A Slow Progressive MPTP Marmoset Model for Idiopathic Parkinson's Disease

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PROGRESSION AND RECOVERY OF PARKINSONISM IN A CHRONIC PROGRESSIVE MPTP-INDUCTION MODEL IN THE MARMOSET WITHOUT PERSISTENT MOLECULAR AND CELLULAR DAMAGE

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ABSTRACT

Chronic exposure to low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in marmoset monkeys was used to model the prodromal stage of Parkinson's disease (PD), and to investigate mechanisms underlying disease progression and recovery. Marmosets were subcutaneously injected with MPTP for a period of 12 weeks, 0,5 mg/kg once per week, and clinical signs of parkinsonism, motor- and non-motor behaviours were recorded before, during and after exposure. In addition, post-mortem immunohistochemistry and proteomics analysis were performed. MPTP-induced parkinsonian clinical symptoms increased in severity during exposure, and recovered after MPTP administration was ended. Post-mortem analyses, after the recovery period, revealed no alteration of the number and sizes of tyrosine hydroxylase (TH) positive dopamine neurons in the substantia nigra (SN). Also the levels of TH in putamen and caudate nucleus were unaltered, no differences were observed in dopamine, serotonin or nor-adrenalin levels in the caudate nucleus, and proteomics analysis revealed no global changes in protein expression in these brain areas between treatment groups. Our findings indicate that parkinsonism symptoms can occur without detectable damage at the cellular or molecular level. Moreover, we show that parkinsonian symptoms may be reversible when diagnosed and treated early.

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder of the central nervous system [35, 196]. PD is characterized by movement dysfunction due to the progressive loss of dopamine (DA) neurons in the substantia nigra (SN) [40, 129, 162]. Various intracellular processes may underlie the demise of DA neurons, including protein misfolding and mitochondrial dysfunction [1, 33]. Patients are diagnosed with PD when multiple symptoms occur and substantial loss of DA neurons is already present [130, 170]. Current treatments focus on relief of symptoms, but have little effect on disease progression and do not prevent neurodegeneration. Consequently, patients benefit more from treatment in the early stages of PD, compared to later stages [152]. It is therefore important to shift research focus from the motor stage to the pre-motor stage, and to characterize in more detail the early disease symptoms and pathology that can be used to diagnose PD in its earliest stages, and to start treatment accordingly. Early non-motor symptoms such as REM-sleep disorder (RBD), constipation, mood disorders and other sleep related abnormalities are often too general to be used as diagnostic markers for early stage PD. However, they can be used as risk indicators, as individuals showing these non-motor symptoms are frequently diagnosed with PD at a later stage [122]. Better knowledge of early symptoms in relation to the onset of the disease will aid the development of novel diagnostic tools and potentially yield novel targets for treatment.

Animal models for preclinical PD research include several genetic mouse models as well as animals injected with neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [44, 163]. At a moderate dose MPTP destroys DA neurons in the SN [142, 163, 164]. Following MPTP treatment, animals develop behavioural, neurochemical and pathological features characteristic of PD. One important aspect of the MPTP model is that it can be used in non-human primates, which are genetically closely related to humans and share with humans the typical neuroanatomical and functional organization of the nigrostriatal pathways [47, 72, 189]. Different MPTP induction protocols have been used in primates that differ in MPTP dosage and frequency of administration, eliciting different onset and severity of parkinsonian symptoms. Acute induction regimens are based on a few injections with a high dose leading to necrotic cell death in the SN and a relative quick onset of parkinsonian symptoms [53, 121]. Sub-acute induction of parkinsonian symptoms is achieved by multiple lower dosages of MPTP administered daily, resulting in apoptotic cell death [83, 94]. Progressive chronic models use low doses of MPTP administered over extended periods of time [90, 150].

It was previously shown that discontinuation of chronic low dose MPTP treatment leads to partial recovery from parkinsonian symptoms in non-human primates [69, 70, 116, 183, 184]. Progressive chronic MPTP models may thus provide a unique opportunity to study early disease progression as well as recovery mechanisms in relation to PD in a controlled environment. Here, we used intermittent low dose MPTP injection in marmosets as a clinically relevant non-human primate model for slowly progressing parkinsonism, which recapitulates the prodromal stage of PD. We studied the MPTP-induced dynamics of behavioural

dysfunction and recovery, including motor as well as non-motor parkinsonian symptoms, and measured molecular and cellular adaptations following treatment and recovery. All behavioural tasks used in the present study have been extensively validated in previous MPTP induction studies [132-134, 177, 181, 182].

EXPERIMENTAL PROCEDURES

Animals

Behavioural experiments were carried out with seven common marmoset monkeys (*Callithrix jacchus*). Animals were between 3.0 and 4.0 years of age (4M/3F). For post-mortem analysis an additional untreated control group of 7 animals was included (3M/4F), so a total of 14 animals was used in this study. Animals were purchased from the purpose-bred colony of the Biomedical Primate Research Centre (BPRC), the Netherlands. Monkeys in experiment were pair-housed, preferably together with their twin-sibling, in spacious marmoset cages (76 x 71 x 190 cm) enriched with branches, ropes, and toys with padded shelter provided on the floor. Animals were housed under controlled conditions of humidity (60%), temperature (23-25 °C) and lighting (12 hour light/dark cycles; lights on at 7:00 am). The daily diet consisted of commercial food pellets for New World monkeys (Special Diet Services, Witham, Essex, UK), supplemented with raisins, peanuts, marshmallows, biscuits, fresh fruit, grasshoppers, Arabic gum and mealworms. Drinking water was provided ad libitum. Bodyweight was measured weekly. All study protocols and experimental procedures were reviewed and approved by the BPRC ethics committee before the start of experiments, according to Dutch law.

