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Establishment of a staging model for Parkinson's disease in mice

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ABSTRACT

Parkinson's disease is the second most common neurodegenerative disease with different pathological mechanisms at each stage. To investigate Parkinson's disease further, this study proposed to develop a continuous staging mouse model of Parkinson's disease to reproduce the pathological features of different stages of Parkinson's disease. We successively treated the mice with MPTP and assessed the behavioral performance of the mice with the open field test and the rotarod test, and detected the aggregation of α-syn and the expression of TH protein in the substantia nigra of the mice with western blot test and immunofluorescence test. The results showed that the mice injected with MPTP for 3 days had no significant behavioral changes, and no significant α-syn aggregation, but reduced TH protein expression and 39.5% loss of dopaminergic neurons in the substantia nigra, similar to the performance in the prodromal phase of Parkinson's disease. However, the behavior of the mice continuously treated with MPTP for 14 days was significantly altered, with significant α-syn aggregation, a significant reduction in TH protein expression, and 58.1% loss of dopaminergic neurons in the substantia nigra, corresponding to the early clinical stage of Parkinson's disease. In the mice that were exposed to MPTP for 21 days, the motor impairment was more obvious, the α-syn aggregation was more significant, the reduction of TH protein expression was more evident, and the loss of dopaminergic neurons reached 80.5% in the substantia nigra, showing a clinical progression similar to that of Parkinson's disease. Consequently, this study found that continuous treatment of C57/BL6 mice with MPTP for 3, 14 and 21 days could construct mouse models of prodromal, early clinical and clinical progressive stages of Parkinson's disease, respectively, providing a promising experimental model foundation for the study of the different stages of Parkinson's disease.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease just after Alzheimer's disease and is the most rapidly growing neurological disease in terms of prevalence, disability and mortality worldwide (1). The PD is characterized by progressive degeneration of substantia nigra (SN) dopaminergic neurons and pathological aggregation of α-syn with motor deficits such as bradykinesia, resting tremor or myotonia or non-motor symptoms such as olfactory disturbances and depression (2). The disease is often diagnosed at an advanced stage with poor treatment outcomes, and an intensive study of the pathology and therapeutic targets in various stages of PD is imperative. According to the classical Braak staging, PD can be classified into Braak stages 1-6 according to the pathological changes (3). In Braak stages 1-3, the lesions mainly involve the lower part of the brainstem and olfactory bulb, with no obvious motor symptoms and less than 50% loss of dopaminergic neurons in the SN, which is regarded as the prodromal stage of PD. In Braak stage 4, the lesion mainly involves the midbrain and forebrain nuclei, and patients begin to develop clinically diagnosable Parkinson's motor symptoms with more than 50% loss of dopaminergic neurons, which is considered an early clinical indication; Braak stages 5-6, the progressive stage, in which the lesion affects the neocortex of the brain, progressively worsen motor symptoms with more than 70% loss of dopaminergic neurons as the disease progresses (4). The pathological mechanisms of the various stages of PD differ and their study relies on an ideal animal staging model, which should reproduce the pathological features of the different stages of the disease.

In order to study PD in detail, researchers have used different methods to construct Parkinson's animal models at different stages of the disease, such as induction with increasing doses of drugs, or induction with different durations of drugs, following the Braak staging principle. Taguchi et al. induced a prodromal Parkinson's monkey model using subcutaneous injections of 1.0, 1.5 and 2.0 mg/kg of MPTP regimen. The number of nigrostriatal DA neurons in the model monkeys was reduced by 10%, 25%, and 30%, respectively, but all animals did not show Parkinson's symptoms (5), confirming that different doses of MPTP can induce different degrees of dopaminergic neuron loss. Researchers gave weekly intramuscular injections of MPTP to rhesus monkeys to construct prodromal and clinical phase PD monkey models: model monkeys

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induced for 10 weeks had <50% loss of SN dopaminergic neurons and did not show Parkinson's motor symptoms, which was considered as prodromal phase; model monkeys induced for 21 weeks had 60% loss of SN dopaminergic neurons and showed Parkinson's motor symptoms, which was regarded as clinical phase (6,7), demonstrating that the loss of SN dopaminergic neurons increased and the condition worsened as the induction time prolonged. In addition, the construction of acute, subacute and chronic PD models with MPTP induction in C57BL mice is a classic animal model for PD research, with 50%-80% loss of SN dopaminergic neurons after induction with Parkinson's symptoms (8-10).

