Measurement of baseline locomotion and other behavioral traits in a common marmoset model of Parkinson?s disease established by a single administration regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Measurement of baseline locomotion and other behavioral traits in a common marmoset model of Parkinson's disease established by a single administration regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: providing reference data for efficacious preclinical evaluations

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Baseline locomotion and behavioral traits in the common marmoset Parkinson's disease model were examined to provide basic information for preclinical evaluations of medical treatments. A single regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine at a cumulative dose of 5 mg/kg as the free base over three consecutive days was administered subcutaneously to 10 marmosets. Data obtained from these marmosets were compared to pre-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine levels or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine free marmosets. After the single regimen, reduced daily locomotion, a measure of immobility (a primary sign of Parkinsonism), was observed for more than a year. A moving tremor was also observed by visual inspection during this period. When apomorphine (0.13 mg/kg, s.c.) was administered, either right or left circling behavior was observed in a cylindrical chamber in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine marmosets, suggestive of unequal neural damage between the two brain hemispheres to different extents. MRI revealed that T1 relaxation time in the right substantia nigra correlated with right circling in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine marmosets. Histology was supportive of dopaminergic neural loss in the striatum. These results increase our understanding of the utility and limitations of the Parkinson's disease model in marmosets with a single 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine regimen, and provide reference data for efficacious preclinical evaluations. *Behavioural Pharmacology* 31: 45–60 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by movement impairments such as immobility, tremor, muscle rigidity, and positional dysfunction. Underlying degeneration is evident in the dopaminergic neurons originating from the substantia nigra and extending to the striatum (Olanow and Tatton, 1999). Movement impairments are mainly associated with degeneration of the nigrostriatal dopaminergic neural system, which in the normal brain plays a key role in smooth movement function.

Although movement impairments are typically the primary topic of PD studies, many other central nervous system-related manifestations and disorders have been reported, such as cognitive impairment, hallucinations, delusions, depression, irritability, sleep disorders, and olfactory dysfunction (Löhle *et al.*, 2009). Autonomic nervous system-related disorders have also been reported, such as orthostatic hypotension, constipation, early satiety, excessive sweating, and urinary urgency (Khoo *et al.*, 2013). These manifestations of PD are considered indicative of the broad linkage not only from the nigrostriatal dopaminergic system to the cortex via the thalamus but also from noradrenergic, serotonergic, cholinergic, and other neural systems (Masilamoni and Smith, 2018). These manifestations may derive from the pathophysiology of PD, or from complications of other neurological disorders, such as Alzheimer's disease and dementia with Lewy bodies, in addition to PD medication.

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Although, it remains unclear whether the above manifestations are directly related to the pathophysiology of PD, some of them have been explored using laboratory animal models. Further investigations of disease manifestations, such as cognitive dysfunction, may be required and have been already reported in preclinical PD studies (Phillips *et al.*, 2017).

A neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been used to establish PD models in various primate species (Langston *et al.*, 1984a; Langston *et al.*, 1984b). In MPTP-treated primates, the neural damage and syndrome is similar to that of human PD patients (Tetrud and Langston, 1989). Thus, primates model have been used to study the pathophysiology, etiology, and pathogenesis of PD, and to evaluate potential medical treatments in the preclinical stage (Fox and Brotchie, 2010).

The common marmoset is a nonhuman primate with a body size comparable to that of a large adult rat. MPTP-treated marmosets show a similar behavioral syndrome and dopaminergic neural degeneration to PD patients, and thus have been used in preclinical studies (Jenner, 2003). Marmosets are easy to handle, compared to macaque monkeys, and show relatively few individual differences in behavioral traits and toxicological sensitivity to MPTP (Ando, 2004). The relative behavioral homogeneity of the marmoset as an experimental primate is considered attributable to the raising of this nonhuman primate species basically under a random non-inbred system in a controlled in-house closed colony over the past 30 years in Japan.

In terms of behavioral traits at baseline, a normal marmoset living in its individual living cage moves freely, leaps between mesh cage walls, clings to the wall in the daytime, and remains still at night (Ando *et al.*, 2012). Infrared motion sensor measurements of daily continuous locomotion of an individual normal caged marmoset remain stable across days and months. Following MPTP administration, a decreased level of locomotion is observed for several weeks or more. A stable level of decreased locomotion after MPTP is considered an objective and quantitative measure of immobility, which is a primary syndrome of Parkinsonism. In addition, locomotion provides a sensitive baseline for evaluating the beneficial effects of drugs (Ando *et al.*, 2008) and other medical treatments such as deep brain stimulation and neural tissue/cell transplantation. Although, the MPTP-induced decrease in locomotion is an important measure, this level recovers spontaneously after MPTP treatment (with some individual variability). This can be avoided if extensive damage is induced by a higher dose MPTP regimen, in which case marmosets show severe manifestations leading to death. The protective effects against MPTP toxicity of a preclinical treatment have also been shown, supporting that the marmoset PD model should use a single regimen of MPTP rather than multiple regimens (Ando *et al.*, 2008).

Multiple regimens of MPTP may complicate the experimental process. Therefore, characterization of behavioral stability after a single, optimal regimen of MPTP is important to understand the utility and limitations of this approach in preclinical studies.

In this report, we investigated baseline locomotion behavior over a period of several months in marmosets that received a single regimen of MPTP in experiment 1. Other PD-like signs were observed in the same experiment using a dysfunction scale. The following experiments were performed by using the same marmosets that received the above MPTP regimen. In experiment 2, influences (direct and indirect effects) of MPTP on food-reinforced choice behavior were investigated with the expectation of detecting cognitive behavioral impairment. In experiment 3, long-lasting but latent influences of MPTP were explored by observing the circling behavior induced by subcutaneous (s.c.) administration of apomorphine (a dopamine receptor agonist), which is suggestive of imbalanced nigrostriatal neural damage in the two brain hemispheres. In experiment 4, in-vivo MRI was performed to detect signal changes in the brain after the MPTP regimen compared to the pre-MPTP condition. In experiment 5, neural degeneration was grossly and macroscopically confirmed in marmosets received the present MPTP regimen by in-vitro immunohistochemical staining with tyrosine hydroxylase (TH) antibody.

Methods

Subjects

Ethics

This study was conducted in strict accordance with recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Research Council (USA). The study protocol was reviewed by the CIEA animal care and use committee and approved by our institute (CIEA; approval no. 07028A). The criteria used by the committee complied with those mandated by the Japanese Law for the Humane Treatment and Management of Animals. This study was designed and conducted under the principle of the three Rs (Replacement, Reduction, and Refinement).

Animals and husbandry

Healthy adult male common marmosets were obtained from CLEA Japan, Inc. (Tokyo, Japan). Their body weight and age were in the range of 339–481 g and 2.8–4.1 years, respectively, at the time of animal selection. Lists of the marmosets used in the present experiments are provided in Table 1, including sample sizes and identification codes. During the experimental period, each marmoset was housed in an individual stainless-steel cage (50 cm high, 30 cm wide, and 48 cm deep) with wire-mesh walls and floor. Animals were administered 50 g/day of a balanced diet (CMS-1M; CLEA Japan, Inc.) and free access to tap water from feed valves. The colony room

Table 1 List of common marmosets used in each experiment

Experiment	Number of MPTP-treated marmosets[a]	ID of MPTP-treated marmosets[b]	Control for MPTP-treated marmosets
Experiment 1: locomotion and dysfunction score	8 (10[b])	CM1[b], CM2, CM3[b], CM4, CM5, CM6, CM7, CM8, CM9, and CM14	Pre-MPTP level of each marmoset listed on the left side
Experiment 2: door-opening choice behavior for food reinforcement	4	CM1[c], CM2, CM4, and CM14	Pre-MPTP level of each marmoset listed on the left side and additional MPTP-free marmosets (CM11, CM16, CM20, and CM22)
Experiment 3: apomorphine-induced circling behavior	6[d]	CM2, CM4, CM5, CM6, CM7, and CM8	Additional MPTP-free marmosets (CM18, CM21, and CM25)
Experiment 4: brain MRI analysis	6[e]	CM2, CM4, CM5, CM6, CM7 and CM8	Pre-MPTP level of each marmoset listed on the left side
Experiment 5: brain histology	4[f]	CM4, CM6, CM8, and CM14	Additional MPTP-free marmosets (CM18 and CM32[g])

[a]All MPTP-treated marmosets in the present study received the single regimen of MPTP at cumulative-free base dose of 5 mg/kg, s.c., over three consecutive days in experiment 1.
[b]Marmosets CM1 and CM3 died 2.7 months after the MPTP-regimen (see text).
[c]Post-MPTP test in CM1 was performed 1 week before its sudden death. This marmoset appeared well enough to be tested at that time. Therefore, the test data were used in experiment 2.
[d]Marmosets with brains scanned by MRI in experiment 4 were used in experiment 3.
[e]Brains of only six marmosets were scanned because of limited MRI machine time.
[f]Only four brains of the MPTP-treated marmosets were examined by tyrosine hydroxylase antibody immunohistochemical staining. Other brain was histologically analyzed in more detailed manner in other advanced MRI studies (Hikishima *et al.*, 2015a; Hikishima *et al.*, 2015b).
[g]Data used and reported in previous study (Ando *et al.*, 2017).

was illuminated from 09:00 to 21:00 hours. The temperature and humidity were held at 25°C–27°C and 42%–58%, respectively.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine preparation

MPTP hydrochloride powder (Lot: 067K1946; Sigma-Aldrich Co., St. Louis, Missouri, USA) was dissolved in physiological saline at a concentration of 1 mg/ml. MPTP doses were calculated and expressed as the free base excluding the weight of hydrochloride. The weight of the MPTP free base was 1.21 times the weight of MPTP hydrochloride.

