Selegiline rescues gait deficits and the loss of dopaminergic neurons in a subacute MPTP mouse model of Parkinson's disease

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Abstract. The monoamine oxidase type-B (MAO-B) inhibitor, selegiline, is often recommended as a first-line treatment for Parkinson's disease (PD) and has been shwon to possess neuroprotective effects. The aim of the present study was to determine whether selegiline increases the levels of the neurotrophic factors (NTFs), glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), and whether it rescues motor dysfunction and the loss of dopaminergic neurons in mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesions. We found that the oral administration of selegiline (1.0 mg/kg/day for 14 days) successfully suppressed the MPTP-induced reduction of nigral dopaminergic neurons and striatal fibers (192.68 and 162.76% of MPTP-exposed animals, respectively; both $P<0.001$). Moreover, improvements in gait dysfunction were observed after 7 and 14 days of a low dose of selegiline that is reported not to inhibit MAO-B. Furthermore, there was a significant increase in GDNF and BDNF mRNA (2.10 and 2.75-fold) and protein levels (143.53 and 157.05%) in the selegiline-treated mice compared with the saline-treated MPTP-exposed mice. In addition, the Bax/Bcl-2 gene and protein expression ratios were significantly increased in the MPTP-exposed mice, and this effect was reversed by selegiline. Correlation analysis revealed that gait measurement and GDNF/BDNF levels positively correlated with the number of dopaminergic neurons. These findings demonstrate that selegiline has neurorescue effects that are possibly associated with the induction of NTFs and anti-apoptotic genes.

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Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAO-B, monoamine oxidase type-B; NTFs, neurotrophic factors; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TH, tyrosine hydroxylase; SNpc, *substantia nigra pars compacta*; ST, striatum; DA, dopamine

Key words: Parkinson's disease, neurorescue, selegiline, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, gait analysis

Introduction

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and their axon terminals in the striatum (ST) associated with a complex, but slow onset of motor symptoms, including bradykinesia, muscular rigidity, resting tremor and gait abnormalities with poor postural balance (1). Monoamine oxidase type-B (MAO-B) inhibitors have returned to the spotlight as an alternative to dopaminergic replacement therapy and studies have demonstrated that they enhance cognitive function (2) and exert neuroprotective effects (3). Disease modification has also been investigated in previous studies, assessing the effects of the MAO-B inhibitors, selegiline and rasagiline (3,4). Although selegiline, the first selective inhibitor of MAO-B, has been widely used in patients with PD as monotherapy and adjuvant therapy, its basic and clinical pharmacological effects have not yet been fully elucidated. There is evidence that its neuroprotective characteristics are mediated through its effects on protein kinase C and mitogen-activated protein kinase signaling pathways (5). Indeed, the improvements observed as regards clinical PD progression following the use of this type of drug have confirmed its neuroprotective activities, which have been previously reported in various cell culture and preclinical *in vivo* models (6-10).

The neuroprotective effects of MAO inhibitors also involve the regulation of cell survival/death pathways, including those involving Bcl-2 family proteins and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) death cascade (11,12). Unlike neuroprotective therapeutic strategies, neurorescue or neurorestorative therapies aim to eliminate neuronal deficits and degeneration after impairment onset. Previous studies have reported that MAO-B inhibitors can facilitate the availability of neurotrophic factors (NTFs) *in vitro*, particularly glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (13,14) and have demonstrated that these outcomes have neurorestorative effects (15,16). However, to our knowledge, there have been no investigations assessing the possible neurorestorative effects of selegiline on

behavioral deficits and molecular alterations associated with NTFs *in vivo*. This gap in the current understanding prompted us to perform experiments assessing the possible neuro-rescue activity of selegiline and the underlying mechanisms in a subacute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD.

Materials and methods

Animal protocols. All procedures were approved by the Animal Ethics Committee of Zhongshan Hospital, Fudan University, Shanghai, China and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were conducted using 10-week-old male C57BL/6 mice weighing 24-26 g purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were maintained in standard conditions (12/12-h light/dark cycle, 21±2˚C and relative humidity of 40%) and allowed access to food and water *ad libitum*.

Administration of MPTP and selegiline. An MPTP model of PD was generated as previously described (17,18). Briefly, the mice received daily intraperitoneal (i.p.) injections of the vehicle (saline) or MPTP (30 mg/kg/day; Sigma, St. Louis, MO, USA) dissolved in physiological saline for 5 consecutive days to induce Parkinsonism. Each treatment group included 10 mice. Selegiline ([(R)-(-)-N,2-dimethyl-N-2-propynylphenethylamine]; L-deprenyl; 1.0 mg/kg/day; Sigma) was dissolved in physiological saline and the pH was adjusted to 7.4 before it was given via intragastric (i.g.) administration. Selegiline or vehicle (saline) treatment commenced 72 h after the final MPTP administration and was administered daily for 14 days. The experimental groups were as follows: group I, normal saline (NS) (i.p.) + NS (i.g.); group II, MPTP (i.p.) + NS (i.g.); group III, MPTP (i.p.) + selegiline 1.0 mg/kg/day (i.g.). The mice were sacrificed by cervical dislocation or perfusion 24 h after the final vehicle or selegiline administration.

Gait test. The gait test was performed according to previously published methods (19-21) with minor modifications. The apparatus was composed of a runway [dimensions: 4.5 cm (w) x 40 cm (l) x 12 cm (h)] illuminated by a light (60 W), and a black wooden box [20 cm (w) x 17 cm (l) x 10 cm (h)] was placed at one end of the runway. The fore and hind paws of the animals were wet with blue ink and they were allowed to trot on a strip of paper (4.5 cm wide, 40 cm long) down the brightly lit runway towards the black goal box. Stride lengths were manually measured as the distance between 2 paw prints. The 3 longest stride lengths (corresponding to maximal velocity) were measured from each run. Paw prints made at the beginning (7 cm) and end (7 cm) of the run were excluded due to changes in velocity. Runs in which the mice were observed making stops or significant decelerations were excluded from the analysis. The behavioral assessment was performed 3 days before the first MPTP injection and on the 7th and 14th day of selegiline or vehicle treatment.

