

DOPAMINERGIC AND BEHAVIORAL CORRELATES OF PROGRESSIVE LESIONING OF THE NIGROSTRIATAL PATHWAY WITH 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE

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Abstract—A number of neurotoxin- and gene-based rodent models of acute neurodegeneration of nigrostriatal dopamine (DA) neurons are used to study Parkinson's disease (PD). The rapid degeneration achieved by many of these current models limits the capacity of the model to develop pathogenic mechanisms and display the various stages of motor degradation representative of the human Parkinsonian condition. Chronic rodent models have been the only ones to reproduce these characteristics, yet do not show correlated progress of DA loss with multiple stepwise behavioral deficits as seen in humans. In the present study, we have developed a progressive model of increasing DA loss and motor dysfunction via progressively increased administration of the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in the C57Bl/6J mouse. Mice were administered a daily (5 d/wk) dose of MPTP that increased weekly over the course of 4 weeks (4 mg/kg, 8 mg/kg, 16 mg/kg and 32 mg/kg). Each treatment group was tested for exploratory and motor behavioral changes after every week leading up to their final dose, as well as changes in tyrosine hydroxylase immunoreactivity (TH-ir) of the substantia nigra pars compacta (SNpc) and caudate putamen (CPu). We detected a 24% decrease in the mean number of TH-ir SNpc neurons/section after 1 week, and a 62% decrease after 4 weeks as compared to the vehicle group. CPu TH-ir began at a 35% loss after 1 week and increased to a 74% loss after 4 weeks compared to the vehicle group. CPu DA content showed an initial decrease of 20% after 1 week, and a final decrease of 70% following week 4 versus the vehicle group. Free-standing rears (versus wall-assisted rears, in a cylinder), decreased from 35% to 8% of total rears as the dose of MPTP increased from 4 mg/kg to 32 mg/kg, respectively. However, motor impairment as measured by a Parallel Rod Activity Chamber test was not significant until week 4 at 32 mg/kg compared to the vehicle group. The present study is the first to show stepwise progression of behavioral deficits which correlate with gradual dopaminergic decline in the nigrostriatal pathway. This progressive lesioning regimen may be appropriate for future investiga-

tion of pathogenic mechanisms and various intervention therapies in PD. Published by Elsevier Ltd on behalf of IBRO.

Key words: dopamine, progressive, tyrosine hydroxylase, motor control, Parkinson's disease.

Parkinson's disease is characterized by a progressive loss of dopamine (DA) neurons associated with increasingly disabling motor impairments such as tremor, rigidity and bradykinesia. These specific behavioral deficits have not been correlated with discrete thresholds of DA loss in the nigrostriatal pathway. It is generally accepted that clinical signs of Parkinson's disease (PD) appear when dopaminergic loss exceeds 70% in the caudate putamen (CPu) nerve terminals and 60% in cell bodies of the substantia nigra pars compacta (SNpc; Bernheimer et al., 1973; Riederer and Wuketich, 1976). Earlier clinical stages of the disease have not been clearly defined. However, a series of sensory and cognitive studies have emerged that suggest pre-motor Parkinsonian decline (Obeso et al., 2010). The concurrence of markers such as cognitive impairment (Cooper et al., 1991), olfactory decline (Wong et al., 2010; Doty et al., 1995), or idiopathic rapid eye movement (REM) sleep behavior disorder (Postuma et al., 2006) have been reported in early-stage human PD patients. With the development of tests for these early-stage markers of PD, it becomes more important to develop animal models that represent both the early and late stages of the disease.

Further development of behavioral tests and biomarkers with sensitivity to the progressive stages of DA loss that occurs in PD is crucial to detecting the disease earlier on where therapeutic intervention might mean long-term recovery as opposed to short-term symptomatic relief. In human patients, several brain-derived cerebral spinal fluid (CSF) biomarkers have been recently suggested as sensitive markers of early-stage Parkinsonism (Sinha et al., 2009; Goldstein et al., 2008; Balducci et al., 2007; Abdi et al., 2006). The correlation of such markers with olfactory and cognitive tests will be very important to the early detection of PD.

Current commonly used animal models of PD lack the concurrence of progressive pathology and behavioral decline in a single model. While progressive pathological markers and behavioral symptoms have been observed in the MitoPark genetic mouse model (Ekstrand et al., 2007; Galter et al., 2010), the mouse strain has not been adopted into common investigation. Conversely, the lipopolysaccharide-induced inflammatory model is commonly used, but has not shown greater than 50% decrease in SNpc DA

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Abbreviations: CPu, caudate putamen; CV, cresyl violet; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; FF/BB, foot faults per beam breaks; FSR, free-standing rears; IHC, immunohistochemistry; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PRAC, parallel rod activity chamber; SN, substantia nigra; SNpc, substantia nigra pars compacta; TBST, tween-20; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase immunoreactivity; WAR, wall-assisted rears.

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neurons (Burguillos et al., 2011; Qin et al., 2007). The caveats of many toxin-induced and genetic models are reviewed elsewhere and will not be discussed in depth in this article (Potashkin et al., 2010; Meredith and Kang, 2006). Several chronic MPTP models exist that achieve a progressive decline of both DA neurons and motor behaviors, but either lack a correlation between pathology and behavioral decline (Meredith et al., 2002; Bezard et al., 1997), do not achieve a depletion of SNpc DA neurons greater than 30% (Schintu et al., 2009) or do not show stepwise progression of either (Blesa et al., 2010; Blume et al., 2009; Schintu et al., 2009; McKnaught et al., 2004; Bezard et al., 1997). In fact, it has been suggested that mice become tolerant to MPTP at a low dose over time (Bezard et al., 1997).

In this study, we have developed a new sequentially increasing MPTP regimen designed to achieve progressive appearance of behavioral symptoms with concomitant denervation in the nigrostriatal pathway. This was based on a study by Fleming et al. (2005), where increasing doses of 6-OHDA were administered into the rat CPu in order to achieve motor behavioral deficits. Olfactory, free-standing rear and locomotor behaviors were assessed following 1 week of each progressively increased MPTP dose (4 mg/kg, 8 mg/kg, 16 mg/kg and 32 mg/kg). At the same intervals, the mean number of DA neurons was assessed by counting the number of TH-immunoreactive (TH-ir) and Cresyl Violet stained neurons/section in the SNpc, and DA terminal changes were measured by TH-ir optical density in the CPu. Additionally, TH and dopamine transporter (DAT) protein expression in the SNpc and CPu tissue were analyzed by Western blot, with CPu DA content and turnover also being assessed.

EXPERIMENTAL PROCEDURES

Animals and drugs

All procedures were approved by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and conducted at the Portland Veterans Affairs Medical Center. Young adult (10 weeks) male C57BL/6J mice (Jackson Labs, Bar Harbor, ME, USA) had *ad libitum* access to standard laboratory chow and water, and were maintained on a 12 hr light-dark cycle. Each of the four MPTP-treated groups was comprised of 12 mice, injected 5 days a week with a weekly increasing daily dose of MPTP (as the base: 4 mg/kg, 8 mg/kg, 16 mg/kg and 32 mg/kg, respectively; Sigma Aldrich, St. Louis, MO, USA) over the course of 4 weeks. Vehicle groups contained eight mice each, and were administered normal saline (0.1 ml/0.1 kg). Half of the animals in each group were analyzed by either TH-ir immunohistochemistry (IHC) or Western immunoblot. After each week of MPTP or saline (5 days with injections followed by 2 days without), olfactory function and motor performance were assessed. In a separate group of animals, the 4 weeks of progressive MPTP was followed by a 3-week washout period. Olfactory latency, rearing behavior and IHC were assessed in these animals to confirm a lasting lesioning effect.

Tyrosine hydroxylase immunohistochemistry

Three days following the last injection of each MPTP dose, animals were anesthetized with 1% ketamine/0.1% mg xylazine (2.0 ml/0.1 kg, i.p.), and euthanized by transcardial perfusion with 1000 U/ml of heparin in 0.1 M phosphate buffer (3 ml total) followed by

fixative [2% paraformaldehyde, 1% acrolein in 0.1 M phosphate buffer; pH 7.4; ~35–50 ml]. Brains were removed and washed for 24 h in 0.1 M phosphate buffer at 4 °C. Consecutive sections through the SNpc (beginning at Bregma–2.9) and CPu (beginning at +1.2), according to Paxinos and Franklin (2004), were cut 70 μ m thick using a vibratome (Ted Pella Inc., Redding, CA, USA). The sections were matched anatomically in each of the animals, verifying that the coronal sections of the SNpc and CPu were similar in all treatment groups. Alternate sections of the rostrocaudal extent of the SNpc and CPu, were incubated in 10 mM sodium citrate (pH 6) for 5 min at 500 W for antigen retrieval, using a PELCO BioWave[®] Pro microwave (Ted Pella Inc., Redding, CA, USA). Sections were incubated with 1% sodium borohydride in phosphate buffer for 30 min, then for 1 h in blocking solution [10% goat serum/0.5% Triton-X 100/0.1 M phosphate buffer; pH 7.4] at room temperature, followed by TH antibody (1:20,000; Immunostar, Hudson, WI, cat. #22941, monoclonal) at 4 °C overnight. The following incubations were carried out in the PELCO microwave: sections were washed for 2 min in 0.1 M phosphate buffer at 150 W, incubated with biotinylated goat anti-mouse secondary antibody at 200 W (1:250; Vector, Burlingame, CA, USA) for 12 min under cycled vacuum, and with avidin-biotin complex solution at 150 W (ABC, diluted according to manufacturer instruction; Vector) for 12 min under vacuum. Sections were then incubated with diaminobenzidine (DAB kit, Vector), mounted on gel-coated slides, and SNpc sections were prepared for Cresyl Violet counter-staining. Optical density of the dorsolateral region of CPu sections, the region receiving the input from the motor cortex (McGeorge and Faulk, 1989), was analyzed using light microscopy by an individual blinded to the treatment group (40 \times magnification, images analyzed using ImagePro 6.3, Media Cybernetics).

