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# Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease

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## Abstract

Despite the widespread use of mice as models of Parkinson's disease there is a surprising lack of validation and characterisation of unilateral lesion models in mice and the extent of behavioural impairments induced by such lesions. The aim of the present study was to characterise the behavioural deficits observed after injection of 6-hydroxydopamine unilaterally into the substantia nigra, and correlate the behavioural impairments with the extent of damage to the mesostriatal dopaminergic pathway. We found that a recently introduced test for assessment of sensorimotor impairment, the corridor task, was particularly useful in determining lesion severity, and that this test, in combination with standard drug-induced rotation tests, can be used to select animals with profound (≥ 80%) dopaminergic lesions that are stable over time. Based on these data we propose criteria that can be used to predict the extent of lesion, classified as severe, intermediate or mild lesions of the mesostriatal pathway. The correlation of cell loss and striatal innervation with the performance in each test provides a useful tool for the assessment of functional recovery in neurorestoration and cell transplantation studies, and for the evaluation of the *in vivo* efficacy and performance of stem cell-derived dopamine neuron preparations.

## Introduction

Damage to the midbrain dopamine (DA) neurons induced by systemic injections of 1-methyl-1,2,3,4-tetrahydropyridine (MPTP) is the most commonly used model of Parkinson's disease (PD) in mice. The MPTP model is highly valuable as a model of neurotoxin-induced oxidative and mitochondrial damage, and is particularly attractive as it avoids the use of more specialised stereotaxic surgery. However, the MPTP model is less useful for functional studies as the lesion-induced behavioural impairments are quite subtle and also strain-dependent (Sedelis *et al.*, 2000), and unless a very heavy treatment regimen is used (e.g., 10 injections of 25 mg/kg + probenecid over 5 weeks; Meredith *et al.*, 2008) the impairments are mostly transient (Sedelis *et al.*, 2001). The bilateral deficits seen in MPTP-treated mice are also more difficult to quantify and distinguish from more general sickness-related behaviour. The experience gained from studies in rats shows that profound and stable PD-like motor impairments are more easily obtained and quantifiable in animals with unilateral lesions, induced by unilateral injections of 6-hydroxydopamine (6-OHDA) into either the medial forebrain bundle (MFB) or the striatum (Kirik *et al.*, 1998; Dowd & Dunnett, 2005).

The unilateral 6-OHDA lesion model has, since its introduction 40 years ago (Ungerstedt, 1968), remained the most widely used PD model in rats. Its application in mice has proved more problematic, at least in part due to the smaller size of the mouse brain, which makes it more difficult to achieve reproducible stereotaxic placements of the toxin injections. Nevertheless, several investigators have explored the possibility of inducing stable behavioural deficits by injection of 6-OHDA into either the striatum (Von Voigtlander & Moore, 1973; Akerud *et al.*, 2001; Lundblad *et al.*, 2004; Alvarez-Fischer *et al.*, 2008) or the MFB (Lundblad *et al.*, 2004; Iancu *et al.*, 2005). When successful, the MFB lesion is clearly very efficient but has a major disadvantage in that it is associated with a very high mortality rate: Lundblad *et al.* (2004) reported a 14% success rate with a mortality rate of 82%, and our own experience (S. Grealish and A. Björklund, unpublished data), using the same lesion parameters as in the Lundblad *et al.* (2004) study, is in line with the results reported here. The experience obtained in studies using intrastriatal 6-OHDA delivery, on the other hand, is that the 'success rate', i.e. the percentage of lesioned animals showing severe nigrostriatal neurodegeneration and behavioural deficits, is generally insufficient in this approach (Lundblad *et al.*, 2004; S. Grealish and A. Björklund, unpublished data).

Intranigral injection of 6-OHDA has emerged as an interesting third alternative. This version, which was introduced by Parish *et al.* (2001), is attractive in that it makes it possible to induce more extensive DA neurodegeneration in the absence of the high mortality rate seen in MFB-lesioned mice. Based on our own preliminary experiments, and results reported in studies from other laboratories (Moses *et al.*, 2008; Parish *et al.*, 2008), we feel that the intranigral 6-OHDA lesion is the one that holds greatest promise for long-term studies in mice.

Regardless of the site of injection, however, 6-OHDA lesions in mice are highly variable, and in any single round of surgery only a sub-portion of the injected animals can be expected to be well lesioned. This has, so far, posed a serious limitation to the usefulness of this mouse PD model. Moreover, the behavioural tests commonly

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used in the 6-OHDA-lesioned rats have as yet not been properly validated for use in mice. The standard amphetamine- and apomorphine-induced rotation tests have been directly applied to 6-OHDA-lesioned mice although it is unclear whether they are equally informative in mice. Indeed, there is no consensus on what protocols to use for evaluation of behavioural impairments following the 6-OHDA lesion. The doses used in amphetamine-induced rotation tests in mice range from 2 mg/kg (Perez *et al.*, 2005) to 10 mg/kg (Offen *et al.*, 2007), and the doses used in the apomorphine-induced rotation tests in mice (ranging from 0.5 to 4 mg/kg (Von Voigtlander & Moore, 1973; Akerud *et al.*, 2001) are much higher than those typically used in rats (0.05–0.25 mg/kg).

The aim of the present study was to perform a more extensive morphological and behavioural characterisation of the unilateral intranigral 6-OHDA lesion mouse model and correlate the extent of damage to the mesostriatal DA projections with the magnitude of impairment seen in a battery of tests commonly used for assessment of motor impairments in rats. Based on this information we have devised a set of behavioural criteria that can be used to indentify well lesioned mice prior to any restorative or disease modifying intervention. In addition to the standard drug-induced rotation, cylinder and stepping tests, we have explored the usefulness of a novel sensorimotor integration test, the corridor task, originally developed for studies in rats (Dowd *et al.*, 2005a), for quantification of behavioural impairments in 6-OHDA-lesioned mice.

## Materials and methods

### Experimental design

A total of 129 female mice (Charles River; NMRI strain, weighing 25–35 g at the time of surgery) were used in this study. 6-OHDA was injected unilaterally in the substantia nigra in 122 mice. The remaining seven mice served as intact controls. All mice were subjected to behavioural analysis in all tests described below and, based on the wide range of motor impairments seen in both drug-induced rotation tests and the corridor task, 40 of the 6-OHDA-lesioned mice were selected for further analysis in the present study, while the others were used in a different experiment. The selection was made so as to include animals that represented the full range of motor deficits induced by the intranigral 6-OHDA lesion, defined as mild, intermediate and severe impairments. In all behavioural tests the animals were numbered without any indication of treatment.

The stability of the motor deficits over time was studied in seven mice that exhibited severe behavioural deficits in the early post-lesion time-point. Beginning 6 weeks after lesioning, these mice were tested at regular intervals in the corridor, drug-induced rotation and stepping tests until 23 weeks post-lesion. In this experiment the seven unoperated mice were included in all tests as intact controls.

The animals were housed under standard conditions with free access to food and water under standard 12-h light–dark regime (light 07.00–19.00 h). All procedures were conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals at Lund University.

### 6-Hydroxydopamine (6-OHDA) injections

6-OHDA (Sigma, Sweden) was injected into the substantia nigra (SN) pars compacta under gaseous anaesthesia and analgesia (2% isoflurane in 2 : 1 oxygen/nitrous oxide), using a stereotaxic mouse frame (Stoelting Germany) and a 5-$\mu$L Hamilton syringe fitted with a fine glass capillary (external diameter 60–80 $\mu$m). The toxin was used at a concentration of 1.6 $\mu$g/$\mu$L (calculated as free base) dissolved in a solution of 0.2 mg/mL ascorbic acid in 0.9% sterile saline, slightly modified from that used by Parish *et al.* (2001). A total volume of 1.5 $\mu$L was injected using the stereotaxic coordinates A/P = −3.0, M/L = −1.2, D/V = −4.5, with a flat skull position (coordinates in mm, with anterior–posterior and lateral measured from bregma, and ventral from dura). Injections were made at a rate of 0.5 $\mu$L/min with a further 2 min allowed for the toxin to diffuse before slow withdrawal of the capillary, followed by cleaning and suturing of the wound.

### Drug-induced rotation

Rotational asymmetry was assessed using an automated rotometer system (AccuScan Instruments, Columbus, OH, USA) based on the design of Ungerstedt & Arbuthnott (1970). Full body turns were counted and data was expressed as net turns per minute, with rotation toward the side of the lesion given a positive value. Amphetamine-induced rotational scores were used as an estimate of the extent of DA depletion and were collected over a 40-min test session following 5 mg/kg of D-amphetamine sulphate, i.p. (dissolved in 0.9% sterile saline). Animals were allowed to habituate for 5 min after injection before the recording of rotations began.

Apomorphine-induced rotation reflects the hypersensitivity of the lesioned striatum and this was assessed by testing over a 40-min test session after challenge with 0.1 mg/kg of apomorphine, s.c. (dissolved in a solution of 0.2 mg/mL ascorbic acid in 0.9% sterile saline). Animals were primed on two separate days prior to performing the rotation test for the first time (i.e. priming on Monday and Wednesday, followed with rotation test on Friday). This avoided a 'wind-up' effect that could obscure the rotational responses observed. Animals were allowed to habituate for 5 min after injection before the recording of rotations began.