Study design

Marmosets were weekly subcutaneously injected with MPTP (0.5 mg/kg) on Fridays for a period of 12 weeks (to a total of 6,0 mg/kg). Behavioural abnormalities were examined prior to (baseline activity), during (MPTP exposure phase), and up to 8 weeks after (recovery phase) MPTP treatment. Behavioural tests were performed weekly on Monday to Thursday, thus excluding acute effects of MPTP due to the injections on Fridays. All tests were executed in the morning and early afternoon between 9:00h and 14:00h. Each animal was scheduled for a fixed time slot. Every task was conducted once a week, with the exception of the daily observation of clinical symptoms and the periodic 24h measurement of home-cage activity. All animals were habituated in the different test setups prior to the start of the experiment. Baseline values were obtained over multiple weeks before disease induction. Previous studies showed no effect of vehicle treatment, and all current animals showed stable baseline performances. Therefore, each monkey served as its own control. At the end of the experiment, 5 weeks after the last MPTP injection, animals were euthanized with an overdose of sodium pentobarbital (intracardiac injection) after deep sedation with alfaxan (i.m.).

Drug treatment

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride, purchased from Sigma Aldrich, St. Louis, USA) was dissolved in 0.9% saline to a concentration of 1 mg/ml (as a

free base). Injection volume was 0.5 ml/kg and administered via subcutaneous injections in the abdominal area.

Observational clinical signs

Marmosets were scored 2-3 times during the week for the presence and severity of parkinsonian symptoms. We recorded clinical scores (CS) using the validated tests described elsewhere [177, 182]: apathy, tremors, immobility, inadequate grooming, mask forming, smack and muscle rigidity. Clinical signs were scored on a scale from 0 (normal/healthy) to 4 (severely affected). The sum of separate observations formed a total clinical score.

Human threat test (HTT)

To test the emotional mood of the monkeys the HTT was applied. The HTT is a documented test of fear and anxiety in non-human primate [177]. In brief, the HTT scores arousal and fear of an animal in the home-cage in response to the presence of a human observer in front of the cage. The observer assessing the behaviour stood approximately 30–50 cm from the cage front and made eye contact with the marmoset during a 2-min test period. Throughout this period, the movements, behaviour and position of the marmoset in the cage were scored as fear postures (rearing, twisting, split staring, jumps in the backside of the cage) and body postures (scent marking, pilo-erection, presenting genitals, forward jumps).

Home-cage activity

Home-cage activity for measuring effects on the circadian rhythm was measured using telemetry allowing continuous remote and non-invasive monitoring of spontaneous activity in the monkeys' home cage over the total period of the experiment. An external actimetry device (Remo 200 series, Remo Technologies, Wiltshire, UK) was attached to the collar of an animal in experiment. An antenna mounted on the ceiling received signals from each device. Data was stored and real-time displayed using eDacq software (EMMS, Bordon, UK). Data is expressed as an averaged arbitrary unit. In addition, periods of resting were derived from these activity patterns. Periods during awake-time lasting for a minimum of 5 min with an activity count below 10 were regarded as resting periods.

Hand-Eye Coordination (HEC)

Reward-related hand-eye coordination (HEC) performance for measuring the fine motor skills was tested with an automated robot-guided test setup [132, 177, 182]. In brief, animals were placed in a test cage in the experimental setup in front of a small panel with a sliding door (5 x 8 cm). A robot arm presented a small marshmallow behind the door, which opened at the beginning of each trial. Subsequently, the monkey had to grab this reward. A balanced design was offered to the monkeys with different speeds of the robot arm: non-moving (0 cm/sec), slow moving (2 cm/sec) and fast moving (4 cm/sec). The test consisted of 24-30 trials depending on the individual performance of the animal. Monkeys were trained to perform > 80% correct trials before the start of the experiment. In addition to accuracy, reaction time needed to grab the marshmallow was recorded.

Hourglass

The hourglass test measures the marmosets' natural motor related righting reflex as a measure of rigidity, analogous to axial motor behaviour in humans. The currently used method was described previously [182]. In short, animals were placed in a Plexiglas cylinder (11 cm x 27 cm) in front of a camera. One trial consisted of a manual 180° turn of the cylinder. The time it took for the marmoset to turn back in the upright position was measured using non-automated video analysis. When an animal did not return to the upright position, the cylinder was turned back after 30 sec allowing animals to sit in their natural postural position. Each session consisted of 10 consecutive trials and sessions were carried out once a week.

Tower

The tower was used to test natural jumping motor behaviour of the animals, as described previously [182]. Minor adjustments were made for this study. In short, a Trespa tower (35 cm x35 cm x 250 cm) with a Plexiglas front contained 5 different horizontal levels. The levels varied in distance from each other, from 10 cm at the lowest levels to 70 cm at the highest levels. Animals entered the tower at the bottom and could freely move for 10 minutes throughout the tower. A camera was placed in front of the tower allowing the scoring of position of the animal using manual video-analysis. Both the location (level) of the animals and the time spent at each location were recorded.

Bungalow

Spontaneous locomotor activity was tested using the bungalow test [132, 182, 198]. The bungalow consisted of four horizontally placed non-transparent boxes (23 x 23 x 23 cm), all interconnected by six PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session and allowed to move freely throughout all compartments in the bungalow for a period of 10 min. Manual video-analysis was used to score the location of the animal and time spent in each of the compartments.

Tissue preparation

Brain tissue of all animals was collected after euthanization as described in section 2.2. Tissue was directly deep frozen in liquid nitrogen without prior perfusion with saline or paraformaldehyde and stored at -80 °C until sample preparation. The left hemisphere was cut into sections of 7 μm for immunohistochemistry. Individual brain areas were isolated from the right hemisphere for immunoblotting and proteomics. Tissue of SN, caudate nucleus and putamen was homogenized in a sucrose buffer (0.32 M sucrose, 5 mM HEPES and 1 tablet of EDTA-free protein inhibitor). Right hemisphere samples of two animals from the control group were classified as outliers and therefor discarded from further analysis, based on aberrant protein expression profiles.

