Original Research

Behavioral characterization in MPTP/p mouse model of Parkinson's disease

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We evaluated the practicability of using the rarely utilized C57BL/6N mouse as a Parkinson's disease model established via the acute MPTP/probenecid (MPTP/p) protocol. We confirmed dopaminergic degeneration in terms of decreased expression levels of tyrosine hydroxylase in the substantia nigra and striatum of MPTP/p-lesioned mice. In addition, acute MPTP/p-lesioned mice demonstrated initial motor dysfunctions followed by spontaneous recovery. Interestingly, these MPTP/p-lesioned mice exhibited anxiolytic and antidepressive behaviors upon recovery from these motor deficits. Additionally, increased expression of norepinephrine transporters in several brain regions, including the hippocampus, medial prefrontal cortex, and striatum, and an elevated rate of adult neurogenesis (in terms of increased numbers of doublecortin-positive neuroblasts) in the hippocampus were observed after recovery from motor dysfunctions. We suggest that the emotional alterations observed under these experimental conditions may be associated with enhanced adult neurogenesis, increased levels of norepinephrine transporters, and/or a possible interplay between these two factors. Consequently, this acute MPTP/p model adequately satisfies the criteria for the validity of a Parkinson's disease model regarding dopaminergic loss and motor impairment. However, the non-motor findings may offer novel evidence against the practicability of utilizing the acute MPTP/p-lesioned mice for modeling the emotional aberrations found in Parkinson's disease patients.

Keywords

Brain regions; Motor symptom; Neurogenesis; Norepinephrine transporter; Non-motor symptom; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders [1]. Unfortunately, a definitive cure for this disease is still elusive; most currently available treatment options rely on palliative symptomatic therapy. Therefore, there is an urgent need to develop medications that prevent the onset or progression of PD. Animal models play a significant role in the preliminary stages of drug development. The model's ability to mimic the disease phenotype is vital in establishing the validity of the disease model. In general, animal models of PD should replicate as many phenotypic characteristics of the disease as possible.

A mouse model commonly used for evaluating PD-like symptoms is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned model [2–4]. Upon uptake in the brain, MPTP is transformed to its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), by astrocytic monoamine oxidase-B. MPP^+ has a strong specificity for the dopamine transporter on dopaminergic neurons, where it is taken up and induces cell death [5]. Administration of the toxin MPTP results in nigrostriatal dopaminergic (DA) cell loss of varying degrees that depend on the regimen used [6]. Three basic MPTP treatment regimens can be used to induce PD-like symptoms in mice: acute, subacute, and chronic administration [7, 8]. Among these, the acute regimen is favored when a shorter time is required to replicate DA loss and the corresponding PD-like motor impairments [6, 9]. Several studies have utilized the acute MPTP model to test the efficacy of PD drug candidates against both its motor and non-motor symptoms; however, the degree to which a protocol can effectively elicit both symptoms varies. Several studies reported the successful impairment of both motor and non-motor functions [10–13], whereas [14] only described the presence of motor symptoms. One reason for the differences between studies could be the variations in MPTP concentrations in the brain. This may be caused by the rapid clearance of the toxin when administered peripherally [15]. MPTP and its active metabolite MPP^+ are rapidly eliminated via renal excretion [16, 17]. Thus, interventions to improve MPTP neurotoxicity have been introduced using agents that inhibit central MPP^+ clearance and/or renal MPTP excretion. One such agent is probenecid, a well-known adjuvant most frequently used in tandem with either subacute or chronic MPTP protocols

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[18, 19]. The exact mechanism of probenecid action is controversial; however, a few proposals have been made, including reductions in renal clearance rate and subsequent increase in MPP^+ concentration available to the brain cells [16, 18] and alterations in the cellular energy metabolism [20]. Nevertheless, these MPTP/probenecid (MPTP/p) protocols have been proven to exacerbate neurotoxicity in mice, resulting in a more pronounced and prolonged DA depletion compared to that with an MPTP-only protocol [18]. Interestingly, the number of studies examining the combined effect of probenecid with acute MPTP administration is limited.