### Corridor task

Lateralized sensorimotor integration was measured using a task that was first established in rats by Dowd *et al.* (2005a) and is based on the classic tests of sensorimotor integration as introduced by Marshall *et al.* (1974). In the current study the corridor test was adapted to mice using a long narrow plastic corridor (60 cm long, 4 cm wide and 15 cm high) with 10 pairs of adjacent pots, each with a diameter of 1 cm (Push cap; LIP Ltd., Galway, Ireland), containing 4-5 sugar pellets (20 mg; TestDiet) that were placed at 5-cm intervals along the length of the corridor (Fig. 1). A clear Perspex lid was placed on top of the apparatus to allow the mice to be observed during testing. Mice were food-restricted and maintained at 85% free-feeding bodyweight throughout habituation and testing. At the first time point, mice were habituated to the corridor by scattering sugar pellets along the floor and allowing them to freely explore for 10 min on two consecutive days prior to testing. When testing began, the mice were first placed in an identical, but empty, corridor for habituation for 5 min, before being transferred to one end of the testing corridor. The number of ipsilateral and contralateral retrievals made by each mouse was counted until the mouse made a total of 20 retrievals, or a maximum time of 5 min elapsed. A 'retrieval' is defined as an exploration into a pot, whether or not a pellet is eaten, and a new retrieval can only be made by investigating a new pot (Dowd *et al.* 2005a). Data are expressed as percentage contralateral retrievals, calculated as the number of contralateral retrievals expressed as a percentage of the total retrievals made from both sides relative to the lesion.

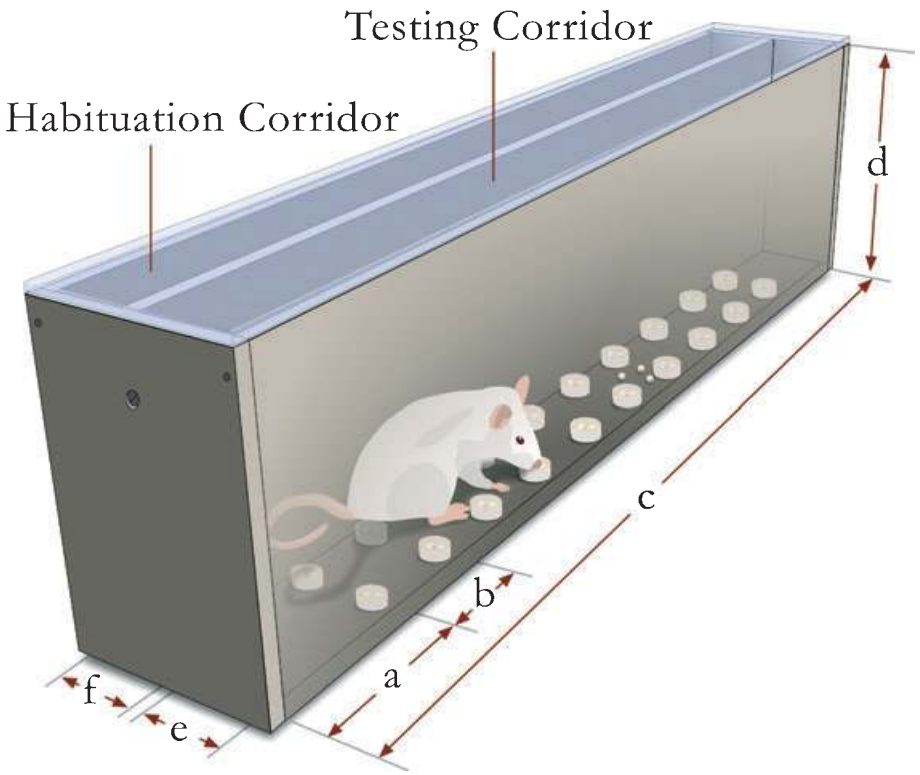


FIG. 1. Design of the corridor task apparatus. A mouse is first placed into an identical but empty habituation corridor for 5 min prior to being placed at the start of the testing corridor, as shown. The testing corridor has adjacent pairs of pots (diameter of 1 cm) that contain sugar pellets (four or five per pot) and the number of retrievals made on each side of the body is counted to determine the lateralised sensorimotor integration deficit induced by the unilateral 6-OHDA lesion. Dimensions are as follows: (a) 7.5 cm, (b) 5 cm, (c) 60 cm, (d) 15 cm, (e) and (f), 4 cm.

### *Stepping test*

Forelimb akinesia was assessed using the stepping test (Olsson *et al.*, 1995), as adapted for mice. Briefly, the mouse was held by the experimenter with one forelimb restrained and the free forepaw placed on a table surface. The number of adjusting steps made by the mouse, using the free forelimb, was counted as it was moved sideways along a table surface over a distance of 30 cm, in both forehand and backhand directions. Data are expressed as the sum of forehand and backhand steps made by each paw.

### *Cylinder test*

Forelimb use was assessed using the cylinder test, as previously described by (Schallert & Tillerson, 2000). Mice were placed in a glass cylinder (diameter 19 cm, height 20 cm), with mirrors placed behind to allow for a 360° view of all touches, until at least 30 weight-bearing paw touches were made by the forelimbs against the side of the cylinder. The session was videotaped and later scored. Paw touches were analysed using freeze-frame analysis of the recording and, in cases where both paws were used simultaneously, these touches were not counted. Data are expressed as percentage contralateral touches, calculated as the number of contralateral touches expressed as a percentage of the total touches made using both paws.

### *Tissue processing and immunohistochemistry*

Once behavioural analysis was complete, mice were terminally anaesthetised with sodium pentobarbitone i.p. (Apoteket, Sweden). Mice were then transcardially perfused with 15 mL of room-temperature (21°C) 0.9% saline, followed by 100 mL of ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were post-fixed for 2 h at 4°C and then transferred to 25% sucrose in PBS at 4°C for cryoprotection overnight. The brains were then sectioned in the coronal plane using a freezing microtome at a thickness of 35 $\mu$m. Sections were collected in six series and stored at −20°C in an antifreeze solution (phosphate buffer containing 30% glycerol and 30% ethylene glycol) until free-floating immunohistochemistry was performed.

Briefly, sections were rinsed three times in potassium phosphate-buffered saline (KPBS) and then endogenous peroxidase activity was quenched in 3% $H_2O_2$ and 10% methanol in KPBS for 20 min. After three rinsing steps in KPBS, the sections were incubated in a blocking solution consisting of 5% normal goat serum in KPBS and 0.25% Triton X-100, to block nonspecific binding sites. Sections were then incubated overnight at room temperature in the same blocking solution as described above with the primary antibody, rabbit anti-tyrosine hydroxylase (TH; 1 : 1000; AB152; Chemicon). On the second day, the sections were rinsed three times in KPBS and then incubated in blocking solution for 20 min before being incubated for 1 h in a 1 : 200 dilution of biotinylated secondary antibody, goat anti-rabbit (Vector Laboratories), in blocking solution. After rinsing three times, the sections were treated with avidin–biotin–peroxidase complex (ABC Elite kit; Vector Laboratories) in KPBS for 1 h before being rinsed again. The colour reaction was developed by incubation in 25 mg/mL 3,3′-diaminobenzidine and 0.01% $H_2O_2$. Sections were mounted on gelatine-coated glass slides, dehydrated in an ascending series of alcohols,

cleared in xylene and cover-slipped with DPX mounting medium (BDH Chemicals).

### Densitometry

High-resolution images were captured from the TH-immunostained sections using a SCANSCOPE GL system with IMAGESCOPE v8.2 software (Aperio Technologies, Oxford, UK). The extent of striatal denervation, as a consequence of lesion, was measured by densitometry in dorsal and ventral halves from three TH-stained sections, as indicated in Fig. 3, corresponding to +0.7, +0.2 and −0.26 mm from bregma, using IMAGE J software (Version 1.32j; National Institutes of Health, USA). The entire striatum was divided into two equal halves along the dorsoventral axis and the measured values were corrected for nonspecific background staining by subtracting values obtained from the corpus callosum. The data are expressed as optical density as a percentage of the corresponding area from the intact hemisphere, and values from all sections were combined to provide a single value for each region.