Immunohistochemistry

The SN was analysed for the presence of DA positive neurons with tyrosine hydroxylase (TH) staining on slices as described [132]. In short, serial sections of 7 μm were stained for TH (Sigma Alderich, 1:100) and subsequently covered with glue and a coverslip. Every 30st

section was taken for analysis. Within seven corresponding sections, the total number of TH positive neurons was manually counted and expressed as a percentage of the number of cells counted in healthy untreated control marmoset brains. Subsequently, sections were analysed using the software package Stream Essentials (Olympus Corporation, Tokio, Japan). Images at 200x magnification were acquired (2560 x 1920 pixels) and TH-positive structures were analysed using the count and measure module of the program. Cell body size was measured by the size in pixels of TH-positive staining with a minimum area of 400 pixels (software parameters: hue 20-53, saturation 125-256 and intensity value 125-256). The area of cell bodies and neurites was measured as TH-positive staining of a minimum of 10 pixels (software parameters: hue 20-53, saturation 25-256 and intensity value 50-256).

Immunoblotting

Protein concentrations were determined using a Bradford assay. Immunoblotting was performed following a standard protocol [178]. In short, samples were heated for 5 minutes at 95 °C in Laemmli sample buffer and 7 μg protein was loaded on a TGX stain-free gradient gel (Biorad, CA, USA). After running of the gel, the proteins were blotted (90V) onto a PVFD membrane for 2h. Subsequently, blots were washed and blocked with 5% non-fat dry milk dissolved in TBS-T for 1h. Primary antibody (rabbit anti-tyrosine hydroxylase (Abcam, Cambridge, UK, 1:10000)) was incubated overnight at 4 °C and secondary anti-rabbit antibody was incubated for 1h at room temperature. Visualization of the bands was performed using FEMTO and a LICOR-scanning device. Intensity of the bands was analysed using Image Studio 2.0.

Monoamine measurement

The monoamines noradrenalin (NA), dopamine (DA) and serotonin (5-HT), and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxy-phenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by high-pressure liquid chromatography. Total cell homogenate of caudate nucleus was examined using HPLC-ECD (Column: Phenomenex C18-XB150x4.6 mm, ECD potential: 460 mV) as described elsewhere [177]. Peaks were identified to calculate monoamine concentrations using a standard solution of 5.06 ng/ml NA (A7257, Sigma Aldrich), 5.1 ng/ml DA (H8502, Sigma Aldrich), 4.79 ng/ml 5-HIAA (H8876, Sigma Aldrich), 9.76 ng/ml 5-HT (H7752, Sigma Aldrich), 4.04 ng/ml HVA (H1252, Sigma Aldrich). Internal standards (Clinrep, Recipe, Munich, Germany) were added to each sample.

Protein separation by electrophoresis and in-gel digestion

Total cell homogenate of SN, caudate nucleus and putamen were incubated with SDS sample buffer at 95 °C for 5 min to denature the proteins, followed by incubation with 50 mM iodoacetamide for 30 min at RT in the dark to alkylate the cysteine residues. To reduce protein complexity samples were size separated on a NuPAGE® 4-12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA, USA) using MOPS SDS running buffer (Invitrogen, Carlsbad, CA, USA) according the manufacturers' protocol. Gels were fixed in a solution containing 50% (v/v) ethanol and 3% (v/v) phosphoric acid in H_2O overnight while shaking at RT and

subsequently stained with Colloidal Coomassie Blue (34% (v/v) Methanol, 3% (v/v) Phosphoric Acid, 15% (w/v) Ammonium Sulphate, and 0.1% (w/v) Coomassie brilliant blue G-250 (Thermo Scientific, Rockford IL, USA). Destaining was performed in ultra-pure water under gentle agitation for several hours to reduce background staining. Each gel lane was sliced into 12 or 6 equal sized parts (12 for SN, and 6 slices for putamen) and each part was cut into blocks of approximately 1 mm3 and collected in an 96 wells filter plate. Gel fragments were destained in ultrapure water with 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile. Gel fragments were dehydrated using 100% acetonitrile for 20 min. The gel parts were rehydrated in 70 µl 50 mM ammonium bicarbonate containing 10 ug/ml of sequence grade trypsin (Promega, Madison, USA) and incubated at 37 °C overnight. Peptides were extracted from the gel pieces twice with a solution containing 0.1% (v/v) trifluoric acid and 50% (v/v) acetonitrile for 20 min each. The samples were dried in a SpeedVac and stored at -20 °C until further analysis.

Mass spectrometry analysis

The peptides were re-dissolved in 15 µL of 0.1% acetic acid of which 10 µL was injected into an Eksigent nano HPLC system coupled to an LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The peptides were initially trapped on a 5 mm Pepmax 100 C18 column (Dionex, 100 µm ID, 5µm particle size). Separation was achieved using a capillary reversed phase C18 column that had been equilibrated with 0.1% acetic acid, 94.9% H_2O, 5% Acetonitril at a flow rate of 400 nL/min. The peptides were eluted by increasing the acetonitrile concentration linearly from 5 to 40% in 80 min and to 90% in 10 min, using the same flow rate. The LTQ Orbitrap mass spectrometer was operated in a data-dependent mode, in which one full-scan survey MS experiment (m/z range 350-2000) was followed by MS-MS experiments on the 5 most abundant ions.

Protein inference and relative protein quantification

MaxQuant software (version 1.3.0.5) was used for spectrum annotation, protein inference, and relative protein quantification [28]. Spectra were annotated against the Uniprot marmoset reference proteome database (Build June 2012). Enzyme specificity was set to Trypsin/P, allowing at most two missed cleavages. Carbamido-methylation of cysteine was set as a fixed modification, and N-acetylation and methionine oxidation were set as variable modifications. Mass deviation tolerance was set to 20 ppm for mono-isotopic precursor ions and 0.5 Da for MS/MS peaks. False-discovery rate cut-offs for peptide and protein identifications were set to 1% for both. The minimum peptide length was seven amino acids. Identified proteins that had the same set of peptides or a subset of peptides compared to another protein, were merged into one protein group. Peptides that were shared between different proteins were assigned to the protein with most peptide evidence (so called 'Razor' peptides). Only protein groups with at least a single unique and a single Razor peptide were included. For relative protein quantification MaxQuant LFQ intensities based on at least a single shared peptide ratio were used [29]. The “Match between runs”- option in MaxQuant was used to allow optimal protein annotation. Label free quantification and protein identification was performed with MaxQuant using a marmoset database. Human protein

and gene identifiers for Marmoset proteins were inferred from best scoring human protein in the results of BLAST alignment [5] of each protein with the Uniprot human proteome database (release 2014).

