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Research report

A partial lesion model of Parkinson's disease in mice – Characterization of a 6-OHDA-induced medial forebrain bundle lesion

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HIGHLIGHTS

- We establish a 6-OHDA partial MFB lesion mouse model.
- We correlate the 6-OHDA dose and degree of nigrostriatal lesion with behavioural impairment.
- Based on a statistical prediction model, we suggest behavioural tests and provide cut-off values to select partially-lesioned mice.

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ABSTRACT

The most frequently used animal models for Parkinson's disease (PD) utilize unilateral injection of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (MFB), which results in total denervation of the dopaminergic nigrostriatal pathway. However, neuroprotective interventions in PD require models resembling earlier stages of PD, where some dopaminergic cells and fibres remain.

The aim of the present study was therefore to establish a MFB partial lesion model in mice.

We tested four different 6-OHDA doses, and our results show a dose-dependent loss of nigral dopaminergic cells and striatal fibres that correlated with behavioural impairment in several behavioural tests. Specifically, doses of 0.7 μg and 1 μg of 6-OHDA induced a partial denervation of the nigrostriatal pathway, associated with a mild but quantifiable behavioural impairment. We identified the amphetamine-induced rotation, stepping, corridor and cylinder test to be sensitive enough to select partial lesion animals. Based on our data, we proposed a range of cut-off values for these different behavioural tests to select partial lesion mice. Using a statistical prediction model we identified two behavioural tests (the stepping test and amphetamine-induced rotation test) that with a high sensitivity and specificity predict the extent of nigral dopaminergic cell loss and select mice with a partial nigrostriatal lesion prior to further interventions.

This model can serve as an important tool to study neuroprotective therapies for PD in mouse models, especially when the treatment targets the substantia nigra and/or the striatum.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting more than six million people worldwide [1]. The motor symptoms in PD are caused by a progressive loss of midbrain dopamine (DA) neurons in the substantia nigra pars compacta (SNpc), leading to a reduction of DA in the striatum [2,3]. This dopaminergic deficit leads to the classical motor symptoms in PD such as resting tremor, slowness of movement, rigidity, and postural instability as well as cognitive and vegetative disturbances.

Experimental models of PD should resemble some of the pathogenic and pathophysiological features of the disease, preferably with a quantifiable behavioural and histological read out in order to study the effect of therapeutic interventions.

One of the most frequently used animal models utilizes the toxin 6-hydroxydopamine (6-OHDA) to lesion the nigrostriatal pathway in order to induce motor impairment [4]. In this model, unilateral injection of 6-OHDA selectively destroys catecholaminergic neurons, thereby inducing a unilateral motor deficit and allowing quantifiable lateralized behavioural tests. Drug-induced rotational and non drug-induced behavioural tests based on the lateralized

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sensorimotor integration and forelimb akinesia correlate well with the degree of the nigrostriatal lesion [5–8]. In addition to a unilateral nigrostriatal defect, 6-OHDA injection leads to a lesion and a motor deficit that is stable over time.

In mice, 6-OHDA has been stereotaxically injected into different brain areas along the nigrostriatal pathways, such as the striatum [9–11], the SNpc [12,13] and the medial forebrain bundle (MFB) [14,15], leading to different types of degeneration and motor symptoms [13–21]. Injections into the MFB have been extensively used in rats (reviewed in [4,22,23]) but to a much lesser extent in mice [14,16–19,21]. With the increasing use of genetically modified mice, there is now a need for well-characterized toxin-induced mouse models of PD. While intrastriatal and intranigral lesions have resulted in variable nigral cell loss [13,15], MFB lesions in mice give a more efficient and predictable lesion, but had initially been complicated by a high mortality rate. However, the latter can now be overcome with appropriate postoperative care [14].

An important advantage of injecting 6-OHDA into the MFB is that the toxin is not injected into the striatum or the SNpc, where potential treatments such as cell grafts or growth factors might later be placed [24–36].

Investigation of potentially neuroprotective or neurorestorative substances such as growth factors requires less severe lesions, where some dopaminergic cells and fibres should remain to demonstrate protective effects. A partial lesion of the nigrostriatal system is more likely to mimic these earlier stages of PD and therefore more appropriate for this type of studies [37,38]. However, the current partial 6-OHDA lesions in mice were achieved by injection of the toxin into the striatum or the SNpc [13,15,16,33], where this may cause confounding reactions and interfere with therapeutic agents such as cell therapies or growth factor treatments. Instead, a partial lesion targeting the MFB would circumvent this problem, but has not been established and characterized in mice so far.

The aim of the present study was to establish and characterize a partial MFB lesion model of PD in mice, by investigating which dose of 6-OHDA results in a predictable degree of partial nigrostriatal lesion that correlates with a clear behavioural impairment. We evaluated nigral tyrosine hydrolase $(TH)^+$ cell loss and striatal TH^+ fibre loss in relation to different doses of 6-OHDA. We also applied a battery of different pharmacological and non-pharmacological behavioural tests and validated their suitability to detect dose-dependent motor impairment and nigrostriatal cell and fibre loss, specifically for partial lesion discrimination. Based on this information, we identified behavioural criteria that can be used to estimate the degree of lesion in this model.

2. Materials and methods

2.1. Mice

Thirty-four male *Rgs5*$^{gfp/+}$ reporter mice were used in this study. These mice have a C57Bl/6 background and show a normal phenotype and behaviour [39]. In this mouse, GFP is expressed under the pericyte-specific regulator of G protein 5 (*Rgs5*) promoter which makes it possible to track pericytes [39]. Mice had an average weight of 30 g at the day of surgery. During the whole experiment mice were housed under a 12 h/12 h light/dark cycle with access to food and water ad libitum. All procedures were conducted in accordance with the Ethical Committee guide for the use of laboratory animals in Lund University.

2.2. 6-OHDA lesion

Thirty-four mice were anaesthetized with a mixture of 4% isofluorane (IsoFlo vet, Apoteksbolaget, Sweden) in 2:1 oxygen/nitrous oxide mixture, placed in a stereotaxic mouse frame and kept on 2% isofluorane. 6-OHDA (Sigma, Sweden) was dissolved in 0.02% ice-cold ascorbate/saline solution and used within 3 h. The toxin was injected as a single bolus of 1 μl into the MFB at the following coordinates (relative to bregma): anterior–posterior (A/P) = −1.2; medio-lateral (M/L) = −1.3 and dorso-ventral (D/V) = −4.75 (from the dura) with a flat skull position [40]. Injections were made using a glass capillary with an outer tip diameter of 50 μm attached to a 10 μl-Hamilton syringe, at a rate of 1 μl over 2 min with a further 3 min before slowly removing the capillary. The following 6-OHDA concentrations were used in this study: 0.3 μg/μl, 0.7 μg/μl, 1 μg/μl and 3.6 μg/μl. The sham group was injected with 0.02% ascorbate/saline solution and used as a control. To minimize mortality, special post-surgery care was conducted during the first two weeks after the surgery [14]. Briefly, mice received sterile glucose-saline solution s.c., and were hand-fed with food pellets soaked in 15% sucrose/water solution and high calorie jelly food (DietGel Boost, Clear H20 Co.).

