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Reduction of Motor Disorder in 6-OHDA-Induced Severe Parkinsonism Rats by Post Treatment with Granulocyte-Colony Stimulating Factor

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Abstract

Granulocyte-colony stimulating factor (G-CSF) induced regeneration of dopaminergic neurons and improved behavior deficit in moderate Parkinson's disease (PD) model mice. Post treatment of G-CSF in severe PD model has not been addressed. A very severe PD model in rats was induced by a high dose 6-hydroxydopamine (6-OHDA) injected into the right medial forebrain bundle to evaluate therapeutic effects of G-CSF. G-CSF (50 μg/kg/day for five days) was given on the 9th day after the 6-OHDA injection. Rotational behavior was examined on the 9th and 28th days. Rats were killed on the 28th day and survival dopaminergic neurons in the substantia nigra, dopaminergic axons and dopaminergic receptor 2 in the striatum were examined. We, for the first time, demonstrated that post treatment with G-CSF reduced abnormal rotational behavior and increased the lesion to non-lesion ratio of dopaminergic fibers in the striatum, but the treatment promoted neither the increase in survival dopaminergic neurons nor the increase in dopaminergic receptor 2 expression. We conclude that post treatment with G-CSF can reduce the abnormal rotational behavior of severe PD rats primarily through relative increases in dopaminergic fibers of the lesion side in the striatum. Results of our study suggest therapeutic potentials of G-CSF for treating severe PD patients.

Key Words: axon, dopaminergic, granulocyte-colony stimulating factor (G-CSF), Parkinson's disease, post treatment, rat, striatum, 6-OHDA

Introduction

Patients with Parkinson's disease (PD) develop various motor dysfunctions which progress to difficulty in walking, talking or completing simple tasks, and finally in self-caring in daily life. The most overt symptoms of PD are attributed to the loss of dopaminergic neurons in the pars compacta of substantial nigra (SNpc) (23), especially in late-stage patients (14). Granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, has been studied for preventing continuous depletion of dopamine neurons in the SNpc in a methylphenyl-tetrahydropyridine (MPTP)-induced PD mice model (9, 11), which may result from altering Bcl-2/Bax expression levels (1). But G-CSF has rarely been addressed in PD rat models,

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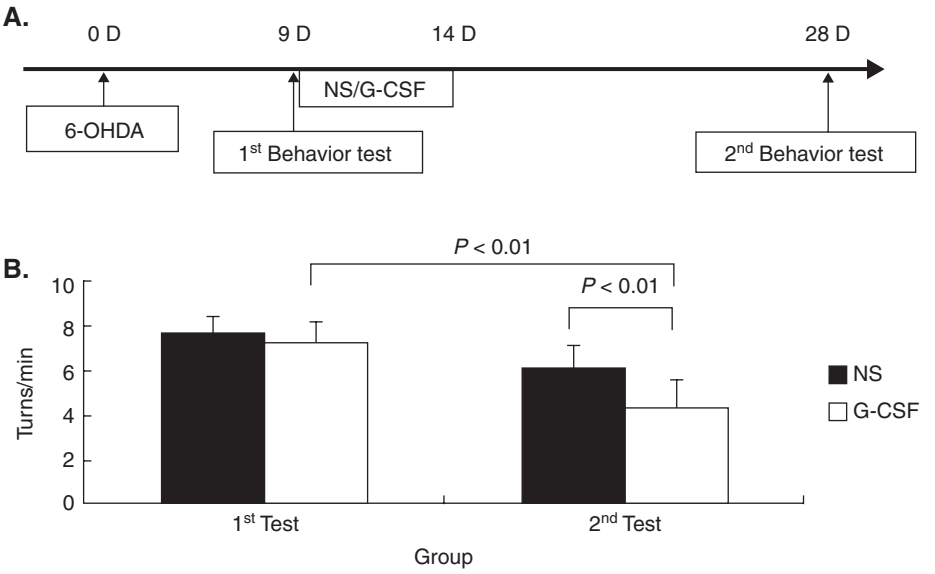