Currently, the most commonly used animals for studying PD are mice, but no investigator has yet clearly proposed a continuous staging model for PD in mice, and there is a lack of clear staging criteria. We proposed to build a mouse model of PD staging using subacute induction of MPTP based on the Braak staging principle, and established staging criteria according to the pathological characteristics of each stage of PD and the previous research: the proportion of dopaminergic neuron loss and motor symptoms in mice were used as criteria. PD mice with no motor symptoms and <50% loss of dopaminergic neurons were defined as prodromal stage; while mice with mild motor symptoms and 50-70% loss of DA neurons were classified as early clinical stage; and mice with severe motor symptoms and >70% loss of dopaminergic neurons were considered as progressive stage (as shown in Table 1).

Materials and Methods

Animal culture

Male C57BL/6 mice (8 weeks, 22-25 g) used in this study were uniformly obtained from the Experimental Animal Center of Southern Medical University, Guangzhou, China. The study of mice was approved by the Ethics Committee of Zhujiang Hospital, Southern Medical University (Animal Ethics Approval No.: LAEC-2021-031FS). Mice lived in an SPF-grade housing environment with a temperature of 22-26°C, air humidity of 40-60%, and a 12-hour light/dark cycle.

Experiment design

To build a staging model of PD, we randomly divided 48 C57/BL6 mice into four groups, normal group, prodromal group, early clinical stage group, and clinical progression group, each group consisted of 12 mice. As shown in Figure 1, we administered MPTP (dissolved in 0.9% saline, 30 mg/kg, ip) intraperitoneally to the mice continuously every day, while an equal volume of saline (ip) was given to the normal control group. The behavioral performance of the mice was observed daily. In the prodromal group, we tested behavioral performance and pathological indicators after continuous injection of MPTP but without obvious motor symptoms; while in the early clinical group, we examined the behavioral performance and pathological indices when motor symptoms started after continuous injection of MPTP; and in the clinical progressive group, behavioral performance and pathological indices were detected when symptoms worsened after continuous injection of MPTP. The behavioral performance of the mice was assessed using the open field test and the rotarod test. After mice were executed, changes in α-syn aggregation and the expression levels of TH protein were measured by WB assay and immunofluorescence assay, respectively.

Open field test

The open field test was used to evaluate the spontaneous activity and anxiety-like behavior in mice as described previously.[11] Animals were placed in the center of a square box (40 × 40 cm, clear 35 cm high walls) equipped with a camera to record activity. The test lasted 5 min per animal. The exploratory behavior of each animal was automatically analyzed using EthoVision XT video tracking software (Noldus, Netherlands) to record the movement distance and movement speed of the mice.

Rotarod test

The mouse rotarod apparatus (Panlab, USA) was used to evaluate motor coordination and balance in mice (11). During training, mice were allowed to explore the non-rotating drum for 2 minutes. Each mouse was then oriented with its head in the opposite direction of rotation and the speed was slowly accelerated from 4 rpm to 40 rpm over a period of 300 seconds. Record the time the mouse stays on the drum (up to 300 seconds) and the speed at which it falls (up to 40 rpm). Test the mice three times at intervals of at least 30 minutes and record the latency time and the falling speed.