Single regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

We focused on a single regimen of MPTP to investigate behavioral changes over more than 1 year. Our rationale for using a single MPTP regimen is described in the Introduction. The MPTP was s.c. to 10 drug-naive marmosets on three consecutive days (free base doses of 2, 2, and 1 mg/kg on days 0, 1, and 2, respectively). Thus, the total cumulative dose of MPTP was 5 mg/kg over three consecutive days. During the 3 days of MPTP administration and the subsequent 2-week period, supplements (orally administered nutrient solution and s.c.-administered electrolyte solution) were administered to marmosets, as described previously (Ando *et al.*, 2012), to compensate for the anorexia and dehydration caused by MPTP toxicity. The present regimen was selected on the basis of our unpublished pilot study, in which MPTP regimens (free base doses of 2, 1, and 1 mg.kg s.c. for group A, and of 2, 2, and 1 mg/kg s.c. for group B, on days 0, 1 and 2, respectively) were tested using three marmosets in each group. The single regimen of MPTP (2, 2, and 1 mg/kg, s.c. on three consecutive days) was used in the present study because marmosets for the group B showed clear manifestations without weakening or death, at least during the 1-month period in which preclinical evaluation tests were performed.

Experiment 1: locomotion and dysfunction score

Marmosets used in this experiment

A total of 10 marmosets that received the single regimen of MPTP were used in this behavioral experiment, which lasted for more than 1 year. Some of these marmosets continued to be used in the other four experiments in the present study.

Continuous measurement of locomotion as an objective, quantitative, and simple measure of immobility, one of the primary signs of Parkinsonism

The daily locomotion count of each marmoset in individual living cages was recorded continuously for 2 weeks before MPTP administration and for more than 1 year after the single regimen. An infrared motion sensor (O'Hara and Co., Tokyo, Japan) attached to the ceiling of each cage detected locomotion by measuring spatial shifts of thermal sources generated by the body parts of the marmosets by CIEA ACT scan computer system (Ando *et al.*, 2008).

Dysfunction score as an overall and qualitative measure based on visual inspection to support the locomotion data

Experienced observers visually inspected gross behavior at least once a week. The inspection was based on items listed on the CIEA dysfunction score form for the

common marmoset. The score included items related to PD-like signs, as well as other dysfunctions (Ando *et al.*, 2012). These items included: staying on the floor, lack of stimulus tracking, no biting, lack of facial expression, no squeak, resting tremor, moving tremor, jerky movements, immobility, hypoactivity, and catalepsy. Each of the 11 items listed on the form was recorded as observed (one point) or not observed (0 points). The dysfunction score represents the total number of observed behaviors out of a maximum of 11 points.

Experiment 2: door-opening choice behavior for food reinforcement

Marmosets used in this experiment

Four marmosets that satisfied the criterion for establishment of door-opening choice behavior during training before the MPTP regimen were selected from those using in experiment 1. Choice behavior data in this MPTP-treated group were compared between the pre- and post-MPTP regimen to examine influences of MPTP on this behavior. A further four healthy and intact male marmosets with body weights and ages comparable to those of the MPTP group were used as the MPTP-free group. They were treated in the same manner as were in the MPTP group, except that marmosets in the MPTP-free group did not receive MPTP.

Equipment

The multiple mailbox-type equipment consisted of 10 small boxes, five of which were placed on top of the other five boxes (see Video 1, Supplemental digital content 1, *http://links.lww.com/FBP/A3*). Each small box was 4.5 cm high, 3.0 cm wide, and 4.5 cm deep. The equipment was temporarily placed in front of a marmoset in its individual living cage when training or testing was performed. A piece of sponge cake (a food piece) was placed inside each box before starting training or testing. This food piece was hidden from the marmoset by a black opaque door.

Choice behavior training

The door-opening choice behavior of each marmoset was trained using food reinforcement in the multiple mailbox-type equipment. When the marmoset pulled a knob attached to any of 10 doors using its fingers, the food piece was available unless it had previously been taken. Thus, the door-opening choice response to each of the 10 boxes was reinforced with a food piece. Experimenters visually observed and recorded every opening response by the marmoset. The choice of a box containing a food piece was termed a correct (reinforced) choice response, while that of a box not containing a food piece (because it had been already taken by the marmoset) was termed an incorrect (non-reinforced) choice response. The experimenters also recorded whether the marmoset obtained and ate, or failed to grasp, the food piece (termed a correct response with eating of a food piece and a correct response with missing of a food piece, respectively). All training sessions were terminated when all food pieces had been obtained by the marmoset, or after 5 minutes (whichever came first). The training was conducted once daily on, generally 3 days per week. Daily food (50 g/day of balanced diet, described in the Animals and husbandry section above) was provided to the marmosets after training as well as after the testing described below. Therefore, the food deprivation procedure typically applied to laboratory animals in food reinforcement experiments was not used in the present experiment. The marmosets were trained until they satisfied the criterion for establishment of choice behavior, which was that all food pieces were obtained within 5 minutes over 5 consecutive training days.

Testing of choice behavior before and after the single regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated group

Once the marmosets had satisfied the criterion for establishment of choice behavior in the repeated training sessions, the pre-MPTP test was performed in the same manner as in the training sessions. This test was performed only once, at 0.5 months before the MPTP regimen.

The post-MPTP test was performed in the same manner as in the pre-MPTP test in the same marmosets, at 1.4 months after the MPTP regimen without any maintenance training. In these MPTP-treated marmosets (MPTP group), influences of MPTP on behavior were examined by comparing test data between the pre- and post-MPTP. Measurements of choice responses are described in the Statistical analysis section.

Testing of choice behavior in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-free group

The stability of door-opening choice responses without maintenance training (as in the MPTP group) was confirmed using control marmosets in the MPTP-free group. Marmosets in the MPTP-free group were selected, trained, and tested in the same manner as the MPTP group. For control MPTP-free marmosets, no maintenance training was performed between the first and second test (separated by a 2-month period), which was comparable to marmosets in the MPTP group.

Experiment 3: apomorphine-induced circling behavior

Marmosets used in this experiment

Long-lasting but latent effects of MPTP at the behavioral level were examined by apomorphine-induced circling behavior in six MPTP-treated marmosets used in experiment 1; brains of these marmosets were also scanned by MRI in experiment 4. Three other MPTP-free male marmosets were used as controls, and were healthy and

intact with body weights and ages comparable to MPTP-treated marmosets.

Apomorphine preparation and administration

Apomorphine hydrochloride (Sigma-Aldrich Co.) was added to physiological saline just before its subcutaneous administration. The mixture then was prepared to pure solution using an ultrasonic crashing apparatus. Apomorphine at 0.13 mg/kg (2 ml/kg) or saline (2 ml/kg) was administered to each marmoset. This test dose of apomorphine was determined in pilot experiments using marmosets that received the present MPTP regimen, and no clear behavioral change or hyper-excitation was observed after administration of apomorphine at 0.06 and 0.25 mg/kg s.c., respectively.

Equipment

A cylindrical chamber (40 cm in diameter and 65 cm high) was used. A transparent plastic cover was placed on top of the chamber to observe circling behavior, and to prevent the marmoset from jumping out. A cone was placed in the center of the circular floor to prevent the marmoset from crossing the center.

Measurement of circling behavior

Circling behavior was observed 14 months after the MPTP regimen. Five minutes after s.c. administration of apomorphine or saline, each marmoset was placed in the chamber. One circle was defined as a 360° full rotation. The numbers of right and left circling were visually counted for 60 minutes.

Experiment 4: brain MRI analysis

Marmosets used in this experiment

Brains of six marmosets were scanned once 5–6 months before, and 2 months after, the MPTP regimen, to obtain T1- and T2-weighted images and T1 and T2 relaxation times. The weighted images and relaxation times were obtained sequentially during the same scan. Not all marmosets were scanned because of the limited availability of MRI machine time. Just before and during each MRI scan, the marmosets were deeply anesthetized and carefully monitored, as described previously (Hikishima *et al.*, 2011; Hikishima *et al.*, 2013).

MRI equipment

A 7T Biospec 70/16 MRI system (Bruker Biospin GmbH, Ettlingen, Germany) with integrated transmitting and receiving coils 62 mm in diameter was used for the brain scan.