Cresyl Violet staining and IHC analysis

Following TH immunohistochemistry, all SNpc sections were counter-stained with Cresyl Violet (CV). TH-labeled sections were mounted on gel-coated slides and dehydrated at room temperature overnight. Slides were dipped in deionized water (dH₂O), incubated for 3 min in Cresyl Violet stain (0.2% in dH₂O) followed by 2 min in differentiator (30 μ l hydrochloric acid in 70% ethanol), 1 min in 95%, then 100% ethanol, and 5 min in xylene. Stained slides were cover-slipped using Pro-Texx[®] medium (Lerner, Pittsburgh, PA). Tissue from all treatment groups was processed on the same day, and all reacted with DAB for the same length of time. TH-ir neurons only at the in-focus surface plane of immunolabeled SNpc tissue were counted using light microscopy (40 \times magnification, images analyzed using ImagePro 6.3, Media Cybernetics) by an individual blinded to the treatment group (Fig. 1A). The number of TH-ir and CV+ neurons from the left and right SNpc sections were averaged for each animal for all six sections analyzed since both sides of the SNpc are affected following systemic administration of this neurotoxin. Mean numbers of TH-ir or CV+ neurons/section were then determined for all animals in a given treatment group. TH-ir neurons within the SNpc were easily delineated from the few, if any, TH-ir neurons in the underlying substantia nigra pars reticulata, and counted from the most lateral edge of the ventral tegmental area to the medial edge of the lateral substantia nigra (SNL). Cresyl Violet-only stained neurons found within the TH-ir layer of the SNpc, and identified as cells of comparable diameter to TH-ir neurons, were counted. All TH-ir neurons were also stained with Cresyl Violet. From these tissue section counts, the total number of labeled neurons was re-evaluated using the Abercrombie correction, which accounts for fragmented nuclei within each section and provides an accurate estimate when tissue thickness exceeds soma thickness by more than 50%, which is the case in this study (70 μ m sections; Clarke, 1992; Smolen et al., 1983).

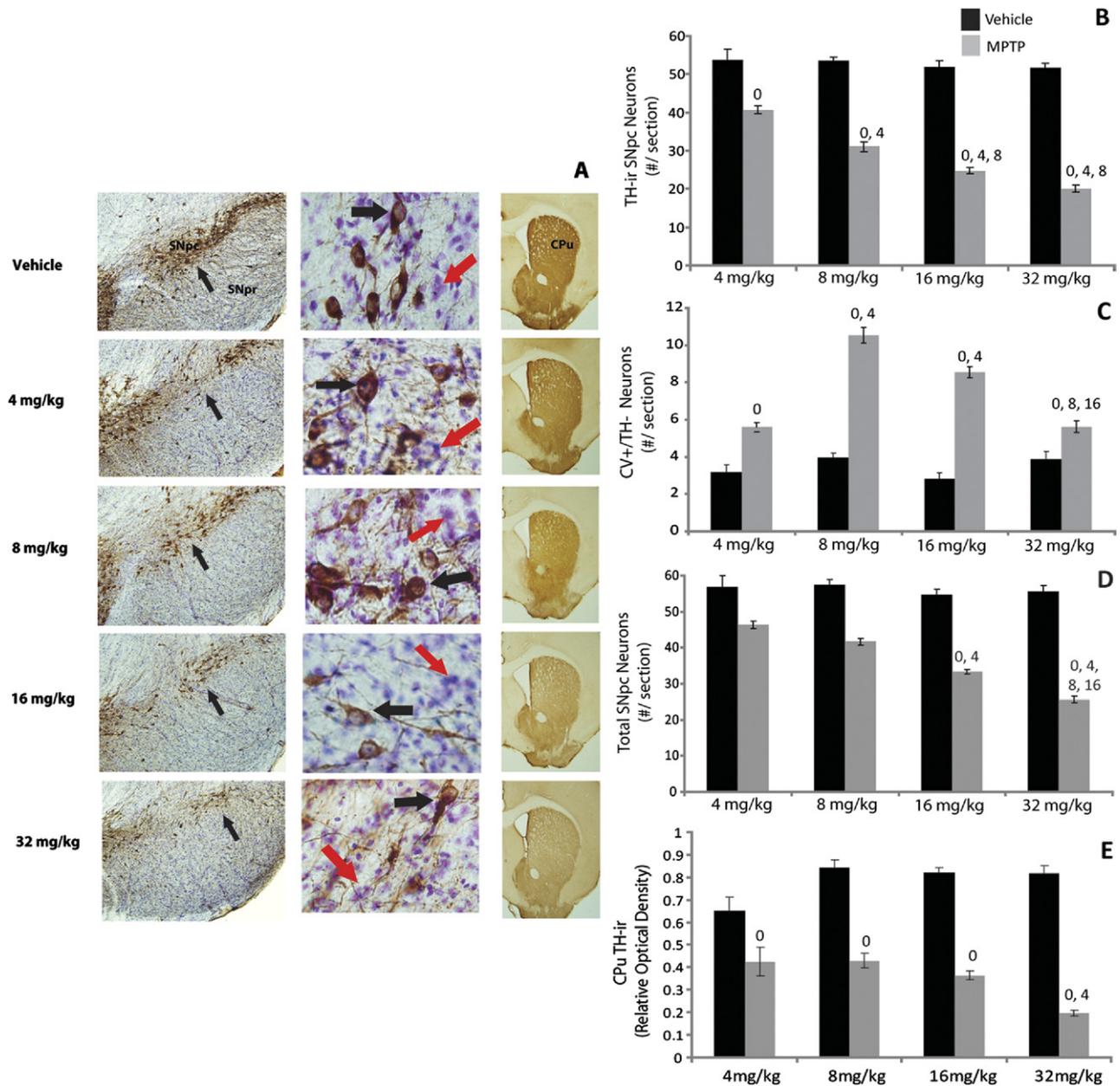


Fig. 1. Changes in the mean number of TH-ir and CV+ SNpc neurons/section, and in the relative optical density of CPU tyrosine hydroxylase immunolabeling. The extent of TH-ir decrease following each dose of MPTP in TH-ir SNpc neurons (black arrow, left and middle columns), Cresyl Violet SNpc neurons (red arrow, middle column) and TH-ir of the CPU is illustrated (A). The mean number of TH-ir SNpc neurons/section decreased following 4 mg/kg, 8 mg/kg, and further at 16 mg/kg MPTP (B). MPTP-treated mice ($n=6$) showed an increase in the mean number of Cresyl Violet positive and TH negative (CV+/TH-) neurons/section after 4 mg/kg and 8 mg/kg, but decreased to vehicle levels ($n=4$) following 32 mg/kg MPTP (C). The mean total SNpc neurons/section, which includes both TH+ and CV+ neurons, decreased after 16 mg/kg, and further after 32 mg/kg MPTP (D). The mean optical density of TH-ir in the CPU decreased after 4 mg/kg, and further after 16 mg/kg and 32 mg/kg MPTP (E). Values represent the mean \pm SEM. ⁰ $P < 0.05$ compared to vehicle, ⁴ $P < 0.001$ compared to 4 mg/kg, ⁸ $P < 0.0001$ compared to 8 mg/kg, ¹⁶ $P < 0.002$ compared to 16 mg/kg.

Western immunoblots

Mice were euthanized by cervical dislocation, and the substantia nigra (SN) and dorsolateral CPU were dissected from the same one side of the brain, and frozen at -80°C . Protein was extracted from the tissue by sonication in lysis buffer [5% 1 M Tris, 2% 0.5 M EDTA, 1% Triton-X 100, 0.5% Protease Inhibitor Cocktail III (Calbiochem, USA)]. Protein concentrations of the tissue from each individual animal were measured using the BCA Protein Assay Kit (Thermo Scientific). Samples were mixed with B-mer-

captoethanol Laemmli Buffer (1:1; Sigma, St. Louis, MO, USA) and electrophoresed on a 7% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA), which were blocked in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) for 45 min. Membranes were then washed three times for 10 min each in TBST, and probed with primary antibodies for tyrosine hydroxylase (TH; Immunostar, Inc., 1:40,000, mouse monoclonal), dopamine transporter (DAT;

Chemicon, 1:6000, rat monoclonal) or β -actin (1:6500; Sigma, St. Louis, MO, USA). After three 10 min washes in TBST, membranes were probed with secondary antibodies for 1 h (goat-anti-mouse for TH and β -actin, and goat-anti-rat for DAT), then washed again in TBST and incubated in ECF substrate (GE Healthcare, Piscataway, NJ, USA) for 10 min. Visualization and quantification of the antigen-antibody binding density were performed using the UltraLum imaging system and Ultraquant 6.0 software, respectively. Protein densities were analyzed relative to individual β -actin densities, and each DAT band was analyzed as a single wider band as typically seen in DAT tissue samples (Chu et al., 2008; Salahpour et al., 2008).

DA tissue content

CPu tissue from the opposite side of the brain (compared to that used for Western blotting) was homogenized in 1 ml of mobile phase (2% sodium dihydrogen phosphate, 0.07% 1-octanesulfonic acid sodium salt, 0.02% triethylamine, 0.05% 100 mM EDTA, 10% acetonitrile; pH 3), then centrifuged at $20,000\times g$ at 4 °C for 30 min and stored at –80 °C. Samples were thawed at 4 °C, centrifugation was repeated and 25 μ l were injected into a high pressure liquid chromatography (HPLC) apparatus, equipped with a reverse-phase column (MD-150, ESA) and coulometric detector (ESA Coulochem III, Chemsfield, MA, USA) to quantify DA and 3,4-dihydroxyphenylacetic acid (DOPAC). Electrodes were set at +220 mV (oxidation) and –150 mV (reduction). Mobile phase was pumped at 0.6 ml/min flow rate. The assay sensitivities for DA and DOPAC were 20.4 fmol/ μ l and 27.8 fmol/ μ l, respectively. Tissue levels for DA were expressed as pg/mg protein, while DA turnover was expressed as a ratio of DOPAC/DA levels (pg/mg protein).

