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Chronic L-DOPA induces hyperactivity, normalization of gait and dyskinetic behavior in MitoPark mice

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Dopamine (DA) replacement therapy continues to be the gold standard treatment for Parkinson's disease (PD), as it improves key motor symptoms including bradykinesia and gait disturbances. With time, treatment induces side effects in the majority of patients, known as L-DOPA-induced dyskinesia (LID), which are often studied in animals by the use of unilateral, toxin-induced rodent models. In this study, we used the progressive, genetic PD model MitoPark to specifically evaluate bilateral changes in motor behavior following long-term L-DOPA treatment at three different stages of striatal DA depletion. Besides locomotor activity, we assessed changes in gait with two automated gait analysis systems and the development of dyskinetic behavior. Long-term treatment with a moderate, clinically relevant dose of L-DOPA (8 mg/kg) gradually produced age-dependent hyperactivity in MitoPark mice. In voluntary and forced gait analyses, we show that MitoPark mice with severe DA depletion have distinct gait characteristics, which are normalized to control levels following long-term L-DOPA treatment. The cylinder test showed an age-dependent and gradual development of bilateral LID. Significant increase in striatal FosB and prodynorphin expression was found to accompany the behavior changes. Taken together, we report that MitoPark mice model both behavioral and biochemical characteristics of long-term L-DOPA treatment in PD patients and provide a novel, consistent and progressive animal model of dyskinesia to aid in the discovery and evaluation of better treatment options to counteract LID.

Keywords: Automated gait analysis system, bilateral dyskinesia model, dyskinesia score, L-DOPA-induced dyskinesia, Parkinson's disease

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Parkinson's disease (PD) is a progressive disease in which motor deficits often begin on one side of the body and later involve both sides. The cardinal motor symptoms, bradykinesia, rigidity, tremor and postural instability, are directly related to dopamine (DA) depletion in the basal ganglia caused by the degeneration of DA neurons in substantia nigra. While treatment with the DA precursor L-DOPA restores motor function, disease progression advances and prolonged treatment leads to the development of side effects called L-DOPA-induced dyskinesia (LID).

To improve and investigate novel PD drug treatments, several animal models have been developed. The toxins 6-hydroxydopamine (6-OHDA) or MPTP are often used to unilaterally or bilaterally deplete DA in the striatum of rodents or primates. Following a short period of time, these animals display various degrees of PD motor symptoms.

The vast majority of dyskinesia studies are performed using unilateral 6-OHDA lesioned rats for which detailed rating scales have been developed that focus on asymmetrical movements of the upper body (Cenci *et al.* 1998). An additional LID model is the Pitx3$^{ak/ak}$ transgenic mouse, in which a deficiency in the homeobox transcription factor Pitx3 induces the bilateral depletion of DA restricted to the dorsal part of the striatum (Ding *et al.* 2007; Hwang *et al.* 2005). In this study, we use MitoPark mice, a tissue-specific knockout model based on the inactivation of the mitochondrial transcription factor (Tfam) specifically in nigral DA neurons. MitoPark mice have an adult-onset degeneration of DA neurons that is followed by motor deficits and they mimic different stages of disease. L-DOPA treatment ameliorates motor deficits in a DA depletion-dependent manner (Ekstrand & Galter 2009; Ekstrand *et al.* 2007; Galter *et al.* 2010). In contrast to unilateral 6-OHDA models, MitoPark mice do not display potential compensatory effects from the unlesioned hemisphere of the brain. Similarly, compared with MPTP models, where severe acute complications of the systemic toxin administration as well as spontaneous recovery complicate LID studies, the genetic PD model presents the advantage of lower inter-individual variability. Very different treatment schemes have been described to induce LID in animal models ranging from 6 to 50 mg/kg L-DOPA per day, and from 6 days to 5 weeks. We chose to study the effects of a moderate dose of L-DOPA (8 mg/kg) administered daily at the beginning of the active period of the mice, to avoid disruption of their circadian rhythm. During the 3 weeks of treatment, a battery of behavioral tests was repeatedly used to study the progression of L-DOPA-induced motor activity. We analyzed changes in gait parameters under voluntary and forced conditions using two different detection systems and compared the effect of long-term L-DOPA treatment at three different ages with increasing severity of DA depletion and describe the gradual development of hyperactivity and LID in this bilateral model of PD.

Material and methods

Animal subjects

MitoPark mice and littermate control mice of both sexes were used. For the generation of MitoPark mice, see Ekstrand *et al.* (2007). Briefly, MitoPark mice (Dat-Cre ± $Tfam^{loxP/loxP}$) were compared with healthy control littermates in which one or both Tfam genes were loxP-flanked ($Tfam^{-/loxP}$ or $Tfam^{loxP/loxP}$). The two mouse strains (Dat-cre and $Tfam^{loxP/loxP}$) are backcrossed to the C57BL/6J background for at least 14 and 10 generations, respectively. In addition, the strains are regularly backcrossed to the C57BL/6J background at least every tenth generation. Mice were housed up to five per cage with a 12/12 h light/dark cycle, 60% humidity and food and water *ad libitum*. At the start of the experiment, mice were 20, 24 or 30 weeks of age to model the increasing severity of PD motor symptoms. The age is used instead of the degree of DA depletion; at 24 weeks of age, DA depletion is ~80% of control littermates and ~95% at 30 weeks of age (Galter *et al.* 2010). Six to nine mice were used per experimental group in all behavior experiments, mostly equal numbers of male and females, with a total of 86 mice. The animal's health status was monitored throughout the experiment and the weight of the mice was measured. Mice were provided with moistened food pellets on the ground of the cage from the age of 20 weeks to counteract the gradual weight loss described earlier in detail (Galter *et al.* 2010). The study protocol was approved by the Stockholm North Ethical Committee on Animal Research.

Drugs administered

In the following, L-DOPA treatment refers to the combination of L-DOPA:benserazide 4:1. Madopar® Quick tablets (Roche AB, Bromma, Sweden) were ground, dissolved in physiological saline solution at the indicated concentration and the pH was adjusted to 7.2. Aliquots were frozen at −20°C until use. Although we have shown in a previous study that using the same doses of pure L-DOPA plus benserazide gave the same behavioral pattern in 30-week-old mice, we acknowledge that injecting (i.p.) the ground Madopar tablets might have produced side effects and increased the variability in the observed behavior. L-DOPA treatment was administered once daily for 3 weeks by an intra-peritoneal injection (i.p.) at the beginning of the dark period at 1800 h. Mice were randomly assigned to treatment with saline or L-DOPA.

Behavioral tests

All behavioral tests were performed at the beginning of the active phase of their circadian cycle (during initial hours of the dark phase). A timeline of the experimental setup is shown in Fig. S1.

Locomotor activity analysis

Horizontal and vertical locomotor activities were recorded on days 1, 10 and 20 of treatment. After a habituation period of 1 h to the dimly lit and noise-controlled room, mice were randomly placed in 40 × 40 cm^2 Plexiglas boxes (30 cm in height) and habituated to the locomotor boxes for 60 min. The mice were removed from the boxes to receive treatment with L-DOPA or saline and were immediately returned. Activity was recorded for 60 min with the VersaMax animal activity monitoring system (AccuScan Instruments, Columbus, OH, USA). Activities are expressed as the number of beam breaks per 5-min intervals. Locomotor activity was studied in 20-, 24- and 30-week-old animals that were treated with saline or L-DOPA.

