The 6-OHDA mouse model of Parkinson's disease – terminal striatal lesions provide a superior measure of neuronal loss and replacement than median forebrain bundle lesions

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Abbreviations
MFB, Medial forebrain bundle; PD, Parkinson's disease; VM, ventral mesencephalon

Abstract
Unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway produce side-biased motor impairments that reflect the motor deficits seen in Parkinson's disease (PD). This toxin-induced model in the rat has been used widely, to evaluate possible therapeutic strategies, but has not been well established in mice. With the advancements in mouse stem cell research we believe the requirement for a mouse model is essential for the therapeutic potential of these and other mouse-derived cells to be efficiently assessed.
This aim of this study focused on developing a mouse model of PD using the 129 P2/OLA Hsd mouse strain as this is widely used in the generation of mouse embryonic stem cells. Both unilateral 6-OHDA medial forebrain bundle (MFB) and striatal lesion protocols were compared, with mice analysed for appropriate drug-induced rotational bias. Results demonstrated that lesioned mice responded to d-amphetamine with peak rotation dose at 5mg/kg and 10mg/kg for MFB and striatal lesions respectively. Apomorphine stimulation produced no significant rotational responses, at any dose, in either the MFB or striatal 6-OHDA lesioned mice. Analysis of dopamine neuron loss revealed that the MFB lesion was unreliable with little correlation between dopamine neuron loss and rotational asymmetry. Striatal lesions however were more reliable, with a strong correlation between dopamine neuron loss and rotational asymmetry. Functional recovery of d-amphetamine-induced rotational bias was shown following transplantation of E13 mouse VM tissue into the lesioned striatum; confirming the validity of this mouse model.

Keywords: 6-hydroxydopamine (6-OHDA), Behavioral rotation, Medial forebrain bundle lesion, Terminal (striatal) lesion, Transplantation.

1. Introduction

Parkinson's disease is a progressive neurological disorder that is characterised by a catalogue of movement impairments such as rigidity, tremor and bradykinesia [1, 2]. The disease is primarily caused by the loss of the nigrostriatal dopamine pathway [1] and therefore highlights the importance of striatal dopamine on motor function.
Experimental evidence for the role of dopamine in the striatum, and its effects on motor function first came from dopaminergic stimulation of the rat striatum, which resulted in marked changes in motor response [3]. The subsequent pioneering investigations on motor effects following unilateral dopamine depletion using 6-hydroxydopamine provided a unique way of analysing dopamine activity by measuring drug-induced motor function [4, 5]. Under these conditions rats display rotational asymmetry with the degree of rotation being proportional to dopamine loss [6] and the direction of rotation being dependent on the dopamine agonist drug used [4, 5].

Since its development, this behavioural model has been used extensively in PD research. It has provided a valuable tool to assess the potential of curative treatments by examining the attenuation of 6-OHDA-lesion-induced behavioural deficits. While most of these models have used rats, behavioral impairment has also been observed in dopamine-depleted mice [7 – 12]. With respect to rotational bias, the methods used to

induce rotational behaviour in these studies vary considerably, with differences in lesion type, dopamine agonist drug, and drug doses used. For instance, the unilateral, 6-OHDA intrastriatal lesions used by Brundin et al, (1986) [7] produced low rotational asymmetry following 2.5mg/kg amphetamine stimulation. In contrast, better rotations were reported by Barberi et al., (2003) [8] following striatal lesions and stimulation by 10mg/kg amphetamine and apomorphine. While good rotations were observed by Barberi and colleagues, the drug doses used seem excessive, particularly with respect to apomorphine. This high-dose apomorphine-induced rotation suggests that no supersensitivity of post-synaptic dopamine receptors occurred, even though increased receptor binding has been previously reported following striatal 6-OHDA lesions in mice [13]. In fact, good rotational bias following 0.5mg/kg apomorphine stimulation in striatal lesioned mice has been demonstrated, indicating supersensitivity can be achieved and a good functioning mouse model can be produced [10]. In addition, over 95% of the mice in this study received reliable lesions unlike the study by Iancu et al., (2005) [9] which showed inconsistent lesions resulting in less than 50% of lesioned animals being used in behavioral tests. More recent studies have shown stable lesions following unilateral 6-OHDA administration which produce deficits in a variety of behavioral tasks [11, 12]. However, whilst improvement is seen in the majority of these behavioral tests, reversal of the rotational bias following transplantation of E12.5 ventral mesencephalon tissue is not [14].

It is clear from these reports that a standard protocol for developing good rotational bias following the unilateral 6-OHDA lesions in mice, which can be reversed following replacement therapy, has yet to be established.
This study addressed this issue by evaluating the suitability of the 129 P2/OLA Hsd mouse strain to exhibit a rotational bias following 6-OHDA lesioning. Here we provide a comprehensive, detailed assessment of lesion type, drug, and drug dose required, to produce a standard, reproducible model where rotational bias reflects both dopamine loss and replacement in the 129 P2/OLA Hsd mouse strain.

2. Materials and Methods

2.1 Subjects

In vivo studies were conducted in young adult female mice of the 129P2/OLA Hsd strain (Harlan Olac, Bicester, UK). Animals were housed in groups of 4-6 mice/cage on a natural 12h:12h light dark cycle and with *ad libitum* access to food and water throughout. Foetal tissues for cell culture or grafts was derived from E13 foetuses (crown-rump length = 11mm) obtained from pregnant female mice of the same strain. All studies were conducted in accordance with full ethical appraisal and licences under the UK Animals (Scientific Procedures) Act 1986.

2.2 Surgical procedures

2.2.1 6-hydroxydopamine (6-OHDA) lesions

Adult female 129P2/OLA Hsd mice (~25-30g), were anaesthetised using gaseous isoflurane (2-5% in 2:1 O2:N2), and received unilateral stereotaxic injections of 1⌠l of 4⌠g/⌠l of 6-hydroxydopamine hydrobromide (6-OHDA, Sigma) dissolved in physiological saline containing 0.01% ascorbic acid. Infusions were delivered over 1 min via a 30 gauge stainless steel cannula. Lesions were placed in either the right medial forebrain bundle (A = –2.0 mm anterior to bregma, L = –0.7 mm lateral to bregma, V

= –4.8 mm ventral to dura; with the incisor bar set at 0.0 mm relative to the interaural line) or the right mid-striatum (A = 0.4 mm, L = -1.8 mm, V = -3.5; with the incisor bar set at 0.0 mm). The cannula was left in place for a further 2 min for diffusion, before being slowly removed and the wound cleaned and sutured.
After lesioning, mice were sutured and injected subcutaneously with 0.5ml 0.9% saline/glucose solution to prevent dehydration. The drinking water was supplemented with Paracetamol for the following 48 h and mice were carefully monitored post-surgery. All mice were allowed to recover for at least 10 days before behavioural testing commenced.

2.2.2 VM tissue transplantation
VM tissue was dissected from E13 mouse embryos and a single cell suspension was prepared as previously described [15]. Briefly, pregnant donors were killed by decapitation under general anaesthesia, and the foetuses removed by caesarian section. The ventral mesencephalon was dissected and pooled from all donors in a litter. Dissected tissue was enzymatically digested using a solution of 0.1% trypsin (Worthington, Lakewood, USA) and 0.05% DNase (Sigma, Poole, Dorset), at 37ºC for 20 mins. After two rinses in Hank's balanced salt solution (HBSS) the tissue was dissociated into a single suspension by mechanical trituration using a 200⌈l Gilson pipette. Cell counts and percentage viable cells were assessed by trypan blue exclusion in a haemocytometer.
Tissue was grafted into the right neostriatum by stereotaxic injection of 500,000 cells in 4⌈l at A = 0.8 mm, L = -1.8 mm, V = -3.0mm and -2.5mm, (2⌈l at each depth) using a 10⌈l Hamilton microsyringe over 4 min. A further 4 min was allowed for diffusion prior to syringe removal, and the wound was then cleaned and sutured. All animals were allowed to recover for at least 10 days before behavioural testing commenced.

