

Research report

An intermittent, controlled-rate, slow progressive degeneration model of Parkinson's disease: antiparkinson effects of Sinemet and protective effects of methylphenidate

Sheila M. Fleming^{a,*}, Yvon Delville^a, Timothy Schallert^{a,b}

^a Department of Psychology, Institute for Neuroscience, University of Texas, Austin, TX 78712, USA

^b Department of Neurosurgery, Center for Human Growth and Development, University of Michigan, Ann Arbor, MI, USA

Received 22 August 2003; received in revised form 18 May 2004; accepted 20 May 2004

Available online 7 July 2004

Abstract

The causes of nigrostriatal neuron degeneration in Parkinson's disease (PD) are not known, but it has been suggested that exogenous or endogenous factors or neurotoxins may play a role. The degree of vulnerability to neurotoxins or other potential mediators of nigral dopamine cell death is thought to be important in understanding Parkinson's disease. In most animal models, the rate of terminal degeneration and corresponding functional impairment is too rapid to investigate effectively either cell vulnerability or the potential benefits of some neuroprotective treatments. In the present study, a new model of Parkinson's disease is described that might help in addressing the issue of nigral cell vulnerability and to evaluate interventions with clinical potential. 6-Hydroxydopamine (6-OHDA) was infused in escalating, intrastriatal doses over several weeks. Control animals received multiple infusions of vehicle at the same volume. Behavioral testing was carried out between each infusion, including forelimb-use and somatosensory function. A symptomatic threshold was established for each animal, indicating the amount of neurotoxin required to induce a stable deficit. Oral administration of L-DOPA (Sinemet) ameliorated limb-use asymmetries acutely. An immunocytochemical assay for tyrosine hydroxylase, a dopamine cell marker, revealed a partial loss of immunoreactive cells in the substantia nigra. Animals that were co-administered methylphenidate (MPH), a dopamine transport inhibitor, along with the 6-OHDA were spared from the behavioral and neurochemical effects of 6-OHDA, despite receiving more than twice as much neurotoxin as controls. These data suggest that establishing a symptomatic threshold preclinically may help researchers evaluate potential treatments and model individual and group resistance to nigrostriatal insults.

© 2004 Elsevier B.V. All rights reserved.

Keywords: 6-Hydroxydopamine; Neuroprotection; Parkinson's disease; Methylphenidate; Sinemet

1. Introduction

Parkinson's disease (PD) is characterized by a progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc). This degeneration results in decreased DA availability, primarily to the terminal regions in the striatum

([23] as cited in [24,4]). When a sufficient number of neurons have been lost, humans display readily detectable motor abnormalities, including akinesia, resting tremor, and rigidity, that worsen as the disease progresses [46,61].

A useful animal model of PD should resemble the disease anatomically and symptomatically, and respond positively to current treatments [13]. Although the cause of most PD cases is unknown, there are many substances that can be toxic to DA neurons in a variety of species. These include 6-hydroxydopamine (6-OHDA; [78,82,85,91]), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; [6,10,44]), paraquat [53], and rotenone [5]. Inducing partial 6-OHDA

* Corresponding author. Present address: Department of Neurology, David Geffen School of Medicine at UCLA, Reed Neurological Research Center B-114, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA. Tel.: +1 310 267 1782; fax: +1 310 267 1786.

E-mail address: sfleming@ucla.edu (S.M. Fleming).

lesions of the nigrostriatal pathway by infusing a single, large dose of 6-OHDA into the terminal region of the striatum has become a well-accepted method for testing treatment strategies [15,17,18,21,33,38,41]. Unilateral delivery is one advantage of this model; another is the existence of a modestly long but practical window in which the cells can be rescued. In humans, however, SNc cells degenerate more slowly, and possibly intermittently, over the course of several years [21]. This pattern of cell loss is associated with the increasing loss of DA terminals. That is, an SNc neuron may survive until a sufficient number of its terminals degenerate or fail to engage receptors left vacant by the loss of other terminals. A reasonable strategy for dealing with PD would be to combine early detection with treatments that could stop or significantly slow its progression. The present study developed an alternative animal model that involves multiple, spaced exposures to 6-OHDA over an extended period of time, leaving ample opportunity to titrate the amount of neurotoxin based on sensitive behavioral markers.

To produce slow, intermittent degeneration of DA neurons, animals were injected intrastrially with multiple, escalating doses of 6-OHDA over several weeks and tested for behavioral impairment after each infusion. To validate the model, animals were treated with Sinemet, the most common treatment for PD, after the lesion was established. In a separate group of animals, the sensitivity of the model was tested using the dopamine transport (DAT) inhibitor methylphenidate (MPH). Sinemet should temporarily ameliorate motor impairments whereas MPH should reduce the entry of neurotoxins such as 6-OHDA or MPTP into DA terminals [43,78] and increase the symptomatic threshold in 6-OHDA-treated animals.

2. Materials and methods

2.1. Experiment 1

The goal of Experiment 1 was to establish an animal model of PD that can determine an individual or group vulnerability to neurotoxic insult. Animals were exposed to multiple, spaced infusions of 6-OHDA and tested for behavioral impairments following each dose. Limb-use asymmetry was examined immediately following the administration of oral Sinemet (L-DOPA:Carbidopa) 2 weeks following the last 6-OHDA infusion to test whether limb-use impairments could be reversed similar to the typical effects of antiparkinson drugs given to PD patients.

2.1.1. Subjects and surgery

All experimental procedures were performed in accordance with the University of Texas Animal Care and Use Committee. Prior to surgery, animals were tamed by frequent handling and tested for limb preference with the limb-use asymmetry test [75,83,84]. Cannulae were implanted in the hemisphere opposite the preferred limb. Seventeen

male (350–400 g) Long-Evans hooded rats were anesthetized with Equithesin (0.35 cm³/100 g, i.p.), and atropine sulfate (0.4 mg/kg, s.c.) was administered to prevent mucus secretions that make breathing difficult. A single cannula (22 gauge, Plastics One, Roanoke, VA) was permanently implanted, with the tip centered in the dorsal aspect of the striatum just ventral to the corpus callosum (AP: +0.2, ML: ±3.5, DV: –3.0), and secured with surgical screws and dental acrylic. A dummy cannula (Plastics One, Roanoke, VA) was attached to each guide cannula to prevent clogging. Animals were allowed at least 1 week of post-operative recovery. Following surgery, awake animals ($n = 12$) were infused with the neurotoxin 6-OHDA every 3 days dependent on the appearance of a limb-use asymmetry (i.e., infusions occurred when there was no detectable asymmetry, if there was an asymmetry then the animal did not receive an infusion). For 6-OHDA infusions, the animal was held by the experimenter, and an injector needle (28 gauge, 5 mm, Plastics One, Roanoke, VA) connected to PE 50 tubing (Clay Adams) and attached to a Hamilton gastight syringe (1 cm³) was lowered into the indwelling cannula. A syringe pump infused the 6-OHDA at a rate of 1 µl/min. An air bubble was introduced in the tubing, far from the cannula, to track the infusion. The injector needle was left in the brain for several minutes after the infusion to allow for diffusion from the tip. The concentration of 6-OHDA increased with each injection (2.72, 5.44, 8.16, 10.88, and 13.60 µg/2 µl, calculated as free base) until the animal displayed a stable deficit (14 consecutive days of impairment) in the limb-use asymmetry test. Control animals ($n = 5$) received multiple vehicle (ascorbic saline) infusions at the same volume as 6-OHDA-treated animals. Extensive pilot work indicated that non-escalating, successive, low-dose infusions did not yield long-lasting behavioral deficits. Because limb-use asymmetry is positively correlated with the level of DA depletion at a wide range of terminal loss, this test was used to determine whether another injection was necessary [78,83,84]. A limb-use asymmetry score of greater than 10 was used as a minimum criterion for deciding whether there was a deficit representative of a partial DA depletion. Animals that displayed a stable score of 10 or above did not receive another infusion of 6-OHDA.

2.1.2. Limb-use asymmetry

Limb-use asymmetry [36,71,75] was measured 2 days after each 6-OHDA infusion. Forelimb-use during explorative activity was analyzed by videotaping rats in a transparent cylinder (20 cm diameter and 30 cm height) for 3–10 min depending on the degree of movement maintained during the trial. A mirror placed behind the cylinder at an angle enabled the rater to record forelimb movements when the animal was turned away from the camera. The cylindrical shape encouraged vertical exploration of the walls with the forelimbs, as well as landing activity. The cylinder was high enough that the animal could not reach the top edge by rearing, and wide enough to permit a 2 cm space between the tip of the snout and the base of the tail when the animal was at rest. A blinded

experimenter scored all trials using a VCR with slow-motion and frame-by-frame capabilities.

Several behaviors were scored to determine the extent of the forelimb-use asymmetry displayed by the animal. These behaviors were recorded during vertical movements along the wall and for landings after a rear, and included independent and simultaneous use of the left and/or right forelimb for contacting the wall during a full rear and landing following a rear. If an experimenter could not determine whether one limb was being used independently or simultaneously, that movement was not scored.

Behaviors were expressed in terms of percent independent use of each forelimb (ipsilateral and contralateral) or use of both forelimbs (simultaneous weight support or alternating stepping along vertical surfaces) relative to the total number of limb-use observations for movements along the wall and while landing (e.g., [contralateral/(ipsilateral + contralateral + both limb-use observations)] \times 100 = % contralateral).

Wall and landing ratios were averaged together for scores that reflected an equal contribution from asymmetries in both. Averaging these scores corrected for variability in the number of wall versus landing movements among animals or between groups. A single overall limb-use asymmetry score that included the percent impaired forelimb movements subtracted from the percent nonimpaired forelimb movements averaged for wall behavior and landing behavior (ipsi minus contra score) was used to determine whether another injection was needed. The minimum score defining a deficit for an individual animal was 10.