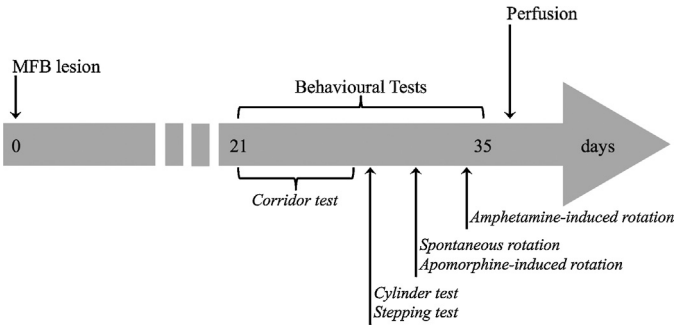

Fig. 1. Study time-line. Three weeks after the lesion, mice were subjected to different behavioural tests and sacrificed five weeks after the lesion.

2.3. Behavioural tests

Behavioural assessments were performed twenty-one days after the surgery when mice achieved a complete post surgery recovery [41]. The behavioural tests were conducted during fourteen days, avoiding more than one test per day and starting with the non-drug behavioural tests, followed by the drug-induced behavioural tests (Fig. 1). Prior to each behavioural test, animals were habituated to the test room for 24 h.

2.3.1. Cylinder test

The cylinder test assesses the spontaneous forelimb lateralization, taking advantage of the natural exploratory instinct of rodents to a new environment [5]. Mice were placed individually inside a glass cylinder (diameter 19 cm, height 20 cm) with mirrors located behind to allow a 360° vision. The session was videotaped during 3 min for later scoring [13,14]. No habituation of the mice to the cylinder was allowed before the recording. Paw touches were analysed using a slow motion video player (VLC software). The number of wall touches (contacts with fully extended digits) executed independently with the ipsilateral and the contralateral forelimb were counted. Simultaneous paw touches were excluded from the analysis. Data are expressed as a percentage of contralateral touches, calculated as (contralateral touches)/(ipsilateral touches + contralateral touches) × 100.

2.3.2. Stepping test

Forelimb akinesia was assessed using a modified version of the stepping test for mice [42]. Briefly, a hand-made structure consisting of a 10 cm-width and 50 cm-length corridor was used. Each mouse was gently lifted by the base of the tail, leaving forepaws touching the substrate and pulled backwards over a distance of

50 cm. All mice were tested 3–5 times in order to achieve enough number of steps per session for the statistical analysis. The session was video recorded for posterior analysis and the number of adjusting steps in both the ipsilateral and the contralateral paws were counted. Data are expressed as a percentage of contralateral steps calculated as (contralateral steps)/(ipsilateral steps + contralateral steps) × 100.

2.3.3. Corridor test

Lateralized sensorimotor integration was assessed using the corridor test [8]. This test has been adapted for mice using a plastic corridor of 60 cm long, 4 cm wide and 15 cm high [13]. Ten pairs of adjacent pots, each one measuring 1 cm in diameter and spaced by 6 cm, containing 2–3 sugar pellets, were present in the test corridor. A clear Perspex lid was placed on top of the apparatus. Mice were food restricted and kept at 85% of the bodyweight. They were first habituated to the apparatus by scattering sugar pellets randomly along the floor where they were free to explore for 10 min for the first two days, and were tested the next 5 days. For each trial, mice were placed on the testing corridor. The number of contralateral and ipsilateral retrievals made by each mouse was counted until a total of 20 retrievals were achieved or a maximum time of 5 min elapsed [13]. Data are expressed as a percentage of contralateral retrievals calculated as (contralateral retrievals)/(ipsilateral retrievals + contralateral retrievals) × 100.

2.3.4. Rotation

Rotational asymmetry was assessed using an automated rotometer system (Omnitech electronics) based on the design of Ungerstedt and Arbuthnott [6]. Full body ipsilateral and contralateral side rotations were automatically counted. Spontaneous rotation in a novel spherical environment was tested for 15 min. This test was based on previous observation assuming mice to turn towards the lesioned side when placed into a novel environment [17,43].

Apomorphine-induced rotation test was performed to study the hypersensitivity of the lesioned striatum, assessed by injecting 0.1 mg/kg of apomorphine s.c. (dissolved in a 0.2 mg/mL ascorbic acid in 0.9% saline solution) and tested over a 40 min session. Mice were primed on two separate days prior to the tests, as previously described [13]. Amphetamine-induced rotation test increases in response to a lesion and results were collected over a 40 min test following i.p. injection of 5 mg/kg of D-amphetamine sulphate [13,33]. Data are expressed as net contralateral turns for the apomorphine test and net ipsilateral turn for the amphetamine test.

2.4. Immunohistochemistry

Five weeks after the lesion, mice were deeply anaesthetized with a sodium pentobarbital i.p. injection (Apoteksbolaget, Sweden) and intracardially perfused first with 25 ml 0.9% sodium saline at room temperature (RT), followed by 100 ml of ice-cold 4% paraformaldehyde (PFA) in phosphate-buffer saline (PBS). Brains were removed, post-fixed in 4% PFA for 24 h and then transferred to 25% sucrose in PBS for tissue cryoprotection. Coronal sections were cut at 30 μm thickness on a freezing sledge microtome. Sections were collected and stored at 4 °C in an antifreeze solution.

For immunohistochemistry, sections were rinsed three times with PBS before endogenous peroxidase activity was quenched with 10% methanol and 3% H_2O_2 in KPBS for 20 min. After washing, sections were transferred to a blocking solution (5% normal goat serum (NGS), 0.25% Triton X-100, KPBS) for 1 h. They were then incubated with polyclonal rabbit anti-mouse tyrosine hydroxylase (TH, 1:1000, Chemicon) diluted in the same blocking solution as described above, overnight at RT. Following twice rinsing with KPBS and once with 2% NGS in a 0.25% Triton X-100-KPBS solution, sections were then incubated with biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories) diluted in blocking solution for 1 h. After rinsing, sections were treated with avidin–biotin–peroxidase complex (ABC Elite kit; Vector laboratories) in KPBS for 1 h. Following washing, the colour reaction was developed using the chromogen 3,3′-diaminobenzadine (DAB kit, Vector Laboratories). Tissue sections were mounted, dehydrated in gradual concentration of ethanol and coverslipped with DPX mounting medium.

2.5. Stereology

The number of TH^+ cell bodies in the SNpc was determined by unbiased stereology counting referred to as the optical fractionator method (Stereo Investigator software) [44]. The medial border of the SNpc and lateral border of the Ventral Tegmental Area (VTA) was defined by a vertical line passing through the medial tip of the cerebral peduncle as described [13]. The average number of SNpc sections per mice was five or six in a 1:5 series. The counting was performed using a 100× oil immersion objective (numerical aperture = 1.30) on a Nikon 80i microscope (Leica) equipped with a *X*–*Y* motorized stage, a *Z*-axis motor and a high precision linear encoder (Leica). The sampling interval was adjusted so that at least 100 cells were counted for each region of interest. The criterion for counting a TH^+ cell was the presence of its nucleus in the focal plane. Data show percentage of TH^+ cell loss, with the intact hemisphere corresponding to 100% for each mouse as previously described [13].