Automated quantitative gait analysis

Gait parameters were analyzed with two different systems: voluntary gait with the CatWalk system (Noldus Information Technology, Wageningen, Netherlands) and forced gait with the TreadScan system (CleverSys Inc., Reston, VA, USA). MitoPark and control mice aged 24 weeks were included in the gait analyses. The tests were conducted at the beginning of the treatment period (on day 3) and repeated 2 weeks later on day 16 of treatment to observe cumulative effects of the treatment. The gait analyses were performed 30 min after L-DOPA or saline injection.

The CatWalk consists of a glass runway, a light bulb and a sensitive LCD camera underneath the glass plate. The light is internally reflected on the runway. When a paw is placed on the glass, light beams can escape the plate illuminating the paw area, which are recorded and afterwards analyzed with the CATWALK software v9.0. Each animal was exposed to four training sessions before the start of the treatment. A test session consists of three trial runs and was rated as compliant if a mouse crossed the runway in at least one of the three trials in 7 seconds. For most of the saline-treated MitoPark mice, it was difficult to complete the task in time; so, we adapted the set-up for this experimental group to analyze the gait parameters whenever more than 11 consecutive steps were performed.

The following parameters were examined: the base of support (BOS), stride length and the swing speed. The BOS is the average widths (cm) of either fore or hind paws. The stride length is the distance (cm) between successive placements of the same paw. The swing speed (cm/second) is the speed of the paw during the swing phase.

The TreadScan device, a transparent belt moving at adjustable constant speed, is illuminated from above and below and is videotaped from underneath with an LCD camera. The analysis of the recorded images depends on the contrast between the color of the paws and the body of the animal. Mice were trained three times before the start of the treatment.

The gait parameters were analyzed if more than 11 consecutive steps were performed, which was rated as compliant. The speed of the treadmill was adjusted to the genotype, treatment and day of treatment. All mice from the same experimental group were recorded at the same speed. Control mice were recorded on both experimental days at a speed of 21.5 cm/second, saline-treated MitoPark mice at 10 cm/second and L-DOPA-treated MitoPark mice at a speed of 10 cm/second at day 2 and 21.5 cm/second on day 16. We chose these velocities after the training sessions to achieve a long sequence of consecutive walking steps without interruption or running. The analyzed parameters were the same as in the voluntary gait analysis, the BOS (cm), stride lengths (cm) and swing speed (cm/second).

Cylinder test

This test was performed on days 2, 11 and 21 of treatment and mice of all three ages were evaluated. Mice were habituated to the dimly lit and noise-controlled room for 60 min before the start of each test.

To properly analyze the movements from all angles, the cylinders were placed in front of a mirror and recorded at a distance and angle that allowed the detailed visualization of the mouse. Thirty minutes after the daily injection of L-DOPA or saline, mice were placed into the cylinder and their behavior was filmed for 3 min. The behavioral response to treatment was analyzed in slow motion after the completion of the study on a computer screen and quantified with help of the JWATCHER software v. 1.2 (Blumstein *et al.* 2006). The following behaviors were evaluated and quantified during the 120 seconds in the middle of the 3-min recording:

1. Duration of rearing: time that mice stood on their hind legs and lifted both forelegs at least 3 cm from the ground, with or without leaning on the cylinder walls.
2. The number of times mice reared.
3. Forepaw dyskinesia: the time mice spent standing on the hind paws and repeatedly touched the cylinder wall with alternating forepaws (Movie S1).
4. Forepaw dyskinesia with turning steps: time mice spent in forepaw dyskinesia with simultaneous stepping movements of the hind paws in one direction, resulting in a combined rearing–turning and rapid forepaw movement along the cylinder wall (Movie S2).
5. Three paw dyskinesia: the time mice spent standing on the hind paws, repeatedly and alternately touching the cylinder wall with the forepaws, and simultaneously repeatedly lifting or touching the wall with one of the hind paws while bearing the weight on the other paw. Occasionally, the behavior changed to repeatedly jumping toward the cylinder wall and

touching it with three or all four paws while the weight was supported only by the tail (Movie S3).
6. Number of third paw wall contacts: number of times mice touched the cylinder wall with one or both hind paws.
7. Dyskinesia score is defined as the sum of durations mice spent with all of the three behaviors forepaw dyskinesia, forepaw dyskinesia with turning steps and three paw dyskinesia.
8. Duration of rotational behavior: defined as repeated rotations in close circles, with all four paws on the floor, indifferent of turning direction.
9. Time mice spent resting: including lying or sitting still, with all four paws on the cylinder floor or occasional grooming.

All behavior categories were also analyzed in controls as comparison but were not included in the statistical analysis.

Tissue preparation and immunohistochemistry

At 20 h after the last of 21 treatment days, mice aged 27 weeks were deeply anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde solution with 14% saturated picric acid in PBS. Brains were dissected, postfixed for 30 min at room temperature and transferred into 10% sucrose for cryoprotection. Sections of 10 μm thickness were mounted on Superfrost microscopy slides (Thermo Fisher Scientific Inc., Gothenburg, Sweden) and incubated overnight at 4°C with anti-FosB antibody (1:300, catalog number H-237; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary biotinylated antibody (Vector Laboratories, Burlingham, CA, USA) and avidin–horseradish peroxidase complex (Elite ABC kit; Vector Laboratories) were applied at room temperature, followed by DAB (Sigma-Aldrich, Stockholm, Sweden) as peroxidase substrate. Images were taken with an Axiophot microscope (Zeiss, Stockholm, Sweden) and the AXIOVISION software v. 4.8 (Zeiss, Stockholm, Sweden).

Tissue preparation and in situ hybridization

At 20 h after the last of 21 treatment days, mice aged 27 weeks were sacrificed by cervical dislocation; brains were dissected and flash frozen. *In situ* hybridization was performed essentially as described before (Westerlund *et al.* 2008). Sections of 14 μm thickness were incubated overnight with radiolabeled specific oligonucleotides, washed, dehydrated and exposed to autoradiographic films (BioMax; Eastman Kodak, Sigma Aldrich, Stockholm, Sweden). The developed films were digitalized and striatal mRNA levels were quantified using IMAGE J v64 program (Schneider *et al.* 2012). Prodynorphin-specific oligonucleotides correspond to nt: 989 to 940 in the reference mRNA sequence for prodynorphin ref/NM_018863_4 and bind to both transcript variants 1 and 2.

Statistical analysis

Statistical analysis was performed using the Prism, GRAPHPAD software version 5.04 (San Diego, CA, USA). Results are expressed as the mean ± SEM for all treatment groups. For the comparison of gait parameters between saline- and L-DOPA-treated MitoPark mice, a Student's *t*-test was used (Figs 2 and 3). Data from the motor activity analysis and from the cylinder test were analyzed with two-way analysis of variance (ANOVA) with treatment and treatment duration as variables (Figs 1, 4 and 5). Striatal FosB and prodynorphin mRNA levels were compared with Student's *t*-test (Fig. 6). Statistical significance was set as $P < 0.05$ for all analyses.

Results

All behavioral tests were performed at the beginning of the active phase of their circadian cycle, during the initial hours of the dark phase. To better model the treatment of PD patients, we used the commonly prescribed medication Madopar Quick. We also analyzed L-DOPA-induced motor behavior changes at three different ages (20, 24 and 30 weeks) to model decreasing DA levels in striatum and an increased severity of PD. To monitor the development of treatment-induced behavioral changes, all motor analyses were performed multiple times. On day 1, mice were randomized to saline or L-DOPA treatment and underwent the first locomotor activity test that was repeated on days 10 and 20. On days 2, 11 and 21 of treatment, motor behavior was analyzed with the cylinder test; and on days 3 and 16, gait parameters were analyzed (Fig. S1).