Fig. 1. The experimental paradigm (A) and rotational behavior (B). Rotation behavior induced by methamphetamine in the 1st and 2nd tests in the normal saline (NS) (n = 5) and G-CSF (n = 6) groups were performed on the 9th and 28th days, respectively, after unilateral (right) 6-OHDA lesion at the medial forebrain bundle (MFB) (B). G-CSF or normal saline was administered daily from the 9th to 13th day.

except in a paper demonstrating its neuroprotective effects on rat dopaminergic neurons injured by 6-hydroxydopamine (6-OHDA) *in vitro* (5). Studies showed that pre-treatment with G-CSF activated the extracellular signal-regulated kinase pathway which may lead to anti-apoptosis (5) and the subsequent production of brain-derived neurotrophic factor (12) in neurons suffering from 6-OHDA neurotoxicity. Whether post treatment with G-CSF may improve very severe PD in rats is still unclear.

Injection of high doses of 6-OHDA to the median forebrain bundle (MFB), the axon of the nigro-striatal projection, selectively destroys dopaminergic neurons in the SNpc and dopaminergic fibers in the striatum (4, 21). Massive loss of dopaminergic neurons results in a very severe PD rat model which may mimic advanced human PD. Abnormal motor behavior manifested as methamphetamine-enhancing rotational behavior can be assessed in unilateral 6-OHDA lesioned rats (7, 20). The therapeutic effects of G-CSF were tested on these animals. The amount of dopaminergic fibers (21) and the number of dopaminergic neurons in the SNpc were identified by tyrosine hydroxylase (TH)-immunohistochemical staining, while the distribution of dopaminergic receptor 2 (D2R) in the striatum was identified by D2R-immunohistochemical staining (10).

Materials and Methods

Experimental Groups and Study Designs

A total of 18 Sprague-Dawley male rats, weighing between 350 to 400 g, were purchased from LASCO (Yilan, Taiwan, ROC). Three rats without treatments with 6-OHDA and G-CSF were sacrificed for immunohistochemical identification of the normal dopaminergic neurons and dopaminergic fibers (normal group, n = 3). Fifteen rats receiving unilateral 6-OHDA injection in the right MFB were subjected to the rotational test on the 9th day after 6-OHDA injection (*i.e.*, the 1st behavior test). A total of 11 rats developing severe abnormal rotational behavior (*i.e.*, methamphetamine-induced rotation behavior > 5 turns/min) were enrolled as the very severe PD rats. The remaining 4 rats that developed less severe rotational behavior (< 5 turns/min) were discarded. After the 1st behavior test, 5 rats that received normal saline (0.5 ml/kg/day, subcutaneously) for 5 days were assigned as the vehicle control group (NS group, n = 5), and 6 rats that received G-CSF (50 μg/kg/day, subcutaneously) for 5 days were assigned as the G-CSF group (G-CSF group, n = 6). Because the dopaminergic neurons were depleted (> 95%) on the 14th day when 6-OHDA was injected into the MFB region (21), we treated the PD rat with G-CSF from the 9th to the 13th days to address its therapeutic effects in the 6 severe PD rats (Fig. 1A). These rats were further subjected to rotational behavior test on the 28th day (*i.e.*, the 2nd behavior test) (Fig. 1A). All the procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tzu-Chi General Hospital, Hualien, Taiwan, ROC.

Model of Severe Parkinson's Disease in Rats

Rats were anesthetized with chloral hydrate (400 mg/kg, Sigma, St. Louis, MO, USA) intraperitoneally before surgery. After fixing the head on a stereotaxic stage (Stoelting, MA, USA), two burr-holes were created on the right side of the skull to facilitate two insertions of a 26-gauge needle that was fixed on a Hamilton syringe and aimed to the right MFB. The coordinates for the two insertion sites were 4.4, 1.2, 7.8 and 4.0, 0.8, 8.0 mm anterior-posteriorly, mediolaterally and dorso-ventrally related to the bregma, respectively. Twelve μg 6-OHDA (Sigma) dissolved in 3 μl sterilized saline containing 0.02% ascorbic acid (Sigma) was slowly administered to each rat. After the injection, the burr-hole was sealed with bone wax to prevent the outflow of the drug. Rectal temperature was maintained at 37°C during the whole surgery period by a feedback-regulated heating pad.