2.1.3. Movement initiation

Movement initiation was measured in each animal 1 day after 6-OHDA injections. To test forelimb movement initiation, stepping movements made with the ipsilateral and contralateral forelimbs were assessed using an isolated forelimb akinesia test [47,58,74,75,83,84]. The rat was held by its torso with its hindlimbs and one forelimb lifted above the surface of a table so that the weight of its body was supported by one forelimb alone. The number of self-initiated steps made in a 10 s trial was recorded for each forelimb for two trials and then averaged. To account for individual differences in the number of steps made between animals, ipsi minus contra scores were calculated [74].

2.1.4. Magnitude of somatosensory asymmetry

Somatosensory asymmetry was measured 1 day after 6-OHDA injections. Asymmetry was assessed using a bilateral tactile stimulation test [72,76,77]. Animals were first tested to indicate the presence of a somatosensory asymmetry. This was done by removing the animal from the home cage and attaching adhesive stimuli (Avery adhesive-backed labels, 113 mm²) to the distal-radial aspect of each forelimb in random order. After being returned to the home cage, rats contacted and removed the stimuli one at a time. The order and latency of stimulus contact and removal was recorded for each of five trials. The order of contact was used to de-

termine whether animals showed a bias for the stimulus on the forelimb unaffected by the injury. If the animal showed an 80% or greater preference for removing the stimulus from the nonimpaired forelimb first, it was then tested to determine the magnitude of the somatosensory asymmetry.

The psychophysical method of limits was adopted in the magnitude of asymmetry phase of the test. That is, the size of the impaired limb stimulus (*I*) was progressively increased over trials and the size of the nonimpaired limb stimulus (*N*) was simultaneously decreased by an equal amount (14.1 mm²). Thus, a sufficient increase in the *I/N* ratio caused a reversal of the original bias if the rat began to respond first to the stimulus placed on the impaired limb. The *I/N* ratio necessary to reverse the initial bias is proportional to the degree of injury [2,70,77]. Seven levels of stimulus pairs were used.

2.1.5. Validation with Sinemet

Sinemet (L-DOPA:Carbidopa) is effective in the early stages of PD and was used here to validate the model. After demonstrating a stable deficit on the limb-use asymmetry test, a subset of 6-OHDA-infused (*n* = 7) and vehicle-infused (*n* = 3) animals was given Sinemet (L-DOPA:Carbidopa, 4:1 ratio) suspended in 2% methylcellulose. The drug was administered orally through infant feeding tubes at a dose of 20 mg/kg [48]. After receiving the drug, the animals were tested for limb-use asymmetry. Because Sinemet lasts approximately 3 to 4 h, they were videotaped at different time points during a 4 h period (prior to Sinemet, 30, 60, 180, and 240 min after administration).

2.1.6. Drug-induced rotation

At least 1 week after Sinemet testing, all animals were administered amphetamine (3–5 mg/ml, i.p.) or (2 days later) apomorphine (0.25 mg/ml, s.c.) [86] and tested for turning response. Animals received the drug and 20 min later were placed into a large bowl where the number and direction of rotations were videotaped for 10 min and later scored.

2.1.7. Tyrosine hydroxylase immunocytochemistry

Immunocytochemical labeling for the DA cell marker tyrosine hydroxylase (TH) was used to estimate the extent of 6-OHDA-induced lesions [37,90]. Animals were sacrificed with CO₂ for 2 weeks following drug-induced rotation testing and their brains were rapidly removed and immersed in 10% Acrolein (Aldrich) in 0.1 M potassium phosphate buffer solution (KPBS, pH 7.2) overnight. They were then transferred to 20% sucrose in 0.1 M KPBS for 48 h. Post-fixed brains were sectioned on a freezing microtome. Forty-micrometer sections were taken at a sequence of 1/3 throughout the substantia nigra. Sections were washed in 0.05 M Tris-buffered saline (TBS, pH 7.6) several times and then washed in 1% sodium borohydride to remove remaining aldehydes. Sections were preincubated in a blocking solution containing 20% normal goat serum (NGS), 0.3% Triton X-100, and 0.3% hydrogen peroxide in 0.05 M Tris-buffered

saline. Sections were transferred to a primary wash containing primary antiserum (mouse anti-tyrosine hydroxylase, 1:20,000; Sigma, St. Louis, MO), 2% NGS, and 0.3% Triton X-100 for 48 h at 20 °C. Subsequent primary incubation sections were washed in TBS and incubated in a secondary antibody solution containing biotinylated goat anti-mouse IgG (Vector, Burlingame, CA), 2% NGS, and 0.3% Triton X-100 in TBS for 45 min. Sections were then transferred to a tertiary solution (Vectastain ABC Elite) in TBS for another 45 min. Visualization was done with diaminobenzidine (0.5 mg/ml, Sigma) and 0.05% hydrogen peroxide in TBS for 10 min. Sections were then mounted onto gelatin-coated slides and coverslipped with Permount.

2.1.8. Substantia nigra cell counts

TH immunoreactive (TH-IR) cells in the substantia nigra pars compacta were counted using camera lucida (10× objective) techniques. Approximately eight sections (representing Figs. 39–44) [59] throughout the substantia nigra were counted for each rat. The area was defined by specific landmarks in the region. Visualization of the cerebral peduncle and medial terminal nucleus of the accessory optic tract defined the medial border and excluded TH-IR cells in the ventral tegmental area from being counted. The lateral border was defined by the lateral terminal nucleus of the accessory optic tract. The slides were coded so the person counting the cells was blind to experimental conditions. Cell counts were added across sections and percent depletion was determined for each animal. This method can result in an over-estimation of tyrosine hydroxylase cell counts; therefore, percent depletion scores were calculated by the following formula: $[100 - ((\text{number of cells on the denervated side} / \text{the number of cells on the intact side}) \times 100)]$. The percent depletion controls for cell number variability between animals due to the staining procedure or number of sections included.

2.1.9. Statistics

Limb-use asymmetry and movement initiation were analyzed using a 2×3 mixed design ANOVA comparing groups (vehicle- and 6-OHDA-infused; between groups measure) across time (pre-injection, threshold, and last injection; within groups measure) with Fisher's Least Significant Difference (LSD) post hoc test. Magnitude of asymmetry and drug-induced rotation scores were analyzed using the Mann-Whitney *U* test. Planned *t* tests of limb-use asymmetry scores were used at each time point prior to (0) and 30, 60, 180, and 240 min after oral Sinemet testing to compare 6-OHDA- and vehicle-infused animals.

2.2. Experiment 2

The goal of Experiment 2 was to test the effects of the DAT inhibitor, MPH, when co-administered with 6-OHDA. Because 6-OHDA enters the cell via DAT, it was hypothesized that MPH would prevent 6-OHDA-induced cell dam-

age and increase the symptomatic threshold for these animals [77].

2.2.1. Subjects, surgery, and behavioral testing

Thirty-two animals received the same cannula implantation surgery, infusion criteria, and behavioral testing as in Experiment 1. Of these, three groups of rats received a co-treatment of methylphenidate (0.6, 1, or 2.5 mg/kg, i.p.) 15 min prior to each intrastriatal 6-OHDA infusion. Groups were labeled "No MPH" ($n = 14$), "0.6 MPH" ($n = 6$), "1.0 MPH" ($n = 7$), and "2.5 MPH" ($n = 5$). Animals in the No MPH group received a pre-treatment of vehicle (i.p.) 15 min before 6-OHDA. Testing of movement initiation, somatosensory asymmetry, and limb-use asymmetry was carried out within 3 days after each 6-OHDA infusion, and drug-induced rotation with amphetamine and apomorphine was measured at least 2 weeks following the last 6-OHDA infusion. At the end of the experiment the animals were sacrificed and their brains stained by immunocytochemistry for tyrosine hydroxylase.

2.2.2. Statistics

A one-way ANOVA was used to compare the cumulative 6-OHDA received by each group (No MPH, 0.6 MPH, 1.0 MPH, and 2.5 MPH). For limb-use asymmetry, movement initiation, and drug rotation, a one-way ANOVA was used to compare groups (No MPH, 0.6 MPH, 1.0 MPH, and 2.5 MPH), followed by Fisher's LSD post hoc tests. Magnitude of asymmetry was analyzed using Mann-Whitney *U*. For histological evaluation, TH-IR cell bodies in the substantia nigra were counted throughout the region for the right and left hemispheres. Percent remaining TH-IR positive neuron scores were compared using a one-way ANOVA followed by Fisher's LSD post hoc tests.

3. Results

3.1. Experiment 1

3.1.1. 6-OHDA required to induce behavioral deficit

The mean (\pm S.E.M.) cumulative amount of 6-OHDA (expressed as free base) required to impart a stable behavioral deficit was 29.47 (\pm 4.56) μ g and the mean number of injections needed was 3.85 (\pm 0.26) over 11.55 (\pm 0.79) days (each infusion being 3 days apart). A total of 17 animals (6-OHDA = 12, vehicle = 5) were included in the analysis. Threshold levels were measured by calculating the highest cumulative amount of 6-OHDA delivered (i.e., tolerated) without the presence of a behavioral deficit. The mean threshold level was 18.59 (\pm 3.54) μ g (Fig. 1). The average highest single dose of 6-OHDA that failed to produce a stable behavioral deficit was 8.16 μ g. This is a dose that, in previous studies in our lab and others, consistently causes severe and stable behavioral deficits when infused into the 6-OHDA-naive rat brain.