2.3 Behavioral testing
2.3.1 Amphetamine and Apomorphine-induced rotations
Mice were placed in cylinders with a diameter of 11.5cm and height of 14cm, in a closed room to avoid any environmental disturbance, and allowed to habituate for 10 min before injection with a specific dose of drug. D-amphetamine (Sigma) was deli-vered intraperitoneally, while apomorphine hydrochloride (Sigma) was delivered sub-cutaneously to the scruff of the neck.
Mice were monitored for either 90 min (d-amphetamine-induced rotations) or 48 min (apomorphine-induced rotations), due to the difference in the metabolism of the two drugs. Each subject was scored for full body rotations in one-minute intervals, every six min, and the net ipsilateral rotation (total right – total left 360° turns) per minute was used as the primary dependent variable. Mice were given at least 7 days to recov-er following each amphetamine dose and at least 4 days following each apomorphine dose to ensure that the drugs had been eliminated from the system prior to behavioural testing.

2.4 Histology
2.4.1 Perfusion and sectioning of 6-OHDA lesioned mouse brains
After behavioural testing had been completed, mice were terminally injected with 0.5ml Euthatal and transcardially perfused with approximately 20 ml phosphate buffered saline (PBS, pH = 7.4), followed by approximately 50 ml 4% paraformaldehyde

(PFA, pH = 7.4). The brains were removed and post fixed in PFA for a further 24 h before being placed in PBS containing 25% sucrose overnight for sectioning.

Brains were coronally sectioned on a microtome at a thickness of 60⌠m and stored at 4°C in Tris buffered 0.9% saline (TBS, pH = 7.4) containing 0.5% sodium azide. A series of 1:3 brain sections were quenched with 10% hydrogen peroxide and 10% methanol in distilled water for 5 min followed by three 10-min washes in TBS. Sections were then blocked in TBS containing 0.2% Triton X-100 (TXTBS) containing 3% normal goat serum (NGS) for 60 min. Without washing, brain sections were incubated overnight at room temperature, or over 2 nights at 4°c in TXTBS containing the primary rabbit anti-Tyrosine hydroxylase (TH) antibody at a concentration of 1:1000 (Chemicon, Temecula, CA) in 1% NGS. After three 10 min washes with TBS, sections were incubated at room temperature for 3 h in the secondary biotinylated goat anti-rabbit antibody at a concentration of 1:200 (DAKO, Denmark) in TBS containing 1% NGS. Sections were washed in TBS before being incubated for a further 2 h at room temperature in streptavidin-biotin complex (DAKO) in TBS with 1% NGS. Following washes in TBS and Tris-Non-Saline (TNS, distilled water containing 0.6% trizma base), TH positive cells in the substantia nigra were visualised by the reaction with vector SG kit (DAKO). Sections were mounted on gelatinised glass slides, air dried overnight, and dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped with DPX.

2.4.2 Histological Analyses

The total numbers of TH positive cells in the substantia nigra were estimated by counting every third 60⌠m coronal brain section at a magnification of 250x using a Leitz Dialux 22 microscope. Only those cells that were clearly identified with dendritic processes were counted on both the lesioned and non-lesioned side of the brain. Segregation of the ventral tegmental area (VTA) and the substantia nigra was achieved by using the medial terminal nucleus of the accessory optic tract as a landmark, as described in Lee et al., 1996 [6] and Bensadoun et al., 2000 [13].

The total number of TH cells present on the lesioned and non-lesioned sides of the brain was estimated using the Abercrombie correction formula [16] and the percentage TH neuron loss on the lesioned side was calculated, to quantify the extent of the lesion.

The numbers of TH positive cells in the transplants were estimated by counting every sixth 60⌠m coronal brain section at a magnification of 250x using a Leitz Dialux 22 microscope. Only those TH cells that were clearly identified and with dendritic processes were counted. The total number of TH cells present in each transplant was estimated using the Abercrombie correction formula (Abercrombie M., 1946). The average TH cell diameter was then calculated by measuring at least 50 random TH positive cells using an Olympus BX50 microscope and an Olympus C.A.S.T grid system.

2.5 Statistical Analyses

Quantitative measures of rotation, and TH-positive cell survival were undertaken by multifactorial analysis of variance (ANOVA) using the Genstat v7.2 statistical package (Rothampstead, Oxon) with post-hoc multiple comparisons by the Neuman Keuls t-test.

3. Results

3.1 Amphetamine-induced rotations in medial forebrain bundle (MFB) 6-OHDA lesioned mice

Twenty-four lesioned mice were tested for their rotational behaviour after the administration of d-amphetamine at 0mg/kg (saline injection), 2.5mg/kg, 5mg/kg and 10mg/kg (Fig. 1a).

Results indicated a significant effect of d-amphetamine dose on rotational response $F(3,69) = 4.63$ $p=0.005$). Mice injected with 0mg/kg d-amphetamine showed no rotational behaviour. Rotation induced by 10mg/kg (approximately 2 rotations/min) was significantly higher than at 0mg/kg (NK: $t_{(2,3)}=5.229$, $p<0.05$). Net ipsilateral rotation induced by 2.5mg/kg and 5mg/kg d-amphetamine (6 rotations/min) was significantly higher than the rotation produced from both 0mg/kg and 10mg/kg d-amphetamine (NK: $t_{(3,3)}=11.896$, $p<0.01$; NK: $t_{(2,3)}=6.667$, $p<0.05$; and NK: $t_{(4,3)}=8.624$, $p<0.01$; NK: $t_{(3,3)}=13.85$, $p<0.01$, respectively).

There was also a significant effect of amphetamine dose across the testing interval ($F(42,966) = 3.67$ $p<0.001$). At 2.5mg/kg, mice showed peak net ipsilateral rotation at 18 min, which was stable until 36 min before decreasing to baseline levels. Mice responded to 5mg/kg amphetamine with similar peak rotation, however the duration of rotational behaviour was more prolonged, with a reduction in circling occurring only after 60 min. At a dose of 10mg/kg, the induced rotational behaviour was not only notably lower than that induced at both 2.5mg/kg and 5mg/kg, but was also delayed (peaking approximately 40 min post-injection).

3.2 Amphetamine-induced rotation in striatal (terminal) 6-OHDA lesioned mice

Seven mice were tested for their rotational behaviour over time after the administration of d-amphetamine at 0mg/kg (saline injection) 2.5mg/kg, 5mg/kg, 10mg/kg and 15mg/kg. Results showed a significant effect of d-amphetamine dose on rotational response ($F(4,24) = 36.77$ $p=<0.001$) (Fig. 1b).

Mice injected with 0mg/kg d-amphetamine showed little rotational behaviour. At 2.5mg/kg, mice showed good ipsilateral rotation during the early intervals with net rotation reaching over 6 ipsilateral rotations/min, significantly higher than behavioural rotation at 0mg/kg (NK: $t(2,4)=14.008$, $p<0.01$). However, this rotational response reduced rapidly after 30 min.

Rotation induced by 5mg/kg d-amphetamine averaged over 8 net ipsilateral rotations/min, significantly higher than rotation induced by 2.5mg/kg (NK: $t(3,4)=8.533$, $p<0.01$) and 0mg/kg (NK: $t(4,4)=22.54$, $p<0.01$) but not significantly different than the rotation induced by 15mg/kg d-amphetamine (NK: $t(2,4)=2.355$, n.s).