T1- and T2-weighted images

For qualitative inspection, T1- and T2-weighted images were obtained before and after the MPTP regimen. Images in the present study were acquired and reported as described previously (Hikishima *et al.*, 2011).

T1- and T2-relaxation time measurements

Different from visual inspection of the above images, objective and quantitative values (ms) of T1 and T2 relaxation times, within the same scan, were obtained before and after the MPTP regimen. T1 relaxation times were acquired using the saturation recovery spin echo method with variable rapid acquisition and relaxation enhancement (RARE) with multiple repetition times (effective echo time: 18 ms; repetition times: 500, 744, 1032, 1384, 1836, 2468, 3527 and 8000 ms, in that order; RARE factor: 4, field-of-view: 23.0 × 34.0 mm on a 128 × 128-matrix; slices: 12, each 1.2 mm in thickness; and total scan time: 7 minutes 48 seconds). T2 relaxation time values were acquired using the multi-slice and multi-spin echo sequence method (echo times: 9, 18, 27, 37, 46, 55, 64, 73, 82, 91, 101, and 110 ms, in that order; repetition time: 3000 ms; total scan time: 4 minutes 48 seconds; field-of-view and slices: the same as for the T1 relaxation time measurement).

Regions of interest (ROIs) for the caudate and putamen in the striatum were drawn as circles with a radius of three pixels (0.54 mm) on the A 9.0 slice of the atlas (Newman *et al.*, 2009), as shown in the upper part of Fig. 5; T1-weighted images of a marmoset were taken before MPTP administration. ROIs for the substantia nigra (SN) compacta and SN reticulata were determined with an ellipse of 15 pixels (2.70 mm) of the major axis and six pixels (1.08 mm) of the minor axis on the A 3.5 slice of the same atlas, as shown in Fig. 5. The upper half of the ellipse in the cranial direction was determined as the SN compacta and the lower half, in the caudal direction, was the SN reticulata.

Experiment 5: brain histology

Marmosets used in this experiment

After completing experiments 1–4, four marmosets (CM4, CM6, CM8, and CM14) were deeply anesthetized with an intraperitoneal administration of sodium pentobarbital (100 mg/kg of somnopentyl; Kyoritsu Seiyaku Co. Ltd, Tokyo, Japan) and euthanized by exsanguination. Brains of these marmosets were used for brief histology in the present study. Detailed histological analysis had already been reported as background data for advanced MRI studies using other marmosets that received the MPTP regimen used in this study (Hikishima *et al.*, 2015a; Hikishima *et al.*, 2015b). Therefore, histological analysis in the present study was performed only to confirm gross brain damage induced by the present MPTP regimen.

The stained brains of MPTP-treated marmosets were compared to the brains of two MPTP-free male marmosets. One (CM32) had been reported in a previous study (Ando *et al.*, 2017). The same staining procedure was used in the previous and the present studies.

Preparation of the brain sample

Brains were removed and fixed in 20% buffered formaldehyde and embedded in paraffin. The coronal section (thickness: 15 μm) was prepared + 10 mm from the interaural line (+6.1 mm from the bregma) by reference to the brain atlas (Yuasa *et al*., 2010). This section was selected because the caudate and putamen were clearly distinguishable in the striatum of MPTP-free marmoset brains. Immunohistochemical staining with TH antibody (1:500, mouse anti-tyrosine hydroxylase MAB318; Merck Millipore Co., Billerica, Massachusetts, USA) was performed using an automated immunostainer (Bond-MAX; Leica Biosystems Co., Mount Waverley, Australia). Images were captured and generated using an upright microscope (Axio Imager; Carl Zeiss Inc., Thornwood, New York, USA).

Quantitative analysis of stained area

Coronal images of the brains of three marmosets (CM4, CM6, and CM8) that received the single MPTP regimen, and of the brains of two MPTP-free marmosets, were quantitatively analyzed using NIH ImageJ software (*https://imagej.nih.gov/ij/*), as described previously (Ando *et al*., 2017). Each brain image was analyzed by the software in the following manner: (1) selecting 'Image → Color → Split Channels' and using only the blue split image, (2) setting the length scale to mm, (3) tracing the outline of the whole coronal brain image as the ROI, (4) selecting 'Image → Adjust → Threshold' and adjusting the minimum threshold to 0 and the maximum threshold to 100 (because these settings yielded clear images of the striatum in the MPTP-free marmoset brain without interference by other areas), and (5) selecting 'Analyze → Measure'. With these settings, the area of the image detected under the fixed thresholds (in pixels) from the whole coronal brain was calculated in mm^2. To address density differences among brain images obtained from different tissue preparations, simplified validation was performed. Each ROI (1.0 × 1.0 mm) was placed in the right and left cortex of the same split blue coronal images and measured in mm^2, with or without thresholds.

Statistical analysis

All averaged data are expressed as means and SDs. In the statistical analysis, $P \leq 0.05$ was considered statistically significant.

Experiment 1

No statistical analysis was performed, although quantitative data are graphically presented in Figs. 1 and 2.

Experiment 2

Data of door-opening choice responses were analyzed in terms of several parameters, as follows: total no. of responses: no. of responses in the daily session; total responses per minute: no. of responses/session time in minutes (time to take 10 food pieces or reaching the 5-minute session-time limit, whichever came first); total correct responses (%): 100 × no. of correct responses including both eating food and missing (failure to grasp) food pieces; correct responses with eating of food piece (%): 100 × no. of correct responses with eating of food piece/no. of total responses; and correct responses with missing of food piece (%): 100 × no. of correct responses with missing of food piece/no. of total responses. All of these measures, presented in Fig. 3, were compared between the post-MPTP and pre-MPTP tests in the MPTP group (n = 4), and between the second and the first tests in the MPTP-free group (n = 4). The percent data were not subjected to arc-sin transformation; instead, they were analyzed statistically using two-tailed Student paired *t*-test in Excel 2011 software (Microsoft Corp., Redmond, Washington, USA), as per the other non-percent data obtained in this experiment.

Experiments 3, 4, and 5

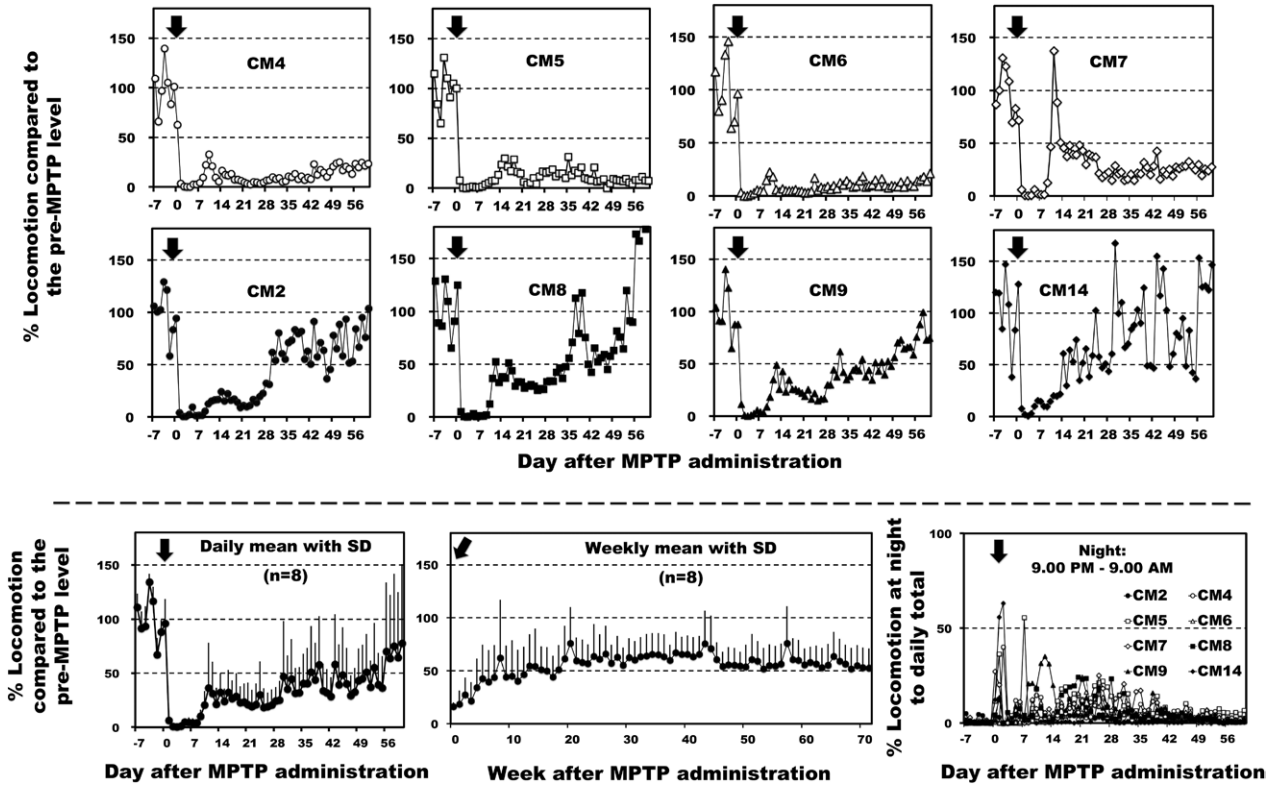
In experiment 3, statistical analysis was not performed, but quantitative data of apomorphine-induced circling behavior (no. of 360° full rotations) are graphically presented in Fig. 4. In experiment 4, all T1 and T2 relaxation times (ms) were obtained from the ROIs in the striatum (putamen and caudate) and the SN (compacta and reticulate) in each brain hemisphere. Values for both hemispheres were averaged for each ROI after confirming the appropriateness of such averaging by analysis of variance (ANOVA) testing using SPSS statistical software (IBM Corp., Armonk, New York, USA). The T1 and T2 relaxation times for each ROI in Fig. 5 were compared between the post- and pre-MPTP tests (n = 6 for each) using two-tailed Student's paired *t*-test in Excel 2011 software. When examining the relationship between the T1 relaxation time and apomorphine-induced circling behavior, the T1 value of each ROI was subjected to Pearson's correlation analysis in terms of apomorphine-induced circling behavior in either direction using SPSS. In experiment 5, only representative coronal brain photos are presented in Fig. 6. Histologically stained areas (mm^2) were also analyzed using ImageJ. The correlation between histological areas of the two coronal hemispheres and circling directions (n = 3) was calculated using SPSS.