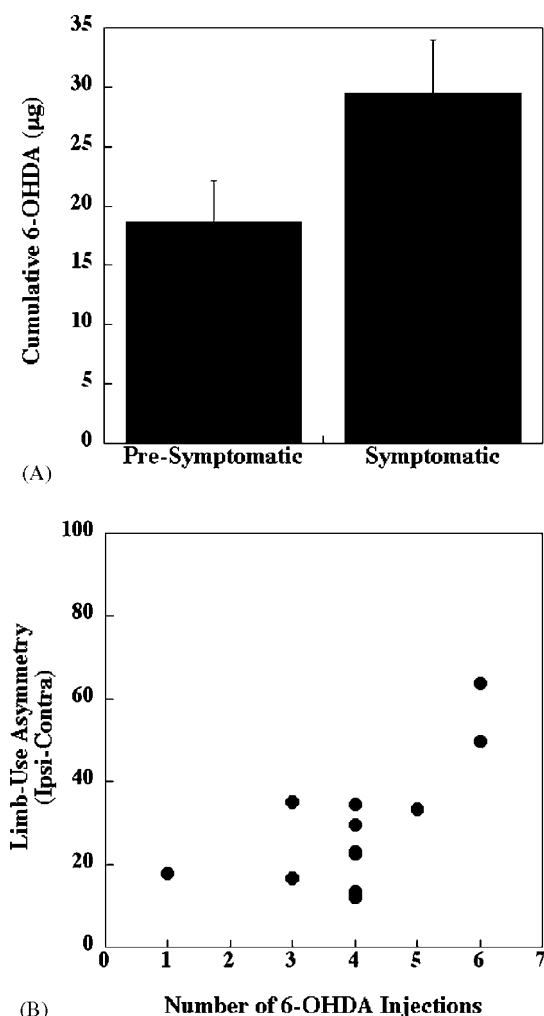


Fig. 1. (A) Cumulative 6-OHDA required to induce a deficit in limb-use asymmetry. The pre-symptomatic bar represents how much neurotoxin animals received just before displaying a deficit (i.e., threshold). The symptomatic bar represents how much neurotoxin was required to induce a stable deficit. (B) Scatter plot illustrating the number of injections required to induce a limb-use asymmetry in each animal receiving intrastriatal infusions of 6-OHDA.

3.1.2. Limb-use asymmetry

The mean \pm S.E.M. limb-use asymmetry scores for 6-OHDA-treated animals were $-3.2 (\pm 3.66)$ pre-6-OHDA, $-1.59 (\pm 3.94)$ threshold, and $29.27 (\pm 4.45)$ last infusion needed for stable deficit. Limb-use asymmetry scores for vehicle-infused animals were $-11.26 (\pm 4.43)$, $-17.97 (\pm 8.20)$, and $-11.62 (\pm 8.46)$ at each time point, respectively. ANOVA indicated a significant main effect of group, $F(1,15) = 30.71$, $P < 0.01$. 6-OHDA-infused animals had significantly higher ipsi minus contra scores, indicating an ipsilateral limb-use bias. There was a main effect of time with $F(2,30) = 6.03$, $P < 0.01$ and a significant group-by-time interaction, $F(2,30) = 4.32$, $P < 0.05$. Post hoc tests (Fisher's LSD, corrected) showed a significant increase in ipsi bias after the last injection for 6-OHDA-infused animals compared to vehicle-infused animals ($P < 0.01$). Within the 6-OHDA

group, ipsi-bias scores were significantly higher following the last injection of 6-OHDA compared to pre-injection scores ($P < 0.01$; Fig. 2A).

3.1.3. Movement initiation

The average number of steps prior to 6-OHDA infusions was $5.69 (\pm 0.39)$ for the ipsi limb and $5.64 (\pm 0.32)$ for the contra limb. After the last infusion of 6-OHDA, mean initiation steps were $6.31 (\pm 0.43)$ for the ipsi limb and $2.92 (\pm 0.49)$ for the contra limb. Ipsi minus contra scores for 6-OHDA-treated animals were $0 (\pm 0.28)$ pre-6-OHDA, $1.77 (\pm 0.98)$ threshold, and $2.04 (\pm 0.73)$ last infusion. Vehicle-infused scores were $-0.3 (\pm 0.66)$, $-1.3 (\pm 1.83)$, and $-1.5 (\pm 0.69)$ for the same time points, respectively. ANOVA showed a significant effect of group with $F(1,15) = 6.13$, $P < 0.05$. Fisher's LSD indicated a significant increase in ipsi minus contra scores for the last injection of 6-OHDA compared to the last injection of vehicle ($P < 0.01$). Within the 6-OHDA-infused group, ipsi minus contra scores were significantly higher after the last injection of 6-OHDA compared to pre-injection scores ($P < 0.05$; Fig. 2B).

3.1.4. Magnitude of somatosensory asymmetry

Somatosensory asymmetry scores for 6-OHDA-treated animals were $0.63 (\pm 0.41)$ pre-6-OHDA, $1.46 (\pm 0.64)$ threshold, and $3.0 (\pm 0.73)$ last infusion. The scores for vehicle-infused animals were $1.3 (\pm 1.3)$, $0.6 (\pm 0.37)$, and $0.6 (\pm 0.49)$ for the same time points, respectively. Mann-Whitney U indicated a significant increase in the magnitude of asymmetry of the 6-OHDA group after the last infusion compared to that of the vehicle group after its last infusion ($P < 0.05$). Within the 6-OHDA group there was a significant increase in magnitude of asymmetry from pre-injection to the last infusion of 6-OHDA ($P < 0.05$; Fig. 2C).

3.1.5. Validation with Sinemet

At least 2 weeks following the last infusion of 6-OHDA, a subset of animals that displayed a deficit on the limb-use asymmetry test was given the antiparkinson drug Sinemet (L-DOPA:Carbidopa). Ten rats (6-OHDA = 7, vehicle = 3) received 20 mg/kg oral Sinemet and were tested for limb-use asymmetry prior to and then 30, 60, 180, and 240 min after drug administration for approximately 3 min at each interval. Planned comparisons revealed a significant difference between 6-OHDA-infused and vehicle-infused groups at the 0 and 30 min time points ($P < 0.01$ and $P < 0.05$, respectively), demonstrating an ipsilateral limb-use bias for 6-OHDA animals. There was a significant reduction in limb-use asymmetry within the 6-OHDA group at the 60 and 180 min time points ($P < 0.05$; Fig. 3A).

3.1.6. Drug-induced rotation

Two weeks after Sinemet testing, all animals received injections of amphetamine (3–5 mg/kg, i.p.), were placed in a standard rotation-behavior bowl and the number and direction of rotations were calculated in a 10 min sample.

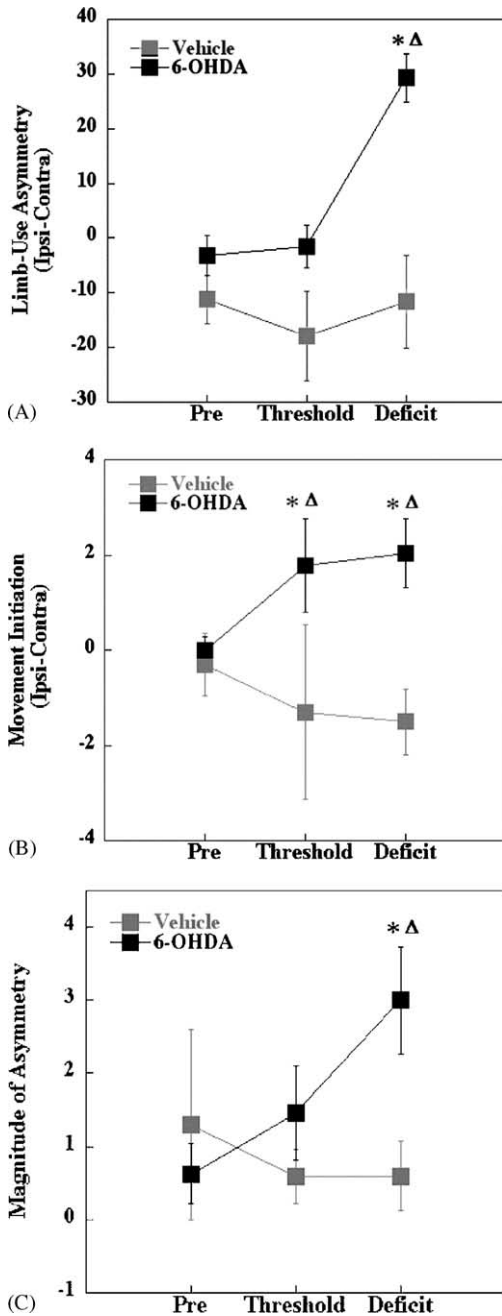


Fig. 2. Effects of 6-OHDA on behavior. (A) Limb-use asymmetry: on average, 6-OHDA animals did not display a deficit after receiving 18.59 (± 3.54) μg of 6-OHDA (threshold) but did show a significant deficit after 29.47 (± 4.56) μg , $P < 0.01$ compared to the vehicle group and to their own pre-infusion and threshold scores. (B) Movement initiation: 6-OHDA animals displayed a significant impairment at threshold levels and following 29.47 (± 4.56) μg of 6-OHDA, $P < 0.01$ compared to the vehicle group and their own pre-infusion scores. (C) Magnitude of asymmetry: 6-OHDA animals displayed a significant somatosensory deficit after 29.47 (± 4.56) μg 6-OHDA, $P < 0.01$ compared to the vehicle group and their own pre-infusion scores. The symbol (*) represents significantly different from the vehicle group and (Δ) represents significantly different from pre-infusion scores.

