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Impact of the lesion procedure on the profiles of motor impairment and molecular responsiveness to L-DOPA in the 6-hydroxydopamine mouse model of Parkinson's disease.

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ABSTRACT

6-hydroxydopamine (6-OHDA) lesions are being used in the mouse for basic research on Parkinson´s disease and L-DOPA-induced dyskinesia. We set out to compare unilateral lesion models produced by intrastriatal or intramesencephalic injections of a fixed 6-OHDA concentration (3.2 µg/µl) in C57BL/6 mice. In the first experiment, toxin injections were performed either at two striatal coordinates (1 or 2 µl per site, termed "striatum$_{2x1µl}$" and "striatum$_{2x2µl}$" models), in the medial forebrain bundle (MFB), or in the substantia nigra pars compacta (SN) (1 µl per site). All the four lesion models produced significant forelimb use asymmetry, but spontaneous turning asymmetry only occurred in the MFB and striatum$_{2x2µl}$ models. After the behavioral studies, the induction of phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK1/2) by acute L-DOPA (30 mg/kg) was used as a marker of post-synaptic supersensitivity. Striatal pERK1/2 expression was sparse in the SN and striatum$_{2x1µl}$ groups, but pronounced in the striatum$_{2x2µl}$ and MFB-lesioned mice. In further experiments, mice with MFB and striatal$_{2x2µl}$ lesions were used to compare behavioral and molecular responses to chronic L-DOPA treatment (12 days at 3 and 6 mg/kg/day). Maximally severe abnormal involuntary movements (AIMs) occurred in all MFB-lesioned mice, whereas only 35% of the mice with striatal lesions developed dyskinesia. Striatal tissue levels of dopamine were significantly lower in the dyskinetic animals (both MFB and striatum$_{2x2µl}$ groups) in comparison with the non-dyskinetic ones. Noradrenaline levels were significantly reduced only in MFB lesioned animals and did not differ among the dyskinetic and non-dyskinetic cases with striatal lesions. In all groups, the L-DOPA-induced AIM scores correlated closely with the number of cells immunoreactive for tyrosine hydroxylase or FosB/ΔFosB in the striatum. In conclusion, among the four lesion procedures examined here, only the MFB and striatum$_{2x2µl}$ models yielded a degree of dopamine denervation sufficient to produce spontaneous postural asymmetry and molecular supersensitivity to L-DOPA. Both lesion models are suitable to reproduce L-DOPA-induced dyskinesia, although only MFB lesions yield a pronounced and widespread expression of post-synaptic supersensitivity markers in the striatum.

Keywords

Motor complications, neurotoxin, 6-hydroxydopamine, rotation, nigrostriatal pathway, monoamine.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) projecting to the striatum, leading to bradykinesia, tremor, rigidity and postural abnormalities (Fahn, 2003). The mechanisms underlying this neuronal degeneration are still unclear and no current treatment can inhibit the progression of the disease. The signs and symptoms of PD are dramatically ameliorated by the DA precursor, L-DOPA. However, the vast majority of PD patients treated with L-DOPA develop dyskinesia (abnormal involuntary movement, AIMs) and motor fluctuations (Ahlskog and Muenter, 2001). L-DOPA-induced dyskinesia is believed to result from fluctuations in central levels of DA, causing aberrant plasticity in dopaminoceptive brain structures, among which the striatum plays a crucial role (reviewed in Bezard et al., 2001; Carta et al., 2008; Cenci and Konradi, 2010; Chase, 1998). Animal models of PD and L-DOPA-induced dyskinesia are essential to investigate pathophysiological mechanisms and identify new potential therapies. Parkinsonian-like motor features and L-DOPA-induced dyskinesia can be reproduced in mammalian species sustaining neurotoxic lesions of nigral DA neurons (reviewed in (Cenci et al., 2002; Fox and Brotchie, 2010). Unilateral injections of 6-hydroxydopamine (6-OHDA) in the nigrostriatal pathway have been widely used and extensively characterized in rats (reviewed in (Blandini et al., 2008; Cenci et al., 2002; Schwarting and Huston, 1996). In the past few years, this lesion procedure has been increasingly used also in mice to obtain stable and pronounced striatal DA denervation, which is required for the animals to exhibit dyskinesia on L-DOPA treatment (Cenci et al., 2002; Darmopil et al., 2008; Lundblad et al., 2004; Lundblad et al., 2005; Santini et al., 2007). The first description of 6-OHDA-lesioned mice exhibiting L-DOPA-responsive motor deficits and developing AIMs with chronic L-DOPA treatment was provided by Lundblad et al. (Lundblad et al., 2004). In this study, unilateral DA-denervating lesions were performed by injecting 6-OHDA either in the striatum or in the MFB, and animals were sacrificed at 7-8 weeks post lesion following treatment with escalating doses of L-DOPA. Both striatal and MFB lesions were found to produce a significant deficit in spontaneous forelimb use, and to confer susceptibility to dyskinesia, although the sensitivity to L-DOPA differed between the two models, and striatally lesioned mice required a 4.5 fold larger dose to reach a severity of AIMs comparable to MFB-

lesioned animals (Lundblad et al., 2004). Because MFB lesions were reported to entail a post-operatve mortality rate of 82% (Lundblad et al., 2004), most of the subsequent studies using mice opted for the intrastriatal 6-OHDA lesion procedure (Kachroo et al., 2005; Lundblad et al., 2005; Pavon et al., 2006; Santini et al., 2007). More recently, the injection of 6-OHDA in the SNpc was proposed as a good model of partial nigrostriatal DA denervation, with negligible post-operative mortality and stable motor deficits (Grealish et al., 2010).

The increasing interest in the use of mice with 6-OHDA lesions, the description of alternative procedures of toxin injection, and our recent refinement of animal nursing protocols, which have greatly reduced the post-operative mortality following MFB lesions (Cenci and Lundblad, 2007) have prompted us to carry out a new and more extensive comparison between different 6-OHDA lesion models in the mouse. The purpose of this study was to provide a complete set of data on the behavioral and histomolecular consequences of intrastriatal and intramesencephalic 6-OHDA injections, which would inform the selection of experimental models for future applications. In a first experiment, toxin injections were performed in the striatum at two different doses, in the MFB, or in the SN. The two models yielding the most reliable outcome were then selected for a second experiment aimed at characterizing the behavioral and molecular effects of repeated L-DOPA exposure.

MATERIALS and METHODS

Subjects

The study was performed in C57BL/6 mice (Charles River Laboratories, Germany) aged approx. 8 weeks when purchased. Mice were housed under a 12-h light/dark cycle with free access to food and water. Housing conditions and experimental treatments had been approved by the Malmö-Lund Ethical Committee on Animal Research. A total of 57, 53 and 41 mice were used in experiments 1, 2 and 3, respectively (see Table 1).

Treatment groups and experimental design

The study consists of three different experiments. Experiment 1 aimed at comparing

four 6-OHDA lesion procedures, namely, a two-site striatal lesion with two different toxin volumes (termed "striatum$_{2x1\mu l}$", "striatum$_{2x2\mu l}$"), versus a lesion in the medial forebrain bundle (MFB) or in the substantia nigra (SN). Behavioral tests were carried out at 3 and 12 weeks post-lesion to evaluate spontaneous turning behavior, horizontal activity, and forelimb use asymmetry. At the end of the behavioral studies mice received an acute challenge injection of L-DOPA (30 mg/kg i.p. complemented with 12 mg/kg benserazide) or saline, and were perfusion-fixed 20 min later for phospho-ERK1/2 (pERK1/2) and tyrosine hydroxylase (TH) immunohistochemistry.

Experiment 2 compared the behavioral and molecular effects of chronic L-DOPA treatment (12 days of single daily injections, ascending doses from 3 to 6 mg/kg/day methyl L-DOPA combined with 12 mg/kg/day benserazide).

In experiment 3, the same experimental groups were prepared for a biochemical determination of dopamine (DA), serotonin (5-HT) and noradrenaline (NA) levels in striatal tissue.

The study design is summarized in Fig. 1 and the total number of animals used in the different experimental phases of experiments 1, 2 and 3 is shown in Table 1.

6-OHDA lesion and post-operative care

Mice were anesthetized with a mixture 4% Isofluorane in air (Isoba®vet., Apoteksbolaget, Sweden) (400 ml/min for 5 min), placed in a stereotaxic frame with a mouse-adaptor (Kopf Instruments, Tujunga, USA), and kept anesthetized using 2% Isofluorane (200 ml/min). 6-OHDA hydrochloride (Sigma Aldrich AB, Sweden) was dissolved at a fixed concentration of 3.2 µg/µl free-base in 0.02% ice-cold ascorbate/saline and used within 2 h. Immediately after surgery, the analgesic Marcain (Bupivacain, 2.5 mg/ml, AstraZeneca, Sweden) was injected subcutaneously (s.c; 10 µl/10 g body weight) in 3-4 sites around the wound.

Injections of 6-OHDA were made at the following coordinates (in mm relative to bregma, sagittal suture and dural surface, cf. (Paxinos and Franklin, 2001): *(a) striatum$_{2x1\mu l}$ lesion*, 2 injections of 1 µl each at (i) AP = + 1.0, L = - 2.1, DV = - 2.9; and (ii) AP = + 0.3, L = - 2.3, DV = - 2.9; (*b) striatum$_{2x2\mu l}$ lesion*, 2 injections of 2 µl each of the same coordinates as above; *(c) MFB lesion*, 1 injection of 1 µl at AP = - 1.2, L = - 1.3, DV = -4.75; *(d) SN lesion*, 1 injection of 1 µl at AP= - 3.6, L= - 1.1,

DV= - 3.75; *(e) sham lesion* was carried out by 1 µl injection of 0.02% ascorbic acid-saline at the two striatal coordinates.

To minimize unspecific tissue damage, the injections were performed at a rate of 0.5 µl/min using a glass capillary with an outer tip diameter of 50 µm attached to a 10-µl Hamilton syringe. The capillary was left in place for 2 min before and 2 min after the injection.

In experiment 1, MFB and striatum$_{2x2µl}$ lesions resulted in 20% mortality during the first 2 post-operative weeks, while the milder lesions (striatum$_{2x1µl}$ and SN) caused less than 10% mortality. In experiments 2 and 3 no animal died thanks to the improved nursing protocol that is described below.

To prevent dehydration mice received sterile glucose-saline solution (50 mg/ml, Baxter Medical AB, Sweden, 0.1 ml/10 g body weight, s.c) immediately after surgery and once a day during the first post-operative week. Where necessary, this treatment was given on alternate days for up to 3 weeks post lesion. In addition, for 2 weeks post-surgery, food pellets soaked in 15% sugar/water solution were placed in a shallow vessel on the floor of the cages twice a day (in the morning and in the evening). Mice that showed difficulties in eating due to severe postural asymmetry were hand-fed (i.e. they were presented with the food while being held by the hands of the investigator). In order to avoid competition for the food, weaker mice were placed in cages other than those containing unimpaired mice. After implementing this protocol (experiments 2 and 3), the post-operative survival rate was 100% in all the groups.

L-DOPA treatment

L-DOPA methyl ester and the peripheral DOPA decarboxylase inhibitor benserazide-hydrochloride (Sigma Aldrich AB, Sweden) were dissolved in physiological saline immediately prior to use. The drugs were injected at the volume of 0.1 ml/10 g body weight in a single intraperitoneal (i.p) injection per day. Throughout the study, each L-DOPA dose was combined with a fixed dose of 12 mg/kg benserazide. In experiment 1, animals received an acute injection of 30 mg/kg L-DOPA, a dose above the threshold for induction of severe dyskinetic behaviors also in mice with intrastriatal 6-OHDA lesions (Lundblad et al., 2004; Lundblad et al., 2005). In

experiments 2 and 3, chronic daily treatment with L-DOPA started 21 days post-lesion and continued for 12 days. The L-DOPA dosage was 3 mg/kg/day for the first 4 days and 6 mg/kg/day for the last 8 days of treatment.