2.6. Densitometry

High resolution images were obtained from the TH-immunostained sections using a 10× objective connected to a Nikon microscope. The extent of striatal denervation was measured in three sections per animal corresponding to a +0.7, +0.2 and −0.26 mm from bregma, using Image J software (Version 1.32; National Institute of health, USA). The entire striatum was divided in two equal halves along the dorso-ventral axis and the measured values were corrected for nonspecific background staining by subtracting values obtained from the corpus callosum [13]. Data show percentage of striatal densitometry, with the intact hemisphere corresponding to 100% for each individual mouse.

2.7. Statistics

All data are expressed as mean ± standard deviation (SD) and were analysed using the Graph Pad Prism Software and the IBM statistical software package for the social science (SPSS v22). Comparison of different groups for the behavioural data, the stereology and densitometry results were analysed using a one-way ANOVA followed by a Tukey post hoc. Statistical significance was set at p-value <0.05. For regression analysis, an Akaike information criterion (AIC) corrected for small sample size (AICc) was calculated from the equation $AICc = AIC + ((2k(k+1))/(n-k-1)$, where k is the number of parameters of the statistical model and n is the sample size, to first select the best fitted curves between three models: linear, log (dose) of a normalized response with variable slopes, and quadratic curves. When the fitted curve selected by the AICc test presented a non-biological representation, the second best regression was used. Then, residuals were tested for normal distribution, R^2 values were calculated to ensure a goodness-of-fit of the models and equation slopes were considered different from zero with a p-value <0.05. Finally, in order to establish a statistical prediction model, a diagnostic test was performed, comparing either partially-lesioned versus sham animals or partially- versus totally-lesioned animals. The sensitivity (i.e. the true positive rate) and specificity (i.e. the false positive rate) were calculated for different cut-off val-

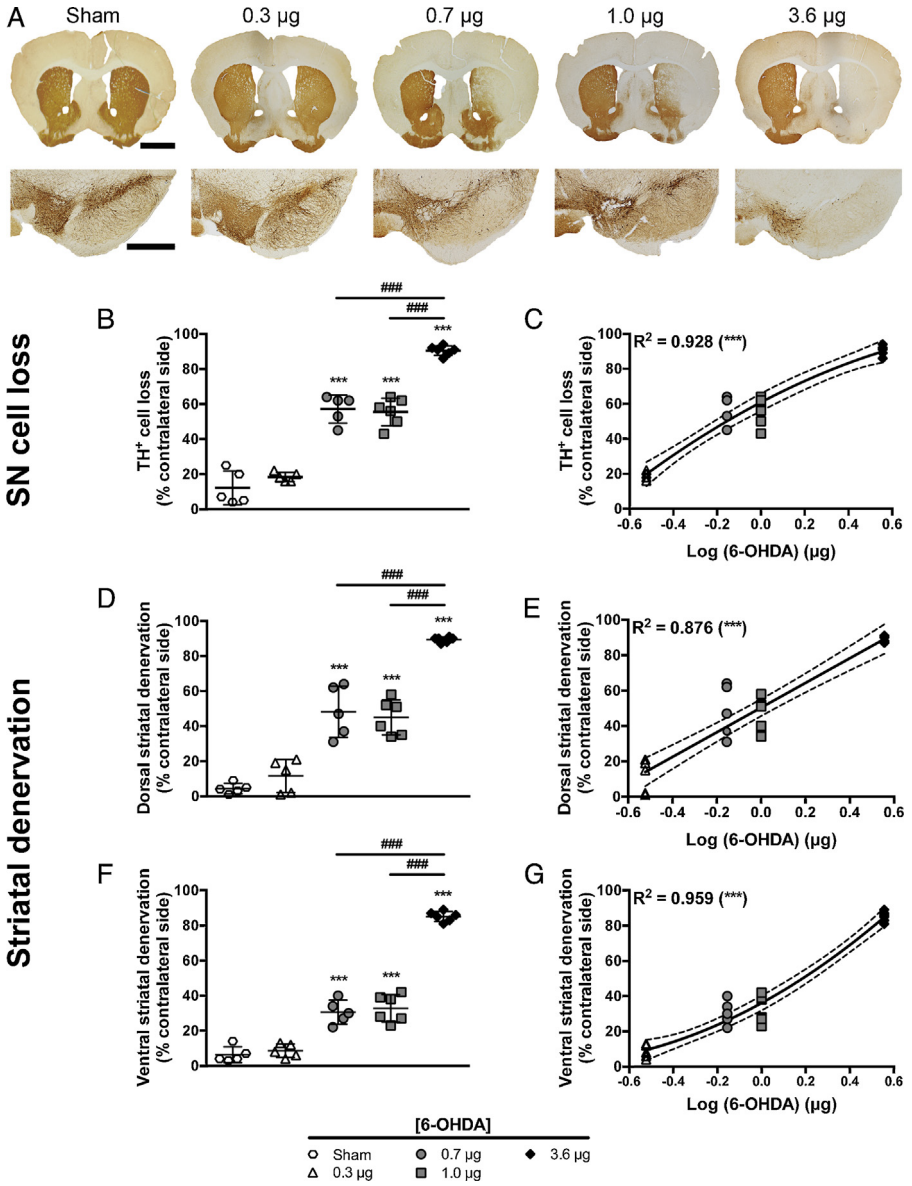

Fig. 2. Extent of nigral TH$^+$ cell loss and striatal fibre denervation correlates to 6-OHDA dose. Bright field photomicrographs of representative coronal brain sections stained for TH showing the striatum (A, upper panel) and the ipsilateral SN (A, lower panel) lesioned with different doses of 6-OHDA. Medium doses of 6-OHDA (0.7 and 1 μg) induced a partial TH$^+$ cell loss (B), a partial dorsal (D) and ventral (F) striatal denervation. Graphs show mean ± SD. The number of symbols denotes the *p*-value when compared to the sham group ($^{***}p < 0.001$) or medium dose group animals ($^{\#\#\#}p < 0.001$). Regression analysis showed the correlation of nigral TH$^+$ cell loss (C), dorsal (E) and ventral (G) striatal fibre loss with a logarithmic 6-OHDA dose. The fitted line is framed within the 95% confidence intervals (dashed line). Scale bars: 800 μm (upper panel) and 400 μm (lower panel).

ues for each behavioural test, thereby drawing a receiver operating characteristic (ROC) curve. The goal of this test was to ensure that all the animals that were identified were actual partial lesion animals, even if this excluded false negative animals. Hence the best cut-off value would provide 100% specificity, and the best sensitivity.

3. Results

3.1. Survival rate

We achieved an overall survival rate of 91% in all groups, with a specific survival rate of 80% in the highest dose group (3.6 μg) that was associated with the highest mortality. Four mice had to be excluded from the study as the lesion was outside the targeted coordinates. The remaining twenty-seven mice were included in the analysis.