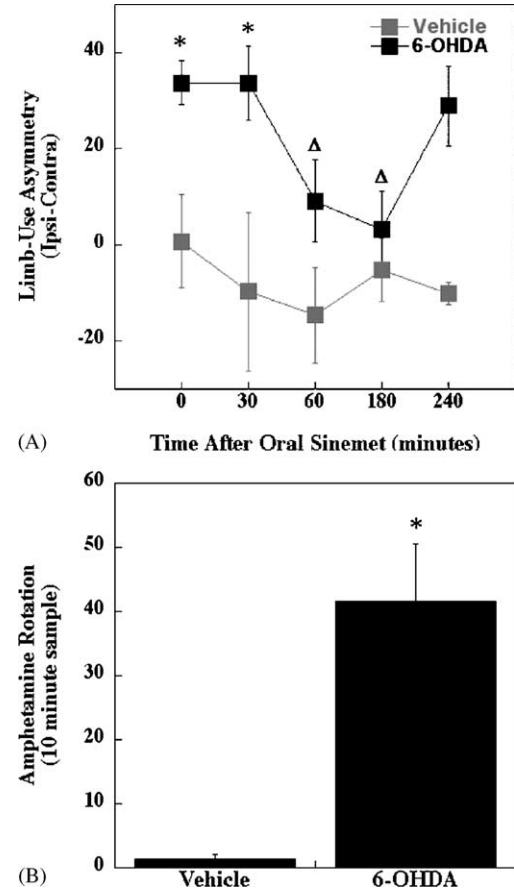


Fig. 3. Sinemet and amphetamine effects. (A) Following multiple infusions of 6-OHDA, a subset of impaired animals was administered oral Sinemet (L-DOPA:Carbidopa, 20 mg/kg) and tested for limb-use asymmetry. Animals were tested prior to (0) and 30, 60, 180 and 240 min after administration. The symbol (*) represents a significant difference from the vehicle-infused group and from the 6-OHDA-infused group at 60 and 180 min ($P < 0.05$) and (Δ) represents a significant difference from time zero (no Sinemet), $P < 0.05$. (B) Amphetamine rotation: animals receiving an average of 29.47 (± 4.56) μg of 6-OHDA rotated significantly more than vehicle-infused animals, $P < 0.01$.

Two days following amphetamine testing, animals were given an injection of apomorphine (0.25 mg/kg, s.c.) and rotations were calculated in a similar manner. The mean number of rotations in response to amphetamine was 41.5 (± 9.03) in a 10-min sample for the 6-OHDA-infused group and 1.4 (± 0.68) for the vehicle-infused group (Fig. 3B). Mann-Whitney U showed a significant difference between 6-OHDA-infused compared to vehicle-infused animals ($P < 0.01$). Animals did not rotate to apomorphine, confirming that the model reflects non-severe DA depletion.

3.1.7. Tyrosine hydroxylase immunocytochemistry

The mean percent of TH-IR cell depletion for animals receiving multiple infusions of 6-OHDA ($n = 6$) was 34.75 (± 10.62). This level of depletion demonstrates a partial loss of TH-IR neurons in the substantia nigra (Fig. 4). There was

no correlation in the DA-depleted animals between level of TH-IR and amount of 6-OHDA tolerated ($r = 0.16$, ns).

3.2. Experiment 2

3.2.1. 6-OHDA required to induce behavioral deficit

The mean (\pm S.E.M.) cumulative amount of neurotoxin tolerated by each group was 24.48 (\pm 3.00) μ g for No MPH, 28.11 (\pm 11.05) μ g for 0.6 MPH, 65.28 (\pm 3.85) μ g for 1.0 MPH, and 58.21 (\pm 7.91) μ g for 2.5 MPH. The 1.0 and 2.5 mg/kg MPH groups tolerated, and therefore received, significantly more 6-OHDA than No MPH and 0.6 MPH animals, $F(3,28) = 13.44$, $P < 0.01$ (Fig. 5). Behaviorally, both 1.0 and 2.5 mg/kg MPH animals showed a reduction or absence in limb-use asymmetry, movement initiation deficits, and somatosensory asymmetry following the last infusion of 6-OHDA. No MPH animals showed significant deficits in all three tests despite the fact that animals in the 1.0 and 2.5 mg/kg MPH groups received over twice as much neurotoxin as they did.

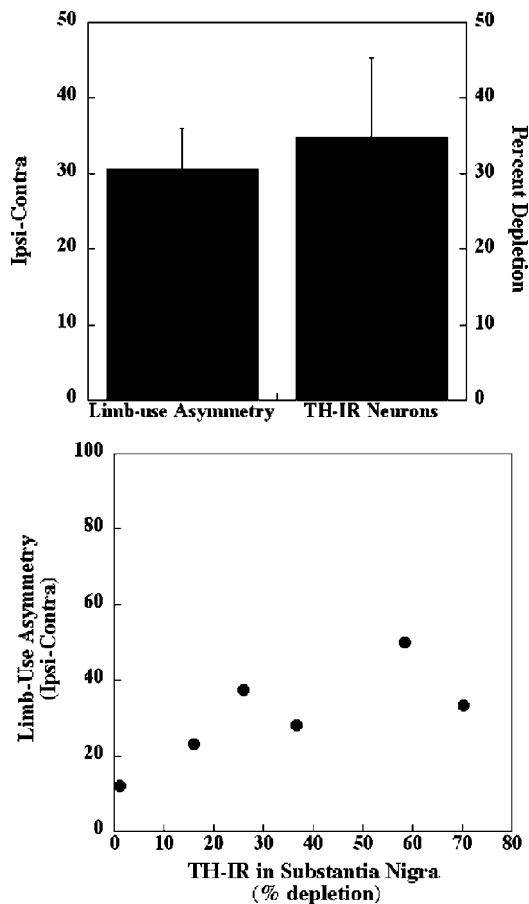


Fig. 4. TH-IR and limb-use asymmetry. (A) Mean limb-use asymmetry score (left) and corresponding mean percent TH-IR cell loss (right) after multiple infusions of 6-OHDA. (B) A scatter plot of the correlation between limb-use asymmetry scores and TH-IR positive neurons in the substantia nigra ($r = 0.74$, ns).

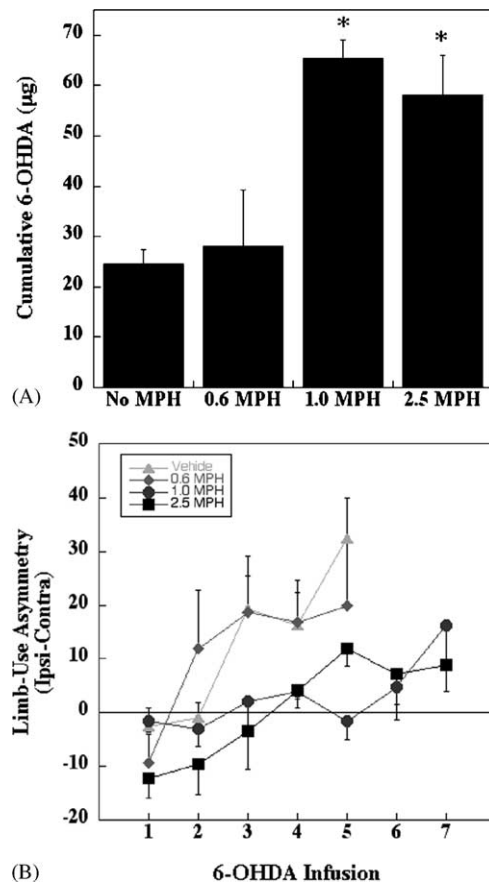


Fig. 5. Cumulative 6-OHDA exposure. (A) Mean amount of 6-OHDA tolerated for animals receiving No MPH (vehicle injection), 0.6 mg/kg (0.6 MPH), 1.0 mg/kg (1.0 MPH), or 2.5 mg/kg methylphenidate (1–2.5 MPH). The 1.0 and 2.5 mg/kg MPH group tolerated significantly more 6-OHDA compared to No MPH, despite having little or no behavioral impairment. The symbol (*) represents $P < 0.01$ compared to No MPH animals. (B) Mean limb-use asymmetry scores at each injection of 6-OHDA.

3.2.2. Limb-use asymmetry

Average limb-use asymmetry scores after the last infusion were 32.10 (\pm 4.23) for No MPH, 27.69 (\pm 6.0) for 0.6 mg/kg MPH, and 13.10 (\pm 3.17) for 1.0 mg/kg MPH, and 16.64 (\pm 3.16) for 2.5 mg/kg MPH animals. ANOVA indicated a significant main effect of group $F(3,28) = 3.99$, $P < 0.05$. Limb-use asymmetry scores were higher in the No MPH group after the last injection, compared to the 1.0 and 2.5 mg/kg MPH groups ($P < 0.05$; Fig. 6A).

3.2.3. Movement initiation

Movement initiation scores after the last infusion were 3.82 (\pm 0.96) for No MPH, 1.58 (\pm 0.94) for 0.6 mg/kg MPH, -0.5 (\pm 0.82) for 1.0 mg/kg MPH, and 0.3 (\pm 0.49) for 2.5 mg/kg MPH animals. ANOVA indicated a significant main effect of group $F(3,28) = 4.34$, $P < 0.05$. Movement initiation deficits were higher in the No MPH group after the last injection compared to the 1.0 and 2.5 mg/kg MPH groups ($P < 0.05$; Fig. 6B).