10mg/kg d-amphetamine produced the highest rotation rate with net ipsilateral rotations of 9.71 per minute by the third interval (18 min); and rotation rate was stable for a long period of time, reaching peak values of 11.29 rotations/min. Rotation induced by 10mg/kg d-amphetamine was significantly higher than rotation produced by 15mg/kg, 5mg/kg, 2.5mg/kg and 0mg/kg d-amphetamine (NK: $t(3,4)=16.68$, $p<0.01$; NK: $t(4,4)=14.324$, $p<0.01$; NK: $t(2,4)=14.324$, $p<0.01$; and NK: $t(5,4)=36.865$, $p<0.01$ respectively).

At 15mg/kg, there was a steady increase in rotational behaviour with time, with a peak net ipsilateral rotation rate of 8.29 rotations/min occurring in the final testing

interval. Rotation induced by 15mg/kg d-amphetamine was significantly higher than the rotation induced by 2.5mg/kg d-amphetamine (NK: $t(2,4)=6.178$, $p<0.05$).

1.3.Amphetamine-induced rotation in non- lesioned (control) mice

Non-lesioned mice were tested for d-amphetamine-induced rotation at all doses (0mg/kg-15mg/kg), to confirm that any rotation observed in the experimental mice was due to the lesioning of the dopamine system and not any other factor (Fig.1c). Following d-amphetamine simulation, the activity of mice was amplified and slight rotational behaviour was seen, this rotation was however insignificant at all doses $F(4,28) = 0.13$ ns.

FIGURE 1 HERE

3.4 Apomorphine-induced rotation in medial forebrain bundle 6-OHDA lesioned mice

Eight mice with unilateral MFB 6-OHDA lesions received doses of apomorphine at 0mg/kg (0.01% ascorbic acid in saline), 0.01mg/kg, 0.05mg/kg, 2.5mg/kg, 5mg/kg and 10mg/kg apomorphine, with rotation measured over 48 min (8 intervals) (Fig. 2a). Results indicated a significant effect of apomorphine dose on rotational response ($F(5,35) = 7.94$ $p<0.001$)

Mice injected with 0mg/kg, 0.01mg/kg and 0.05mg/kg showed little rotational response across the whole eight intervals (average rotations of 0.19, 0.08 and 0.05 rotations/min respectively). Following 2.5mg/kg apomorphine, contralateral rotational behaviour was observed and averaged 2.76 net contralateral rotations/min. At a dose of 5mg/kg, rotation was more stable for a longer period of time, with rotation rates of around 3 net contralateral rotations/min being established and maintained from the second interval (12 min) until the final testing interval. 10mg/kg apomorphine produced rotations that were stable throughout the 48 minute testing session but these were relatively low (less than 2 net contralateral rotations/min).

Analysis of data showed that net contralateral rotation induced by 5mg/kg apomorphine was significantly higher than rotations produced by 10mg/kg, 0.05mg/kg, 0.01mg/kg and 0mg/kg apomorphine (NK: $t_{(3,5)}=6.519$, $p<0.05$; NK: $t_{(6,5)}=11.704$, $p<0.01$; NK: $t_{(5,5)}=11.593$, $p<0.01$; and NK: $t_{(4,5)}=11.185$, $p<0.01$ respectively), but not to 2.5mg/kg (NK: $t_{(2,5)}=1.667$, n.s).

Rotation induced by 2.5mg/kg apomorphine was significantly higher than rotations induced by 10mg/kg (NK: $t_{(2,5)}=4.852$, $p<0.05$), 0.05mg/kg (NK: $t_{(5,5)}=10.037$), $p<0.01$), 0.01mg/kg (NK: $t_{(4,5)}=9.926$, $p<0.01$) and 0mg/kg (NK: $t_{(3,5)}=9.519$, $p<0.01$).

3.5 Apomorphine-induced rotations on striatal 6-OHDA lesioned mice

Seven mice with unilateral striatal 6-OHDA lesions, received subcutaneous injections of apomorphine at doses of 0mg/kg (0.01% ascorbic saline injection), 0.05mg/kg, 2.5mg/kg, 5mg/kg, 10mg/kg and 15mg/kg (Fig. 2b). Results indicated a significant effect of apomorphine dose on rotational response ($F(5,30) = 12.32$ $p<0.001$).

There was zero rotational response with 0mg/kg or 0.05mg/kg apomorphine. 2.5mg/kg, 5mg/kg or 15mg/kg apomorphine administration produced only a small contralateral rotational response. Rotations at 10mg/kg apomorphine produced the highest number of rotations: averaging 3 net contralateral rotations/min within the first four intervals; but then the response fell sharply after 30 min. Average scores over the entire eight intervals were 1.89 net contralateral rotations/min.

Analysis of results showed that 10mg/kg apomorphine produced significantly more contralateral rotation than 15mg/kg, 5mg/kg, 2.5mg/kg, 0.05mg/kg and 0mg/kg (NK:

$t_{(2,5)}$=6.902, p<0.01; NK: $t_{(3,5)}$=8.754, p<0.01; NK: $t_{(4,5)}$=11.111, p<0.01; NK: $t_{(5,5)}$=13.847, p<0.01; and NK: $t_{(6,5)}$=15.001, p<0.01 respectively). Doses of 15mg/kg apomorphine produced significantly more contralateral rotation than 0.05mg/kg (NK: $t_{(4,5)}$=6.944, p<0.05), and 0mg/kg (NK: $t_{(5,5)}$=8.098, p<0.05), but not 5mg/kg (NK: $t_{(2,5)}$=1.852, n.s) and 2.5mg/kg (NK: $t_{(3,5)}$=4.209, n.s).

3.6 Apomorphine-induced rotation in non- lesioned (control) mice

Non-lesioned mice were tested for apomorphine-induced rotation at all doses (0mg/kg-15mg/kg). This was necessary to confirm that the rotation observed in the experimental mice was due to the lesioning of the dopamine system and not any other factor. Results showed no significant rotational bias at any dose (F(6,42) = 3.19 p=0.011 ns) (Fig. 2c).

FIGURE 2 HERE

3.7 Comparison of dose-response curves in MFB and Striatal lesions

The dose-response curves for MFB and striatal lesioned mice have been superimposed for direct comparison, as shown in Figure 3.

Statistical analysis of the results showed a significant difference in rotation after d-amphetamine stimulation, dependent on the lesion the mice had received (F(3,122) = 3.10 p=<0.05).

With MFB lesions 5mg/kg d-amphetamine induced the peak rotational response. However, at 5mg/kg d-amphetamine, the rotational behavior for both lesion models was similar, with no significant difference of rotation seen between the two groups (NK: $t_{(2,3)}$=1.189, n.s). In contrast, the striatal lesioned animals exhibited significantly more ipsilateral rotations compared to MFB lesioned animals when given a higher dose of 10mg/kg amphetamine (NK: $t_{(2,3)}$=7.544, p<0.05).

Although apomorphine produced a relatively low rotational response, it was more effective on MFB lesions than on striatal lesions (F(4,78) = 3.6 p=<0.05), with doses of 2.5mg/kg and 5mg/kg inducing the best rotational responses, which were more pronounced the MFB lesioned animals compared to the striatal lesioned mice, with averages of 2.78 and 3.18 compared to 0.66 and 0.72 net contralateral rotations/min respectively (NK: $t_{(2,4)}$=4.198, p<0.05; and NK: $t_{(2,4)}$=4.871, p<0.05) (Figure 3).

FIGURE 3 HERE

3.8 Rotation vs. dopamine loss

The extent of dopamine loss was analysed in both lesion models by counting and comparing the number of TH positive cells in the substantia nigra (SNr), on both the lesioned and non-lesioned side of the brain (Supplemental figure). Counts were obtained from at least three 60 µm brain sections, and the average percentage of SNr dopamine neuron loss was calculated. Although the mean neuron losses for MFB and striatal 6-OHDA lesions were similar (65.4 $\pm$ 29.2 % and 68.3 $\pm$ 4.4 % respectively), MFB lesions showed significant variability in the degree of lesioning, with a much larger standard deviation about the mean, compared to the striatal lesion group (F(20,5)=63.66, p<0.001).

