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Characterization of long-term motor deficits in the 6-OHDA model of Parkinson's disease in the common marmoset

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Research aimed at developing new therapies for Parkinson's disease (PD) critically depend on valid animal models of the disease that allows for repeated testing of motor disabilities over extended time periods. We here present an extensive characterization of a wide range of motor symptoms in the 6-OHDA marmoset model of PD when tested over several months. The severity of motor deficits was quantified in two ways: i) through manual scoring protocols appropriately adapted to include species specific motor behavior and ii) using automated quantitative motion tracking based on image processing of the digital video recordings. We show that the automated methods allow for rapid and reliable characterization of motor dysfunctions, thus complementing the manual scoring procedures, and that robust motor symptoms lasting for several months could be induced when using a two-stage neurotoxic lesioning procedure involving one hemisphere at a time. This non-human primate model of PD should therefore be well suited for long-term evaluation of novel therapies for treatment of PD.

1. Introduction

When investigating new therapeutic approaches for PD, researchers crucially depend on valid animal models of the disease and reliable methods to assess the symptoms displayed. Although several different animal models of PD exist, the preferred choices by many labs today are the 6-hydroxydopamine (6-OHDA) lesioned rat or the MPTP-lesioned non-human primate [1][2], since these models have proven to capture several important features of the disease. However, MPTP is a severe safety hazard to the personnel handling the animals and strict procedures and appropriate laboratory safety equipment are an absolute requirement [3]. Consequently, there have also been a number of studies aimed at developing a primate model of PD based on intracerebral 6-OHDA lesions which would minimize the risk of inadvertent toxic exposure for researchers and animal care taking personnel that is associated with systemic MPTP treatment [4][5][6][7]. In parallel with the ongoing efforts to improve the reliability and validity of PD animal models, more sophisticated and diverse methods to assess severity of PD symptoms in animals has also been a key objective in the methodological development for several labs [8][9]. Given that the relevance of preclinical research ultimately is dictated not only by the validity of the model, but also to a great extent by the reliability and sensitivity of the testing methods used, this work aims towards further improvement of the procedures used to assess symptoms in animal models of PD. In particular, when evaluating new potential therapies, for example, neuromodulatory approaches like deep brain stimulation [10][11], or spinal cord stimulation [12][13], robust testing procedures are needed to allow researchers to repetitively assess the severity of the symptoms displayed over long time periods in response to changes in therapeutic interventions.

To this end, we have here developed new methods for behavioral assessment of PD symptoms in the 6-OHDA lesioned common marmoset (*Callithrix jacchus*). These procedures include manual scoring of PD symptoms according to an adapted PD motor rating scale, and automated movement tracking procedures based on digital video recordings. Using these methods, a thorough characterization of changes in motor behavior in nine 6-OHDA lesioned marmoset monkeys were conducted over a time period of several months. By testing the animals in four different symptomatic stages in a step-wise lesioning procedure, different levels of motor symptom severity could be characterized. The stages evaluated were: 1) intact state prior to lesion; 2) after unilateral lesion; 3) after bilateral lesion; and 4) after bilateral lesion plus treatment with the dopamine synthesis blocker alpha-methyl-p-tyrosine (AMPT). The order of successive lesions and testing procedures in PD model are shown in Fig. 1A.

2. Material and Methods

2.1.Animals and housing conditions

Nine adult male common marmosets (*Callithrix jacchus*) 300-550g were used in the study. The animals were housed in pairs in cages (1.0 x 1.0 x 2.3 m^3) in a vivarium with natural light cycle (~12/12 hours). Each cage have cover for rain and direct sun light, and the vivarium has a mobile roof that can be opened or closed according to weather changes such as heavy rain. Common marmosets are endemic to Northeast Brazil where the vivarium is located; thus ensuring suitable temperature, humidity and light conditions. To enrich the housing environment, cages are supplemented with elements such as sticks, tubes, ropes and ladders. Each cage has a small wooden box used as nest for protection and sleeping. Animals are offered two meals a day consisting of primate chow, local fruits, vegetables, mealworm larvae, gum arabic, dairy products, grains, eggs, and meat under the supervision of a veterinarian.

All animal procedures were carried out according to approved protocols by AASDAP Ethics Committee and strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). This project was approved by SISBIO/Brazilian Institute of Environment and Natural Resources (IBAMA) (No. 20795-2).

2.2 Procedure for 6-OHDA injections

The animals were initially sedated with ketamine (10-20 mg/kg ,i.m.) and atropine (0.05 mg/kg i.m.) followed by deep anesthesia with isoflurane inhalated though a nose cone, to be finally intubated with an endotracheal tube and ventilated with artificial ventilator to be maintained with isoflurane 1-5% in oxygen at 1-1,5 L/min during the surgery. One mL of 6-OHDA hydrochloride (4 mg/mL dissolved in 0.05% ascorbate/saline solution) was freshly prepared and stored protected from light on ice before use. Five injections (2 µL each) were made with a 32 gauge Hamilton syringe at 0.5 µL per minute into the medial forebrain bundle (MFB) in the following locations (Anteroposterior/Mediolateral/Dorsoventral from the interaural midpoint): 6.5/1.2/6.0; 6.5/1.2/7.0; 6.5/2.2/6.5; 6.5/2.2/7.5; 6.5/3.2/8.0, which corresponds to a slightly modified version of the protocol used by Annett et al. 1992 [5]. Anteroposterior coordinates were corrected according to the dimensions of the skull of each animal based on the anatomy atlas by Stephan et al., 1980 [14]. After each infusion, the needle was left in place for another 3 min to allow the spread of the solution through the cerebral tissue at the exact area of interest.

During the 3-5 days following the surgery, the animals received non-steroid anti-inflammatory analgesic, flunixin meglumine (1 mg/kg, s.c.) and dexamethasone (0.5 – 1 mg/kg, i.m.), and a supplementary high-energy liquid diet. After eight weeks, the same procedures were repeated for the second 6-OHDA lesion in the other hemisphere, as previously described by Mitchell et al. 1995 [4]. The second 6-OHDA lesion was made in the contralateral hemisphere to the preferred limb.

2.3 Adaptation to box and tower behavioral testing set-ups

Animals were accustomed to the behavioral testing procedures in a step-wise manner. First, while in their home cage, three times a week for two weeks, the animals were habituated to the food rewards used: 2x5x10 mm^3 (~60 mg) marshmallow pieces or mealworm (*Tenebrio molitor)* depending on the preference of each marmoset. Second, animals were accustomed to the transportation box (animals were allowed to explore the transportation box containing food baits while being free to return to their home cage at any time). Once showing interest in the transportation box, the animals were accustomed to a sound signaling entrance and another sound signaling exit from the transportation box. Animals were then trained to exit from the transportation box and explore the two different behavioral testing set-ups used in the study – a transparent cubic acrylic box (0.45 x 0.45 x 0.45 m^3) and a vertical tower (width x depth x height: 0.36 x 0.37 x 2.20 m^3) with seven horizontal bars located at different distances above ground (0.1, 0.2, 0.4, 0.6, 0.9, 1.25, and 1.75 m; Fig. 1B) [15]. In this training, pieces of marshmallows were placed on the floor of the box or on the bars in the tower testing set-up to encourage the animal to explore the environment. A disposable white sheet of ethylene-vinyl acetate foam covered the floor to preventing the animal from slipping. This training scheme was performed twice a week for two weeks (in a parallel study animals were trained to reach and grasp food rewards through holes in one of the walls - this behavior was not evaluated in the current study and the shelves were not baited). All the procedures were performed either between 10:30 and 12:00 or between 14:00 and 15:30, corresponding to the natural peak of motor activity (cf. Fig. 3C). The food rewards obtained during training of the task replaced the juice portion that the animals would normally receive in their home cages.