Sampling

After behavioral performance testing, mice were anesthetized with sodium pentobarbital (50 mg/kg, ip) and executed, and the midbrain was rapidly dissected, rapidly frozen in liquid nitrogen and stored at -80°C for biochemical analysis. For histological analysis, mice were

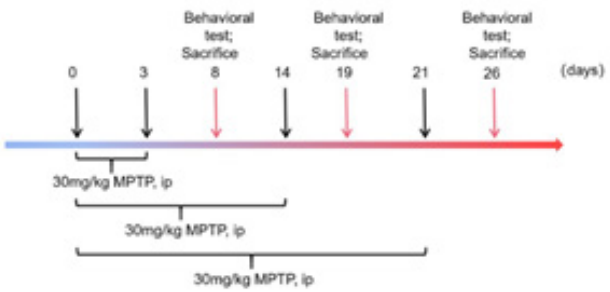

Figure 1. Flow chart of experimental design of this study.

Table 1. Staging criteria for PD.

Stage	Parkinson's motor symptoms	Loss of dopaminergic neurons (%)
Prodromal stage	No motor symptoms	Loss <50%
Early clinical stage	Mild motor symptoms	Loss of 50-70%
Clinically progressive stage	Severe motor symptoms	Loss >70%

anesthetized and perfused transcardially with 0.1 mol/L phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Afterward, mouse brains were collected and fixed in 4% PFA for 24 hours. After fixation, brain tissue was dehydrated with various concentrations of sucrose (from 20%, to 30%) until it was at the bottom of the solution and subsequently embedded for immunofluorescence staining.

Protein extraction and Western blot assay

Brain tissues were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor and phosphatase inhibitor according to the manufacturer's instructions. The lysed tissue mixture was then centrifuged at 12,000 g for 20 min at 4°C to obtain the supernatant. The protein concentration of the supernatant was measured using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). The proteins were subsequently denatured with sodium dodecyl sulfate solution and cooked at 100°C for 15 min. Equal amounts of protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were closed with 5% skim milk (Dogesce, Beijing, China) for 1 h at room temperature. Add mouse monoclonal β-actin antibody (1:10,000, Proteintech, Rosemont, IL, USA), rabbit polyclonal α-synuclein antibody (1:1000, Abcam, Cambridge, MA, USA) and rabbit polyclonal TH antibody (1:1000, Abcam, Cambridge, MA, USA) and incubate overnight at 4°C. After washing the membrane with 1× tris buffered saline-tween (TBST) solution (5 min, 3 times), add HRP-coupled anti-mouse secondary antibody or HRP-coupled anti-mouse secondary antibody (1:200, Abcam, Cambridge, MA, USA) and incubate for 1.5 hr. Protein band intensity was detected using ECL Ultrasensitive Luminol Solution (MERCK MILLIPORE, Billerica, MA, USA) and chemiluminescence instrument (UVITEC, England). Finally, the intensity of each protein band was quantified using Image J v1.47 software (National Institutes of Health, Bethesda, MD, USA). Relative band intensities were measured using β-actin as an internal control.

Immunofluorescence experiment

The SN tissue was cut into successive 10-μm-thick sections. After fixing the sections with methanol, the tissue antigens were repaired in sodium citrate solution. 0.3% Triton solution was used to rupture the cell membranes. After blocking the tissue antigen with 10% goat serum, chicken polyclonal TH antibody (1:200, Abcam, Cambridge, MA, USA) was added and incubated overnight at 4°C. The next day, goat anti-chicken IgY H&L (Alexa Fluor® 647) (1:200, Abcam, Cambridge, MA, USA) was added in a dark room for 1 h. After imaging the sections with a Nikon inverted fluorescence microscope, we used Image J to count TH-positive neurons.

Statistical analysis

Statistical analysis was performed to analyze the data obtained from the behavioral tests and biochemical assays. For the behavioral tests, one-way analysis of variance (ANOVA) was used to compare the differences in movement distance, movement speed, falling latency, and falling rotarod speed among the different groups. Post-hoc tests, such as Tukey's honestly significant difference (HSD) test, were conducted to determine specific group differences if a significant overall difference was detected. In the biochemical analysis, protein band intensities were quantified using Image J software, and statistical comparisons were made using one-way ANOVA followed by post-hoc tests. The results of the statistical analysis provided valuable insights into the motor impairment and pathological changes observed in the staged mouse model of Parkinson's disease.