Open field test

In experiment 1, the open field test was performed in a cardboard box (64 x 64 cm), where the floor was divided into squares (8 x 8 cm) by a grid of black lines (Lundblad et al., 2004). The mouse was placed in the center of the box and videotaped for 10 min (corresponding to the period of maximal activity in the open field). The number of 180° turns (scored as one turn) and 360° turns (scored as two turns) ipsilateral and contralateral to the lesion was counted manually, then summed and expressed as absolute number of ipsilateral and contralateral turns. The number of line crossings during the monitoring period was also counted.

In experiments 2 and 3, the open field activity was monitored using a video tracking system with customized software (*Viewer*^{2}, Biobserve GmbH, Bonn, Germany) which can detect and track the position of the animal's head, body and tail and provides measures of both rotational and horizontal activity. Turning movements were detected based on the rotation of the mouse body axis and expressed as number of 360° turns.

Horizontal activity was quantified by a parameter called "rated zone crossing" (RZC), which rates the total number of line crossing events with an algorithm that emphasizes linear movements and discards oscillations at each zone's margin.

Cylinder test

To examine side bias in spontaneous forelimb use, mice were placed individually inside a glass cylinder (10 cm diameter, 14 cm height), which was located in front of two vertical mirrors in order to be able to view the mice from all angles. Mice were immediately videorecorded for 3 min using a digital video camera (Sony Handcam, DCR-HC90E PAL) (Lundblad et al., 2002). No habituation of the animals to the testing cylinder was allowed before videorecording them. Videorecordings were then examined to count the number of supporting wall touches (contacts with fully extended digits) executed independently with the forelimb ipsilateral and contralateral to the lesion. All the animals performed a minimum of 9 supporting wall contacts per

testing session. A measure of forelimb use asymmetry was obtained by expressing the touches performed by the paw contralateral to the lesion (left paw) as a percentage of the total number of touches in each session.

Behavioral testing during chronic L-DOPA treatment

In experiments 2 and 3, all mice (both saline and L-DOPA-treated) underwent 3 types of behavioral tests during the L-DOPA treatment period (i.e. open field, cylinder test and rating of AIMs). Open field recordings and AIMs ratings were performed alternately every other day for a total of 5 and 6 sessions, respectively. The cylinder test on L-DOPA was performed only once (day 9, L-DOPA dose 6 mg/kg) in order to avoid habituation of the animals to the test. Open field recordings were carried out using a video tracking system and *Viewer*^{2} software. The animals were placed in glass bowls (9.5 cm diameter at their base) and videotaped for 20 min starting 20 min after L-DOPA injection, in order to cover the period of maximal drug effect. Horizontal activity was estimated by the RZC parameter.

To avoid motor interferences due to dyskinesias (Lundblad et al., 2002; Picconi et al., 2003), animals were put in the cylinder during the rapidly declining phase of the AIMs curve, corresponding to 120 and 100 min post-injection for MFB- and striatal lesioned mice, respectively.

AIMs rating

Abnormal involuntary movements (AIMs) were rated as previously described (Lundblad et al., 2004). Each mouse was observed individually for 1 min every 20 min for 3 h, starting 20 min after L-DOPA/benserazide (or saline) administration. Only hyperkinetic and dystonic movements that could be clearly distinguished from naturally occurring behaviors (i.e. grooming, sniffing, rearing and gnawing) were considered in the ratings. The AIMs were classified into three different subtypes based on their topographic distribution: (i) axial AIMs, i.e. twisting of the neck and upper body towards the side contralateral to the lesion; (ii) orolingual AIMs, i.e. jaw movements and contralateral tongue protrusion; (iii) forelimb AIMs, i.e. purposeless movements of the contralateral forelimb, sometimes combined with grabbing movement of the paw. Examples of the three subtypes of dyskinetic behavior are

shown in Supplemental material, movies 1-3. Each AIM subtype was scored on a severity scale from 0 to 4: 0, absent; 1, present during less than half of the observation time; 2, present during more than half of the observation time; 3, present all the time but suppressible by sensory stimuli; 4, continuous, severe and not suppressible. Each mouse could thus reach a theoretical maximum score of 108 in one session (maximum score per monitoring period = 12; number of monitoring periods per session = 9). The mice were evaluated on this test a total of 6 times during the chronic L-DOPA treatment period.

Immunohistochemistry

Mice were killed by transcardial perfusion 20 min (experiment 1) or 24 h (experiment 2) after the last L-DOPA injection. Animals were deeply anesthetized with sodium pentobarbital (240 mg/kg, 10 ml/kg of body weight, i.p; Apoteksbolaget, AB, Sweden) and perfusion-fixed with 0,9% saline, followed by 4% ice-cold, buffered (pH 7.4) paraformaldehyde (PFA) (Merck via VWR, Stockholm, Sweden) (dissolved in 0.1 M phosphate buffer, PB, pH 7.4). The fixative solution was delivered by a peristaltic pump at the speed of 20 ml/min during 5 min. Brains were rapidly extracted and post-fixed in the same fixative solution for 2 h, and then cryoprotected in ice-cold 25% phosphate-buffered sucrose (in 0.1 M PB) over night. Coronal sections of 30 µm thickness were cut on a freezing microtome and stored in a non-freezing solution (30% ethylene glycol and 30% glycerol in 0.1 M PB) at -20°C until being used for the immunohistochemical staining.

Bright-field immunohistochemistry for TH and FosB/ΔFosB-related proteins was performed according to our standard protocols. Because the FosB primary antibody recognizes both full-length FosB and ΔFosB-related proteins, the immunostaining obtained with this antibody will be referred to as FosB/ΔFosB positivity. Sections were rinsed three times in 0.02 M potassium phosphate buffered saline (KPBS) pH 7.4 and pretreated with 3% hydrogen peroxide (H_2O_2) in 10% methanol/water to quench endogenous peroxidase activity (this step was omitted for FosB/ΔFosB). Sections were then preicubated for 1h in blocking buffer, consisting of 5% normal serum in KPBS containing 0.25% Triton-X (KPBS/T). This was followed by overnight incubation at 4 °C with one of the following primary antibodies: rabbit

anti-tyrosine hydroxylase (TH) (1:1000; Pel-Freez, Rogers, AR), mouse anti-FosB/ΔFosB (1:15000, preadsorbed on normal mouse brain tissue for 6 h; Santa Cruz Inc, USA). After incubation with the primary antibody, sections were rinsed and incubated with the biotinylated goat anti-rabbit (BA1000) or horse anti-mouse (BA2001) secondary antibodies (1:200; Vector Laboratories, Burlingame, CA). This was followed by incubation in an avidin-biotin-peroxidase solution (Vectastain Elite ABC; Vector Laboratories) for 1 h at room temperature. The immunocomplexes were visualized using 3,3-diaminobenzidine (DAB) and H_2O_2 (both from Sigma-Aldrich). Sections were then rinsed in KPBS/T, mounted onto chromalum-coated slides, and coverslipped using DPX mounting medium (Sigma-Aldrich).

For pERK1/2 immunohistochemistry, the sections were kept on ice through all the steps preceding the incubation with secondary antibodies, as in Westin et al. (Westin et al., 2007). After a quenching step in 3% H_2O_2 and 10% methanol sections were preincubated for 1 h with 5% normal goat serum (NGS) in Tris-HCl buffered saline (TBS)/0.1% Triton. The primary antibody recognized ERK1/2 when phosphorylated on the threonine 202 and tyrosine 204 residues (polyclonal rabbit anti phospho-p44/42 MAP-Kinase; Cell Signaling Technology, Inc., USA; 1:200). Incubation with this antibody was carried out overnight at 4°C. On the following day the sections were incubated for 2 h with a goat anti-rabbit antibody (Vector Labs, Burlingame, USA, dilution 1:200). Detection of the bound antibodies was carried out using a standard peroxidase-based method (ABC-kit, Vectastain, Vector Labs, USA; 2 h incubation), and diaminobenzidine (DAB) as a chromogen.

Image analysis and expression of the data

Densitometric measurements of TH immunostaining and counts of immunoreactive cells on regions of interest (experiments 1 and 2) were performed using the freeware, *NIH Image J 1.43* (National Institute of Health, Bethesda, MD, 2007; downloadable from http://rsbweb.nih.gov/). Sample areas were digitized through a videocamera (Nikon DMX 1200F) connected to a Nikon Eclipse 80i microscope using a 10x objective. In experiment 1, counts of pERK1/2-immunoreactive cells and measurements of TH optical density (O.D.) were carried out separately in the medial and lateral parts of the striatum in two sections per animal, corresponding to the mid-

caudal level of the caudate-putamen (bregma +0.74 to +0.14). Data were expressed as number of pERK1/2-positive cells per mm^2 or percentage of residual TH O.D. on the lesion side relative to the contralateral intact side in each section.

In experiment 2, TH O.D. was analyzed in the striatum, SN and VTA using the same software (see Supplement 1 for a definition of rostrocaudal levels and sample areas). The same four rostrocaudal levels through the striatum used for TH O.D. analysis (I, bregma +1.34/+1.18; II, bregma +0.74/+0.50; III, bregma +0.14; IV, bregma -0.34/-0.50) (Paxinos and Franklin, 2001) were used to count the number of TH- and FosB/ΔFosB-positive cells with *NIH Image J* software. FosB/ΔFosB cell counts were carried out separately in the medial and lateral parts of the striatum, whereas the number of TH-positive cells was determined in the whole striatum. Images were acquired using a 4x objective and digitized through the Nikon DMX 1200F videocamera. Data were expressed as number of cells/mm^2.

Stereological cell counts

In experiment 2, the number of TH-positive cell bodies in the SNpc and VTA was determined by unbiased stereology according to the optical fractionator method (West, 1999). Following TH-immunostaining and mounting, sections through the midbrain were counterstained with Cresyl Violet (Nissl staining). Stereological analysis was carried out on 18 animals treated with L-DOPA: 5 MFB-lesioned dyskinetic mice (MFB-LDdys), 5 striatally lesioned animals that developed dyskinesia (ST-LDdys), 8 non-dyskinetic mice with striatal lesions (ST-LDnd), and 5 sham-lesioned mice (sham). Eight 30 µm thick coronal sections throughout the rostrocaudal extent of SNpc and seven throughout the VTA were sampled from each animal. The SNpc was delineated from -2.7 to -3.88 mm posterior to bregma and the VTA from -2.92 to -3.88 mm posterior to bregma, according with the mouse atlas (Paxinos and Franklin, 2001). Analysis was performed using a Nikon 80i microscope with an x-y motor stage controlled by the NewCAST software (Visiopharm, Hoersholm, Denmark, 2008). The SNpc (excluding the SN pars lateralis) and VTA were delineated at low magnification (4x objective). Cell counting was performed using a 100x oil immersion objective with a numerical aperture of 1.4. The border between SNpc (A9 cell group) and VTA (A10 cell group) was defined using a 4x

objective by drawing a vertical line passing through the medial tip of the cerebral peduncle and the medial terminal nucleus of the accessory optic tract. The counting frame area was 1066.2 μm^2 and guard volumes of 5 µm from the top and from the bottom of the section were excluded from both the surfaces. The sampling fraction was varied to generate at least 100 sampled neurons in each structure analyzed per animal and side. The criterion for counting a TH-positive neuron was the presence of its nucleus in the focal plane either within the counting frame or touching the right or top frame lines, but not touching the left or bottom lines. The total number of TH-labeled cells in the SNpc and VTA was estimated according to the optical fractionator formula (West, 1999).