Development of L-DOPA-induced hyperactivity

In previous studies, we demonstrated that L-DOPA-induced locomotor activity crucially depends on the age of animals due to the progressive DA depletion in MitoPark mice over time (Galter *et al.* 2010). In this study, we used repeated treatment with 8 mg/kg L-DOPA. Three weeks of daily treatment induced a gradual increase of horizontal activity in all age groups of MitoPark mice (Fig. 1). Except for the first day in 20-week-old mice, the horizontal activity of L-DOPA-treated mice was always significantly higher compared with saline-treated MitoPark mice (20-week-old: time: $F_{2,26} = 18.2$, $P = <0.001$; treatment: $F_{1,26} = 71.82$, $P < 0.001$; interaction: $F = _{2,26} = 15.45$, $P < 0.001$; 24-week-old: time: $F_{2,44} = 28.31$, $P < 0.001$; treatment: $F_{1,44} = 496.52$, $P < 0.001$; interaction: $F_{2,44} = 10.74$, $P < 0.001$; 30-week-old: time: $F_{2,42} = 3.73$, $P = 0.032$; treatment: $F_{1,42} = 344.08$, $P < 0.001$; interaction: ns) (Fig. 1a–c). In 20-week-old animals, the vertical activity of L-DOPA-treated MitoPark mice increased successively and reached statistical significance after 20 days of L-DOPA treatment compared with the saline-treated group (time: $F_{2,26} = 4.52$, $P = 0.021$; treatment: $F_{1,26} = 13.04$, $P = 0.001$; interaction: $F_{2,26} = 5.09$, $P = 0.014$) (Fig. 1a′). In contrast, at the age of 24 weeks (time: $F_{2,44} = 5.63$, $P = 0.007$; treatment: $F_{1,44} = 112.1$, $P < 0.001$; interaction: $F_{2,44} = 5.36$, $P = 0.008$) (Fig. 1b′) and 30 weeks (time: $F_{2,46} = 7.20$, $P = 0.002$; treatment: $F_{1,46} = 79.76$, $P < 0.001$; interaction: $F_{2,46} = 6.48$, $P = 0.003$) (Fig. 1c′), the vertical activity was significantly increased for saline-treated MitoPark mice from day 1 forward even above control levels. The highest levels of vertical activity were detected in 24-week-old MitoPark mice following 10 days of L-DOPA treatment that reached 15 times the vertical activity of saline-treated littermates. The detailed representation of locomotor activity over time, before and after L-DOPA administration, in saline- and L-DOPA-treated mice is shown in Fig. S2. The L-DOPA-induced change in locomotor activity was entirely dependent on the MitoPark genotype, in which control littermates treated with L-DOPA for 3 weeks showed no increase in horizontal or vertical activity (Fig. S3).

L-DOPA-induced changes in voluntary gait

For voluntary gait, we assessed stride length, swing speed, BOS, as well as compliance. Compliance was defined as the ability of an animal to perform more than 11 consecutive steps without prompting for short-term (L-DOPA or saline treatment for 3 days) and long-term (L-DOPA or saline treatment for 16 days) treatments. Short-term L-DOPA treatment had no effect on swing speed or BOS of either the fore or hind limbs as compared with saline-treated 24-week-old

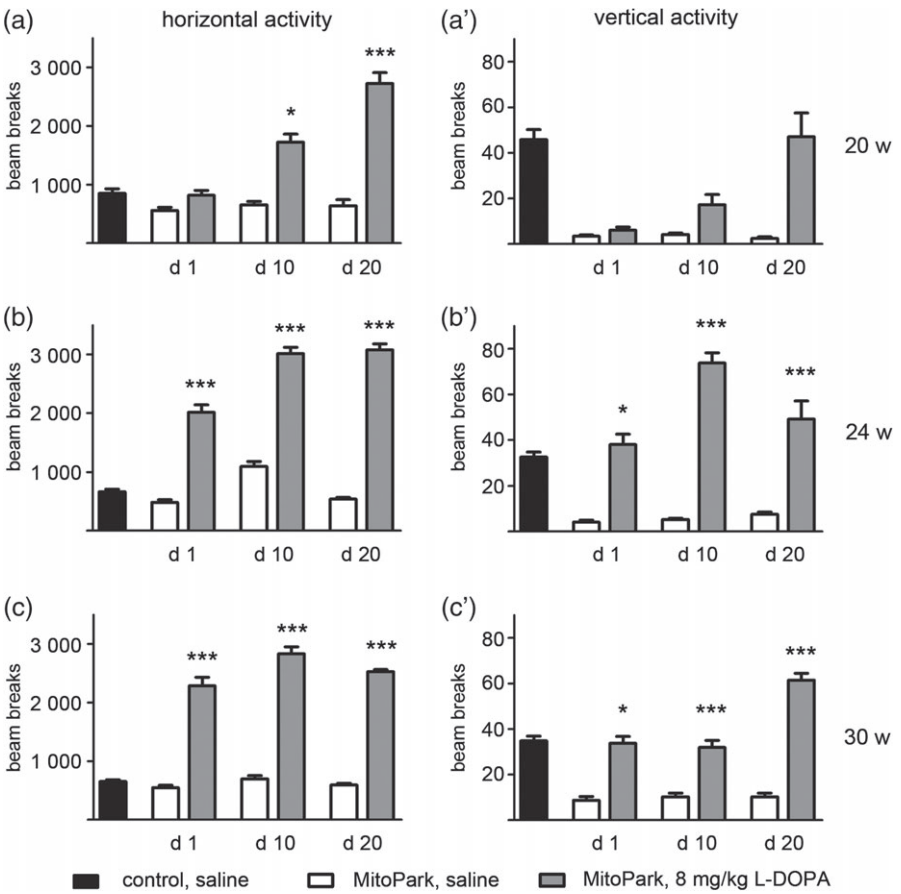

Figure 1: Comparison of locomotor activity. Following daily L-DOPA or saline treatment for 21 days, locomotor activity was examined in animals of 20, 24 and 30 weeks of age. The drug-induced horizontal and vertical activity during 60 min is expressed as beam breaks per 5 min (mean ± SEM). The black bar in each graph represents the activity of saline-treated control littermates for comparison. L-DOPA increases horizontal and vertical locomotor activity dependent on DA depletion and treatment duration. Statistical analysis comparing the activity of saline- and L-DOPA-treated MitoPark mice detected significant treatment effects and the significance levels of the Bonferroni *post hoc* test are indicated (*$P < 0.05$; ***$P < 0.001$).

MitoPark mice (Fig. 2b,c). L-DOPA treatment did, however, result in an unexpected significant reduction in stride length in hind limbs ($t_{(34)} = 2.13$, $P = 0.04$) (Fig. 2a) compared with saline treatment. Further analysis of stride length data for the saline-treated MitoPark mice showed that stride length was highly variable (Fig. S4), which is most likely the cause of the unexpected result of L-DOPA-induced reduction of stride length.