Results

Experiment 1: baseline locomotion and dysfunction score

Changes in the daily locomotion of each marmoset treated with the single regimen of MPTP at the cumulative-free base dose of 5 mg/kg s.c. on three consecutive days are presented in Fig. 1. The locomotion count was expressed as the percentage relative to the baseline of each marmoset (the preceded 1-week mean of daily counts recorded during the 2 weeks before the single

Fig. 1

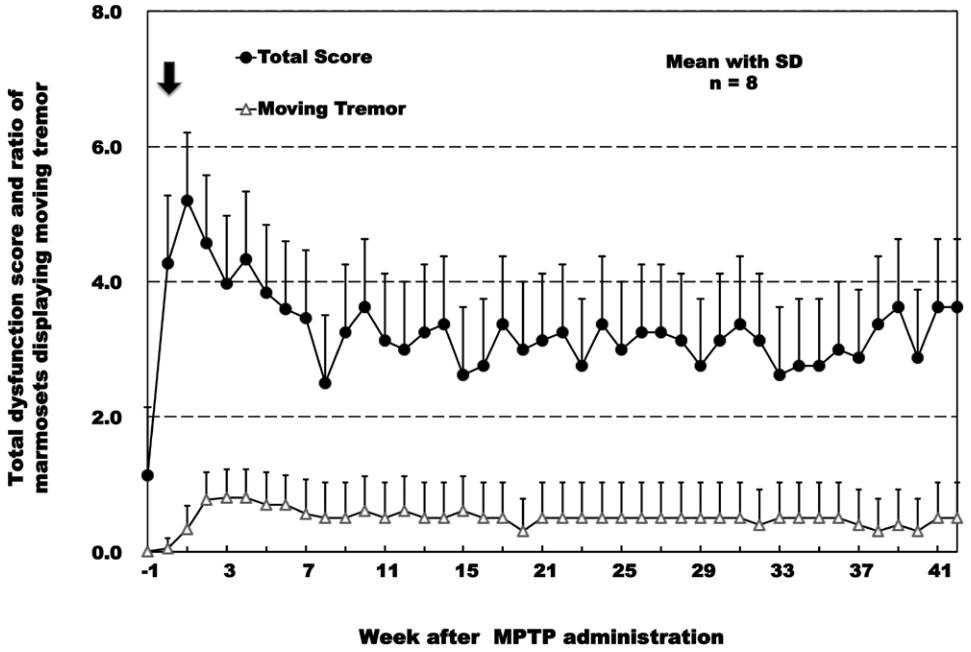


Long-term changes in daily locomotion of common marmosets that received a single regimen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Decreased locomotion is an objectively quantifiable measure of immobility, one of the main signs of Parkinsonism. This measure is considered sensitive and useful for preclinical evaluations of drug therapy and brain transplantation of neural cells in the marmoset of Parkinson's disease model. A single MPTP regimen is convenient for preclinical evaluation studies if the decreased level of locomotion is properly characterized over the long-term. In the present single regimen, subcutaneous (s.c.) MPTP at free base doses (not included HCl in its calculation) of 2, 2, and 1 mg/kg on days 0–2, respectively, was administered to 10 marmosets. The locomotion count of each marmoset in its individual living cage was recorded continuously, every hour for 1 year or more, after the single MPTP regimen (cumulative dose of 5 mg/kg). The count was expressed as the percentage relative to the pre-MPTP baseline level of each marmoset, which was defined as the preceded 1-week mean of daily counts recorded during the 2 weeks before single MPTP regimen. Downward arrows represent the 3-day MPTP regimen. Each of the upper eight blocks in this figure indicates the locomotion count of each of eight marmosets. Data for the other two marmosets are not presented in this figure because these marmosets died 2.7 months after the MPTP regimen (see text for explanation). The bottom left and middle blocks represent means and standard deviations of the eight marmosets over 60 days and 70 weeks, respectively. The bottom right block indicates the percentages of nightly locomotion (21:00–09:00 hours) as proportions of the daily total locomotion of each marmoset.

regimen). Four marmosets in the top blocks were relatively stable, with low locomotion counts for 8 weeks or more after the MPTP regimen, whereas the other four marmosets in the middle blocks showed spontaneous recovery over several weeks. Two marmosets showed low and stable levels of locomotion, similar to the four marmosets in the top blocks, but these animals died so their data are not presented in the figure. Marmoset CM1 died 2.7 months after the single MPTP regimen, with occasional vomiting and lying down exhibited for a few days before death. According to the autopsy, there was an abnormality in the digestive system. Marmoset CM3 was euthanized by exsanguination under deep anesthesia by sodium pentobarbital at 100 mg/kg, i.p., because this marmoset gradually showed decreased food intake, body weight loss, and physical weakness for an undetermined reason.

The left and middle bottom blocks represent mean counts with SDs from eight marmosets, excluding the two dead marmosets, over 2 months (60 days) and over more than 18 months (72 weeks), respectively, after the single MPTP regimen. Mean locomotion counts indicated gradual recovery over days, weeks, or months, but did not attain the mean pre-MPTP baseline levels (100%) after 18 months. To explore influences of MPTP on the circadian rhythm of locomotion, the percentages of locomotion at night (21:00–09:00 hours) in terms of total daily (24-hour) locomotion were calculated, and are shown in the right bottom graph. This percentage at night increased significantly in some marmosets for a few weeks after the single regimen. In some cases, these increases went back to the zero percent level, but generally the levels remained high for several weeks after the single regimen compared to pre-MPTP levels (Fig. 1).

Fig. 2



Changes in dysfunction scores after the single MPTP regimen. The dysfunction score represents the weekly mean and SD values in eight marmosets over 40 weeks, excluding two marmosets which died in the middle of the experiment. The score data were based on visual inspection by experienced observers according to the items of the Central Institute for Experimental Animals (CIEA) dysfunction score. The score was composed of 11 items related to Parkinsonism and some other signs of dysfunction in marmosets after the MPTP regimen. These items include moving tremor, resting tremor, jerky reaction, immobility, catalepsy, hypoactivity, lack of facial expression, lack of squeaking, lack of eye-tracking, lack of biting a pencil, and lying on the floor. Each item was recorded as observed (one) or not observed (0). Thus, the dysfunction score was defined as the total number of observed items (max = 11). For reference, total dysfunction score 1 week before MPTP regimen (indicated as '−1' week on the horizontal axis) was 1.1 and 0.6 (mean and SD). The moving tremor, one of the most clearly evident signs of PD, is represented as the ratio of marmosets with moving tremors out of the total eight marmosets, with weekly mean and SD data. The ratio for the moving tremor ranged between 0.0 (moving tremor not observed in any marmoset) and 1.0 (moving tremor observed in all eight marmosets). For reference, the ratio of marmosets displayed moving tremor 1 week before MPTP regimen was 0.0 and 0.0 (mean and SD). Downward arrow represents the 3-day MPTP regimen. PD, Parkinson's disease.