Biochemical analysis

In experiment 3 a total of 13 MFB-lesioned mice (n=7 L-DOPA, n=6 saline), 17 mice with striatum$_{2x2µl}$ lesions (n=4 L-DOPA-dyskinetic, n=7 L-DOPA-non-dyskinetic and n=6 saline) and 11 sham-lesioned controls were killed 24 h after the last injection of L-DOPA or saline and used for biochemical measurements of dopamine (DA), 5-hydroxytryptamine (5-HT) and noradrenaline (NA) tissue levels in the striatum. After decapitation, the brains were rapidly extracted, frozen at -80ºC and mounted onto a cryostat chuck. Tissue punches (2 mm diameter) were taken from the dorsolateral striatum and part of the medial region on both hemispheres, spanning rostrocaudal levels +1.18 to -0.34 mm relative to bregma. The samples were kept frozen at -80º C until analysis. After ultrasound homogenization (Sonifier Cell Disruptor B30; Branson Sonic Power Co.) in 0.1 M perchloric acid, 2.5 mM of Na_2EDTA and subsequent centrifugation, the supernatant was used for high pressure liquid chromatography (HPLC) followed by electrochemical detection as described in (Lindgren et al., 2010).

Statistical analysis

Data obtained from a single time point (behavioral data in experiment 1 and all histomolecular and biochemical data) were compared using one-factor analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls test. AIMs scores and other behavioral data recorded on multiple testing sessions during the chronic L-DOPA

treatment period were compared using repeated measures ANOVA and Tukey´s honestly significant difference (HSD) test. Relations between variables (AIMs, FosB/ΔFosB, TH cell counts, DA and NA striatal contents) were examined by linear regression (log-transformed values were used for DA and NA tissue concentrations). The alpha level of statistical significance was set at $p < 0.05$. All data are expressed as group means ± standard error of the mean (SEM).

Results

Comparison of motor deficits in four lesion models

In the first experiment, mice sustaining four types of lesion (MFB, striatum$_{2x2\mu l}$, striatum$_{2x1\mu l}$, SN) and sham-lesioned controls were evaluated in an open field test of rotational and horizontal activity, and in the cylinder test of forelimb use asymmetry at 3 and 12 weeks post-surgery. For the analysis of rotational behavior, only turns in the direction ipsilateral to the lesion were considered because contralateral turns were very few (below 0.5 turn/min in all cases). Significant group differences in ipsilateral rotation were found at both 3 weeks (Fig. 2A; $F_{(4,52)} = 6.04$, $p < 0.001$) and 12 weeks (Fig. 2A', $F_{(4, 51)} = 4.85$, $p = 0.002$). On the 3-weeks test, both MFB- and striatum$_{2x2\mu l}$– lesioned mice showed a significant rotational asymmetry, whereas the striatum$_{2x1\mu l}$ and SN groups did not differ from sham-lesion controls. The MFB-lesioned mice, but not the striatum$_{2x2\mu l}$ ones, displayed a significant rotational asymmetry also at 12 weeks post-lesion (Fig. 2A'). The number of line crossings in the open field revealed significant group differences at 3 weeks (Fig 2B; $F_{(4,52)} = 9.07$, $p < 0.001$) but not 12 weeks post-lesion (Fig. 2B', $F_{(4,51)} = 1.63$, $p = 0.179$). On the 3-weeks test, the number of line crossings was significantly reduced below sham-lesion values in all the 6-OHDA models but the SN one, and the reduction was greatest in mice with striatum$_{2x2\mu l}$, and MFB lesions (-45% and -56% vs sham, respectively). The lack of significant group differences at 12 weeks post-surgery depended on the low levels of horizontal activity displayed by the sham-lesioned controls (in fact, the number of line crossings was similar to the 3-weeks values in all the other groups, Fig. 2B'). In the cylinder test, highly significant group differences occurred at both 3 weeks (Fig. 2C, $F_{(4, 52)} = 6.75$, $p < 0.001$) and 12 weeks (Fig. 2C'; $F_{(4,51)} = 4,05$, $p = 0.006$). On the 3 weeks test,

striatum$_{2x1\mu l}$, striatum$_{2x2\mu l}$, and MFB groups showed a similar degree of forelimb use asymmetry, performing approximately 30% wall contacts with the paw contralateral to the lesion ($p < 0.05$ vs sham-lesion controls in each group). In contrast, SN-lesioned mice did not differ significantly from controls (42% contralateral paw use). At 12 weeks post-surgery, all the four lesion types differed significantly from sham-lesion controls (< 40% contralateral paw use; Fig. 2C').

Post-synaptic supersensitivity to L-DOPA in the four lesion models

Following the behavioral studies, animals were given one acute injection of a relatively high dose of L-DOPA (30 mg/kg) and transcardially perfused 20 min later for an immunohistochemical analysis of active ERK1/2 (pERK1/2) in the striatum. Cells immunoreactive for pERK1/2 were counted in two regions, a lateral one showing the largest extent of TH loss in all groups (Fig. 3A; Fig. 4 A-E) and a medial one showing a significant (≥ 60%) residual TH-innervation in the striatal and SN lesion models (Fig. 3C). In the medial striatal region, only MFB-lesioned mice showed a significant upregulation of pERK1/2 (Fig. 3D, $F_{(4,25)} = 67.96$, $p < 0.001$; $p < 0.05$ for MFB vs all the groups in the post-hoc comparisons). In the same region, the levels of TH immunoreactivity were greatly reduced in MFB-lesioned animals compared to all the other groups (Fig. 3C). In the lateral region, all lesion models but the SN one showed a significant upregulation of pERK1/2, although marked group differences occurred ($F_{(4, 25)} = 27.67$, $p < 0.001$; Fig. 3B, Fig. 4 F-J). The largest number of pERK1/2 positive cells was found in the MFB mice (Fig. 4I, $p < 0.05$ vs all the other groups), followed by the striatum$_{2x2\mu l}$ group (Fig. 4H, $p < 0.05$ vs striatum$_{2x1\mu l}$, SN and sham lesions). Interestingly, despite clear differences in pERK1/2 immunoreactivity, the amount of residual TH innervation in this lateral region did not differ significantly between MFB, striatum$_{2x2\mu l}$, and striatum$_{2x1\mu l}$ groups (Fig. 3A; $p < 0.05$ for each of these groups vs sham lesions and SN group).

Taken together, the behavioral and immunohistochemical data from experiment 1 showed that the striatum$_{2x2\mu l}$ and MFB lesion models had produced comparable levels of short-term motor impairment and a robust post-synaptic supersensitivity to L-DOPA in the lateral striatum. These two lesion models were therefore selected to examine the effects of chronic L-DOPA treatment.

L-DOPA-induced abnormal involuntary movements

In experiment 2, mice with striatum$_{2x2\mu l}$ and MFB lesions received a chronic course of L-DOPA treatment (3 and 6 mg/kg/day L-DOPA for 4 and 8 consecutive days, respectively). A comparison of the axial, limb and orolingual AIM scores between groups and testing sessions showed significant overall differences (Fig. 5A; repeated measures ANOVA, group effect: $F_{(3,130)} = 89.01$, $p < 0.001$; time effect: $F_{(5,130)} = 34.75$, $p < 0.001$; interaction: $F_{(15,130)} = 19.64$, $p < 0.001$). While all the MFB-lesioned animals developed marked dyskinesia, mice with striatal lesions clustered in two groups, one of which showed very low AIM scores (severity grade 0-1) and did not differ significantly from saline-injected 6-OHDA-lesioned mice and sham-lesioned control animals. This group (consisting of about 60% of all the striatal lesioned mice treated with L-DOPA in both experiment 2 and 3) will be named ST-LDnd (Striatum-LDOPA-non-dyskinetic) in all the following descriptions. The other group of striatally lesioned mice displayed consistent dyskinesia from treatment day 5 onwards (ST-LDdys, Fig. 5A). However, AIM scores in these animals were significantly lower than those recorded from MFB lesioned mice. Indeed, while the ST-LDdys group reached AIM severity grade 2 (indicating alternation between dyskinetic movements and normal activity), mice with MFB lesions exhibited continuous dyskinetic behaviors, reaching the highest severity grade (3-4) on the rodent AIM scale ($p < 0.05$ for MFB-LDdys vs all the other groups). Moreover, the temporal profile of the AIMs post L-DOPA dosing differed between MFB and striatal lesions (Fig. 5B; repeated measures ANOVA: group effect: $F_{(3,156)} = 109.19$, $p < 0.001$; time effect: $F_{(6,156)} = 37.64$, $p < 0.001$; interaction: $F_{(18,156)} = 15.57$, $p < 0.001$). In both groups, the peak AIM severity occurred between 40 and 60 min post injection, but AIMs had a longer duration in the MFB-LDdys group vs the ST-LDdys one (160 min vs 100 min, respectively; $p < 0.05$).

L-DOPA-induced rotational asymmetry, horizontal activity and cylinder test

In experiment 2, a comparison of L-DOPA-induced rotational counts between groups and testing sessions revealed significant overall differences (Fig. 6A; repeated measures ANOVA group effect: $F_{(5,160)} = 87.30$, $p < 0.001$; time effect: $F_{(4,160)} = 1.35$, $p > 0.05$; interaction: $F_{(20,160)} = 2.39$, $p = 0.001$). All lesioned groups treated with L-

DOPA (tested 20-40 min after drug administration) showed increased rotational activity compared with saline-treated groups. Dyskinetic animals with MFB or striatal lesions (MFB-LDdys and ST-LDdys groups) showed the highest levels of rotational activity ($p < 0.05$ vs ST-LDnd), and did not differ significantly from each other up to the fourth day of treatment (3 mg/kg L-DOPA dose). From day 6 to 10, when the L-DOPA dose was increased to 6 mg/kg, the rotational counts gradually increased in MFB-LDdys mice while remaining stable in ST-LDdys group ($p < 0.05$ MFB-LDdys vs ST-LDdys). Striatally lesioned mice with no or low AIM scores (ST-LDnd group) showed rotational counts intermediate between the saline-treated animals (MFB-sal and ST-sal) and the L-DOPA-treated, dyskinetic ones ($p < 0.05$ for ST-LDnd vs MFB-sal and ST-sal on days 2, 6, 8 and 10; $p < 0.05$ vs MFB-LDdys and ST-LDdys mice on days 2, 4, 6, 8 and 10). Sham-lesioned animals treated with L-DOPA did not show any rotational activity.

On the counts of horizontal activity (rated zone crossings), all 6-OHDA lesioned groups treated with L-DOPA showed a significant motor activation compared with saline-injected 6-OHDA-lesioned mice (MFB-sal and ST-sal) and L-DOPA-treated sham-lesioned controls (Fig. 6B; repeated measures ANOVA, group effect: $F_{(5,160)} = 34.88$, $p < 0.001$; time effect: $F_{(4,160)} = 0.52$, $p > 0.05$; interaction: $F_{(20,160)} = 1.67$, $p = 0.042$). The two dyskinetic groups (MFB-LDdys and ST-LDdys) showed comparable levels of horizontal activity on all the sessions. The L-DOPA-treated non-dyskinetic mice (ST-LDnd) exhibited levels of horizontal activity intermediate between the two above groups and saline-treated controls up to the fourth day of treatment ($p < 0.05$ for ST-LDnd vs MFB-LDdys, ST-LDdys, MFB-sal, ST-sal, sham). When the L-DOPA dose was increased to 6 mg/kg, rated zone crossings scores from ST-LDnd mice did not differ significantly from those of the dyskinetic groups (cf. days 8 and 10 in Fig. 6B).

The percentage use of the contralateral forepaw in the cylinder test showed significant overall differences between groups and testing sessions (Fig. 6C; repeated measures ANOVA group effect: $F_{(5,47)} = 24.89$, $p < 0.001$; time effect: $F_{(1,47)} = 14.57$, $p < 0.001$; interaction: $F_{(5,47)} = 9.22$, $p < 0.001$). All the lesioned groups exhibited a significant forelimb use asymmetry when examined off L-DOPA (Fig. 6C, left panel). Following the administration of 6 mg/kg L-DOPA (100-120 min interval, cf. Methods), all lesion groups showed a similar reversal of forelimb akinesia, regardless

of the presence or absence of AIMs (Fig. 6C, right panel, $p < 0.05$ for MFB-LDdys, ST-LDdys, and ST-LDnd vs MFB-sal and ST-sal).