In contrast, long-term treatment (16 days) significantly reduced hind limb BOS and brought these values closer to control levels ($t_{(10)} = 3.44$, $P = 0.006$) (Fig. 2c′), as well as increased both the stride length and swing speed in fore and in hind limbs of L-DOPA-treated MitoPark mice (stride length $t_{(22)} = 6.95$, $P < 0.001$ and $t_{(22)} = 9.85$, $P < 0.001$; swing speed $t_{(22)} = 3.83$, $P < 0.001$ and $t_{(22)} = 3.02$, $P = 0.006$) (Fig. 2a′,b′). The compliance of saline-treated MitoPark mice to voluntarily perform 11 consecutive steps drastically deteriorated between treatment days 3 and 16. L-DOPA treatment fully restored compliance on both days (Fig. 2d,d′). Taken together, long-term L-DOPA treatment produced beneficial and significant changes in three gait parameters restoring them to control levels.

L-DOPA-induced changes in forced gait

For forced gait, we analyzed the same parameters as for voluntary gait (stride length, swing speed and BOS) and compared saline- and L-DOPA-treated 24-week-old MitoPark mice. On day 3 of treatment (short-term treatment), stride length and BOS were not changed (Fig. 3a,c); only the swing speed of forelimbs was reduced in L-DOPA compared with saline-treated MitoPark mice ($t_{(4)} = 7.84$, $P = 0.001$) (Fig. 3b). After 16 days of treatment (long-term treatment), the stride lengths of both the forelimbs ($t_{(8)} = 3.03$, $P = 0.016$) and the hind limbs ($t_{(8)} = 4.48$, $P = 0.002$) were increased and reached control levels (Fig. 3a′). In addition, the swing speed of the forelimbs was significantly increased after L-DOPA treatment ($t_{(8)} = 3.37$, $P = 0.01$) (Fig. 3b,b′). No treatment effects were detected for BOS at either 3 or 16 days of treatment (Fig. 3c,c′) and the compliance (the ability to perform more than 11 consecutive steps without stopping) was stable over time with approximately two thirds of the L-DOPA-treated mice exhibiting compliance similar to control animals, whereas only around 20% of the saline-treated MitoPark mice were compliant (Fig. 3d,d′). Taken together, forced gait parameters in MitoPark mice were normalized by long-term but not by short-term L-DOPA treatment and the changes were entirely dependent on the MitoPark genotype, as L-DOPA-treated control littermates displayed no differences in stride length, swing speed or BOS compared with saline-treated mice (Fig. S5).

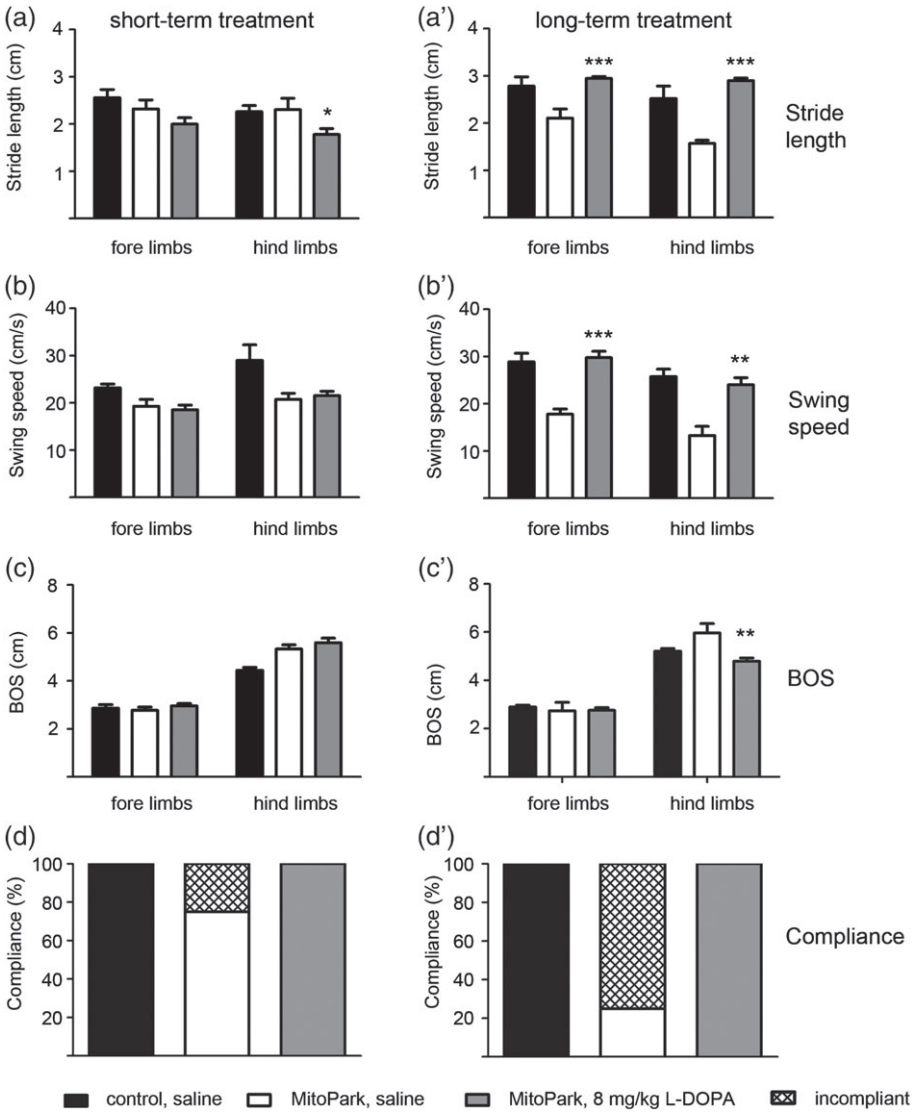

Figure 2: Automated analysis of voluntary gait with the CatWalk system. MitoPark mice treated with saline or L-DOPA and saline-treated control littermates were tested on days 3 (short-term) and 16 (long-term) of treatment. Stride length (in cm) (a and a′), swing speed (cm/s) (b and b′) and BOS (cm) (c and c′) are shown for fore and hind limbs and were compared between saline- and L-DOPA-treated MitoPark mice. The black bars depict the respective values for saline-treated control littermates and were not included in the statistical analysis. Long-term treatment increases stride length and swing speed of both hind and fore limbs were significantly similar to levels as control littermates. The compliances of control and L-DOPA-treated MitoPark mice are not changed from short- to long-term treatment, whereas the compliance of saline-treated MitoPark mice decreases (d and d′). Values are expressed as the mean ± SEM and the significance levels are indicated (*$P < 0.05$; **$P < 0.01$).

L-DOPA-induced motor behavior analyzed in the cylinder test

The beneficial effect of L-DOPA on motor deficits of PD patients ceases after a few years and many patients develop motor fluctuations and dyskinesia. We used the cylinder test to analyze peak-dose motor behavior induced by repeated L-DOPA treatment in MitoPark mice over time at three different ages (Figs 4 and 5).