Results

Motor impairment in mice increased with an increasing induction time of MPTP.

In this study, the field test and the rotarod test were used to measure motor function in mice. The field test assesses spontaneous movements and anxiety-like behaviors, while the rotation test focuses on coordination and motor endurance. Here, no significant decrease in movement distance and movement speed was observed in the open field test in mice that were given MPTP for 3 days (Figure 2a&b, movement distance: One-Way ANOVA, P=0.6267, n=6; movement speed: One-Way ANOVA, P=0.9964, n=6), while those that were given MPTP for 14 and 21 consecutive days mice were significantly decreased. At the same time, the movement distance and movement speed were significantly lower in mice intervened by MPTP for 21 days than those intervened for 14 days (movement distance: One-Way ANOVA, P=0.0102, n=6; movement speed: One-Way ANOVA, P=0.0183, n=6). The results of the rotarod test showed that the falling latency and falling rotarod speed of mice that were treated with MPTP for 3 consecutive days were not statistically different from those of normal controls (Figure 2c&d, falling latency: One-Way ANOVA, P=0.3038, n=6; falling rotarod speed: One-Way ANOVA, P=0.2663, n=6). However, significant decreases were observed in mice that were intervened by MPTP for 14 and 21 consecutive days, and the falling latency and falling rotarod speed were significantly lower in the latter than in the former (falling latency: One-Way ANOVA, P=0.0372, n=6; falling rotarod speed: One-Way ANOVA, P=0.0443, n=6).

As the induction time of MPTP increased, α-syn aggregation increased and protein expression of TH decreased in the SN of mice.

Loss of dopaminergic neurons and deposition of α-syn are the pathological basis of PD. To explore the pathological changes in mice, we examined the α-syn aggregation and TH protein expression levels in the SN of normal control mice and modeled mice using WB experiments. The results showed that compared with the normal control group, α-syn accumulation in the SN of mice intervened by MPTP for 3, 14 and 21 days showed a gradual increase, while TH protein expression exhibited a gradual decrease, indicating a stable progression of the pathology with the increased administration of MPTP. However, the levels of α-syn aggregation and TH protein were not statistically significantly different in mice intervened by MPTP for 14 consecutive days compared to those intervened for 21 days (TH: One-Way ANOVA, P=0.2923; α-syn: One-Way