Behavioral tests

Each behavioral test was performed following 1 week at each dose of MPTP. Because MPTP has been shown to induce acute physiological and behavioral changes (Sedelis et al., 2000, 2001; Linder et al., 1987), rearing and foot fault tests were performed 2 days following the last injection; olfactory testing was performed on the third day following the last injection of a specific dose of MPTP.

Olfactory test

Olfactory testing was carried out as previously described, with modifications (Schintu et al., 2009). In brief, mice were deprived of food for 20 h prior to testing in order to provoke motivation to find the hidden food. Mice were individually acclimated to a large, clean cage (45 \times 45 \times 30 cm³) for 15 min, then transferred to a clean testing cage of the same parameters where a 5 g corn chip was buried 1 cm beneath the cage bedding. Each mouse was positioned in the center of the testing cage, and latency to bite the corn chip was measured within a 5 min testing period.

Rearing

In a plastic cylinder (13 cm in diameter, 16 cm height), mice were allowed to explore freely for 2 min. Rears were counted as elevations to an erect stance, and separated by forelimb contact to the horizontal base of the cylinder. Free-standing rears (FSR), wall-assisted rears (WAR) and total rears were assessed; FSR and WAR were evaluated as percents of total rears. Mice were not acclimated to the cylinder prior to baseline testing.

Foot faults and total activity

The parallel rod activity chamber (PRAC), as previously characterized by Kamens and Crabbe (2007), was modified from the original apparatus with the addition of photo beams to record locomotor activity. When the mouse paw slips through the parallel

rods and contacts the metal plate, a circuit is closed and a foot fault is recorded by the computer. The sensitivity of the metal plate was designed such that slips of the tail or feces through the rods are not recorded (Kamens and Crabbe, 2007). The apparatus is not designed to distinguish between slips of the forepaw versus hind paw. Videotaping each mouse would make this possible to determine, but was not done in the current study. When a mouse passes in front of a photo beam, the beam breaks and is recorded as a horizontal movement by the computer. Mice were acclimated to the PRAC, each in an individual dark sound-proof box, for 10 min, once daily for 2 days prior to recording baseline levels. Foot faults per total activity are expressed as a ratio of foot faults per beam breaks (FF/BB) in order to normalize the data against possible changes in locomotor (BB) activity (i.e. to evaluate whether changes in foot faults are due to changes in movement or in motor control).

Statistics

All assessments were analyzed by repeated measures ANOVA, using two-way comparisons between vehicle and MPTP-treated groups and one-way comparisons between different MPTP doses. Significant differences were then analyzed using post-hoc Tukey-Kramer HSD test for multiple comparisons and Pearson correlations, considered significant at $P<0.05$. Pearson correlations chosen for graph report had r values significant at $P<0.05$ and were correlated with behaviors; correlations between non-behavioral assessments are reported in Table 2.

RESULTS

Immunohistochemistry and Cresyl Violet

Changes in SNpc TH-ir neurons, CV neurons and CPu TH-ir due to progressively increased MPTP are shown in Fig. 1A. The main effect of treatment ($F_{(4,23)}=27.8$; $P<0.0001$) on the decrease in the mean number of TH-ir SNpc neurons/section suggests that TH-ir neurons were significantly lower in MPTP mice at each dose compared to the respective vehicle group. Repeated measures ANOVA indicated that increasing the dose of MPTP significantly influenced the degree of TH-ir neuron decrease ($F_{(3,6)}=5.22$; $P<0.05$). Post-hoc analysis of the MPTP groups showed that the mean number of TH-ir neurons/section of the SNpc was significantly decreased from levels at 4 mg/kg MPTP following 1 week of 8 mg/kg MPTP ($P<0.001$). TH-ir neurons decreased again from levels at 8 mg/kg after 1 week of 16 mg/kg (Fig. 1B; $P<0.005$). After 1 week of 32 mg/kg, the 62.3% decrease in mean number of TH-ir neurons/section was similar to the 53.8% decrease found after the 16 mg/kg dose, but significantly greater than the decrease at 8 mg/kg ($P<0.0001$). In a separate set of animals, after 4 weeks of progressive MPTP, toxin treatment was discontinued for three additional weeks. At the end of this 3-week withdrawal period, there were significantly fewer TH-ir SNpc neurons/section compared to vehicle treated animals ($F_{(2,12)}=156.3$; $P<0.0001$; Table 1). This decrease in TH-ir neurons after drug withdrawal is similar to what was observed after the final week of treatment with 32 mg/kg (Fig. 1B). This demonstrates the continued effect of MPTP treatment and the decrease in TH-ir neurons is not due to an acute effect of the toxin on TH immunoreactivity.

Table 1. Effect of a 3-week washout period after the final dosing of MPTP (32 mg/kg) on TH-labeled neurons, olfactory and rearing behavior

Treatment	TH-ir SNpc neurons	Olfactory latency (s)	FSR (% of total)	WAR (% of total)	Total rears (#)
Vehicle	44.73±1.31	28.8±6.2	44.71%±2.5%	55.28%±1.3%	24±2.2
3 weeks off MPTP	21.44±1.74	495.6±39.4	12.01%±5.8%	87.99%±5.3%	13.4±2.2
<i>P</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Values are means±SEM.

A significant effect of treatment ($F_{(4,23)}=35.8$; $P<0.0001$) indicated that the mean number of CV-stained but TH negative (TH−) SNpc neurons/section increased compared to vehicle levels beginning after 4 mg/kg, and increased again following 1 week of 8 mg/kg ($P<0.0001$). However, following 1 week of 32 mg/kg MPTP, the mean number of CV+/TH− neurons/section decreased back to the values seen after 1 week of 4 mg/kg, but remained significantly higher compared to the vehicle group (Fig. 1C; $P<0.003$).

When TH-ir neurons were included in CV analysis of the SNpc, the mean number of total neurons/section did not decrease significantly until after 1 week of 16 mg/kg MPTP, but this loss progressed after 32 mg/kg (Fig. 1D; $P<0.002$); the mean number of total neurons/section was fewer in number compared to the vehicle, 4 mg/kg, 8 mg/kg and 16 mg/kg MPTP groups. This trend was similar to that observed for the mean number of TH-ir only SNpc neurons/section.

Densitometric analysis of dorsolateral TH-ir in the CPu sections showed a significant effect of MPTP beginning after 4 mg/kg ($F_{(2,23)}=17.8$; $P<0.0001$). There was a further decrease following 32 mg/kg MPTP (Fig. 1E; $P<0.01$).

Western blot analysis

The effects of progressively increased MPTP on TH and DAT protein expression in the SN and CPu are depicted in Fig. 2. There was a significant effect of treatment ($F_{(1,10)}=5$; $P<0.05$) on TH protein expression in the SN (Fig. 2A, B). Repeated measures ANOVA indicated that increasing the dose of MPTP had a significant effect on decreasing protein expression ($F_{(3,8)}=0.77$; $P<0.008$; Fig. 2B). Post-hoc analysis revealed that following 4 mg/kg MPTP, ex-

pression was not different compared to vehicle values. However, following 8 mg/kg, 16 mg/kg and 32 mg/kg, TH protein expression in the SN was significantly lower versus the respective vehicle group ($P<0.03$). TH protein levels did not decrease from 4 mg/kg MPTP until 32 mg/kg ($P<0.05$). Vehicle levels of DAT protein expression in the SN were significantly lower than levels seen in the CPu ($P<0.01$; data not shown), but were not different between treatment groups, and did not change over the course of the experiment (Fig. 2A, C).

TH protein expression in the dorsolateral CPu was significantly lower in MPTP mice compared to vehicle mice ($F_{(1,10)}=2.18$; $P<0.0001$; Fig. 2D, E). Repeated measures ANOVA revealed a significant effect of increased dose ($F_{(3,8)}=14.9$; $P<0.002$), suggesting that a higher MPTP dose resulted in a greater decrease in TH protein expression. A significant interaction between treatment and dose ($F_{(3,8)}=15$; $P<0.002$) on TH protein expression in the CPu suggested that MPTP-treated mice had increasingly lower expression compared to vehicle mice (Fig. 2D, E). Expression of TH protein in the dorsolateral CPu did not decrease significantly below vehicle levels until after 8 mg/kg MPTP (Fig. 2E; $P<0.005$). This decrease continued following 16 mg/kg ($P<0.03$), but showed no further decrease.

Densitometric analysis of CPu DAT protein expression also showed an effect of treatment ($F_{(1,8)}=35.1$; $P<0.0004$; Fig. 2D, F). A significant effect of increased dose was also indicated by repeated measures ANOVA ($F_{(3,6)}=7$; $P<0.02$). A significant interaction between treatment and dose suggests there was a continued decrease in CPu DAT protein expression due to MPTP (Fig. 2F; $F_{(3,6)}=8.2$; $P<0.02$). There was a significant dose-dependent decrease beginning after 1 week of 4 mg/kg MPTP ($P<0.008$). Following the 16 mg/kg MPTP dose, protein expression was lower compared to the 4 mg/kg group ($P<0.001$). DAT protein expression following 32 mg/kg was decreased compared to 4 mg/kg and 8 mg/kg ($P<0.02$), but similar to 16 mg/kg levels.