Perfusion and tissue processing. At the end of the experiment, half of the animals (n=5) in each group were sacrificed under 10% chloral hydrate overdose anesthesia (360 mg/kg) then perfused via intracardial infusion with saline (0.9%) followed by 4% paraformaldehyde (PFA), pH 7.4. Following intracardial perfusion, the brains were collected and post-fixed in 4% PFA for 24 h at 4˚C, embedded in paraffin and cut into 5-μm-thick coronal sections encompassing the entire SNpc and ST (antero-posterior levels: -3.64 to -2.92 mm and +0.86 to +0.02 mm) as previously described (22).

Another 5 animals in each group were sacrificed by cervical dislocation and the tissue of their ventral midbrain was dissected rapidly on ice, frozen in liquid nitrogen and stored at -80˚C until use.

Tyrosine hydroxylase (TH) immunohistochemistry. Immunohistochemistry was performed as previously described (23,24) with minor modifications. Briefly, the fixed brain sections were incubated with 0.3% hydrogen peroxide (H_2O_2) for 10 min at room temperature to quench endogenous peroxidase activity and then placed in blocking buffer containing 10% goat serum with 0.2% Triton X-100 in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 30 min at 37˚C. In each treatment, the slides were washed at least 3 times with 0.01 M PBS for 5 min each, followed by incubation at 4˚C overnight with mouse anti-TH monoclonal antibody (Sigma) at 1:2,000 dilution in 0.01 M PBS containing 1% goat serum and 0.2% Triton X-100. The following day, the sections were treated for 30 min with biotinylated anti-mouse IgG and then processed with streptavidin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA). The peroxidase reaction was visualized by 0.05% diaminobenzidine (DAB) with 0.03% H_2O_2 in Tris-HCl buffer. Adjacent sections were stained with cresyl violet to confirm cell vitality.

Quantification of TH-immunoreactive neurons and fibers. The number of dopaminergic neurons was determined as previously described (25). Briefly, we manually counted TH-positive cells under bright-field illumination in the right SNpc using a x10 or x20 objective with a DP71 camera (Olympus, Center Valley, PA, USA). Cell counts were determined blindly on 5 anatomically matched sections from each of the animals (n=5/group). It should be noted that the analyses of the TH-immunoreactive profiles were restricted to the SNpc and thus excluded the ventral tegmental area. In addition, neurons were only counted if they contained a nucleus that was surrounded by cytoplasm.

The optical density (OD) of the striatal dopaminergic fibers was analyzed using Image-Pro Plus Software (Media Cybernetics, Inc., Rockville, MD, USA), according to a previously described optical dissector method (26,27). The average labeling for each area was calculated on 5 anatomically matched brain sections (n=5/group). For further determination of the number of TH-positive axons in the ST, we selected the section corresponding to bregma +0.260 mm at high magnification in a 45-μm^2 area according to a previously described method (25).

Real-time polymerase chain reaction (PCR). Total RNA was isolated by homogenizing frozen ventral midbrain (left side) tissue in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by isopropanol precipitation (n=5). The resulting pellets were washed with 70% ethanol and suspended in RNase-free water and the concentration of RNA was determined using a GeneQuant RNA/DNA Calculator (Amersham Biosciences,

Table I. Primer sequences used for real-time PCR.

Gene name	Gene ID	Primer sequence	
GDNF	NM_010275.2	Sense:	5'-AAG GTC ACC AGA TAA ACA AGC GG-3'
		Antisense:	5'-TCA CAG GAG CCG CTG CAA TAT C-3'
BDNF	NM_007540.4	Sense:	5'-ACT ATG GTT ATT TCA TAC TTC GGT T-3'
		Antisense:	5'-CCA TTC ACG CTC TCC AGA-3'
Bax	NM_007527.3	Sense:	5'-CGG CGA ATT GGA GAT GAA CTG-3'
		Antisense:	5'-GCA AAG TAG AAG AGG GCA ACC-3'
Bcl-2	NM_177410.2	Sense:	5'-ACC GTC GTG ACT TCG CAG AG-3'
		Antisense:	5'-GGT GTG CAG ATG CCG GTT CA-3'
β-actin	NM_007393.3	Sense:	5'-CCT CTA TGC CAA CAC AGT GC-3'
		Antisense:	5'-GTA CTC CTG CTT GCT GAT CC-3'

PCR, polymerase chain reaction; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor.

Piscataway, NJ, USA). RNA (1 μg) was taken as a template and total cDNA synthesis was performed using a PrimeScript™ RT Reagent kit (Takara, Shiga, Japan). SYBR-Green PCR [using SYBR Premix Ex Taq™ (Takara)] amplification was performed in a Realplex4 S Real-time PCR instrument (Eppendorf, Hamburg, Germany). β-actin was labeled with a reporter dye and used as an endogenous control. The relative fold changes were determined using the $2^{-\Delta\Delta Ct}$ method as previously described (28). All primers (Table I) were designed according to the relevant literature and synthesized by Genemed Biotechnologies, Inc. (South San Francisco, CA, USA).