Extent of the dopaminergic lesions and their relationship with dyskinesia

In experiment 2, the extent of dopaminergic lesion was examined by densitometric analyses of TH in the striatum, VTA and SNpc (see Fig. S1 A-F and Table S1 in Supplement 1). In summary, TH levels were generally more depleted in MFB-lesioned mice compared to those with striatum$_{2x2\mu l}$ lesions, but within the latter group there was no significant difference between mice that developed AIMs (ST-LDdys group) and those that remained free from dyskinesia (ST-LDnd group) in any region.

To better compare the extent of DA cell loss between dyskinetic and non-dyskinetic animals, stereological counts of TH-positive cells in the SNpc and VTA were performed in all the L-DOPA-treated mice. This analysis revealed a significant difference between the lesion groups on the side ipsilateral to the lesion (Table 2; VTA, $F_{(2,16)} = 7.49$, $p < 0.001$; SNpc, $F_{(2,16)} = 17.42$, $p < 0.001$). MFB-lesioned mice showed the lowest number of TH-positive cells (approx. 1% and 39% of sham control values in the SNpc and VTA, respectively), differing significantly from striatally lesioned animals (approx. 24-30% and 56-62% of controls in the SNpc and VTA, respectively; Table 2). Among the mice with striatal lesions, no significant difference was observed between dyskinetic and non-dyskinetic cases (ST-LDdys and ST-LDnd groups), which is quite in agreement with the TH O.D. data from the same regions (cf. Table S1). No significant differences between groups were found on the side contralateral to the lesion (Table 2; intact side; one-factor ANOVA, VTA $F_{(3,19)} = 0.27$, $p > 0.05$; SNpc $F_{(3,19)} = 1.85$, $p > 0.05$).

FosB/ΔFosB positive cells in the striatum

Because of its gradual increase during chronic L-DOPA treatment (Andersson et al., 2001; Cenci et al., 1999; Valastro et al., 2007) and persistent expression (Andersson et al., 2003), FosB/ΔFosB immunoreactivity was used as a marker of striatal post-synaptic supersensitivity in experiment 2. The number of FosB/ΔFosB-positive cells differed significantly among the groups in both medial and lateral striatum (Table 3,

medial striatum, $F_{(5,47)} = 13.57$, $p < 0.001$; lateral striatum, $F_{(5,47)} = 82.72$, $p < 0.001$). In the medial striatum, MFB-LDdys mice showed a several-fold larger number of FosB/ΔFosB positive cells than did L-DOPA-treated mice with striatal lesions and saline-injected animals (Table 3). Among striatally lesioned mice, FosB/ΔFosB cell counts in the medial striatum did not differ between dyskinetic and non-dyskinetic cases, and neither of these groups differed significantly from saline-injected lesioned animals (Table 3). In the lateral striatum, the number of FosB/ΔFosB-immunoreactive cells was largest in MFB-LDdys mice (Fig. 7A; $p < 0.05$ for MFB-LDdys vs ST-LDdys), but a significant elevation of FosB/ΔFosB immunoreactivity was found also in striatally lesioned animals that had developed dyskinesia ($p < 0.05$ for ST-LDdys animals vs ST-LDnd, MFB-sal, ST-sal and sham; cf. Fig. 7B with Figs. 7C-F).

In agreement with our previous study in the mouse (Lundblad et al., 2004), the number of FosB/ΔFosB-like positive cells was positively correlated with the L-DOPA-induced AIMs scores (Fig. 8A; $R^2 = 0.824$, $p < 0.001$).

TH-positive cells in the striatum and their correlations with markers of dyskinesia

A previous study reported the occurrence of TH-immunoreactive cells in the mouse striatum following 6-OHDA lesion, and this cell population appeared to be modulated by L-DOPA treatment (Darmopil et al., 2008). We therefore counted the number of TH-immunoreactive neurons in the DA-denervated striata in all the animals from experiment 2. The number of TH-positive cells/mm^2 differed significantly between groups (Figs. 7 G-L; Table 3; $F_{(4,33)} = 71.29$, $p < 0.001$). A clear increase in TH-positive cell numbers above saline controls values occurred in both MFB-lesioned mice and striatally lesioned mice that had developed AIMs (Figs. 7 G-H; $p < 0.05$ vs all the other groups). Among the L-DOPA-treated mice with striatal lesions, the number of TH-positive cells was significantly larger in dyskinetic cases (ST-LDdys) compared to the non-dyskinetic ones (ST-LDnd; Table 3). A very low number of TH-positive cells were counted in saline-treated animals (Fig. 7 J-K), whereas cell counting was not possible in the sham-lesioned mice because of the very high TH fiber density (Fig. 7L).

Interestingly, the number of TH-immunoreactive cells showed tight linear correlation with the number of FosB/ΔFosB positive cells (Fig. 8B, $R^2 = 0.779$, $p <$

0.001), with the L-DOPA-induced AIM scores (Fig. 8C, $R^2 = 0.819$, $p < 0.001$) and with the number of L-DOPA-induced contralateral rotations (Fig. 8D, $R^2 = 0.684$, $p < 0.001$). These results raised the question, whether a striatal induction of dopaminergic neurons by L-DOPA may be involved in the generation of dyskinesia by providing an aberrant source of DA. Such question prompted the biochemical analyses described below.

Striatal tissue concentration of DA, 5-HT and NA

An additional set of animals with identical lesions, treatments and group allocations were prepared for a biochemical determination of monoamine levels (DA, NA and 5-HT) in striatal tissue during the "off L-DOPA" condition (experiment 3). Striatal DA concentrations differed significantly among the groups on the side ipsilateral to the lesion (Table 4; $F_{(6,34)} = 514.659$, $p < 0.001$), being reduced by > 99.5% in MFB-lesioned mice, and by 90-97% in mice with the striatum$_{2x2\mu l}$ lesion ($p < 0.05$ for each 6-OHDA-lesioned group vs sham-lesioned controls). Among the L-DOPA-treated groups, striatal DA levels were significantly lower in dyskinetic mice (both MFB- and striatally lesioned ones) than in non-dyskinetic cases (ST-LDnd group). Moreover, a significant, inverse logarithmic relationship ($y = b_1 - b_0 * \log[x]$) was found between DA contents and L-DOPA-induced AIM scores ($R^2 = 0.451$; $p < 0.001$; Fig. 9A). No significant group difference in DA levels was found on the intact sides (Table 4; one-factor ANOVA $F_{(6,34)} = 1.94$, $p = 0.102$).

Striatal NA tissue contents showed a significant overall group difference on the DA-denervated side (Table 4; $F_{(6,31)} = 3.70$, $p = 0.006$), where the MFB lesions had reduced NA levels by approximately 60% ($p < 0.05$ for both saline-treated and L-DOPA-treated MFB–lesioned animals vs sham). A trend towards reduced NA levels also occurred in the mice with striatal lesions, although no post-hoc comparison involving any of these groups reached statistical significance. Striatal NA contents showed a weak, inverse logarithmic relationship with the L-DOPA-induced AIM scores ($R^2 = 0.259$; $p = 0.015$; Fig. 9B). No significant group difference in NA levels was found on the intact side (Table 4; $F_{(6,34)} = 0.61$, $p=0.720$).

The tissue concentrations of 5-HT did not show any significant group differences or either side of the striatum (Table 4; lesion side $F_{(6,34)} = 1.62$, $p = 1.692$; intact side $F_{(6,33)} = 1.780$, $p = 0.1338$).

Discussion

Following its introduction by Ungerstedt (Ungerstedt, 1968), unilateral 6-OHDA lesions of the nigrostriatal pathway in the rat became the most widely used animal model of PD (reviewed in Cenci et al., 2002; Schwarting and Huston, 1996). Even today, intracerebral injections of 6-OHDA provide the most reliable procedure to induce stable and reproducible damage to the nigrostriatal system, and can be virtually applied to any species. One of the advantages of this procedure lies in its versatility, because the degree and the temporal course of nigrostriatal DA degeneration can be controlled and standardized by choosing appropriate toxin concentrations and injection sites (Blandini et al., 2008).

Thanks to a high level of genomic homology with humans (Bradley, 2002) and to a vast availability of genetically engineered strains, the mouse currently represents the preferred mammalian species for detailed molecular investigations on disease mechanisms. In the past few years, an increasing number of laboratories have applied 6-OHDA lesions to mice in order to obtain models of PD-like motor deficits and L-DOPA-induced dyskinesia. Our laboratory was the first to report that mice with 6-OHDA injections in the striatum or the MFB, treated chronically with L-DOPA, developed dyskinetic movements that exhibited marked phenomenological and molecular similarities to those of more complex animal models (Lundblad et al., 2004). In this previous study we had identified some problems associated with the use of 6-OHDA in mice. In particular, we had reported a high post-operative mortality (82% and 30% following MFB and striatum$_{2x2\mu l}$ lesions, respectively), which also was encountered by other authors using these types of lesion (Grealish et al., 2010). We had therefore pointed to the need for further studies evaluating alternative injection sites and/or toxin concentrations. In addition to behavioral data, further studies should also provide a biochemical characterization of the lesions, aimed at defining the precise extent of DA depletion and the possible damage produced to other monoaminergic systems.

This study represents the largest systematic evaluation of different 6-OHDA lesion models in the mouse. Such a large study, in which 151 mice reached all the predetermined behavioral and biochemical endpoints, would not have been possible without a significant improvement in our surgical and post-operative routines. At least 80% of the mice in experiment 1 and 100% of the animals in experiment 2 and 3 survived the critical 3-weeks post-operative period, even after the most severe lesions. This survival rate is strikingly higher than that previously reported (Lundblad et al., 2004) and depends on two main technical improvements, (i) the use of gaseous instead of injectable anesthesia, yielding a fast post-surgical awakening; (ii) a much improved protocol of post-operative care, including daily subcutaneous injections of glucose-saline solution, placing sweetened smashed food inside the home cages, and hand-feeding the mice, where necessary, during the first post-operative days. The first post-operative week is particularly critical for the mice, because the severe and sudden unilateral DA loss seems to cause transient dystonic postures that are evidently disabling. During this week, daily animal care is needed in order to prevent a fast loss of body weight and dehydration. Thanks to the excellent post-operative recovery of the mice and large number of animals, we were able to perform extensive comparisons between different 6-OHDA procedures using not only partial lesion models (as the striatal or the nigral one), but also MFB lesions.

In the first experiment, we set out to find a manageable lesion procedure yielding a degree of DA denervation sufficient to mimick a symptomatic state of PD, associated with molecular supersensitivity to L-DOPA. Of the four procedures examined, only the striatum$_{2x2\mu l}$ and the MFB one offered these features. We thus performed additional experiments aimed at determining the behavioral, molecular and biochemical responses to chronic L-DOPA treatment in these two models. Following the administration of a therapeutic dose of L-DOPA (6 mg/kg), all mice with striatum$_{2x2\mu l}$ or MFB lesions showed similar reversal of forelimb akinesia and increases in horizontal activity, although severe axial, limb and orolingual AIMs only occurred in the MFB group. Among the mice with striatum$_{2x2\mu l}$ lesions, 65% did not develop dyskinesia, whereas 35% exhibited AIMs of mild severity. These two groups differed significantly with respect to their striatal DA tissue contents and their expression of post-synaptic supersensitivity markers in striatal neurons, as will be discussed below.

Comparison between four lesion models

The results obtained in the first experiment showed that the MFB lesion had yielded severe and uniform DA depletion in both the lateral and medial striatum, whereas the striatal lesions ($striatum_{2x2\mu l}$ and $striatum_{2x1\mu l}$) had caused comparable DA denervation only in the lateral striatum. The SN lesions had resulted in a depletion of TH not exceeding 30% even in the most affected striatal regions. The MFB lesion produced the most stable behavioral deficits, while a recovery of spontaneous rotational asymmetry occurred in mice with striatal lesions by 12 weeks post lesion. These results suggest that striatally lesioned mice are more prone to undergo spontaneous behavioral recovery than MFB-lesioned ones. This conclusion is in line with observations from previous studies performed in both rats and mice. Stanic et al. (Stanic et al., 2003a; Stanic et al., 2003b) described a long-term (16 weeks) compensatory axonal sprouting and behavioral recovery in rats that underwent partial 6-OHDA nigrostriatal lesions not exceeding 70%. Moreover, mice treated with MPTP can exhibit spontaneous histochemical, behavioral and biochemical recovery that seems to depend on a compensatory sprouting of catecholamine nerve terminals (Date et al., 1990; Hallman et al., 1985; Mitsumoto et al., 1998). A partial recovery of behavioral parameters also has been reported in a study using mice with striatal 6-OHDA lesions, where the animals showed an improvement in rotarod performance over a 2 months period (Alvarez-Fischer et al., 2008). The occurrence of dopaminergic axonal sprouting in the striatum may be a positive feature of the striatal 6-OHDA model in studies assessing neurorestorative and neuroprotective treatments (Biju et al., 2010; Schwarting and Huston, 1996). The same phenomenon may, however, represent a confounding variable in studies assessing the effects of chronic symptomatic treatments.