2.4 Automated assessment of motor activity in home cage

Spontaneous motor activity of two animals were collected using actimeters (Actiwatch Mini, CamNtech) worn in custom made vests inside theirs home cage. The actimeters collected data every two seconds for three consecutive days (72h) during the baseline, unilateral and bilateral periods. For the panel in Fig. 3B, the average raw motor activity of two consecutive 4am-6pm periods of the 72h-recording session is represented in relation to the date of the second 6-OHDA lesion (except for the unilateral lesion period of Monkey 6 where only one 4am-6pm period was used, since the data from the second period was not available). For the graphics of Fig. 3C, each recording was smoothed with a one-hour (1800 samples) moving average window sliding at every sample, divided in two full 24-hour periods, and the periods finally averaged.

2.5 Manual PD scoring

To evaluate the motor disability of the parkinsonian animals, we adapted the Unified Parkinson's Disease Rating Scale developed by Fahn and colleagues for the clinical setting [18] to fit aspects of non-human primate behavior based on previously developed procedures [16], [17].

The adapted scale consists of 16 categories scored from zero to three, which corresponds to absence of altered state to more intense symptomatology, respectively. Some categories involve

symptoms that were evaluated for each body part individually (i.e., limbs, trunk, head), each receiving a maximum score of 3, thus, these categories could reach a maximum of 15 (“*Tremor at rest”* and *“Tremor in motion”*) or 9 points (“*Fine motor skills”*), and were therefore subsequently normalized to 3 in order to facilitate the direct comparison of different categories of symptoms. Hence, the maximum total score of the scale is 16x3 = 48 points (Table 1).
The motor examination was performed in the animal’s home cage. Assessments occurred at two times of day: in the morning (~ 9 am) or afternoon (~ 5 pm). All tests were done before meals.

The quantified categories are the following:

i) Tremor at rest

[0]: Absent

[1]: Occasional or detected rarely

[2]: Frequent or easily detected

[3]: Continuous and intense

ii) Tremor in motion

[0]: Absent

[1]: Rarely detected, present during action

[2]: Moderate amplitude, present during action

[3]: Moderate amplitude, can interfere with feeding

iii) Freezing

[0]: Unhindered to move the body and show normal use of the limbs, e.g., in finding and grasping marshmallows in the reaching task

[1]: Difficulties in starting to walk, or in the initiation of particular movements. For example, when reaching for a marshmallow, the start of the reaching movement is delayed. In these cases the freezing episodes are short

[2]: Same as in [1], but the freezing episodes have a longer duration - between 5 and 10 seconds

[3]: Same as in [1], but freezing episodes last over 10 seconds

iv) Gait and locomotion

[0]: Walks normally according to pre-lesion locomotion patterns, with symmetrical limb use

[1]: Shows reduced walking activity and walks with mild asymmetry

[2]: Walks slowly, with asymmetry, and occasionally drags a limb (usually a hindlimb)

[3]: Unable to walk

v) Fine motor skills (scored for each arm independently)

[0]: Normal ability to grasp marshmallows

[1]: Grasps with difficulty

[2]: Grasps with difficulty and requires one arm to support the stance while using the other to grab the marshmallow

[3]: Totally unable to grasp marshmallows

vi) Bradykinesia (scored independently for limbs and trunk)

[0]: No difficulty in initiating or performing rapid and precise movements.

[1]: Difficulties in initiating movements and displays smoother and slower movements when reaching for marshmallows or moving around spontaneously

[2]: Clear delay in initiating movements and shows a marked slowing of movements in reaching and in spontaneous motor activity

[3]: Totally immobile

vii) Hypokinesia

[0]: Moves freely and is alert and responsive

[1]: Reduced activity, moves with less speed

[2]: Low spontaneous activity, moves when provoked

[3]: Totally immobile

viii) Rigidity

[0]: Moves freely; coordinated actions, absence of rigidity

[1]: Mild rigidity or rigidity apparent only when other body parts are moving

[2]: Striking stiffness, yet some complete movements are performed easily

[3]: Severe rigidity, no movements are performed or movements appear incomplete

ix) Body balance (Spontaneous behavior)

[0]: Normal stance and coordination

[1]: Compromised coordination, but is able to change from quadrupedalism to bipedalism without falling

[2]: Compromised coordination, unstable locomotion with occasional falls

[3]: Face down or lying in supine position unable to maintain any kind of stance

x) Body balance (Induced behavior elicited by food offering)

[0]: Normal stance and coordination

[1]: Compromised coordination but changes from quadrupedalism to bipedalism, without falling

[2]: Compromised coordination, unstable locomotion with occasional falls

[3]: Face down or lying in supine position unable to maintain any kind of stance

xi) Posture

[0]: Normal posture

[1]: Somewhat altered posture when standing, such as wider positioning of limbs. Resting with limbs and tail in abnormal body position

[2]: Hunched posture, abnormal trunk position; abnormal head posture (neck flexed or inclined to one side)

[3]: Unable to maintain posture, lying in supine or lateral position

xii) Startle response

[0]: Immediate, robust threat response

[1]: Slightly diminished or delayed response, threats with open mouth

[2]: Minimal or much delayed response, no open mouth threat

[3]: No response to provocation

xiii) Climbing

[0]: Normal

[1]: Climbs with difficulty. Slow on the branches and home cage mesh. No falling

[2]: Very compromised. Climbs branches and cage mesh with great effort. Falling may occurs

[3]: Not able to climb

xiv) Gross motor skills (scored for each arm independently)

[0]: Normal limb use when grasping larger objects

[1]: Reduced ability to grasp larger objects to support body weight

[2]: Rarely is able to grasp larger objects to support body weight

[3]: Unable to grasp and hold large objects/structures

xv) Facial Expression

[0]: Normal

[1]: Slightly apparent decrease of facial expression (hypomimia)

[2]: Moderate hypomimia with lips separated during brief moments

[3]: Fixed face, severe or total loss of facial expression, lips separated in 6 mm or more

xvi) Vocalization

[0]: Normal quantity

[1]: Spontaneous vocalization reduced

[2]: Induced vocalization reduced

[3]: Absent

For the categories *"Climbing"*, *"Bradykinesia"*, *"Fine Motor Skills"* and *"Body Balance (Induced),"* mealworms or a piece of marshmallow were offered with tweezers to induce the desired motor behavior. For the evaluation of the category *"Rigidity"*, a blunt forceps was presented to the animal. Since the animal associates the forceps with food offering, it would normally grab it. Following grasping of the forceps, gentle ‘push and pull’ movements were made to evaluate the level of stiffness of the forelimb. The procedure was repeated for both forelimbs.

In experiments involving AMPT-treatment, manual PD-scoring was performed six hours after the first injection.

