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Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model

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Dear Editors:

We would like to submit the enclosed manuscript entitled "Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model", which we wish to be considered for publication in "JoVE".

In this work, we evaluated how to establish a Murine Model for Parkinson's Disease by using MPTP and two effective behavioral tests, modified open field test and cylinder test, to measure this sort of lesion, which attaches vital importance to the study of Parkinson's Disease. These two behavioral tests are used to measure motor ability traditionally. Here we modify them to adapt them in Parkinson's disease study. And we took L-DOPA, one of the most commonly used PD drugs, as one example to show how to apply this model in the study of PD drugs. Our results indicate that MPTP neurotoxicity induces motor deficit and it can be reversed by L-DOPA. I hope this paper is suitable for "JoVE".

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

We deeply appreciate your consideration of our manuscript, and we look forward to receiving comments from the reviewers. If you have any queries, please don't hesitate to contact me at the address below.

Thank you and best regards.

Yours sincerely,

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TITLE:

Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model

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KEYWORDS:

Parkinson's disease, MPTP, behavioral test, cylinder test, open field test, animal model

SHORT ABSTRACT:

We describe establishment of a murine model for Parkinson's disease using MPTP, and behavioral assessments using cylinder and open field tests to measure motor function. We then use L-DOPA as one example to show how to apply this model in the study of PD drugs.

LONG ABSTRACT:

Parkinson's disease (PD) is a common neurodegenerative disorder disease, causing the phenomenon of shaking, rigidity, slowness of movement and dementia. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can lead to some Parkinson's-like symptoms by destroying dopaminergic neurons in the substantia nigra of the brain. It has been thus used to establish PD models in various animal studies. Here, mice receive MPTP injections (20 mg/kg/day) for seven days and the behavioral tests are performed on the eighth day. This model is adapted efficiently in the study of PD. The behavioral tests here include the cylinder test and the open field test. The cylinder experiment is used to detect the animals' ability to lift their front paws when put into a different environment. As the PD model mice show arching—the mouse arches its back—the number of paw liftings decrease. This test is easy to execute. The open field test is used to detect the amount of time the mice spend on running, walking, and remaining immobile. We analyze animals' movements in open field using software and obtain data. Lastly, we use L-DOPA, one of

the most commonly used PD drugs, as one example to show how to apply this model to the study of PD drugs. Our results indicate that MPTP neurotoxicity induces motor deficit which can be mitigated by L-DOPA.

INTRODUCTION:

Parkinson's disease (PD), one of the most common diseases among older individuals, is a long-term neurodegenerative disorder¹. Patients always show the phenomenon of shaking, rigidity, slowness of movement and dementia that worsen over time². Other symptoms including sensory, sleep, and emotional problems are also commonly observed². The cause of PD is still unclear, but it is generally believed to involve both genetic and environmental factors, which induce loss of dopaminergic neurons in the substantia nigra³, and development of Lewy bodies and Lewy neurites in various regions of the brain⁴.

Among the studies of PD, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)⁵ is adapted widely in recreating some PD symptoms in experimental models. In 1984, Langston *et al.* first found that injections of MPTP in squirrel monkeys resulted in Parkinsonism⁶. Although the MPTP rodent model doesn't show the presence of Lewy bodies, which is the biomarker of PD, MPTP causes Parkinson's-like symptoms by destroying dopaminergic neurons in the substantia nigra of the brain⁷. Compared to other drug model for PD such as those induced by 6-hydroxydopamine (6-OHDA)⁸ and 1-methyl-4-phenylpyridinium (MPP+)⁹, injection of MPTP is easy to execute and the MPTP model takes less time. Mice receive MPTP injections (20-30 mg/kg/day) for seven days, and the behavioral tests are performed on the eighth day¹⁰.

The open field test¹¹ was first developed by Calvin S. Hall, an American psychologist who studied the fields of dream research and analysis. In this experiment, an animal is placed in an arena marked with a grid and square crossings. Video cameras are used to record the movements of the animal and associated software is used to process the recorded video data. This method is mostly used by scientists to test general locomotor activity levels, and the willingness of animals (usually rodents) to explore¹². In various studies, different kinds of behaviors are tested. In research which focuses on Parkinson's diseases, behaviors like locomotion activities and the speed of locomotion are tested to see if the animal's ability to move around is affected. Compared with other methods used to test the establishment of PD animals, open field test is easy to carry out because the equipment needed is simple, and prototyping and data analysis software (*e.g.*, MATLAB, Excel) can be used to easily collect and graph the data. Also, the coefficient of variation is relatively small¹³, which means that the result of open field test is reliable. Another advantage over other methods is that the behaviors included in this experiment are easy to distinguish; the mice can be either running, walking or standing still. Usually the open field test can be used on rodents when the researcher needs to evaluate the subject's mobility.