3.2. 6-OHDA-dose-dependent lesion of the nigrostriatal system

First, we investigated whether different 6-OHDA doses could damage the nigrostriatal pathway to different extent (Fig. 2). We tested four different doses of 6-OHDA injected in a volume of 1 μl: 3.6 μg ($n = 6$), 1 μg ($n = 6$), 0.7 μg ($n = 5$) and 0.3 μg ($n = 5$) and compared to a sham lesion ($n = 5$). We found a dose-dependent reduction of both TH$^+$ dopaminergic fibres and neurons in the ipsilateral striatum and SNpc, respectively (Fig. 2A). A 6-OHDA dose of 3.6 μg induced a TH$^+$ cell loss of 90.5 ± 2.7% in the ipsilateral SNpc, consistent with a total lesion, which was significantly different from all the other groups (Fig. 2B). Interestingly, the two medium doses

(0.7 and 1 μg) induced a partial reduction of the number of TH+ cells in the ipsilateral SNpc (57.2 ± 8.0 and 55.5 ± 7.8%, respectively) that could be discriminated from the sham (12.2 ± 9.6%) and the total lesion group, but did not show significant differences between them. The lowest 6-OHDA dose (0.3 μg) induced a TH+ cell loss of 18.4 ± 2.6%, giving no significant difference when compared to the sham group. This 6-OHDA dose-dependent TH+ cell loss was confirmed by regression analysis that detected a strong non-linear relationship between 6-OHDA dose and TH+ cell loss, with a high R^2 value (0.928) (Fig. 2C).

Injection of 6-OHDA into the MFB also induced a loss of TH+ fibres in the striatum (Fig. 2D–G). Densitometry evaluation of the dorsal striatal TH+ innervation revealed that a 6-OHDA dose of 3.6 μg reduced the dorsal striatal TH+ fibre density up to 89.3 ± 1.5%, which was significantly different from all the other groups (Fig. 2D). Medium 6-OHDA doses (0.7 and 1 μg) gave a less severe dorsal striatal TH+ fibre loss than the total lesion group, reaching 48.2 ± 14.7 and 45.0 ± 10.0% of the contralateral side, respectively. However, this reduction was more pronounced when compared to the sham or low dose group (0.3 μg) (4.4 ± 3.0 and 11.6 ± 9.5% of the contralateral side, respectively). No significant difference was observed between medium dose groups. The 0.3 μg 6-OHDA dose did not cause any significant dorsal striatal denervation, when compared to sham injections. Regression analysis revealed a linear correlation (R^2 = 0.876) between 6-OHDA doses and the dorsal striatal denervation (Fig. 2E), validating a 6-OHDA dose-dependent effect on the dorsal striatal denervation.

Similar results were observed on the ventral striatum, with a strong reduction of the TH+ fibre density after 6-OHDA injection of 3.6 μg (85.2 ± 2.9% of the contralateral side; Fig. 2F). Medium doses of 6-OHDA (0.7 and 1 μg) resulted in a significant reduction of the TH+ fibres (30.6 ± 6.8 and 32.8 ± 7.8%, of the contralateral side, respectively) and could be distinguished from the sham (6.4 ± 4.5% of the contralateral side) and the total lesion group. The 0.3 μg 6-OHDA dose did not cause any significant ventral striatal denervation (8.6 ± 3.8% of the contralateral side), when compared to sham injections. Regression analysis indicated a strong (R^2 = 0.959) non-linear relationship between 6-OHDA doses and ventral striatal fibre loss (Fig. 2G).

3.3. Behavioural assessment of 6-OHDA degree of lesion

We next examined whether the 6-OHDA dose-dependent damage of the nigrostriatal system is reflected in behavioural changes in the lesioned mice (Fig. 3).

3.3.1. Cylinder test

The 3.6 μg 6-OHDA group showed a marked deficit in use of the contralateral paw as compared to the ipsilateral paw (touches with the contralateral paw = 2.0 ± 2.8% of total touches, mean ± SD) (Fig. 3A). 0.7 and 1 μg 6-OHDA lesions induced a moderate use of the contralateral side (24.6 ± 12.0 and 26.7 ± 5.3%, respectively) and could be separated from the sham (49.6 ± 4.0%) and 3.6 μg groups, but did not show significant differences between them. The lowest dose of 6-OHDA (0.3 μg) did not induce any behavioural impairment in the cylinder test (45.8 ± 9.1%).

3.3.2. Stepping test

Animals lesioned with 3.6 μg 6-OHDA only rarely used the contralateral paw (6.0 ± 2.8% of total steps performed), which was significantly different when compared to all the other dose groups (Fig. 3B). Animals lesioned with medium 6-OHDA doses showed 22.4 ± 4.7% (0.7 μg) and 27.3 ± 8.7% (1 μg) contralateral adjusting steps, which were significantly different from the sham (52.6 ± 2.7%) and 3.6 μg groups. There was no significant difference between the two medium dose groups. The lowest dose of 6-OHDA (0.3 μg) did not induce any behavioural impairment in the stepping test (43.4 ± 11.3%).

3.3.3. Corridor test

Sham mice showed approximately the same number of contralateral and ipsilateral retrievals in the corridor test (59.0 ± 2.5% contralateral retrievals) (Fig. 3C). Mice in the 3.6 μg dose group showed a marked deficit in use of the contralateral paw (11.2 ± 5.2% of total retrievals) compared to all the other groups. Medium 6-OHDA dose animals showed a mild preference for ipsilateral side retrievals, with contralateral retrievals of 24.2 ± 7.2 and 25.7 ± 8.4% of total retrievals for the 0.7 and 1 μg 6-OHDA dose, respectively, and could be discerned from the sham and high dose groups. Interestingly, also the 0.3 μg 6-OHDA dose induced a slight impairment of the corridor test performance when compared to the sham group, showing 37.8 ± 9.5% contralateral retrievals.

3.3.4. Rotation

Assessment of spontaneous rotation showed that animals in the highest dose group rotated spontaneously towards the lesion side (34.8 ± 8.9 net ipsilateral turns) (Fig. 3D), when compared to the sham (0.1 ± 9.1) and 1 μg. However, 0.3 μg, 0.7 μg and 1 μg 6-OHDA doses did not induce any significant spontaneous rotation when compared to the sham group (−7.7 ± 11.6, 17.0 ± 16.2 and 3.6 ± 14.4, respectively).

The apomorphine-induced rotation test revealed two clear groups (Fig. 3E). Sham-lesioned and 0.3 μg 6-OHDA dose groups did not rotate when triggered with apomorphine (−3.4 ± 4.3% and −0.6 ± 1.3 net contralateral turns, respectively). On the other hand, 0.7 μg, 1 μg and 3.6 μg 6-OHDA groups showed a significant increase in contralateral rotations after apomorphine injection, when compared with the sham group (−149.3 ± 45.1, −218.2 ± 75.1 and −204.1 ± 48.8, respectively). However, no difference between medium and high dose groups could be observed.

Assessment of the amphetamine-induced rotation test indicated an increase in net ipsilateral turns with increasing cell loss (Fig. 3F). Mice in the 3.6 μg dose group had the highest score (202.5 ± 20.7 net ipsilateral turns) that was significantly different from the sham (7.1 ± 6.0 net ipsilateral turns) and medium dose groups (64.5 ± 22.6 and 90.6 ± 34.0 net ipsilateral turns for 0.7 and 1 μg, respectively). Medium dose group scores were significantly different from both sham and high dose groups but not between them.