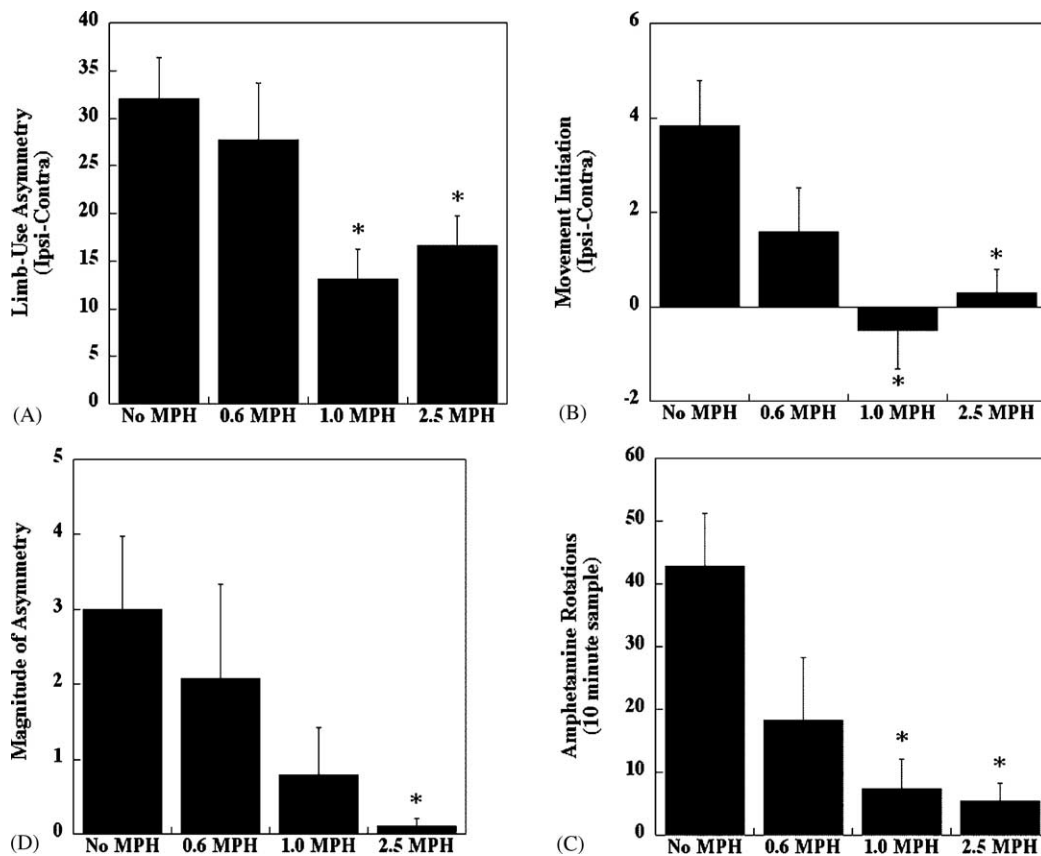


Fig. 6. MPH effects on behavior. (A) Despite receiving twice as much 6-OHDA, the 1.0 and 2.5 MPH groups' limb-use asymmetry scores were significantly reduced compared to the No MPH group. (B) No MPH animals displayed a significant impairment in movement initiation compared to the 1.0 and 2.5 mg/kg MPH animals. (C) 2.5 MPH mg/kg animals displayed a reduced somatosensory asymmetry compared to No MPH animals. (C) The No MPH group rotated significantly more than 1.0 and 2.5 mg/kg MPH animals. The symbol (*) represents $P < 0.01$ compared to No MPH animals.

3.2.4. Magnitude of somatosensory asymmetry

Average magnitudes of asymmetry scores after the last infusion were $3.0 (\pm 0.98)$ for No MPH, $2.08 (\pm 1.25)$ for 0.6 mg/kg MPH, $0.79 (\pm 0.63)$ for 1.0 mg/kg MPH, and $0.1 (\pm 0.1)$ for 2.5 mg/kg MPH animals. Mann-Whitney U indicated that somatosensory asymmetry scores were higher in the No MPH group after the last injection compared to the 2.5 mg/kg MPH group ($P < 0.05$; Fig. 6C).

3.2.5. Drug-induced rotation

Following behavioral testing, all animals received injections of amphetamine (3–5 mg/kg, i.p.) and the number and direction of rotations were calculated in a 10 min sample. The mean number of rotations in response to amphetamine was $42.77 (\pm 8.43)$ for No MPH, $18.33 (\pm 9.95)$ for 0.6 mg/kg MPH, $7.33 (\pm 4.77)$ for 1.0 mg/kg MPH, and $5.5 (\pm 2.75)$ for the 2.5 mg/kg MPH animals (Fig. 6D). No-MPH animals rotated significantly more than those in the 1.0 and 2.5 MPH groups ($P < 0.05$).

3.2.6. Tyrosine hydroxylase immunocytochemistry

Average percent remaining scores were $50.87 (\pm 7.35)$ for No MPH, $52.09 (\pm 7.99)$ for 0.6 mg/kg MPH, $80.84 (\pm 7.79)$ for 1.0 mg/kg MPH, and $89.73 (\pm 4.36)$ for 2.5 mg/kg

MPH. ANOVA showed that the No MPH group had significantly fewer remaining TH-IR neurons compared to the 1.0 and 2.5 MPH combined group, $P < 0.05$ indicating an estimated sparing of DA neurons by MPH co-treatment (Figs. 7 and 8).

4. Discussion

The results of this study show that unilateral terminal degeneration, induced by escalating, multiple infusions of 6-OHDA into the striatum, produces subtle behavioral impairments and a partial loss of the TH-IR neurons in the SNc. Functionally, animals displayed significant deficits in limb-use asymmetry, movement initiation, and somatosensory asymmetry. Animals rotated in response to amphetamine but not to apomorphine, which is consistent with partial lesions of the nigrostriatal pathway [1,38,45]. A unique aspect of this study is that the dosing regimen was individualized to each animal according to its functional outcome. This approach controls for differences in vulnerability to the neurotoxin and any variability due to the infusion procedure or location of the cannula tip. Because the animals required varying amounts of neurotoxin, a threshold for symptoms could be established.

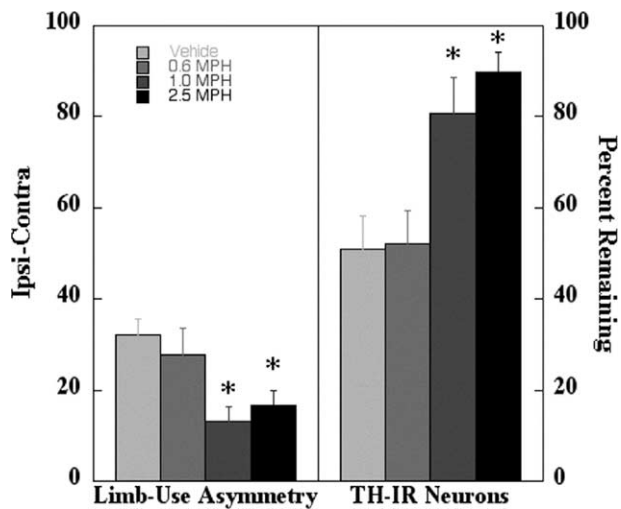


Fig. 7. Limb-use asymmetry and percent remaining TH-IR in the SNc. Values are expressed as means \pm S.E.M. Animals receiving the 1.0 and 2.5 mg/kg MPH prior to 6-OHDA displayed an attenuated behavioral deficit and TH-IR neuron sparing in the SNc compared to animals that received No MPH prior to 6-OHDA infusions. It is important to note that animals administered 1.0 and 2.5 mg/kg MPH received almost twice as much neurotoxin by the end of the experiment as animals that were not administered MPH.

Once animals were symptomatic, administration of oral Sinemet alleviated limb-use asymmetry deficits for several hours, which parallels the effects of this drug in humans with PD. Finally, symptomatic animals showed an approximately 35% loss of TH-IR neurons in the SNc, suggesting a partial lesion. The benefit of such a model is that potential therapeutic treatments can be tested at varying stages of degeneration.

The intermittent 6-OHDA delivery approach provides a thorough assessment of motor and somatosensory abilities following each infusion of the drug. Frequent monitoring of behavior allows for early detection of deficits and the development of a symptomatic threshold. We established a maximum sub-threshold for which animals are asymptomatic after low concentrations of 6-OHDA and a threshold for which they become more chronically symptomatic following the next increased concentration of 6-OHDA. After the final infusion of 6-OHDA, all animals displayed significant limb-use and somatosensory asymmetries compared to pre-infusion and sub-threshold doses. Additionally, preconditioning may have played a role in rendering resistance across multiple neurotoxin exposures. In the present study, that is, it seems possible that the striatum was partially preconditioned by the prior intermittent exposures to 6-OHDA so that deficits did not appear until a much higher than expected dose was sustained. We have found that the highest non-symptomatic dose in the present study would have produced a very severe and stable deficit in toxin-naïve rats. Preconditioning is a well-established effect in hypoxia/ischemia models and may be due to transient activation of multiple neuroadaptive and neuroprotective mechanisms [31,40].