The Cylinder test is also called the test of asymmetric use of forelimbs. When this test was first designed, it was used to test the asymmetric use of the rat's forelimbs¹⁴. Here, we use this test to analyze the animal's ability to stretch out and use both of its forelimbs to explore new surroundings. When the substantia nigra and corpus striatum are damaged by MPTP in the brain, the animal tends to arch its back and becomes less likely to stretch out and explore the unknown

environment. This test is easy to execute and can give a preliminary result. However, this test has high internal variability, so it is generally used with along with other behavior experiments.

Taking L-DOPA, which is also known as levodopa or L-3,4-dihydroxyphenylalanine, is a common way to treat Parkinson's disease since one cause of PD is the decrease of dopamine in the substantia nigra¹⁵. L-DOPA is the precursor to dopamine. But unlike dopamine, it can cross the blood-brain barrier, which means that it will be more efficient in increasing the concentration of dopamine in the brain area. After it crosses the blood-brain barrier, L-DOPA is converted into dopamine by L-amino acid decarboxylase¹⁵.

Here we describe the measurement and analysis of motor function in MPTP-induced-PD model mice using a cylinder test¹⁴ and a modified open field test. We administer L-DOPA as one example to show how to apply this model in the study of PD drugs. Our results indicate that MPTP induces motor deficit that can be mitigated by L-DOPA.

PROTOCOL:

This study was performed according to the international, national and institutional rules considering animal experiments. The study protocol was approved by the animal ethics committee of Nankai University.

1. MPTP and L-DOPA administration

Note: Ten-week-old female BALB/c mice were provided by the Institute of Zoology, Chinese Academy of Sciences. Mice were housed six per cage under a 12 h light/dark cycle (lights on at 08:00-20:00), a constant temperature of 21-22 °C and a relative humidity of 55% ± 5%. Autoclaved standard mice chow of the same formulation and water *ad libitum* was given to all animals.

1.1. After one week of acclimatization, divide the animals into three groups of six mice each.

1.2. To **Group 1**, administer intraperitoneal injections (see step 1.5) of 250 µL/mouse/day of saline from day 1 to day 7 and then perform intragastric administration (see step 1.6) of 250 µL/mouse of saline on day 8.

1.3. To **Group 2**, administer intraperitoneal injections of 20 mg/kg/day of MPTP every day from day 1 to day 7 and then perform intragastric administration of 250 µL/mouse of saline on day 8.

1.4. To **Group 3**, administer intraperitoneal injections of 20 mg/kg/day of MPTP from day 1 to day 7 and then perform intragastric administration of 5 mg/kg of L-DOPA on day 8.

1.5. Perform the intraperitoneal injection as follows.

1.5.1. Load the drug into a sterile 1 mL syringe with a 26 G needle. Eliminate air from the syringe.

1.5.2. Scruff the mouse with its belly facing up. Keep the head, neck and body of the mouse in a straight line as well with the head fixed.

1.5.3. Angle the needle to penetrate the peritoneum. Push the needle for a proper distance until there is little resistance, and then inject the drug. Pull out the needle smoothly.

1.6. Perform the intragastric administration as follows.

1.6.1. Prepare the medication in a sterile 1 mL syringe with an oral-gavage needle. Eliminate air from the syringe.

1.6.2. Hold the mouse with its belly facing up. Keep the head, neck and body of the mouse in a straight line with the head fixed.

1.6.3. Keep the needle parallel to the body of the mouse, and insert it from the corner of the mouse's mouth, pressing the tongue and pushing inward against the upper jaw.

1.6.4. With little resistance, which shows the needle is entering the esophagus smoothly, carefully push the needle in a proper distance. Before the tip of the needle reaches the bottom part of chest, inject the medication.