Taken together, the behavioural impairment was 6-OHDA-dose-dependent, and 4 out of the 6 behavioural tests investigated could clearly distinguish animals with a partial lesion from animals with a sham or total lesion.

3.4. Nigrostriatal denervation correlates with behavioural deficit in 6-OHDA mice

We next investigated whether the behavioural responses of 6-OHDA mice also mirrored the dopaminergic system alteration. Linear or non-linear regression analyses were performed to identify any possible correlations between the nigrostriatal damage and the behavioural impairment (Fig. 4). All regression analysis presented a normal distribution of their residuals, thereby providing a statistical strength for the correlations (data not shown). When compared with the TH+ cell loss (Fig. 4A–E), all tests presented strong correlations, with an R^2 value reaching from 0.744 (apomorphine-induced rotation, Fig. 4D) to 0.913 (amphetamine-induced rotation, Fig. 4E). Similar results were found when comparing the dorsal striatal denervation with the different behavioural tests (Fig. 4F–J), with correlations reaching from 0.594 (apomorphine-induced rotation, Fig. 4I) to 0.878 (cylinder test, Fig. 4F). Strong correlations could

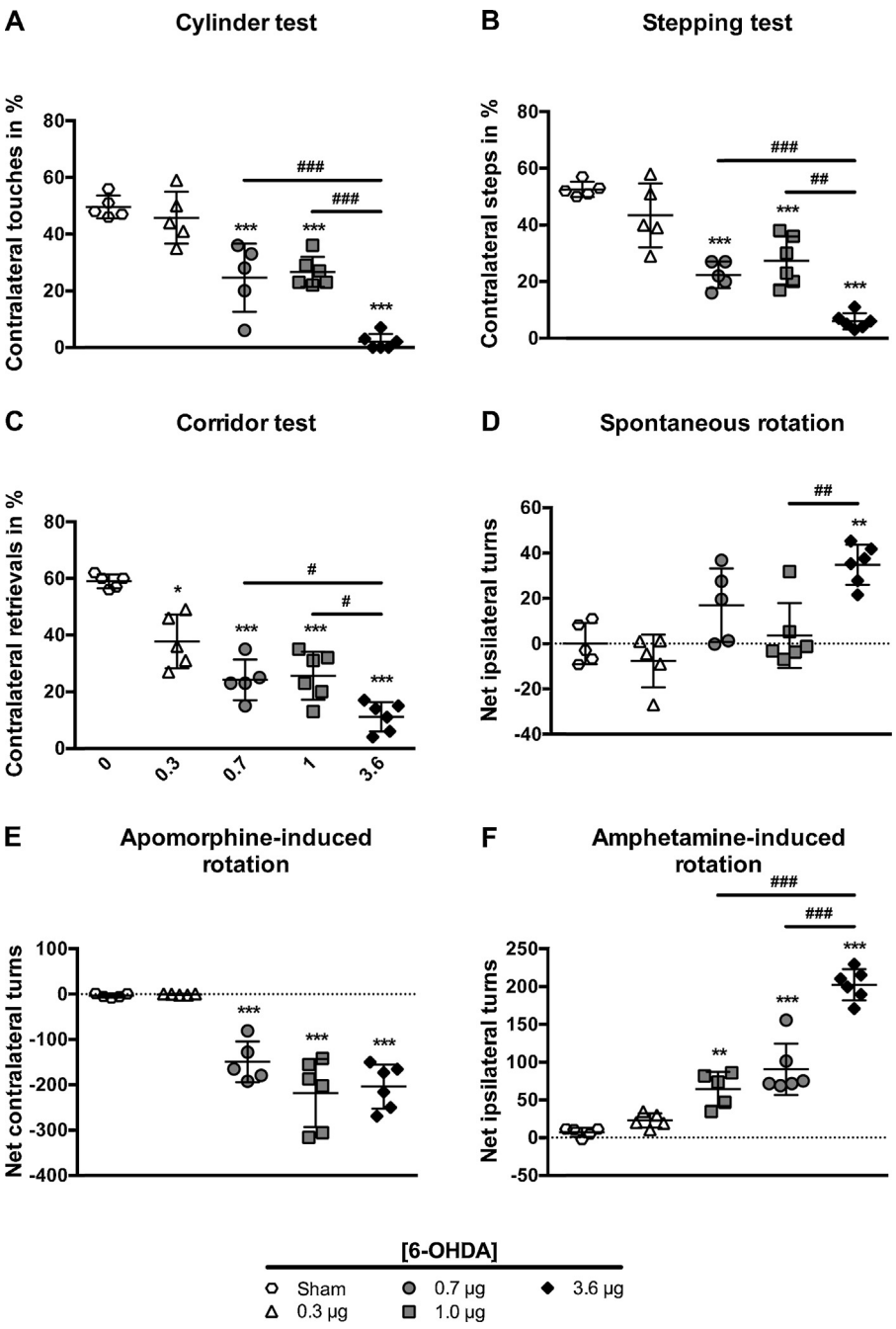

Fig. 3. Behavioural impairment in different 6-OHDA dose groups. Different 6-OHDA doses induced varying degrees of behavioural deficits depending on the test applied. Cylinder test (A), stepping test (B), corridor test (C) and amphetamine-induced rotation test (F) could distinguish the medium dose groups from the sham and high dose group. Neither spontaneous rotation (D) nor apomorphine-induced rotation (E) tests could clearly differentiate animals in the medium dose groups from animals in the sham or high dose groups. Graphs show mean ± SD. The number of symbols denotes the p-value when compared to the sham group ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) or medium dose groups ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$).

also be observed when comparing the ventral striatal denervation with the different behavioural tests, with significant R^2 values from 0.430 (apomorphine-induced rotation, Fig. 4N) to 0.885 for the amphetamine-induced rotation test (Fig. 4O). Only the correlation between the nigrostriatal lesion and the apomorphine-induced rotation showed a wide 95% confidence interval associated with the lowest R^2 values among all behavioural tests (Fig. 4D, I and N).

These data provide evidence for a strong correlation between the degree of nigrostriatal lesion and the behavioural impairment.