The large difference in rearing behavior between the treatment groups in the cylinder test was similar to the vertical activity observed in locomotor activity (Fig. 4). Saline-treated MitoPark mice did not rear throughout the duration of the cylinder test, while control animals reared ∼20 seconds and L-DOPA-treated MitoPark mice increasingly spent more time rearing (Fig. 4a). In the 20-week-old group, rearing behavior slowly increased over time starting from no rearing at all on day 2 of treatment, and reached levels similar to control animals following 11 days of L-DOPA treatment (time: ns; treatment: ns; interaction: ns). In contrast, in 24-week-old MitoPark mice, L-DOPA treatment induced rearing behavior that exceeded levels of control mice already at the second day of treatment, and continued treatment increased the rearing time to around 100 seconds of the 2 min scored (time: ns; treatment: $F_{1,8} = 79.63$, $P < 0.001$; interaction: $F_{2,16} = 3.67$, $P = 0.049$). In 30-week-old mice, rearing was increased similar to 24-week-old mice (time: $F_{2,14} = 5.59$, $P = 0.016$; treatment: $F_{1,7} = 20.64$, $P = 0.003$; interaction: $F_{2,14} = 5.59$, $P = 0.016$). We also quantified the total number of times mice reared during the cylinder test (Fig. 4b); control mice exhibit an exploratory rearing behavior, standing on their hind legs for a brief moment, often leaning with one forepaw on the cylinder wall before returning all four paws to a sitting position, whereas MitoPark mice treated with saline were almost completely immobile and never reared. In contrast, L-DOPA-treated MitoPark mice increased the number of rearing in an age-dependent

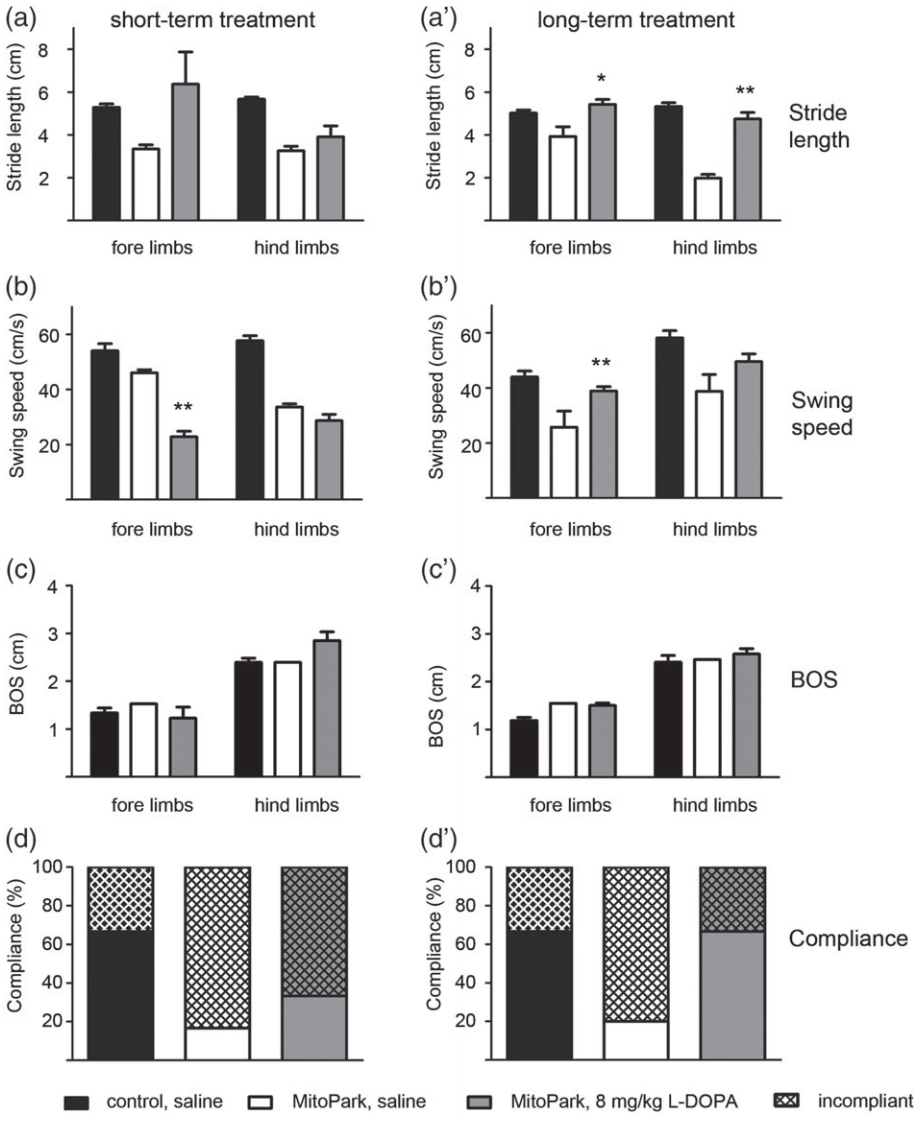

Figure 3: Automated analysis of forced gait with the Tread-Scan system. MitoPark mice treated with saline or L-DOPA and saline-treated control littermates were tested on days 3 (short-term) and 16 (long-term) of treatment. Stride length (in cm) (a and a′), swing speed (cm/s) (b and b′) and BOS (cm) (c and c′) are shown for fore and hind limbs and were compared between saline- and L-DOPA-treated MitoPark mice. The black bars depict the respective values for saline-treated control littermates and were not included in the statistical analysis. Following long-term treatment, stride length and swing speed of L-DOPA-treated MitoPark mice were significantly increased compared with saline-treated MitoPark mice. The compliance of control and saline-treated MitoPark mice was similar on day 3 and on day 16 (d and d′). Long-term treatment with L-DOPA increases the compliance of MitoPark mice to control levels. Values are expressed as the mean ± SEM and the significance levels are indicated (*$P < 0.05$; **$P < 0.01$).

manner: in 20-week-old mice, the number of rearing did not exceed control levels even on day 21 (time: ns; treatment: ns; interaction: ns), in 24- and 30-week-old animals, the number of rearing reached control levels on day 11 and continued to increase (time: $F_{2,16} = 9.98$, $P = 0.002$; treatment: $F_{1,8} = 22.63$, $P = 0.001$; interaction: $F_{2,16} = 9.34$, $P = 0.002$ for 24 week-old mice; time: $F_{2,14} = 7.02$, $P = 0.008$; treatment: $F_{1,7} = 60.38$, $P < 0.001$; interaction: $F_{2,14} = 7.02$, $P = 0.008$ for 30-week-old mice).

Motor activities only observed in long-term L-DOPA-treated MitoPark mice include: forepaw dyskinesia with or without turning and three paw dyskinesia. These activities increased in duration and intensity with treatment duration and age of mice. A variety of the forepaw dyskinesia was observed when the alternating forepaw movements were unidirectional and hind paws are following with stepping movements in the same direction resulting in rearing–turning movements. At the same time, we often observed axial instability. At any age analyzed, forepaw dyskinesia did not exceed 50 seconds and decreased in duration after 3 weeks of treatment (Fig. 4c,d).

The motor activity pattern that increased the most during long-term treatment was the three paw dyskinesia (Fig. 4e,f). In 20-week-old mice, only one mouse developed this behavior, whereas at 24 and 30 weeks, all MitoPark mice showed different degrees of this repetitive abnormal behavior (24 weeks time: $F_{2,16} = 10.26$, $P = 0.001$; treatment: $F_{1,8} = 21.49$, $P = 0.002$; interaction: $F_{2,16} = 10.26$, $P = 0.001$ and 30 weeks: ns). The number of third paw wall contacts (Fig. 4f) increased with the duration of treatment and with age (24-week-old: time: $F_{2,16} = 17.17$, $P < 0.001$; treatment: $F_{1,8} = 16.67$, $P = 0.004$; interaction: $F_{2,16} = 17.17$, $P < 0.001$; 30-week-old: ns) and reached over 60 contacts during the 2 min in mice with highly dyskinetic behavior.