1.6.5. Pull out the needle smoothly.

2. Cylinder test

Note: The behavioral tests were performed on day 8. L-DOPA was injected to the third group of mice 40 min before the behavioral tests. If the behavioral testing is not done in the same room where the animals are housed, animals need to be acclimated to the new room for 30-60 min before test.

2.1. Perform the test 24 h after the last dose of MPTP.

2.2. Place a transparent glass cylinder (height = 19.5 cm, diameter = 15 cm, weight ≥ 1 kg) at the center of a table. Surround the cylinder on three sides with black cardboard to reduce the effect of environmental lighting. Leave one side of the cylinder facing the camera for video recording.

NOTE: The distance between the cardboard and cylinder should be about 4 to 8 cm.

2.3. Attach a camera (> 1 million-pixel resolution) at ~ 40 -60 cm away from the cylinder to ensure that the full cylinder is visible.

NOTE: This is neither too near nor too far so as to not disturb the mouse while recording the video of its movement.

2.4. Place one mouse into the cylinder at a time and start filming at once. Stop filming after 3 min.

NOTE: During this process, try to avoid noise or light changes in order avoid influencing the mouse's behavior.

2.5. Place the mouse back into the home cage after testing.

2.6. Clean the cylinder with water and then spray 70% v/v ethanol over the inner wall to sanitize it and remove mouse scents. Wipe the cylinder dry before another mouse is placed into.

2.7. Play back the video at a rate of 0.5x the regular speed and count the number of paw lifts against the wall of each mouse.

NOTE: Paw lifts occur when the mouse rears up on its hindlimbs, raises both forelimbs above shoulder level and lands. Usually, a mouse raises its forelimbs to touch the cylinder walls. If the mouse raises its forelimbs above shoulder level several times continuously without landing, it should be counted only as once.

3. Open field test

3.1 Perform the open field test 24 h after MPTP dosing.

NOTE: It can be carried out at the same time of the cylinder test.

3.2. Prepare a transparent open field reaction box (45 cm L x 45 cm W x 25 cm H) with a wooden plate cover at the bottom by a black cloth. Fix a camera over the field at a height of 1 m.

NOTE: The color of open field box bottom should be different from the tested mouse color to ensure color contrast in the video.

3.3. Adjust the camera to make sure that the open field reaction box is right in the center of the video.

3.4. Put one test mouse into the box, and let the mouse familiarize itself with the environment for about 1 min.

3.5. Record a 5 min video using the camera connected to the computer.

3.6. Analyze the video using software tools (*e.g.*, MATLAB) to get the movement trace figure, the distribution of static (velocity <1 cm/s), walking (velocity 1-20 cm/s) and running (velocity >20 cm/s) time, the total traveled distance, and average speed of each tested mouse.

NOTE: When analyzing the video, we track the whole body of the mouse. The average speed of a body means the total length of path covered, divided by the elapsed time. Therefore, if a mouse is not moving, its instantaneous velocity would be considered as zero.

3.7. Clean up any feces in the open field reaction box. Spray 70% ethanol on the box and wipe it.

REPRESENTATIVE RESULTS:

In the cylinder test, the decrease of rears against the wall was observed in mice (group 2) treated with MPTP from day 1 to day 7 and saline on day 8 as compared with saline-treated mice (group 1), while an increase of rears was observed in the mice (group 3) treated with MPTP from day 1 to day 7 and L-DOPA on day 8 as compared with the mice (group 2) treated with MPTP from day 1 to day 7 and saline on day 8 (**Figure 1**).

Figure 2 shows the representative traces and distribution of static time, walking time and running time of the three groups of mice. In (A) mice were treated with saline. In (B) mice treated with MPTP from day 1 to day 7 and saline at day 8. In (C) mice treated with MPTP from day 1 to day 7 and L-DOPA at day 8. When a mouse was moving in the open field arena with speed <1 cm/s, 1-20 cm/s, or >20 cm/s, it was judged as static, walking or running respectively. Mice treated with only MPTP showed a lower movement speed, a shorter movement distance, a longer static time and a shorter running time than saline-treated mice, which indicated the motor deficit induced by MPTP. Mice treated with both MPTP and L-DOPA showed a higher movement speed, a longer movement distance, a shorter static time and a longer running time than the mice treated with just MPTP (**Figure 2, Figure 3**), which showed that L-DOPA mitigated the MPTP-induced motor deficit.