2.6 Automated tracking procedures

Digital video recording were performed in the two testing set-ups. Two cameras (AVT – Stingray F033C, 80 fps) were used for digital video recordings in the acrylic box, from top and side views whereas tower activity was recorded using a single front view camera (AVT – Stingray F033C, 80 fps) (Fig. 1B). Motion tracking in the two setups was performed using similar methods. Software tools were developed in MATLAB and included mex-implementations (MATLAB compiled c-code; Mathworks Inc.). Constant light conditions during each recording session eliminated the need for advanced background models. Hence, a simple algorithm where each pixel is modeled as belonging to one of two Gaussian distributions was employed. The two distributions were estimated for each pixel by iterating through a sufficient number of frames of the video and updating the estimated parameters of the most probable distribution. In this case the background is contained in the brighter distributions, as the animals' image in these experiments was always darker than the actual background. After subtracting the background, the resulting foreground images were used in the shape analysis. By assuming that the two-dimensional image of the monkey in each camera plane is approximately elliptically shaped, the position and orientation of the animal could be estimated by the position and orientation of the three-dimensional ellipsoid that best fitted the foreground images. Given a measured foreground image F in a given camera and an estimated foreground image M generated by projection of the ellipsoid onto the camera plane, the matching quality is defined as

$$q = \frac{\sum_{i,j} \min\big(F(i,j), M(i,j)\big)}{\sum_{i,j} \max\big(F(i,j), M(i,j)\big)}$$

where i and j are the pixel coordinates. When multiple cameras were used, the combined quality measure was created by multiplying the individual q-scores. Note that the foreground image M is not actually computed, but instead the quality measure was computed using the projected quadric matrix for the ellipsoid. Movement tracking in time was then carried out by using the last known location to initiate estimation for a given frame followed by step-wise improvements of the matching quality by gradual adjustments of the parameters of the estimated ellipsoid. These calculations were performed for every frame in the video, resulting in the vectors ($\boldsymbol{x}, \boldsymbol{y}, \boldsymbol{z}, \boldsymbol{\theta_x}, \boldsymbol{\theta_y}, \boldsymbol{\theta_z}$) describing the position and orientation of the estimated ellipsoid. Each vector will therefore be of the length N, where N is the number of frames in the video. Note that in the tower experiments, the z-coordinate was fixed and not estimated due to the use of only one camera (see http://homepages.inf.ed.ac.uk/rbf/VAIB14PAPERS/palmer.pdf for technical details on tracking procedures).

2.7 Automated extraction of kinematic parameters presented in plots

Relevant metrics summarizing changes in kinematic parameters over the different experimental conditions were constructed from the tracking data. From the (x,y,z) position vectors, speed was estimated as the Euclidean distance between (x_i, y_i, z_i) and $(x_{i+k}, y_{i+k}, z_{i+k})$, divided by k, frame number difference, and multiplied by the time resolution. Locomotion bouts were detected by applying a threshold on the acquired speed vectors. A locomotion bout was defined as the period of time where instantaneous speed was uninterruptedly greater than the chosen threshold. To improve robustness, multiple values of k were used for this detection, and all different estimates of the speed at a time have to be greater than the chosen threshold (approximately corresponding to a speed of 0.04 m/s).

From each locomotion bout, a number of different parameters were obtained: maximum speed, average speed, distance covered, duration and maximal acceleration.

2.8 Tyrosine-hydroxylase staining and quantification

After the period of the experiments, the animals were sacrificed by intracardiac perfusion after deep sedation with ketamine (40 mg/kg i.m.); (xylazine 0.04 mg/kg i.m.) and atropine (0.05 mg/kg i.m.). Intracardiac perfusion was performed with 0.9% saline solution and heparin at 37 °C, followed by 4% paraformaldehyde in phosphate buffer, 0.1 M (pH 7.4), cooled to 4 °C. The brains were removed and postfixed in the same solution for 2h, washed in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 24 hours, cryoprotected in 20% following 30 % sucrose solution at 4 °C, and finally rapidly frozen for cryostat embedding in Tissue-Tek medium. The brains were kept in a freezer at -80 °C until sectioned coronally at 50 µm in a cryostat.

Immunohistochemical staining was performed free-floating or on sections mounted directly on electrically charged glass slides. The sections were processed for immunohistochemical detection of tyrosine hydroxylase (TH) in substantia nigra and in striatal regions using modifications of the protocol of Eslamboli et al. (2003) [19].

The sections were washed in 0.1M phosphate buffer (PB) for 5 minutes. Then, incubated in 1% hydrogen peroxide/methanol solution for 20 minutes to remove endogenous peroxidase activity, and rinsed in 0.05% phosphate buffer-Tween 0.05% (PB-T) for 5 minutes. Thereafter, the sections were confined with the aid of a hydrophobic PAP pen and incubated in 10% goat normal serum diluted in 0,1 PB for 30 minutes. Excess serum was removed and sections were incubated in the primary anti-TH (rabbit polyclonal antibody; 1:500; diluted in normal serum/PB) overnight at room temperature in a humidity chamber to prevent air-drying of the tissue sections. The sections were washed with PB-T (5 min) and incubated in biotinylated goat anti-rabbit secondary antibody (1:200, diluted in PB; Vector Labs) for two hours. After that, the sections were washed again with PB-T (5 min) and incubated in avidin-biotin-peroxidase solution (Vectastain Standard ABC kit, Vector Laboratories) for one hour.

After removal of the ABC solution, the sections were washed in PB (5 min) and placed in a solution containing 0.03% 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma) and 0.001% hydrogen peroxide in 0.1M PB. The reaction was monitored in a light microscope. The sections were washed and slides were left to dry overnight. After dehydration through a series of graded alcohols and clearance in xylene, the slides were cover-slipped using Entellan mounting medium.

2.9 Quantification of striatal and nigral tyrosine hydroxylase immunoreactivity

The tissue samples were mounted and photographed using a microscope with the same camera configuration and under identical illumination conditions. TH reactivity in both striatum (caudate and putamen) and in substantia nigra pars compacta (SNc) was assessed by computer densitometry using digital images captured from a camera (CX9000, MBF Bioscience) attached to the microscope (light field Nikon Eclipse 80i - 10x and 20x objectives). TH-reactivity across the striatum was assessed by optical densitometry using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). Measurements were obtained using a 0.2 mm^2 square window positioned in different regions throughout the striatum (60 samples per striatum and animal). To reduce the effects of within-group variability, a normalized scale based on the reactivity for TH of the internal capsule (white matter) was adopted (average over measurements of 10 different sites using the same window). For each animal, a contrast index was calculated according to the equation: $C = (G–W)/(G+W)$ [20], in which G is the average optical density of striatal tissue, and W is the optical density of the white matter (internal capsule). To count TH-labeled cells, we used at least three sections per animal. For the different positions along the rostral-caudal axis (rostral, central and caudal area), the boundaries of the SNc were defined in each section according to the atlas by Paxinos et al. [21] and the area of the SNc was calculated using the sections from control animals (it was not possible to identify the SNc contours in the lesioned animals because of the substantial loss of dopaminergic neurons from 6-OHDA treatment). Cells labeled with TH within the defined areas were subsequently counted (StereoInvestigator system, MBF Bioscience Inc) and the resulting cell densities were expressed as TH^+ cells/mm^2.

2.10 Statistical analyses

The statistical tests used in the study are specified in the main text and in the figure legends together with the data used for the respective test. Analyses of significance were performed using either Matlab functions or GraphPad Prism 5.01 software.

