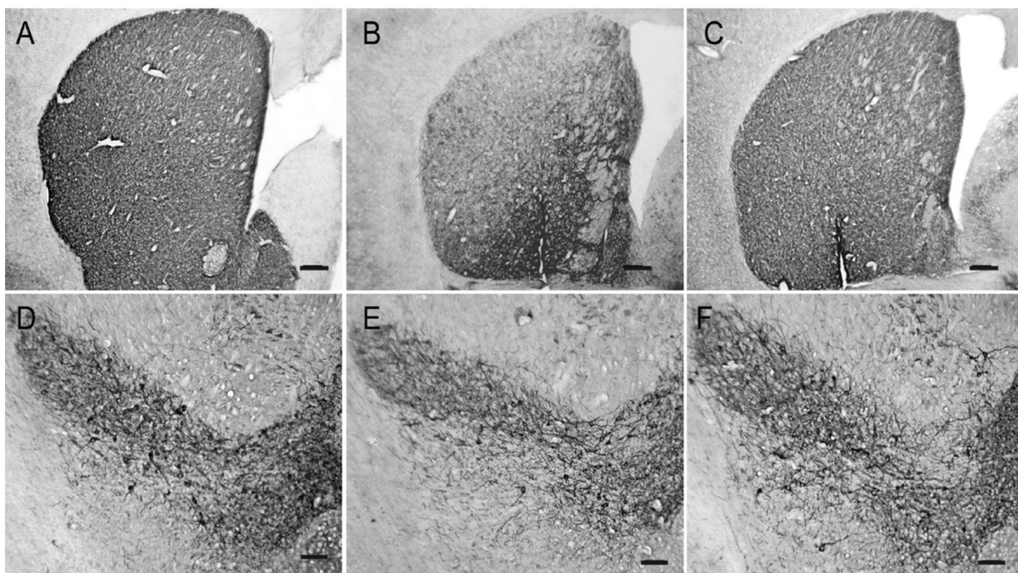


Fig. 2. (Upper panel) Mean striatal DA and DA metabolite levels in each group of mice. One-way ANOVA followed by Bonferroni's multiple comparison test showed DA levels were significantly decreased by MPTP when compared to controls (* $p < 0.05$). DA levels in the MPTP/G-CSF group were not significantly different than control levels. Same analysis for DOPAC showed statistically significant decreases in MPTP group compared to controls, but not in the MPTP/GCSF group. HVA levels in the MPTP-group and the MPTP/GCSF groups were significantly lower than controls. Lower panel shows examples of TH immunostaining in striatum (A–C) and SN (D–F) hemi-sections from each group of experimental mice. A, D = control; B, E = MPTP; C, F = MPTP/G-CSF. Bars = 100 μm for SN and 200 μm for striatal sections.