### Stereology

Unbiased stereological analysis was conducted, using the optical fractionator principle (West, 1999) to estimate the number of $TH^+$ cell numbers in the SN and ventral tegmental area (VTA). The borders defining the SN and VTA on all levels along the rostrocaudal axis were delineated by using a low-power objective lens (4×; SPlan). The medial border of the SN and lateral border of the VTA was defined by a vertical line passing through the medial tip of the cerebral peduncle (and by the medial terminal nucleus of the accessory optic tract, when present in sections). The ventral border followed the dorsal border of the cerebral peduncle, thereby excluding the $TH^+$ cells in the pars reticulata, and the area extended laterally to include the pars lateralis in addition the pars compacta. The sections used for counting covered the entire SN and VTA from the rostral tip of the pars compacta back to the caudal end of the pars reticulata. This typically yielded five or six sections in a 1 : 6 series. The counting was done using a 60× Plan-Apo oil objective (numerical aperture = 1.4) on a Nikon 80i microscope equipped with an *X-Y* motorise stage (Märzhauser, Wetzlar, Germany), a *Z*-axis motor and a high-precision linear encoder (Heidenhain, Traunreut, Germany). All three axes and the input from the digital camera were controlled using a PC running software that utilised a random start systematic sampling routine (NewCast Module in VIS software; Visiopharm A/S, Horsholm, Denmark). The sampling interval in the *X-Y* axis was adjusted so that at least 100 cells were counted for each region of interest. Coefficient of error attributed to the sampling was calculated according to Gundersen & Jensen (1987). Errors = 0.10 were accepted. In Figs 4 and 6 data are expressed as percentage surviving cells and in Table 1 as percentage lost, with the intact hemisphere corresponding to 100% for each individual mouse. The average number of $TH^+$ cells counted in the intact SN was 2698 ± 699.57 and the average in the VTA was 2645 ± 782.94.

### Statistics

All data are expressed as mean ± SEM unless stated otherwise. All statistical analyses were conducted using the Statistical Package for the Social Sciences 17 (SPSS Inc.). A paired Student's *t*-test was used to compare the number of midbrain $TH^+$ neurons on the intact and 6-OHDA-injected side. Linear regression was performed on the densitometric values and cell counting in Fig. 4, the correlations between the performances in the different behavioural tests in Fig. 5 and the correlations between behavioural impairments and densitometric values and cell counts in Fig. 6. A one-way ANOVA with a Tukey *post hoc* was performed on the behavioural data comparing subgroups of lesioned mice in Fig. 7. The long-term deficits observed in lesioned and intact animals (Fig. 8) were compared using a two-way ANOVA using the generalised linear model and the Wald chi-square test, with main effects of group and time. A one-way ANOVA with a Student–Newman–Keuls *post hoc* was performed for all of the parameters described in Table 1, with all contrasts at least $P < 0.05$.

## Results

### Extent of damage to the nigrostriatal DA pathway

The 6-OHDA injection was targeted at the mediolateral/anterior–posterior mid-point of the SN pars compacta, as illustrated in a composite, horizontal TH-immunostained section in Fig. 2. The lesion caused in most cases a substantial loss of the A9 cells in the SN, while

TABLE 1. Summary of the extent of denervation of the mesostriatal pathway and the resultant behavioural impairments assessed in each of the five tests

| | | Lesion type | | | Statistics | |
|---|---|---|---|---|---|---|
| Parameter | Intact mice | Mild | Intermediate | Severe | *F*-values | *Post hoc* |
| Striatal Denervation (reduction in % of intact side) | | | | | | |
| Dorsal | N/A | 53.7 ± 17.2 | 81.0 ± 7.4 | 96.7 ± 2.9 | $F_{2,33} = 44.31$ | M < I < S* |
| Ventral | N/A | 27.2 ± 7.9 | 52.5 ± 9.1 | 79.0 ± 9.9 | $F_{2,33} = 105.11$ | M < I < S* |
| Total | N/A | 43.1 ± 10.3 | 69.1 ± 5.5 | 88.8 ± 4.8 | $F_{2,33} = 113.93$ | M < I < S* |
| $TH^+$ cell loss (reduction in % of intact side) | | | | | | |
| SN | N/A | 71.4 ± 17.9 | 92.5 ± 6.9 | 94.4 ± 7.1 | $F_{2,33} = 13.26$ | M < I = S* |
| VTA | N/A | 17.3 ± 16.9 | 33.1 ± 13.2 | 59.3 ± 14.6 | $F_{2,33} = 7.88$ | M < I = S$^\dagger$ |
| Total | N/A | 44.4 ± 16.0 | 62.6 ± 9.5 | 67.5 ± 8.9 | $F_{2,33} = 13.26$ | M < I = S* |
| Corridor task (% contralateral retrievals) | 52.0 ± 6.4 | 36.6 ± 5.9 | 21.5 ± 16.3 | 8.5 ± 6.7 | $F_{3,45} = 47.44$ | In > M > I > S* |
| Apomorphine-induced rotation (net contralateral turns per minute) | 0.0 ± 0.0 | −0.1 ± 0.4 | −3.1 ± 4.3 | −7.2 ± 3.9 | $F_{3,45} = 18.63$ | In = M > I > S* |
| Amphetamine-induced rotation (net ipsilateral turns per minute) | 1.2 ± 3.6 | 0.9 ± 5.9 | 9.1 ± 3.0 | 8.6 ± 5.3 | $F_{3,45} = 11.12$ | M = In < S = I* |
| Stepping test (number of contralateral steps) | 15.1 ± 0.9 | 11.8 ± 3.1 | 10.1 ± 3.8 | 8.8 ± 3.5 | $F_{3,39} = 9.42$ | S = I = M < In* |
| Cylinder test (% contralateral touches) | 49.8 ± 3.7 | 43.2 ± 7.7 | 39.3 ± 11.9 | 36.5 ± 10.2 | $F_{3,39} = 5.10$ | S = I < In$^\dagger$ |

Data are presented as mean ± SD. One-way ANOVA with a Student–Newman–Keuls *post hoc*. *$P < 0.0001$, $^\dagger P < 0.05$. I, intermediate; In, intact; M, mild; S, severe.

the A10 cells in the VTA were less affected. In the 40 mice included in the present study the total $TH^+$ cell loss, in SN and VTA combined, ranged from −12 to −82% (mean, −58.5 ± 15.9%), which was highly significant compared to the intact hemisphere ($t_{35} = -21.5$; $P < 0.0001$). The loss of $TH^+$ cells in the SN (−85.8 ± 15.7%; $t_{35} = -31.2$, $P < 0.0001$) was more severe than in VTA (−31.6 ± 18.6%; $t_{35} = -9.8$, $P < 0.0001$) when compared to the respective structures in the intact hemisphere. Representative examples of the extent of $TH^+$ cell loss in animals with varying degrees of degeneration are illustrated in Fig. 3.

As illustrated in the TH-stained sections in Fig. 3, the loss of $TH^+$ cell bodies was accompanied by a substantial loss of $TH^+$ innervation in the striatum. Densitometry of the $TH^+$ innervation of the entire striatum [caudate–putamen (CPu) and nucleus accumbens (NAc) combined] showed a $TH^+$ fibre loss that ranged from 25 to 95% (mean, 65 ± 20.7%), which was significant compared to the intact hemisphere ($t_{35} = -18.8$, $P < 0.0001$). The denervation was most pronounced in the dorsal part, including to the CPu, which is the main target of the $TH^+$ cells in the SN (−75.2 ± 21.6%; $t_{35} = -20.9$, $P < 0.0001$), and overall less severe in the ventral part, corresponding