3. Results

3.1 Acute effects of 6-OHDA lesions

Immediately following the first lesion, animals showed a rigidity in the limbs and visuospatial neglect contralateral to the lesioned hemisphere and head position deviation ipsilateral to it [22]. In addition, animals showed ipsilateral body rotation while trying to ambulate, and difficulty to use the forelimb contralateral to the lesioned hemisphere. In spite of these evident motor symptoms the animals were still able to feed themselves in their home cages (as indicated by a < 10% weight loss following surgeries). At least eight weeks later, the animals were exposed to a second injection of 6-OHDA in the opposite hemisphere (Fig. 1A). Directly following this second lesion, animals generally showed similar but more severe motor impairments, in some cases requiring special care when animals had difficulties feeding themselves to ensure weight loss would not exceed 10% of total body weight during the first two weeks following surgery [23]. Animals were allowed to recover for two weeks before assessments of PD symptoms commenced.

3.2 Evaluation of motor symptoms using an adapted PD motor disability rating scale

In the manual assessment of PD-symptoms a total of 16 different categories were evaluated: (1) resting tremor, which was not observed in this model; (2) tremor in motion and sporadic postural tremor; (3) episodes of freezing - brief periods of sudden immobility when initiating quadripedal locomotion or goal directed reaching; (4) uncoordinated gait - inaccurate positioning of the limbs and wobbling of the trunk during locomotion (in the literature referred to as clumsy, poor-balanced gait; Eslamboli, 2003); (5) deficits in fine motor skills - difficulty in using arms to grab any food offered (in some animals the weakness was exacerbated by a worsening of gross motor skills, see below); (6) bradykinesia - noticeable slowing of the execution of movements; (7) hypokinesia - a general reduction in motor activity (motility, grooming, climbing); (8) rigidity - particularly noticeable in forelimbs during extension; (9 and 10) body balance - abnormal body positions and difficulty to rest on branches; (11) hunched posture; (12) a slowed startle response

- animals would not respond to alarm vocal signals from mates; (13) slowed climbing; (14) loss of gross motor skills - for example, inability to grasp branches; (15) episodes of hypomimia - reduction of the marmoset's typical behavior of maintaining eye contact and impaired inability to display facial expression in response to interaction with care givers; and (16) lack or decrease of vocalizations (marmosets use vocalizations abundantly to communicate between them).

For each of these 16 categories, the severity of motor disability was repeatedly evaluated in every individual in a total of eight animals under different degrees of Parkinsonism. Following the first lesion, stable parkinsonian symptoms were observed in all individuals over the more than 8 week long testing period (average score [mean±SEM], week 1-8: 6.9±1.0; Fig. 2A, left). After the second lesion, symptoms were on average more severe compared to the first unilateral lesion during the corresponding assessment period (average score week 1-8: 12.6±0.7, Fig. 2A, right). Animals were then monitored for another few months and persistent symptoms were confirmed. However, during these extended testing periods a certain degree of spontaneous recovery was observed resulting in a gradual decline of the total PD score over a 32 week period (Fig. 2A). Severe Parkinsonism could, however, nevertheless always be transiently reinstated for ~18 h through systemic treatment with the dopamine synthesis blocker AMPT (average score under AMPT effect for week 1-16: 24.6±1.8; week 17-32: 19.7±1.0. Interestingly, the degree of functional recovery varied substantially between different types of motor symptoms. When analyzing the PD-scores for each category of symptoms divided into 8-week periods following the second lesion it became evident that for example symptoms related to locomotion and body balance during spontaneous behavior showed negligible improvements over time (Fig. 2B). These findings indicate that quantitative assessments of spontaneous locomotor behavior could be particularly useful in experiments where testing periods lasting over several months are required.

3.3 Twenty-four hour recordings of motility in the home cage

As a complement to the detailed manual assessments of dysfunctions in motor behavior, the overall spontaneous motor activity during 72h periods in the home cage was also recorded in two animals. It was found that the absolute amount of motor activity was clearly decreased following the first and second lesion, with a relative decrease after unilateral lesion corresponding to: -44% and -39%, and after bilateral lesion: -78% and -36% for the two monkeys, respectively (Fig. 3A, B). At the same time, the characteristic variations in the relative amount of motor activity displayed throughout the day-night cycle was comparatively preserved also in the parkinsonian state (Fig. 3C).

3.4 Automatic assessment of locomotive activity in the Tower testing set-up

In each testing session, the spontaneous locomotion of the animal was recorded for 5 min in 120 testing sessions in a total of 7 animals. The distance travelled during the testing session was subdivided into vertical and horizontal translation (Fig. 4A). It was evident that intact animals were considerably more active than lesioned animals and that the distance travelled successively declined in the more severe PD models (Fig. 4B). On average the distance traveled (horizontal/vertical) in meters per minutes for animals in the four different stages of Parkinsonism were (mean±SD), intact: 1.51±0.52/2.54±1.41, hemilesion: 0.81±0.41/1.16±0.85, bilateral: 0.50±0.28/0.49±0.29, bilateral+AMPT: 0.17±0.06/0.18±0.08 (Fig. 4B; $p<0.05$, Kruskal-Wallis). Furthermore, healthy individuals preferred staying on the bars positioned relatively higher up in the tower in contrast to the parkinsonian animals resulting in significant differences in mean expectation values in height over ground for the four groups (Fig. 4C; $p<0.05$, Kruskal-Wallis). Finally, we also observed that when moving between different heights, healthy individuals often displayed longer uninterrupted movement bouts involving multiple transitions between different levels, whereas the lesioned animals moved more frequently one level at a time (fraction of multi-level transitions for the four groups were: 0.23±0.14, 0.09±0.07, 0.03±0.03, 0.05±0.10; $p<0.05$, Kruskal-Wallis Fig. 4D).

3.5 Automatic assessment of locomotive activity in the Box testing set-up

Spontaneous locomotion in the transparent cubical box was quantified from ~5min recordings in a total of 120 testing sessions in 4 animals. Similarly to the tower test, the distance travelled was subdivided into vertical and horizontal translation and in agreement with the behavior in the tower the distance travelled was clearly reduced in the more severe PD models. On average the distance travelled (horizontal/vertical) per minute for the four groups were (mean±SD), intact: 2.34±0.86/0.55±0.39, hemilesion: 0.96±0.40/0.36±0.41, bilateral: 0.23±0.12/0.04±0.03, bilateral+AMPT: 0.14±0.10/0.04±0.05 (Fig. 5A; $p<0.01$ for both horizontal and vertical distance, Kruskal-Wallis). A more detailed analysis of the locomotion bouts revealed further differences in the pattern of locomotion. We found that 1) bout duration, 2) bout maximum speed, 3) bout distance, as well as 4) frequency by which bouts of locomotion were displayed were all reduced in parkinsonian animals (Fig. 5B). Finally, in order to verify that the motor deficits observed were stable over extended time periods, the individual experiments were ordered and analyzed with respect to the time of assessment in relation to the two lesion procedures. To eliminate any inter-individual variability, all the analyzed features of the locomotive behavior were normalized to the motor behavior displayed by each individual during baseline conditions. While slight variations were found between different recording sessions during each of the three conditions, a much greater difference was observed between intact, hemilesioned and bilaterally lesioned animals. Notably, these differences persisted over several months and were found to be particularly evident for bout distance and the frequency by which bouts of locomotion were displayed (Fig. 5C).