Correlations were made between dopamine loss and peak drug-induced rotations for each animal with either a MFB 6-OHDA lesion or a striatal 6-OHDA lesion. MFB lesioned animals showed no specific correlation between dopamine loss and rotation following induction with either d-amphetamine or apomorphine (Figs. 4a and 4c). Linear regression analysis correlating neuronal loss with d-amphetamine and apomorphine-induced rotation for MFB lesioned animals were r^2=0.007 and r^2=0.005 respectively. Striatal lesioned animals however, showed stronger correlations between dopamine loss and rotation following d-amphetamine stimulation with an r^2 value of 0.72 (Fig 4b). No significant correlation was seen between neuronal loss and apomorphine-induced rotation in striatal-lesioned animals, (r2 = 0.0012; Fig 4d).

FIGURE 4 HERE

3.9 Dopamine neuron grafts induce functional recovery of rotational bias

Grafts of dopamine neurons were clearly visible with TH immunohistochemistry (Fig.5a-d). Using the Abercrombie correction formula, grafts were calculated to contain an average of 1273 ± 227 dopamine neurons.

Rotational asymmetry in response to 10 mg/kg amphetamine, was assessed in mice receiving unilateral 6-OHDA striatal lesions and VM grafts over a 6-week period post-transplantation (Figure 6e). This time period was sufficient for grafts to significantly reverse 6-OHDA lesion-induced rotational deficits ($F(3,28) = 31.06$, $p<0.001$). Mice receiving grafts of E13 mouse VM tissue (n=8), showed a significant decline in rotational bias at 4 weeks (NK: $t_{(3,3)}$=13.125, $p<0.01$) and 6 weeks (NK: $t_{(4,3)}$=13.546, $p<0.01$) post-transplantation when compared to pre-graft scores. Lesion only mice (n=8, striatal 6-OHDA lesion) showed good, stable rotational behaviour with no difference in rotational scores throughout the 6 weeks of testing. When compared to rotation in mice receiving grafts, lesion only animals had significantly higher rotation at 2 weeks (NK: $t_{(2,2)}$=7.707, $p<0.05$), 4 weeks (NK: $t_{(2,2)}$=17.099, $p<0.01$) and 6 weeks (NK: $t_{(2,2)}$=17.892, $p<0.01$).

FIGURE 5 HERE

4. Discussion

The 129 P2/OLA Hsd mouse strain exhibited rotational bias following unilateral 6-OHDA lesioning of either the medial forebrain bundle (MFB) or the striatum. However, there was little correlation between dopamine loss and rotation following d-amphetamine and apomorphine stimulation in MFB lesioned animals. Striatal lesioned animals were more reliable, with consistent lesions, consistent rotation, and stronger correlations with d-amphetamine-induced rotation.

The data reported here suggest that the optimal model for Parkinson's disease in the 129 P2/OLA Hsd mouse strain is produced following striatal 6-OHDA lesions, which can be measured most effectively using drug-induced rotation with an optimal dose of 10mg/kg d-amphetamine. The validity of this model was confirmed by the observation of functional recovery of rotational bias following transplantation of E13 mouse VM tissue into the lesioned striatum. To our knowledge we are the only authors to describe transplantation of dopamine neurones into mice with intrastriatal lesions and demonstrate functional recovery.

Although the striatal 6-OHDA lesion model is not novel, the model established here is fundamentally different and more reliable than previous mouse models since the conditions that produce optimal rotational behaviour have been experimentally defined. The development of this model is of great importance because it allows the therapeutic potential of mouse embryonic stem cells, and other mouse-derived cell lines to be assessed efficiently in a same species host (allogeneic transplants) without the added complications associated with immunosuppression when transplanting mouse cells to adult rats (xenogeneic transplants).

4.1 Drug-induced rotations

A short period of time was taken for the effects of the drugs to induce rotation, but once established a clear pattern emerged with d-amphetamine and apomorphine-induced rotation declining with time, presumably because of metabolism of the drugs. However, at high doses of d-amphetamine (10mg/kg and 15mg/kg for MFB and striatal lesions respectively), the reverse was observed, with the number of rotations/min increasing with time. The reason for this increased rotational activity is unclear, but perhaps these high doses of d-amphetamine stimulated not only the denervated dorsal striatum, but also, other intact dopaminergic regions, including the ventral striatum. Since this striatal region is involved in the initiation of stereotypical behaviour, such as repetitive limb and head movements, sniffing, and oro-facial stereotypies [9], which are dopamine dependent [17], it is likely that stereotypy would have been induced at these high d-amphetamine doses. Such stereotypic behaviour would have decreased rotational bias in a similar fashion to the way that high-dose amphetamine-induced stereotypy has been reported to decrease locomotor activity in non-lesioned rats [18]. As time elapsed and amphetamine is metabolised, stereotypy would decrease and rotational behaviour would be enhanced, thus explaining why rotational behaviour response to high drug doses was delayed post-injection.

4.2 MFB vs. Striatal lesions

The two different lesion models used in this study produced either near total (MFB) or partial (striatal) unilateral lesions of the nigro-striatal dopamine pathway. As a result there was a marked difference in the amount of rotation between the two groups, with MFB lesion animals responding to both d-amphetamine and apomorphine stimulation, while striatal lesion mice responded significantly more to d-amphetamine than apomorphine. When the two lesion groups were directly compared, the degree of d-amphetamine-induced rotational bias was generally higher for the striatal lesion group, and significantly higher at 10mg/kg.

Since at least 50% dopamine loss is necessary for amphetamine-induced rotation to be observed [19-21], it is unsurprising that both lesion groups showed rotations following d-amphetamine stimulation. It is somewhat surprising however, that d-amphetamine was more effective at producing a rotational bias in the striatal lesion group when compared to the MFB lesion group. MFB lesions produced higher dopamine depletion, and therefore it would seem logical that these lesions would result in animals displaying more rotational bias than animals receiving striatal lesions, due to the greater imbalance in dopamine release between the intact and lesioned hemispheres. The reason why this does not occur could be explained by the severity of the MFB lesion. In addition to depleting the nigrostriatal dopamine pathway, MFB lesions also deplete the dopaminergic neurons of the ventral tegmental area (VTA), which project to the nucleus accumbens, whereas with striatal lesions the nucleus ac-cumbens is spared [22]. Although we have not quantified the number of TH positive

cells in the VTA following 6-OHDA lesioning or transplantation, it can be seen from the microphotographs that the VTA is depleted in MFB lesioned mice compared to striatal lesions (Supplementary figure 1). Studies have reported that the reduction in dopaminergic cells following MFB lesions in mice in as much as 50% with no increase post-intrastriatal transplantation (14).

Although the nucleus accumbens is not involved in the direction of rotation, it is involved in locomotor activity [23] and therefore may influence rotational bias. This has been clearly demonstrated following 6-OHDA lesioning of the nucleus accumbens, which subsequently results in a significant reduction in amphetamine-induced locomotor activity and rotational behavior [24, 25]. In addition, dopamine grafts into the lesioned accumbens significantly increase both of these motor behaviors [24, 26]. The decrease in locomotor activity following the loss of neurons in the VTA caused by an MFB lesion may therefore explain why a d-amphetamine challenge causes greater rotational bias in striatal lesioned animals when compared to MFB lesioned animals.