Tissue DA content and turnover in the CPu

The effects of increasing the dose of MPTP on CPu DA tissue content and turnover are depicted in Fig. 3. Both tissue DA content and DA turnover (DOPAC/DA) were significantly affected by MPTP treatment ($F_{(1,8)}=14.5$; $P<0.005$). Repeated measures ANOVA indicated an effect of increasing MPTP dose on decreasing DA content and increased turnover ($F_{(3,6)}=6.2$; $P<0.03$). Tissue DA content only showed a significant decrease compared to vehicle levels after the 16 mg/kg dose ($P<0.009$). The levels of DA following 32 mg/kg were decreased compared to vehicle ($P<0.001$), 4 mg/kg

Table 2. Correlations of non-behavioral measurements

Variable 1 compared to variable 2	<i>r</i> (Pearson coefficient)	<i>P</i>	
CPu TH IHC	SNpc TH IHC	0.8299	<0.0001
CPu DA content	CPu TH IHC	0.5857	0.0007
CPu DA content	SNpc TH IHC	0.5367	0.0022
CPu DA turnover	SNpc TH IHC	−0.5493	0.0025
SNpc TH West	CPu TH IHC	0.4759	0.0078
CPu DA turnover	CPu TH IHC	−0.4517	0.0158
CPu DAT West	CPu TH West	0.3995	0.0287
SNpc TH West	CPu DA HPLC	0.3964	0.0301
CPu TH West	CPu DA HPLC	0.3855	0.0354
SNpc TH West	CPu TH West	0.3838	0.0363
SNpc TH West	SNpc TH IHC	0.373	0.0423

IHC, Immunohistochemistry optical density for TH-ir in the CPu or cell counts in the SNpc; West, Western immunoblot.

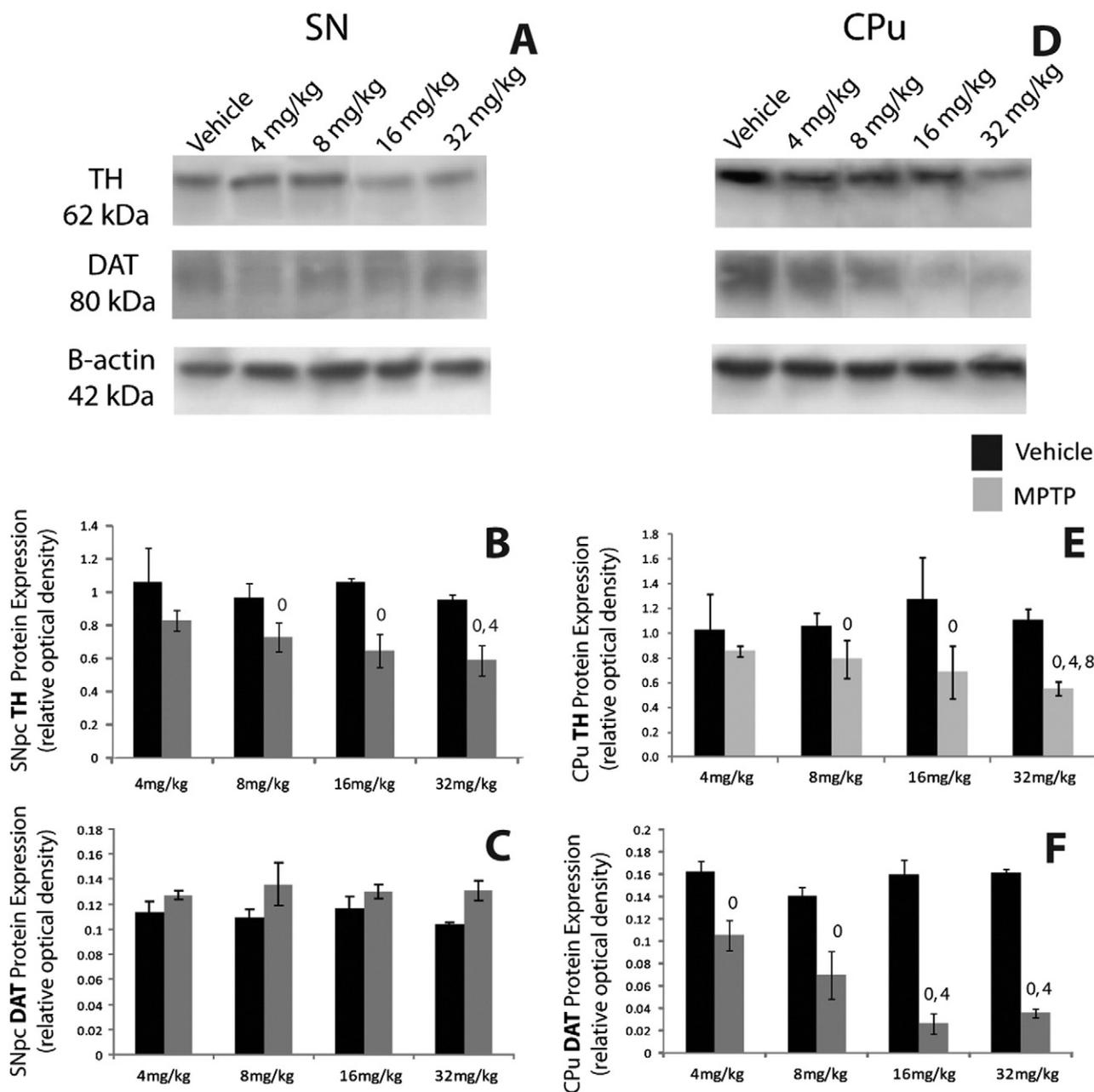


Fig. 2. Changes in the mean relative optical density of SN (substantia nigra) and CPu TH and DAT protein expression using Western immunoblotting. Immunoblots for both TH and DAT in the SN (A) and CPu (D) are shown. Following 16 mg/kg and 32 mg/kg, MPTP-treated mice ($n=5-6$) showed TH protein expression in the SN which was similar to that seen in 4 mg/kg and 8 mg/kg treated groups, but significantly decreased compared to their respective vehicle groups (B). DAT protein expression in the SNpc showed no changes after even the highest dose of MPTP (C). TH protein expression in the CPu decreased following 32 mg/kg MPTP compared to vehicle levels (E). In the CPu, DAT protein expression decreased significantly following 4 mg/kg, and significantly further following 16 mg/kg; levels in the 8 mg/kg and 32 mg/kg treatment groups were similar to their respective previous doses (F). Values represent the mean \pm SEM and are normalized relative to the individual β -actin optical density. * $P < 0.05$ compared to respective vehicle group, ⁴ $P < 0.004$ compared to 4 mg/kg, ⁸ $P < 0.03$ compared to 8 mg/kg.

($P < 0.05$), 8 mg/kg (0.0008), but not 16 mg/kg MPTP (Fig. 3A; $P < 0.03$). This observation is confirmed by analyzing DA turnover, which showed a significant effect of dose ($F_{(3,6)} = 5.23$; $P < 0.04$). Following 32 mg/kg MPTP, turnover was greater compared to vehicle ($P < 0.0006$), 4 mg/kg ($P < 0.02$), and 8 mg/kg (Fig. 3B; $P < 0.02$).

Olfactory performance

Because olfactory impairment is considered a pre-motor deficit in PD (Obeso et al., 2010; Wong et al., 2010), olfactory function was evaluated both prior to any vehicle or MPTP treatments, and following each consecutive week

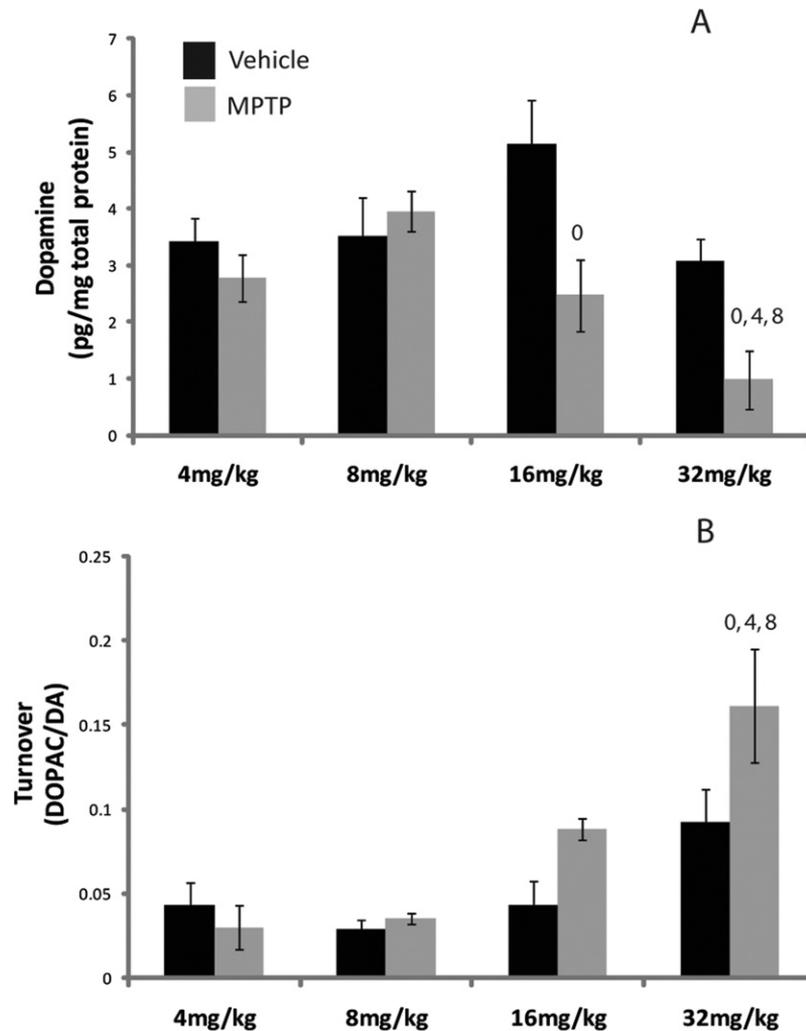


Fig. 3. DA tissue content and turnover in the CPu. DA tissue content showed a decrease following 16 mg/kg, which was significantly different from vehicle, 4 mg/kg and 8 mg/kg doses. Levels at 32 mg/kg were decreased compared to all other treatment groups (A). DA turnover (DOPAC/DA) increased following 32 mg/kg compared to vehicle, 4 mg/kg and 8 mg/kg MPTP doses (B). Values represent the mean \pm SEM. * $P < 0.0006$ compared to vehicle group, ⁴ $P < 0.02$ compared to 4 mg/kg, ⁸ $P < 0.02$ compared to 8 mg/kg.