Western blot analysis. For western blot analysis, isolated tissues from the ventral midbrain (right side) were homogenized in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 % sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and proteinase inhibitors; Beyotime, Shanghai, China] and centrifuged at 13,000 rpm at 4˚C for 5 min. Total protein content in the supernatant was determined using a BCA Protein Assay kit (Beyotime) with a spectrophotometer (Labomed, Inc., Culver City, CA, USA), and it was diluted to an appropriate final concentration with homogenization buffer and a protein solubilization solution. The sample was boiled for 3 min and 30 μg of protein from each sample was electrophoresed on a 10% SDS polyacrylamide gel then electrophoretically transferred onto a nitrocellulose membrane in transfer buffer using a Trans Blot SD apparatus (Bio-Rad, Hercules, CA, USA). The membrane was then blocked by immersion in Tris-buffered saline containing Tween-20 (TBST) and 1% BSA for 4 h at room temperature and incubated at 4˚C overnight with mouse primary antibodies: anti-GDNF and anti-BDNF (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA USA) and anti-Bax, anti-Bcl-2 and anti-β-actin (1:1,000; Santa Cruz Biotechnology, Inc.). After rinsing 3 times in TBST for 10 min, blots were incubated for 2 h at room temperature with an anti-rabbit IgG-peroxidase conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc.). Immunoreactivity was visualized with an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). The blots were scanned with a KODAK *In-Vivo* Multispectral Imaging System FX (Carestream Health, Rochester, NY, USA) during a 5-min exposure time and images were automatically acquired with a CCD camera. The intensity of the protein bands was measured by densitometry and expressed as a ratio to β-actin intensity as previously described (29).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL assays were performed according to previously described methods (23,30) with minor modifications. Briefly, an *In Situ* Death Detection kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions on serial coronal brain sections encompassing the SNpc (bregma, -2.92-3.64 mm). The total number of TUNEL-positive cells within the SNpc was counted in 14-15 slides/animal under a light microscope equipped with a x20 objective lens.

Statistical analyses. All data are presented as the means ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by post-hoc analyses of Tukey's honestly significant difference (HSD) and Student-Newman-Keuls multiple comparisons tests were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant difference. Linear regression analysis was applied to assess the correlations between 2 parameters.

Results

Selegiline improves gait dysfunction in a subacute MPTP mouse model of PD. Shortened stride length is one of the chief characteristics of abnormal gait in patients with PD (31). Accordingly, we observed a significant decrease in fore- and hindlimb stride length in the MPTP-exposed mice treated with vehicle (saline) on the 9th day after the final MPTP administration (7th day of vehicle treatment), compared with the normal control group (P=0.023 and P=0.014, respectively) (Fig. 1). The fore- and hindstride lengths of the mice in the selegiline (1.0 mg/kg/day) treatment group were longer than those in the MPTP-vehicle group (P=0.024 and P=0.029,

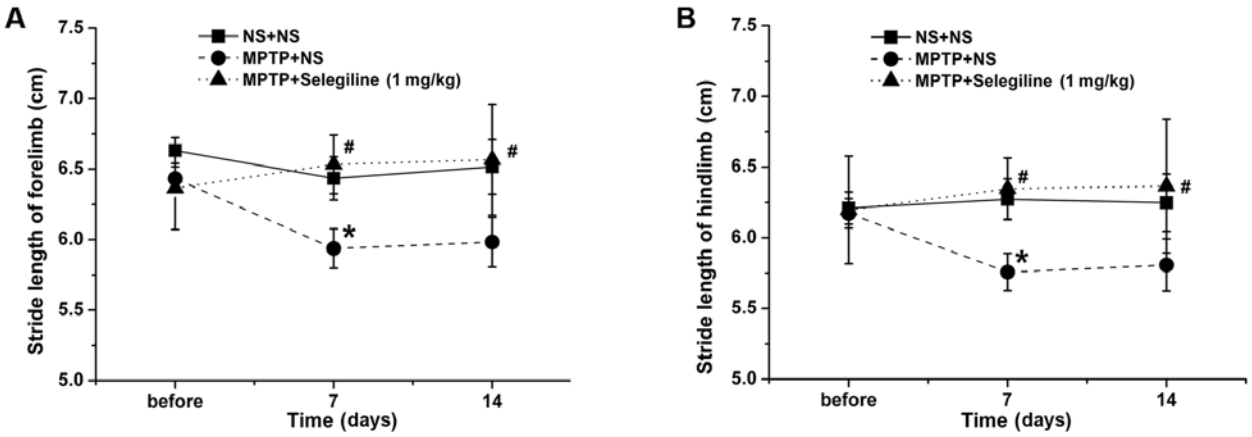

Figure 1. Selegiline improves subacute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced gait impairment. (A) Forelimb stride length. We observed a significant reduction in stride length in the MPTP + NS group compared with the NS + NS group on the 7th day of MPTP-vehicle treatment; however, these deficits were prevented by selegiline (1.0 mg/kg/day) on the 7th and 14th day of treatment. (B) Hindlimb stride length. We observed a significant decreased in stride length in the MPTP + NS group compared with the NS + NS group on the 7th day of the MPTP-vehicle treatment; however, this was significantly improved by selegiline. Data are expressed as the means ± SEM (n=10 each). *P<0.05 vs. NS + NS group; #P<0.05 vs. MPTP + NS group. NS, normal saline.

respectively). Improvement in the selegiline-treated group was also observed on the 14th day of treatment compared with the MPTP-exposed mice (P=0.032 and P=0.044, respectively).