The difference in apomorphine-induced rotational behaviour between the two lesion groups may be explained by up-regulation and hypersensitivity of post-synaptic dopaminergic receptors [19-21]. Global receptor hypersensitivity occurs when more than 90% of the nigrostriatal system is destroyed [19, 20], so will not have occurred in the striatal partial lesion model. Instead, only localised areas of post-synaptic receptor hypersensitivity may have occurred, with the majority of compensation being provided by increased dopamine synthesis from the remaining neurons [19].
While receptor hypersensitivity and up-regulation would explain why the MFB model produced better rotational bias than the striatal model following apomorphine stimulation, hypersensitivity of receptors probably did not occur to a great extent since low doses of apomorphine (0.01-0.05mg/kg) did not result in any rotational behaviour in the MFB lesion group. In addition, most of the MFB lesions resulted in less than 90% dopamine neuron loss, therefore receptor hypersensitivity may not have been as pronounced as is seen normally in the rat MFB 6-OHDA lesion model. Instead, the contralateral rotation seen at high apomorphine doses was probably a collective result of increased excitation of the lesioned striatum and increased rotational drive initiated by the activation of the nucleus accumbens, both which were probably mediated primarily through receptor up-regulation rather than receptor hypersensitivity.

4.3 Dopamine loss and replacement vs. rotation

Unilateral 6-OHDA MFB lesions in mice have recently been used to produce a model of PD, which shows good rotational asymmetry in response to amphetamine and apomorphine challenge [9]. However, while this model shows good rotational behaviour, less than 50% of the total number of animals that were lesioned in the study by Iancu et al., (2005) [9] were selected to participate in behavioral tests as the extent of MFB lesion was variable.
Histological examination of the MFB lesioned animals in our study also shows that MFB lesions are variable. In contrast, the striatal lesions show more reliable and consistent dopamine loss. Like the study by Iancu, we could also have selected a proportion of MFB lesion mice that showed good rotational behaviour, and only used these animals in the rotation tests. However, while this would have certainly increased the average rotational bias of this lesion group, it would not have reflected the inconsistent dopamine loss produced by the MFB lesion.

The level of inconsistency of the MFB lesions is extremely relevant when attempting to reproduce this model since it would mean that more animals would need to be lesioned, so that sufficient numbers of animals would display sufficient dopamine neuron loss. The most likely explanation for inconsistent MFB lesions is that the MFB is relatively small and is therefore difficult to target in the adult mouse brain. The striatum on the other hand, is easier to pinpoint and therefore it is unlikely that such variability would result from striatal lesions.

In the current study, transplants of embryonic VM to mice with terminal striatal lesions led to recovery from rotational asymmetry. Surprisingly, this is in contrast to the findings from a recent study that has also been shown to produce a stable mouse-model of Parkinson's disease, but has failed to demonstrate significant reversal of rotational behavior following transplantation of fetal dopamine neurons, despite a significant recovery in the choice reaction time task, the rotarod test and corridor test [14]. Heuer et al. argue that this may be attributed to underestimating rotation due to the difficulties with measuring rotational behavior in mice rather than a difference in anatomical motor pathways. When compared to our study, Heuer et al., measured ro-tational behaviour using automated rotometer bowels, whereas rotational bias was calculated post hoc after reviewing video footage of the testing intervals in our study. Although the method used by Heuer et al., may theoretically restrict subject move-ment (because of the use of a harness apparatus) and therefore result in reduced activi-ty, we believe that this is unlikely to be responsible for the failure to observe a rever-sal in rotational behaviour. More importantly, a crucial difference which may explain the difference in rotational recovery between the two studies is the lesion placement. Heuer et al., performed MFB lesions whereas the reversal of rotational behaviour in our study is seen in terminal striatal lesioned mice. This suggests that the placement of the lesion may not only be a critical factor in determining the precise behavioural deficit produced, but more significantly, it also determines whether transplanted do-pamine neurons can alleviate rotational deficits. Again, as previously mentioned (sec-tion 4.2) the failure to reverse rotational bias in the paper by Heuer et al may reflect the failure to replenish non-striatal areas which are depleted following MFB lesions and are important in behavioural tasks (i.e the VTA and nucleus accumbens).

4.4 The importance of a mouse model of PD

The 6-OHDA animal model of PD is a powerful tool which allows the potential of dopamine replacement treatments to be assessed by examining the attenuation of 6-OHDA-lesion-induced behavioural deficits. To date, the most promising alternative to human foetal tissue for dopamine replacement therapy in PD are embryonic stem cells (ES cells) since these have shown the generation of dopamine neurons *in vitro* [27, 28] and the attenuation of rotational bias when transplanted into 6-OHDA rat model of PD [29].

Assessing the potential of mouse-derived ES cells to develop into functional dopamine neurons via transplantation into the 6-OHDA rat model of PD is, however, far from ideal since rats receiving the xenografts require immunosuppression to avoid graft rejection. This is normally achieved by the administration of the drug Cyclosporin A (CsA), which suppresses the immune response by inhibiting T-cell activation [30]. While CsA promotes xenograft survival [31], its use is problematic because not only is prevention from cell rejection not absolute [32] but also, CsA treatment is as-

sociated with toxic side effects such as hepatotoxicity or even death [33]. Importantly, continued injections of CsA into unilateral 6-OHDA-lesioned rodents have also been shown to elevate striatal dopamine levels, perhaps by having neuroprotective effects or by promoting regeneration nigro-striatal dopamine neurons thus interfering with accurate measures of transplant function [34]. These problems of treatment with CsA could be avoided if the species of the donor and recipient are the same.
The development of a reliable 6-OHDA mouse model therefore allows the therapeutic potential of mouse embryonic stem cells, and other mouse-derived cell lines to be efficiently assessed, and is therefore of absolute importance especially with the current advancements in embryonic stem cell research.

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Figure Legends

Fig. 1. Graphs show the rotational behaviour of MFB (a), striatal 6-OHDA lesioned mice (b), and intact (control) mice (c), across different testing intervals following stimulation with d-amphetamine at various doses. There is a significant effect of amphetamine dose on rotational response in both MFB and striatal 6-OHDA lesion groups but not in the control group. Negative values indicate contralateral rotation. Data expressed as mean ± S.E.M.

Fig. 2. Graphs show the rotational behaviour of MFB (a), striatal 6-OHDA lesioned mice (b), and intact (control) mice (c), across different testing intervals following stimulation with apomorphine at various doses. There is a significant effect of apomorphine dose on rotational response in both MFB and striatal 6-OHDA lesion groups but not in the control group. Negative values indicate contralateral rotation. Data expressed as mean ± S.E.M.

Fig. 3. Dose-response curve of d-amphetamine and apomorphine for both MFB and striatal 6-OHDA lesion groups. Graph shows a right shift of the dose-response curve for the striatal 6-OHDA lesion group. Negative values indicate contralateral rotation. Data expressed as mean ± S.E.M; * $p<0.05$.

Fig. 4. Comparison of dopamine loss and rotation for each animal. Graphs show the correlation between dopamine neuron loss in MFB lesioned animals and rotations induced by 5mg/kg d-amphetamine (a) and 5mg/kg apomorphine (c) challenge; and the correlation between the dopamine neuron loss in striatal lesioned animals and rotations following 10mg/kg d-amphetamine (b) and 10mg/kg apomorphine (d) challenge. Linear regression indicates a stronger correlation between dopamine neuron loss and rotation with the striatal 6-OHDA lesions compared to MFB lesions. Negative values indicate contralateral rotation.

Fig. 5:
TH immunohistochemical staining in brain sections moving rostral-caudal from left to right through the levels of the striatum and the substantia nigra.
(a) The lesion alone group showed partial depletion of TH staining on right hand lesioned side, in both the striatum and the substantia nigra. (b) The control (non-lesioned) group showed normal levels of TH expression in the striatum and substantia nigra on both sides of the brain. (c) The VM Graft can be clearly seen in the striatum.