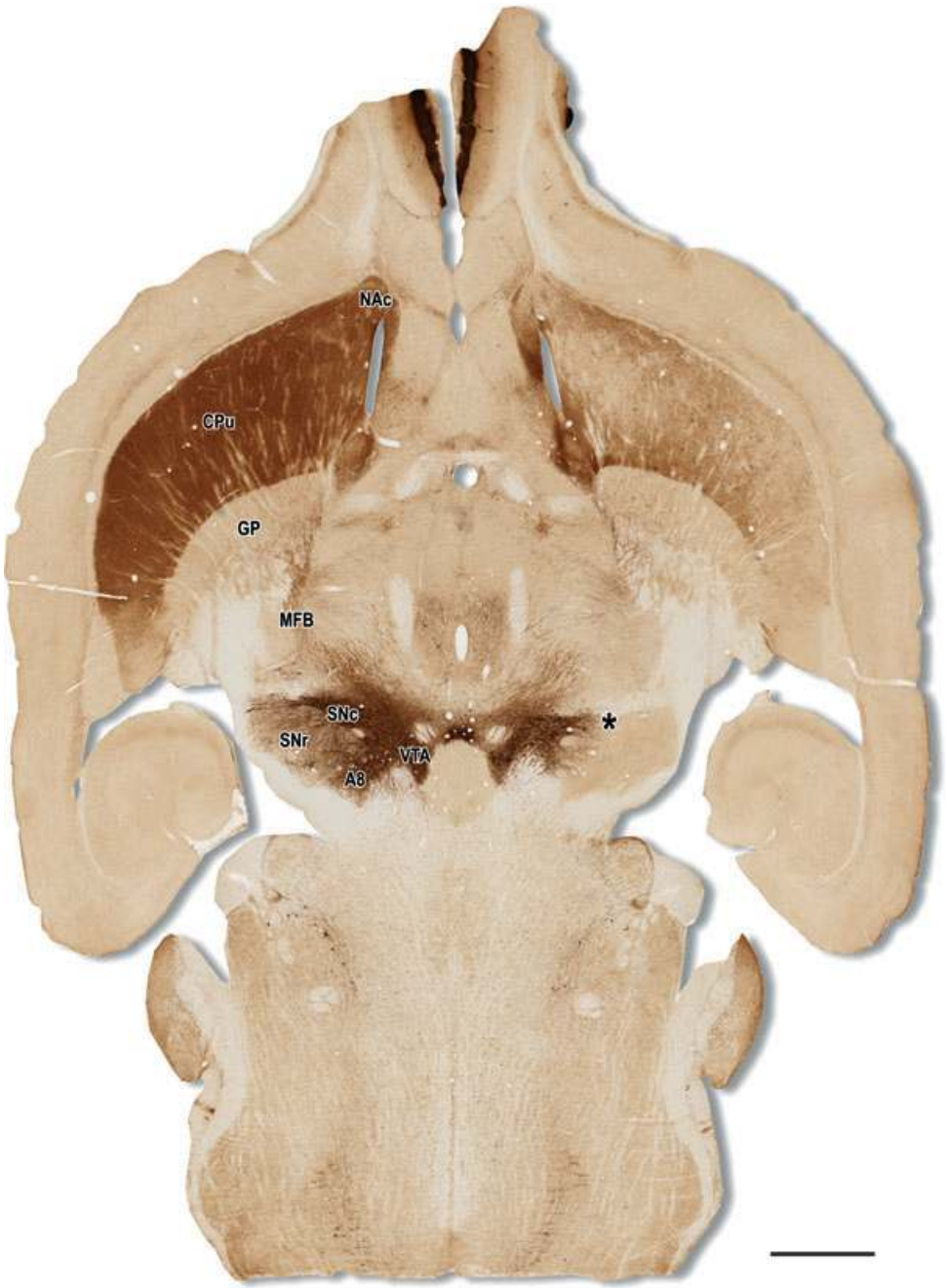


FIG. 2. The extent of damage to the ascending mesostriatal dopamine pathway. This is a composite image of two different dorsoventral levels of a mouse brain immunostained for TH, and depicts the pathways of both the nigrostriatal and mesolimbocortical system, which compose the mesostriatal pathway on the lesioned (right) and the intact (left) side. In the animal illustrated here, the intranigral 6-OHDA injection (*) has killed off most of the $TH^+$ neurons in the SN and group A8 while the VTA is mostly spared, and as a result the $TH^+$ innervation of the dorsal striatum (shown here) is substantially reduced. *, injection site; CPu, caudate–putamen unit; GP, globus pallidus; MFB, medial forebrain bundle; NAc, nucleus accumbens; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area. Scale bar, 1 mm.

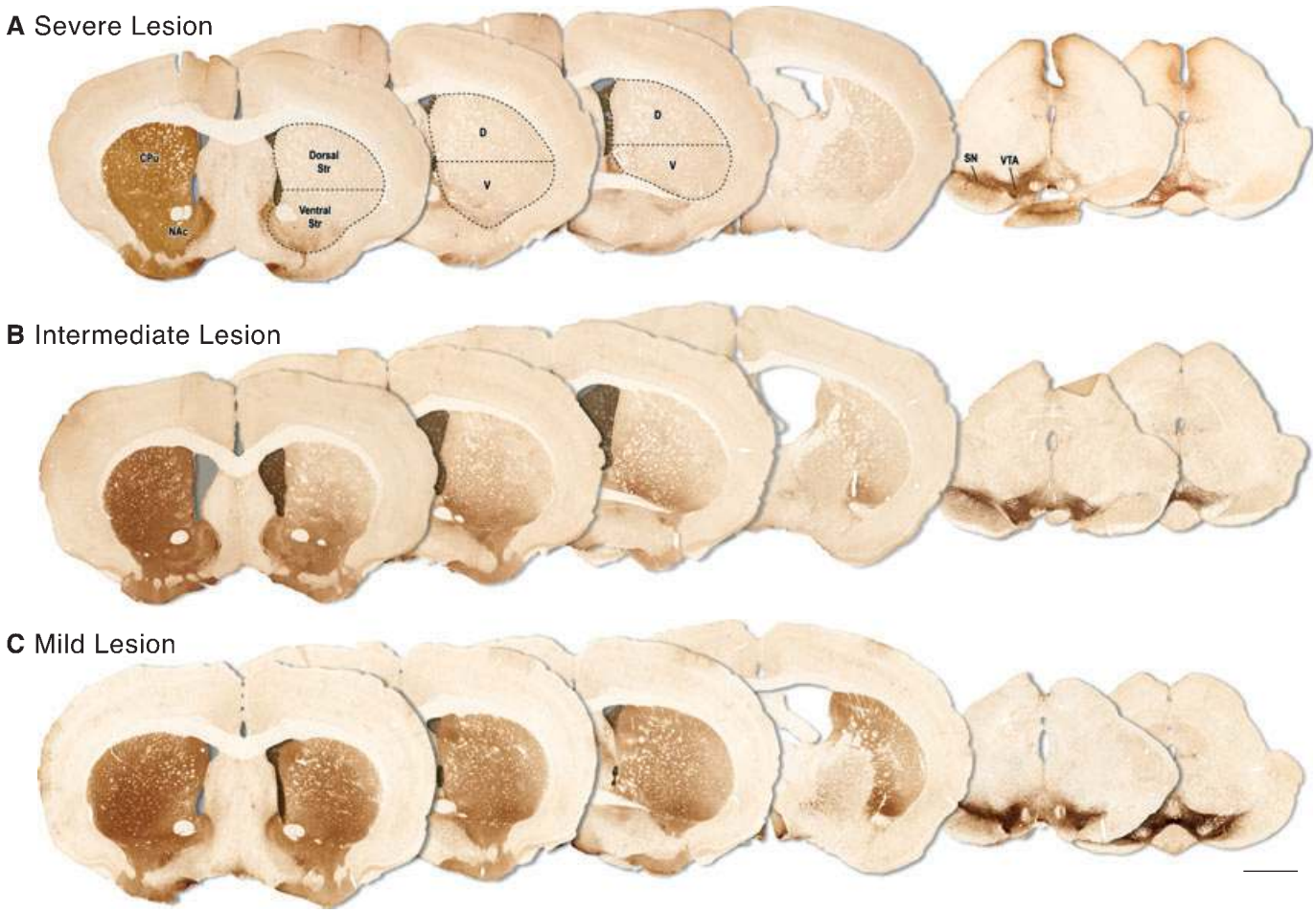


FIG. 3. Extent of degeneration after intranigral 6-OHDA lesion. Overlays of coronal sections immunostained for TH display the varying degrees of degeneration in the mesostriatal pathway observed after injection of 2.4 $\mu$g of 6-OHDA directly into the SN. (A) A severe lesion of the mesostriatal pathway results in a near-complete loss of dopaminergic neurons at the level of the midbrain, notably in the SN and the VTA, and also complete loss of dopaminergic innervation of the striatum. The mouse depicted here had behavioural deficits as follows: apomorphine rotation, −8 turns/min; amphetamine rotation, 7 turns/min; stepping test, 6 steps; cylinder, 45%; and corridor task, 0%. (B) An intermediate lesion results in a prominent loss of $TH^+$ cells in the SN, but less so in the VTA, which is also reflected in the remaining $TH^+$ innervation of the ventral striatum. The mouse depicted here had behavioural deficits as follows: apomorphine rotation, 0 turns/min; amphetamine rotation, 6 turns/min; stepping test, 14 steps; cylinder, 47%; and corridor task, 26%. (C) A mild lesion showing loss of $TH^+$ cells in the SN, while the VTA is mostly spared and a less prominent loss of $TH^+$ innervation in the striatum. The mouse depicted here had behavioural deficits as follows: apomorphine rotation, 0 turns/min; amphetamine rotation, 1 turn/min; stepping test, 16 steps; cylinder, 37%; and corridor task, 40%. CPu, caudate–putamen unit; NAc, nucleus accumbens; SN, substantia nigra; Str, striatum; VTA, ventral tegmental area. Scale bar, 1 mm.

to the VTA-innervated NAc (−50.8 ± 23.4%; $t_{35} = -13$, $P < 0.0001$). From the scatter plots in Fig. 4 one can see that the loss of $TH^+$ innervation in the whole striatum was highly correlated with the overall cell loss measured by stereology in the midbrain (SN and VTA combined; $R^2 = 0.52$, $P < 0.0001$; Fig. 4A), and that the loss of $TH^+$ innervation in the dorsal striatum (CPu) was highly correlated with the $TH^+$ cell loss in the SN ($R^2 = 0.61$, $P < 0.0001$; Fig. 4B). The denervation of the ventral striatum, on the other hand, was less well correlated with the $TH^+$ cell loss in the VTA ($R^2 = 0.34$, $P < 0.0001$; Fig. 4C).

### *Correlation of behavioural impairments with the extent of $TH^+$ striatal denervation and cell loss*

Deficits in motor function were evaluated in the two drug-induced rotational asymmetry tests, amphetamine- and apomorphine-induced rotation, which are the most commonly used motor tests in unilaterally lesioned mice, and in two tests of spontaneous motor performance, the stepping and cylinder tests, which are standard tools in 6-OHDA-lesioned rats but are less commonly used in mice. In addition, we wanted to validate a novel motor performance test, the so-called corridor task (Dowd *et al.*, 2005a), which so far has not been used for assessment of motor impairments in mice.