FIGURE AND TABLE LEGENDS:

Figure 1. Effects of MPTP on motor performance in cylinder test. This shows the analysis of the numbers of rearing on the 8th day. * represents $p < 0.05$, ** represents $p < 0.01$ for a one-way ANOVA test. All values represent mean \pm SD ($n = 6$).

Figure 2. Movement path of mice and the distribution of time that mice stay, walk, and run during the 5 min in the open field test. (A) Untreated mice. (B) Mice treated with MPTP. (C) PD model mice rescued by L-DOPA. Run: the mouse was moving with a velocity of more than 20 cm/s. Walk: the mouse was moving in the open field and with a velocity of 1-20 cm/s. Static: the mouse was stationary (speed <1 cm/s) in the open field ($n = 6$).

Figure 3. The motor deficit induced by MPTP and the therapeutic effect of L-DOPA on motor performance in open field test. (A) Analysis of static, walking and running time. (B) Analysis of average movement distance. (C) Analysis of average movement speed. *** represents $p < 0.001$, **** represents $p < 0.0001$ for a one-way ANOVA test. All values represent mean \pm SD ($n = 6$).

DISCUSSION:

Due to destruction of dopaminergic neurons in the substantia nigra of the brain, MPTP causes Parkinson's-like symptoms in mice⁷. L-DOPA is the most preferred drug for PD ever since its clinical use, because it helps in maintaining normal daily activities in patients with PD, with effective suppression of motor abnormalities including akinesia and rigidity¹⁵. The mice treated with MPTP showed impairments in the behavioral tests like cylinder test and open field test, which could be used to study the MPTP model. Cylinder test and open field test are usually used to measure spontaneous action. Here, we modified these two behavioral tests to apply them in the study of PD. As shown in our results, L-DOPA can mitigate the impairments in behavior by improving the animals' motor abilities.

Normally, mice explore their surroundings when they kept introduced to a new environment. Thus, when kept in a transparent cylinder, they explore within the cylinder by moving around and raising their bodies to touch the cylinder walls with their forelimbs. Mice intoxicated with neurotoxic agents such as MPTP, reduce this action. This allows us to apply the cylinder test in the PD study. Paw lifts quantities indicate forelimb use for body support and are thus used to evaluate the effect of new chemical entities on motor performance¹⁶. The cylinder test also provides a measure for the forelimb motor function¹⁷ in many neurodegenerative diseases involving motor cortex lesions, such as amyotrophic lateral sclerosis and spinocerebellar atrophy¹⁸.

The open field test is an experimental test conventionally used to assay general locomotor activity levels, and the willingness of rodents in terms of the time that they stay at the center of the box. Measurement of movement path, speed and the time mice stay, walk, and run respectively in the whole field rather than specific area in open field can evaluate motor function in PD¹⁹. Leveraging simple and readily available coding software (see the **Table of Materials**), we can obtain and analyze the data related to the locomotor function efficiently.

The cylinder test and the open field test were chosen to measure spontaneous locomotive function in PD model mice for the following reasons. The mice do not need to be trained before the behavioral tests. Only several minutes are required for the response, which ensures the efficiency. The cost of the equipment needed to perform the tests is low. However, a major disadvantage of this measure is that the animals' activity levels can vary significantly due to environment changes and individual differences. To reduce the amount of variation in the outcome data, caution should be taken. Firstly, animals of the same age, sex, and genetic background^{20,21,22} should be assessed at the same time. Secondly, the equipment, cylinder and open field, need to be cleaned before and after testing each mouse. Finally, keep the environment quiet and undisturbed during the test.

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DISCLOSURES:

The authors have nothing to disclose.