Methamphetamine-Induced Rotation Test

The rotation behavior which was enhanced by methamphetamine was used as the behavioral assessments for the motor function in the PD animal model (7, 19). The rats were challenged with methamphetamine (2 mg/kg, Sigma) subcutaneously. After the injection, the rotation number of each rat was recorded by Rotameter (TSE, Chesterfield, MO, USA) for two hours. The maximum number of turns in sixty minutes of each rat was taken as its behavioral performance. The rotational number increases proportionally to the severity of the lesion.

Preparation of Brain Sections

One day after the 2nd behavior test, rats were anesthetized with chloral hydrate (800 mg/kg, intraperitoneally) and perfused with 200 ml of normal saline followed by 200 ml of 4% para-formaldehyde (Sigma). Brains were removed and soaked in a 30% sucrose (Sigma) solution at 4°C for 3 days, then frozen to -20°C and sectioned coronally with a cryomicrotome (CM3050S, Leica, Wetzlar, Germany) into 20-μm thick sections. The brain sections were mounted on slides coated with silane, and were fixed in 95% alcohol.

Dopaminergic Neurons and Dopaminergic Receptor 2 (D2R) Analysis

TH is the specific marker for identifying dopaminergic neurons or fibers (20, 21). Dopaminergic neurons in the SNpc and their projection fibers (dopaminergic fibers) in the striatum were demonstrated by TH-immunohistochemical staining. D2R in the striatum, however, was identified by D2R-immunohistochemical staining (10). The brain sections on slides were rinsed in TBS with 0.1% Tween-20 (Sigma) and blocked by normal goat serum (GIBCO, Carlsbad, CA, USA) for 2 h at room temperature to reduce nonspecific binding. These sections were then incubated with the following primary antibodies for 2 h: mouse anti-TH (1:400, Sigma) or rabbit anti-dopamine receptor 2 (1:50, Chemicon, MA, USA). Fully-rinsed sections were incubated with a second antibody conjugated with labeled polymer-HRP (Cytomation EnVision, DAKO, CA, USA) for 30 min and the immunoreactions were detected by a DAB kit (Cytomation EnVision DAKO) according to the manufacturer's instruction. Hematoxylin stains were applied as the counter stain, and the sections were mounted with mounting medium (Assistent, PAN-SUN, Taipei, Taiwan, ROC).

Image Analysis

Images were taken by a microscope (Axiovert 200M, Carl Zeiss, AG, Germany) equipped with a digital camera (E5000, Nikon, Tokyo, Japan). The numbers of TH positive cells in the SNpc were counted under a high-power view (400×). TH-positive cell number in the SNpc was presented as the average of cells counted from 3 consecutive brain sections. Cells loss on the lesion side was presented as the ratio of lesion to non-lesion cell count in the SNpc to avoid variations in the neuronal number of the individual rats.

Images of TH and D2R immunoreactivity (THir and D2Rir) at the striatum were photographed, and analyzed with the Image J software (NIH, Bethesda, Maryland, USA). The software can quantitate the intensity of regions of interest (21). To normalize variations of immunoreactivity on each slide, the background value (intensity of the cortex) was deducted, and a ratio of lesion to non-lesion intensities of THir and D2Rir was calculated, respectively.

Statistical Analysis

All data were presented as means ± SD. The analysis of variance (ANOVA) for multiple comparisons and Student's *t*-test was used to compare the indicated index between two groups. All statistical analyses were performed using the SPSS software for Windows (version 10.0; SPSS, Chicago, IL, USA).