Several studies in animal models of PD have led to the identification of plastic changes occurring in the basal ganglia during the development of L-DOPA-induced dyskinesia (reviewed in (Cenci and Konradi, 2010; Cenci et al., 2009; Jenner, 2008). The induction of pERK1/2 in striatal neurons provides an early marker of aberrant neuroplasticity and post-synaptic D1 receptor supersensitivity in dyskinetic animals (Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007). The striatal expression of pERK1/2 is transiently induced by each L-DOPA dose, and subsides completely within 24 hours (Westin et al., 2007). In the present work, the expression pattern of pERK1/2 was examined in the four lesion models following acute

administration of a high dose of L-DOPA (30 mg/kg). Within each lesion group, the distribution of pERK1/2 immunoreactivity was found to provide a mirror image of that of TH. Animals with striatal 6-OHDA lesions showed a significant number of pERK-positive cells only in the most denervated lateral striatal regions, whereas areas with more than 60% residual TH staining (medial striatum) did not show any ERK activation. In contrast, MFB-lesioned mice exhibited a widespread upregulation of pERK1/2 throughout the striatum, matching their pronounced and uniform loss of TH. Interestingly, although the amount of residual DA innervation in the lateral striatum did not differ significantly between MFB- and striatally lesioned animals, the levels of pERK immunoreactivity were significantly higher in the former compared to the latter model. We interpret these results as indicating that, in striatal 6-OHDA models, denervation-induced DA receptor supersensitivity in the lateral striatum is mitigated by volume transmission of DA from residual fibers located in the medial striatum. The SN lesion model showed low levels of ERK activation also in the most affected region (the lateral striatum), indicating that this type of lesion is not suitable for studies aimed at reproducing L-DOPA-induced dyskinesia in the mouse.

Effects of chronic L-DOPA treatment in mice with striatum$_{2x2\mu l}$ or MFB lesions

In further experiments, we selected the MFB and striatum$_{2x2\mu l}$ lesion model to compare the behavioral and molecular effects of chronic L-DOPA treatment. During the course of the treatment, the animals underwent AIM ratings, cylinder test, and measurements of horizontal and rotational activity using a new videotracking system. Interestingly, all the MFB-lesioned mice developed pronounced dyskinesia, whereas the severity of axial, limb and orolingual AIMs varied markedly among the striatally lesioned animals, many of which remained totally free from AIMs. The absence of dyskinesia was not due to a lack of response to L-DOPA, because the non-dyskinetic mice showed levels of horizontal activity and cylinder test performance on L-DOPA that were comparable with those of the dyskinetic groups.

Following the behavioral studies, we investigated the expression pattern of ΔFosB-related proteins, stable transcription factors that accumulate in the brain in response to chronic perturbations (Hope et al., 1994). The striatal expression of FosB/ΔFosB immunoreactivity provides a robust correlate of dyskinesia in rats (Andersson et al., 1999), mice (Fasano et al., 2010; Lundblad et al., 2004; Pavon et

al., 2006) and non-human primate models of PD (Berton et al., 2009; Fasano et al., 2010). As expected, the number of FosB/ΔFosB-immunoreactive cells showed a strong positive correlation with the L-DOPA-induced AIM scores, being significantly higher in dyskinetic mice than in non-dyskinetic cases. Remarkably, we also found a strong correlation between AIM scores and number of TH-positive cell bodies in the striatum. Previous studies have demonstrated the presence of dopaminergic neurons in the striatum both in parkinsonian patients (Huot and Parent, 2007) and in mice with striatal 6-OHDA lesions (Darmopil et al., 2008). In the latter study, these neurons were seen between 3 days and 1 week after the lesion, and then progressively declined, but their number increased again following L-DOPA treatment (Darmopil et al., 2008). This is the first study reporting a correlation between striatal TH-positive neurons, AIM scores and a well-established molecular markers of dyskinesia such as FosB/ΔFosB. The distribution of these cells resembled those of pERK and FosB/ΔFosB, occurring throughout the striatum in the MFB-lesioned dyskinetic mice, but mostly in the lateral regions in mice with striatal lesions. Importantly, dyskinetic mice with striatal lesions showed a significantly higher number of striatal TH-positive cell bodies than did non-dyskinetic cases within the same lesion model. Since these cells don't express the dopamine transporter (Darmopil et al., 2008), we initially hypothesized that they could play a role in the development of dyskinesia by providing an unregulated source of DA production and release. This hypothesis was however contradicted by the results of the biochemical analyses, which did not evidence any increase in striatal DA tissue contents "off L-DOPA" in the dyskinetic groups. On the contrary, DA levels were significantly reduced in the dyskinetic animals compared to non-dyskinetic ones.

Biochemical analysis of NA striatal concentrations revealed a significant decrease below sham-control values only in MFB-lesioned mice, but a trend towards reduction was observed also in striatally lesioned animals. Although an inverse logarithmic relationship was found between striatal NA contents and AIM scores, no difference in NA levels occurred between dyskinetic and non-dyskinetic cases within the striatal lesion group, nor between either of these groups and the MFB one. These results suggest that variations in striatal NA concentrations due to the lesion procedure are unlikely to have a major impact on the susceptibility to L-DOPA-induced dyskinesia, at least under the present experimental conditions. Such a

conclusion would be supported by a study in MFB-lesioned rats, where the behavioral response to L-DOPA was not affected by a concomitant lesion of the locus coeruleus (Marin et al., 2008).

Since striatal 5-HT contents did not differ significantly between the groups, we conclude that neither the MFB lesion nor the striatum$_{2x2\mu l}$ one had affected the serotonin system. This situation is at variance with our results from the rat model of L-DOPA-induced dyskinesia. In this model, the MFB lesion causes partial damage to the ascending serotonin projections, and chronic L-DOPA treatment causes sprouting of 5-HT axon terminals, associated with an increase in striatal 5-HT levels in animals that develop dyskinesia (Lindgren et al., 2010; Rylander et al., 2010). The lower 6-OHDA concentration used in mice compared to rats (~ 3 vs 7 μg/injection), along with a shorter duration of L-DOPA treatment, provide a likely explanation to the lack of biochemical changes in striatal 5-HT projections in the mouse model.

Summary and concluding remarks

Taken together, our results indicate that the MFB lesion should be viewed as the procedure of choice to obtain a mouse model of severe and evenly distributed DA denervation, stable behavioral deficits, and maximal supersensitivity to L-DOPA. In contrast, striatal lesions yield a heterogeneous distribution of DA denervation and post-synaptic supersensitivity to L-DOPA. At the behavioral level, these features are associated with some degree of long-term spontaneous recovery and with a varying susceptibility to dyskinesia upon L-DOPA treatment. The two lesion models also differ from each other with regard to the extent of VTA involvement, probably resulting in a different pattern of extrastriatal DA denervation. The relative sparing of midbrain DA cells bodies and their ascending fibers upon intrastriatal 6-OHDA injection makes this lesion model particularly suitable to evaluate neuroprotective/neurorestorative treatments for PD. Moreover, the large interindividual variation in L-DOPA-induced AIM scores represents an advantage for studies aimed at defining correlations between molecular/biochemical parameters and dyskinesia severity. For this sort of application, it is important to rule out that a varying susceptibility to dyskinesia simply depends on interindividual differences in the extent of nigrostriatal lesion. In the present study, the levels of TH immunoreactivity in the striatum and midbrain DA cell groups did not differ between

dyskinetic and non-dyskinetic animals within the striatal lesion model. However, striatal DA contents were significantly lower in the dyskinetic cases. This observation indicates that biochemical determinations of DA tissue levels provide a more sensitive method than TH O.D. analyses and/or stereological cell counts to detect subtle differences in the extent of dopaminergic damage following striatal 6-OHDA lesions. Of all the parameters under investigation, those exhibiting strongest correlations with the L-DOPA-induced AIM scores proved to be the number of FosB/ΔFosB-immunoreactive cells and TH-positive cell bodies in the striatum, both of which reflect post-synaptic adaptations to L-DOPA treatment. Accordingly, a large difference in the expression of these markers was found between dyskinetic and non-dyskinetic mice in the striatum$_{2x2\mu l}$ lesion model. The primary determinants of such a pronounced variation in postsynaptic supersensitivity among mice remain elusive. Although the level of striatal DA depletion sets the sensitivity threshold, additional sources of inter-individual variation are likely to depend on genetic, epigenetic, or stochastic differences in the activity of transmembrane receptors and intracellular modulators implicated in DA signaling (reviewed in (Cenci and Konradi, 2010).

In conclusion, the present results will guide the selection of lesion procedure, behavioral and biochemical end-points in future studies using 6-OHDA to reproduce parkinsonism and/or L-DOPA-induced dyskinesia in the mouse. These neurotoxic models will be instrumental to the utilization of genetically engineered mice in basic research on PD.

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Figure legends:

Fig. 1. *Experimental design.* The upper and lower horizontal arrows represent the time course of experiment 1 and experiments 2-3, respectively. In experiment 1, mice were tested for spontaneous rotational activity, horizontal activity and forepaw use at 3 and 12 weeks post lesion. They were then perfusion-fixed (vertical arrow) and processed for IHC after an acute L-DOPA challenge (30 mg/kg). In experiments 2 and 3, mice were tested for spontaneous rotations, horizontal activity, forepaw use and AIMs. All of these behavioral tests (except for AIMs scoring) were applied both "off" (week 3) and "on" L-DOPA (weeks 4 and 5). During weeks 4-5, chronic L-DOPA treatment was administered using an escalating L-DOPA dosage (3 and 6 mg/kg/day). Mice were killed 24 hours after the last L-DOPA injection (vertical arrow) and their brains were processed for IHC or HPLC.
IHC, immunohistochemistry; HPLC, high pressure liquid chromatography; SN, substantia nigra; ST, striatum; MFB, medial forebrain bundle. Behavioral testing sessions are indicated by open circles (rotation and horizontal activity), open squares (cylinder test) and open triangles (AIMs ratings).

Fig. 2. *Motor deficits induced by different 6-OHDA lesion types (experiment 1).* Values give the total number of spontaneous ipsilateral rotations (A, A´) and line crossings (B, B´) recorded in an open field test at 3 and 12 weeks post lesion. Forelimb use asymmetry was evaluated in the cylinder test at the same post-lesion intervals (C, C'). Test duration was 10 min in A and B, and 3 min in C. Values report the percentage of supporting wall contacts performed by the paw contralateral to the lesion (left paw). One-factor ANOVA and *post-hoc* Student-Newman-Keuls test; $p < 0.05$, (*d*) vs. sham, SN and $ST_{2x1\mu l}$; (*e*) vs. all other groups; (*f*) vs. sham and MFB; (g) vs. sham and SN; (*h*) vs. sham. MFB, medial forebrain bundle; ST, striatum; SN, substantia nigra; sham, sham lesioned.