Statistics

Statistical analyses of behavioural data and post-mortem analyses were performed using R (version 3.0.2). Data in all graphs is expressed as averaged values with a standard error of the mean (SEM). Data was first tested for normal distribution using a Shapiro-Wilk test. All test showed non-parametric distribution of one ore more test parameters, excluding repeated measures ANOVA for data analysis [52]. Also Friedman's test was not applicably since we measured only one group across multiple time points. Therefore behavioural data was reduced using a linear mixed model [107, 194]. The R-package 'nlme' (Linear and non linear mixed effect models, version 3.1-118) was used to acquire slopes and intercepts for each parameter over the three different phases (baseline, MPTP exposure and recovery) [95, 107, 138]. Subsequently, intercepts and slopes were tested for significant differences between stages. Slope and intercept data were first tested for normality (Shapiro-Wilk). Parametric data was then analysed using ANOVA and Tukey as post-hoc test. Non-parametric data was tested using Friedman and Mann-Whitney tests. Immunohistochemical data was tested using Students t-test. Results were regarded significant when $p < 0.05$. Statistical analysis for proteomics was performed using SAM-analysis [172] in MeV (Multi experiment Viewer, www.tm4.org; [151]). Results were regarded significant if $q < 5\%$.

RESULTS

Clinical expression: Non-motor symptoms

Throughout the experiment, three experimental phases were distinguished: I) baseline activity patterns recorded before MPTP injection, II) activity patterns recorded during MPTP exposure, and III) recovery of disease symptoms recorded after MPTP injections were halted. Behavioural parameters were examined using linear modeling to determine slope and intercepts of all three phases, and statistical significance was determined accordingly (Table 1, page 31). Averaged parameter values per phase were also calculated, either for the entire phase (as indicated the in the text), or for the last three weeks only of the treatment and recovery phases (as summarized in Table 2, page 32). Bodyweight measurements remained unchanged during the whole experiment (pre exposure: 0.379 ± 0.022 kg, during MTPT exposure: 0.379 ± 0.023 kg, after MPTP exposure: 0.389 ± 0.026 kg), indicating that there were no obvious general adverse health effects of the MPTP treatment.

No parkinsonian symptoms were observed during the baseline period. Parkinsonian behaviour increased significantly within a week after the start of MPTP injections (baseline vs. MPTP exposure, $p = 0.028$) (Figure 1A, next page). After the MPTP injections ended, animals showed recovery and a significant reduction to 9.0 ± 0.6 at week 17 in parkinsonian related symptoms compared to the highest CS reached of 14.7 ± 0.6 at week 13 (MPTP exposure vs. recovery phase, $p < 0.001$). The earliest recognizable symptoms were changes in

grooming, movement and smacking behaviour, followed by apathy and tremors, and finally rigidity and facial mask formation.

MPTP-injected animals showed decreased anxiety scores (Figure 1B), i.e. body postures related to arousal-behaviour in the Human Threat Test, with disease progression (baseline vs. MPTP exposure, $p = 0.018$), but not between the MPTP exposure and recovery (baseline: 8.2 ± 1.3; MPTP exposure: 6.0 ± 0.9; recovery: 7.3 ± 0.8). Fear-related behaviour remained unaffected during disease progression and recovery (baseline: 2.6 ± 0.4; MPTP exposure: 1.7 ± 0.3; recovery: 1.9 ± 0.3).

Normal circadian rhythms were observed throughout the experiment. MPTP exposure caused a reduction from 147 ± 42 to 58 ± 6 activity counts of daytime activity with disease progression (baseline vs. MPTP exposure, $p < 0.001$), which increased again to 104 ± 15 activity counts during the recovery phase (MPTP exposure vs. recovery phase, $p = 0.017$) (Figure 1C). Nighttime activity was unaffected by MPTP (baseline activity: 5.9 ± 0.9; MPTP exposure: 7.8 ± 2.3; recovery: 7.8 ± 2.1) (Figure 1C). An increase was observed in the number and total duration of daytime resting periods after MPTP exposure until week 8 (count: baseline 2 ± 1, week 8: 8 ± 2, duration: baseline 0.20h ± 0.08h, week 8: 1.22h ± 0.17h) (Figure 1D). From week 9 after MPTP exposure and onwards, we observed a decrease in resting periods (week 17: count: 4 ± 1 duration 0.48h ± 0.08h). The overall slopes of duration and total number of resting periods were, however, not significantly different between baseline, MPTP exposure or recovery phase.