Selegiline attenuates the loss of TH-positive nigral neurons and striatal axons in subacute MPTP-exposed mice. After 14 days of treatment with selegiline or the vehicle and the completion of the behavioral assessment, half of the mice in each group were sacrificed and the brains were prepared for TH-immunoreactivity experiments. Representative coronal mesencephalon sections containing TH-positive neurons and fibers in the SNpc and ST are shown in Fig. 2A-D. There was a significant decrease in the number of TH-positive nigral dopaminergic neurons in the vehicle-treated, MPTP-exposed group compared with the non-exposed control mice (42.93% of saline control, P=0.000) (Fig. 2A, B and E). In the mice receiving daily oral selegiline treatment, the number of TH-positive neurons was significantly higher than that in the MPTP/vehicle-treated animals (192.68% of MPTP control, P=0.001) and did not differ compared with the non-exposed control mice (82.72% of saline control, P>0.05).

We also observed a reduction in the number of TH-positive axons and fibers throughout the dorsal ST of the MPTP-exposed animals; however, this damage improved in the selegiline-treated group. Both the number and OD analysis of TH-positive fibers revealed a significant loss of dopamine (DA) terminals in the MPTP/vehicle-treated group (58.44 and 47.37% of saline control; P=0.000 and 0.001, respectively) (Fig. 2F and G). By contrast, the number and density of TH-positive axons and fibers were clearly increased in the MPTP-treated mice that received selegiline compared with those that received saline (143.41 and 162.76% of MPTP control; P=0.015 and 0.038, respectively) (Fig. 2C, D, F and G), bringing them to 83.90 and 76.69% of the normal control levels (P>0.05). Linear regression analysis revealed that there was a strong positive correlation between forelimb stride length and the number of TH-positive SNpc neurons, as well as the OD of TH-positive striatal fibers (Fig. 5A and B).

Selegiline increases the relative mRNA and protein levels of GDNF and BDNF in the SNpc of subacutely MPTP-exposed mice. We performed real-time PCR and western blot analyses to assess changes in GDNF and BDNF expressions at the mRNA and protein level following treatment with selegiline. We observed a significant increase in the relative mRNA and protein levels of GDNF in the MPTP/selegiline-treated animals compared with the MPTP/vehicle-treated mice (2.10-fold in mRNA and 143.53% in protein of MPTP control; P=0.017 and 0.009, respectively). There were similar changes in BDNF expression; we observed significantly higher relative mRNA and protein levels in the MPTP/selegiline-treated group compared with the MPTP/vehicle-treated animals (2.75-fold and 157.05% of MPTP control; P=0.048 and 0.004, respectively) (Fig. 3A-C). These results demonstrate that selegiline induces the gene and protein expression of GDNF and BDNF. Linear regression analysis revealed that there was a strong positive correlation between GDNF/BDNF protein levels and the number of TH-positive SNpc neurons (Fig. 5 C and D).

Selegiline attenuates the relative mRNA and protein ratios of Bax/Bcl-2 in the SNpc of subacutely MPTP-exposed mice. The effects of selegiline on apoptosis were assessed by analyzing Bax and Bcl-2 expression by real-time PCR and western blot analyses. The relative mRNA level of the pro-apoptotic factor, Bax, increased in the ventral midbrain of MPTP-exposed mice (2.14-fold of saline control, P=0.037), while that of the anti-apoptotic factor, Bcl-2, did not differ significantly between the treated mice and the normal control mice (P>0.05). However, the mRNA ratio of Bax/Bcl-2 increased significantly in the MPTP-exposed mice (1.99-fold of saline control, P=0.002) (Fig. 4A); however, this increase was reversed within 14 days of selegiline treatment (58.79% of MPTP control, P=0.004) (Fig. 4A). Similarly, the protein ratio of Bax/Bcl-2 significantly increased in the MPTP-exposed mice (265.97% of saline control, P=0.000), and this increase was reversed by selegiline (52.91% of MPTP control, P=0.000) (Fig. 4B and C), due to an obvious downregulation of Bax (73.70% of MPTP control,