Results

The Severe PD Rat Model

To select for the very severe PD rats, amphetamine-enhanced rotational behavior and the lesion

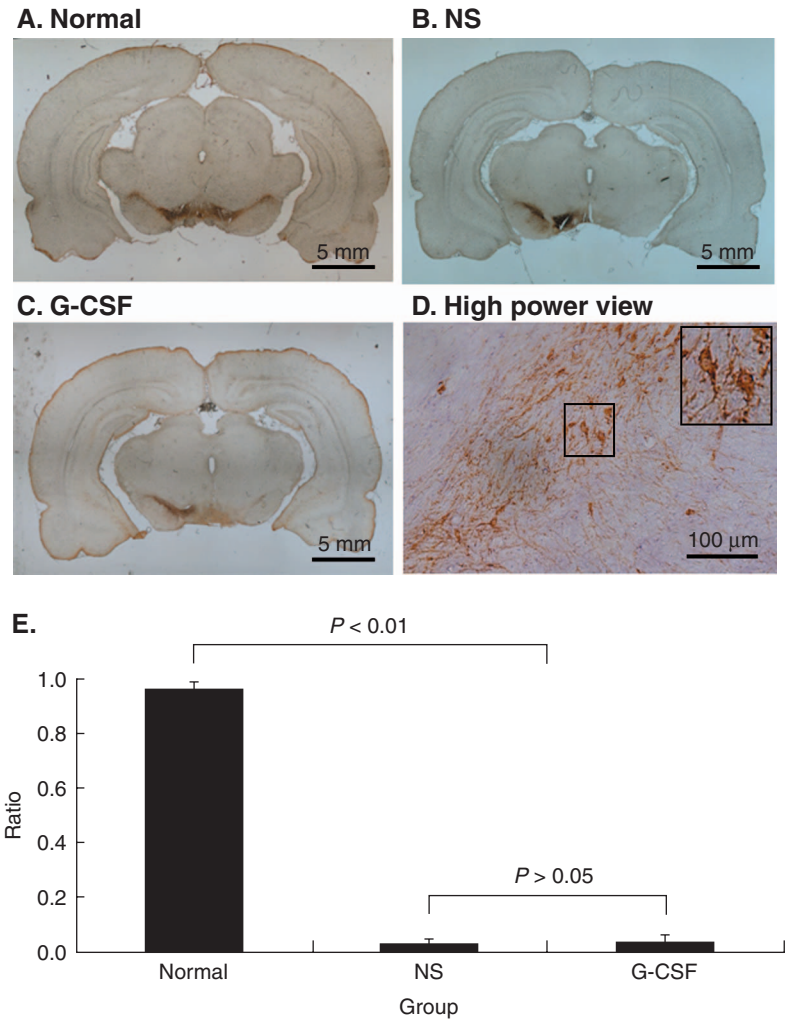

Fig. 2. Dopaminergic neurons in substantia nigra (SNpc) in the Normal, Normal Saline (NS) and G-CSF (G-CSF) groups. Rats in the Normal group were killed without lesion nor any treatment, while rats in the NS and G-CSF groups were killed on the 28th day after 6-OHDA lesion. Brain sections were stained with tyrosine hydroxylase (TH) for enumerating the dopaminergic cells. Dopaminergic neurons in low-power view are shown in the Normal (A), NS (B) and G-CSF (C) groups; dopaminergic cells were counted in a high-power view after TH staining in SNpc (D). The survival rate is expressed by the right to left (lesion to non-lesion) ratio of TH-positive cells (E).

to non-lesion ratio of dopaminergic neurons in the SNpc were determined. The severe PD rats were characterized with very severe rotational behavior tested on the 9th day in the NS (7.61 ± 0.82 turns/min) and the G-CSF groups (7.29 ± 0.93 turns/min) (Fig. 1B), respectively. The severity was inversely correlated with the ratio of dopaminergic neurons in the SNpc of both the NS (0.032 ± 0.030) and the G-CSF groups (0.028 ± 0.018) examined on the 28th day after the 6-OHDA lesion (Fig. 2, B, C and E). In addition, dopaminergic fibers in the striatum on the lesion sides in the NS group (Fig. 3B) and even in the G-CSF group (Fig. 3C) were markedly reduced.