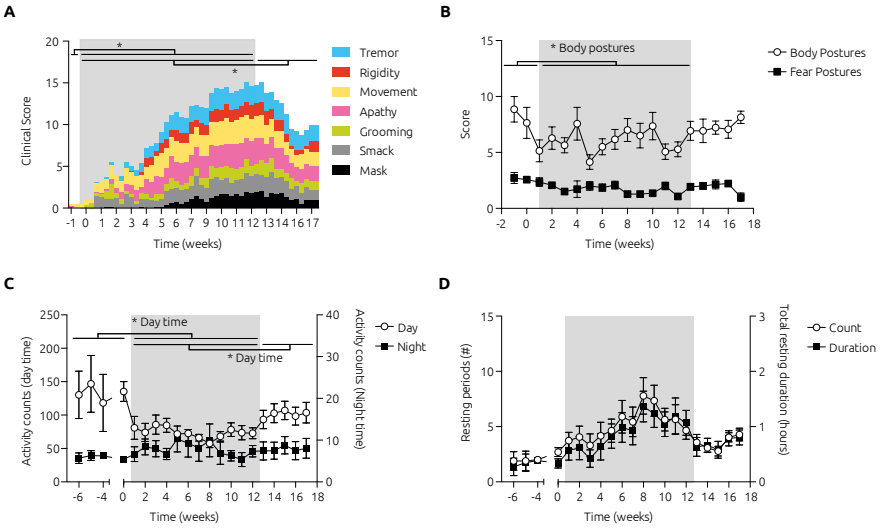

Figure 1: Non-motor parkinsonian symptoms. MPTP admission is reflected by the grey shaded areas. Asterisks indicate significant differences between slopes of baseline compared to disease phase and disease compared to recovery phase, when $p < 0.05$. (A) Summation of clinical observation of parkinsonian symptoms. (B) Body postures (open circles) and fear related postures (closed squares) measured in the human threat test. (C) Daytime (open circles, left y-axis) and night time activity (closed squares, right y-axis) measured in the home cage. (D) Resting periods measured in the home-cage are expressed as duration (closed squares, right y-axis) and number of periods (open circles, left y-axis).

Table 1: Linear modeling-derived slopes, intercepts and statistical significance of behavioral parameters

Test	N	Intercept			Slope			P-value (slope)	
		Baseline	**MPTP exposure**	**Recovery**	**Baseline**	**MPTP exposure**	**Recovery**	**Baseline vs MPTP exposure**	**MPTP exposure vs recovery**
Bodyweight	7	0,38±0,02	0,38±0,02	0,35±0,02	0±0	0±0	0±0	n.s.	n.s.
Bungalow	7	18,41±3,12	14,44±2,53	-16,4±2,45	-3,28±0,01	-0,03±0,03	2,63±0	<0,001	<0,001
Clinical Score	7	0,57±0,27	0,06±0,27	31,38±0,42	0,43±0,24	1,29±0	-1,26±0	0,028	<0,001
HEC – Acc at 0 cm/s	7	7,44±0	7,15±0,55	6,65±0,03	-0,42±0	0,08±0,02	0,09±0,02	n.s.	n.s.
HEC – Acc at 2 cm/s	7	7,08±0	6,42±0,63	7,87±0	-0,16±0,1	0,04±0,06	-0,05±0,03	n.s.	n.s.
HEC – Acc at 4 cm/s	7	6,56±0	4,83±0,9	2,7±1,34	0,43±0,38	0,18±0,05	0,23±0,04	n.s.	n.s.
HEC – RT at 0 cm/s	7	3419,39±618,6	3310,84±520,4	6644,29±1916,38	90,08±135,74	-20,01±12,96	-203,62±95,5	n.s.	n.s.
HEC – RT at 2 cm/s	7	2157,12±132,2	2565,02±93,94	2418,15±378,08	-105,96±18,63	-2,36±7,78	6,18±36,04	n.s.	n.s.
HEC – RT at 4 cm/s	7	1715,77±55,7	1962,11±54,94	1931,34±42,77	-106,6±17,27	-3,96±0,26	4,38±1,53	n.s.	n.s.
Homecage activity – Daytime activity	7	175,03±24,27	90,47±8	47,74±29,44	6,71±0	-2,34±0,34	3,47±2,08	0,005	0,017
Homecage activity – Nap count	5	-0,95±0	4,08±0,83	3,27±2,01	-0,5±0,13	0,18±0,06	0,03±0,11	<0,001	n.s.
Homecage activity – Nighttime activity	6	5,55±0	9,1±2,08	7,68±5,77	-0,08±0,12	-0,19±0,15	0,02±0,38	n.s.	n.s.
Homecage activity – Total nap duration	5	0,02±0,01	0,02±0,01	0,03±0,03	0±0	0±0	0±0	n.s.	n.s.
Hourglass	7	1,68±0	2,48±0,51	4,11±0	-0,33±0,06	-0,01±0,01	-0,12±0,01	<0,001	<0,001
HTT – Body postures	7	7,64±1,27	5,82±0,64	3,4±2,37	-1,21±0,48	0,02±0	0,26±0,12	0,018	n.s.
HTT – Fear postures	7	2,57±0,17	2,16±0,01	4,32±0	-0,14±0,6	-0,07±0,01	-0,16±0	n.s.	n.s.
Tower – Highest level	7	5±0	4,55±0,15	2,99±0	0±0	0,01±0,01	0,12±0	n.s.	0,002
Tower – Level changes	7	13,69±0	7,98±1	18,53±6,06	2,5±0	0,4±0,23	-0,45±0,3	<0,001	0,036

NB. Acc = accuracy, RT= Reaction Times

Table 2. Averaged values of behavioral parameters over selected time points

Test	N	Baseline (all datapoints)	MPTP exposure (last 3 weeks)	Recovery (last 3 weeks)
Bodyweight	7	0,38 ± 0,02	0,38 ± 0,02	0,39 ± 0,03
Bungalow	7	21,10 ± 4,56	15,38 ± 3,46	25,57 ± 3,67
Clinical Score	7	0,36 ± 0,22	13,67 ± 0,45	10,87 ± 0,63
HEC - accuracy at 0 cm/s	7	91,85 ± 5,74	94,71 ± 3,75	95,44 ± 2,9
HEC - accuracy at 2 cm/s	7	85,61 ± 7,95	83,53 ± 8,08	85,69 ± 6,22
HEC - accuracy at 4 cm/s	7	70,93 ± 12,38	81,42 ± 7,62	81,14 ± 7,64
HEC - reaction time at 0 cm/s	7	3239 ± 686	3309 ± 842	3437 ± 812
HEC - reaction time at 2 cm/s	7	2369 ± 239	2476 ± 241	2491 ± 271
HEC - reaction time at 4 cm/s	7	1966 ± 156	1947 ± 119	1979 ± 126
Homecage activity – daytime activity	7	128,73 ± 33,72	74,91 ± 9,73	103,05 ± 14,24
Homecage activity – nap count	5	2,44 ± 1,21	5,31 ± 1,06	3,7 ± 0,48
Homecage activity – nighttime activity	6	5,55 ± 1,08	6,32 ± 1,44	8,11 ± 2,21
Homecage activity – total nap duration	5	0,33 ± 0,13	1,10 ± 0,26	0,72 ± 0,14
Hourglass	7	2,34 ± 0,42	2,30 ± 0,85	1,98 ± 0,34
HTT - body postures	7	9,05 ± 1,49	5,9 ± 0,86	7,48 ± 0,65
HTT - fear postures	7	2,75 ± 0,64	1,48 ± 0,25	1,79 ± 0,33
Tower - highest level	7	100 ± 0	76 ± 10	100 ± 0
Tower - level changes	7	11 ± 4	16 ± 3	12 ± 2