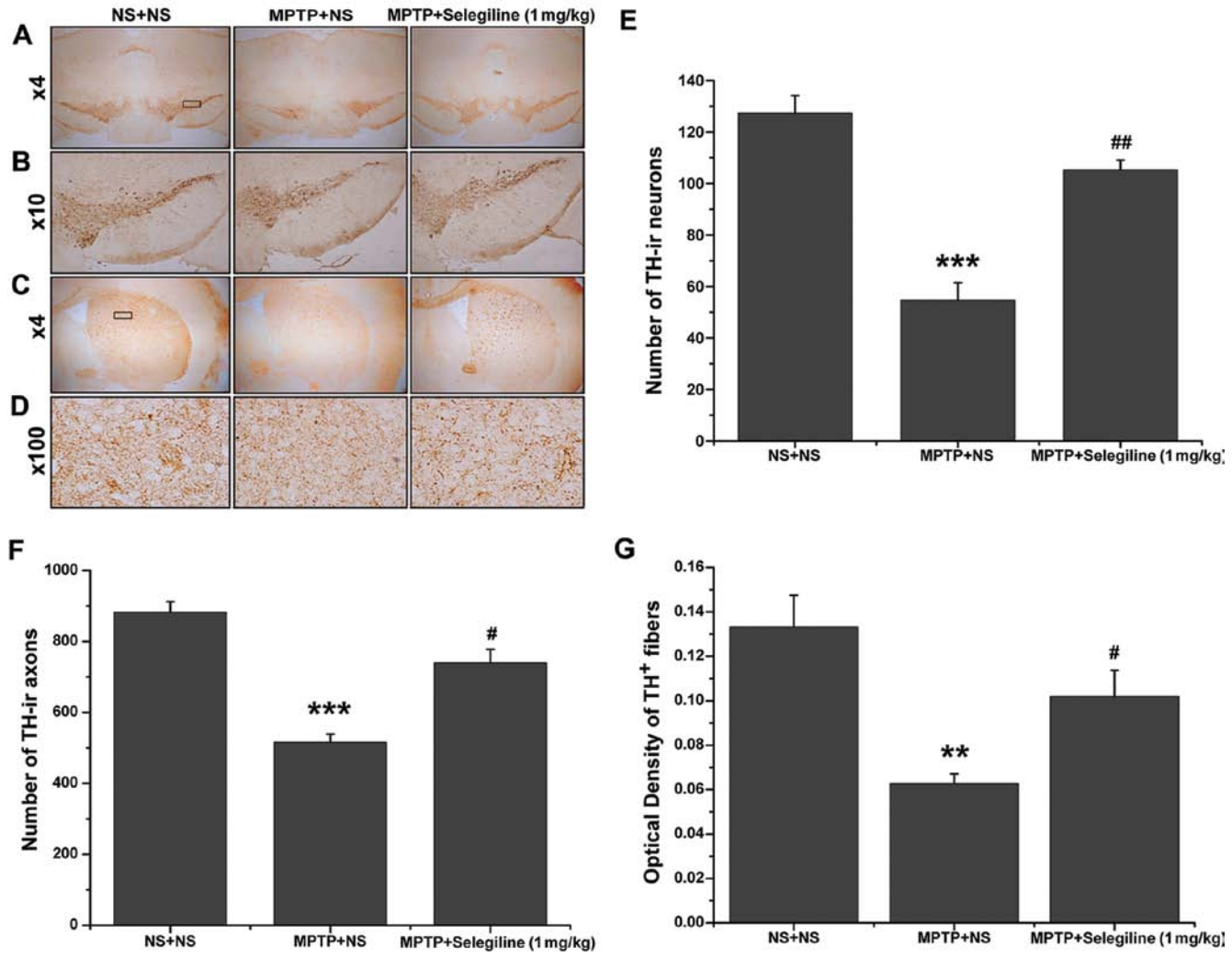

Figure 2. Neurorescue effects of selegiline against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) detected by tyrosine hydroxylase (TH) immunohistochemistry. (A and B) Representative photomicrographs of TH-positive neurons in the *substantia nigra pars compacta* (SNpc). (C and D) Representative photomicrographs of TH-positive striatal fibers. (E) Following MPTP injection, the number of TH-positive neurons in the SNpc was significantly reduced; however, a marked recovery was observed in the selegiline-treated (1.0 mg/kg/day) group compared with the vehicle-treated MPTP-exposed mice. (F and G) MPTP decreased the number of TH-positive axons and the optical density (OD) of striatal fibers, which were preserved by selegiline treatment. Data are expressed as the means ± SEM (n=5 each). **P<0.01, ***P<0.001 vs. NS + NS group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. MPTP + NS group. NS, normal saline; TH-ir axons, axons showing TH-like immunoreactivity.

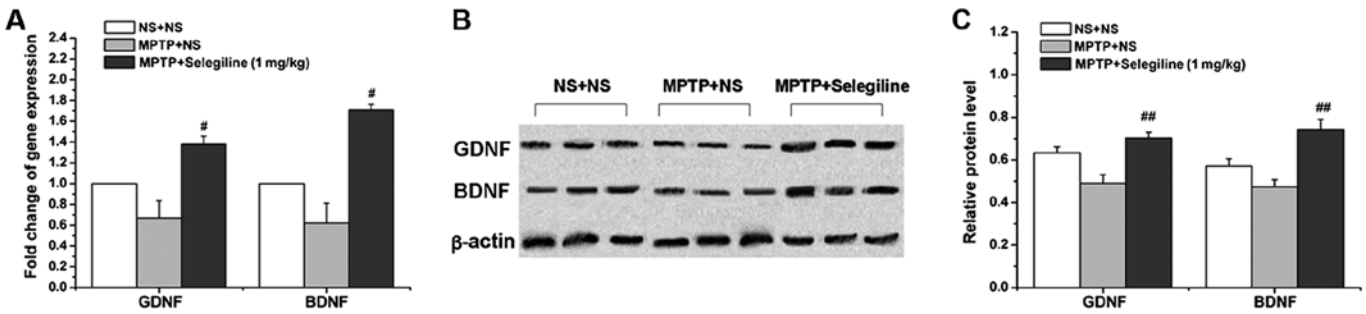

Figure 3. Real-time polymerase chain reaction and western blot analyses of relative mRNA and protein expression levels of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the ventral midbrain. (A) The relative mRNA expression of GDNF and BDNF did not differ significantly in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) + NS and NS + NS groups; however, selegiline (1.0 mg/kg/day) enhanced the expression levels significantly compared with the MPTP + NS group. (B) Representative GDNF and BDNF protein bands, control lanes are β-actin. (C) The relative protein levels of GDNF and BDNF did not differ significantly in the MPTP + NS group compared with the NS + NS group; the expression levels were significantly increased in the selegiline-treated group compared with the MPTP + NS group. Data are expressed as the means ± SEM (n=5 each). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. MPTP + NS group. NS, normal saline.

P=0.023) and an upregulation of Bcl-2 (140.38% of MPTP control, P=0.001). Linear regression analysis revealed a strong negative correlation between the Bax/Bcl-2 protein ratio and the number of TH-positive SNpc neurons (Fig. 5E).