3.6 Immunohistochemical verification of 6-OHDA lesions

Subsequent to these extensive characterizations of behavioral changes during parkinsonian conditions, *post mortem* tissue analyzes were performed. Immunohistochemistry for tyrosine hydroxylase (TH) was used to quantify the extent of the lesions (Fig. 6A, B). A reduction in the number of TH-positive cells of the midbrain dopaminergic neurons projecting to the forebrain in the lesioned hemispheres was confirmed. The cell densities (number of cells/mm^2) were (mean±SEM), 57.66 ± 6.23 and 139.01±12.13 in bilaterally lesioned and control animals, respectively ($P<0.0001$, $U=28$, Mann-Whitney U-test; Fig. 6C, bottom panel). The axonal terminal density of TH positive cells projecting to the caudate-putamen was also quantified. A contrast index was used to quantify the TH-staining in relation to background staining (see Methods for detail) showing a significant reduction of TH-immunoreactivity in lesioned animals vs. controls in both the caudate nucleus (0.155±0.01 vs. 0.254±0.02; $P<0.05$, U=112, Mann-Whitney U-test) and in putamen (0.135±0.02 vs. 0.213±0.02; $P<0.05$, U=109, Mann-Whitney U-test; Fig. 6C, top panel). Taken together, the average staining intensity of terminals in the caudate-putamen of lesioned animals was 44%, and the density of stained midbrain cells 41% compared to intact animals.

4. Discussion

Non-human primate models have a key role in PD-research aimed at understanding the underlying pathophysiology of the disease, as well as for the development of new treatment strategies. Whereas experiments in rodents in many cases can provide important insights in the early phase of basic PD-research, results are not always transferable to humans. In particular, the large difference in overall neuroanatomical complexity between the rodent and primate central nervous system can sometimes make findings in rats and mice less clinically relevant. The possibility to perform large scale experiments in the MPTP-treated macaque - which by many researchers is regarded as the most valid model of PD - is on the other hand very limited due to the high costs associated with housing and treating these larger primates and the safety precautions required for safe handling of these animals in order to avoid inadvertent neurotoxic exposure. In this perspective the 6-OHDA marmoset model of PD, which we here thoroughly characterized, may present a valuable complement.

Investigations aimed at developing prospective treatments for PD generally demand long evaluation periods, it was therefore important to systematically evaluate the marmosets with respect to a range of motor deficits over a time period of several months following lesions. While a recovery of certain motor functions was observed after about three months in the detailed manual PD-scoring assessments, other symptoms remained stable also after more than six months following lesions, indicating that this model may indeed be useful for the purpose of evaluating novel PD therapies under chronic disease conditions. Moreover, in experimental situations where severe Parkinsonism is desired, the additional pharmacological treatment with the dopamine synthesis inhibitor AMPT reproducibly induced marked motor disability in all animals tested. In spite of the comparatively severe symptoms that are transiently induced under such conditions, these tests were well tolerated and could be repeated multiple times in all animals.

The use of automated procedures for the analysis of spontaneous locomotive behavior provided important information on motor dysfunctions adding to the outcome of the manual scoring of PD symptoms. Both in 24h home cage recordings and in the shorter testing sessions in the Tower and Box set-ups, consistent differences between the different parkinsonian states were observed. In fact, in certain respects the automated procedures showed a greater sensitivity than the manual scoring, as evident from the persistent reduction in locomotor-related kinematic parameters such as bout frequency and total distance travelled, which could be established over very long time periods following the second lesion, even when a functional recovery of some other motor functions resulted in a gradual decrease of the total PD-score over time. Taken together, given the complex pattern of motor dysfunctions revealed by the manual scoring procedures, on the one hand, and the robust identification of motor symptoms using the automatic techniques, on the other, the current findings suggest that a combined automatic/manual approach is preferable in order to capture the full range of PD motor symptoms over extended time periods in this model of PD. It can be concluded that, using the methods developed herein, the two-stage 6-OHDA marmoset model of PD provides a robust and reliable primate model of PD lasting for periods of months that can potentially have an important role in the future development of novel therapies.

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LEGENDS

Table 1

Summary of assessments performed in the nine male marmosets included in the study. Animals exposed to bilateral lesions were generally also assessed following the first lesion providing additional data to the hemilesioned group. When multiple tests were performed in the same animal in the Tower/Box set-up and through manual scoring, assessments were made during the same day to facilitate direct comparisons.

Fig. 1. Description of experimental procedures. (**A**) Timeline of experimental procedures. The two-stage 6-OHDA bilateral lesion procedure allowed for repetitive assessment of motor symptoms at gradually more severe stages of Parkinsonism over extended time periods. (**B**) Spontaneous locomotion was evaluated in two testing chambers designed to capture different types of locomotive behavior, including both horizontal and vertical locomotion in both set-ups. Left: Tower – two examples of typical movement bouts between bars locates at different heights are illustrated, blue lines denote the tracked movement traces and the red ellipses the position of the thorax as estimated by the image system. 3D image shows the total amount of locomotion displayed during a typical 5 min recording period in a healthy individual (Time represented in color code ranging from dark blue (t=0) to red (t=5 min), Right: Box - side and top view, respectively (color codes as in Tower).

Fig. 2. Manual scoring of motor impairments. **(A)** Total motor disability score of individual animals during ten weeks following the first unilateral lesion (n=4, yellow), 32 weeks following

the second lesion (n=6), and under additional treatment with the dopamine synthesis inhibitor AMPT (6h after 2x250mg/kg AMPT; n=6). On the y-axis, 48 points represents the highest possible total score when the partial scores of the 16 categories are added and zero corresponds to pre-lesion behavior for each individual. The average score during each testing period and condition is represented by the thick horizontal lines. (**B**) Normalized scores of motor impairment after the second lesion divided by symptom category and testing period, week: 1-8, 9-16, and 17-24 after lesion, and under the additional effect of AMPT (week 1-32 after lesion; mean values shown, error bars represents S.E.M.). Significant differences in average scores were found week [1-8] vs. [9-16] (α, $p<0.01$), [1-8] vs. [17-24] (β, $p<0.01$), [1-8] vs. [AMPT] (*, $p<0.05$), [9-16] vs. [AMPT] (#, $p<0.01$), and [17-24] vs. [AMPT] (*d*, $p<0.05$; ANOVA for repeated measures ($p<0.05$) with post hoc Bonferroni-corected paired tests).

Fig. 3. General activity in home cage before and after 6-OHDA lesions, measured in two animals during 72h-recordings using accelerometers. **(A)** Green panels show data collected during baseline conditions prior to lesioning surgery, yellow and red panels represent the activity displayed after unilateral and bilateral lesions, respectively. **(B)** Average activity recorded during the active periods of the day (4am-6pm) during the three different conditions (baseline, unilateral and bilateral lesion). On the x-axis, day zero corresponds to the day of the second lesion, days -50 to 0 to unilateral lesion, and earlier than day -50 represents baseline recordings. **(C)** The average activity displayed during circadian cycle for baseline and bilateral lesion conditions.