Clinical expression: Motor symptoms

A significant decrease was observed in spontaneous locomotor activity measured in the bungalow test from averaged baseline activity of 23 ± 5 compartment changes to an average of 14 ± 3 compartment changes during the total period of MPTP exposure (baseline vs. MPTP exposure, $p < 0.001$) (Figure 2A). Marmosets did not have a preferred compartment and spent equal amounts of time in each compartment throughout the whole experiment (compartment A: 148.7 ± 7.4, B: 130.5 ± 9.6, C: 103.0 ± 7.0, D: 124.9 ± 7.9). An increase back to baseline levels of compartment changes was observed after discontinuation of MPTP to an average of 27 ± 3 compartment changes during the 4 last weeks of recovery period (MPTP exposure vs. recovery phase, $p < 0.001$).

The time that a healthy animal needs to regain an upright position in the Hourglass system was less than 2 seconds (baseline: 1.6 ± 0.2 sec). Slope analysis revealed a significant difference between all three phases ($p < 0.001$ for both), but the averaged time to turn upright in the hourglass during the different phases did not change (MPTP exposure: 1.7 ± 0.5 sec, recovery: 1.6 ± 0.4 sec). However, there was an overall increase in the variation among animals between baseline and MPTP exposure (Levene's test, $p = 0.002$), but not between MPTP exposure and recovery phase.

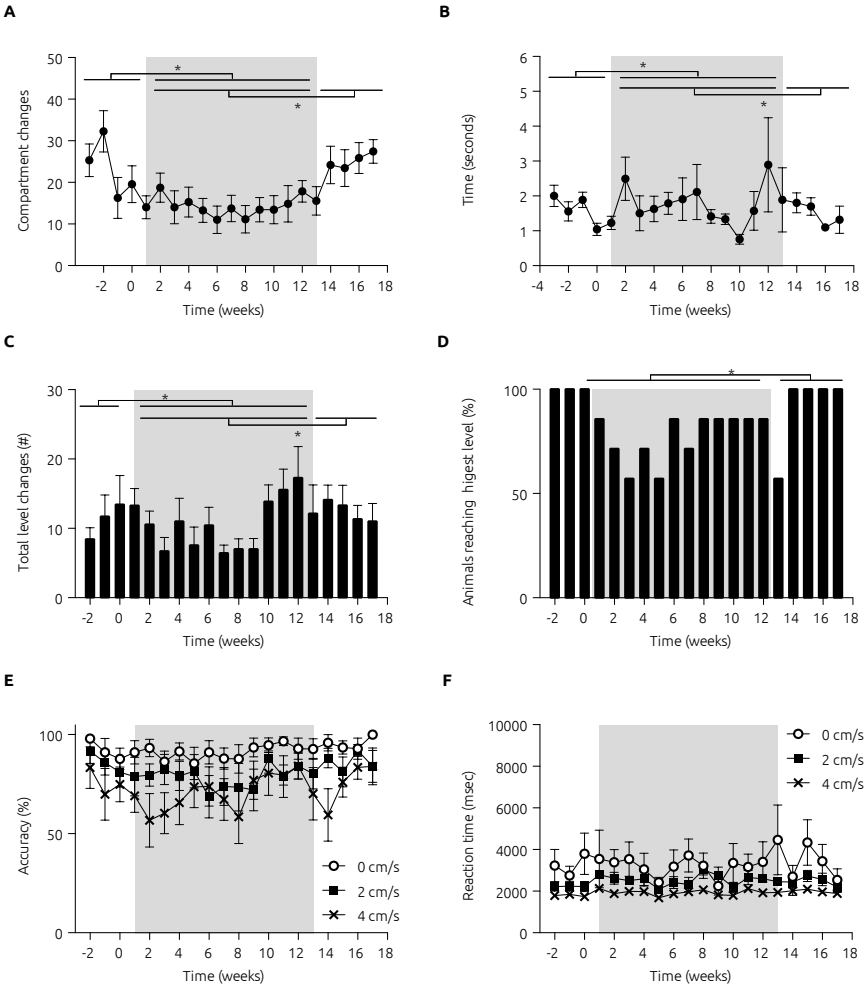

Figure 2: Motor related parkinsonian symptoms. MPTP admission is reflected by the grey shaded areas. Asterisks indicate significant differences between slopes of baseline vs. disease phase, and disease vs. recovery phase, when $p < 0.05$. (A) Spontaneous locomotor activity was measured in the bungalow by between compartment changes. (B) Time needed to return to an upright position in the hourglass. (C) Total level changes in the tower as measurement of natural jumping behaviour. (D) The amount of animals that reached the highest level of the tower. (E and F) Accuracy and reaction times measured the reward related hand-eye coordination task.