Detection of functional deficits with partial loss of nigrostriatal DA neurons varies across studies [1,12,14,20,38]. Sev-

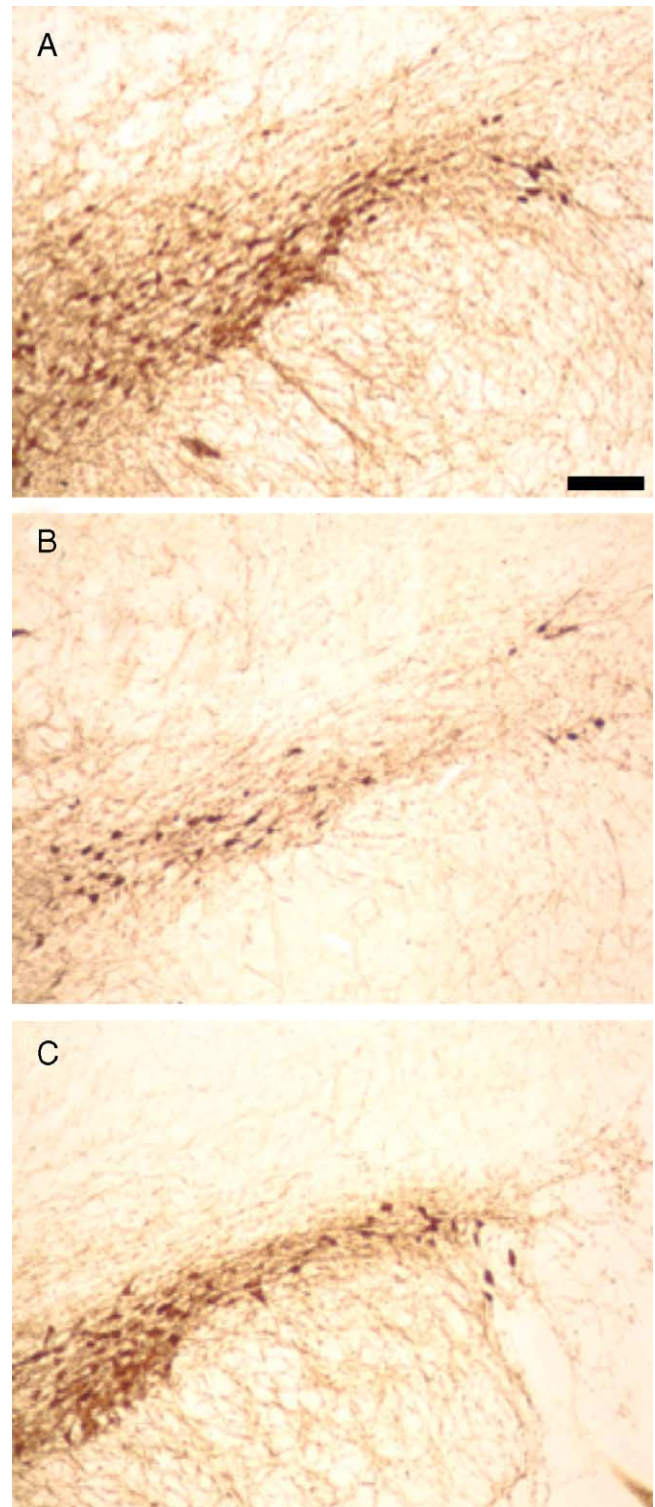


Fig. 8. Photomicrographs of representative TH-IR cells in the SNc. (A) Vehicle only, 10 \times , scale bar equals 100 μ m. (B) Multiple infusions of 6-OHDA. (C) 2.5 mg/kg MPH co-administered with 6-OHDA.

eral studies have demonstrated that there is a protracted loss of DA neurons over the course of several weeks following intrastriatal infusions of 6-OHDA [32,33,69], and that variable denervation of DA cell bodies occurs despite similar dener-

vation of DA terminals depending upon the site of neurotoxin injection in the brain and route of delivery [20,38] and may account for the variable behavioral impairments. The 35% loss of TH-IR neurons in the substantia nigra observed in Experiment 1 is similar to that recorded in studies performed on nonhuman primates despite the use of a different neurotoxin and route of administration [6]. In the progressive MPTP macaque model of PD, detectable symptoms occurred at 43.2% TH-IR positive neuron loss. In mice treated with the same low dose of MPTP for 20 days, TH-IR neuron loss in the substantia nigra increased with each injection up until approximately injection 15. However, no behavioral testing was performed to establish a symptomatic threshold in these mice [7]. In the current study, the battery of non-drug-induced tests was sensitive enough to detect subtle sensorimotor deficits in animals with somewhat less than 50% TH-IR neuron loss.

In the present study there was no significant correlation between TH-IR positive neurons and behavior or between TH-IR positive neurons and cumulative 6-OHDA required to induce a behavioral deficit. Given this lack of correlation, it is possible that compensatory changes within the nigrostriatal system, including sprouting of DA terminals and normalization of extracellular DA, may have prevented detection of such correlations [67,80]. Animals were sacrificed at the same time after implantation of the cannula, and therefore at different intervals after the last exposure to 6-OHDA. These data are consistent with the possibility that the vulnerability of striatal DA terminals varies for individual animals, which is apparent from studies in which DA content varies widely from animal to animal despite exposure to similar doses of 6-OHDA [78]. It is also possible that variations in cannula placement or levels of preconditioning influenced the number of 6-OHDA infusions required to induce a limb-use asymmetry. It has been shown that discrete lesions in the dorsomedial striatum do not affect bracing of the contralateral forelimb in 6-OHDA-treated rats, whereas lesions in the dorsolateral region of the striatum have significant effects on bracing [14].

Oral administration of Sinemet, the gold standard therapy for PD [49], was investigated for its acute antiparkinson effects. Sinemet (L-DOPA:Carbidopa) increases striatal DA content by being converted to DA by dopa decarboxylase and thus enhancing DA activity in surviving neurons. Limb-use asymmetry was temporarily reversed following a dose of 20 mg/kg of Sinemet. The results indicated a peak beneficial effect of Sinemet between 60 and 180 min after oral administration. This is in agreement with previous studies showing reversal of limb-use asymmetry in the cylinder following either intraperitoneal injection or rAAV-mediated gene transfer of L-DOPA [39,51]. As has been shown previously for the akinesia and somatosensory asymmetry tests, DA agonists such as apomorphine and amphetamine and indirect agonists such as L-DOPA can ameliorate the 6-OHDA-induced deficits without producing dyskinesias as long as the DA depletion is not severe [13,47,59,74,75,77].

An intermittent, ultra-slow, progressive degeneration model could prove to be uniquely valuable in the development

of effective neuroprotective strategies for PD. One could use the mean total level of neurotoxin tolerated to assess treatment effects on vulnerability to neurotoxin exposure. This would provide a novel dependent variable for determining how well a treatment strategy alters the risk of developing symptoms. Clinically, there is considerable interest and urgency in finding behavioral tests that can be used to identify individuals who have otherwise-undiagnosed PD, ideally long before the classic motor symptoms appear. Positive screens could be followed by striatal DA functional imaging tests. Protecting DA neurons early in the disease process would certainly be an advance over current symptom treatment with drugs such as L-DOPA, which become less effective and produce increasing side effects in the late stages of the disease [35,57]. Once there is a severe loss of neurons, trying to replace the neurons and ensure accurate connections and functionally adequate firing patterns becomes a formidable task. Pre-symptomatic treatments that would render DA neurons resistant to multiple mechanisms of degeneration are a rational alternative [15,75]. Some progress has been made already in devising functional screens that might potentially detect in humans non-classic behavioral signs that reflect a high likelihood of an eventual diagnosis of PD [3,11,47,52,56,73,89].

Experiment 2 further investigated the slow lesion model and demonstrated neuroprotection against the neurotoxic effects of 6-OHDA by MPH, a DAT inhibitor. In the slow degeneration model, animals receiving 1.0 or 2.5 mg/kg of MPH prior to 6-OHDA infusions displayed attenuated behavioral deficits and significant sparing of TH-IR neurons in the SNc, despite receiving over twice as much neurotoxin compared to controls. MPH co-treatment resulted in significantly attenuated deficits in limb-use, movement initiation, somatosensory function, and reduced rotation following amphetamine, which suggests a specific effect on the DA system. Although neuroprotection by MPH is not surprising, it demonstrates the specificity and sensitivity of the slow lesion model, and suggests a novel approach for assessing neuroprotective therapies.

Methylphenidate is a stimulant drug used to treat attention deficits in children and adults [9,30]. Although it would not be expected to relieve symptoms of PD once the number of terminals lost is high, in some cases it might be capable of protecting dopamine neurons if administered in a controlled release form on a daily basis prior to the appearance of the classic PD symptoms. Moreover, stimulant drugs can enhance neurotrophic factor expression [16–18], which might be additionally advantageous because it could render neurons resistant to a broad spectrum of possible causes of cell death, as well as provide protection against secondary loss of non-DA cells.

MPH, in addition to its noradrenergic uptake-blocking mechanism, binds to the DAT and prevents DA re-uptake with a potency similar to that of cocaine [87]. Presynaptic DAT activity plays an important role in mediating DA behaviors in animals [28] and is involved in the toxicity of the DA neurotoxins 6-OHDA and MPTP that enter the cell

through the transporter [29,60]. For example, mice lacking DAT are resistant to MPTP toxicity [8,34] whereas mice with increased DAT show increased toxicity to MPTP [22]. Blockade of DAT should therefore prevent toxins like 6-OHDA and MPTP from entering DA terminals and reduce DA cell death [78]. Pharmacologically, oral MPH at a dose of 0.25 mg/kg occupies more than 50% of the DA transporters, while a dose of 0.07 mg/kg administered intravenously has the same occupancy rate [87,88]. In addition, MPH dose dependently increases extracellular DA levels [88]. The current effective doses used in this study are comparable to those used in the treatment of attention disorders and are below the threshold for producing hyperactivity and cross-sensitization to methamphetamine [42].

Recently, MPH has been shown to increase vesicular monoamine transporter-2 (VMAT2) uptake of DA [68], in vitro, after only one exposure to the drug. Increase of vesicular uptake may be neuroprotective by sequestering 6-OHDA into vesicles. Studies have shown that VMAT2 can sequester DA toxins and metabolites into vesicles and prevent damage that would otherwise occur [50,54,55,81]. It has been argued by some that MPH or MPH-like drugs might prevent PD in some individuals because MPH upregulates VMAT2 and inhibits the DAT [50,54,55,81]. Regardless of whether MPH given clinically to pre-symptomatic patients might be neuroprotective, it would be expected that in the early stages of PD the drug might be relatively harmless in comparison to L-DOPA and possibly effective for symptom management via the large numbers of terminals that are as yet undamaged. Moreover, its effects on attention and enhanced task salience might provide motivation to engage in physical exercise and motor enrichment therapies, which in animal models have been shown to be neuroprotective [16,83,84].