The total dyskinesia score, comprised of forepaw dyskinesia, forepaw dyskinesia with turning steps and three

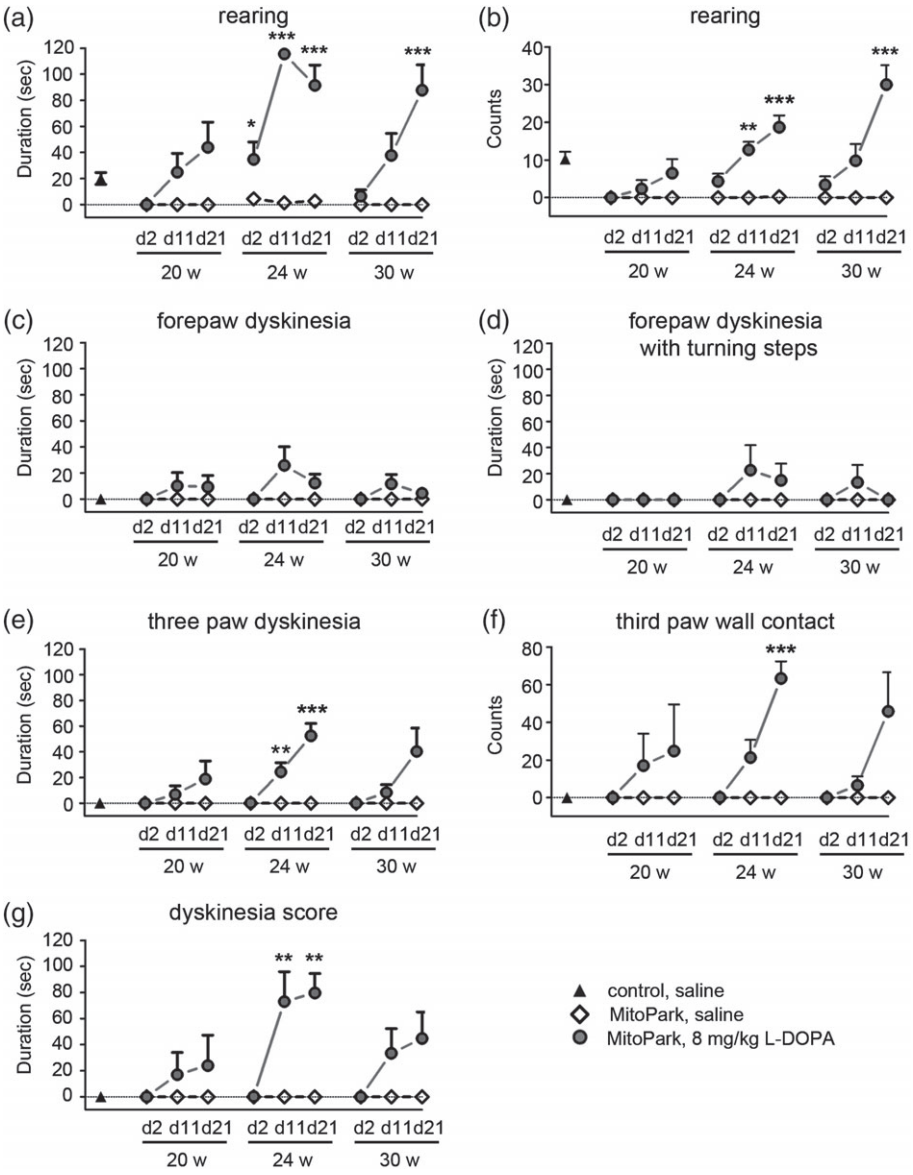

Figure 4: Quantification of L-DOPA-induced motor behavior in the cylinder test. MitoPark mice or control littermate mice of different ages were treated daily with L-DOPA or saline and evaluated in the cylinder test on days 2, 11 and 21 of treatment. The following behavior parameters were measured during the sampling period of 120 seconds: time spent in rearing position (a), the number of rearing events (b), duration of forepaw dyskinesia (c), duration of forepaw dyskinesia with turning steps (d), duration of three paw dyskinesia (e), number of third paw wall contacts (f) and dyskinesia score (g). Long-term L-DOPA treatment gradually increased the time MitoPark mice spent with repetitive, abnormal and purposeless behaviors that were not observed in saline-treated MitoPark mice or in controls. Statistical analysis: Two-way ANOVA with repeated measures (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

paw dyskinesia, shows the progressive nature of LID development at all three ages (Fig. 4g). In 20-week-old mice, the dyskinesia score only reached 24 and the variability between animal responses was large. In contrast, in 24-week-old animals, the dyskinesia score was already 80 on day 11 and the response was very stable (time: $F_{2,16}=7.45$, $P=0.005$; treatment: $F_{1,8}=12.25$, $P=0.008$; interaction: $F_{2,16}=7.45$, $P=0.005$). In 30-week-old mice, the dyskinesia score increased over time but did not quite reach significance (time: $F_{2,20}=3.20$, $P=0.062$; treatment: $F_{1,10}=4.72$, $P=0.055$; interaction: $F_{2,20}=3.20$, $P=0.062$).

An additional abnormal and repetitive behavior that was only detected in L-DOPA-treated MitoPark mice was rotational behavior (Fig. 5a). At 24 weeks of age, rotations were occasionally observed. A few MitoPark mice with severe DA depletion, at 30 weeks of age, displayed persistent rotational behavior on days 11 and 21 of treatment. Each mouse had a preferred turning direction which was maintained; both right- and left-turning mice were observed.

The differences in resting time most clearly express the differences between genotype, treatment and age at the onset of treatment: it decreased from 120 seconds in saline-treated MitoPark mice and around 100 seconds in control mice to intermittently under 10 seconds in L-DOPA-treated MitoPark (Fig. 5b) (20-week-old: ns; 24-week-old: time: $F_{2,16}=22.83$, $P<0.001$; treatment: $F_{1,8}=141.75$, $P<0.0001$; interaction: $F_{2,16}=25.4$, $P<0.001$; 30-week-old: time: ns; treatment: $F_{1,7}=225.21$, $P<0.001$; interaction: ns).

To examine whether any of these behaviors were independent of the cylinder, we monitored L-DOPA-treated MitoPark mice in their home cages and observed similar movement patterns on the cage wall.

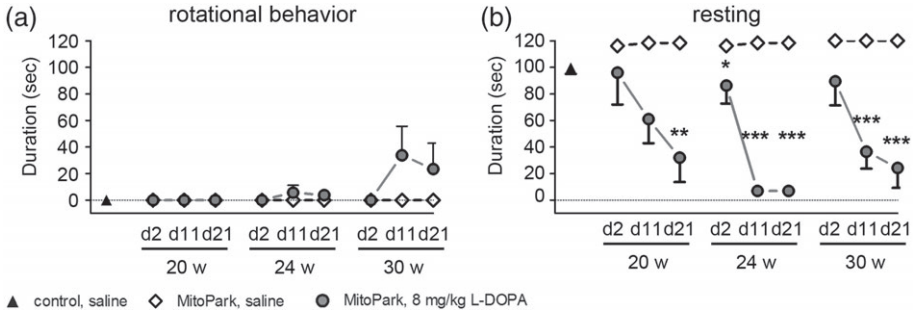

Figure 5: Rotational behavior and resting time. In some mice, L-DOPA treatment induced rotational behavior in which mice maintained the same preferred turning direction (a). The time spent resting decreased in all ages with the days of L-DOPA treatment (b). Only on day 2 of L-DOPA treatment, MitoPark mice showed a similar or lower resting time as control mice. Statistical analysis: Two-way ANOVA with repeated measures (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

In summary, with the cylinder test, we can follow the development of L-DOPA-induced motor behavior in qualitative and quantitative terms.