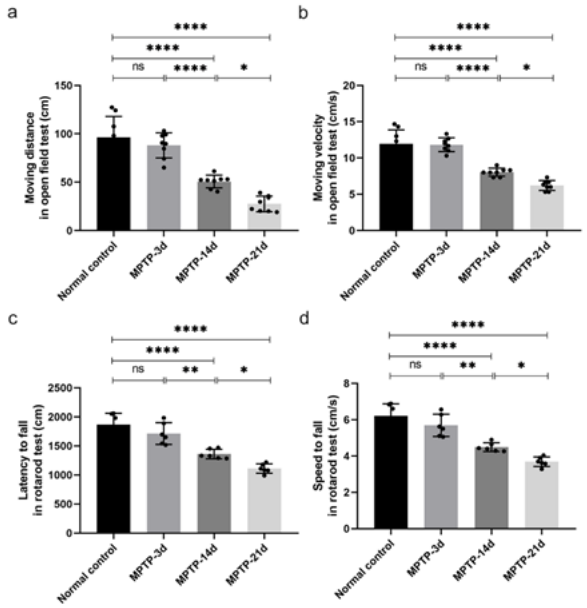

Figure 2. Increased motor deficits in mice with increased MPTP administration. (a,b) In the open field test, compared to normal controls, the movement distance (One-Way ANOVA, p=0.0626, n=6 mice) and movement velocity (One-Way ANOVA, P=0.9964, n=6) were not significantly decreased in mice that were intervened for 3 days, while they were significantly decreased in mice that were intervened for 14 and 21 days. Also, the movement distance (One-Way ANOVA, P=0.0102, n=6) and movement velocity (One-Way ANOVA, P=0.0183, n=6) were significantly decreased in mice that were intervened for 21 days compared to 14 days of intervention. (c,d) In the rotarod test, falling latency (One-Way ANOVA, P=0.3038, n=6) and falling rotarod speed (One-Way ANOVA, P=0.2663, n=6) were not significantly decreased in the mice that were intervened for 3 days compared to the normal controls. However, the mice that were intervened for 14 and 21 days had significantly lower falling latency and falling rotarod speed, and the latter was lower than the former in terms of the falling latency (One-Way ANOVA, P=0.0372, n=6) and falling rotarod speed (One-Way ANOVA, P=0.0443, n=6).

ANOVA, P=0.5965), which may be attributed to the fact that they were already relatively low or high in mice intervened by MPTP for 14 consecutive days.

With the increase of MPTP administration, the loss of dopaminergic neurons in the SN of mice increased and the pathological damage intensified.

To further examine the number of dopaminergic neurons in the model mice, we measured TH-positive cells (dopaminergic neurons) in the normal and modeled groups by immunofluorescence assay, and the results demonstrated that compared with the normal control group, mice subjected to MPTP for 3 consecutive days lost 39.5% TH-positive cells in the SN (One-Way ANOVA, P<0.0001, n=6). While the loss of TH-positive cells was 58.1% in mice treated with MPTP for 14 days (One-Way ANOVA, P<0.0001) and 80.5% in mice exposed to MPTP for 21 days (One-Way ANOVA, P<0.0001, n=6). Meanwhile, mice intervened by MPTP for 21 consecutive days lost substantially more TH-positive cells than those intervened for 14 days (One-Way ANOVA, P=0.0060, n=6), suggesting more severe pathological damage in the former. So far, according to the PD staging criteria we set, combined with the behavioral manifestations and the proportion of dopaminergic neuron loss, we can conclude that the prodromal, early clinical and clinical progressive models of PD in mice can be constructed by successive treatment of mice with MPTP for 3, 14 and 21 days, respectively.

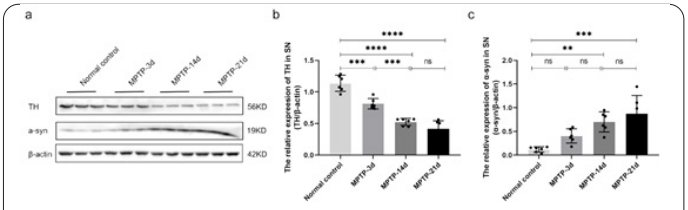

Figure 3. As the induction of MPTP was prolonged, α-syn aggregation increased and TH protein expression decreased in the SN of mice. (a) Western blot assay was applied to detect α-syn aggregation and TH protein expression levels in the SN of mice. Quantitative analysis of α-syn and TH in the SN: compared with normal controls, the expression of TH protein in the SN of mice with 3, 14 and 21 days of continuous MPTP intervention showed a gradual decrease (b) and α-syn accumulation with a gradual increase (c). However, there was no statistically significant difference in the levels of α-syn aggregation (One-Way ANOVA, P=0.5965, n=6) and TH protein (One-Way ANOVA, P=0.2923, n=6) in mice with 14 and 21 consecutive days of intervention.