Higher magnification of graft (in c) shows the presence of TH neurons (d). Scale bar represents 2 mm for panels a-c.

e**)** Amphetamine-induced rotational behaviour following transplantation of embryonic VM cells into the 6-OHDA-lesioned striatum. A significant difference in rotational bias with groups was observed ($p<0.001$), with VM grafted groups showing a reduction in rotation at 4 and 6 weeks post-transplantation when compared to pre-graft rotation scores. Lesion only mice showed stable rotations throughout the 6-week testing period and non-lesioned mice showed no significant rotational bias. ***a* $p<0.01$ vs. pre-graft scores, ***b* $p<0.01$ vs. lesion only group, **b* $p<0.05$ vs. lesion only group. Negative values indicate contralateral rotation. Data expressed as mean ± S.E.M.

Supplemental Figure: TH immunohistochemical staining in brain sections moving rostral-caudal from left to right through the levels of the striatum and the substantia nigra. MFB lesions show clear depletion of TH staining on right hand side, in the striatum, substantia nigra & VTA (A). Striatal lesions show partial depletion of TH staining on right hand side in the striatum and the substantia nigra but not the VTA (B). Scale bar = 1.5mm.

Figure 1.

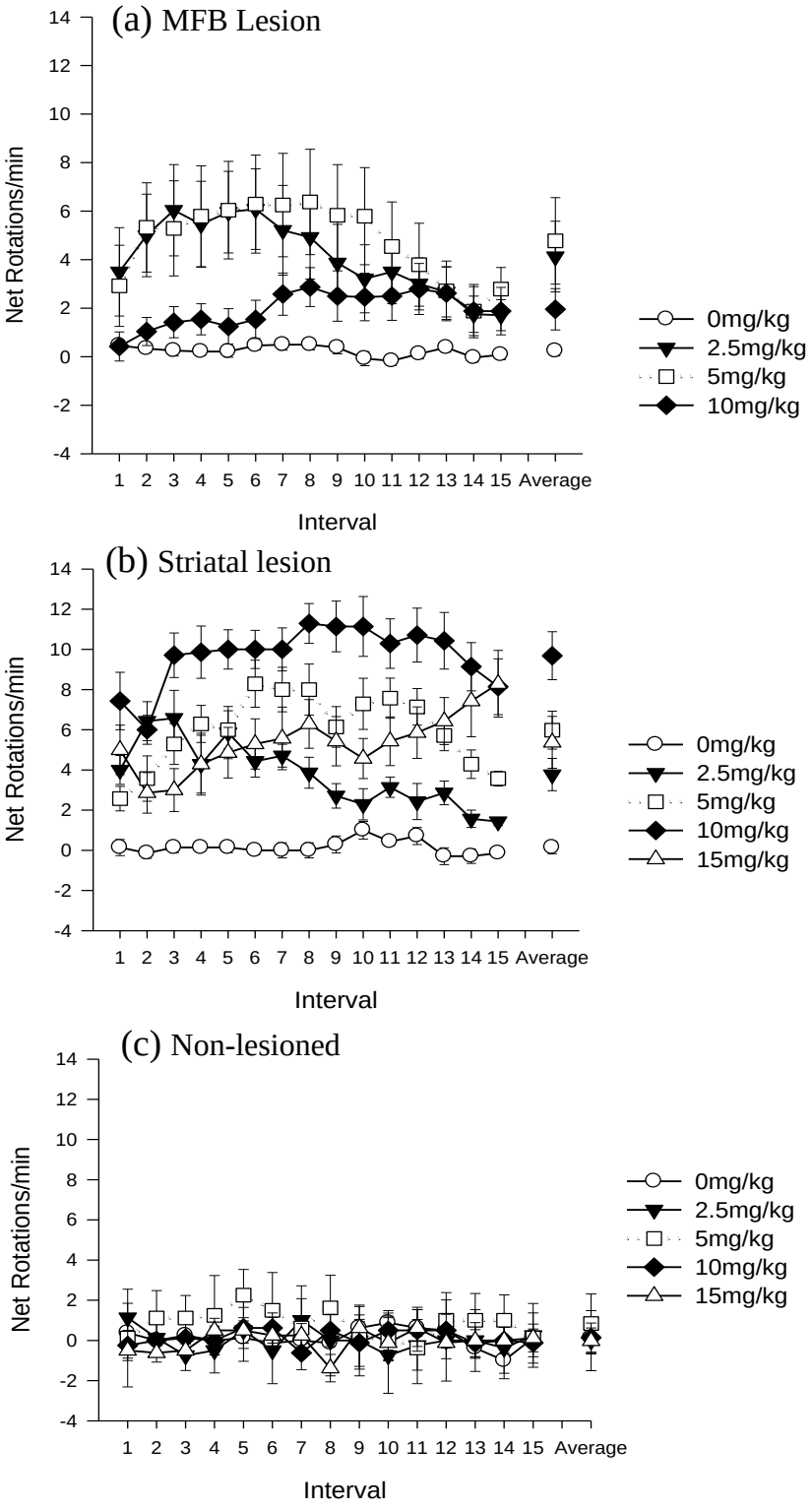
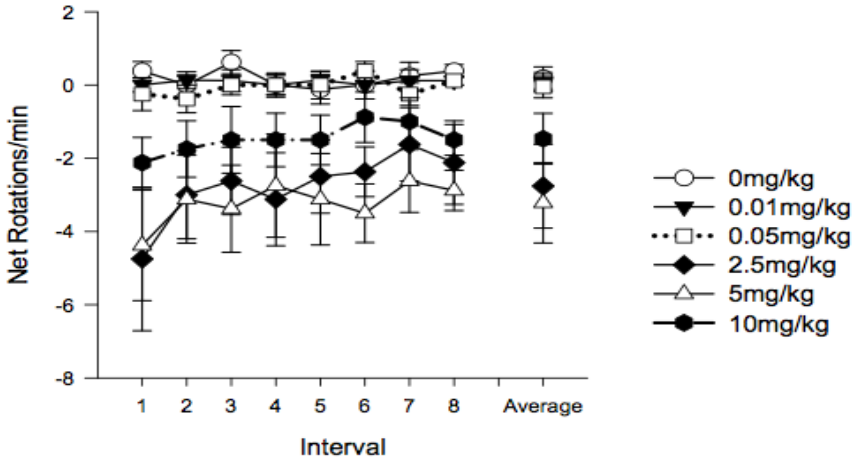
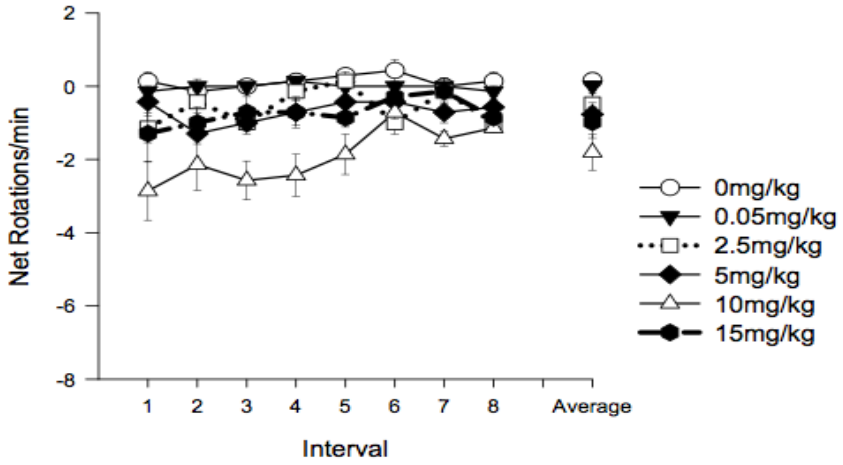