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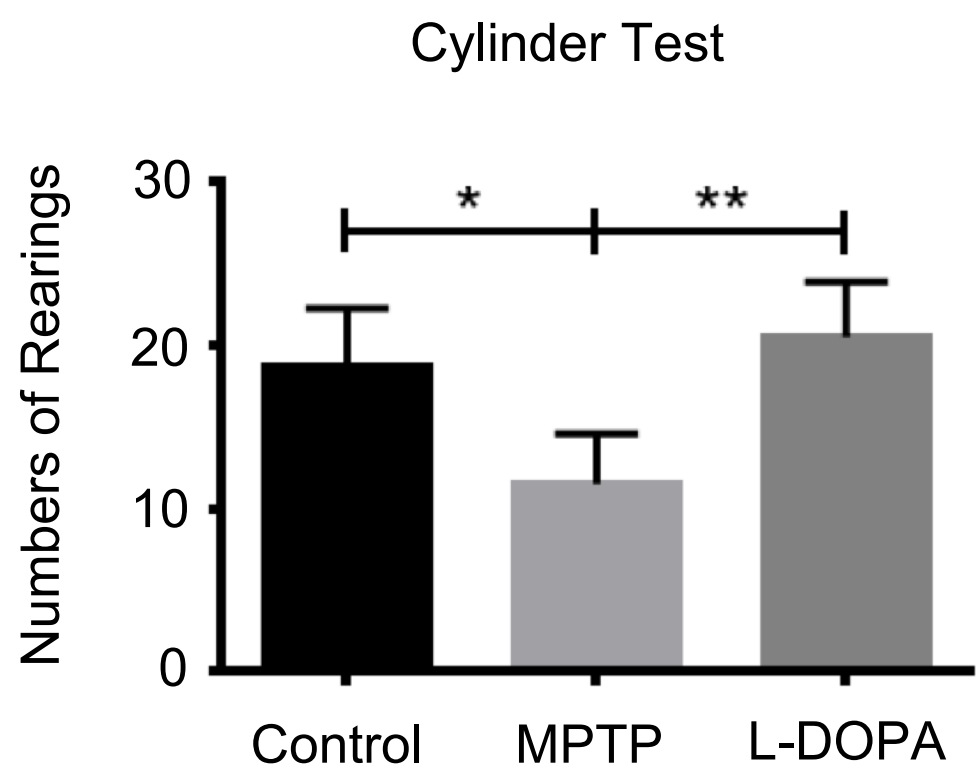
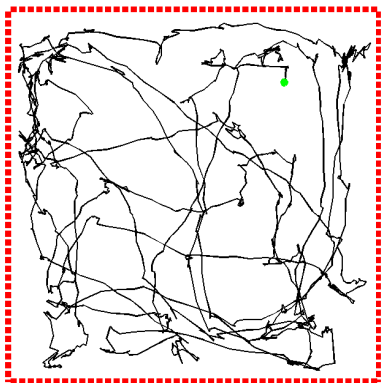
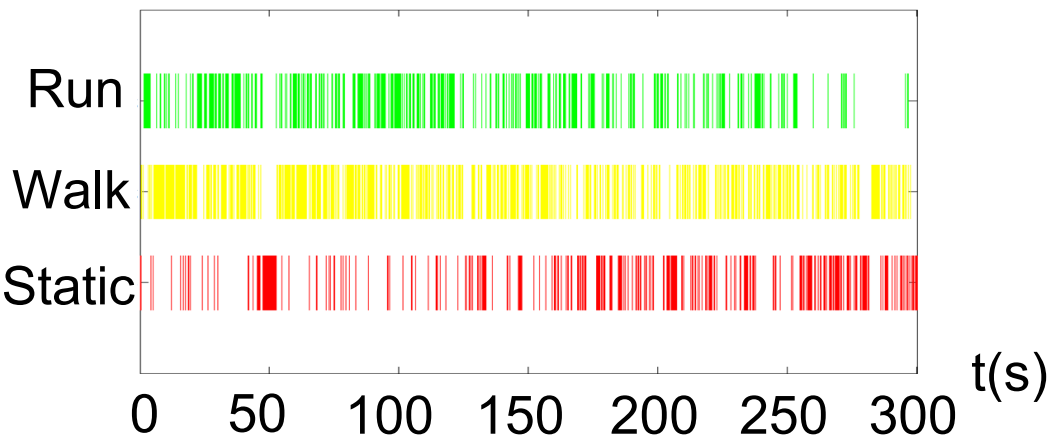