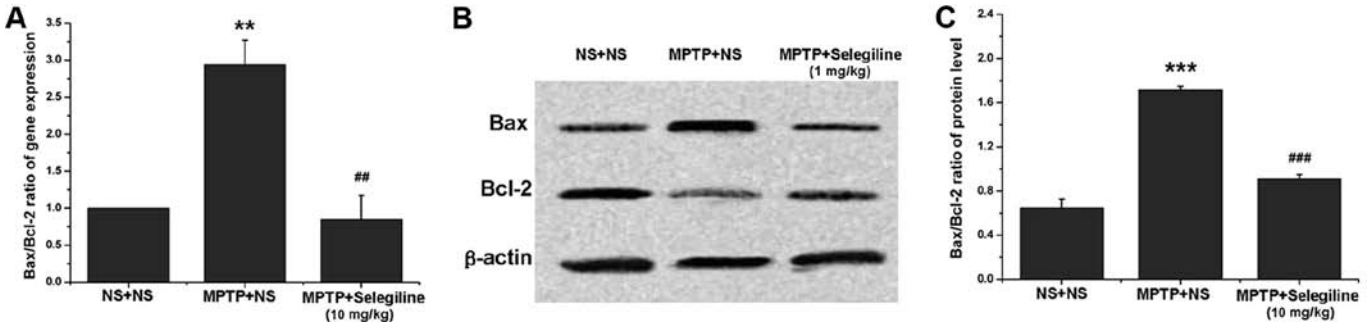

Figure 4. Real-time polymerase chain reaction and western blot analyses of relative mRNA and protein levels of Bax/Bcl-2 in the ventral midbrain. (A) The Bax/Bcl-2 ratio of relative mRNA expression in the MPTP + NS mice increased significantly compared with the saline-treated control mice; however, this increase was reversed by selegiline treatment compared with the MPTP-vehicle mice. (B) Representative Bax and Bcl-2 protein bands, control lanes are β-actin. (C) The Bax/Bcl-2 ratio of relative protein level in the MPTP + NS group increased significantly compared with the NS + NS control group and this increase was significantly decreased by selegiline. Data are expressed as the means ± SEM (n=5 each). **P<0.01, ***P<0.001 vs. NS + NS group; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. MPTP + NS group. NS, normal saline.

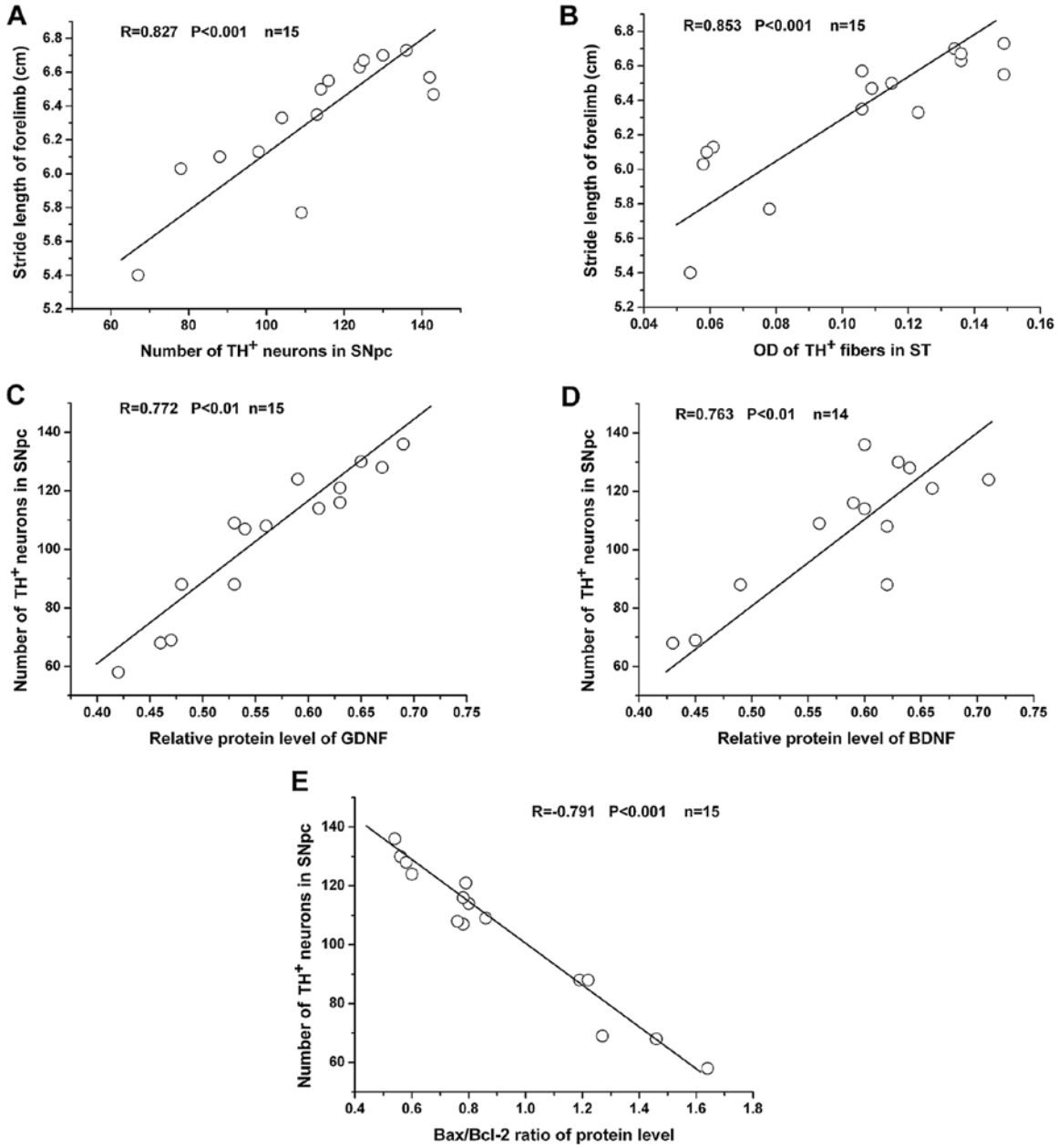

Figure 5. Correlation analyses. (A and B) Linear regression analysis of gait measure vs. tyrosine hydroxylase (TH)-positive *substantia nigra pars compacta* (SNpc) neurons and optical density (OD) of TH-positive striatal (ST) fibers. (C and D) Linear regression analysis of TH-positive neurons vs. relative glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) protein levels. (E) Linear regression of TH-positive neurons vs. Bax/Bcl-2 ratio. Correlation coefficient, P-value and number of individual pairs are shown.