Fig. 4. Behavioral testing in Tower. Quantification of spontaneous horizontal and vertical locomotion in the tower testing set-up reveals clearly different behavior in the four different stages of Parkinsonism. **(A)** Example of vertical (green trace) and horizontal (brown trace) displacement of the animal during a 5 min recording session (height over ground for the different bars are denoted on the axis to the left, tracking data quantized to the levels are shown in the thick lines and the original tracking data are shown as thinner lines). **(B)** Summary of the average horizontal and vertical distance travelled in all recordings (median, 25% and 75% percentiles shown in boxes, whiskers denote range). Note the successive decrease in distance travelled in the

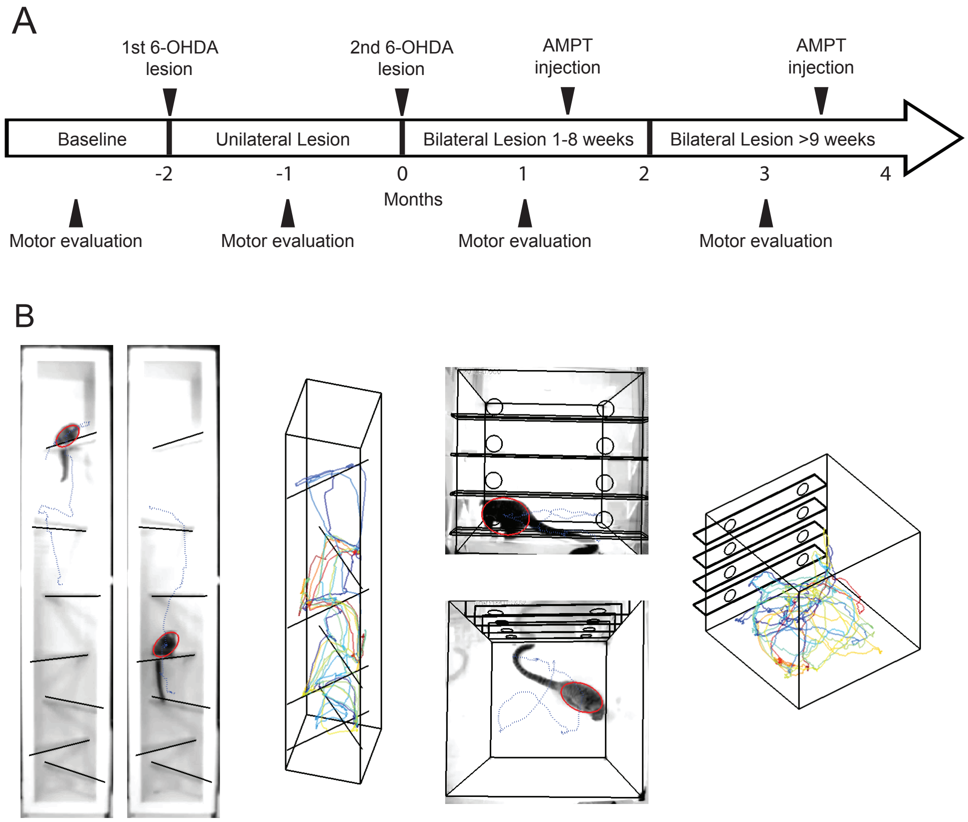
more severe PD models [green=intact (I), yellow=hemilesion (H), red=bilateral lesion (B), black= bilateral lesion + AMPT (A)]. **(C)** A change in the preference for bars located relatively higher up to bars at lower levels with increasing severity of Parkinsonism. The relative amount of time spent on the respective level is indicated by colored bars. **(D)** Transition matrices describing the probability that the animal will move from a certain level (row) to another level (column). Levels are denoted from G to 7, where G is ground and 7 is the highest bar, each treatment group is normalized to the total number of transitions observed in that condition. It can be noted that animals move less frequently more than one level at a time and between the higher bars with more severe parkinsonian symptoms.

Fig. 5. Behavioral testing in Box. Quantification of spontaneous locomotive behavior in the box set-up reveals marked differences between the different degrees of Parkinsonism. **(A)** The average horizontal and vertical and distance travelled per minute for animals grouped according to severity of Parkinsonism. **(B)** Differences in bout duration, distance, speed and frequency, shown for the different groups in histograms representing the relative frequency of observed parameter values in four equally sized intervals of the full range for the respective parameters. **(C)** Bout frequency and total distance travelled shown for all recorded sessions divided according to lesion group (mean and SD indicated by horizontal line and box, respectively). Note the robust reductions following both the first and second lesion which persist throughout each >8 week long testing period. [Color code: green=intact (I), yellow=hemilesion (H), red=bilateral lesion (B), black= bilateral lesion + AMPT (A)].

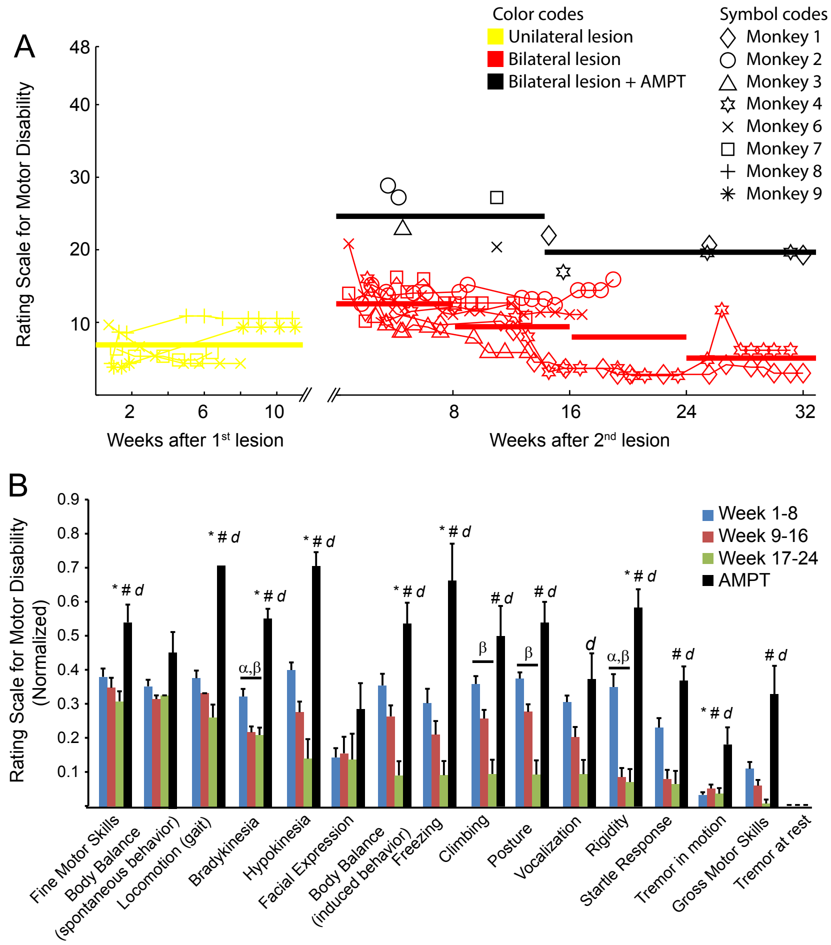
Fig. 6. Histological confirmation of dopaminergic lesions. **(A)** Examples of tyrosine hydroxylase (TH) immunolabeling in the caudate (Cd), putamen (Put). Immunohistochemistry of TH showed intense labeling of Cd-Put in both hemispheres in control animals (top panel). Lesions performed in the left hemisphere induced a pronounced loss of labeling in Cd/Put on this side (middle panel). In bilaterally lesioned animals, both sides of Cd/Put were strongly affected, showing much weaker TH-staining (bottom panels) compared to controls. **(B)** Examples of TH immunolabeling in the substantia nigra (SN). In SN, TH-labelling of cell bodies of midbrain

dopaminergic neurons in the intact brain is evident but is strongly reduced in lesioned hemispheres. (**C**) Quantitative summary of TH-immunolabeling of terminals in the caudate-putamen (top) and of cell-bodies in substantia nigra (bottom) confirming extensive dopaminergic lesions.