In Fig. 5, the performance of the individual 6-OHDA-lesioned mice in each of the five tests is plotted against the striatal $TH^+$ innervation density (in panels A–E), and against the total number of $TH^+$ cells in SN and VTA combined (in panels F–J). Linear regression analysis showed that the corridor task had the best predictive value for both striatal denervation ($R^2 = 0.46$, $P < 0.0001$; Fig. 5A) and $TH^+$ cell loss in the midbrain ($R^2 = 0.29$, $P < 0.0001$; Fig. 5F), followed by the apomorphine-induced rotation test (striatal denervation: $R^2 = 0.45$, $P < 0.0001$; $TH^+$ cell loss: $R^2 = 0.28$, $P < 0.0001$; Fig. 5B and G). The scores recorded in the amphetamine-induced rotation test showed a significant correlation with both striatal denervation ($R^2 = 0.44$, $P < 0.0001$; Fig. 5C) and $TH^+$

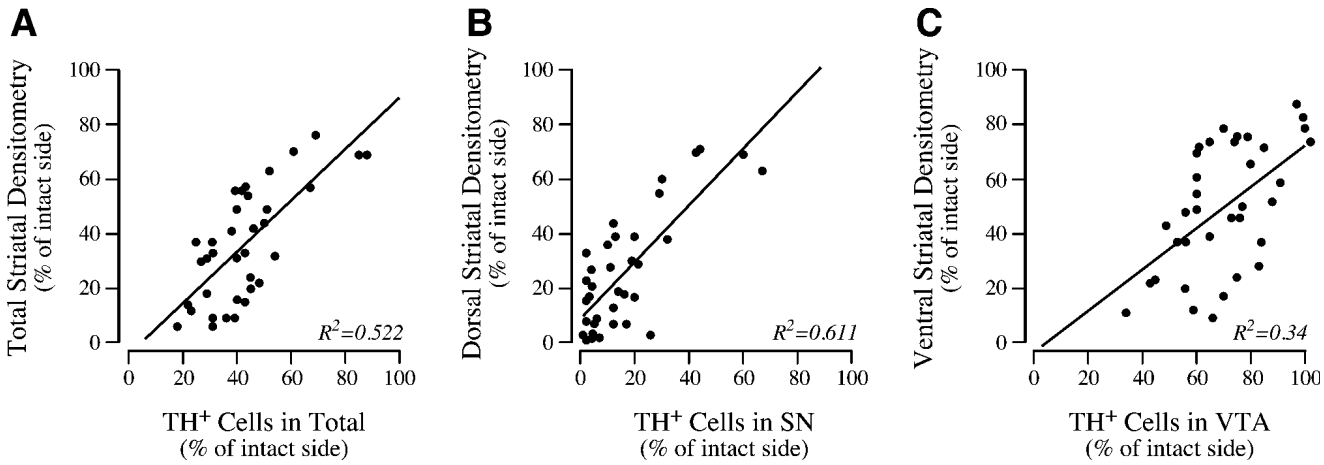


FIG. 4. Midbrain dopaminergic cell loss correlates with the loss of $TH^+$ innervation in striatum. The extent of 6-OHDA lesion was assessed by calculating the number of $TH^+$ cells in the SN and VTA using unbiased stereological methods, while the $TH^+$ innervation in dorsal and ventral striatum was measured using semiquantitative densitometry. (A) The loss of $TH^+$ cells in the two structures combined correlated well with the loss of $TH^+$ innervation in the whole striatum. (B and C) The loss of $TH^+$ cells in (B) SN and (C) VTA, and $TH^+$ innervation of their respective terminal fields, (B) dorsal and (C) ventral striatum, also showed a direct relationship.

cell loss ($R^2 = 0.23$, $P < 0.05$; Fig. 5H). Closer inspection of the plots, however, reveals that this measure has much less predictive value than the two other tests. The impairment seen in the stepping test showed no correlation with striatal denervation ($R^2 = 0.08$, $P = 0.14$, n.s; Fig. 5D) and only very weak correlation with the $TH^+$ cell loss ($R^2 = 0.16$, $P < 0.05$; Fig. 5I). The cylinder test, finally, showed only weak correlation with striatal denervation ($R^2 = 0.14$, $P < 0.05$; Fig. 5E) and no correlation with $TH^+$ cell loss ($R^2 = 0.04$, $P = 0.24$, n.s; Fig. 5J).

We also investigated to what extent the behavioural impairment correlated with the extent of denervation in the dorsal striatum (corresponding to the CPu) or ventral striatum (comprising above all the NAc), with the loss of $TH^+$ cells in SN or VTA, when analysed separately. These data are summarised in Supporting information Figs S1 and S2. Consistent with the data for the whole striatum, above, the performance in the corridor, apomorphine and amphetamine tests showed strong correlation with the extent of denervation in both dorsal and ventral striatum (corridor: $R^2 = 0.30$, $P < 0.001$ and $R^2 = 0.57$, $P < 0.0001$; apomorphine rotation: $R^2 = 0.30$, $P < 0.001$ and $R^2 = 0.56$, $P < 0.0001$; amphetamine rotation: $R^2 = 0.48$, $P < 0.0001$ and $R^2 = 0.33$, $P < 0.0001$, respectively), while the impairments in the stepping and cylinder tests were poorly correlated with any of these parameters ($R^2 = 0.15$, $P < 0.05$, or less). The correlations with $TH^+$ cell loss in SN or VTA, analysed separately, showed a similar pattern as for $TH^+$ innervation density (right-hand panels in supporting Figs S1 and S2).

### *Correlation of the lesion-induced behavioural impairments in the five tests*

The results summarised in Fig. 5 suggest that the impairments seen in the different tests are poorly correlated. To corroborate this impression further we studied how the scores in the five different tests correlated with each other. The behavioural impairments observed in the corridor task were well correlated with the apomorphine rotation scores ($R^2 = 0.73$, $P < 0.0001$; Fig. 6D), and to a lesser extent also with the impairments observed in the stepping test ($R^2 = 0.43$, $P < 0.0001$; Fig. 6B), but not with the scores recorded in the cylinder and amphetamine rotation tests ($R^2 = 0.09$, $P = 0.09$, n.s; $R^2 = 0.10$, $P < 0.05$, respectively; Fig. 6A and C). It is notable that the amphetamine and apomorphine rotation scores showed no correlation to one another ($R^2 = 0.09$, $P = 0.06$, n.s; Fig. 6G), and that the impairments seen in the cylinder test were modest overall, and were poorly correlated with the performance in any of the other tests used ($R^2 \leq 0.16$).

### *Selection of severity of the lesion based on behavioural impairments*

One of the main purposes of the present study was to develop criteria for the *in vivo* selection of well-lesioned 6-OHDA-lesioned mice based on their performance in selected behavioural tests. In order for a test to be of useful for this purpose it must be able to differentiate between animals with various degrees of lesion. To pursue this further the 40 6-OHDA-lesioned mice included in the present study were allocated to three subgroups, based on the extent of striatal denervation recorded in each animal: severe lesion (80–100% denervation; example shown in Fig. 3A), intermediate lesion (60–79% denervation; Fig. 3B) and mild lesion (< 60% denervation; Fig. 3C). (For an overview of $TH^+$ cell loss, striatal denervation and behavioural deficits of the mice in the three subgroups, see Table 1.) We then compared the behavioural deficits of these three subgroups to see whether each of the tests could discriminate between the extent of lesion (Fig. 7).

The corridor task proved to be the one most able to distinguish between lesions of different severity (Group, $F_{2,33} = 23.05$, $P < 0.0001$; Fig. 7A). Tukey *post hoc* analysis revealed that the severe lesion was significantly different from the intermediate lesion ($P < 0.05$) and highly significantly different from the mild lesion ($P < 0.0001$), while the intermediate lesion was significantly different from the mild lesion ($P < 0.05$). Apomorphine-induced rotation was also able to differentiate between the three subgroups (Group, $F_{2,33} = 15.09$, $P < 0.0001$; Fig. 7B). The *post hoc* analysis revealed that the severe lesion was significantly different from the intermediate lesion ($P < 0.05$) and highly significantly different from the mild lesion ($P < 0.0001$), while the intermediate lesion was significanly different from the mild lesion ($P < 0.05$). Amphetamine rotation was clearly less informative and could only differentiate between the animals with a mild lesion and those with > 60% striatal denervation