of treatment. Latency to locate and bite the buried corn chip was significantly affected by MPTP treatment ($F_{(1,9)}=89.9$; $P < 0.0001$). Repeated measures ANOVA revealed that an increasing dose of MPTP also had a significant effect on the latency ($F_{(3,7)}=19.4$; $P < 0.0009$). A significant interaction between treatment and dose suggests that increased MPTP had an effect on increasing latency (Fig. 4A; $F_{(3,7)}=19$; $P < 0.0009$). Post-hoc analysis showed that the latency was greater compared to the respective vehicle group after 1 week of 4 mg/kg MPTP ($P < 0.03$), after 1 week of 8 mg/kg ($P < 0.02$), after 1 week of 16 mg/kg ($P < 0.0001$) and after 32 mg/kg MPTP ($P < 0.0001$). Following the 16 mg/kg dose, the latency was also increased relative to 4 mg/kg and 8 mg/kg MPTP doses ($P < 0.02$). Following 32 mg/kg MPTP, latency to bite the corn chip was significantly increased compared to 4 mg/kg ($P < 0.0002$), 8 mg/kg ($P < 0.0004$) and 16 mg/kg ($P < 0.02$). Following the 3-week washout period, the MPTP group showed significantly greater latency to find the corn chip

compared to the vehicle group ($F_{(2,12)}=19.9$; $P < 0.0001$; Table 1). This finding is comparable to that seen after the final week of toxin treatment with 32 mg/kg of MPTP (Fig. 4A).

Free-standing rears (FSR) and wall-assisted rears (WAR)

FSR and WAR were recorded 2 days following the final MPTP administration for each increasing dose (Fig. 4B, C). The observed significant effect of treatment on FSR suggests that MPTP negatively influenced this behavior ($F_{(1,18)}=297$; $P < 0.0001$). A repeated measures ANOVA indicated that increasing the dose of MPTP resulted in a continuing decrease in FSR ($F_{(3,10)}=38.5$; $P < 0.0001$). An interaction between treatment and dose suggested that the higher MPTP dose resulted in a greater FSR deficit ($F_{(3,10)}=23.4$; $P < 0.0001$; Fig. 4B). Post-hoc analysis indicated that FSR were significantly decreased

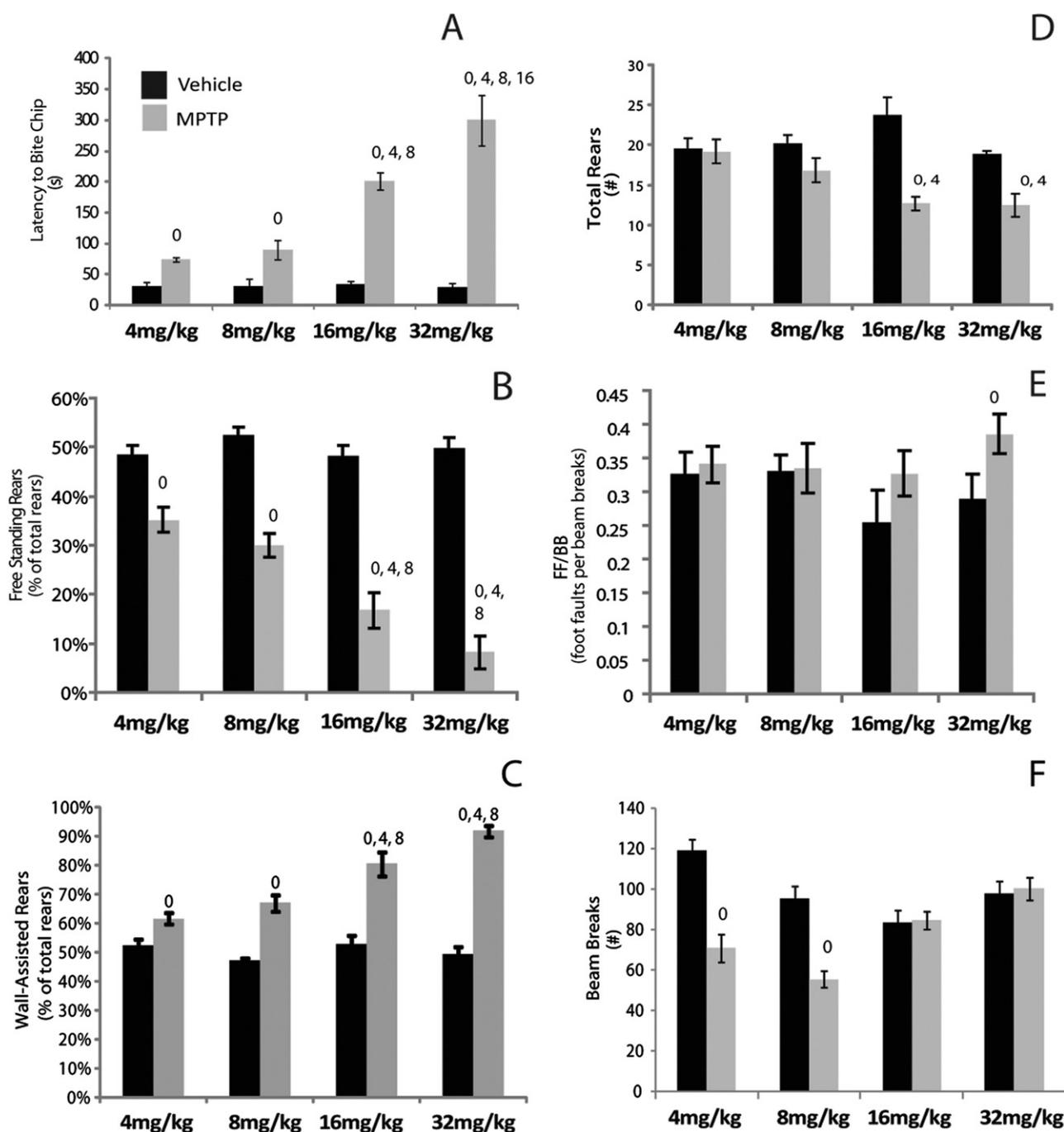


Fig. 4. Effects of progressively increased MPTP on olfactory, rearing and PRAC behavior. Latency to bite the corn chip in the olfactory test increased from baseline level beginning at 4 mg/kg, and showed a progressive increase following 8 mg/kg, 16 mg/kg and 32 mg/kg relative to the respective previous doses (A). Free-standing rears (% of total rears) were significantly lower than both their respective vehicle group levels and all previous MPTP dose levels following each week (B). Wall-assisted rears (% of total rears) were significantly increased following 4 mg/kg, 16 mg/kg and 32 mg/kg MPTP (C). Number of total rears showed a progressive decrease but was statistically different compared to its respective vehicle, 4 mg/kg and 8 mg/kg groups only after administration of 16 mg/kg or 32 mg/kg of MPTP (D). After administration of 32 mg/kg during week 4, the foot fault/beam break (FF/BB) ratio was significantly higher compared to its respective vehicle treated group and the 4 mg/kg group (E). Beam breaks were decreased following 4 mg/kg and 8 mg/kg MPTP but returned to vehicle levels at later doses (F). Values represent the mean \pm SEM ⁰ $P < 0.05$ compared to vehicle or baseline, ⁴ $P < 0.05$ compared to 4 mg/kg, ⁸ $P < 0.02$ compared to 8 mg/kg.

compared to their respective vehicle groups following 4 mg/kg ($P < 0.002$), 8 mg/kg ($P < 0.0001$), 16 mg/kg ($P < 0.0001$) and 32 mg/kg ($P < 0.0001$). Compared to 4

mg/kg, further decreases were significant beginning at 16 mg/kg ($P < 0.0004$). Compared to 8 mg/kg MPTP, 16 mg/kg and 32 mg/kg showed a greater decrease in

free-standing rears ($P < 0.005$ and 0.0001 , respectively). There was no further impairment beyond the 16 mg/kg dose.

The effect of MPTP treatment on WAR was also significant ($F_{(1,18)} = 79.2$; $P < 0.0001$). Repeated measures ANOVA showed that increasing the dose of MPTP resulted in increasing WAR ($F_{(3,10)} = 22$; $P < 0.0001$). The interaction between treatment and dose suggested that WAR were increasingly greater compared to the vehicle group ($F_{(3,10)} = 24.7$; $P < 0.0001$). Following 4 mg/kg, WAR increased compared to the vehicle group ($P < 0.02$; Fig. 4C). While WAR remained significantly greater compared to the vehicle group following 8 mg/kg ($P < 0.0001$), there was no further increase compared to the 4 mg/kg MPTP dose. There was a further increase in the 16 mg/kg group, where WAR was significantly different compared to the vehicle ($P < 0.0001$), 4 mg/kg ($P < 0.0008$) and 8 mg/kg ($P < 0.004$) groups. Finally, following 32 mg/kg, WAR further increased compared to the vehicle ($P < 0.0001$), 4 mg/kg ($P < 0.0001$), 8 mg/kg ($P < 0.0001$) treatment groups, but not 16 mg/kg MPTP. The group that discontinued MPTP treatment for 3 weeks maintained significantly fewer FSR compared to the vehicle group ($F_{(2,11)} = 26.7$; $P < 0.0001$; Table 1). These data are similar to those seen after the final week of treatment with 32 mg/kg of MPTP (Fig. 4B).