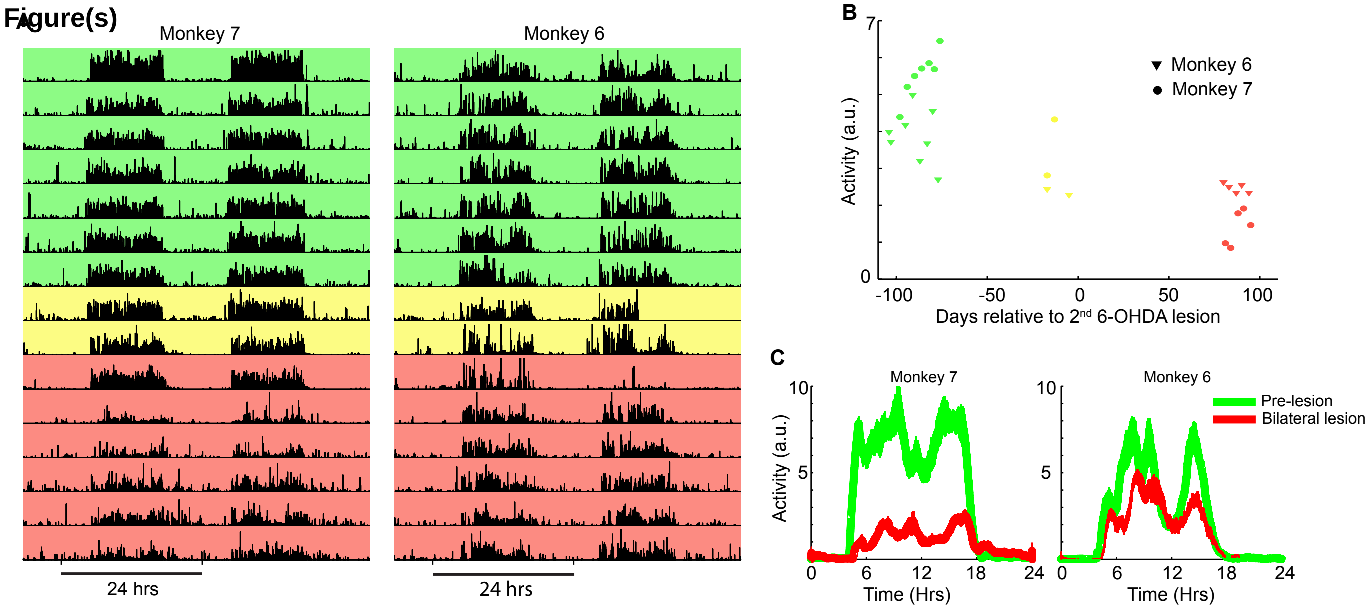
Figure(s)


A
1st 6-OHDA lesion
2nd 6-OHDA lesion
AMPT injection
AMPT injection
Baseline
Unilateral Lesion
Bilateral Lesion 1-8 weeks
Bilateral Lesion >9 weeks
-2
-1
0
1
2
3
4
Months
Motor evaluation
Motor evaluation
Motor evaluation
Motor evaluation
B

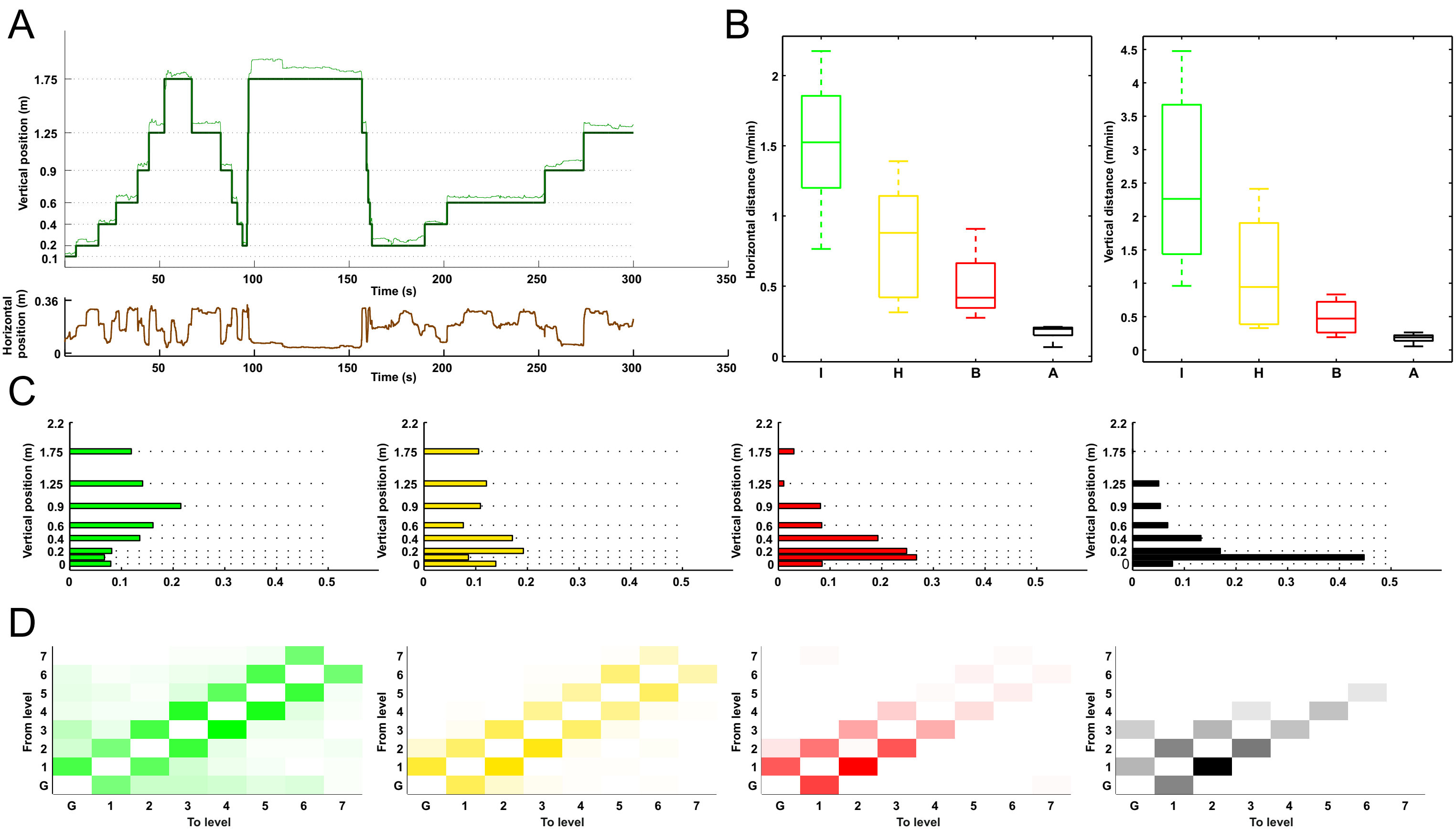
Figure(s)


A
Color codes
Unilateral lesion
Bilateral lesion
Bilateral lesion + AMPT
Symbol codes
Monkey 1
Monkey 2
Monkey 3
Monkey 4
Monkey 6
Monkey 7
Monkey 8
Monkey 9
Rating Scale for Motor Disability
Weeks after 1st lesion
Weeks after 2nd lesion
B
Rating Scale for Motor Disability (Normalized)
Week 1-8
Week 9-16
Week 17-24
AMPT
Fine Motor Skills
Body Balance (spontaneous behavior)
Locomotion (gait)
Bradykinesia
Hypokinesia
Facial Expression
Body Balance (induced behavior)
Freezing
Climbing
Posture
Vocalization
Rigidity
Startle Response
Tremor in motion
Gross Motor Skills
Tremor at rest