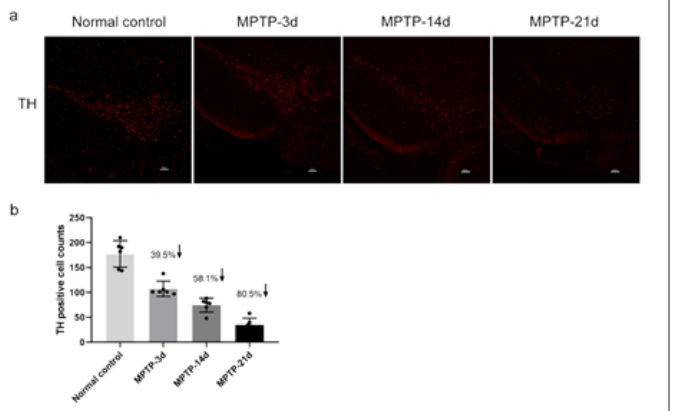

Figure 4. With the increased use of MPTP, the loss of dopaminergic neurons in the SN of mice increased and the pathological damage worsened. (a) Immunofluorescence experiments were performed to examine the loss of TH-positive cells (dopaminergic neurons) in the SN of mice. (b) In comparison with normal controls, 39.5% of TH-positive cells were lost in the SN of mice treated with MPTP for 3 days (One-Way ANOVA, P < 0.0001, n = 6), while 58.1% of TH-positive cells were lost in the SN of mice treated for 14 days (One-Way ANOVA, P < 0.0001); and mice with 21 days of continuous intervention lost 80.5% of TH-positive cells in the SN (One-Way ANOVA, P<0.0001, n=6). At the same time, mice treated with MPTP for 21 days lost significantly more TH-positive cells than those treated for 14 days (One-Way ANOVA, P=0.0060, n=6).

Discussion

PD is often diagnosed in the later stages with poor treatment outcomes, and early diagnosis and prevention are effective ways to slow the progression of the disease and reduce its harmful effects. The pathological manifestations of PD differ in each stage, but there is no ideal mouse PD staging model to reproduce the pathological features and clinical manifestations of each stage of PD. The purpose of this study was to explore a continuous staging model of PD in mice and to provide a model base for further studies of PD. In this study, we examined the

motor behavior of mice with the open-field test and the rotarod test and detected the α-syn aggregation and loss of dopaminergic neurons in the SN of mice with the WB test and immunofluorescence test. We found that, compared with normal control mice, the motor function of mice was not significantly changed after 3 days of continuous MPTP injection, but the α-syn aggregation was not obvious, TH protein expression was significantly decreased, and TH-positive cells in their SN were lost by 39.5%; after 14 days of continuous MPTP injection, the motor function of mice was significantly impaired, the α-syn aggregation in their SN was noticeable, TH protein expression was significant. Upon continuous injection of MPTP for 21 days, the motor function of mice was more severely impaired than that of mice injected for 14 days, and the decrease of α-syn aggregation and TH protein expression in their SN was more significant, and the loss of dopaminergic neurons reached 80.5%. Therefore, based on the set staging model criteria, continuous intervention of mice with MPTP for 3, 14 and 21 days was able to construct mouse models of prodromal, early clinical and clinical progressive stages of PD, respectively. This provides a reliable experimental model for the study of different periods of PD.

In previous studies, mice that were intervened by MPTP for 5 consecutive days were often found to have or not altered behavioral performance and dopaminergic neuron loss of about 50% (12). In the present study, we observed no visible behavioral changes and 39.5% loss of dopaminergic neurons in mice subjected to MPTP for 3 days, and mice exposed to MPTP for 14 days showed significant behavioral alterations and 58.1% loss of dopaminergic neurons, consistent with previous findings. Increased α-syn accumulation leads to degeneration and loss of dopaminergic neurons (13), which was also verified in our study. As the induction time of MPTP increased, the aggregation of α-syn gradually increased, while the expression of TH protein and the number of dopaminergic neurons gradually decreased.