Total rears

FSR and WAR were combined to assess the effects of increasing doses of MPTP on total rears (Fig. 4D). There was a significant effect of treatment, suggesting that MPTP influenced the total number of rears ($F_{(1,10)} = 18.6$; $P < 0.002$). An interaction between treatment and dose indicated that increasing doses of MPTP resulted in a decrease in total rears ($F_{(3,8)} = 6.8$; $P < 0.02$). Post-hoc analysis revealed that following the 16 mg/kg MPTP dose, this group showed significantly fewer total rears compared to the vehicle, 4 mg/kg ($P < 0.01$). In the 32 mg/kg MPTP group, total rears maintained a significant difference compared to the vehicle and 4 mg/kg MPTP ($P < 0.01$). Following the 3-week washout period, the MPTP group showed significantly fewer total rears compared to the vehicle group ($F_{(2,11)} = 21.1$; $P < 0.0001$; Table 1). This finding is comparable to what is observed after the final week of treatment with MPTP at a dose of 32 mg/kg (Fig. 4D).

Foot faults per total activity (parallel rod activity chamber: PRAC)

There was an effect of treatment ($F_{(1,18)} = 8.2$; $P < 0.01$) on foot faults per total activity (FF/BB). The FF/BB ratio increased significantly compared to vehicle treated group following 32 mg/kg MPTP ($P < 0.03$). Compared to the other MPTP-treated groups, the FF/BB ratio in the 32 mg/kg MPTP group was only significantly increased compared to the 4 mg/kg group (Fig. 4E; $P < 0.05$). When analyzed separately, total activity as measured by BB revealed a significant effect of MPTP following 4 and 8 mg/kg ($F_{(1,18)} = 17.6$; $P < 0.0006$), but no difference be-

tween vehicle and MPTP groups following 16 mg/kg or 32 mg/kg (Fig. 4F).

Correlation of behavioral impairments and deficits of the nigrostriatal pathway due to increasing dose of MPTP

Correlation analyses of SNpc and CPU measurements with the behavioral data are shown in Fig. 5. The results summarized in Fig. 5A, B suggest a significant correlation between viability of the nigrostriatal pathway and olfactory latency (A) or FSR (B). Column A indicates significant negative correlations between increasing olfactory latency and FSR ($r = -0.74$, $P < 0.0001$; Fig. 5A₁), SNpc TH-ir neurons ($r = -0.74$, $P < 0.0001$; Fig. 5A₃), CPU TH-ir ($r = -0.73$, $P < 0.0001$; Fig. 5A₄) or CPU DA content ($r = -0.44$, $P < 0.02$; Fig. 5A₂), and a positive correlation with FF/BB ($r = 0.5$, $P < 0.0007$; Fig. 5A₅). Correlations in column B show a negative relationship between FSR and CPU DA Turnover ($r = -0.51$, $P < 0.006$; Fig. 5B₁), and positive relationships between FSR and SNpc TH-ir neurons ($r = 0.86$, $P < 0.0001$; Fig. 5B₂), CPU TH-ir ($r = 0.76$, $P < 0.0001$; Fig. 5B₃), CPU DA content ($r = 0.58$, $P < 0.0007$; Fig. 5B₄) or CPU TH protein expression ($r = 0.46$, $P < 0.02$; Fig. 5B₅).

The correlations summarized in Fig. 5C, D suggest significant relationships between nigrostriatal protein markers and total rears (C) or FF/BB (D). Column C indicates significant positive correlations between total rears and FSR ($r = 0.67$, $P < 0.0001$; Fig. 5C₁), SNpc TH-ir neurons ($r = 0.59$, $P < 0.0005$; Fig. 5C₂), CPU TH-ir ($r = 0.36$, $P < 0.05$; Fig. 5C₃), CPU DA content ($r = 0.53$, $P < 0.003$; Fig. 5C₄) or CPU TH protein expression ($r = 0.36$, $P < 0.05$; Fig. 5C₅). Negative correlations were significant between PRAC (i.e. FF/BB) and FSR ($r = -0.6$, $P < 0.0004$; Fig. 5D₁), SNpc TH-ir neurons ($r = -0.66$, $P < 0.0001$; Fig. 5D₂) and CPU TH-ir ($r = -0.64$, $P < 0.000$; Fig. 5D₃).

Correlation analyses of SNpc and CPU measurements showed several significant positive relationships between TH-ir, TH protein expression and DA content, and negative correlation with DA turnover. Significant correlations between cellular deficits are summarized in Table 2.

DISCUSSION

The present study indicates that a progressively increasing dose of MPTP results in a stepwise progressive decline of some behaviors, which correlates with a gradual decline in nigrostriatal DA. Though motor disturbances, measured by FF/BB, did not appear until there was a >70% loss of TH-ir in the CPU and >60% decrease in the mean number of SNpc TH-ir neurons/section, olfactory decline indicated the onset of Parkinson-like features much earlier on. The stepwise decline in FSR in the mouse may indicate onset of sensory or autonomic deficit, and may be useful for investigating future drug therapies. The results of the present study suggest that this progressive MPTP induced dopaminergic loss may be representative of both early and late stages of Parkinson-like decline. Although PD is gener-

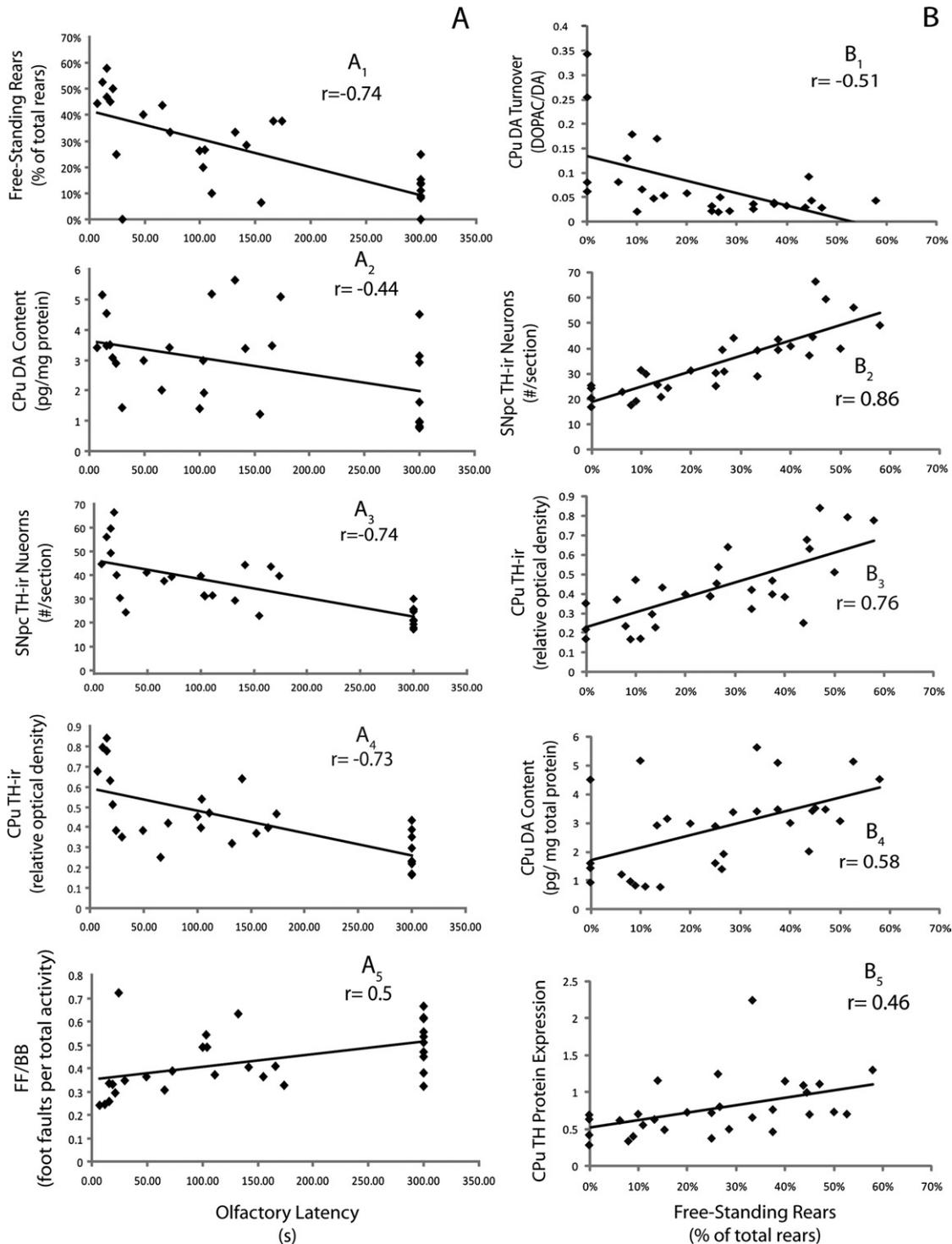


Fig. 5. Correlation of behavioral impairments and degeneration of the nigrostriatal pathway. The changes in free standing rears (% of total rears) (A₁) and foot fault/beam break behaviors (A₅), SNpc TH-ir neurons (A₃), CPU TH-ir (A₄) and DA content (A₂) were statistically correlated with increases in olfactory latency (A). SNpc TH-ir neurons (B₂), CPU TH-ir (B₃), CPU DA content (B₄), CPU TH protein expression (B₅) were all positively correlated with free standing rears while DA turnover (B₁) was negatively correlated with free standing rears (B). Free-standing rears (C₁), SNpc TH-ir neurons (C₂), CPU TH-ir (C₃), CPU DA content (C₄) and CPU TH protein expression (C₅) were all positively correlated with changes in total rears (C). Free-standing rears (D₁), SNpc TH-ir neurons (D₂) and CPU TH-ir (D₃) were all negatively correlated with the foot fault/beam break ratio (D). See Results section for a description of the statistical analyses.