Animals showed significant alterations in natural jumping behaviour measured in the Tower with disease progression (Figures 2C and 2D). The total number of level changes observed between baseline (11.2 ± 3.0) and MPTP exposure decreased until week 9 (7.0 ± 1.6) and increased from week 10 onwards. Significant differences were observed between baseline and MPTP exposure (slope analysis, $p < 0.001$) and MPTP exposure and recovery

(p = 0.036). The percentage of animals that reached the highest level only differed between MPTP exposure (77%) and recovery (91%) (p = 0.002).

Basal performance showed differences between the three different velocities in accuracy and reaction times in the HEC, which is a normal observed behaviour, and which continued throughout the experiment (Figures 2E and 2F). Moreover, slope analysis revealed no differences in accuracy or reaction times between experimental phases after Post hoc correction for the three different velocities.

Pathology

Histological and biochemical analyses were performed to examine neurodegeneration in the nigrostriatal dopamine system after MPTP treatment. Immunohistochemical analysis showed no difference in TH-expression in the SN between MPTP-treated and untreated animals in the number of dopaminergic neurons present in the SN (81 ± 9% of control) (Figure 3A). Also the areas of TH-positive cell bodies (119 ± 46% of control) and neurites (97 ± 32% of control) in the SN were not different between the groups. Immunoblot analysis of TH in the SN confirmed these findings (94 ± 8% of control), and also TH protein levels in the putamen (109 ± 15% of control) and caudate nucleus (93 ± 9% of control) were not significantly different between treatment groups (Figure 3B). HPLC analysis of total homogenate of the caudate nucleus showed no difference between treatment groups in levels of the neurotransmitters DA, 5-HT and NA, or the dopamine metabolites DOPAC, HVA and 5HIAA (Figure 3C).

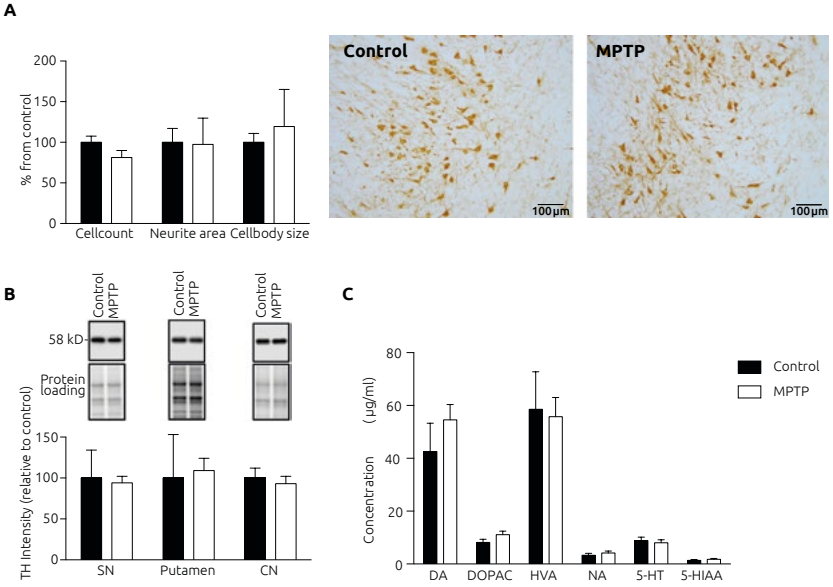

Figure 3: Histological analysis. Open bars represent MPTP-treated animals, closed bars represent control animals. (A) Immunohistochemistry with TH staining in SN (maginification: 200x). (B) Immunoblots of TH in SN, Putamen and caudate nucleus. (C) Catecholamine levels of the caudate nucleus as measured with HPLC.

Proteomics

Proteome analysis of putamen and SN was performed using tandem mass spectrometry. Because not all annotations for marmoset protein sequences were available in the Uniprot database, missing annotations were derived from best-matching human protein sequences as obtained by BLAST analysis. Averaged homology of sequences was 95.4% and 96.0% for the putamen and SN, respectively.

A total of 3491 different proteins were identified in samples of the SN. 1312 of these proteins were present with a maximum of two missing values per group. For the putamen, 2222 unique proteins were identified, of which 828 proteins were present in at least 60% of the animals per treatment group. Proteins with a maximum of two missing values in each group were selected for further statistical analysis. Figure 4 shows protein quantities per brain area in control versus MPTP-treated conditions. Statistical evaluation of differential protein expression resulted in only 3 differentially expressed genes in putamen and 2 in the SN, which amounts to 0.15-0.36% of all measured proteins. In the putamen, PDE10A and FOLH1 were lower expressed, 1.9- and 1.5-fold, respectively, and MAP6 level was increased by 1.7-fold. In the SN CAPS and CADM4 showed lower levels, 2.4- and 1.6-fold, respectively.

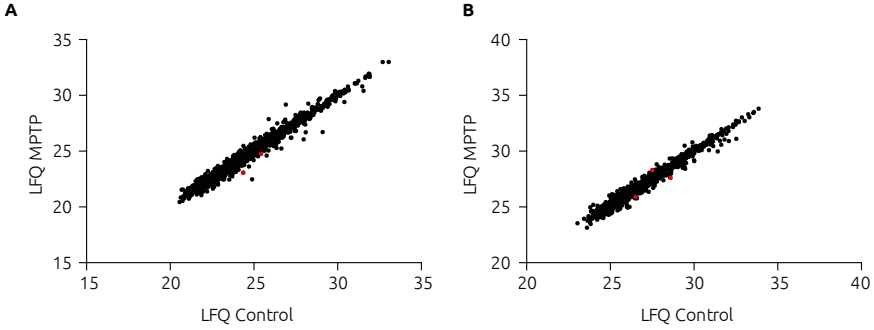

Figure 4: Proteome analysis. Label Free Quantification (LFQ) values of control (x-axis) and MPTP-treated (y-axis). Every dot represents a single protein in SN (A) and putamen (B), with red dots depicting significant proteins.