Figure 2

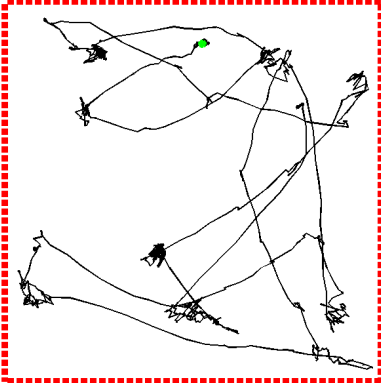
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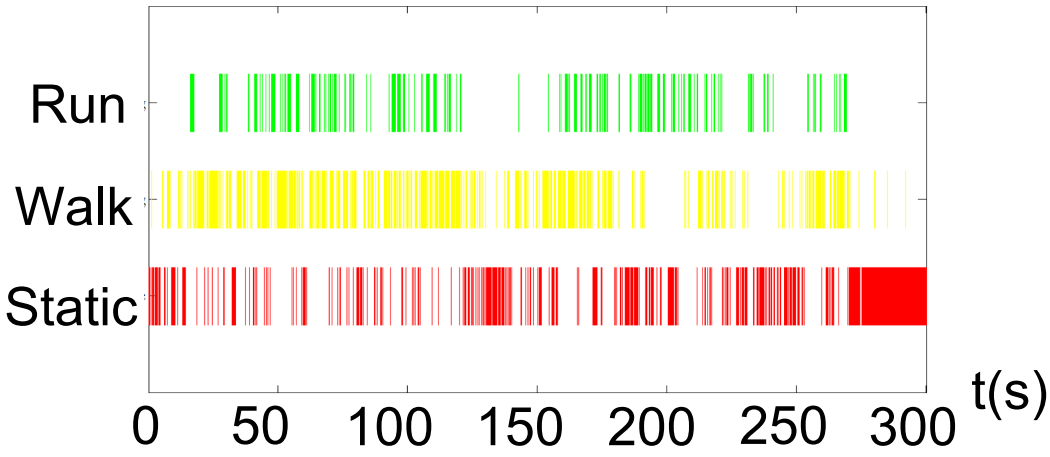
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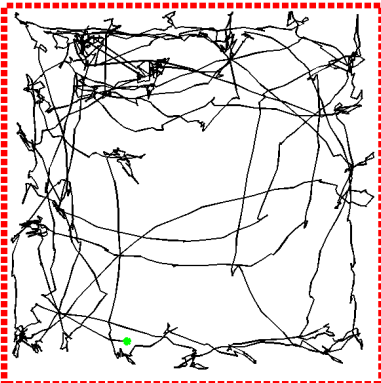
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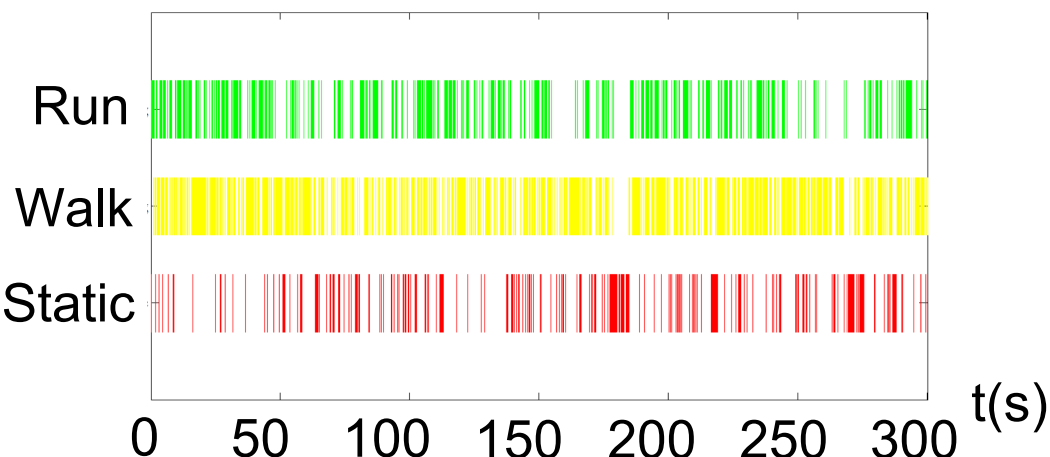
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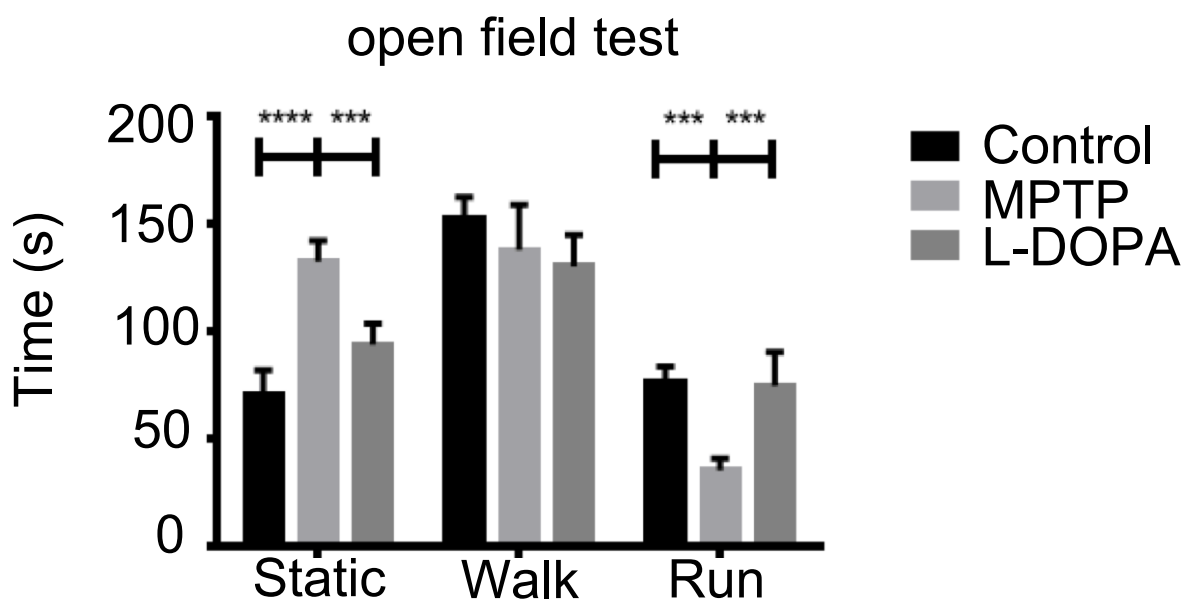
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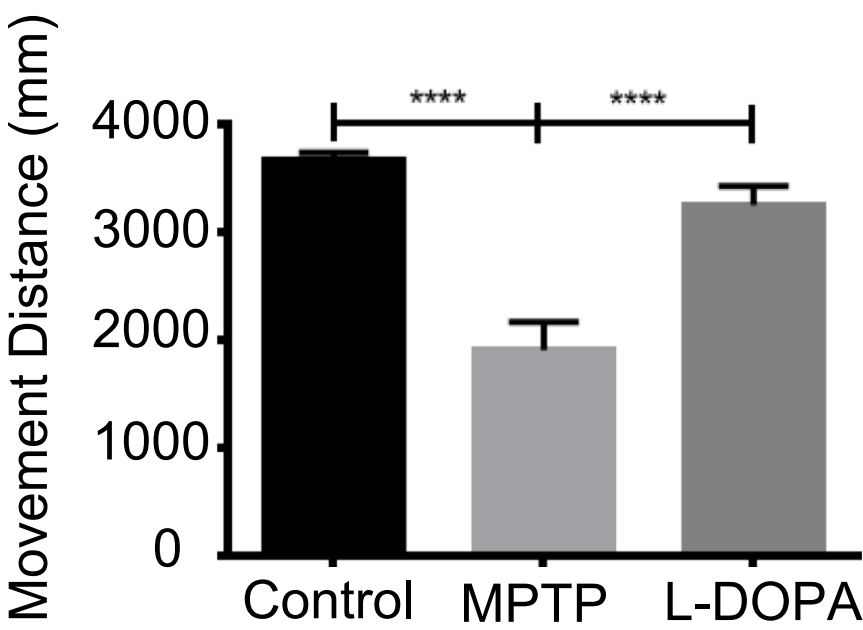
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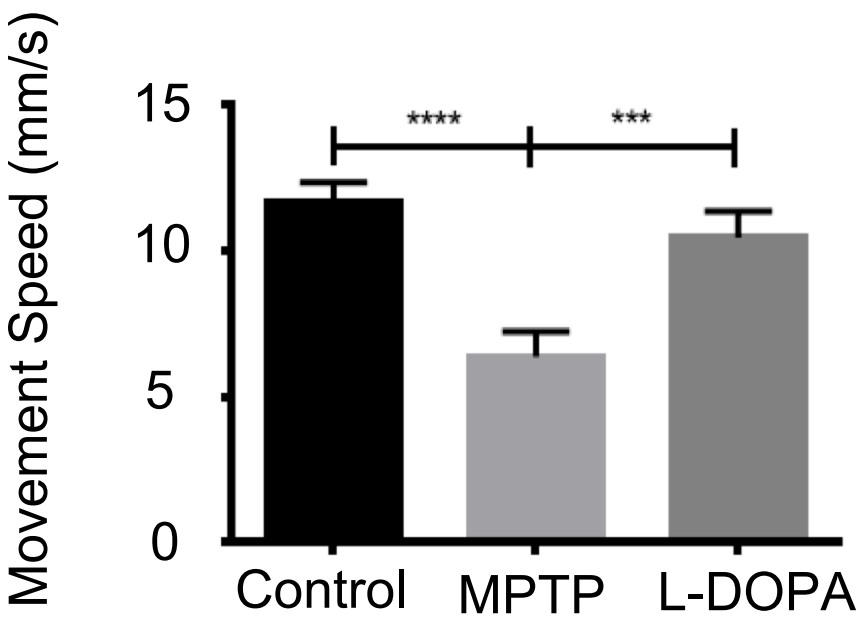
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
70% Ethanol	Ruiboxing Company	RBX-64175	
Camera	BASLER	acA645-100gm	
Cylinder test	Made in-house at Nankai University	N/A	
Excel	Microsoft	N/A	
Levodopa	Sigma-Aldrich	72816	
Matalb 2017a	Mathworks	N/A	
Mice	Institute of Zoology, Chinese Academy of Sciences	Balb/c	Adult female mice(10 weeks)
MPTP	Yuanye Biological Technology Company Ltd., Shanghai	S31504-500mg	
Open field test	Made in-house at Nankai University	N/A	
Syringe	Solelybio	S-xsgwz-w	Irrigation
Syringe	Jiangxi Fenglin Medical Application Co.	hc3824	Intraperitoneal injection