Fig. 3. *pERK1/2 upregulation and dopaminergic denervation in different types of 6-OHDA lesions (experiment 1).* The most pronounced depletion of TH-positive fibers (A, C) and the most elevated expression of pERK1/2 (B, D) were detected in MFB lesioned mice, both in the lateral (A, B) and the medial striatum (C, D). In the lateral striatum, significant pERK activation was also observed in striatally lesioned mice

(B), whereas the SN lesion model did not differ significantly from sham-lesioned controls. One-factor ANOVA and *post-hoc* Student-Newman-Keuls test; $p < 0.05$ (*e*) vs. all other groups, (*f*) vs. sham and SN, (*g*) vs. MFB, $ST_{2x2\mu l}$, $ST_{2x1\mu l}$.
MFB, medial forebrain bundle; ST, striatum; SN, substantia nigra; sham, sham lesion.

Fig. 4. *TH immunoreactivity and pERK1/2 upregulation in different types of 6-OHDA lesions (experiment 1)*. Photomicrographs were obtained from the lateral part of the striatum on the side ipsilateral to the 6-OHDA injection (A-J). Scale bar, 100 µm. * indicates corpus callosum.

Fig. 5. *L-DOPA-induced AIMs (experiment 2)*. The sum of axial, limb and orolingual AIM scores per session during chronic treatment with L-DOPA is shown in A (the L-DOPA dose was 3 and 6 mg/kg/day on days 1-3 and days 5-11, respectively). The duration of dyskinesia after one single injection of L-DOPA is shown in B. Values in B give axial, limb and orolingual AIM scores per monitoring period during the last dyskinesia test (day 11). Repeated measures ANOVA and post hoc Tukey's test; $p < 0.05$ (*d*) vs. sham, MFB-sal and ST-sal; (*e*) vs. all other groups; (*f*) vs. MFB-LDdys.
MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; MFB-sal, MFB lesions treated with saline; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non dyskinetic; ST-sal, striatal lesions treated with saline; sham, sham lesions.

Fig. 6. *Motor improvement induced by chronic L-DOPA treatment (experiment 2)*. Contralateral rotations (A) and rated zone crossings (B) were measured on 5 testing sessions during chronic treatment with L-DOPA (3 mg/kg/day on days 2 and 4, and 6 mg/kg/day on days 6-10). Animals were recorded at 20-40 min following the injection of L-DOPA or saline. Forelimb use asymmetry in the cylinder test (C) was assessed both prior to the start of chronic L-DOPA treatment (OFF L-DOPA) and on the 9^{th} day of chronic L-DOPA treatment (ON L-DOPA). This test was applied for 3 min starting at 100 min and 120 min after drug administration in striatum- and MFB-lesioned mice, respectively (these time points corresponded to the rapidly declining phase of the dyskinesias). The results are expressed as the percentage of supporting wall contacts performed by the paw contralateral to the lesion (left paw). Repeated measures ANOVA and post hoc Tukey's test; $p < 0.05$ (*d*) vs. sham, ST-LDnd, MFB-

sal and ST-sal; (*e*) vs. all; (*f*) vs. MFB-LDdys and ST-LDdys; (*g*) vs. sham, MFB-sal and ST-sal; (*h*) vs. sham; (*i*) vs. sham and on L-DOPA; (*k*) vs. sham, MFB-LDdys, ST-LDdys, ST-LDnd on L-DOPA.

MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; MFB-sal, MFB lesions treated with saline; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non dyskinetic; ST-sal, striatal lesions treated with saline; sham, sham lesions.

Fig. 7. *FosB/ΔFosB and TH-immunoreactive cells in the striatum (experiment 2).* Photomicrographs of FosB/ΔFosB (A-F) and TH-positive cell bodies (G-L) were taken from the dorsolateral striatum. Scale bar, 100 μm.

MFB-LDdys, MFB lesion treated with L-DOPA and dyskinetic; MFB-sal, MFB lesion treated with saline; ST-LDdys, striatal lesion treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesion treated with L-DOPA and non dyskinetic; ST-sal, striatal lesion treated with saline; sham, sham lesion.

Fig. 8. *Correlations between postsynaptic markers of dyskinesia (experiment 2).* The number of FosB/ΔFosB immunoreactive cells/mm^2 (average of medial and lateral striatum) was plotted on the cumulative AIMs scores during the treatment period (A) and on the number of TH immunoreactive cells/mm^2 counted in the entire striatum (B). The number of striatal TH immunoreactive cells correlated positively with the cumulative AIM scores (C) and with the number of L-DOPA-induced contralateral (left) rotations (D).

MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; MFB-sal, MFB lesions treated with saline; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non dyskinetic; ST-sal, striatal lesions treated with saline; sham, sham lesions.

Fig. 9. *Plots of AIM scores on striatal DA and NA levels (experiment 3).*

Values indicate cumulative AIM scores (y axis) and actual tissue concentrations of the monoamines (x axis) from L-DOPA-treated animals.

DA, dopamine; NA, noradrenaline; AIMs, abnormal involuntary movements, MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non dyskinetic; sham, sham lesions.

Table legends

Table 1. *Experimental groups and number of animals.* The number of animals used in the different phases of experiments 1, 2 and 3 is reported above. Further details are given in Material and Methods.
IHC, immunohistochemistry; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; MFB, medial forebrain bundle; ST, striatum; SN, substantia nigra; Sham, sham lesion.

Table 2. *Stereological counts of TH-positive neurons (experiment 2).* Values represent the total number of cells in the SNpc and VTA from 8 and 7 sections per animal, respectively. One-factor ANOVA and *post-hoc* Student-Newman-Keuls; $p < 0.05$ (b) vs. sham and all ST groups; (c) vs. sham and MFB groups. MFB-LDdys, MFB-lesioned mice treated with L-DOPA and dyskinetic; ST-LDdys, striatally lesioned mice treated with L-DOPA and dyskinetic; ST-LDnd, non-dyskinetic mice with striatal lesions and L-DOPA treatment; sham, sham-lesioned controls. SNpc, substantia nigra pars compacta; VTA, ventral tegmental area.

Table 3. *Computer-assisted counts of cells immunoreactive for FosB/ΔFosB and TH in the striatum (experiment 2)*. Values represent the average number of cells/mm2 from 4 sections per animal. One-factor ANOVA and *post-hoc* Student-Newman-Keuls; $p < 0.05$ (e) vs. all; (f) vs. MFB-LDdys and ST-LDdys; (g) vs. MFB-LDdys; n.c., not counted.
MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; MFB-sal, MFB lesions treated with saline; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non-dyskinetic; ST-sal, striatal lesions treated with saline; Sham, sham lesions.

Table 4. *Striatal tissue levels of DA, 5-HT and NA (experiment 3).* Values represent the monoamine concentrations as ng/mg of striatal tissue. One-factor ANOVA and *post-hoc* Student-Newman-Keuls; $p < 0.05$ (a) vs. Sham, (d) vs. Sham and ST-LDnd, and (h) vs. Sham, MFB-LDdys and ST-LDdys.

DA, dopamine; 5-HT, 5-hydroxytryptamine; NA, noradrenaline; MFB-LDdys, MFB lesions treated with L-DOPA and dyskinetic; MFB-sal, MFB lesions treated with saline; ST-LDdys, striatal lesions treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesions treated with L-DOPA and non-dyskinetic; ST-sal, striatal lesions treated with saline; Sham, sham lesions.

Fig.1

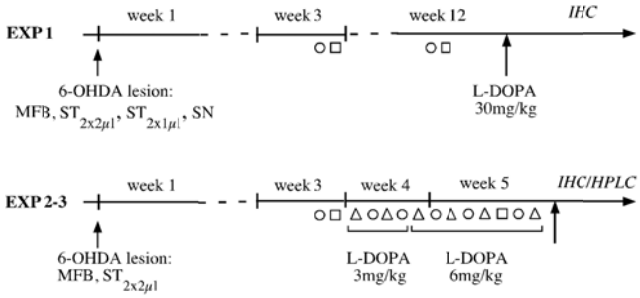


Fig.2

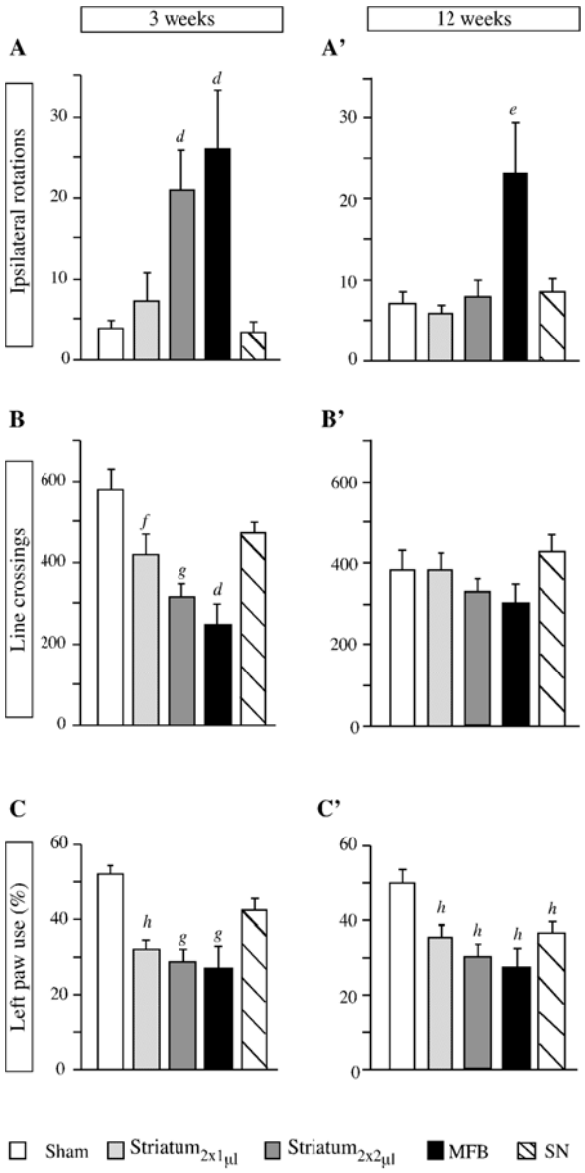


Fig. 3

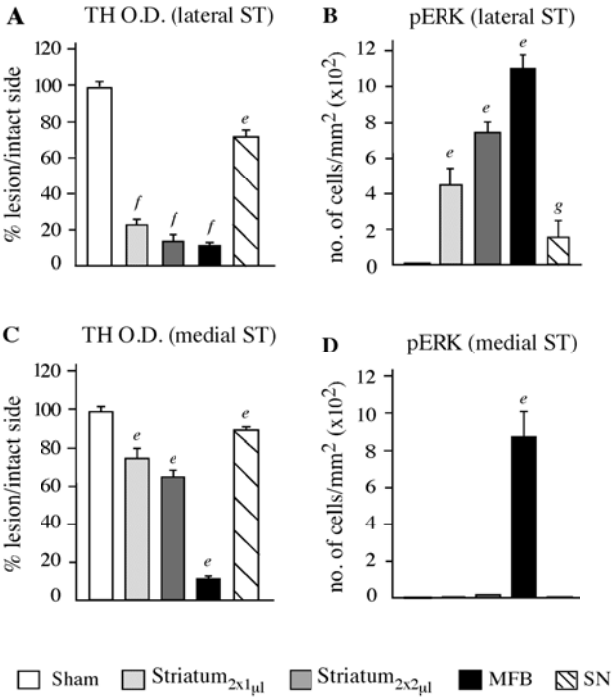

A
TH O.D. (lateral ST)
% lesion/intact side
120
100
80
60
40
20
0
f
f
f
e
B
pERK (lateral ST)
no. of cells/mm2 (x102)
12
10
8
6
4
2
0
e
e
e
g
C
TH O.D. (medial ST)
% lesion/intact side
120
100
80
60
40
20
0
e
e
e
e
D
pERK (medial ST)
no. of cells/mm2 (x102)
12
10
8
6
4
2
0
e
Sham
Striatum2x1µl
Striatum2x2µl
MFB
SN