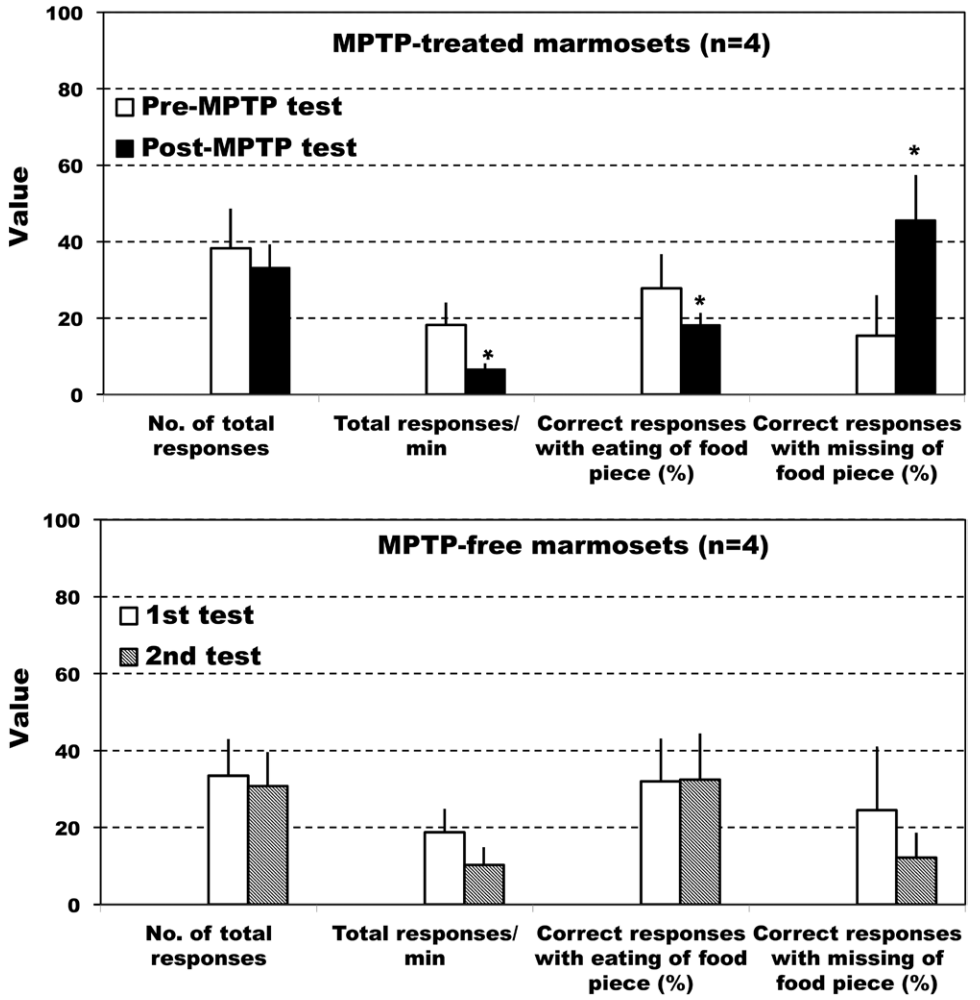
Gross behavioral observation revealed various changes, including the development of PD-like signs such as immobility, lack of facial expression, and jerky movements after the single regimen of MPTP, but these changes were variable across animals and time. Total dysfunction scores and extent of moving tremor (motion tremor) >40 weeks after the regimen are shown in Fig. 2. The dysfunction scores of MPTP-treated marmosets, based on visual inspection of gross behavior, increased after the MPTP regimen. The mean scores did not decrease to pre-MPTP levels even ≥40 weeks after the regimen. For reference, total dysfunction score 1 week before MPTP regimen was 1.1 and 0.6, each mean and SD, respectively. Of the observational items, moving tremor was one of the most distinct and clearly observable signs, exhibiting minimal variability across observation times within marmosets. Once the moving tremor appeared in a specific marmoset, this persisted consistently for a relatively long period. Therefore, moving tremor data are especially significant, and are presented here in addition to the total dysfunction scores. As shown in Fig. 2, the ratio for moving tremor ranged between 0.0 (moving tremor not observed in any marmoset) and 1.0 (moving tremor observed in all eight marmosets). Moving tremor persisted in more than half of the eight marmosets observed weekly for 40 weeks after the single regimen of MPTP. For reference, ratio of marmosets displayed moving tremor 1 week before MPTP regimen was 0.0 and 0.0, each mean and SD, respectively.

Experiment 2: door-opening choice behavior for food reinforcement

Training

Marmosets were trained for door-opening choice behavior for several months. Marmosets that satisfied the establishment criterion for this behavior, as described in the Methods, completed the pre- and post-MPTP tests as the MPTP group (n = 4). Marmosets in the MPTP-free group that satisfied the same establishment criterion were also selected for the first and second tests (n = 4). An example of door-opening choice behavior is presented in Video 1 (Supplemental digital content 1, *http:///links.lww.com/FBP/A3*).

Fig. 3



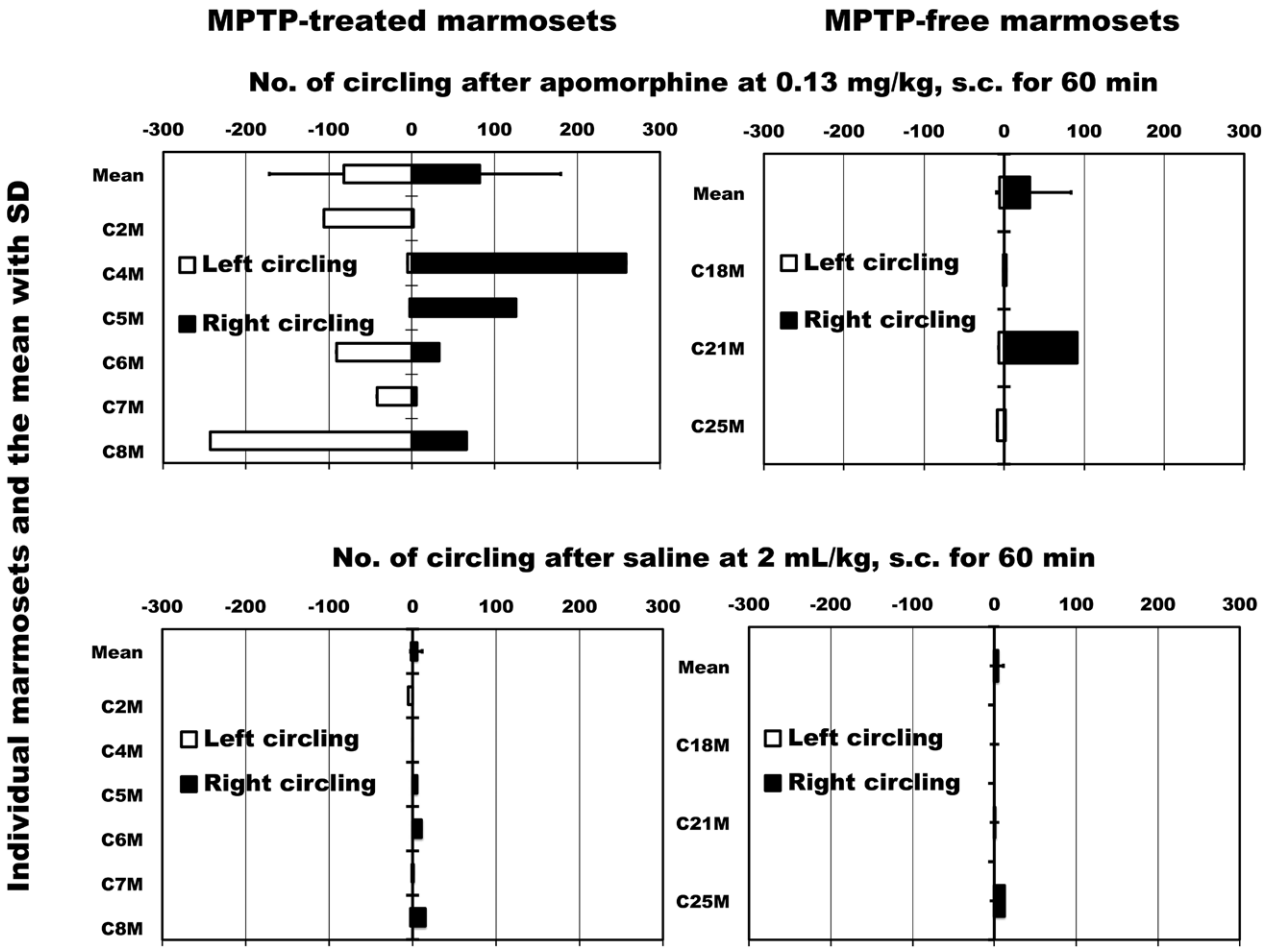
Influences (direct and indirect effects) of MPTP on food-reinforced door-opening choice behavior in marmosets, using mailbox-type equipment. Before performing the tests described below, marmosets were repeatedly trained for choice behavior for a food piece, obtained by opening one of 10 opaque doors in which a food piece was placed inside (unless it had already been taken). The MPTP-treated marmosets and MPTP-free marmosets (n = 4 in each group) that satisfied the choice behavior establishment criterion were selected and used for two tests, which were separated by a 2-month interval. The two tests were named pre- and post-MPTP tests in the MPTP group and the first and second tests in the MPTP-free group. The marmosets in the MPTP group were selected from those that received the single regimen of MPTP in experiment 1. Maintenance training was not provided between the two tests in either group. The total response represents the number of total door-opening responses by each marmoset in one test session, in which all 10 food pieces had been obtained or 5 minutes had elapsed (whichever came first). Response percentages for each measure are expressed relative to the total number of responses. A correct response was defined as the choice of a box that contained a food piece. The mean and SD data for measure are expressed in the graphs. Within-group data for each measure were compared between the two behavioral tests using a two-tailed paired Student *t*-test (*$P \leq 0.05$).