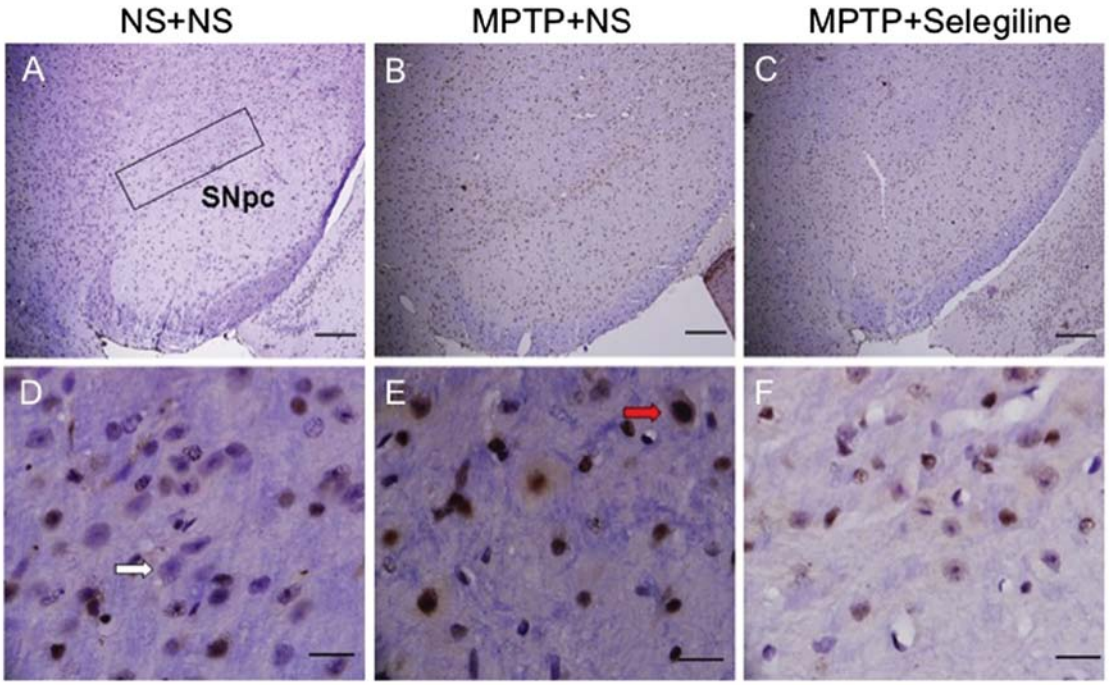

Figure 6. TUNEL analysis of apoptotic cells. (A-C) Low- and (D-F) high-power representative photomicrographs of TUNEL-positive cells in the *substantia nigra pars compacta* (SNpc). Normal cells possess integrated nuclear membranes and soma (D, white arrow), while typical apoptotic cells have nuclear chromatin clumps and shrunken soma (E, red arrow). Scale bars, 200 μm (A-C); 20 μm (D-F). NS, normal saline.

Selegiline effectively reverses apoptosis in the SNpc of MPTP-treated animals. We performed TUNEL staining (Fig. 6) and observed that the SNpc of the MPTP/vehicle-treated animals contained more apoptotic nuclei (Fig. 6E, red arrow) than the control animals. Notably, the MPTP/selegiline-treated mice did not show any evidence of apoptosis (Fig. 6F).

Discussion

Our results demonstrate that selegiline, the first MAO-B inhibitor, rescues motor deficits and induces NTF expression in a subacute MPTP mouse model of PD, which is the most commonly used model of PD. The magnitude of the MPTP-induced lesion is dependent on the administration regimen (17). The subacute regimen induces a 40-50% depletion of striatal DA levels and a 30-40% SNpc neuronal loss (18). Compared to the more severe acute regimen, the subacute regimen was more appropriate for our experiments, in which we sought to identify neurorestorative effects.

We observed that 14 days of oral selegiline restored the number of nigral dopaminergic neurons, the number and density of striatal dopaminergic terminals and improved gait dysfunction compared with the vehicle/MPTP-treated mice. Moreover, our results suggest that the neurorescue effects of selegiline are mediated by the induction of GDNF and BDNF expression, as well its regulatory effects on Bax and Bcl-2, 2 key molecules of the Bcl-2 family involved in the apoptosis of dopaminergic neurons in PD pathogenesis.

MAO-B inhibition is known to diminish the rapid turnover of striatal DA, allowing for it to accumulate. For a patient with PD, blocking endogenous DA catabolism provides symptomatic relief through enhanced neurotransmission (32). Increasing endogenous DA concentrations may be a practical alternative to dopaminergic replacement therapy (33). Clinical studies have shown that compared with dopaminergic therapy, MAO-B inhibitors, including selegiline and rasagiline, offer limited symptomatic improvement when administered as monotherapy (34-36). Thus, it remains unclear whether the motor effects of selegiline are associated with MAO-B inhibition or with its neuroprotective activities. We noted a decrease in stride length in the MPTP-exposed mice, similar to the characteristic shuffling gait in patients with PD. Indeed, this results from a combination of hypokinesia, rigidity and posture and equilibrium defects. However, post-treatment selegiline reversed the shortening of the stride lengths. Moreover, this was not associated with other neurorescue mechanisms apart from MAO-B inhibition; the dose of selegiline (1.0 mg/kg/day) used in our study was lower than the dose reported to inhibit MAO (37). Furthermore, the delayed start of administration in our experiment ensured that any observed effects were not due to the compound interfering with the conversion of MPTP to its active metabolite, MPP^+, a reaction that is mediated by MAO-B (15). Previous studies have demonstrated that selegiline protects against MPP^+ toxicity, even in cell lines that lack MAO-B (38,39). Moreover, MAO-B-knockout mice are not protected from damage caused by hypoxia or MPP^+ (40). In short, the neurorescue effects on gait dysfunction observed suggest novel molecular mechanisms of action of selegiline that are independent of MAO-B inhibition.