G-CSF Post-Treatment Reduced Rotational Behavior in Very Severe PD Rats

The rotation behavior in the 1st test in the NS group was 7.61 ± 0.82 turns/min, whereas that in the G-CSF group was 7.29 ± 0.93 turns/min with no significant difference ($P > 0.05$) (Fig. 1B). After post-treatment with normal saline or G-CSF, the rotation behavior at the 2nd test in the NS group was 6.14 ± 0.97 turns/min, whereas that in the G-CSF group was 4.33 ± 1.25 turns/min, with significant difference ($P < 0.01$) (Fig. 1B). In the G-CSF group, the rotational behavior of the 2nd test was significantly less than that of the 1st test ($P < 0.01$).

G-CSF Post-Treatment Promotes Surviving Dopaminergic Cells

To quantify the surviving dopaminergic neurons in ipsilateral SNpc, THir-positive neurons on both

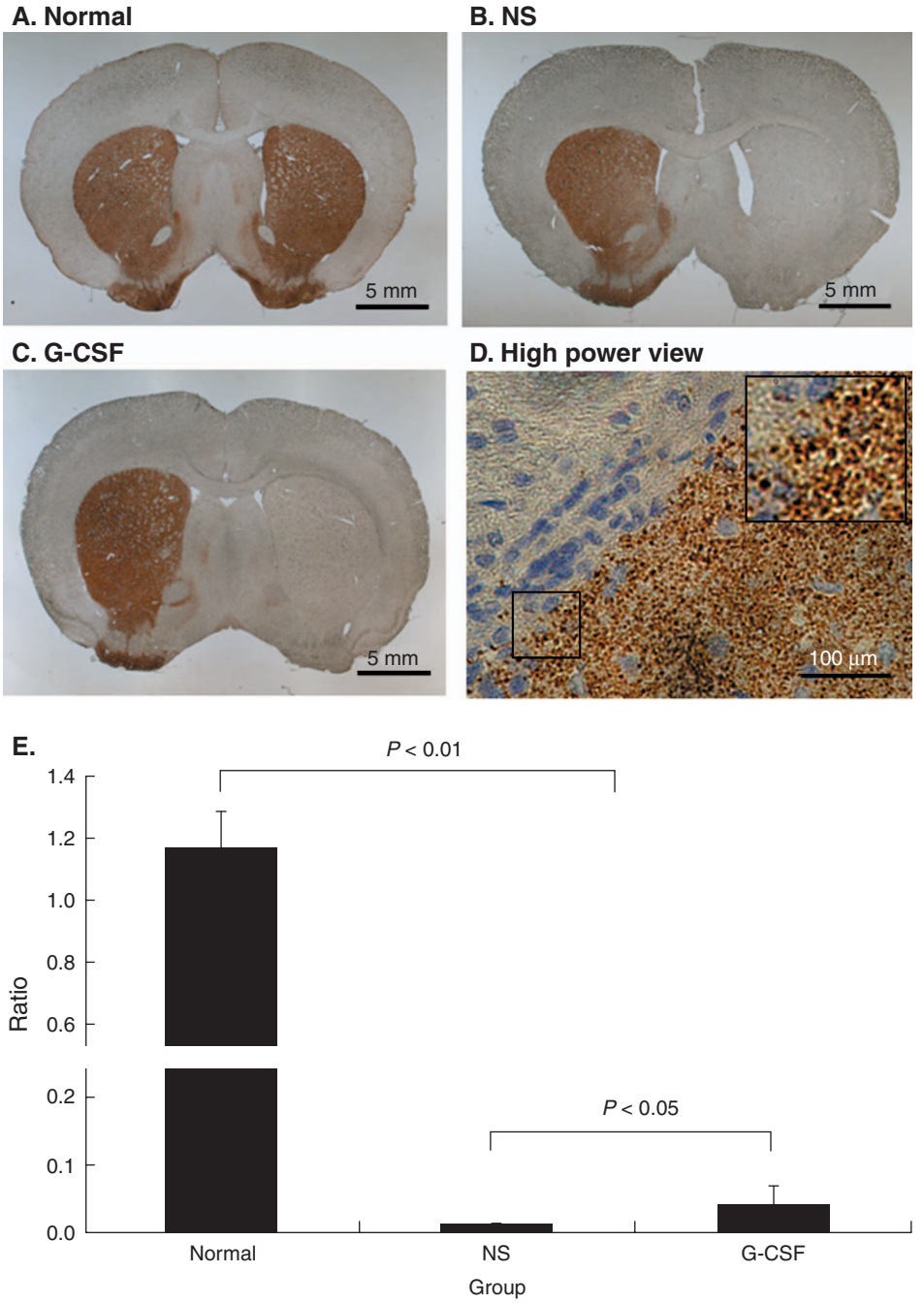

Fig. 3. Tyrosine hydroxylase immunoreactivity (THir) in the striatum of the Normal, NS and G-CSF groups. Rats were killed for THir immunohistochemical staining of the dopaminergic fibers. THir staining in the Normal (A), NS (B) and G-CSF (C) groups is shown. The high-power view of dopaminergic fiber shows high density of TH staining (D). The bar graph shows the right to left (lesion to non-lesion) ratio of THir in all the groups (E). Note that the stains on the lesion (right) side of both the NS and G-CSF groups are very light so that the difference between two groups was hard to be distinguished from each other.

sides (Fig. 2D) were counted and were presented as survival ratio of the lesion to non-lesion cell count (Table 1). The ratio in the G-CSF (0.032 ± 0.030) and the NS groups (0.028 ± 0.018) has no significant difference ($P > 0.05$) on the 28th day after 6-OHDA lesion (Fig. 2E).

G-CSF Post-Treatment Induced More Sprouting of Dopaminergic Fibers in the Striatum of the Very Severe PD Rat