Comparison between the post- and pre-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine tests of door-opening choice behavior in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine group

Results of food-reinforced choice behavior in the MPTP group are presented in the upper graph of Fig. 3. The total number of door-opening choice responses in the post-MPTP test did not change significantly after the single regimen of MPTP, even though 2 months had passed between the pre- and post-MPTP tests without maintenance training. On the other hand, there was a decrease in the total number of responses per minute in the post-MPTP test compared to the pre-MPTP test.

The percent total correct responses in the post-MPTP test did not significantly differ from the pre-MPTP test (data not shown in Fig. 3). On the other hand, the percent correct responses when food pieces were eaten decreased significantly compared to the pre-MPTP test. The percent correct responses when food pieces were missed (failed to be grasped) increased significantly compared to the pre-MPTP test. Eating and missing of food pieces were confirmed by visual inspection by the

Fig. 4



Circling behavior of marmosets in a cylindrical chamber. The number of 360° full rotations was recorded in the MPTP and MPTP-free groups within 60 minutes after s.c. administration of apomorphine at 0.13 mg/kg or saline. Circling behavior in the MPTP group was recorded at 14 months after the single MPTP regimen in experiment 1.

experimenters. The missing indicates impairment of skilled finger motion after the single MPTP regimen.

Comparison between the first and second tests of door-opening choice behavior in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-free group

As shown in the lower graph of Fig. 3, the four measures of door-opening choice behavior and the total correct responses (%; data not shown) did not change significantly between the first and second tests. No maintenance training was given to marmosets between the two tests (2 months apart), and stability of this door-opening choice behavior was observed in the MPTP-free marmoset group. Therefore, changes in the MPTP group are likely associated with MPTP administration.

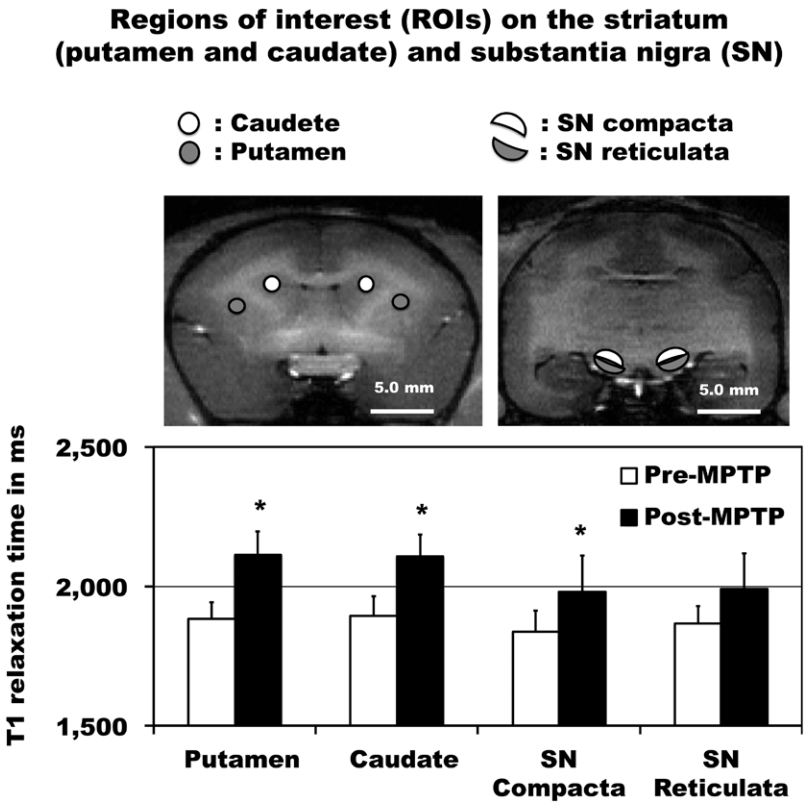
Experiment 3: apomorphine-induced circling behavior

The six marmosets used in this experiment showed moving tremors in their individual living cages over 1 year after the single regimen of MPTP. These marmosets did not show any tendency for circling behavior in their individual living cages over a long period of observation. When apomorphine was administered at 0.13 mg/kg s.c., either right or left circling (one circle = 360° full rotation) was observed in the cylindrical chamber (Fig. 4). The direction of circling differed depending on the marmoset, although all marmosets had previously received the same MPTP regimen by the subcutaneous route. On the other hand, they did not show any clear circling after saline administration. In MPTP-free marmosets, no such circling tendency as in the case of MPTP-treated marmosets was observed after subcutaneous administration of either apomorphine at 0.13 mg/kg or saline. The circling behavioral data were analyzed in relation to the MRI data, as described in of experiment 4.

Experiment 4: MRI analysis

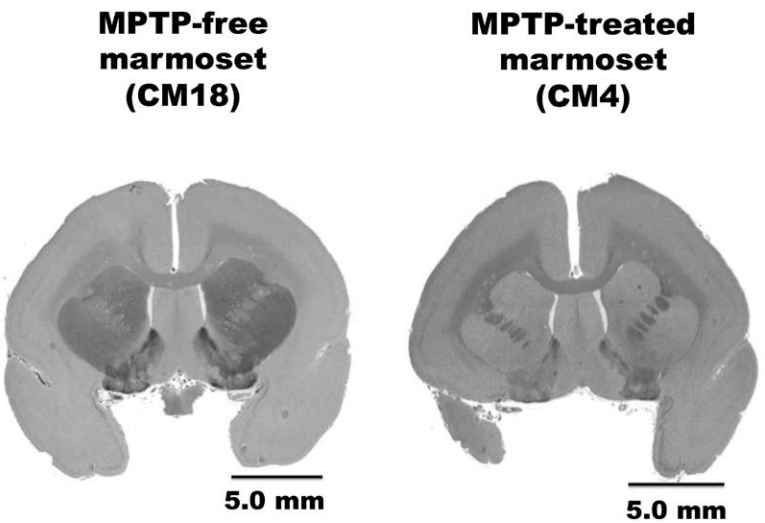
Global and qualitative brain images of six marmosets were compared between pre- and post-MPTP regimen by visual inspection (data not shown). No qualitative

Fig. 5



MRI analysis of marmoset brains. In the upper part, regions of interest (ROIs) on the striatum (putamen and caudate) and the substantia nigra (compacta and reticulata) are delineated on the T1-weighted coronal images of an MPTP-free marmoset. In the lower graphs, means and SDs of T1 relaxation times (ms) are presented on each ROI in the pre- and post-MPTP scans. MRI scans were performed for six marmosets, 4–5 months before and 2 months after, the MPTP regimen in experiment 1. The mean T1 relaxation times on each ROI were compared between pre- and post-MPTP scans using the two-tailed paired Student *t*-test (*$P \leq 0.05$).

Fig. 6



Histology of marmoset brains. Tyrosine hydroxylase immunohistochemical staining was performed on the coronal slice taken +10 mm from the interaural line (+6.1 mm from the bregma) of a representative brain of an MPTP-free marmoset, and from marmoset that received the present MPTP regimen in experiment 1.

difference was visually evident in the coronal views of T1- and T2-weighted images between pre- and post-MPTP administration, with a focus on ROIs in the striatum (putamen and caudate) and the SN compacta and SN reticulata.

Next, T1- and T2-relaxation times (ms) for the above ROIs were compared between pre- and post-MPTP regimen. The T1- and T2-values for each ROI were averaged between the right and left hemispheres of each marmoset brain. As shown in the bottom graph of Fig. 5, T1 values averaging the ROIs of the two hemispheres significantly increased in the putamen, caudate, and SN compacta in the post-MPTP scan compared to the pre-MPTP scan, but did not increase significantly in the SN reticulata. The average T2 values of both hemispheres for all post-MPTP ROIs did not differ significantly from pre-MPTP values (data not shown).

Concerning the appropriateness of averaging the values between the right and left hemispheres, three-way ANOVA [factor 1, timepoint (pre- and post-MPTP); factor 2; ROIs; and factor 3; laterality (right and left)] did not reveal any significant difference in the laterality [n = 6, F (7, 80) = 0.50, *NS* the T1 values; n = 6, F (7, 80) = 0.04, *NS* for the T2 value].

In the analysis of the relationship between the T1 values and apomorphine-induced circling behavior, the correlation coefficients between the post-MPTP T1 values in the two brain hemispheres and the number of circling behaviors in either direction were calculated. A significant correlation was observed between the T1 value of the right SN compacta in the post-MPTP value and the right circling behavior (n = 6, r = 0.85, P < 0.05). On the other hand, no significant correlation was observed between circling in either direction and any post-MPTP T1 value in the striatum (putamen and caudate) of either hemisphere.