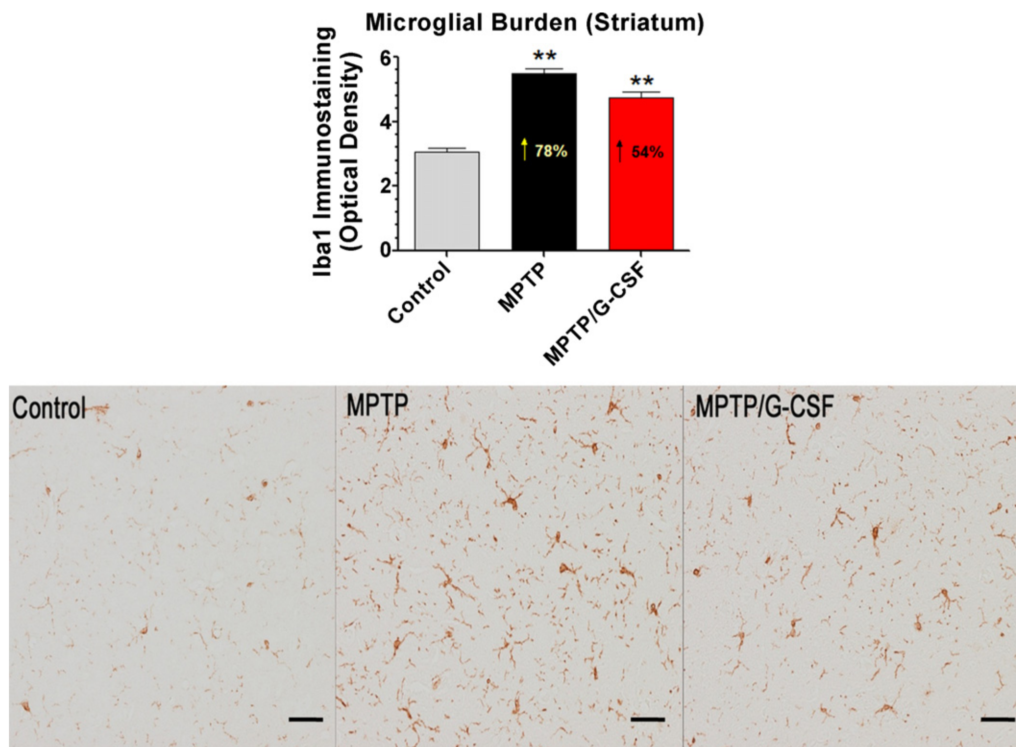


Fig. 3. Microglial burden in striatum indicated by Iba1 immunostaining. Upper panel shows the mean area of striatal Iba1 immunostaining in each group of mice. MPTP treatment alone increased total microglial burden and treatment with G-CSF after the course of MPTP decreased total microglial burden. One-way ANOVA followed by Newman–Keuls multiple comparison test showed all groups were significantly different from each other (**$p < 0.05$). Lower panel shows representative microglial immunostaining (Iba1) from each of the 3 groups of animals. Bar = 50 μm.

15 (Test 3). The MPTP/G-CSF (250 μg/kg) group performed significantly better than mice given MPTP alone (Test 3). Lower doses of G-CSF (50 and 125 μg/kg) given 2 days after MPTP did not restore or worsen locomotor activity (data not shown).

After Test 3 on the rotometer was completed (day 15), mice were euthanized and half-brain was removed and dissected for determination of striatal DA and metabolites. The other half-brains and midbrains were fixed and processed for immunohistochemistry. Striatal DA, HVA and DOPAC levels were reduced significantly in the MPTP-treated group compared to controls (Fig. 2). The group of mice in which G-CSF (250 μg/kg) treatment followed MPTP revealed a statistically significant attenuation of DA and DA metabolite depletion. Lower doses of G-CSF did not replete levels of striatal DA and metabolites (data not shown). Qualitative assessment of TH immunostaining showed corresponding decreases in TH intensity in striatum, and to a lesser extent the SN, in the MPTP group. TH immunostaining appeared to be partially restored in the MPTP/G-CSF group.

Following MPTP injections, the total microglial burden in striatum was significantly increased by 78% compared to control (Fig. 3). When G-CSF was administered after MPTP treatment, the total microglial burden was significantly reduced by 24% relative to the MPTP treatment alone group. In the SN, the increase in microglial burden following MPTP was only modestly increased (17.4%) compared to controls (data not shown). Unlike results observed in the striatum, the MPTP/G-CSF group did not decrease the microglial burden in the SN.

Two previous studies have demonstrated neuroprotective effects of G-CSF when administered before and during the injection of the neurotoxicant MPTP [3,14]. The present study extends those observations to demonstrate the capacity of G-CSF to partially rescue injured striatal DA terminals shortly after the lesion had been induced. Repletion of striatal DA levels was associated with a small but statistically significant recovery of performance of the G-CSF-treated mice on the rotometer compared to mice that received MPTP alone. Lower doses of G-CSF were not effective in restoring DA levels or improving locomotor function.