The intensity of THir in the striatum has been reported as the axonal projection of dopaminergic

Table 1. THir neurons in the SNpc of normal and Parkinsonism rats treated with or without G-CSF

Group	Normal (n = 3)		NS (n = 5)		G-CSF (n = 6)	
Side	Left	Right	Left	Right	Left	Right
	51.6 ± 7.6	52.0 ± 10.0	91.7 ± 11.5	1.3 ± 1.5	76.0 ± 6.0	1.0 ± 0.0
	65.3 ± 14.5	68.7 ± 15.3	102.0 ± 11.5	2.0 ± 1.0	92.7 ± 17.8	2.0 ± 0.0
	77.7 ± 15.5	82.0 ± 16.6	69.3 ± 4.0	4.0 ± 2.6	69.0 ± 7.9	3.3 ± 1.5
			85.3 ± 16.6	2.7 ± 0.6	40.0 ± 21.0	0.7 ± 0.6
			89.3 ± 21.0	1.7 ± 0.6	60.7 ± 18.4	0.3 ± 0.6
					70.0 ± 6.6	6.0 ± 3.5
AVG	64.9 ± 13.0	67.6 ± 15.0	87.5 ± 16.7	2.3 ± 1.4	68.1 ± 12.2	2.2 ± 1.2

TH-positive cell number in the SNpc was presented as the average of cells counted from 3 consecutive brain sections. The counts of left (non-lesion) side among the Normal, NS and G-CSF groups and those of the right (lesion) side between the NS and G-CSF showed no significant difference ($P > 0.05$).

neurons in SNpc (21). To compare the amount of residual dopaminergic (THir) fibers in the striatum of each group, a lesion to non-lesion ratio of THir-fibers in the striatum was examined (Fig. 3E). The ratio of THir-fibers significantly increased in the G-CSF group (0.044 ± 0.027) as compared to the NS group (0.012 ± 0.003, $P < 0.05$), indicating more sprouting of the dopaminergic axons in the striatum.

G-CSF Post-Treatment Did not Affect D2Rir in the Striatum of the Very Severe PD Rats

To compare the amount of dopaminergic receptors in the striatum of each group, a lesion to non-lesion ratio of D2R in the striatum was performed (Fig. 4, A, B and C). The lesion to non-lesion ratio of D2Rir in the striatum significantly increased in the G-CSF (1.78 ± 0.23) and NS groups (1.90 ± 0.56), respectively, as compared to the Normal group (1.18 ± 0.10) (Fig. 4D).