Experiment 5: brain histology

A representative coronal slice of an MPTP-free intact marmoset shows dopaminergic (catecholaminergic) neurons in the putamen and caudate of the striatum (Fig. 6). A representative slice of a marmoset that received the present single MPTP regimen was indicative of neural loss in the striatum as shown in the same figure. In the present study, the coronal slices of four marmosets (CM4, CM6, CM8, and CM14) that received the same MPTP regimen were obtained and examined histologically. The other marmoset brain in the present study was made available for advanced MRI studies (Hikishima *et al.*, 2015a; Hikishima *et al.*, 2015b), in which detailed histological analysis was performed to validate the MRI results.

Based on quantitative analysis using ImageJ software in the present study, stained coronal images of the brains of four marmosets that received the present MPTP regimen were captured and digitalized (by pixels) as well as in the case of the brains of two MPTP-free marmosets. The chosen thresholds (minimum = 0 and maximum = 100) allowed for visualization of only the striatum in the MPTP-free marmoset brains. These areas were 37.4 and 43.5 mm^2 in MPTP-free marmosets CM18 and CM32, respectively. However, areas within the same thresholds ranged from 0.1 to 6.1 mm^2 in the four marmosets that received the MPTP regimen.

Using the same software, TH-stained coronal areas in the right and left hemispheres were measured separately in three marmosets (CM4, CM6, and CM8) received MPTP regimen and used in the apomorphine-induced circling behavior experiment. There was no statistically significant correlation between the stained coronal area in either hemisphere and circling behavior in either direction.

Discussion

Objectively recorded locomotion as a measure of immobility to evaluate protective effects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity for future preclinical treatments

The present study investigated the baseline locomotion behavior over a period of several months of marmosets in individual living cages after receiving a single regimen of 5 mg/kg s.c. MPTP over three consecutive days (The MPTP dose was calculated and expressed as the free base excluding HCl). The purpose of this experiment was to characterize the usefulness and limitations of this behavioral baseline for future preclinical evaluations of drug therapies or neural cell transplantations, in terms of protection against MPTP toxicity. Within this framework, the single regimen of MPTP is considered preferable for efficacy evaluation compared to repeated MPTP regimens over week-long intervals, because repeated and multiple MPTP regimens may result in unpredicted complications. The single MPTP regimen is also desirable because the delivery of treatment regimens is time-consuming for experimenters, with important safety considerations associated with MPTP toxicity. However, if the MPTP-induced PD model is used to investigate the acute dose effects of test drugs, repeated MPTP regimens can be used until marmosets show marked and desirable PD-like manifestations.

To the best of our knowledge, no previous reports have continuously recorded the locomotion of PD model marmosets in individual living cages (not socially housed) for more than 1 year after a single regimen of MPTP. Other studies have performed detailed characterizations of MPTP-treated marmosets in terms of motor and non-motor functions, such as olfaction and social interaction (Philippens *et al.*, 2010; Phillips *et al.*, 2017). It is important to compare these functions between marmosets that received full versus partial MPTP dose regimens to obtain a basic understanding of the marmoset model. On the other hand, our study described a practical model for preclinical evaluation of medical treatments,

in which multiple factors with complicated relationships, such as those involved in social interaction, and multiple regimens of MPTP administration were not considered.

Decreased levels of locomotion are considered a measure of immobility, one of the main signs of Parkinsonism. In preclinical evaluation studies, a clear conclusion regarding the efficacy of a given medical treatment is required, although that conclusion is based on specific experimental conditions. A decreased level of locomotion is considered an objective, quantitative, simple, and sensitive measure for characterizing the protective effects of drugs against MPTP toxicity (Ando *et al.*, 2008). However, the MPTP-induced decrease in baseline locomotion showed some fluctuations and spontaneous recovery among the animals in this study. The usefulness and limitations of the locomotion measure were clarified by observing these changes over a period of months. Evaluation tests should be performed within a 2-month period after the MPTP regimen: two of the 10 marmosets in this study died within 2.7 months. In future studies examining protective effects of medical treatments against MPTP toxicity, preclinical evaluations could employ analyses such as area under the curve (x-axis: days after MPTP regimen; y-axis: locomotion level) between an MPTP plus vehicle control group and an MPTP plus preclinical treatment group.

Other points for consideration regarding locomotion measurement

The locomotion counts of marmosets were continuously recorded for 24 hours over consecutive days, weeks, and months in this study. In the control MPTP-free marmosets, high counts were consistently recorded during light-on daytime but very low levels or almost no locomotion were recorded at night, indicative of a circadian rhythm similar to humans rather than rodents. Daily locomotion counts were stable for multiple days before MPTP administration, with some variability across marmosets. When the daily locomotion counts for each marmoset were expressed as percentages of the mean daily counts for a predetermined period (e.g. for 1 week) before the MPTP regimen, the percentage locomotion measure represented a stable behavioral baseline with less individual variability among marmosets. In MPTP-treated marmosets, the percentage locomotion counts decreased for several weeks, with less variability within all marmosets when expressed as the percentages rather than actual locomotion counts. Therefore, the decreased level of locomotion in the MPTP-treated marmoset is considered a valid, objective, quantitative, and stable behavioral baseline.

Although the baseline locomotion was stable within a limited period, spontaneous recovery to pre-MPTP levels was observed in some marmosets. It is possible that the present MPTP regimen did not induce long lasting damage to the dopaminergic neural system in marmosets. Thus, the remaining neural system in the brains of these marmosets may be able to compensate for the damaged system, leading to behavioral recovery. Other individual differences in recovery among marmosets may be related to variations in the metabolic processes involved in converting MPTP to MPP^+ in dopaminergic cells of the SN, although many other factors may be involved in individual differences in MPTP toxicity (Przedborski and Jackson-Lewis, 1998; Bajpai *et al.*, 2013). The individual differences and recovery in the present model may provide important information for understanding the etiology of PD and for considering rehabilitation methods for PD patients by activating remaining neural networks. Aside from the recovery issue, two marmosets died or were euthanized during the study. The cause of their gradual weakening was thought to be MPTP toxicity, although all marmosets were provided with several weeks of intensive care. These two marmosets were considered more sensitive than the others in terms of toxicity to MPTP or MPP^+.

It should also be noted that the proportion of locomotion at night increased for several weeks after the MPTP regimen in some marmosets. This phenomenon persisted for a limited period (several weeks) after the MPTP regimen, but is reminiscent of the sleep disturbances seen in PD patients with dopaminergic neural activity changes (Barraud *et al.*, 2009).

Parkinson's disease like signs observed based on the dysfunction score

Various PD-like and other signs of dysfunction were measured in this study. Monitoring of these signs was considered a secondary purpose of our present study. PD-like signs in marmosets that received MPTP have already been reported in detail previously (Jenner *et al.*, 2000; Fox and Brotchie, 2010).

Dysfunction scores in the present study increased for several weeks after the MPTP regimen, and then decreased but remained high for more than 40 weeks after single regimen of MPTP. The increased level of dysfunction (subjectively observed) is in accordance with the decreased level of locomotion (objectively measured). Visually observable PD-like signs in the present study were scored as ‘yes'or ‘no'by observers. Among the various signs, moving tremor (motion tremor) is one of distinct PD-like signs in MPTP-treated marmosets, although the resting tremor reported in PD patients was hardly observed in MPTP-treated marmosets.

Although objectively recorded locomotion is considered preclinically useful as a measure of immobility, other PD-like signs should be considered. Visual inspection of the general and gross behavior of experimental animals by experienced observers is important and useful in preclinical studies (Nomoto *et al.*, 1998; Fox and Brotchie, 2010).

Without this, it may be difficult to determine whether increased marmoset locomotion reflects an improvement due to treatment or merely an adverse reaction (such as hyper-sensitization or dyskinesia) (Ando *et al.*, 2014). Detection of genuine therapeutic recovery from objectively measurable immobility requires evaluation of total and general behavioral signs as in the present dysfunction score.

Food-reinforced choice behavior

Influences (direct and indirect effects) of MPTP on door-opening operant behavior were investigated in marmosets that received the MPTP regimen. Initially, we expected that some types of cognitive impairment would be detected in MPTP-treated marmosets using the present behavioral method (Vezoli *et al.*, 2011). However, the correct response rate (%) in the post-MPTP test (a possible measure of cognitive impairment) did not show a statistically significant decrease compared to the pre-MPTP test. Therefore, cognitive impairment in the MPTP-model marmosets was not detected using the present method. However, impairment of the skilled motion of fingering was detected based on the higher percentage of correct responses with missed (failure to grasp) food pieces. Slowed motion in the present choice behavior was also detected at 3 months after the MPTP regimen, as shown by the decrease in total responses per minute in the MPTP group.