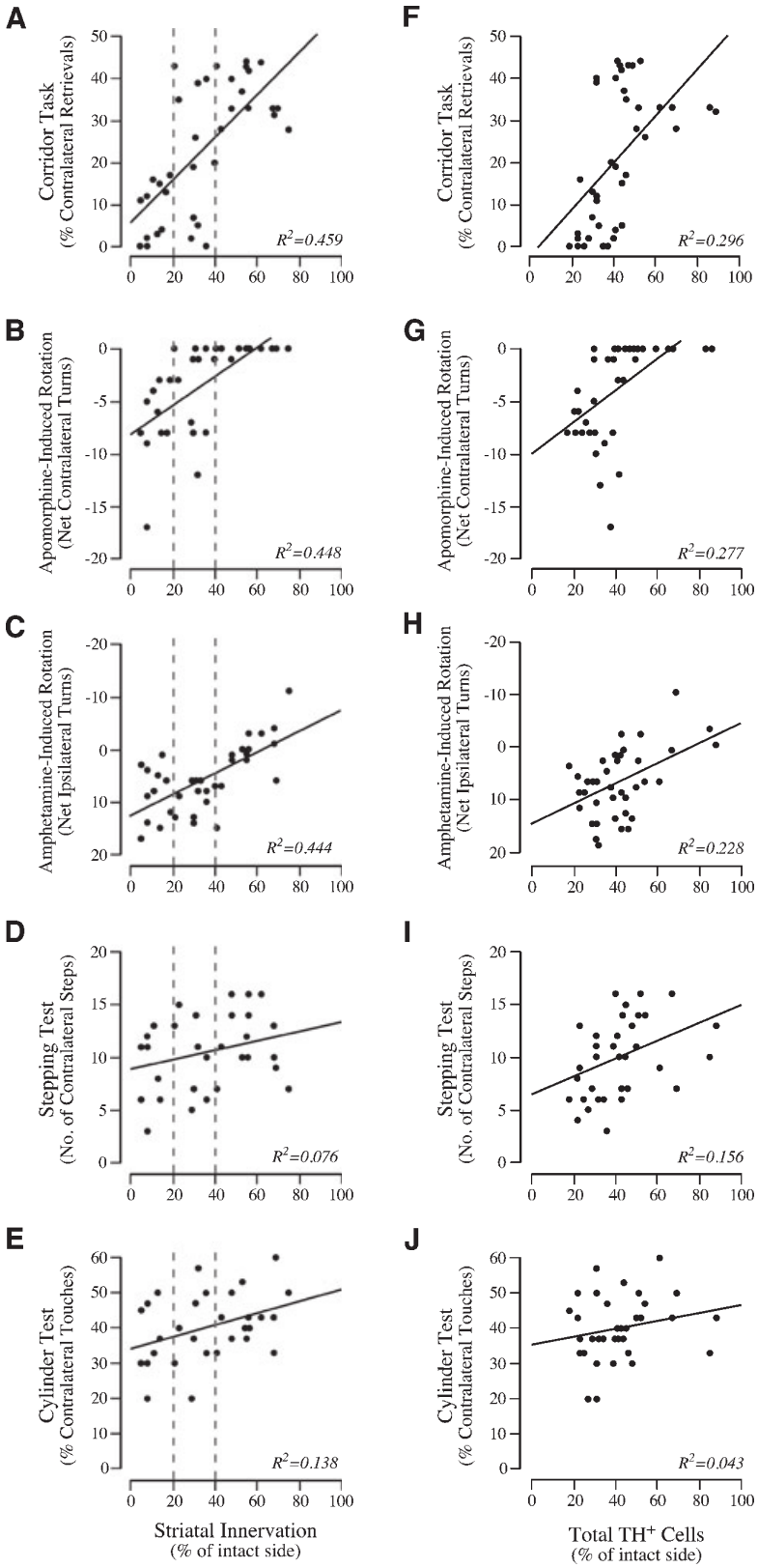


FIG. 5. Correlation of behavioural impairments and degeneration of the mesostriatal pathway. The loss of (A–E) $TH^+$ striatal innervation and (F–J) $TH^+$ cells in the midbrain were correlated with the behavioural impairments observed in the tests used in this study, in order to determine which tests were best in predicting the extent of degeneration after 6-OHDA lesion. The corridor task showed the best correlation with both (A) striatal denervation and (F) $TH^+$ cell loss. This was followed by (B and G) the apomorphine-induced and (C and H) amphetamine-induced rotation scores. The behavioural deficits observed in the stepping (D and I) and cylinder (E and J) tests showed poor correlation with the integrity of the mesostriatal pathway. See text for description of statistical analyses. The dashed grey lines at 20 and 40% striatal innervation density indicate the levels of discrimination used in this study to classify severe, intermediate and mild lesions.

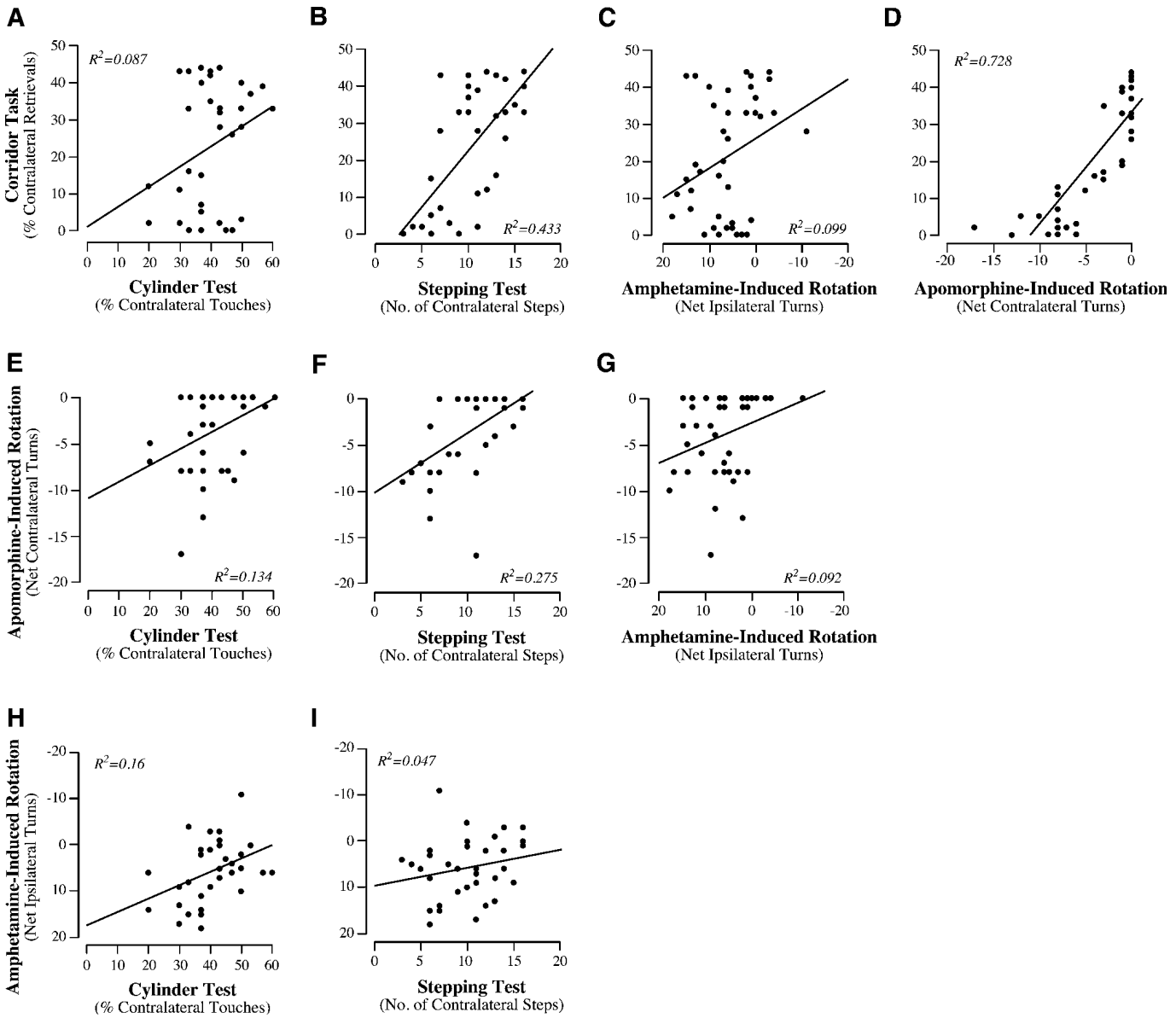


FIG. 6. Relationship between behavioural impairments observed in each test. The impairments assessed in each behavioural test are compared to one another in order to validate their predictive power. (A–D) The newly described corridor task is compared against all other behavioural tests. (E–G) Apomorphine-induced rotation is compared to the remaining behavioural tests and so is (H and I) amphetamine-induced rotation. (D) The corridor task and apomorphine-induced rotation showed the best correlation, while the impairments observed in the stepping test showed good correlation with those assessed by (B) the corridor task and (F) apomorphine-induced rotation. (E) Apomorphine-induced rotation showed a moderate correlation with deficits measured by the cylinder test. (A) The cylinder test showed little correlation with the corridor task. Amphetamine-induced rotation showed poor correlation with all other tests: (C) the corridor task, (G) apomorphine-induced rotation, (H) cylinder and (I) stepping tests. See text for description of statistical analyses.

(Group, $F_{2,33} = 10.69$, $P < 0.0001$; Fig. 7C); Tukey *post hoc* analysis revealed that the mild lesion was significantly different from both the intermediate and the severe lesions ($P < 0.001$ and $P < 0.05$, respectively). By contrast, neither the stepping test nor the cylinder tests were able to distinguish between any of the lesion types (Group, $F_{2,33} = 2.08$, $P = 0.15$, n.s; Group, $F_{2,27} = 1.31$, $P = 0.29$, n.s, respectively; Fig. 5D and E).