Increased FosB and prodynorphin expression in dorsal striatum

We analyzed striatal FosB expression in MitoPark mice after 21 days of saline and L-DOPA treatment and compared it to the expression in saline-treated control mice. Saline-treated MitoPark mice had a low number of FosB immunoreactive cells in dorsal striatum (Fig. 6a). Following repeated L-DOPA treatment (Fig. 6b,c), the total amount of FosB-positive cells was significantly increased ($t_{(8)} = 3.34$, $P = 0.01$) and the striatal area of FosB expressing cells expanded compared with saline-treated MitoPark mice.

Striatal expression of prodynorphin is also modulated in response to the development of LID as shown in rat and mouse models (Cenci *et al.* 1998; Ding *et al.* 2007; Lundblad *et al.* 2004). We also found a significant increase in striatal prodynorphin levels in MitoPark mice treated for 3 weeks with L-DOPA compared with saline-treated MitoPark mice ($t_{(11)} = 3.68$, $P = 0.004$) (Fig. 6d). Control littermates showed no significant change after L-DOPA treatment.

Discussion

This study is the first to show that long-term L-DOPA treatment induces significant increases in stride length and swing speed in a rodent PD model. Two automated gait analysis systems were used to show that voluntary and forced gait parameters are improved in MitoPark mice aged 24 weeks. Gait parameters have been previously studied in rodent models with partially conflicting results. A study in 2005 reported significantly reduced stride length and high stride-to-stride variability in MPTP-treated mice walking on a treadmill (Amende *et al.* 2005). A later study reported no significant differences in gait dynamics in MPTP-treated mice (Guillot *et al.* 2008), which might be related to the lower MPTP doses used (Hampton & Amende 2010). In long-term L-DOPA-treated MitoPark mice, we detected an increase close to values found in control mice in stride length under forced gait conditions. These findings correlate well with observations made in humans. It has been reported that drug naïve PD patients treated for 12 weeks with L-DOPA gradually improve gait scores over several weeks (Olanow *et al.* 1991). To date, voluntary gait parameters have only been studied in rat models. Reduced swing speed and the increased BOS were reported in the unilateral 6-OHDA model, as well as a partial recovery following the engraftment of dopaminergic stem cells (Chuang *et al.* 2010). In bilateral 6-OHDA-lesioned rats, a single administration of L-DOPA increased stride length and swing speed in both fore and hind limbs to the levels of unlesioned rats (Westin *et al.* 2012). In MitoPark mice, we found a similar increase in stride length and swing speed produced by long-term treatment. It is likely that the inconsistency of the observations in PD rodent models is related to differences between unilateral and bilateral models and that alterations in gait dynamics are more easily detectable and more robust in bilateral models irrespective of species or automated gait analysis system used. In summary, our results demonstrate that 24-week-old MitoPark mice recapitulate important features of gait deficits observed in PD patients, and that long-term L-DOPA treatment improves the gait parameters reported to be L-DOPA responsive in PD, stride length and swing speed (Blin *et al.* 1991).

In addition to gait parameters, there are many other motor functions that change in PD patients following long-term L-DOPA treatment. In a previous study, we analyzed the effect of a single administration of variable L-DOPA doses to MitoPark mice at different ages (Galter *et al.* 2010). In this study, we monitor the development of motor activity changes due to chronic L-DOPA treatment in MitoPark mice modeling symptomatic and late stage PD (20–30 weeks) by using the locomotor activity and the cylinder test. Starting from low levels of locomotor activity comparable to hypokinesia present in PD patients, daily L-DOPA treatment gradually normalized the motor activity of MitoPark mice to the levels of control littermates and later, with continued treatment, progressed to hyperactivity. The time that it took for hyperactivity to develop critically depended on the age of the mice when L-DOPA treatment was initiated. In 24-week-old MitoPark mice, when striatal DA levels are known to drop below 20% of control

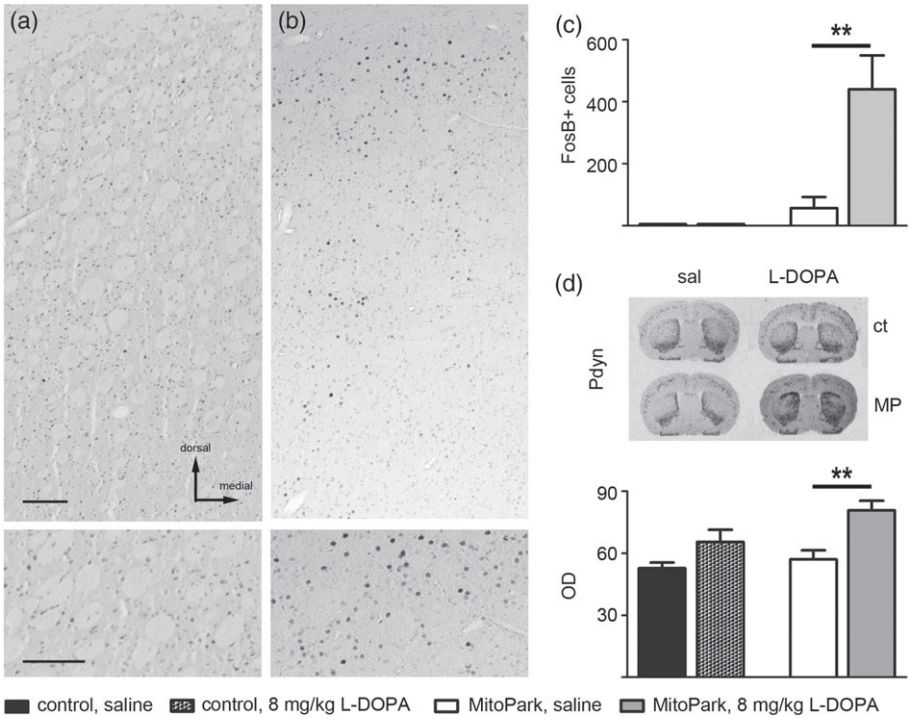

Figure 6: Changes in FosB and prodynorphin expression in striatum of MitoPark mice after 3 weeks of treatment. Compared with saline-treated MitoPark mice (a), there is an increased FosB expression in dorsal striatum of MitoPark mice after chronic L-DOPA treatment (b). Scale bar = 200 μm. Quantification of FosB expression ($n = 4–5$ for each condition) showed a significant increase of FosB expression (c). Prodynorphin mRNA expression in striatum of saline- and L-DOPA-treated control and MitoPark mice (d). Quantification of mRNA levels in 6–7 mice per group showed a significant increase in L-DOPA-treated MitoPark mice but not in control mice (d, Student's *t*-test, $^{**}P < 0.01$).

levels (Galter *et al.* 2010), we found a robust horizontal and vertical hyperactivity after 10 days of L-DOPA treatment. The excessive vertical activity was also reflected in the progressive increase in rearing time and rearing counts detected in the cylinder test.