Discussion

The objective of this study was to determinate whether post-treatment with G-CSF has therapeutic effects in a very severe PD rat model. We used 6-OHDA to create a very severe PD model in rats characterized by very severe rotational behavior (> 5 turns/min) (Fig. 1B), very few survival dopaminergic neurons in the SNpc (Fig. 2), and very scanty dopaminergic fibers (very low THir intensity) in the striatum on the lesion side (Fig. 3). We, for the first time, demonstrated that post-treatment with G-CSF led to a reduction of the abnormal rotational behavior (Fig. 1B) and an increase of the lesion to non-lesion ratio of dopaminergic fibers in the striatum (Fig. 3D). However, post-treatment of G-CSF did not promote the survival of dopaminergic neurons (Fig. 2). In addition, the lesion to non-lesion ratio of D2R amount (intensity) both increased in the NS and G-CSF groups with no significant difference ($P > 0.05$, Fig. 4). These findings suggest that post-treatment with G-CSF may favor a relative increase in the dopaminergic fibers in the striatum of the lesion side to compensate for the severely loss of dopaminergic neurons.

We are the first to establish the very severe PD model in rats for evaluating the therapeutic effects of G-CSF post-treatment (50 μg/kg/day, subcutaneously for 5 days). These rats were characterized by very severe rotational behavior (> 5 turns/min) tested on the 9th and 28th day (Fig. 1). The severity was confirmed with few survival dopaminergic neurons (ratio < 0.04) in the SNpc of both the NS and G-CSF groups on the lesion side examined on the 28th day after the 6-OHDA injection (histological staining in Fig. 2A-D, cell counts in Table 1 and statistical data in Fig. 2E), and very scanty dopaminergic fibers in the striatum on the lesion side in the NS group (histological stain in Fig. 3, A-C, and statistical data in Fig. 3E). Therefore, selection of the very severe PD rats with rotational behavior > 5 turn/min was sufficient and reliable for this model.

Post-treatment with G-CSF reduced the abnormal motor behavior of the very severe PD rats (Fig. 1). This raised the question on whether the G-CSF treatment might have preserved survival of the dopaminergic neurons. In fact, other investigators have inferred in a mouse PD model that post-treatment with G-CSF markedly increases the number of dopaminergic neurons in the SNpc (9), but the authors failed to identify any newly generated dopaminergic neurons in SNpc. However, our results demonstrated that post-treatment with G-CSF could not increase the survival dopaminergic neurons (Fig. 2E). It was, therefore, interesting to investigate whether other factors, such as sprouting of dopaminergic fibers and changes in D2R in the striatum, may compensate to reduce the abnormal motor behavior in the very