### *Stability of behavioural impairments over time*

A subset of seven severely lesioned mice was followed long-term in four of the tests that showed profound deficits at the early post-lesion time-point (6–7 weeks), and were compared to a group of seven intact control animals (Fig. 8A–D). In all four tests the two groups showed stable performance over the entire test period (20–23 weeks), and the lesioned and intact mice performed significantly different from one another in all four tests, including the corridor test (Group, $\chi^2_{1,48} = 827.14$, $P < 0.0001$; Fig. 8A), apomorphine-induced rotation (Group, $\chi^2_{1,48} = 159.69$, $P < 0.0001$; Fig. 8B), amphetamine-induced rotation (Group, $\chi^2_{1,48} = 26.91$, $P < 0.0001$; Fig. 8C) and the stepping test (Group, $\chi^2_{1,36} = 208.26$, $P < 0.0001$; Fig. 8D). There was no significant effect of time measured in any of the behavioural tests, thus confirming the stability performance in both the intact and lesioned groups (data not shown).

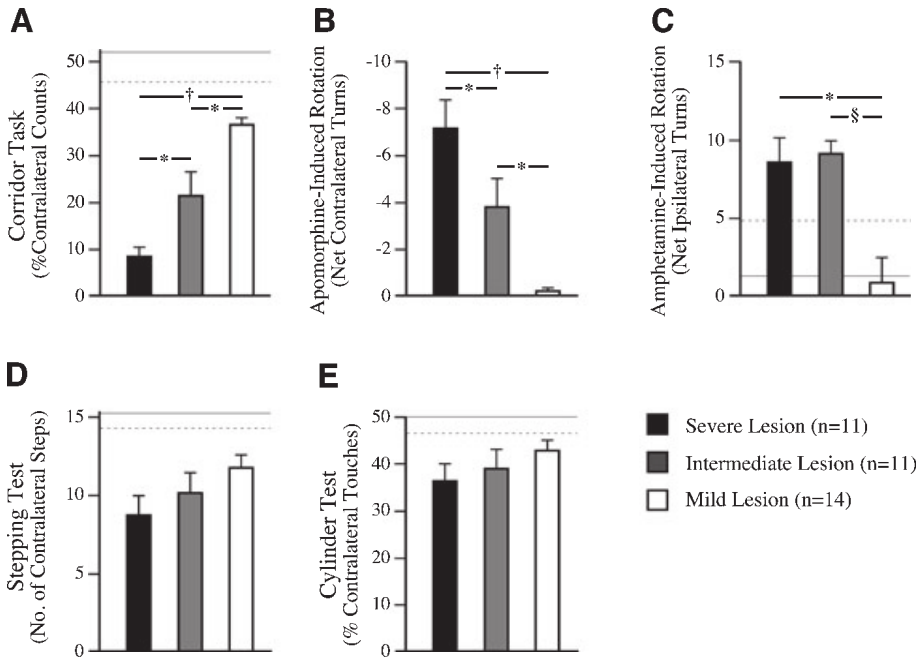


FIG. 7. Identifying subcategories of lesion extent. The behavioural deficits assessed by all tests for each of the three classes of lesion: severe (black bars), intermediate (grey bars) and mild (white bars) were compared against one another to see whether tasks could differentiate between the subcategories of lesion. Only (A) the corridor task and (B) apomorphine-induced rotation test could successfully discriminate between all three classes of lesion. (C) Amphetamine-induced rotation could separate mild lesions from more extensive ones. Neither (D) the stepping nor (E) cylinder tests were able to discern between the three subcategories. The solid horizontal line represents the mean performance of intact mice, while the dashed line represents the SD. $^{*}P < 0.05$, $^{\S}P < 0.001$, $^{\dagger}P < 0.0001$. See text for description of statistical analyses.

## Discussion

The results show that intranigral 6-OHDA lesions can be used to induce profound loss of midbrain dopaminergic (DAergic) neurons, accompanied by extensive denervation of the striatum and behavioural impairments in a range of drug-induced and spontaneous motor tests. Based on the extent of striatal $TH^+$ denervation we allocated the mice into three subgroups, exhibiting severe, intermediate and mild lesions of the mesostriatal pathway. From the behavioural impairments seen in these subgroups, it was possible to predict the severity of the lesion, i.e. the extent of striatal $TH^+$ denervation and $TH^+$ cell loss, based on the degree of impairment seen in the corridor task and rate of turning in the apomorphine-induced rotation test. The standard tests commonly used for this purpose in 6-OHDA-lesioned rats, the cylinder and stepping tests and amphetamine-induced rotation, were found to be less useful as tools to monitor lesion severity in mice. Based on the present data we have devised a set of behavioural criteria that can be used to distinguish between mice with varying degrees of cell loss induced by 6-OHDA lesions of the nigrostriatal pathway.

Our study is the first to characterise in detail the intranigral 6-OHDA lesion model in the mouse. The commonly used drug-induced rotation tests, cylinder test and stepping test were evaluated and compared, along with a novel task, the corridor task, for the assessment of sensorimotor deficits on the side opposite to the lesion. The results confirm the usefulness of the intranigral lesion model in mice. The intranigral 6-OHDA lesion compares favourably with available alternatives, i.e. injections of 6-OHDA into the MFB, which are highly effective but complicated by a high death rate among the injected mice, and injections of 6-OHDA into the striatum, which tend to be less effective overall in inducing stable and severe behavioural deficits. Due to the small size of the mouse brain the 6-OHDA lesions tend to be much more variable in mice than in rats, regardless of the injection site. This is a serious problem in experimental studies, particularly in studies that involve functional recovery over time, where profound and stable baseline deficits are important. In 6-OHDA-lesioned rats behavioural tests (most commonly amphetamine or apomorphine rotation) are generally used to preselect animals that exhibit sufficiently severe nigrostriatal lesions to be included in the study. Similar selection criteria have so far been lacking for 6-OHDA-lesioned mice.

### *Assessment of lesion severity*

In the mild lesion group the average loss of $TH^+$ neurons in the SN was 72%. These animals showed no deficits in any of the behavioural tests, which may be explained by the fact that the VTA remained largely intact (mean cell loss 17%). As a consequence, the overall density of the $TH^+$ innervation in the striatum was only reduced by 36%, insufficient to induce any detectable deficits in either drug-induced or spontaneous motor tests. Inspection of the scatter plots in Fig. 5 and supporting Figs S1 and S2 suggests that significant motor asymmetry in the apomorphine and amphetamine rotation tests, and significant deficits in the corridor test, are seen only in mice with $> 60\%$ loss of striatal $TH^+$ innervation (dorsal and ventral parts combined, including NAc), caused by the loss of $> 75\%$ of the $TH^+$ cells in the SN and a $> 20\%$ loss of $TH^+$ cells in the VTA. Only apomorphine-induced rotation and the corridor task were able to further subdivide mice with more extensive lesions and distinguish between the intermediate and severe lesion groups. The corridor task and the apomorphine-induced rotation test were the only behaviours that showed a sufficiently graded response to allow the identification of animals with various degrees of DAergic neurodegeneration based on their performance scores (see Table 1).

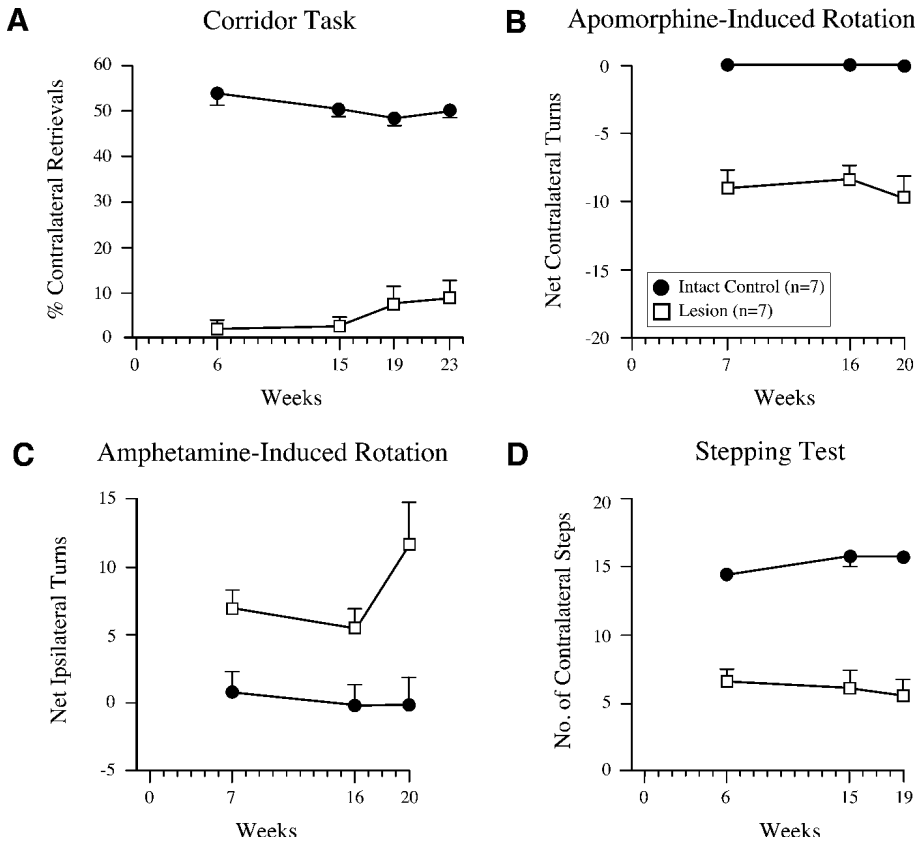


FIG. 8. Long-term stability of behavioural impairments. A subgroup of severely-lesioned animals ($n$ = 7; open squares) and intact controls ($n$ = 7; filled circles) were monitored at regular intervals over a period of 23 weeks to determine whether the impairments observed in each of the tasks were stable over time. In (A) the corridor task, (B) apomorphine-induced rotation, (C) amphetamine-induced rotation and (D) stepping test the lesion group was statistically significant from the intact group at all time points, and showed no significant change in performance over the time points analysed. See text for description of statistical analyses.