MPH may also be neuroprotective by possibly altering dendritic morphology within the nigrostriatal system and upregulating neuroprotective growth factors. Intermittent administration of stimulant drugs like amphetamine and cocaine, albeit at high doses, can lead to sensitization [62,63]. It has been shown that neuroadaptations, like increased branching and density of dendritic spines, occur after repeated administration of various stimulant drugs [64–66]. In addition, intermittent exposure to stimulant drugs is associated with long-lasting increases in the growth factor FGF-2 [25–27], known to be beneficial in animal models of PD [19,79]. In the present study, MPH-treated animals received between five and seven intermittent injections of the drug, which could potentially induce sensitization contributing to the neuroprotective effects of MPH. However, it is not known if sensitization occurred in the present experiment, and a recent study suggests that MPH administered in low doses that do not increase extracellular DA levels does not cause sensitization [42].

The development of a symptomatic threshold sensitive to pharmacological application may be a valuable tool for use in preclinical research. Early detection is crucial to the success of protective therapies and it will be important to establish

screening methods through brain imaging and thorough neurological assessment to maximize the benefit of protective treatments.

Acknowledgements

We would like to acknowledge the valuable assistance of J. Tsai, J. Chang, T. Lin, M. Woodlee and G. Redwine. Funded by NS23979.

References

- [1] Barneoud P, Descombris E, Aubin N, Abrous DN. Evaluation of simple and complex sensorimotor behaviours in rats with a partial lesion of the dopaminergic nigrostriatal system. *Eur J Neurosci* 2000;12(1):322–36.
- [2] Barth TM, Jones TA, Schallert T. Functional subdivisions of the rat somatic sensorimotor cortex. *Behav Brain Res* 1990;39(1):73–95.
- [3] Berendse HW, Booij J, Francot CM, Bergmans PL, Hijman R, Stoof JC, et al. Subclinical dopaminergic dysfunction in asymptomatic Parkinson's disease patients' relatives with a decreased sense of smell. *Ann Neurol* 2001;50:34–41.
- [4] Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci* 1973;20(4):415–55.
- [5] Betarbet R, Sherer TB, Mackenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 2000;3(12):1301–6.
- [6] Bezard E, Dovero S, Prunier C, Ravenscroft P, Chalon S, Guilloteau D, et al. Relationship between appearance of symptoms and the level of nigrostriatal degeneration in a progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned macaque model of Parkinson's disease. *J Neurosci* 2001;21(17):6853–61.
- [7] Bezard E, Dovero S, Bioulac B, Gross CE. Kinetics of nigral degeneration in a chronic model of MPTP-treated mice. *Neurosci Lett* 1997;234(1):47–50.
- [8] Bezard E, Gross CE, Fournier MC, Dovero S, Bloch B, Jaber M. Absence of MPTP-induced neuronal death in mice lacking the dopamine transporter. *Exp Neurol* 1999;155:268–73.
- [9] Biederman J, Quinn D, Weiss M, Markabi S, Weidenman M, Edson K, et al. Efficacy and safety of Ritalin LA, a new, once daily, extended-release dosage form of methylphenidate, in children with attention deficit hyperactivity disorder. *Paediatr Drugs* 2003;5(12):833–41.
- [10] Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ. A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci USA* 1983;80:4546–50.
- [11] Camicioli R, Grossmann SJ, Spencer PS, Hudnell K, Anger WK. Discriminating mild parkinsonism: methods for epidemiological research. *Mov Disord* 2001;16:33–40.
- [12] Carman LS, Gage FH, Shults CW. Partial lesion of the substantia nigra: relation between extent of lesion and rotational behavior. *Brain Res* 1991;553(2):275–83.
- [13] Cenci MA, Whishaw IQ, Schallert T. Animal models of neurological deficits: how relevant is the rat? *Nat Rev Neurosci* 2002;3:574–9.
- [14] Chang JW, Wachtel SR, Young D, Kang UJ. Biochemical and anatomical characterization of forepaw adjusting steps in rat models of Parkinson's disease: studies on medial forebrain bundle and striatal lesions. *Neuroscience* 1999;88(2):617–28.
- [15] Choi-Lundberg DL, Lin Q, Schallert T, Crippens D, Davidson BL, Chang Y-N, et al. Behavioral and cellular protection of rat dopamin-

- ergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. *Exp Neurol* 1998;154:261–75.
- [16] Cohen AD, Tillerson JL, Smith AD, Schallert T, Zigmond MJ. Neuroprotective effects of prior limb use in 6-hydroxydopamine-treated rats: possible role of GDNF. *J Neurochem* 2003;85:299–305.
- [17] Conner B, Kozlowski DA, Schallert T, Tillerson JL, Davidson BL, Bohn MC. The differential effects of adenoviral vector mediated glial cell line-derived neurotrophic factor (GDNF) in the striatum vs substantia nigra of the aged parkinsonian rat. *Gene Ther* 1999;6:1936–51.
- [18] Conner B, Kozlowski DA, Unnerstall JR, Elsworth JD, Tillerson JL, Schallert T, et al. Glial cell line-derived neurotrophic factor (GDNF) gene delivery protects dopaminergic terminals from degeneration. *Exp Neurol* 2001;169:83–95.
- [19] Date I, Yoshimoto Y, Imaoka T, Miyoshi Y, Gohda Y, Furuta T, et al. Enhanced recovery of the nigrostriatal dopaminergic system in MPTP-treated mice following intrastriatal injection of basic fibroblast growth factor in relation to aging. *Brain Res* 1993;621(1):150–4.
- [20] Deumens R, Blokland A, Prickaerts J. Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp Neurol* 2002;175(2):303–17.
- [21] Di Paola R, Uitti RJ. Early detection of Parkinson's disease. In: Palmer KJ, editor. *Drug treatment issues in Parkinson's disease*. Hong Kong: Adis International Ltd.; 2000. p. 1–11.
- [22] Donovan DM, Miner LL, Perry MP, Revay RS, Sharpe LG, Przedborski S, et al. Cocaine reward and MPTP toxicity: alteration by regional variant dopamine transporter overexpression. *Brain Res Mol Brain Res* 1999;73(1/2):37–49.
- [23] Ehringer H, Hornykiewicz O. Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. *Klin Wochenschr* 1960;38:1236–9.
- [24] Fahn S. Description of Parkinson's disease as a clinical syndrome. *Ann N Y Acad Sci* 2003;991:1–14.
- [25] Flores C, Rodaros D, Stewart J. Long-lasting induction of astrocytic basic fibroblast growth factor by repeated injections of amphetamine: blockade by concurrent treatment with glutamate antagonist. *J Neurosci* 1998;18(22):9547–55.
- [26] Flores C, Samaha A-N, Stewart J. Requirement of endogenous basic fibroblast growth factor for sensitization to amphetamine. *J Neurosci* 2000;20(RC55):1–5.
- [27] Flores C, Stewart J. Changes in astrocytic basic fibroblast growth factor expression during and after prolonged exposure to escalating doses of amphetamine. *Neuroscience* 2000;98(2):287–93.
- [28] Garris PA, Budygin EA, Philips PEM, Venton BJ, Robinson DL, Bergstrom BP, et al. A role for presynaptic mechanisms in the actions of nomifensine and haloperidol. *Neuroscience* 2003;118:819–29.
- [29] Glinka Y, Gassen M, Youdim MB. Mechanism of 6-hydroxydopamine neurotoxicity. *J Neural Transm Suppl* 1997;50:55–66.
- [30] Greenhill L, Beyer DH, Finkleson J, Shaffer D, Biederman J, Conners CK, et al. Guidelines and algorithms for the use of methylphenidate in children with attention-deficit/hyperactivity disorder. *J Attention Disord* 2002;6(Suppl 1):S89–100.
- [31] Hua Y, Schallert T, Keep RF, Wu J, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 2002;33(10):2478–84.
- [32] Ichtani Y, Okamura H, Matsumoto Y, Nagatsu I, Ibata Y. Degeneration of the nigra DA neurons after 6-hydroxydopamine injection into the rat striatum. *Brain Res* 1991;549(2):350–3.
- [33] Ichtani Y, Okamura H, Nakahara D, Nagatsu I, Ibata Y. Biochemical and immunocytochemical changes induced by intrastriatal 6-hydroxydopamine injection in the rat nigrostriatal DA neuron system: evidence for cell death in the substantia nigra. *Exp Neurol* 1994;130(2):269–78.
- [34] Jabor M, Jones S, Giros B, Caron MG. The dopamine transporter: a crucial component regulating dopamine transmission. *Mov Disord* 1997;12:629–33.
- [35] Jankovic J. Levodopa strengths and weaknesses. *Neurology* 2002;58(4 Suppl 1):S19–32.
- [36] Johnson RE, Schallert T, Becker JB. Akinesia postural abnormality after unilateral dopamine depletion. *Behav Brain Res* 1999;104(1/2):189–96.
- [37] King JA, Barkley RA, Delville Y, Ferris CF. Early androgen treatment decreases cognitive functioning and catecholamine innervation of the frontal cortex in an animal model of ADHD. *Behav Brain Res* 2000;107:35–43.
- [38] Kirik D, Rosenblad C, Bjorklund A. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal DA system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp Neurol* 1998;152(2):259–77.
- [39] Kirik D, Georgievska B, Burger C, Winkler C, Muzyczka N, Mandel RJ, et al. Reversal of motor impairments in parkinsonian rats by continuous intrastriatal delivery of L-DOPA using rAAV-mediated gene transfer. *Proc Natl Acad Sci USA* 2002;99(7):4708–13.
- [40] Kleim JA, Jones TA, Schallert T. Motor enrichment and the induction of plasticity before or after brain injury. *Neurochem Res* 2003;28(11):1757–69.
- [41] Kozlowski DA, Conner B, Tillerson JL, Schallert T, Bohn MC. Delivery of a GDNF gene into the substantia nigra after a progressive 6-OHDA lesion maintains functional nigrostriatal connections. *Exp Neurol* 2000;166:1–15.
- [42] Kuczenski R, Segal DS. Exposure of adolescent rats to oral methylphenidate: preferential effects on extracellular norepinephrine and absence of sensitization and cross-sensitization to methamphetamine. *J Neurosci* 2002;22(16):7264–71.
- [43] Langston JW. MPTP neurotoxicity: an overview and characterization of phases of toxicity. *Life Sci* 1985;36(3):201–6.
- [44] Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 1983;219(4587):979–80.
- [45] Lee CS, Sauer H, Bjorklund A. Dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by intrastriatal 6-hydroxydopamine in the rat. *Neuroscience* 1996;72(3):641–53.
- [46] Lees AJ. When did Ray Kennedy's Parkinson's disease begin? *Mov Disord* 1992;7:110–6.
- [47] Lindner MD, Plone MA, Francis JM, Emerich DF. Validation of a rodent model of Parkinson's disease: evidence of a therapeutic window for oral Sinemet. *Brain Res Bull* 1995;39(6):367–72.
- [48] Lindner MD, Winn SR, Baetge EE, Hammang JP, Gentile FT, Doherty E, et al. Implantation of encapsulated catecholamine and GDNF-producing cells in rats with unilateral dopamine depletions and parkinsonian symptoms. *Exp Neurol* 1996;132(1):62–76.
- [49] Lindner MD, Plone MA, Mullins TD, Winn SR, Chandonait SE, Stott JA, et al. Somatic delivery of catecholamines in the striatum attenuate parkinsonian symptoms and widen the therapeutic window of oral sinemet in rats. *Exp Neurol* 1997;145(1):130–40.
- [50] Lui Y, Peter D, Roghan A, Schuldiner S, Prive GG, Eisenberg D, et al. A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter. *Cell* 1992;70:539–51.
- [51] Lundblad M, Andersson M, Winkler C, Kirik D, Wierup N, Cenci MA. Pharmacological validation of behavioural measures of akinesia and dyskinesia in a rat model of Parkinson's disease. *Eur J Neurosci* 2002;15(1):120–32.
- [52] Mazzoni P, Ford B. The freezing of time as a presenting symptom of Parkinson's disease. *N Engl J Med* 1999;341:1317–8.
- [53] McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, et al. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol Dis* 2002;10(2):119–27.