The cellular and molecular mechanisms that lead to neuronal death in PD are still not fully understood. The exploration of the pathophysiological mechanisms of PD cannot be conducted without ideal animal models of PD, which are generated by toxins or genetic interventions (14-17). Researchers have developed different toxicological animal models to reproduce the SN degenerative features of PD, including using specific neurotoxins such as 6-hydroxydopamine (18), insecticides such as paraquat (19) or rotenone (20) and proteasome inhibitors (21), etc. However, all these Parkinson's models have major limitations in reproducing the patho-physiology of PD. For example, acute topical 6-hydroxydopa injections do not reproduce the progressive and bilateral neuronal degeneration that occurs in PD, paraquat, rotenone and proteasome inhibitor models are prone to animal death and often non-dopaminergic damage and proteasome inhibitor models also lack reproducibility (17, 22-25). MPTP has been found to selectively damage dopaminergic neurons in the SN, producing Parkinson's symptoms similar to the changes seen in human PD (26), and therefore this neurotoxin has been widely used in mice and monkeys to mimic PD.

Because it is difficult to study the pathology of the prodromal phase of PD directly in humans, some investigators have simulated the manifestation of different stages of PD on monkey models. Using MPTP induction for 10 or 21 weeks, investigators have reproduced the pathological and behavioral manifestations of model monkeys during the prodromal and clinical symptom phases of PD, respectively (6,7). Although this model can better simulate the natural course of PD in humans, apes are expensive, take a long time to model, are difficult to obtain and difficult to manipulate. Therefore, the monkey model is hard to promote as a Parkinson's continuous staging model. Meanwhile, researchers have observed that C57/BL6 line mice are well sensitive to MPTP and are often used to study PD (27). In the present study, MPTP was used to intervene in C57/BL6 mice for modeling, and the subjects of this method are cheap, easily available, and easy to manipulate. Meanwhile, this method can reproduce the pathological changes and clinical symptoms of PD, which can be easily replicated.

Three MPTP induction regimens are currently available (8-10): acute, subacute, and chronic. Acute MPTP administration produces extensive dopaminergic neuronal damage and rapid cell death in the SN, and acute MPTP-induced dopaminergic damage shows a high degree of variability and reversibility (28,29), in contrast to the continuous progression of PD. The chronic MPTP induction approach uses lower doses of neurotoxin for a longer duration and can recapitulate the slow progressive features of PD (30). However, investigators found that the chronic MPTP induction model evoked slow neurodegeneration of the nigrostriatal pathway with 25%, 45%, and 55% loss of dopaminergic neurons at 1, 2, and 3 months of induction, respectively, with a long model-building period and limited use (31). In the subacute MPTP induction model, continuous injection of MPTP for 5 days results in approximately 50% dopaminergic neuronal damage and behavioral deficits.[10] Taking into account the model cycle and similarity to PD, we chose a subacute MPTP induction approach for intervention modeling.

In addition, for a long time, clinicians and researchers have often relied on patients' clinical symptom scores such as the Hoehn & Yahr score and pathological changes. The Hoehn & Yahr score is mainly based on motor symptoms for staging and is not sensitive enough to judge PD in the prodromal or early clinical stage. Although the pathological alterations staging method is accurate and reliable, experimental animals must be executed to know their pathological characteristics and staging. Staging PD on a time scale is simple and easy to perform, providing a convenient and reliable model basis for PD research.

Conclusion

Our study concluded that continuous intervention of C57/BL6 mice with MPTP for 3, 14 and 21 days could build mouse models of prodromal, early clinical and clinical progressive stages of PD, respectively, providing a good experimental model foundation for the study of different stages of PD.

Author Contributions

Yueqin Tian and Xiaoya Gao conceived and designed the study. Yueqin Tian performed the experiments. Jianhua Xian and Yuehua Tian finished the statistical analysis and provided general support and coordination for the study. Yueqin Tian wrote and revised the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval
All animal experiments and procedures were reviewed and approved by the Ethics Committee of Zhujiang Hospital, Southern Medical University (Animal Ethics Approval No.: LAEC-2021-031FS).

Competing Interests
The authors have no relevant financial or non-financial interests to disclose.

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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