3.5. ROC curve analysis identifies amphetamine-induced rotation and the stepping tests as best predictors of partial nigral cell loss

With the aim to predict the degree of the dopaminergic system degeneration from the results obtained in the behavioural tests, we run a diagnostic test. This test reports the efficacy to identify a partially lesioned animal (referred as the sensitivity, or true positive rate) and the ability to discard animals that are not partially lesioned (referred as specificity, or false positive rate). This is done for different cut-off value and each behavioural test. A ROC curve is drawn that represents the sensitivity as a function of the

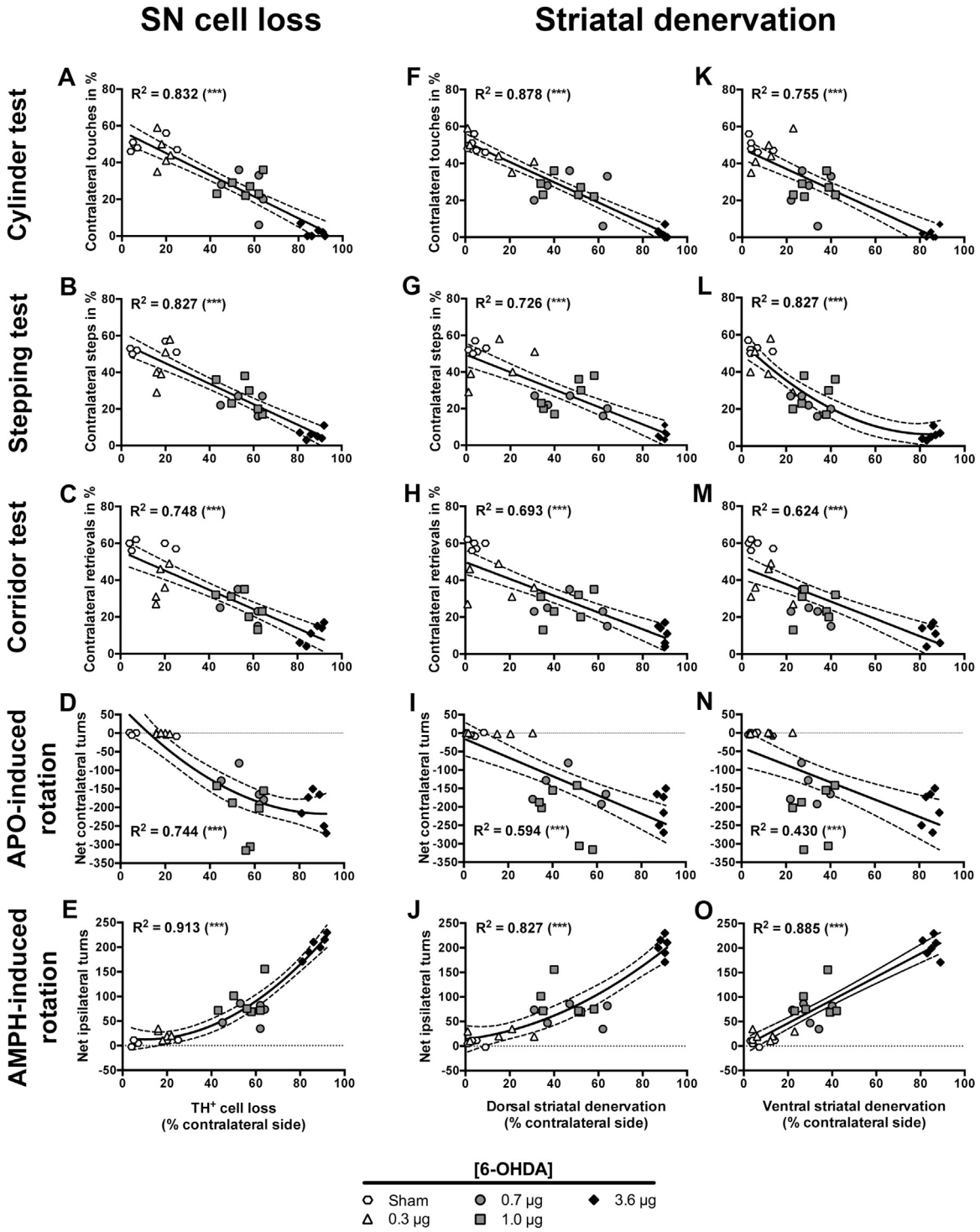

Fig. 4. Behavioural impairment correlates with the degree of nigrostriatal denervation. Linear or non-linear regressions were calculated to identify correlations between behavioural tests and the nigral TH^+ cell loss (A–E), the dorsal striatal denervation (F–J) and the ventral striatal denervation (K–O). The fitted line is framed within the 95% confidence intervals (dashed lines). The number of * denotes a slope significantly different from zero (***$p < 0.001$). APO: apomorphine; AMPH: amphetamine.

specificity. The area under the curve (AUC) indicates the global accuracy for the behavioural test to separate the two groups, where an AUC of 100% would represent a perfect test, while an AUC of 50% would mean that the test failed to separate both groups.

We defined cut off values in the different behavioural tests, that ensured that only partial lesion animals are identified, hence the need for the specificity to be 100%.

Here, we compared the TH^+ cell loss of either partial lesion with sham animals, or partial with total lesion groups, for each behavioural test, thereby obtaining different ROC curves (Fig. 5). It appeared that, when comparing partial lesion with the sham groups, all tests gave the best ROC curve, reaching an area under the curve (AUC) of $100 \pm 0.0\%$, and with a specific cut-off value giving 100% specificity and sensitivity (Fig. 5A–E). The best cut-off values for each behavioural test would provide 100% specificity and 100% sensitivity. Therefore, a score <41% of contralateral touches for the cylinder, <44% of contralateral steps for the stepping test, <45.50% of contralateral retrievals for the corridor test, <−44.82 net