- [54] Miller GW, Erickson JD, Perez JT, Penland SN, Mash DC, Rye DB, et al. Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. *Exp Neurol* 1999;156:138–48.
- [55] Miller GW, Gainetdinov RR, Levey AI, Caron MG. Dopamine transporter and neuronal injury. *Trends Pharmacol Sci* 1999;20:424–9.
- [56] Montgomery Jr EB, Koller WC, LaMantia TJ, Newman MC, Swanson-Hyland E, Kaszniak AW, et al. Early detection of probable idiopathic Parkinson's disease: development of a diagnostic test battery. *Mov Disord* 2000;15:467–73.
- [57] Obeso JA, Grandas F, Vaamonde J, Luquin MR, Artieda J, Lera G, et al. Motor complications associated with chronic levodopa therapy in Parkinson's disease. *Neurology* 1989;39(11 Suppl 2):11–9.
- [58] Olsson M, Nikkha G, Bentlage C, Bjorklund A. Forelimb akinesia in the rat Parkinson model: differential effects of DA agonists and nigral transplants as assessed by a new stepping test. *J Neurosci* 1995;15(5 Pt 2):3863–75.
- [59] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. San Diego, CA: Academic Press Inc.; 1998.
- [60] Pifl C, Giros B, Caron MG. Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium. *J Neurosci* 1993;13(10):4246–53.
- [61] Riederer P, Wuketich S. Time course of nigrostriatal degeneration in parkinson's disease. A detailed study of influential factors in human brain amine analysis. *J Neural Transm* 1976;38(3/4):277–301.
- [62] Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Rev* 1993;1:247–91.
- [63] Robinson TE, Berridge KC. Incentive-sensitization addiction. *Addiction* 2001;96(1):103–14.
- [64] Robinson TE, Kolb B. Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *J Neurosci* 1997;17:8491–7.
- [65] Robinson TE, Kolb B. Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *Eur J Neurosci* 1999;11:1589–604.
- [66] Robinson TE, Gorny G, Mitton E, Kolb B. Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. *Synapse* 2001;39(3):257–66.
- [67] Robinson TE, Mocsary Z, Camp DM, Whishaw IQ. Time course of recovery of extracellular dopamine following partial damage to the nigrostriatal dopamine system. *J Neurosci* 1994;14(5 Pt 1):2687–96.
- [68] Sandoval V, Riddle EL, Hanson GR, Fleckenstein AE. Methylphenidate redistributes vesicular monoamine transporter-2: role of dopamine receptors. *J Neurosci* 2002;22:8705–10.
- [69] Sauer H, Oertel WH. Progressive degeneration in nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience* 1994;59(2):401–15.
- [70] Schallert T. Aging-dependent emergence of sensorimotor dysfunction in rats recovered from dopamine depletion sustained early in life. *Ann N Y Acad Sci* 1988;515:108–20.
- [71] Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology* 2000;39(5):777–87.
- [72] Schallert T, Hernandez TD, Barth TM. Recovery of function after brain damage: severe and chronic disruption by diazepam. *Brain Res* 1986;379(1):104–11.
- [73] Schallert T, Leasure JL, Kolb B. Experience-associated structural events, subependymal proliferation activity, and functional recovery after injury to the central nervous system: a review. *J Cereb Blood Flow Metab* 2000;20:1513–28.
- [74] Schallert T, Norton D, Jones TA. A clinically relevant unilateral rat model of parkinsonian akinesia. *J Neural Transplant Plast* 1992;3:332–3.
- [75] Schallert T, Tillerson JL. Intervention strategies for degeneration of DA neurons in parkinsonism: optimizing behavioral assessment of outcome. In: Emerich DF, Dean III RL, Sandberg PR, editors. *Central nervous system diseases*. Totowa, NJ: Humana Press; 2000.
- [76] Schallert T, Upchurch M, Lobaugh N, Farrar SB, Spirduso WW, Gilliam P, et al. Tactile extinction: distinguishing between sensorimotor and motor asymmetries in rats with unilateral nigrostriatal damage. *Pharmacol Biochem Behav* 1982;16(3):455–62.
- [77] Schallert T, Upchurch M, Wilcox RE, Vaughn DM. Posture-independent sensorimotor analysis of inter-hemispheric receptor asymmetries in neostriatum. *Pharmacol Biochem Behav* 1983;18(5):753–9.
- [78] Schallert T, Wilcox RE. Neurotransmitter-selective brain lesions. In: Boulton AA, Baker GB, editors. *Neuromethods (series 1: neurochemistry), general neurochemical techniques*. Totowa, NJ: Humana Press; 1985. p. 343–87.
- [79] Shults CW, Ray J, Tsuboi K, Gage FH. Fibroblast growth factor-2-producing fibroblasts protect the nigrostriatal dopaminergic system from 6-hydroxydopamine. *Brain Res* 2000;883(2):192–204.
- [80] Song DD, Haber SN. Striatal responses to partial dopaminergic lesion: evidence for compensatory sprouting. *J Neurosci* 2000;20(13):5102–14.
- [81] Staal RG, Hogan KA, Liang CL, German DC, Sonsalla PK. In vitro studies of striatal vesicles containing the vesicular monoamine transporter (VMAT2): rat versus mouse differences in sequestration of 1-methyl-4-phenylpyridium. *J Pharmacol Exp Ther* 2000;293(2):329–35.
- [82] Stricker EM, Zigmond MJ. Effects on homeostasis of intraventricular injections of 6-hydroxydopamine in rats. *J Comp Physiol Psychol* 1974;86(6):973–94.
- [83] Tillerson JL, Cohen AD, Philhower J, Miller GW, Zigmond MJ, Schallert T. Forced limb-use effects on behavioral and neurochemical effects of 6-hydroxydopamine. *J Neurosci* 2001;21(12):4427–35.
- [84] Tillerson JL, Cohen AD, Caudle WM, Zigmond MJ, Schallert T, Miller GW. Forced nonuse in unilateral parkinsonian rats exacerbates injury. *J Neurosci* 2002;22(15):6790–9.
- [85] Ungerstedt U. Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol Scand Suppl* 1971;367:95–122.
- [86] Ungerstedt U, Arbuthnott GW. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res* 1970;24(3):485–93.
- [87] Volkow ND, Wang G-J, Fowler JS, Fischman M, Foltin R, Abumrad NN, et al. Methylphenidate and cocaine have a similar in vivo potency to block dopamine transporters in the human brain. *Life Sci* 1999;65(1):7–12.
- [88] Volkow ND, Wang G-J, Fowler JS, Gatley SJ, Logan J, Ding Y-S, et al. DA transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. *Am J Psychiatry* 1998;155(10):1325–31.
- [89] Wolters EC, Francot C, Bergmans P, Winogrodzka A, Booij J, Berendse HW, et al. Preclinical (premotor) Parkinson's disease. *J Neurol* 2000;247(Suppl 2):II 103–9.
- [90] Wommack JC, Delville Y. Chronic social stress during puberty enhances tyrosine hydroxylase immunoreactivity within the limbic system in golden hamsters. *Brain Res* 2002;933:139–43.
- [91] Zigmond MJ, Stricker EM. Supersensitivity after intraventricular 6-hydroxydopamine: relation to dopamine depletion. *Experientia* 1973;36(4):436–8.