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Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model and the Harvest of Brain

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Article Title:

Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model and the Harvest of brain

Signature:

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Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Behavioral Assessments of Spontaneous Locomotion in a Murine MPTP-induced Parkinson's Disease Model" (ID: JoVE58653).

Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied these comments carefully and have made correction which we hope to meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the editor comments are as following:

Responses to the editorial comments:

1. Comment: This sentence cannot be understood. Unclear what is meant by "lesion of spontaneous locomotion". There are grammatical errors.

Response: We're sorry for the incorrect writing. We've rewritten this sentence in line 40.

2. Comment: I'm not sure what is meant by this phrase. It is grammatically incorrect. Please revise

Response: We're sorry for the incorrect writing. We've changed the word "lesion" into "motor deficit" in line 96

3. Comment: This sentence needs revision, it is unclear what is being said.

Response: Thank you for your suggestion. We've rewritten this sentence in line 96.

4. Comment: Instead of the previous note, I have made the administration of drugs into steps to ensure that it is filmed. This appeared to be important to show. Please verify that the steps are correct.

Response: Thank you for your suggestion. We have checked the steps from your suggestion and made some small changes to it.

5. Comment: Please describe how intragastric administration is performed.

Response: Thank you for your suggestion. We have added a description of how intragastric administration is performed in line 130.

6. Comment: Lesion on which anatomical region? This phrasing is incorrect. Why don't you show a figure showing the MPTP-induced lesion as a supplementary figure? If you do not have this, I am not sure how you can claim that there is a lesion without validation.

Response: We're sorry for the incorrect writing. The word "lesion" has been replaced by "deficit", which is shown in the motor function of mice. We tried to reorganize the language

to make it clear.

7. Comment: Lesion on which anatomical region? Substantia nigra? Please provide figures showing the MPTP-induced lesion and its treatment using L-DOPA as a supplementary figure? If you do not have this, I am not sure how you can claim that the lesion is treated without validation. All supplementary figures need figure legends.

Response: We're sorry for the incorrect writing. The word "lesion" has been replaced by "deficit", which is shown in the motor function of mice.

8. Comment: Again, this phrasing is incorrect. Please change this. Also, you do not show that there is a lesion on the substantia nigra.

Response: We're sorry for the incorrect writing. The word "lesion" has been replaced by "deficit", which is shown in the motor function of mice.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes but marked in red in revised paper.

We appreciate for Editor's warm work earnestly and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions ,

Best regards ,

Peien Jiang

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