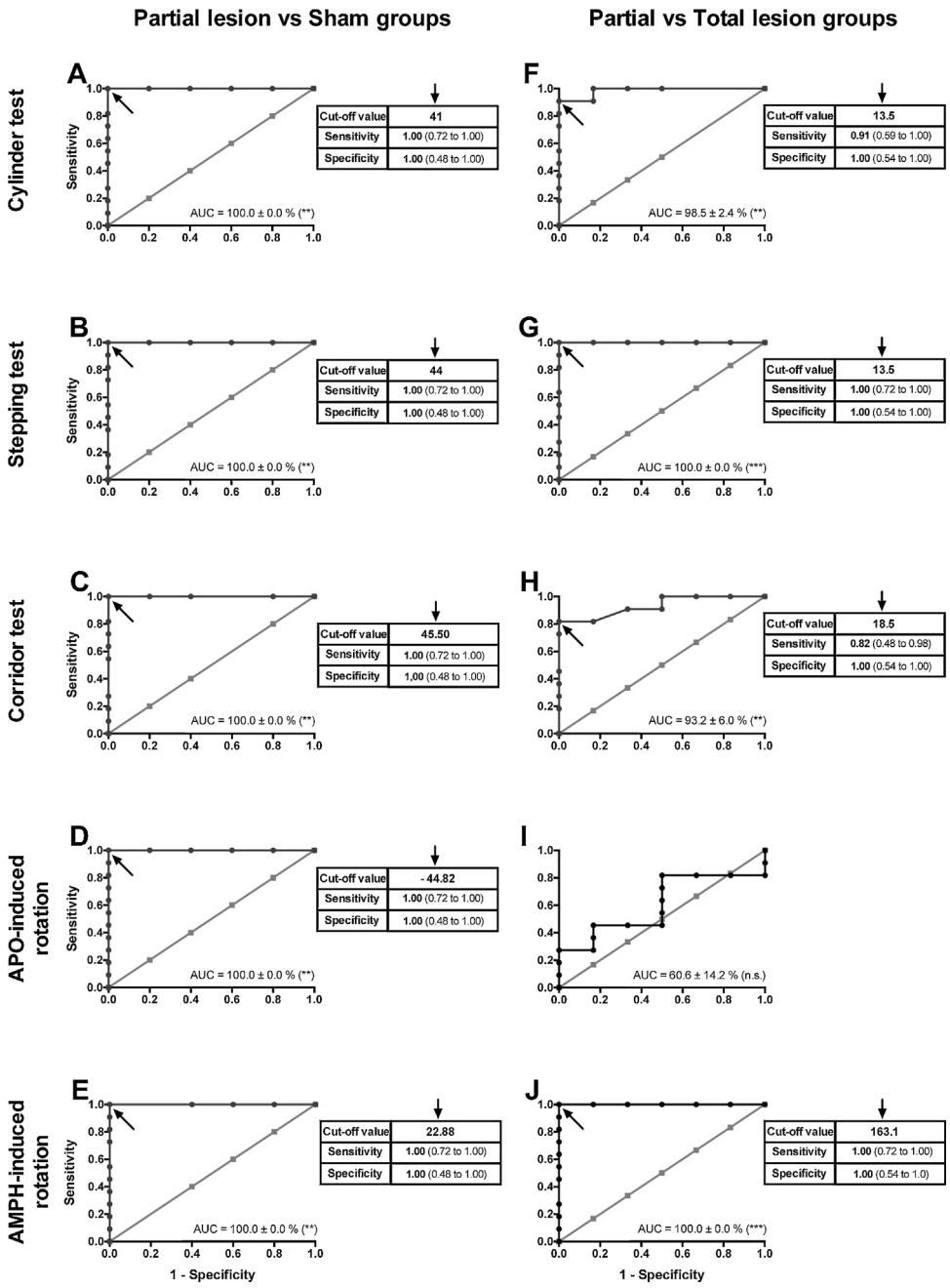

Fig. 5. Sensitivity and specificity of different behavioural tests to identify partially-lesioned animals. The figure illustrates the ROC curves (black lines) between partial lesion group with sham group (A–E) or total lesion group (F–J), for each behavioural test. The number of * denotes whether the area ± SD under the curve (AUC) is significantly different from 50% (grey line) (**$p < 0.01$; ***$p < 0.001$; n.s., non-significant). The arrow indicates a specificity of 100% and the best sensitivity for each behavioural test. The corresponding cut-off values to separate groups, the specificity and sensitivity with their respective confidence interval are presented in the tables within the panels. APO: apomorphine; AMPH: amphetamine.

ipsilateral turns for the apomorphine-induced rotation test and >22.88 net contralateral turns for the amphetamine-induced rotation test would distinguish 100% of the partial lesion animals from the sham group, without any false positive animals. The results were more scattered when examining ROC curves based on the comparison between partial and total lesion groups (Fig. 5F–J). Our data showed that most tests gave a ROC curve covering an AUC significantly higher than 90%, except for the apomorphine induced-rotation test (AUC = 60.6 ± 14.2%; n.s.), and achieving 100 ± 0.0% for the stepping test and the amphetamine-induced rotation test. Therefore, a score >13.5% of contralateral touches for the cylinder test and >18.5% of contralateral retrievals for the corridor test would give a perfect specificity in both tests, and a near-to-perfect sensitivity, where 91% and 82% of partial lesion animals would be distinguished from the total lesion groups, respectively. A score >13.5% of contralateral steps for the stepping test, and <163.1 net contralateral turns for the amphetamine-induced rotation test would thus select all partial-lesion animals, without including any false-positive total-lesion animals.

4. Discussion

4.1. Summary

Here we established and characterized for the first time a partial MFB lesion model of PD in mice. Four different 6-OHDA doses

were tested, and our results showed a dose-dependent loss of nigral dopaminergic cells and striatal fibres that correlated with several drug-induced and non drug-induced behavioural tests. Specifically, we identified the amphetamine-induced rotation test, stepping test, corridor test and cylinder test to be sensitive enough to discriminate partial lesion animals from sham or total lesion mice. On the other hand, we also determined several tests that were less useful to distinguish a partial MFB lesion (spontaneous rotation and apomorphine-induced rotation tests). Based on our data, we proposed a range of cut-off values for these different behavioural tests that enable to select partial lesion mice. We applied a statistical prediction model that identified two behavioural tests (the stepping test and amphetamine-induced rotation test) that can be used to predict the extent of TH^+ cell loss and select mice with a partial nigrostriatal lesion with a high sensitivity and specificity prior to further interventions. Partial MFB 6-OHDA lesion in mice could be used to assess neuroprotective and neurorestorative interventions, especially when the treatment would target the SN and/or striatum.

4.2. Survival rate

MFB lesion is one of the most common PD models, however the use of this model in mice has been strongly criticized due to the elevated mortality rate. Implementation of intensive post-operative care reduces the mortality to a very low level [14,16–20]. We report a survival rate of 80% in the total lesion mice that is in accordance with previous studies [14,16,17,45].

4.3. Dose-dependant effect of 6-OHDA on the dopaminergic system

Injection of 3.6 μg of 6-OHDA in the MFB produced a marked degeneration of the dopaminergic neurons in the SNpc as well as denervation of the TH^+ fibres in the dorsal striatum. The depletion achieved in our study is in the same range as previous findings [15,19]. Most importantly, we identified a dose range of 6-OHDA (0.7–1.0 μg) that induced a partial lesion in mice and gave a distinct and moderate alteration of the nigrostriatal pathway. This is the first report of a dose-dependent nigrostriatal denervation in a unilateral 6-OHDA MFB mouse model, with an emphasizis on a partial degeneration of the nigrostriatal pathway.

In this study, we refrained from assessing the TH^+ cell loss in the ventral tegmental area (VTA) as the impact of the VTA on different behavioural tests has been shown to be small, and behavioural deficits were mainly driven by the dopaminergic cell loss in the SNpc [16]. Therefore, behavioural tests will more likely predict SNpc cell loss and striatal fibre loss.

Regression analysis confirmed a dose-dependant effect of 6-OHDA on the dopaminergic system that is consistent with findings in a rat model of PD where increasing 6-OHDA dosages were used [37].

The stereotaxic coordinates used in this study are in accordance with coordinates used by others to induce a complete MFB lesion in mice [14–18,21,41,45], suggesting that the degree of lesion in the nigrostriatal pathway depends on the amount of 6-OHDA injected.

4.4. Behavioural impairment correlates with the degree of nigrostriatal denervation

We have, for the first time, described an MFB partial lesion mouse model and correlated the degree of DA denervation with the degree of behavioural impairment using a wide battery of different tests. Accurate MFB lesion has been a challenge in mice, which makes it mandatory to use appropriate behavioural tests to predict the extent of TH^+ nigral cell and striatal fibre loss. As a step further we wanted to investigate whether the behavioural tests used in this study could distinguish partial lesion animals from sham or total lesion animals.

Similarly to a partial MFB lesion model in rats [37], we observed a behavioural impairment that corresponded to the dopaminergic nigrostriatal denervation and the 6-OHDA doses. Partial lesion mice showed a mild yet quantifiable behavioural impairment that could be identified using several behavioural tests. We found that four out of six behavioural tests used in this study were able to discriminate partial lesions from both total and sham groups.