The main difference in design of the present study from the previous study [14] is timing and dosage of G-CSF administration. In the present study, G-CSF was administered 250 μg/kg, s.c. daily for 8 days starting 2 days after the last dose of MPTP was given (and 7 days from the initiation of MPTP injections). By contrast, in the earlier studies, G-CSF was administered 40 μg/kg, s.c. one day before and during MPTP administration but not during the intervening 7 days before analysis of striatal DA [14]. If DA depletion and TH immunoreactivity are prevented by G-CSF pretreatment and concurrent administration with MPTP, a neuroprotective effect can be inferred. When G-CSF is given after a course of MPTP, a rescue and repair phenomenon can be inferred. In the present study, high doses of G-CSF were given during the period of time when terminals were degenerating and depletion of DA had not reached its nadir. Earlier studies have demonstrated that maximal depletion of striatal DA occurs 7 days after MPTP treatment (in 12-month-old C57BL mice) [6]. Therefore, the partial repletion of striatal DA levels and restoration of locomotor function conferred by post-treatment with G-CSF can be considered, at least in part, a neuroprotective action. Repletion of striatal DA might also be attributed to striatal DA terminal sprouting, a neurotrophic action. This is unlikely because the first markers of terminal sprouting are observed from day 14 onwards [9] and the analysis of DA levels in the present study occurred on the 15th day after initiation of MPTP treatment. In any case, the present study did not quantify striatal terminal density, so the extent to which re-innervation explains recovery of DA levels cannot be answered here.

Neuroprotective effects of G-CSF have been attributed to activation of several anti-apoptotic pathways. G-CSF prevented neuronal death triggered by methyl-phenylpyridinium (MPP+) in cell cultures of primary midbrain neurons and PC12 cells by increasing

expression of the anti-apoptotic protein Bcl-2 while decreasing the pro-apoptotic Bax [3,14]. A similar mechanism of neuroprotection was reported in a rat model of stroke after treatment with G-CSF [11]. Activation of the PI3K/Akt pathway by G-CSF in cultured rat cortical neurons was reported to be another anti-apoptotic pathway responsible for neuroprotection [20].

Among its many actions, G-CSF modulates the immune system [8] leading to the hypothesis that its neuroprotective effects in the MPTP model relate to modulation of neuro-inflammation. Markers of neuroinflammation, including activated microglia and increased levels of circulating inflammatory cytokines have been reported in brains and cerebrospinal fluid of PD patients [1,16,24]. In the animal model, agents that reduced inflammation provided some protection against MPTP[5]. Mice that lack the iNOS (inducible nitric oxide synthase) gene exhibited a dampened microglial response to MPTP and were resistant to its neurotoxic effects [12]. Mutant mice that lack the caspace-11 gene (caspace11$^{-/-}$) exhibited less neurotoxicity, microglial activation and inflammation than wild-type mice following acute MPTP treatment (5 injections of 20 mg/kg in 24 h) but not when MPTP was given “chronically” over 5 days [7]. Under the “chronic” regimen of MPTP, caspace11$^{-/-}$ mice lost nigral DA neurons to the same extent as wild-type mice. The primary cascade of inflammatory and apoptotic pathways appears to depend on schedule of MPTP administration [7].

To assess the role of G-CSF in modulating microglial activation in the MPTP-treated mouse brain, total microglial burden was measured in the striatum and SN. MPTP-treated mice exhibited significant microgliosis indicated by Iba1 immunostaining on day 15 and this was reduced by 24% in the G-CSF treated mice. The elevation of microglial burden in the SN was much less than striatal microgliosis on day 15 and G-CSF treatment did not reduce the microgliosis in the SN. The time course of microglial activation following MPTP has been previously studied in 10- to 12-month-old C57BL mice [6]. Four injections of MPTP (10 mg/kg at 1-h intervals) resulted in a time-dependent increase in microglial activation in the striatum and SN. In addition to changes in microglial morphology, the numbers of microglia (stained with Isolectin B4) increased to a maximum 24 h later and gradually decreased but remained elevated above control for 14 days. In the present study, striatal microglial burden remained elevated on day 15 (10 days after last dose of MPTP) but midbrain microglial burden was only slightly elevated above control. Both studies clearly showed a significant increase in microglial burden after MPTP even though the dosage regimen of MPTP and the time course of microglial analysis were different.

The present data extend previous pre-clinical studies in mouse models of PD that demonstrate neuroprotective effects of G-CSF. Administration of a course of high dose G-CSF after the nigrostriatal lesion was induced by MPTP resulted in partial recovery of locomotor function and striatal DA levels. Previous studies suggest that G-CSF triggers anti-apoptotic and inflammatory processes to mitigate ongoing neurodegeneration. Modulation of microglial activation by G-CSF is also likely to be involved in the neuroprotective and restorative effect.

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