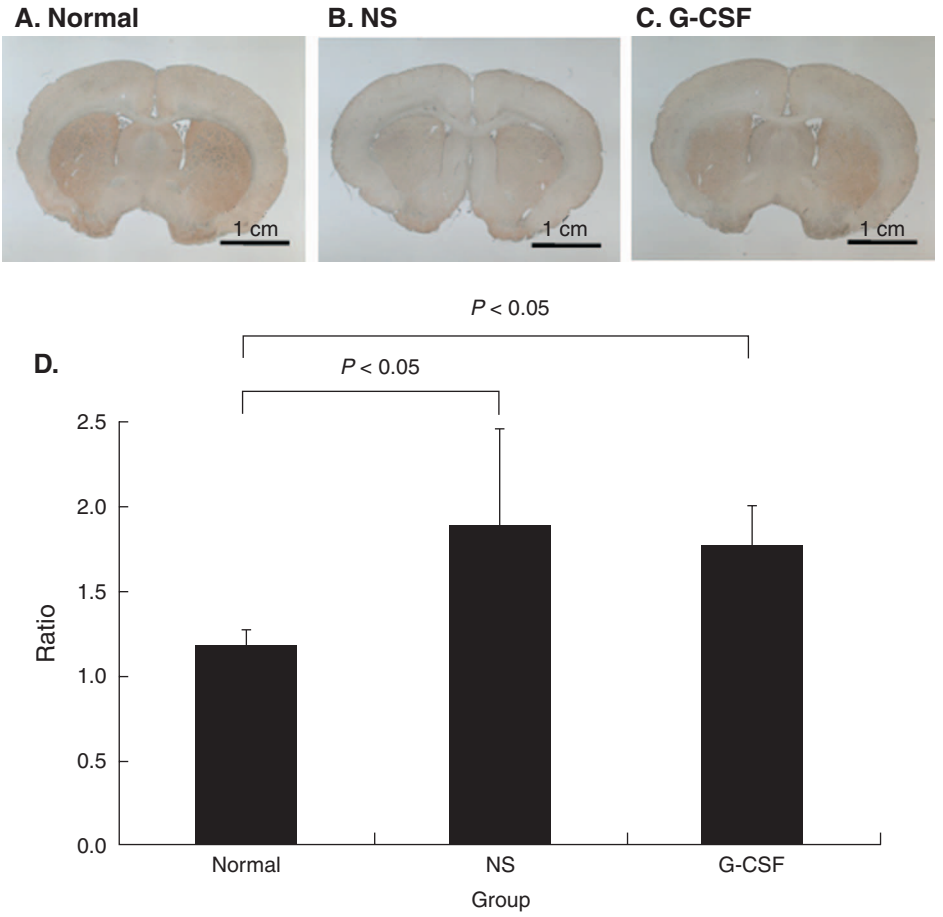

Fig. 4. Dopamine 2 receptor immunoreactivity (D2Rir) in the striatum of the Normal, NS and G-CSF groups. D2Rir staining in the Normal (A), NS (B) and G-CSF (C) groups is shown. The lesion to non-lesion ratio of D2Rir significantly increased in the NS and G-CSF group as compared to the Normal group ($P < 0.05$) (D). No significant difference ($P > 0.05$) was found between the NS and G-CSF groups (D).

severe PD rats.

The intensity of THir in the striatum has been reported as the axonal projection of dopaminergic neurons in SNpc (21). Data in Fig. 3E demonstrate that the ratio of dopaminergic fibers significantly increased ($P < 0.05$) in the G-CSF group compared with the NS group, but data in Fig. 2E show that post-treatment with G-CSF could not increase the survival dopaminergic neurons (Table 1 and Fig. 2E), indicating that more sprouting of the dopaminergic axons had occurred in the striatum. This result is consistent with a parallel study of our research group which showed that treatment of G-CSF preserved more axonal branches of dopamine neurons and dopamine uptake in mesencephalic culture treated with 6-OHDA (5). An increased ratio, however, can simply be the result of an increase in the lesion side, or a decrease in the normal side, or the result of relative increase in the lesion side due to simultaneous increases or decreases in both the lesion and non-lesion sides.

Beside the pre-synaptic axonal sprouting, the activity of the post-synaptic neurons may influence the rotation behavior by the increase in supersensivity (15) or the amount of dopaminergic receptors (16, 18). The D2R, but not D1R, plays a role in modulating the extent of the sprouting of SNpc neurons (3, 18). We, therefore, examined the intensity of D2Rir in the striatum, and demonstrated that the lesion to non-lesion ratio of D2R was equally increased in both the NS and G-CSF groups with no significant difference between the groups ($P > 0.05$, Fig. 4D), indicating that G-CSF may not affect the supersensitivity or the amount of D2R in the very severe PD rat. The improvement of rotation behavior in the G-CSF group is, therefore, due primarily to the pre-synaptic axonal sprouting but not due to the expression of D2R.

On the other hand, there are many nervous circuits involving the motor behavior of PD, such as the nigropallidal dopamine pathway to the internal segment of the globus pallidus (22), gama-aminobutyric acid (GABA) neurons in the striatum (8), cortico-striatal glutamatergic projection and its glutamatergic

receptors (2, 6), or thalamo-striatal glutamatergic projection and their receptors (13, 17). Whether G-CSF affects these projections and their receptors needs further investigation.

In conclusion, in the absence of promoting survival dopaminergic cells, post-treatment with G-CSF can reduce (improve) the abnormal rotational behavior of the very severe PD rats primarily through a relative increase in dopaminergic fibers of the lesion side in the striatum. Our study supports therapeutic potential of G-CSF for very severe PD patients.

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