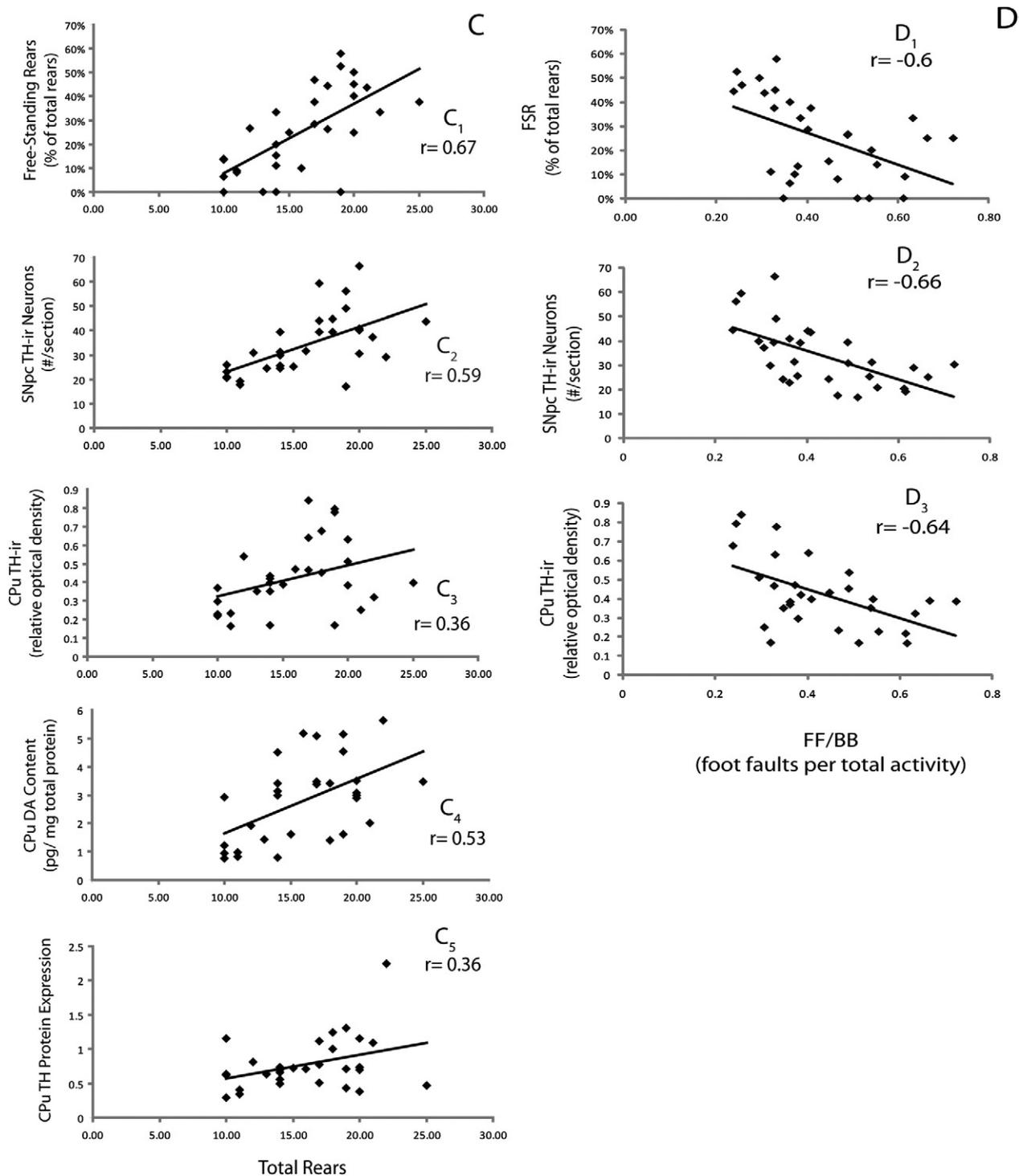


Fig. 5. (Continued).

ally considered a disease of aging, there has been recent progress in identifying pre-motor symptoms in patients who eventually develop the classical motor deficits. Some of these pre-motor indications can develop as early as 20 years prior to the manifestations of the motor signs (Hawkes et al., 2010), further justifying the

use of younger mice in this study. These pre-symptomatic clinical biomarkers include loss of olfaction, sleep abnormalities and autonomic dysfunction (Berendse and Ponsen, 2009; Siderowf and Stern, 2008). The current study equates to studying humans who are most likely mildly symptomatic and undiagnosed.

Tyrosine hydroxylase-labeling of the SNpc and CPu decreases similarly, but surviving CV-stained neurons fluctuate

The results of the present study show a gradual but not dose-dependent decrease in the mean number of TH-ir SNpc neurons/section. However, the relative optical density of TH-ir in the CPu was significantly decreased only following 4 mg/kg and 32 mg/kg MPTP. Therefore, during the continued decrease in TH-ir SNpc neurons with the higher dose of MPTP there was not an equivalent loss of TH-ir within the CPu. It is possible that as the number of DA neurons decreases, there are active compensatory mechanisms such as sprouting of DA terminals (Finkelstein et al., 2000) that results in a steady density of TH-ir within the CPu until the highest dose of MPTP is administered. Together, these results suggest that there is a progressive degeneration of the nigrostriatal pathway.

The immunohistochemical methodology of the current study may have yielded an underestimation of the mean number of TH-ir and CV+ neurons/section in the SNpc. However, according to recent comparisons of 2D and 3D analyses of brain tissue, our approach is appropriate (Baquet et al., 2009; Benes and Lange, 2001). Bezard et al. (1997) have reported that a 4 mg/kg dose of MPTP, administered daily over the course of 20 days, also results in a gradual loss of TH-ir SNpc neurons up to 70%. However, their study found that the decline of TH-ir neurons was greatest following each of the first five injections, but was asymptotic and no longer showed any progressive loss after 14 days of treatment. In comparison, the present study showed a slow and continued decrease in TH-ir out to 21 days in the SNpc and out to 28 days in the CPu, with final decreases of 62% SNpc TH-ir neurons, and 74% CPu TH-ir optical density. We are aware that in the current study, 4 weeks of a progressive decrease in DA markers in either the SNpc or CPu is still a shorter period compared to the time of progression observed in human Parkinson's disease. However, by administering a progressively higher dose of MPTP every other week, extending the study out to 8 weeks, we find an even greater effect on all of the behaviors and DA markers that were measured in the current study (Goldberg and Meshul, unpublished observation). We also have preliminary data that a further increase in the weekly dose of MPTP to 48 mg/kg/d results in a further decrease in the mean number of TH-ir neurons/sections (Goldberg and Meshul, unpublished observation). This suggests that for future studies, a further progression in terms of DA cell loss is possible.

The maintained decrease in the mean number of SNpc TH-ir neurons/section and the effects on specific behaviors following the 3-week washout period suggests that the effects of progressive MPTP lesioning were sustained. It has been reported that MPTP administration can transiently inactivate the TH enzyme by inducing the production of the superoxide peroxynitrite (Smeyne and Jackson-Lewis, 2005). Because the progressively treated MPTP mice did not recover TH-ir SNpc neurons following the washout period, it is unlikely that inactivation of the TH

protein is responsible for the decreases seen during progressive administration. A similarly sustained loss of SNpc TH-ir neurons has been observed in the previously characterized MPTP/probenecid model (Petroske et al., 2001). It was shown that with a similar 3-week washout period following chronic MPTP/probenecid, there was a 50% decrease in TH-ir SNpc neurons with no significant recovery.

The most successful MPTP models span 7 weeks, though some lack representative DA neuron decline (Blume et al., 2009; Schintu et al., 2009) or behavioral correlates (Meredith et al., 2008, 2002; Yazdani et al., 2006; Fornai et al., 2004). In addition, the current study achieved a 62% decrease in the mean number of TH-ir cells/section in the SNpc even after the highest dose of 32 mg/kg MPTP, while Bezard et al. (1997) reported a 70% decrease using just 4 mg/kg. At best, we find a 25% decrease in TH-ir SNpc cells following this same low dose of MPTP (Goldberg and Meshul, unpublished observation). It should be noted that we analyzed the rostro-caudal extent of the SNpc, while Bezard et al. (1997) specifically analyzed the median plane of the region. However, other progressive MPTP models have shown a similar discrepancy in MPTP susceptibility within the C57Bl/6 strain. The MPTP/probenecid mouse model of Parkinson's has been shown to achieve both 70% (Meredith et al., 2008), or a 30% (Schintu et al., 2009) decrease in TH-labeled SNpc neurons following 5 weeks of treatment.

The counter-staining of SNpc tissue with CV showed an increase in the mean number of CV+/TH− neurons/section following 1 week of 4 mg/kg, 8 mg/kg and 16 mg/kg MPTP doses, but decreased significantly after the 32 mg/kg dose. This indicates that after 8 mg/kg and 16 mg/kg MPTP, a certain percent of DA neurons stopped expressing TH, but were still viable as suggested by the increase in CV+ neurons. These CV+/TH− neurons (see Fig. 1A, middle column) did not appear necrotic or swollen, but cell shape or area was not quantified. After 32 mg/kg, CV+/TH− neurons decreased back to levels similar to the vehicle group, suggesting that this population of CV+ neurons once expressed TH, but were TH negative following the 16 mg/kg dose and most likely degenerated by the end of the 32 mg/kg dose. This pattern is a new phenomenon, as most acute MPTP models demonstrate the simultaneous loss of TH-ir and Thionin or other neural markers (Haque et al., 2009; Benner et al., 2004; Jackson-Lewis et al., 1995; Seniuk et al., 1990). Our results are in agreement with previous findings that TH mRNA in PD patients is decreased in surviving DA neurons compared to controls (Javoy-Agid et al., 1990). However, the total mean number of CV+ neurons/section, including TH-ir neurons, showed a decreasing trend only following 16 mg/kg MPTP in the current study. Because there were so many more CV+/TH+ neurons than CV+/TH− neurons, the fluctuation of the CV+/TH− surviving neurons is less apparent in the total neuron counts. To our knowledge, this is the first time that CV+/TH− neurons have been reported in a progressive MPTP study. However, the observation of a small population of surviving CV+/TH− neurons has been re-

ported in both an acute and a sub-acute MPTP mouse models (Anderson et al., 2008).