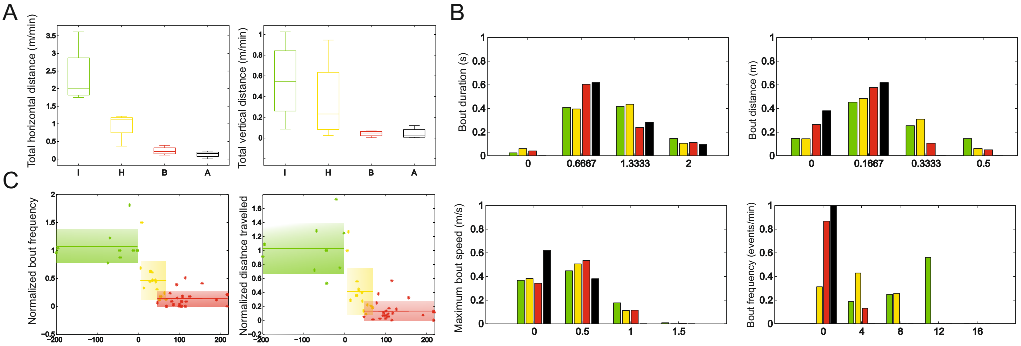
Figure(s)


A
Monkey 7
Monkey 6
24 hrs
24 hrs
B
Activity (a.u.)
Monkey 6
Monkey 7
Days relative to 2nd 6-OHDA lesion
7
0
-100
-50
0
50
100
C
Monkey 7
Monkey 6
Pre-lesion
Bilateral lesion
Activity (a.u.)
Time (Hrs)
Time (Hrs)
10
5
0
6
12
18
24

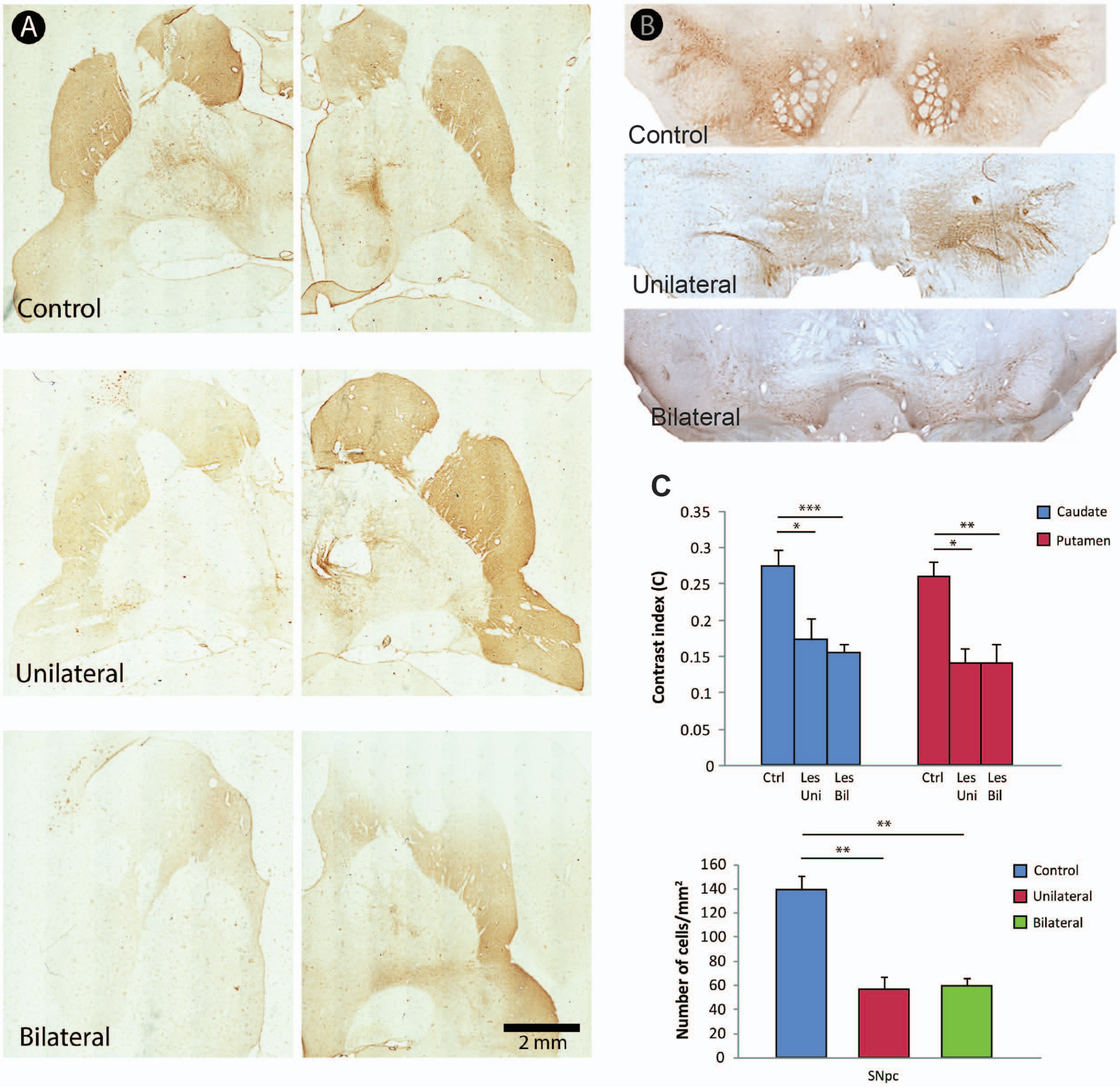
Figure(s)


A
Vertical position (m)
1.75
1.25
0.9
0.6
0.4
0.2
0.1
50
100
150
200
250
300
350
Time (s)
Horizontal position (m)
0.36
0
B
Horizontal distance (m/min)
Vertical distance (m/min)
I
H
B
A
C
Vertical position (m)
2.2
0
0.1
0.2
0.3
0.4
0.5
D
From level
To level
G
1
2
3
4
5
6
7

Figure(s)


A
B
C
Total horizontal distance (m/min)
Total vertical distance (m/min)
I
H
B
A
Bout duration (s)
Bout distance (m)
0
0.6667
1.3333
2
0
0.1667
0.3333
0.5
Normalized bout frequency
Normalized disatnce travelled
Maximum bout speed (m/s)
Bout frequency (events/min)
0
0.5
1
1.5
0
4
8
12
16

Figure(s)



A
Control
Unilateral
Bilateral
2 mm
B
Control
Unilateral
Bilateral
C
Contrast index (C)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
*
**
*
Caudate
Putamen
Ctrl
Les Uni
Les Bil
Ctrl
Les Uni
Les Bil
Number of cells/mm²
160
140
120
100
80
60
40
20
0
**
**
Control
Unilateral
Bilateral
SNpc

Table(s)

Animal	Lesion	Manual PD assessment	Tower test	Box test	Activity in home cage
1 - Beto	bilateral	X	X		
2 - Dedé	bilateral	X		X	
3 - Max	bilateral	X			
4 - Tom	bilateral	X	X		
5 - Kaká	bilateral		X	X	
6 - Pele	bilateral	X	X	X	X
7 - Zeca	bilateral	X	X	X	X
8 - Deco	unilateral	X	X		
9 - Kadu	unilateral	X	X		