Fig. 4

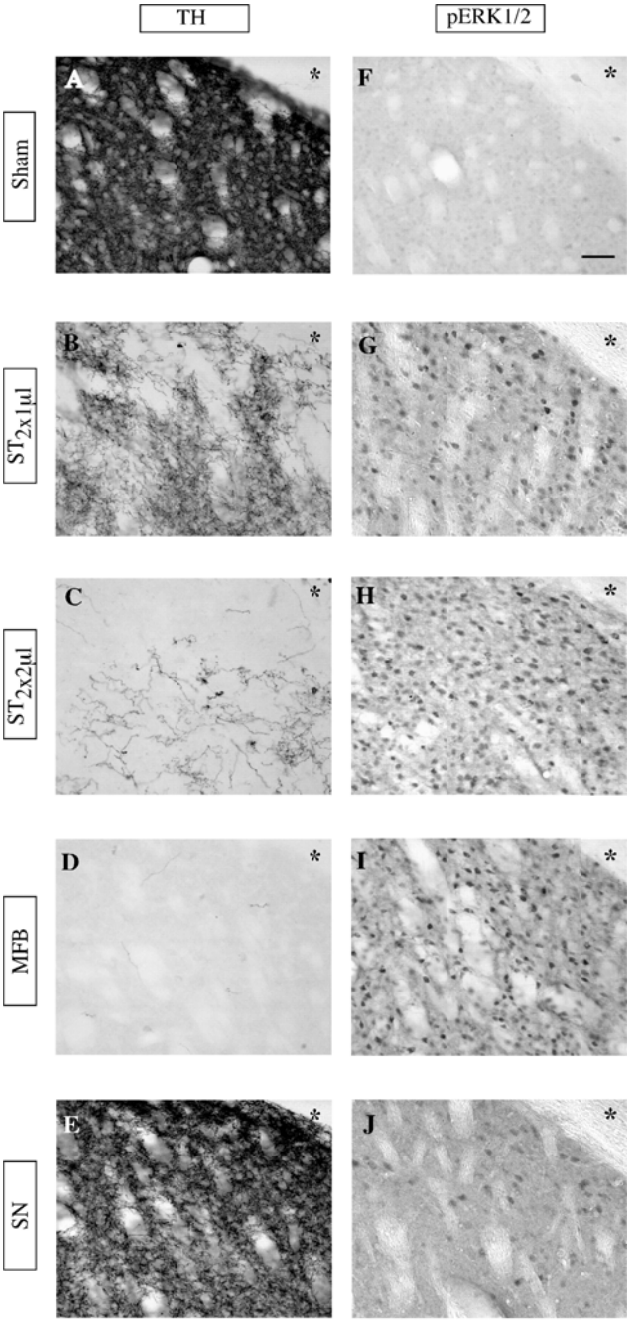

TH
pERK1/2
Sham
ST2x1µl
ST2x2µl
MFB
SN
A
B
C
D
E
F
G
H
I
J
*

Fig. 5

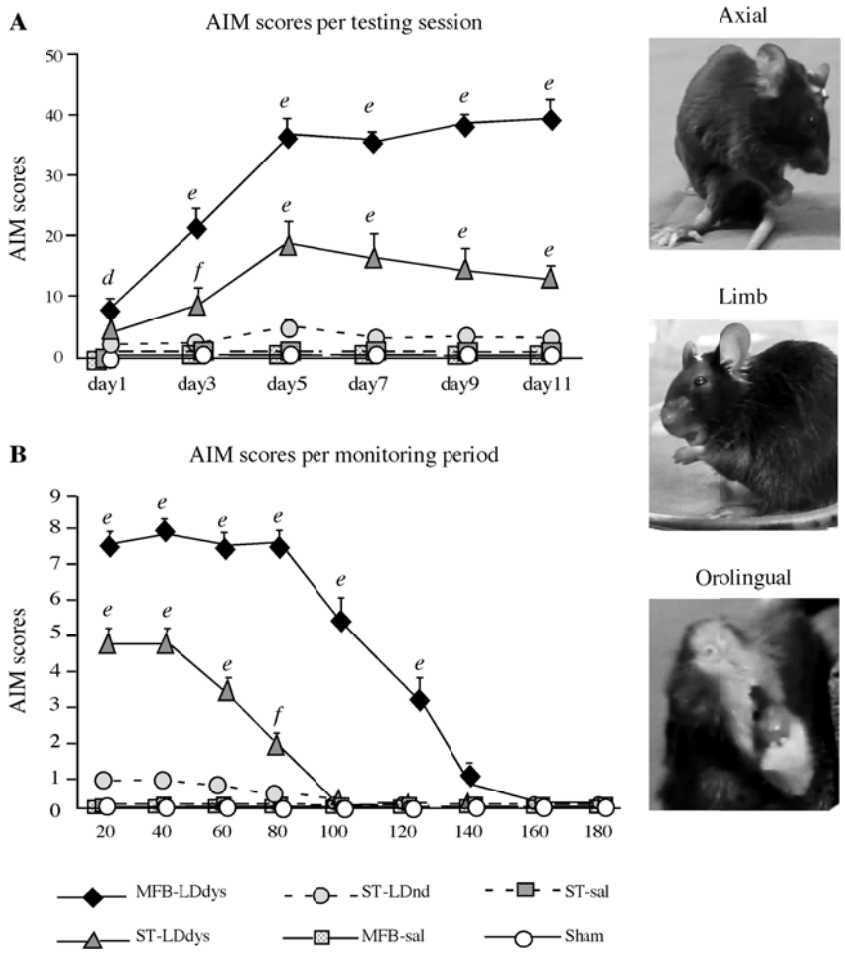

A
AIM scores per testing session
AIM scores
day1
day3
day5
day7
day9
day11
B
AIM scores per monitoring period
AIM scores
MFB-LDdys
ST-LDnd
ST-sal
ST-LDdys
MFB-sal
Sham
Axial
Limb
Orolingual

Fig. 6

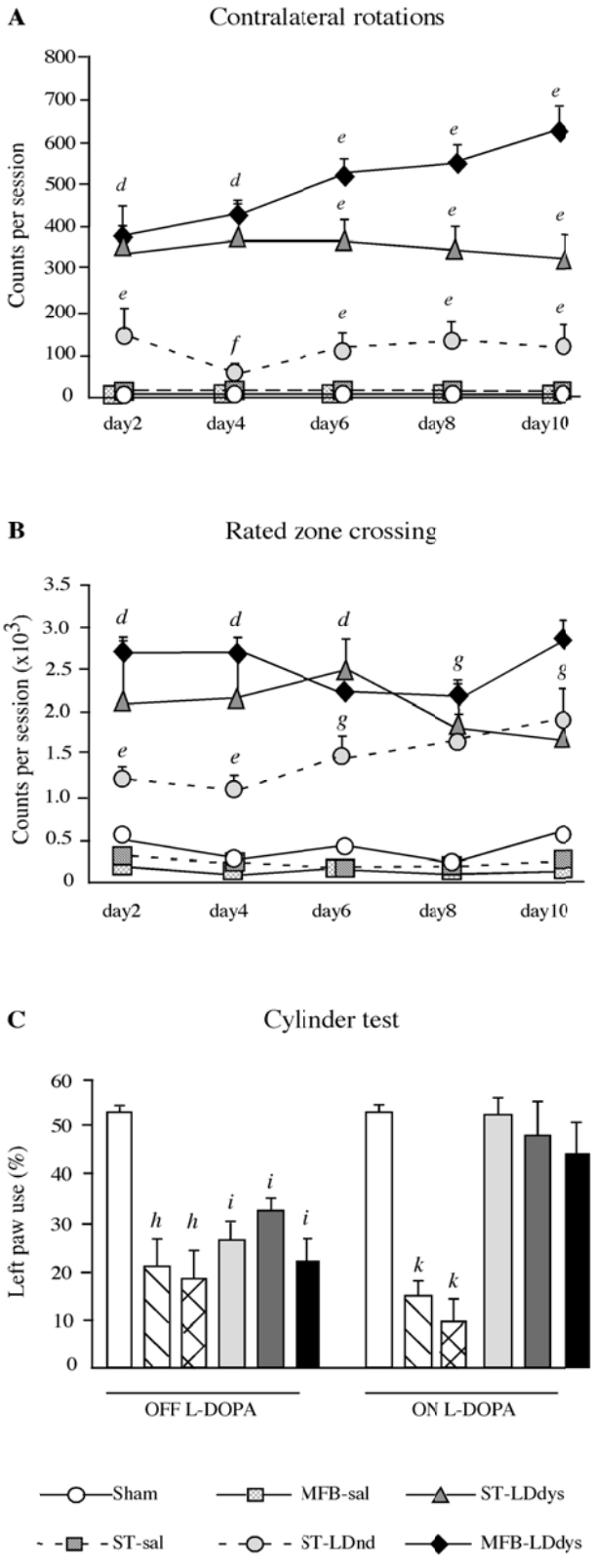
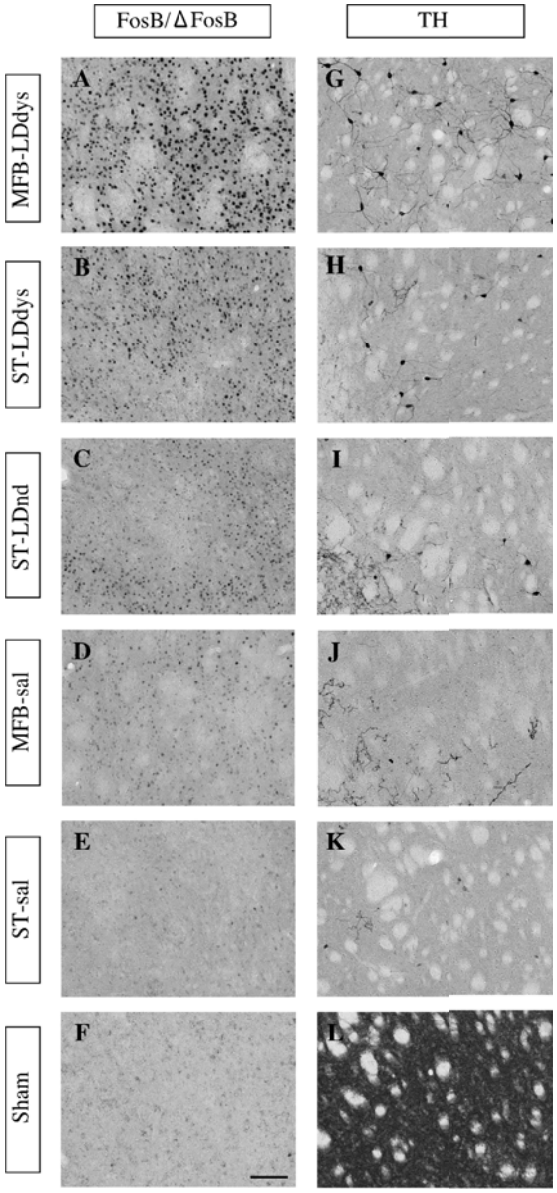

A
Contralateral rotations
Counts per session
800
700
600
500
400
300
200
100
0
day2
day4
day6
day8
day10
B
Rated zone crossing
Counts per session (x10^3)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
C
Cylinder test
Left paw use (%)
60
50
40
30
20
10
0
OFF L-DOPA
ON L-DOPA
Sham
MFB-sal
ST-LDdys
ST-sal
ST-LDnd
MFB-LDdys

Fig. 7


FosB/ΔFosB
TH
MFB-LDdys
ST-LDdys
ST-LDnd
MFB-sal
ST-sal
Sham
A
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D
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Fig. 8