Tests that assess for forelimb akinesia (cylinder and stepping tests) effectively discriminated partial lesion groups from sham and total lesion groups. The cylinder test is one of the most common behavioural test used to identify unilateral lesions in the striatum [16], the SNpc [13,15] and the MFB [16–18]. In our study, this test showed a high correlation with the degree of nigrostriatal denervation. Mice that received the highest 6-OHDA dose (3.6 μg) showed a strong impairment in the cylinder test performance, similar as reported by Iancu and co-workers [18]. However, this is at variance with other results from Lundblad and co-workers [15] and Francardo and co-workers [17], where the same dose of 6-OHDA induced a less pronounced impairment in the contralateral paw. We speculate that this difference may be due to either difference between strains or the fact that, in our study, mice were recorded only for a 3-min period.

Our results suggest the stepping test as one of the best tests to discriminate between different degrees of nigrostriatal lesion. Mice with a total lesion showed a strong akinesia in the ipsilateral paw when compared to mice with a partial lesion and sham lesioned mice. Interestingly, partial lesion mice showed a moderate impairment in this test that correlated well with the partial degeneration of the dopaminergic nigrostriatal pathway. The strong correlation observed between the stepping test and the nigrostriatal pathway damage are in accordance with the findings of Heuer and co-workers, who suggested a correlation between dorsal striatal TH^+ fibre density and the performance in the stepping test [16]. This correlation is not found in an other study where the lesion was located in the SNpc [13].

Lateralized sensorimotor integration was assessed by the corridor test, originally used for rats and adapted for mice by Grealish and co-workers [13]. This is a useful tool to examine behavioural asymmetry as no direct contact with the animal is required (compared to the stepping test), no specific training is necessary and animals are guided by their motivation [13].

The corridor test has been shown to be one of the most sensitive behavioural tests for unilateral 6-OHDA lesion. Several authors have found a significant correlation in the proportion of dopaminergic cell bodies remaining in the ipsilateral SNpc with the performance on the corridor test in an MFB model [16] as well as in an intranigral lesion model [13], consistent with our findings. The highest dose of 6-OHDA (3.6 μg) induced a strong impairment in the corridor test that correlates with the high number of TH^+ cell loss in the SNpc. Partial lesion mice showed a mild impairment in the corridor test that corresponded with the degree of lesion in the nigrostriatal pathway. Interestingly, a mild impairment could also be observed in the group receiving the lowest 6-OHDA dose (0.3 μg), reinforcing the idea of the corridor test to be the most informative test for the assessment of the lesion severity in 6-OHDA-lesioned mice, as suggested by Grealish and co-workers [13].

Rotational asymmetry was assessed by a non-drug and drug-induced approach [6]. Only mice that received the highest dose of 6-OHDA displayed a significant spontaneous rotation towards the ipsilateral side. Partial lesion mice did not rotate spontaneously, indicating that this test is not sensitive enough to detect partial

lesions. Therefore, spontaneous locomotor behaviour was judged as not useful to identify partial lesion in mice, confirming findings by others [15,16], and in concordance with Heuer and co-workers, who showed that spontaneous rotations correlate more with the cell loss in the VTA [16].

Drug-induced rotations are the most commonly used test in assessing 6-OHDA unilateral lesions [6,13,15,16,18,38,45]. Our result showed that apomorphine-induced rotation had the weakest correlation with the nigrostriatal pathway damage. The test was not able to distinguish partial from total lesion mice. This may be due to the apomorphine dose and injection paradigm we used, as the animals are primed four and two days before the test day, as previously described [13]. Thus, the three doses of apomorphine may induce a hyperstimulation of dopamine receptors in the denervated side, evoking ipsilateral turning in mice with an average TH^+ cell loss of 55–57% [46].

Amphetamine induces DA release and inhibits DA reuptake causing ipsilateral turning behaviour in animals with unilateral nigrostriatal lesions [6]. Amphetamine-induced rotation test has been shown to correlate with the degree of DA depletion in rats [47–49] and in mice [11,16,18], which is in concordance with our study where regression analysis showed strong correlation coefficients between amphetamine-induced rotation and the DA system alteration. This may indicate that rotational behaviour is mainly driven by the TH^+ cell loss, as suggested by Heuer and co-workers [16].

4.5. Stepping test and amphetamine-induced rotation test as predictors for MFB partial lesion in mice

For each behavioural tests, we determined cut-off values based on a ROC analysis to specifically identify partial lesion animals, thereby providing 100% specificity and the highest corresponding sensitivity, whether or not false negative result would be discarded. We showed that, when comparing sham with partially lesioned mice, all behavioural tests presented a very good sensitivity and specificity and, for partial and total lesion group comparison, near-to-perfect ROC curves were drawn for all tests, except for the apomorphine-induced rotation test. This is in concordance with other results in this study, as apomorphine-induced rotation test could not distinguish between 6-OHDA dose of 0.7 μg or 1 μg, with 3.6 μg dose, and gave the weakest correlation, compared to all the other tests, with broad confidence intervals, reflecting its lack of specificity.

Stepping and amphetamine-induced rotation tests were the only tests to give excellent sensitivity and specificity in both comparisons, enabling to isolate mice with a partial TH^+ cell loss from sham and total TH^+ cell loss mice. Based on our findings, we suggest the amphetamine-induced rotation test as a useful behavioural test, together with the stepping test, to discriminate partially lesioned animals. Therefore, a partial TH^+ cell loss in an MFB-lesioned mice would present a score reaching from 13.5% to 44% of contralateral steps for the stepping test, and a score within 22 and 163 net ipsilateral turns for the amphetamine-induced rotation test.

While this result is surprising for the stepping test, as contradiction in the literature would render it less reliable [13], amphetamine-induced rotation test is in concordance with previous data, where it highly correlates with nigral dopaminergic cell loss [16,18]. Interestingly, even though the corridor test has been identified as one of the best behavioural test to predict the degree of lesion in 6-OHDA unilateral models [13], its potential strength to discriminate partially lesioned animals was weaker than expected, indicating that, at least using our paradigm, this test was not suitable to distinguish between partial and total lesioned mice.

4.6. Application of a partial MFB animal model

Regenerative therapies such as cell replacement or growth factor treatments are usually placed either into the striatum or the SN. Injection of a toxin to create a model may interfere with the effect of the respective intervention. Furthermore, potentially neuroprotective interventions require models resembling earlier stages of PD, thus less severe lesions, where some dopaminergic cells and fibres remain. The partial MFB model has the advantage that the toxin is injected in a non-interest area; the unilateral nigrostriatal lesion results in behavioural and histological readouts and the behavioural impairment allows selection of partially lesioned mice based on a statistical prediction model. Using a partial MFB lesion in mice opens up the possibility to combine studies using reporter mice or otherwise genetically modified mice and hence presents a valuable research tool.

5. Conclusion

In this study we have characterized, for the first time, a partial lesion model of Parkinson's disease with injection of 6-OHDA in the MFB resulting in a ca. 50% nigrostriatal lesion. The degree of lesion is severe enough to be reflected in behavioural impairments that can be used to select animals and evaluate interventions.

Finally, we have identified a combination of specific behavioural tests and cut-off values that can be used to reliably predict the degree of nigral dopaminergic cell loss and to select mice with a partial MFB lesion prior to any therapeutic intervention.

Conflict of interest

The authors declare no conflict of interest.

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