Western immunoblotting and correlation with immunohistochemistry

Western immunoblot analysis was performed on SN and dorsolateral CPu tissue to compare TH protein expression to TH-ir neuron and nerve terminal optical density levels as shown in the immunohistochemical (IHC) analysis. Though the mean number of TH-ir neurons/section began to decrease following 1 week of 4 mg/kg MPTP and continued to decrease after 1 week of 8 mg/kg and 16 mg/kg, TH protein expression by Western immunoblot in the SN did not decrease until after 8 mg/kg compared to vehicle levels. This suggests that the amount of TH protein in the CPu may not be accurately represented in the number of TH-ir neurons. However, the decrease in the relative optical density of TH-labeled nerve terminals is similar to the decrease in TH protein expression. It is possible that the remaining TH-ir neurons are employing compensatory mechanisms such as increased DA synthesis (Zigmond et al., 1990) or sprouting (Finkelstein et al., 2000; Song and Haber, 2000), resulting in greater levels of TH protein/neuron at the terminals.

Expression of DAT protein in the CPu terminals, as analyzed by Western immunoblot, decreased significantly following 4 mg/kg and 16 mg/kg MPTP. The decrease seen in CPu DAT occurred earlier on and was more pronounced than the decrease seen in TH. This is consistent with the finding that the decrease in CPu DAT binding is greater than the decrease in TH expression in pre-symptomatic stages of MPTP-treated Macaque monkeys (Meissner et al., 2003). Additionally, it has been reported that DAT decreases similarly to TH protein expression following sub-acute MPTP (Chen et al., 2009).

Changes in CPu DA content and correlation with Western immunoblotting

When DA tissue content of the CPu was determined, a significant decrease in DA was found only after 16 mg/kg and 32 mg/kg MPTP. This was consistent with the finding by Western immunoblot that TH did not decrease significantly until a higher dose. This was confirmed by the delayed increase of turnover until the highest dose of MPTP, 32 mg/kg, was administered.

However, the delayed decrease in CPu DA tissue content is inconsistent with previous findings using the MPTP/probenecid progressive model, where DA content in the CPu dropped drastically after one dose of 25 mg/kg, and when the relative optical density of TH was decreased either by 30% (Schintu et al., 2009), or by 70% (Novikova et al., 2006). However, the tissue preparation used by Schintu et al. (2009) was notably different from that used in the present study which could have resulted in different sensitivity to changes in DA levels. Additionally, Alvarez-Fischer et al. (2008) (Fig. 3), showed that after 5 weeks of MPTP/probenecid, which was infused by osmotic minipump, CPu DA content decreased by ~25% whereas optical density of TH-ir CPu fibers decreased by ~58%.

These data from the current studies and others (Schintu et al., 2009; Alvarez-Fischer et al., 2008; Novikova et al., 2006; Sedelis et al., 2001, 2000; Seniuk et al., 1990) suggest that measuring several markers of DA integrity is crucial in understanding how a progressive dosing of MPTP affects the nigrostriatal pathway and production of DA.

It is noteworthy that changes in CPu DA content due to an increasing dose of MPTP show a similar trend to CPu TH protein expression. These measurements corroborate to suggest that while TH-ir SNpc neurons may be partially degenerated following only 4 mg/kg MPTP, the remaining neurons are able to compensate by increasing TH protein and DA content in the CPu. This may occur by several mechanisms, including decreasing DA uptake (Zhang et al., 1988) and increased synthesis (Zigmond et al., 1990). Additionally, it has been reported that DA neurons can sprout terminals within the CPu up to an 80% lesion, which implies that the neurons can compensate for partial DA neuron loss up to this point (Finkelstein et al., 2000, their Fig. 8; Song and Haber, 2000). This explanation supports the findings in the current study that DA content did not decrease until a dose of 16 mg/kg MPTP. At this higher dose, the decrease in SNpc neurons was ~60%, with a decrease in CPu TH-ir of ~70%.

Changes in olfactory, rearing and locomotor behaviors

The earliest detection of behavioral impairment due to MPTP treatment was observed in the olfactory test. The trend in increased latency to bite the corn chip followed the same time course as the decrease in TH-ir SNpc neurons. This correlation indicates a sensitivity of the olfactory test to the decrease in TH-ir neurons in the SNpc, and suggests that 1 week of 4 mg/kg MPTP is sufficient to impair olfactory sensitivity. Similar MPTP models have also seen olfactory impairment with less than a 30% decrease in TH-ir neurons (Schintu et al., 2009). Following 1 week of 16 mg/kg MPTP, 60% of the mice score above 5 min on the latency test, and following 32 mg/kg, 80% of the mice scored beyond 5 min (data not shown). This suggests that after 32 mg/kg MPTP, the 74% CPu and 62% SNpc decrease in TH-ir was sufficient to significantly alter olfactory sensitivity. While it is possible that the initial increases in olfactory latency at 4 mg/kg and 8 mg/kg MPTP were related to decreases seen in total locomotor activity (BB) as compared to the vehicle groups, the number of BB increased to vehicle levels at during the 16 mg/kg and 32 mg/kg doses of MPTP. This suggests that the latency to bite the corn chip following the higher doses of MPTP was not due to decreased motor activity.

The correlation of olfactory decline with the decrease in DAT protein expression in the CPu is similar to a report in elderly patients with nigrostriatal degeneration that olfactory decline correlates with decreased CPu DAT binding (Wong et al., 2010, their Fig. 4). Additionally, it has been suggested that PD can be distinguished from subjects without evidence of dopaminergic deficit (SWEDDs) by olfactory impairment (Silveira-Moriyama et al., 2009). This suggests that the correlation is a strong representation of

early Parkinson-like behavioral/sensory deficits. Alteration in olfactory sensitivity is relatively specific to PD, where other movement disorders such as multiple system atrophy and supranuclear palsy express olfactory impairment infrequently or not at all (Mckinnon et al., 2010; Doty et al., 1993; Wenning et al., 1993). Both olfactory sensitivity and FSR decreased following 4 mg/kg and 16 mg/kg MPTP. This further suggests that both olfactory and FSR behaviors are sensitive to the decrease in TH-ir in the SNpc and CPu.

It has been established that the decreases in nigrostriatal DA and TH-ir due to MPTP result in mice rearing less frequently (Fornai et al., 2004; Sundstrom et al., 1990), in agreement with the decline in total rears in the current study. However, the distinction between free-standing and wall-assisted rears has not been thoroughly investigated, and to our knowledge, has not yet been comparatively reported in other sub-chronic MPTP models (Goldberg et al., 2010). Behavioral markers such as olfactory function and free-standing rears, which we show to be progressively consistent with the decline of nigrostriatal DA, are important because they can be correlated with the incidence of CSF biomarkers (see Introduction; Van Dijk et al., in press; Sinha et al., 2009; Goldstein et al., 2008; Balducci et al., 2007; Abdi et al., 2006).

Because FSR decreased in a progressive manner similar to the decline in total rears, it was necessary to examine changes in WAR in order to report whether the decrease in FSR was due to increased dependence on wall support or general decline in rearing. A significant increase in WAR following doses of 4 mg/kg and 16 mg/kg of MPTP compared to the other groups suggests that decreased total rearing during the latter half of the study was due to both a decrease in FSR, and an increased dependence on the wall for support while rearing. It is possible, therefore, that the decline in FSR and total rears was associated with a decline in blood pressure, which has been reported extensively in human Parkinson's patients as orthostatic hypotension, and results in dizziness when standing upright from a sitting position (Oka et al., 2007; Senard et al., 1997). It will be of interest in future studies to measure blood pressure of mice before and after MPTP treatments to assess the possible role of orthostatic hypotension in differential FSR percentage and total rears.

The continued impairment of olfactory and rearing behavior in MPTP treated animals following the 3-week washout period corroborates the sustained SNpc TH-ir deficit. This suggests that the effect of the progressive MPTP regimen produced a stable lesion. In the chronic MPTP/probenecid model, rotorod performance showed a similar maintenance of motor deficit 3 weeks following the last MPTP/probenecid injection (Petroske et al., 2001).

The observed lack of change in FF/BB with the lower doses of MPTP is consistent with other animal and clinical studies, which suggest that sensory deficits such as olfactory and exploratory behavior precede the appearance of motor symptoms (Juri et al., 2010; Gierthmuhlen et al., 2009; Schneider and Kovelowski II, 1990). Only at the higher doses of MPTP, resulting in a greater decrease in

TH-ir neurons in the SNpc compared to the current study, are alterations in fine motor movements observed (as measured in the grid test; Meredith and Kang, 2006; Tillery and Miller, 2003; Petroske et al., 2001). Additionally, delayed motor decline in the current progressive MPTP study is appropriate since PD patients are not typically diagnosed until motor symptoms are apparent, and when 60–70% of nigrostriatal DA is depleted (Bernheimer et al., 1973; Riederer and Wuketich, 1976). In the current study, both FF/BB behavior and CPu TH protein expression showed significant deficit only after 32 mg/kg MPTP, and DA content decreased only following 16 mg/kg MPTP. These correlations suggests that the delay in FF/BB deficit may have been due to compensatory mechanisms that were successful until a >70% lesion was achieved. In addition, we are currently pursuing studies to investigate the effectiveness of L-DOPA in reversing the observed motor symptoms.

The advantage of the progressive decline in TH-ir neurons in the SNpc over the 4-week time period is that therapies can be tested at any time point. Interventions could be initiated at an early time point (i.e. after 4 or 8 mg/kg MPTP) in order to observe their effect on slowing the progression of nigrostriatal degeneration, or at a later time point (i.e. 16 mg/kg or 32 mg/kg MPTP) to determine whether there is a restorative effect. The intervention would take place during continued MPTP administration. These are stages at which the decrease in TH-ir neurons in the SNpc is equivalent to that seen in patients with both early stage and late stage Parkinson's disease (Mori et al., 2006).

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