The detailed analysis of motor behavior in the cylinder test also showed the gradual development of dyskinesia in MitoPark mice. To qualitatively and quantitatively measure LID in MitoPark mice, we used a dyskinesia score similar to that reported for Pitx3$^{ak/ak}$ mice (Ding *et al.* 2007) and show that 24-week-old MitoPark mice develop the highest LID score. An important difference between the two bilateral LID models is the L-DOPA dose used; whereas the Pitx3$^{ak/ak}$ model developed dyskinesia following treatment with 20 and 50 mg/kg per day in two injections (Ding *et al.* 2007, 2011), we report LID development in MitoPark mice at a much lower and clinically relevant dose. Two different L-DOPA threshold doses for the induction of dyskinesia have been reported for unilateral 6-OHDA mouse models: 6 mg/kg for mice lesioned in the medial forebrain bundle and a threefold higher dose for mice lesioned in striatum (Lundblad *et al.* 2004). Recent studies use either 20 or 25 mg/kg L-DOPA (Gonzalez-Aparicio & Moratalla 2014; Suarez *et al.* 2014) or 10 mg/kg L-DOPA (Lopez *et al.* 2011) to induce LID. Despite producing comparable behavioral changes scored as LID, different doses of L-DOPA might produce diverse effects on the cellular and molecular level hampering the discovery of new drugs aimed to alleviate dyskinesia in PD patients.

The unilateral 6-OHDA rat model is the oldest and still most commonly used dyskinesia model in which a well-defined rating scale exists (for a recent review, see also Morin *et al.* 2013). The four composing subscales are axial dystonia, orolingual dyskinesias, forelimb dyskinesias and locomotor dyskinesia. Since all AIM subscales are based on scoring asymmetrical movements or postures, they cannot be easily adapted to bilateral models. Occasionally, we have observed axial dystonia to either side of L-DOPA-treated MitoPark mice, causing a loss of equilibrium and animals rolling over. Orolingual dyskinesias have also been detected, mostly in aged MitoPark mice, but again, lacking lateralization they are difficult to score. We focused on quantifying limb dyskinesias in MitoPark mice, which closely resemble the unilateral forelimb dyskinesias reported for unilateral mouse models and the dyskinesia subscales described in the bilateral Pitx3$^{ak/ak}$ model. Unexpected for a bilateral model, L-DOPA treatment produced rotational behavior in three aged MitoPark mice, possibly due to a not entirely symmetrical striatal DA depletion that may lead to differences between sensitized DA receptors in either hemisphere. The exact cause of this rotational behavior in severely DA-depleted MitoPark mice is not clear, but it might reflect a variability in the model which becomes apparent only in aged mice. Furthermore, compared with rats, where a very robust contralateral turning behavior develops when supersensitive receptors on the DA-depleted side of the brain are activated, mice show a more diverse and complicated rotational behavior (Lundblad *et al.* 2004) and many recent studies avoid the scoring of the fourth AIM subscale in mice (Suarez *et al.* 2014). We have quantified the turning behavior, which was almost exclusively observed in 30-week-old mice and report it in Fig. 5a, but have not included the time animals spent with turning behavior in the total dyskinesia score reported in Fig. 4g. The exclusion of rotational behavior from the dyskinesia score explains the reduced LID score for the 30-week-old MitoPark

group as well as the lack of significance in the comparison to saline-treated MitoPark mice. The use of 24-week-old MitoPark mice to study LID is therefore recommended.

A correlation between striatal FosB protein and the dyskinesia score has been reported for several rodent models (Andersson *et al.* 1999; Ding *et al.* 2007; Pavon *et al.* 2006). For MitoPark mice, we also demonstrated significantly increased levels of FosB expression and prodynorphin mRNA following chronic L-DOPA treatment. Similar to other studies, we found that control littermates showed no significant change after L-DOPA treatment and that saline-treated controls and MitoPark mice had similar striatal prodynorphin levels indicating that DA depletion is not associated with changes in dynorphin gene expression.

In several regards, the effects of long-term L-DOPA treatment in PD patients are more closely modeled in MitoPark mice compared with other models. In toxin-induced models, LID already appears after a couple of days and stabilize at a given score, whereas in PD patients LID develops slowly and increases in severity and duration with time (Fox & Brotchie 2010). MitoPark mice reproduce LID starting from a score of zero on the second day of treatment to around 60 after 10 days of treatment and finally around 80 after 3 weeks of treatment. In the bilateral Pitx3$^{ak/ak}$ model, the first L-DOPA treatment already triggered dyskinetic behavior (Ding *et al.* 2007). In the unilateral 6-OHDA mouse model, total dyskinesia scores of 6 are reported on day 1, representing 66% of the final score of 9 on day 11 (Suarez *et al.* 2014). An additional advantage of a genetic, bilateral model is the lack of confounding factors due to the use of a toxin or compensatory mechanisms (Morin *et al.* 2013; Terzioglu & Galter 2008), whereas unilateral models often use the non-lesioned brain hemisphere as a control for both behavior and biochemical evaluation with the advantageous cost reduction, the importance of compensatory mechanisms cannot be ignored. For example, a recent comparison of PET and microdialysis in the unilateral 6-OHDA rat model not only found a high correlation between the results of the two methods for the lesioned brain hemisphere, but also reported changes in the contralateral side (Walker *et al.* 2013).

Establishing the MitoPark mouse as a dyskinesia model will further benefit LID research and allow cross breeding with other transgenic lines leading to new sets of tools to investigate the mechanisms involved in dyskinesia.

In conclusion, MitoPark mice show both the beneficial and the negative side effects produced by chronic L-DOPA treatment at a therapeutic dose. We report a significant improvement in the gait parameters for voluntary and forced gait conditions following long-term L-DOPA treatment. Furthermore, we show that a therapeutic dose of L-DOPA induces the progressive development of LID, whose total score was dependent upon the length of treatment and the DA degenerative state of MitoPark mice.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Timeline of the experimental setup. Sacrifice of mice on day 22, 20 h after the last treatment.

Figure S2: Locomotor responses over time following saline or L-DOPA treatment in mice aged 20, 24 and 30 weeks. Saline or L-DOPA was injected after 30 min (arrow) of habituation to the locomotor boxes and the response was measured for another 60 min. Horizontal activity on day 1 (d1), day 10 (d10) and day 20 (d20) of experiment of saline-treated 20-week-old control and MitoPark mice are depicted in panel (a), and the horizontal activity of L-DOPA-treated 20 week-old MitoPark mice on days 1, 10 and 20 are shown in panel (b). The vertical activity of the same mice is shown in panel (c) and (d). Horizontal and vertical activities of 24-week-old mice are shown in a similar order in panels (e–h), and data from 30-week-old mice are shown in panels (i–l).

Figure S3: Locomotor response over time of L-DOPA-treated control mice. Horizontal activity (a) and vertical activity (b) of 24-week-old control mice on day 1 (d1), day 10 (d10) and day 20 (d20) of treatment.

Figure S4: Variation of stride length of 24-week-old MitoPark mice analyzed with the CatWalk following short-term treatment (day 3). Control mice (black bars), saline-treated MitoPark mice (white bars) and L-D-DOPA-treated MitoPark mice (gray bars). Values are expressed as the mean ± SEM.

Figure S5: Automated analysis of forced gait with the TreadScan system for control mice treated with saline (black bars) or L-DOPA (gray bars with pattern) tested on days 3 (short-term) and 16 (long-term) of treatment. Values are expressed as the mean ± SEM, and no significant change was detected between the groups (Student's *t*-test).

Movie S1: Forepaw dyskinesia (avi format).

Movie S2: Forepaw dyskinesia with turning steps (avi format).

Movie S3: Three paw dyskinesia (avi format).