(a) MFB Lesion
Net Rotations/min
Interval
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Average
0mg/kg
2.5mg/kg
5mg/kg
10mg/kg
(b) Striatal lesion
Net Rotations/min
Interval
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Average
0mg/kg
2.5mg/kg
5mg/kg
10mg/kg
15mg/kg
(c) Non-lesioned
Net Rotations/min
Interval
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Average
0mg/kg
2.5mg/kg
5mg/kg
10mg/kg
15mg/kg

Figure 2 revised

(a) MFB Lesion



(b) Striatal lesion



(c) Non-lesioned

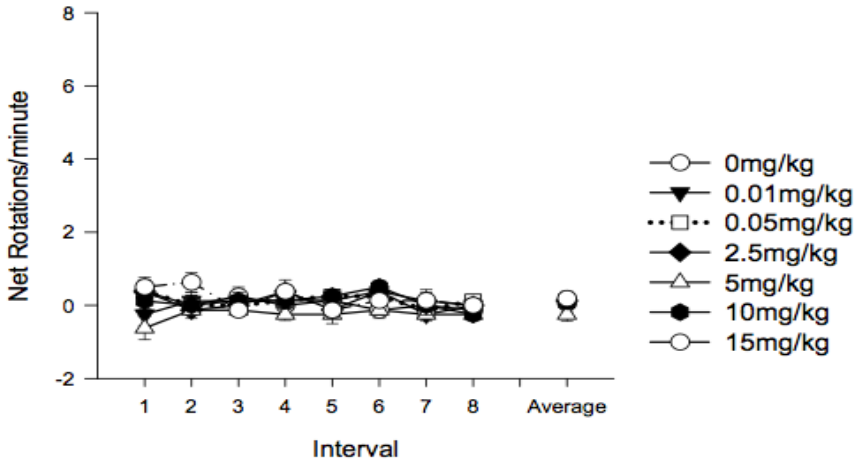


Figure 3

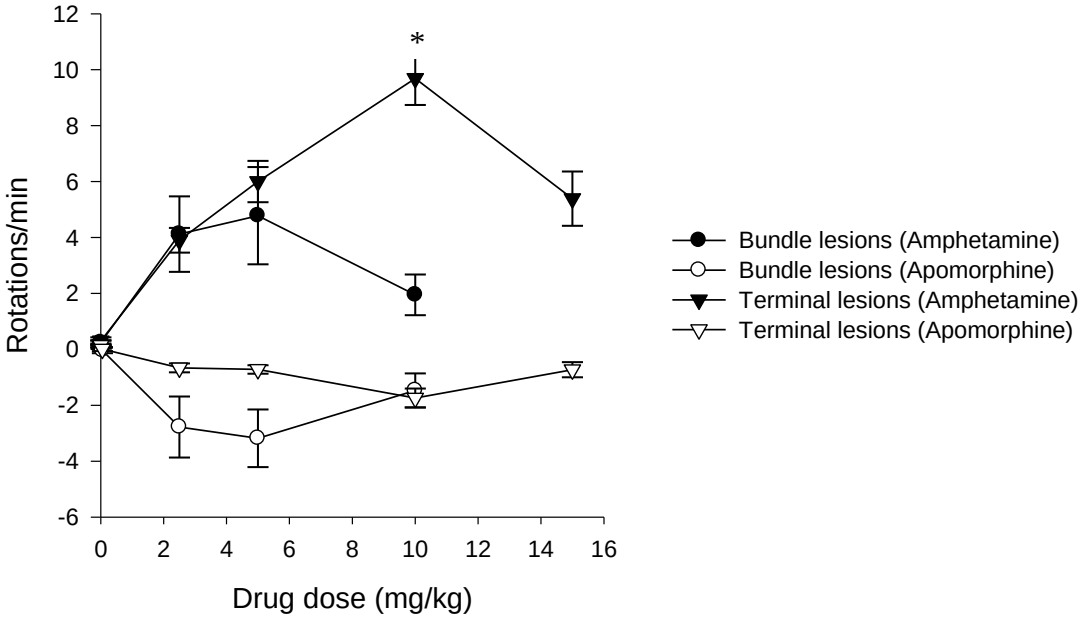

12
10
8
6
4
2
0
-2
-4
-6
Rotations/min
0
2
4
6
8
10
12
14
16
Drug dose (mg/kg)
*
Bundle lesions (Amphetamine)
Bundle lesions (Apomorphine)
Terminal lesions (Amphetamine)
Terminal lesions (Apomorphine)