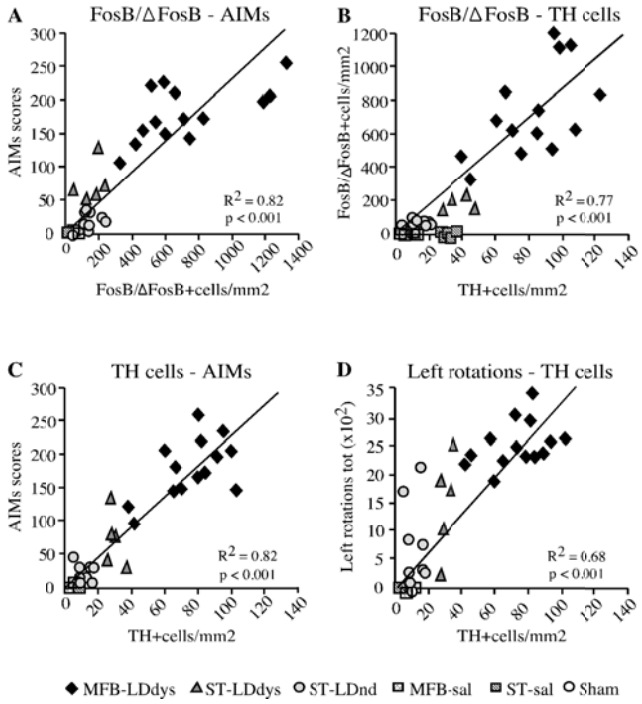
A
FosB/ΔFosB - AIMs
AIMs scores
FosB/ΔFosB+cells/mm2
R2 = 0.82
p < 0.001
B
FosB/ΔFosB - TH cells
FosB/ΔFosB+cells/mm2
TH+cells/mm2
R2 = 0.77
p < 0.001
C
TH cells - AIMs
AIMs scores
TH+cells/mm2
R2 = 0.82
p < 0.001
D
Left rotations - TH cells
Left rotations tot (x10-2)
TH+cells/mm2
R2 = 0.68
p < 0.001
MFB-LDdys
ST-LDdys
ST-LDnd
MFB-sal
ST-sal
Sham

Fig. 9

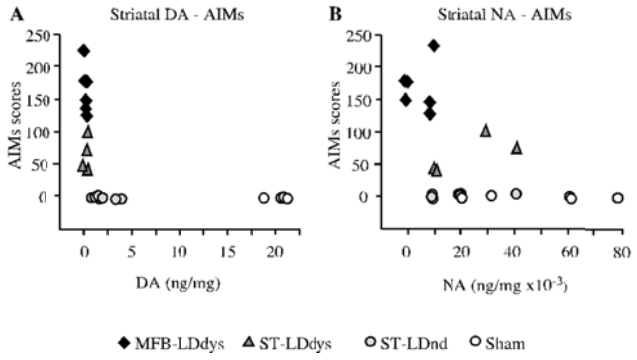
A
Striatal DA - AIMs
AIMs scores
DA (ng/mg)
B
Striatal NA - AIMs
AIMs scores
NA (ng/mg x10-3)
MFB-LDdys
ST-LDdys
ST-LDnd
Sham

Table 1. Experimental groups and number of animals

Experiment	Lesion groups	Behavioral tests	Treatment		IHC/HPLC
			L-DOPA	Saline	
1	Sham (n=8)	(n=8)	(n=6)	(n=2)	(n=8)
	SN (n=14)	(n=14)	(n=8)	(n=5)	(n=13)
	MFB (n=12)	(n=12)	(n=4)	(n=5)	(n=9)
	$ST_{2x1\mu l}$ (n=11)	(n=11)	(n=5)	(n=4)	(n=9)
	$ST_{2x2\mu l}$ (n=12)	(n=12)	(n=7)	(n=2)	(n=9)
2	Sham (n=9)	(n=9)	(n=5)	(n=4)	(n=9)
	MFB (n=23)	(n=23)	(n=17)	(n=6)	(n=23)
	$ST_{2x2\mu l}$ (n=21)	(n=21)	(n=15)	(n=6)	(n=21)
3	Sham (n=11)	(n=11)	(n=6)	(n=5)	(n=11)
	MFB (n=13)	(n=13)	(n=7)	(n=6)	(n=13)
	$ST_{2x2\mu l}$ (n=17)	(n=17)	(n=11)	(n=6)	(n=17)

Table 2. Stereological counts of TH-positive neurons

	Sham	ST-LDnd	ST-LDdys	MFB-LDdys
SNpc				
les side	4283±226.3	131±11.8[c]	102±10.1[c]	44±2.6[b]
int side	4155±160.3	3620±227.4	3575±207.7	3325±215.2
VTA				
les side	5786±736.4	3592±235.4[c]	3266±277.9[c]	2267±157.3[b]
int side	5919±509.7	5776±416.1	5374±379.5	5692±394.1

Table 3. Computer-assisted counts of cells immunoreactive for FosB/ΔFosB and TH in the striatum

	Sham	ST-Sal	MFB-Sal	ST-LDnd	ST-LDdys	MFB-LDdys
FosB/ΔFosB+ cells						
Medial striatum	1±2.0	8±2.6[g]	19±2.9[g]	21±3.8[g]	48±12.7[g]	260±41.6[e]
Lateral striatum	1±0.9	5.4±1.3[f]	14±1.5[f]	35.4±5.1[f]	106.7±37[e]	394±22.1[e]
TH+ cells	n.c.	5±0.4[f]	7±1.3[f]	13±1.9[f]	37±2.4[e]	85±5.2[e]

Table 4. Striatal tissue levels of DA, 5-HT and NA

	Sham	ST-Sal	MFB-Sal	ST-LDnd	ST-LDdys	MFB-LDdys
DA						
les side	19.04±0.5	1.03±0.3[d]	0.11±0.06[d]	1.84±0.46[h]	0.47±0.08[d]	0.08±0.02[d]
int side	19.19±0.8	17.66±1.05	20.48±1.24	19.03±0.83	16.68±0.61	21.39±1.23
5-HT						
les side	0.40±0.03	0.52±0.03	0.51±0.02	0.52±0.04	0.48±0.05	0.54±0.06
int side	0.40±0.04	0.43±0.03	0.46±0.02	0.35±0.05	0.40±0.02	0.57±0.06
NA						
les side	0.05±0.01	0.02±0.005	0.013±0.004[a]	0.015±0.003	0.02±0.007	0.02±0.01[a]
int side	0.07±0.01	0.43±0.03	0.46±0.02	0.35±0.05	0.40±0.02	0.52±0.04

Supplement 1: TH optical density analysis in the striatum, SN and VTA (experiment 2)

Methods

In experiment 2, the optical density (O.D.) of TH-immunostaining was analyzed in all animals in the striatum, SN pars compacta (SN) and ventral tegmental area (VTA) by a blinded investigator using NIH Image J software 43u (2007). Sections were digitized under a 4x objective in a Nikon Eclipse 80i microscope connected to a Nikon DMX 1200F videocamera. Cells were counted both in the 6-OHDA-lesioned hemisphere and on the contralateral intact side. For the striatum, four different rostrocaudal levels were examined, corresponding to the following coordinates, in mm relative to bregma, according to the rat brain atlas by Paxinos and Franklin (Paxinos and Franklin 2001): +1.34/+1.18 (level I), +0.74/+0.50 (level II), +0.14 (level III), -0.34/-0.50 (level IV). At these striatal levels, separate measurements were taken in the lateral and medial half of the section. Optical density analysis was carried out also on the SN and VTA at the following rostrocaudal levels: -2.92/-3.08 (level I), -3.16/-3.28 (level II), -3.40/-3.52 (level III), bregma -3.64 (level IV). The optical density on white matter tracts (tissue background) was subtracted from the values obtained in the regions of interest, and measurements from the lesion side were expressed as a percentage of the values from the intact side in each animal.

Results

The MFB lesion reduced TH O.D. by more than 85% in both the lateral and the medial striatum (Table S1, Fig. S1A). By contrast, the striatum$_{2x2\mu l}$ lesion resulted in > 85% TH loss only in the lateral region ($p < 0.05$ vs. Sham, non-significant difference vs. MFB), whereas the medial region showed a reduction by 77-79% ($p < 0.05$ vs. both sham and MFB groups; Table 1S, Fig. S1B). Importantly, striatal TH levels did not differ between mice that developed severe AIMs (ST-LDdys group) and those that remained free from dyskinesia (ST-LDnd group), nor did TH O.D. values in the SNc and VTA differ between these two groups (Table S1); (one-factor ANOVA, lateral striatum $F_{(5,47)} = 2107.27$, $p < 0.001$; one-factor ANOVA, medial striatum $F_{(5,47)} = 429.87$, $p < 0.001$). Striatally lesioned animals showed a significantly

higher residual TH innervation both in the VTA (about 54%) and in the SNc (about 23%) in comparison with the MFB lesioned mice (about 24% in the VTA and 10% in the SNc) (Table S1, Fig. S1 D, E, F; one-factor ANOVA, VTA, $F_{(6,46)} = 36.77$, $p < 0.001$; SN, $F_{(6,46)} = 117.99$, $p < 0.001$). In none of the regions examined was there any difference in the extent of DA denervation between the three groups with striatal 6-OHDA lesions (ST-sal, ST-LDnd, and ST-LDdys). Thus, the extent of TH residual innervation was dependent only on the type of lesion, and was not conditional on L-DOPA treatment. Among the animals with striatal lesions, the intensity of TH immunostaining did not predict which animals developed dyskinesia and which did not.

Figure legend

Fig. S1. *TH immunostaining (experiment 2)*. Representative photomicrographs of TH immunoreactivity in the striatum, VTA and SN were taken from MFB (A and D), striatal (B and E) and sham-lesion animals (C and F). The side ipsilateral to the lesion is shown to the right. Scale bar, 1 mm.

Table legend

Table S1. *Densitometric analysis of TH immunoreactivity (experiment 2).* Values represent the mean ± SEM per 4 sections throughout striatum, VTA and SN (experiment 2). They are expressed as percentage of the lesioned side/intact. One-factor ANOVA and post-hoc Student-Newman-Keuls, $p < 0.05$; (a) vs. Sham; (b) vs. Sham and all ST groups; (c) vs. Sham and all MFB groups; (d) vs. Sham and STnd.
SN, substantia nigra pars compacta; VTA, ventral tegmental area; MFB-LDdys, MFB lesion treated with L-DOPA and dyskinetic; MFB-sal, MFB lesion treated with saline; ST-LDdys, striatal lesion treated with L-DOPA and dyskinetic; ST-LDnd, striatal lesion treated with L-DOPA and non-dyskinetic; ST-sal, striatal lesion treated with saline; Sham, sham lesion.

Fig. S1

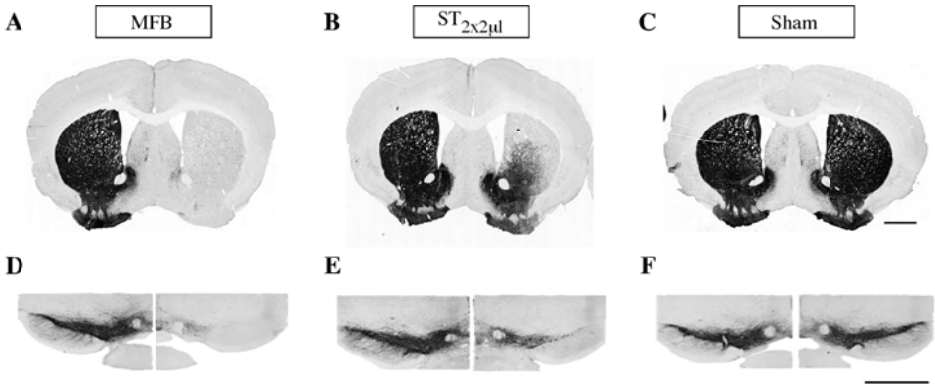

Table S1. Densitometric analysis of TH immunoreactivity

	Sham	ST-Sal	MFB-Sal	ST-LDnd	ST-LDdys	MFB-LDdys
Lateral ST	100.4±0.5	10.6±0.5[a]	10.9±0.8[a]	10.8±1.2[a]	12.0±1.3[a]	12.7±1.7[a]
Medial ST	100.7±0.6	23.1±3.5[c]	10.5±0.6[b]	22.9±3.0[c]	21.4±1.6[c]	11.6±0.2[b]
SNpc	100.3±2.0	20.5±5.4[a]	10.6±1.6[d]	29.3±5.4[c]	19.3±1.2[a]	11.2±0.7[d]
VTA	99.9±1.8	55.3±9.7[c]	26.3±3.1[b]	59.3±6.9[c]	50.9±5.7[c]	22.9±1.3[b]