In the MPTP-free group, no significant change was detected in any measure between the first and second tests, which were separated by 3 months. Therefore, the learning behavior was well-maintained during this period without any maintenance training. Thus, changes in measures in the MPTP group observed after the MPTP regimen compared to those before the regimen were not related to time-dependent changes or the lack of maintenance training between the two tests, but instead to influences of MPTP on behavior.

Food-reinforced learning behavior using the present mailbox-type equipment was established in a limited number of training sessions within a few months, and without food deprivation. The criterion for establishment of this behavior was not as strict as in our previous operant behavioral studies of the rhesus monkey and rat. In a delayed matching-to-sample experiment using the rhesus monkey, and a two-lever drug discrimination experiment using the rat (under water and food deprivation conditions, respectively), the behavior establishment criterion was set at 80% correct responses over several consecutive daily sessions (Ando and Yanagita, 1992; Ando *et al.*, 2003). Based on these results, we established several liquid-reinforced operant behaviors in the marmoset using the modified test chamber originally designed for the rat. In this way, it was possible to establish similar lever-pressing operant behavior in the marmoset. However, it was not easy to obtain stable baseline behavior in this small primate during similar tasks given to the rhesus monkey and rat. This may be because the marmoset is behaviorally restless and very sensitive to external stimuli. These behavioral traits make it difficult for the marmoset to concentrate on a specific task during experimental sessions. It should also be noted that some degree of food or water deprivation are required to establish stable operant baseline behavior in laboratory animals, but deprivation above a certain limit is not appropriate for the marmoset because this small monkey species is likely to be physically weaker than the rhesus monkey and rat after deprivation. Furthermore, the deprivation procedure resulting in physical weakness in marmosets may not be ethically appropriate.

We used a simple choice task with neither deprivation nor strict behavior establishment criterion. In laboratory animal experiments, cognitive functions must be examined according to motor responses that differ from those in humans, in which verbal responses and minimum motor functions can be used based on an experimenter's oral instructions. Motor functions play important roles in cognitive function tests in laboratory animals, such as maze learning and delayed matching-to-sample tests; our present behavioral test is situated in between these two tests in terms of the contribution of motor functions. In addition, other monkey species with a higher tolerability for deprivation procedures and high concentration ability, such as the macaque monkeys, may be more appropriate to examine higher order cognitive functions using behavioral methods. The rationale for assessing choice behavior in our experiments was based on the purpose of our study, that is, to investigate multiple behaviors (including those affected by motor dysfunction) in MPTP-treated marmosets.

Apomorphine-induced circling behavior as a possible measure of asymmetrical neural damage in the brain hemispheres

Persistent but latent behavioral influences of MPTP were examined in this study based on apomorphine-induced circling behavior. More than 1 year after the MPTP regimen, right or left circling of the marmosets in a cylindrical chamber was observed after subcutaneous administration of apomorphine. Using 6-hydroxidopamine (another dopaminergic neurotoxin), circling behavior has been extensively studied in both rodents and marmosets (Przedborski *et al.*, 1995; Ekesbo *et al.*, 2000; Eslamboli, 2005). Since 6-hydroxidopamine does not pass the blood-brain barrier, this toxin must be infused directly into the brain. When 6-hydroxidopamine is infused into the nigrostriatal dopaminergic neurons in either brain hemisphere, the neurons are specifically damaged on that side. Thus, denervation hypersensitivity occurs at the dopaminergic receptors only on the damaged side. When apomorphine (a dopaminergic

receptor agonist) is peripherally administered to laboratory animals with unbalanced neural brain damage, the behavioral manifestation is contralateral circling, which is attributable to hyper-stimulated dopaminergic receptors on the damaged brain side. In our present study, MPTP was not infused into either brain hemisphere but was administered peripherally (s.c.). Thus, MPTP was expected to damage the dopaminergic neurons in both brain hemispheres equally. However, either right or left circling, which varied among the MPTP-treated marmosets, was observed after subcutaneous administration of apomorphine. Furthermore, right circling correlated significantly with the post-MPTP T1 value in the right SN compacta. Therefore, this circling may be attributable to asymmetrical dopaminergic neural activity in the brain hemispheres. Although the relationship between circling behavior and neural imbalance is interesting, the implication of the T1 value increase in the SN compacta remains unclear, and asymmetrical neural damage to the two brain hemispheres in the present MPTP-treated marmosets was not detected by TH histology. Further studies are required to explore relationship between this circling behavior and imbalanced brain damages. We expect that apomorphine-induced circling behavior may play a role in MPTP-treated marmosets as a sensitive behavioral probe to detect asymmetrical neural brain damage only if the other background data are to be more accumulated.

MRI analysis of neural brain damage

Two MRI studies have been already performed on marmosets that received the present MPTP regimen, to detect neural damage caused by the peripheral administration of MPTP. In these already published reports, voxel-based morphometry showed that the volumes of SN and the locus coeruleus decreased after the present MPTP regimen (Hikishima *et al.*, 2015a). Another study using diffusion tensor analysis and tractography, on the same MPTP-treated marmosets used in the present study, revealed fiber disruption in the nigrostriatal pathway from the SN to the striatum (Hikishima *et al.*, 2015b), backed by histological evidence, in marmosets that received the present MPTP regimen. These two studies were started after the present MRI analysis using only T1 and T2 relaxation times. Therefore, the basic and grounded data on these relaxation times in the present study was considered to be worth reporting here although the order was the other way around.

When the marmoset brains were scanned via 7T-MRI, neither T1- nor T2-weighted post-MPTP images were visually different from the pre-MPTP images. On the other hand, quantitative examination of T1 relaxation time showed significant increases in the bilateral means of T1 values (ms) in ROIs of the SN compacta and the striatum after the present MPTP regimen, relative to the pre-MPTP administration data. No significant change was observed in T2 relaxation times in any of the ROIs.

The cause of the increase in T1 in the dopaminergic neurons in the ROIs remains unclear, but may reflect an MPTP-induced decrease in melanin concentration in these neural cells. Melanin in normal dopaminergic neurons associates with metals to create a ferromagnetic field. The MPTP-induced decrease in melanin level may increase the T1 value of neurons in the SN.

In contrast to PD model marmosets, iron accumulation in PD patients triggered regional T1 and T2 value changes compared to healthy subjects (Vymazal *et al.*, 1999). However, the implications of increased T1 values reported in various studies in PD patients must be viewed with caution because T1 values depend on the molecular environment and the strength of the magnetic field.

Our MRI data on MPTP-treated marmosets were compared to the results of our earlier in-vivo PET study (Ando *et al.*, 2012) using other MPTP-treated marmosets. This PET study showed that the binding potential of a radioligand tracer ([^{11}C]PE2I) to dopamine transporters was decreased in the striatum of MPTP-treated marmosets compared to MPTP-free marmosets. It should be emphasized that a BP decrease at the putamen is strongly correlated with a decreased level of locomotion ($r = 0.98$). Together with the present MRI study, our data showed that in-vivo imaging of marmoset brains is important and useful to characterize the MPTP model in terms of its utility for preclinical evaluation of medical treatments.

Histology of brain damage

After experiments 1–4 had been completed, only four of 10 marmoset brains were examined by immunohistochemical staining with TH antibody. The purpose of the histology analysis in the present behavioral study was to confirm that brain damage was caused by the present MPTP regimen. The histological analysis confirmed that the dopaminergic neural system of the striatum was clearly and extensively damaged, as has been reported in previous studies (Waters *et al.*, 1987; Phillips *et al.*, 2017).

For the brains of marmosets (e.g. CM7) that received the present MPTP regimen, detailed histological analysis was reported in other MRI studies, wherein decreased volumes of tissue in the SN (Hikishima *et al.*, 2015a) and a reduction in nigrostriatal fibers between the SN and the striatum (Hikishima *et al.*, 2015b) were observed as described above.

In addition to the histological results in the present study, we found that the decreased TH-stained area in the striatum, calculated using ImageJ software, correlated with the decreased level of locomotion (correlation coefficient of 0.83) in marmosets ($n = 5$) that received two regimens of MPTP HCl at 2, 2, and 1 mg/kg s.c. on three consecutive days, respectively (unpublished study by Nishime C, Inoue R, Nishinaka E, and Ando K). Although the role of TH staining in the present study was for gross

confirmation of neural damage, the present histological analysis corroborated that neural damage in the striatum was clear by the present MPTP regimen.

Conclusion

Baseline locomotion and some behavioral traits in a common marmoset PD model with a single MPTP regimen were characterized. The results increase our understanding of the utility and limitations of the PD marmoset model in preclinical evaluations of medical treatments.

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K.A. conceived and designed the experiments. K.A., T.I., K.H., Y.K., and K.K. performed the experiments. K.A., T.I., K.H., Y.K., and K.K. analyzed the data. K.A. wrote the first draft of the manuscript. T.I., K.H., Y.K., K.K., R.I., C.N., E.N., K.U., and H.O. reviewed and critiqued the manuscript.

Conflicts of interest

H.O. is a paid member of the Scientific Advisory Board of SanBio Co. Ltd and K Pharma Inc. For the remaining authors, there are no conflicts of interests.

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