Figure 4

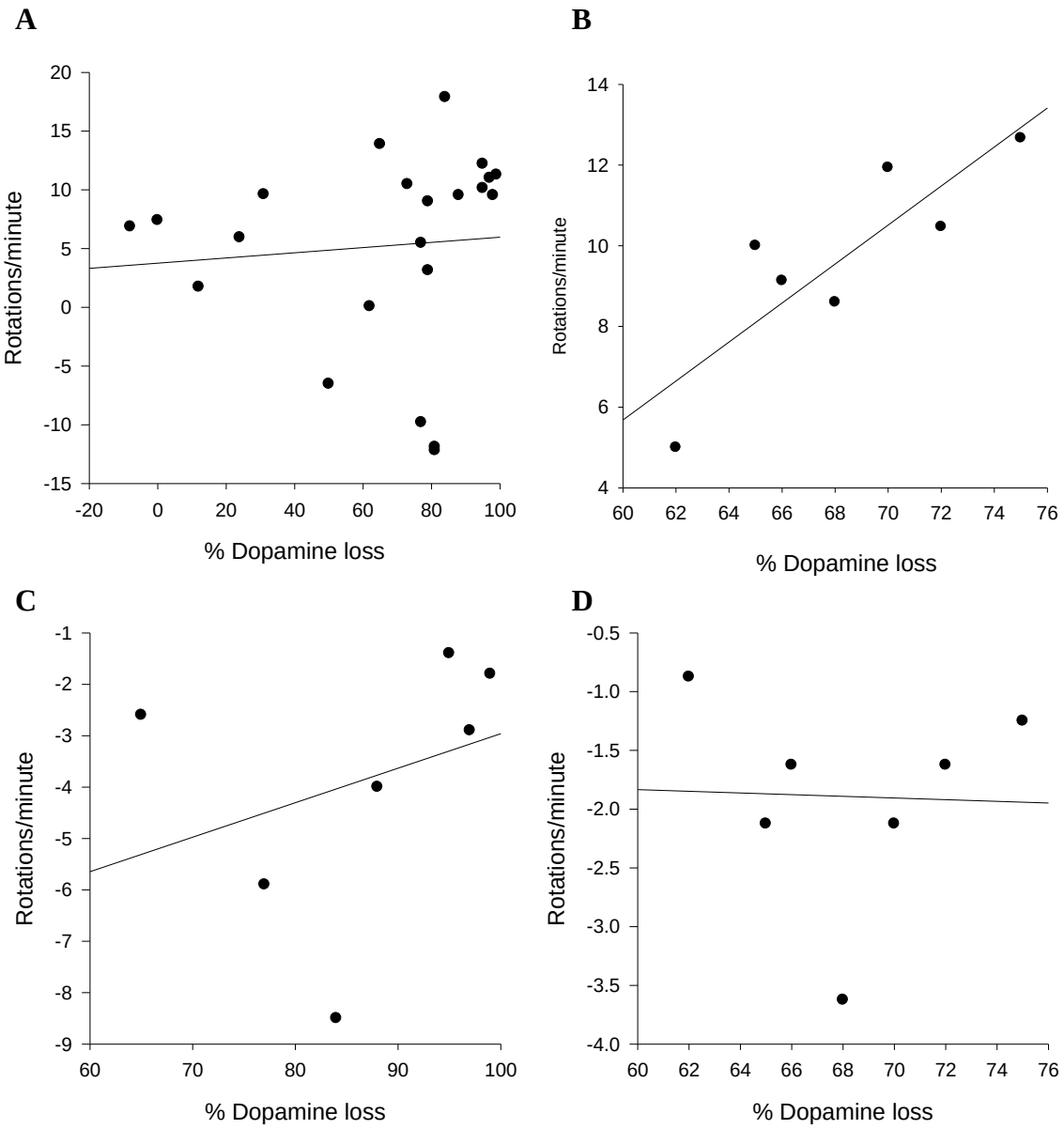
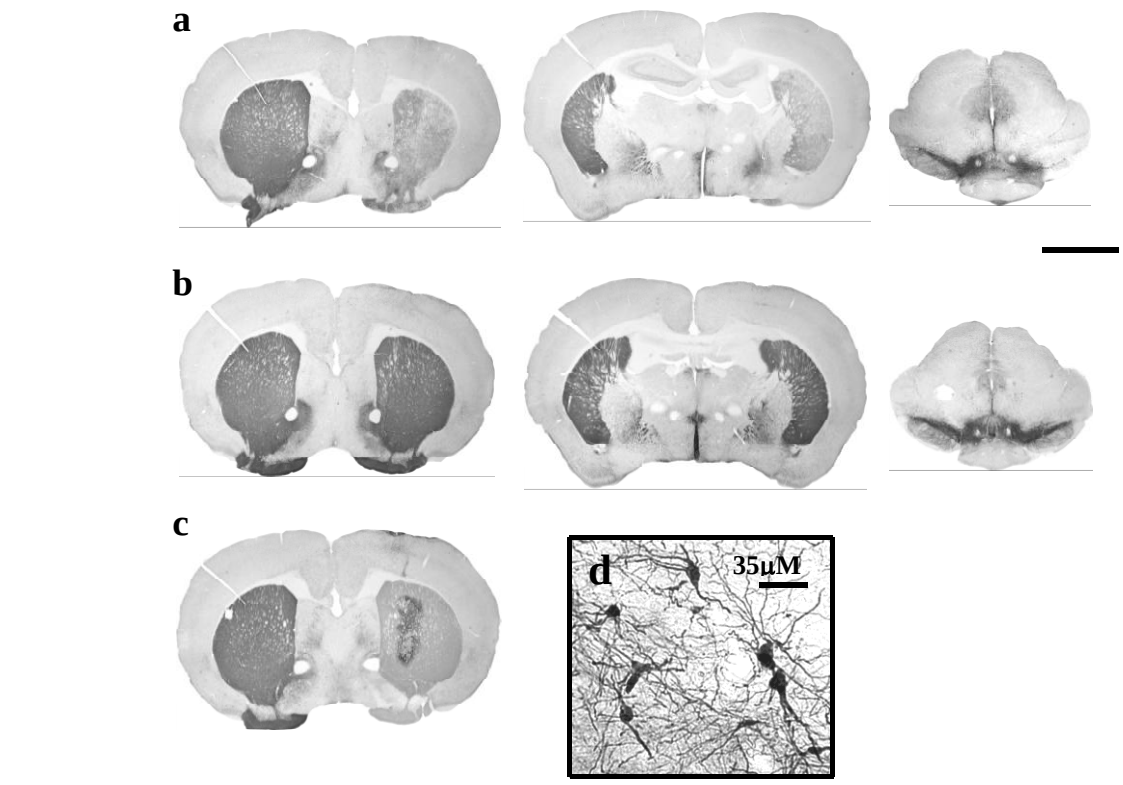
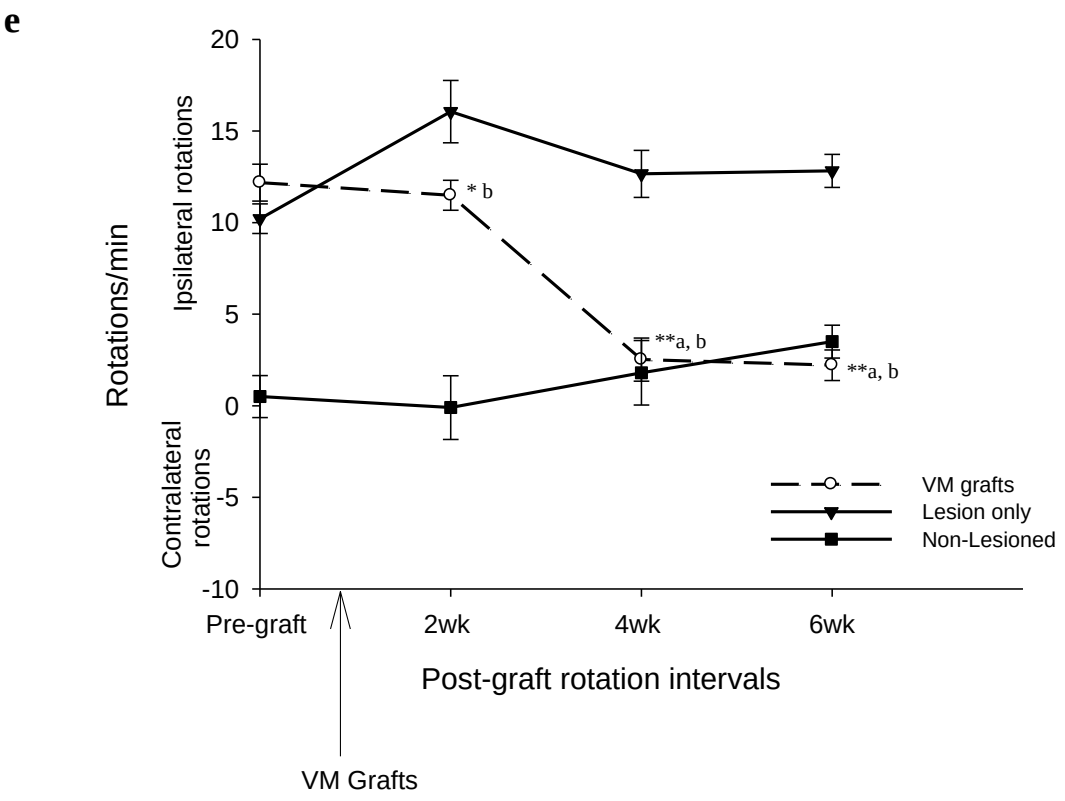

A
Rotations/minute
% Dopamine loss
B
Rotations/minute
% Dopamine loss
C
Rotations/minute
% Dopamine loss
D
Rotations/minute
% Dopamine loss

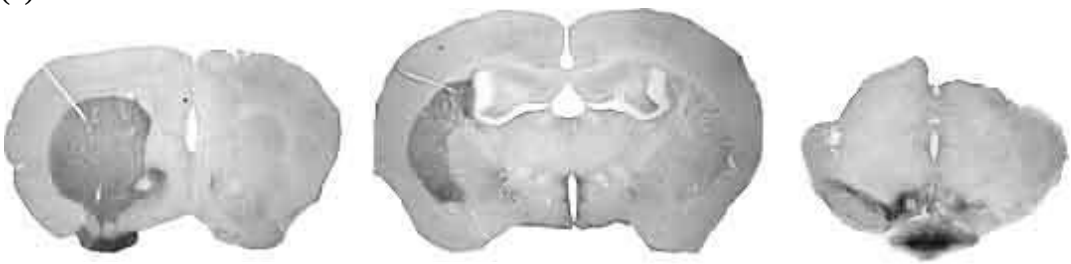
Figure 5


a
b
c
d
35µM


e
Rotations/min
Ipsilateral rotations
Contralateral rotations
20
15
10
5
0
-5
-10
* b
**a, b
**a, b
VM grafts
Lesion only
Non-Lesioned
Pre-graft
2wk
4wk
6wk
Post-graft rotation intervals
VM Grafts

Supplemental Figure 1

(a) Median forebrain bundle lesion



(b) Striatal lesion