The stepping and cylinder tests, which are highly useful for assessment of deficits in paw use in unilaterally-lesioned rats, were remarkably uninformative in the mouse. All lesion subgroups showed similar, minor deficits in the stepping test, without any clear correlation to lesion size, and significant impairments in the cylinder test (i.e. < 30% touches by the contralateral paw) was seen in only two of the 40 mice included in the present study. This is at variance with two previous reports that have reported more pronounced deficits in the cylinder test (Iancu *et al.*, 2005; Lundblad *et al.*, 2005). In the Iancu *et al.* study contralateral paw touches were reduced to 0% in some animals, while the lowest score seen in the current study was 20%, despite the fact that the degeneration of the nigrostriatal pathway was similar in the two studies. It seems possible that this discrepancy may be due to differences between strains used, or to the fact that we, in the current study, used a minimum of 30 total paw touches for each test session, while the Iancu *et al.* (2005) and Lundblad *et al.* (2005) studies only recorded the mice for a maximum time of 3 min, not stating the total number of touches made. It seems possible that side bias in paw use observed over such short observation times may not be representative of a larger sample collected over a longer observation period. The Iancu *et al.* (2005) study also reported that apomorphine-induced rotation was a poor indicator of successful lesion. This is in contrast to the results in the present study, showing that apomorphine rotation is one of the most informative tests for determining the size of the 6-OHDA lesion. In the present study we used a dose of apomorphine that was five times lower than that used by Iancu and colleagues (0.1 vs. 0.5 mg/kg). We have previously observed that repeated injections of apomorphine at higher doses (0.25 mg/kg) will induce dyskinetic, abnormal involuntary movements in lesioned mice (S. Grealish and A. Björklund, unpublished results). To avoid this confounding factor we have reduced the dose to 0.1 mg/kg, which is still high enough to induce a strong rotational response. At higher doses, as used by Iancu *et al.* (2005), it seems possible that the induction of dyskinesia could mask, or interfere with, the rotational response. Our recommendation, therefore, is to perform the apomorphine rotation test in mice at the 0.1 mg/kg dose in combination with a priming dose regimen (two priming injections 4 and 2 days before the first actual rotation test; see Materials and Methods).

### *Use of the corridor task for assessment of sensorimotor deficits in 6-OHDA-lesioned mice*

From the present data the corridor test stands out as the single most informative test for the assessment of lesion severity in 6-OHDA-lesioned mice, and that performance in this test, in combination with one of the standard drug-induced rotation tests, can be used as reliable screening tests for experimental studies. The corridor test, which was originally developed for studies of unilateral sensorimotor impairments in rats, was adapted here for experiments in mice. This test has several attractive features: it does not require any specialised training or equipment and, in contrast to, e.g., the stepping test, does not involve any direct contact with the animal during testing. Moreover, the motivational aspect of the task (sugar pellets) makes it useful for

repeated testing and does not require any time-consuming off-line assessment, which is the case with the cylinder test. These features make the corridor task attractive for studies involving assessment of functional changes over time, such as in neurorestorative studies and cell transplantation experiments, which have already been reported for rats (Dowd *et al.*, 2005a,b; Torres *et al.*, 2008). Our own preliminary observations suggest that the deficits observed in intranigral 6-OHDA-lesioned mice in the corridor task and the apomorphine- and amphetamine-induced rotation tests can be at least partially rescued with an intrastriatal transplant of embryonic ventral mesencephalic tissue (S. Grealish and A. Björklund, unpublished results). This is consistent with a recent study that has reported recovery in amphetamine- and apomorphine-induced rotation following intrastriatal transplantation of midbrain neural stem cells (Parish *et al.*, 2008).

### *Criteria for the determination of lesion severity in 6-OHDA-lesioned mice*

Based on the results presented here we propose the following criteria for the determination of lesion severity in 6-OHDA-lesioned mice:

Mice with severe lesions, defined as an overall loss of > 80% of the $TH^+$ innervation in the striatum (dorsal and ventral striatum combined), are characterised by 20% retrievals of pellets in the corridor task on the side contralateral to the lesion and 3 contralateral turns/min in response to 0.1 mg/kg apomorphine, s.c.. These mice will in most, but not all, cases score 6 ipsilateral turns per minute in response to an i.p. injection of 5 mg/kg amphetamine. Mice exhibiting this magnitude of impairment are expected to display > 85% $TH^+$ cell loss in SN and > 45% $TH^+$ cell loss in VTA.

Mice with intermediate lesions, defined as an overall 60–80% $TH^+$ denervation of striatum, are defined by 21–40% retrievals of pellets, contralaterally, in the corridor task. These mice will show a similar response to amphetamine as mice with severe lesions, and may or may not display contralateral rotations in response to apomorphine. The magnitude of $TH^+$ cell loss in these animals is likely to be > 85% in the SN and > 20% in the VTA.

Mice with mild lesions, defined as < 60% denervation of the striatum, are difficult to distinguish from intact mice as they show only minor deficits in the corridor task (40–45% contralateral pellet retrievals) and little to no rotational asymmetry in the apomorphine and amphetamine tests. In these mice $TH^+$ cell loss in the midbrain is typically < 50%.

In our original cohort of 122 mice, and with the lesion parameters used here, 34% of the lesioned mice showed deficits consistent with a severe lesion and 29% with an intermediate lesion, while the remaining mice showed mild or no deficits.

In this classification lesion severity is defined on basis of the extent of striatal $TH^+$ denervation rather than the degree of $TH^+$ cell loss. The reason for this choice is that the behavioural deficits in the corridor and rotation tests were more closely correlated with extent of striatal denervation than cell loss. This is particularly the case for the identification of mice with severe lesions: all mice with > 80% loss of striatal $TH^+$ innervation showed < 20% pellet retrieval in the corridor test and scored at least 3 turns/min in the apomorphine test (see Fig. 5). Mice with severe lesion-induced deficits were not as easily identified based on the extent of $TH^+$ cell loss. It is notable that mice with almost complete, 90%, $TH^+$ cell loss in SN pars compacta displayed highly variable performance in the corridor and rotation tests (0–40% retrievals in the corridor task and 0–20 turns/min in the rotation tests; supporting Fig. S1). Maximal behavioural impairment was obtained only when the 6-OHDA lesion involved also part of the VTA: in the cohort of mice studied here, all mice with < 20% pellet retrieval in the corridor test showed a significant (20–70%) loss of $TH^+$ neurons in the VTA (supporting Fig. S2). This suggests that the entire mesostriatal projection, including cells distributed throughout the SN and VTA, has to be involved by the lesion in order to induce profound motor performance deficits in mice. Once this extent of lesion is achieved, however, our results show that the deficits are highly stable over time.

Our data suggest that these selection criteria can reliably be used to identify mice with > 60% lesion of the mesostriatal projection. The identification of mice with more severe lesions, however, is less perfect. In the cohort studied here 4 of the 17 mice that showed a combined score consistent with a severe, > 80%, lesion (< 20% pellet retrieval in the corridor test and 3 contralateral turns/min in the apomorphine test) had a less severe lesion than predicted by this level of impairment, i.e. in the range of 60–80% striatal denervation, as determined by densitometry.

In conclusion, we show that the novel corridor task is a highly useful test for the evaluation of lesion-induced sensorimotor deficits in mice with unilateral lesions of the mesostriatal dopamine system, and that this test, in combination with conventional drug-induced rotation tests, can be used to select animals with profound DAergic lesions that are stable over time. The correlation of DAergic cell loss and striatal innervation with the performance in each test provides a useful tool for the assessment of functional recovery in neurorestoration and cell transplantation studies, and for the evaluation of the *in vivo* efficacy and performance of dopamine neuron preparations generated from, e.g., transgenic reporter mice (Jonsson *et al.*, 2009) or pluripotent stem cells (Takahashi & Yamanaka, 2006; Tabar *et al.*, 2008; Lindvall & Kokaia, 2009).

## Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Correlation of behavioural impairments and degeneration of the nigrostriatal pathway.

Fig. S2. Correlation of behavioural impairments and degeneration of the mesolimbocortical pathway.

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## Abbreviations

6-OHDA, 6-hydroxydopamine; CPu, caudate–putamen unit; DA, dopamine; DAergic, dopaminergic; KPBS, potassium phosphate-buffered saline; MFB, medial forebrain bundle; MPTP, 1-methyl-1,2,3,4-tetrahydropyridine; NAc, nucleus accumbens; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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