Another significant effect of selegiline was the recovery of TH-immunopositive neurons and fibers in the MPTP-exposed mice. This finding is similar with the results of previous studies on rasagiline, a second-generation irreversible, selective MAO-B inhibitor. However, the effects of rasagiline on

striatal DA content did not correlate with its MAO-B inhibitory activity (41). Proteomic and genomic methods subsequently demonstrated that rasagiline induced the activation of cell signaling mediators associated with an NTF-responsive tyrosine kinase receptor (Trk) pathway and a downstream increase of phosphatidylinositol 3 kinase (PI3K) protein. The induction of NTFs, such as GDNF and BDNF seems to be associated with the neurorescue mechanism(s) of rasagiline (41). As regards selegiline, 'trophic-like' action or NTF induction has been reported in both *in vitro* and *in vivo* neuroprotective studies (13,42). Our data demonstrate the rescue effects of low-dose selegiline on dopaminergic neurons and fiber loss in MPTP-exposed mice and confirm that this subacute MAO inhibitory dose also induces GDNF and BDNF mRNA and protein expression, even after neuronal cell death has begun. These results support and extend those of previous studies, showing that both the gene and protein expression of several Trk-ligands (including GDNF and BDNF) are induced by selegiline and rasagiline. Moreover, they demonstrate the involvement of GDNF and BDNF in neurorescue or restorative treatment for neurodegenerative diseases, particularly PD. In our study, both the GDNF and BDNF protein levels were significantly positively correlated with the number of TH-positive SNpc neurons, which suggests that NTF reduction may play a role in pathological changes underlying PD and suggests that increasing NTF levels may be a useful therapeutic strategy.

Selegiline also increased neuronal survival by interfering with the apoptotic signaling pathway, independent of MAO-B inhibition. Previous studies have indicated that the neuroprotective effects of selegiline are associated with the decreased synthesis of pro-apoptotic proteins, such as Bax, c-jun and GAPDH, and the increased synthesis of anti-apoptotic proteins, such as Bcl-2, Cu-Zn superoxide dismutase and heat shock protein 70 (42). Bcl-2 pro-apoptotic family members are known to participate in neuronal death in a variety of PD models (43) and the ratio of Bax/Bcl-2 is used to determine whether cells will live or die (44). Thus, we investigated anti-apoptotic signaling in the subacute MPTP mouse model, in which dopaminergic neurodegeneration occurs through apoptosis. Similar with pre-treatment studies on selegiline (42), we found that the post-administration of selegiline inhibited the increase in the Bax/Bcl-2 ratio at the gene and protein level compared with the untreated MPTP-exposed group. In addition, the strong negative correlation between the Bax/Bcl-2 protein ratio with the number of TH-positive neurons further confirmed the involvement of Bcl-2 family members in the pathogenesis of a subacute MPTP-induced mouse model of PD. TUNEL assays further demonstrated that selegiline successfully prevented apoptosis, even when administered after MPTP. Although it remains unclear whether there are common pathways with respect to the correlation between Bcl-2 family members and NTF expression, our current results suggest that increasing endogenous GDNF and/or BDNF levels and regulating the expression of Bcl-2 pro-apoptotic family members may be a useful strategy for neuronal rescue therapies.

Finally, it seems prudent to discuss the clinical implications for the observed improvement in gait dysfunction in our study and the possible correlation with NTF induction. The effects of selegiline on gait dysfunction in the MPTP mouse model are not dependent on its MAO inhibitory effect, which is currently the focus of promising clinical investigations. In PD, motor symptoms, such as bradykinesia and rigidity respond well to DA replacement medications. Although balance and gait problems may also be reversed by dopaminergic agents early in the course of the disease, they usually become resistant to these therapies as the disease progresses (45). The effects of selegiline on gait dysfunction and the significantly positive correlation between stride length and pathological characteristics in our study further support the efficacy of the compound on PD-related motor dysfunction. Indeed, several studies have reported that NTFs delay neuronal degeneration and the progression of abnormal gait or walking patterns in rats (46-48). In patients with PD, intraputaminal GDNF infusion resulted in the significant, sustained improvement of bilateral motor functions, including gait and balance (49). These findings are in agreement with our presumption that selegiline ameliorates gait impairment and rescues the loss of dopaminergic neurons, mostly likely through the induction of GDNF and BDNF expression.

In conclusion, the present study demonstrates that selegiline exerts neurorescue effects on MPTP-induced gait dysfunction and the loss of dopaminergic neurons and fibers *in vivo*. These effects appear to correlate with the multifactorial activities of this compound, including the enhancement of GDNF and BDNF expression levels and the suppression of apoptosis in the ventral midbrain of a subacute MPTP-exposed mouse model through the regulation of Bcl-2 family members. Combined with the results of previous *in vitro* and *in vivo* studies regarding the neuroprotective effects of selegiline, we further demonstrate the efficacy of selegiline in delaying PD symptom progression and reversing existing neurodegenerative damage, even at a dose that does not inhibit MAO-B.

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