DISCUSSION

Marmosets were treated with low doses of MPTP to study the progression of PD-related behaviour during induction and the recovery of parkinsonian symptoms. As expected the MPTP-injected marmosets showed a gradual increase of parkinsonian symptoms after low doses of MPTP injections. After MPTP injections were stopped, the marmosets showed functional recovery from MPTP-induced parkinsonian symptoms, including disease score, jumping behaviour, locomotor activity, righting reflex and circadian rhythm. Importantly, at necropsy neither decrement of DA neurons nor alteration in their protein content could be found. To our knowledge, such a clinic-pathological paradox has not been documented in marmosets before.

The behavioural tasks used in the current study test different aspects of parkinsonism. Impairments in motor-related behaviour during disease progression are well documented in other studies in both primates [30, 47, 81, 182, 189] and rodents [90, 94, 126, 153], but have not been demonstrated with so many different behavioural parameters within one study design. Interestingly, slow development of parkinsonian symptoms was observed at the level of motor as well as non-motor related behaviours. Motor skills were tested in the tower, bungalow, hourglass and hand-eye coordination tests [182, 197, 198]. These motor-related tasks were proven to be affected after MPTP induction and resemble motor problems seen in patients [40]. Tasks such as the human threat test and home-cage activity, which reflect non-motor parkinsonian symptoms, were also compromised [177, 181].

In the current study, marmosets showed behavioural impairments during MPTP exposure at the level of spontaneous locomotor activity, jumping behaviour, daytime activity in the home-cage and arousal-related behaviour in response to human interaction, whereas an increase was observed in the number and duration of resting periods in the home-cage. Hand-eye-coordination was not affected, which may be due to interference of non-motor related aspects of the task, or to compensatory mechanisms. Reward expectancy for instance is known to increase dopamine release in the striatum [8] and could therefore interfere with the dopaminergic pathways required to execute the HEC-task. Also the involvement of a strong cognitive compensatory component in this task might explain the lack of effects, since cognitive impairment is only observed later in disease development [100]. In previous studies, more severe pathology was shown to significantly decrease HEC performance [132], which supports the idea that the current model indeed mimics a prodromal stage of PD. Differences found in the righting reflexes were statistically significant but small, which might be explained by the fact that automated behaviours in general are known to be affected only later in the course of PD, possibly due to compensatory involvement of non-dopamine-dependent brainstem-mediated processes [166].

The expression of parkinsonian symptoms seen after recovery was not associated with detectable cellular changes in the nigrostriatal system. Given the clear display of clinical signs during MPTP exposure, the SN was first examined for dopamine cell loss. Immuno-histochemistry and immunoblotting showed no significant damage to dopamine neurons in terms of the number of TH-positive cells, size of the cells, density of the dendritic arbors and TH-levels. Also TH levels measured with immunoblotting in the major projection areas of the

SN, the putamen and caudate nucleus, were unaffected by the MPTP administration. Furthermore, the levels of several monoamines, i.e. DA, 5-HT and NA, as well as their metabolites in the caudate nucleus were not different between MPTP-treated and untreated animals. These data do not exclude possible effects on NA neurons in the LC as previously observed by others [55, 136, 147]. However, recent research suggests that thalamic, prefrontal and somatosensory NA systems are not affected after recovery from MPTP treatment, even when DA loss in the nigro-striatal regions is evident [119, 135], and it is thus not very likely that our slow, low dose, MPTP induction protocol, which does not result in DA cell loss, would affect NA neurons. Finally, proteomics analysis of the putamen and SN did not reveal major changes in protein levels compared to control animals. These findings indicate that dopamine producing neurons were not severely compromised by low dose MPTP treatment, and likely experienced only mild mitochondrial stress that nevertheless led to a well recognizable clinical manifestation. Under these conditions, MPTP induced mitochondrial stress apparently falls within the normal plasticity boundaries of the cells, with the possibility to recover when treatment is discontinued. In addition, it could also well be that synaptic connections of the striatal regions were (temporally) disturbed as suggested by Schulz-Schaeffer [156]. Absence of dopaminergic cell loss after low doses of MPTP was previously observed in mice [90]. It would be of value to collect tissue samples at the time when highest clinical score are observed, however, this was beyond the scope of the present study.

Recovery from parkinsonian symptoms has previously been reported in different animal models, including marmoset and rhesus monkeys [69, 70, 149, 183, 184]. Several mechanisms that may compensate for impaired dopamine neuron function and dopamine neuron loss have been proposed. For instance, increased activity in the nucleus accumbens may enable the recovery of compromised axon terminals of the nigrostriatal circuitry [70]. More recently, Vezoli and Bezard [10, 184] argued that compensatory mechanism may not be mediated via the dopamine system itself, as they observed improvement of behaviour, even in the presence of dopaminerigic cell loss [10]. In our study we did not detect any major molecular or cellular alterations in the nigrostriatal dopamine system resulting from low dose MPTP treatment, supporting the idea that early typical parkinsonian symptoms occur at a stage that still allows a cell-autonomous recovery process to be activated and to result in the restoration of normal nigrostriatal function.

Our data also suggest that a combination of subtle changes in several motor and non-motor behaviours that are typical for PD may allow earlier and more accurate diagnosis. Indeed, PD-like symptoms are also seen in patients without the accompanied dopaminergic cell loss (e.g., scans without evidence of dopaminergic deficits; SWEDD), however these patients are often misdiagnosed with PD whereas they have essential tremor or dystonia [36, 45]. Early diagnosis might be followed by treatment that is specifically targeted at preventing neurodegeneration and restoring normal nigrostriatal function. The present low dose MPTP induction model can complement research in patients to identify early biomarkers as well as possible underlying mechanisms, and allows testing of new hypotheses in a controlled environment. In addition, anti-PD drugs such as L-DOPA that are now typically given in advanced PD, can be tested for possible beneficial effects when administered earlier in the disease.

In conclusion, we report presence of Parkinson symptoms in the absence of detectable Parkinson pathology in a chronic progressive PD model induced with frequent low doses of MPTP. As explanation for this clinic-pathological paradox we propose that mild pathological changes in this model are reversible, as can be seen from the recovery of clinical symptoms.

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