Another reason for the variability of acute MPTP regimens in inducing PD-like non-motor symptoms may be the inconsistent use of mouse strains in different studies. Evidence suggests that mice of different genetic backgrounds exhibit different physiological [21–23] and behavioral [24, 25] phenotypes. So far, the majority of studies utilizing an acute MPTP regimen have used the inbred strain C57BL/6 owing to its high susceptibility to the MPTP toxin compared to other strains [26]. However, there is growing evidence that even for the most common substrains of C57BL/6, including BL/6J and BL/6N, there are still notable differences in their metabolic [27] and behavioral [28] phenotypes. Remarkably, compared to studies using BL/6J, studies that specifically use the BL/6N substrain for establishing acute MPTP models are lacking.

To assess and validate the ability of the acute MPTP regimen to replicate the pathogenic mechanisms of PD, we sought to establish a model by using a protocol with a few variations from that usually employed by previous reports and evaluated the practicability of using the C57BL/6N substrain in creating an acute MPTP/p-lesioned model of PD. We describe the profile of motor impairments and the timeline of their onset and eventual recovery, along with the corresponding changes in DA signaling in the nigrostriatal region. Furthermore, we characterize the non-motor behaviors observed upon recovery from motor dysfunction and their possible underlying mechanisms.

2. Materials and methods

2.1 Animals

C57BL/6N male mice aged 12 weeks and weighing 25–28 g were procured from Daihan-Biolink Co. (Chungbuk, South Korea) and allowed to acclimatize for 1 week before their use. The animals were housed in a room maintained at 23 ± 2 °C with a relative humidity of 50 ± 5%, 12 h light-dark schedule, and 13–18 hourly changes in air volume. The animals were provided access to commercial rodent chow (Jeil Feed Co., Daejeon, Korea) and water *ad libitum* throughout the experiment. Upon acclimatization, the mice were divided randomly into control (vehicle-treated) and MPTP/p-treated groups. The procedures and protocols followed were approved by the Institutional Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2019-22). Animal care was in accordance with the National Institute of Health (NIH) guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978). In addition, care was taken to reduce the suffering of the animals throughout the experiments.

2.2 MPTP treatment

The acute MPTP/p protocol is similar to that described previously [29–31]. Briefly, probenecid (250 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected 30 min before the first MPTP injection. Then, the MPTP/p-lesioned group received four intraperitoneal injections of MPTP (Sigma-Aldrich), at 22 mg/kg per dose, an empirically derived dosage, and administered at 2-h intervals single day for a cumulative dose of 88 mg/kg. This resulted in a 50–80% survival rate while still achieving a ~50% decrease in motor function. The control group (CON), on the other hand, received saline injections. The mice were sacrificed, and brain samples were obtained for immunohistochemical analyses at 1, 2, 8, and 16 days and western blot analyses at 16 days after the last injection of MPTP or saline. Behavioral tests (open field, rotarod, elevated plus maze [EPM], and marble burying tests; grid test and tail suspension test [TST]) were performed on different days, as described in Fig. 1. A total of 74 mice were divided among three separate experiments; in experiment 1, n = 21 per group, in experiment 2, n = 7 per group, and in experiment 3, n = 9 per group. The same set of animals was used for the battery of behavioral tasks indicated in experiments 2 and 3, as shown in Fig. 1.

2.3 Immunohistochemical analysis

Immunohistochemical analysis of free-floating sections was performed as per protocols described in previous studies [32–34]. After sacrificing the animals, brain tissues were collected and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Subsequently, the brain tissues were stored in 30% (w/v) sucrose for 4 days. After that, 30-μm-thick sections were made along the coronal plane using a sliding microtome (SM2010R; Leica Microsystems, Wetzlar, Germany) and stored in PBS at 4 °C. After three washes with PBS, endogenous peroxidase activity was inactivated by incubating the sections in 0.3% (v/v) hydrogen peroxide in distilled water for 20 min at room temperature (RT; 22 ± 2 °C). The sections were then blocked with 1% (v/v) bovine serum albumin (BSA; Sigma-Aldrich) with 2% (v/v) normal goat serum (NGS; Vector Laboratories, Burlingame CA, USA) in 0.3% (v/v) Triton X-100 for 1 h at RT. The sections were then incubated with the primary antibodies rabbit anti-tyrosine hydroxylase (TH; 1 : 500; Millipore, Darmstadt, Germany) and anti-doublecortin (DCX; 1 : 500; Cell Signaling Technology, Beverly, MA, USA) in an antibody dilution buffer (Invitrogen, Carlsbad, CA, USA) overnight at 4 °C. After washing in PBS, the sections were allowed to react with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit; Vector Laboratories) for 2 h at RT. The sections were then washed and incubated with avidin-biotin-

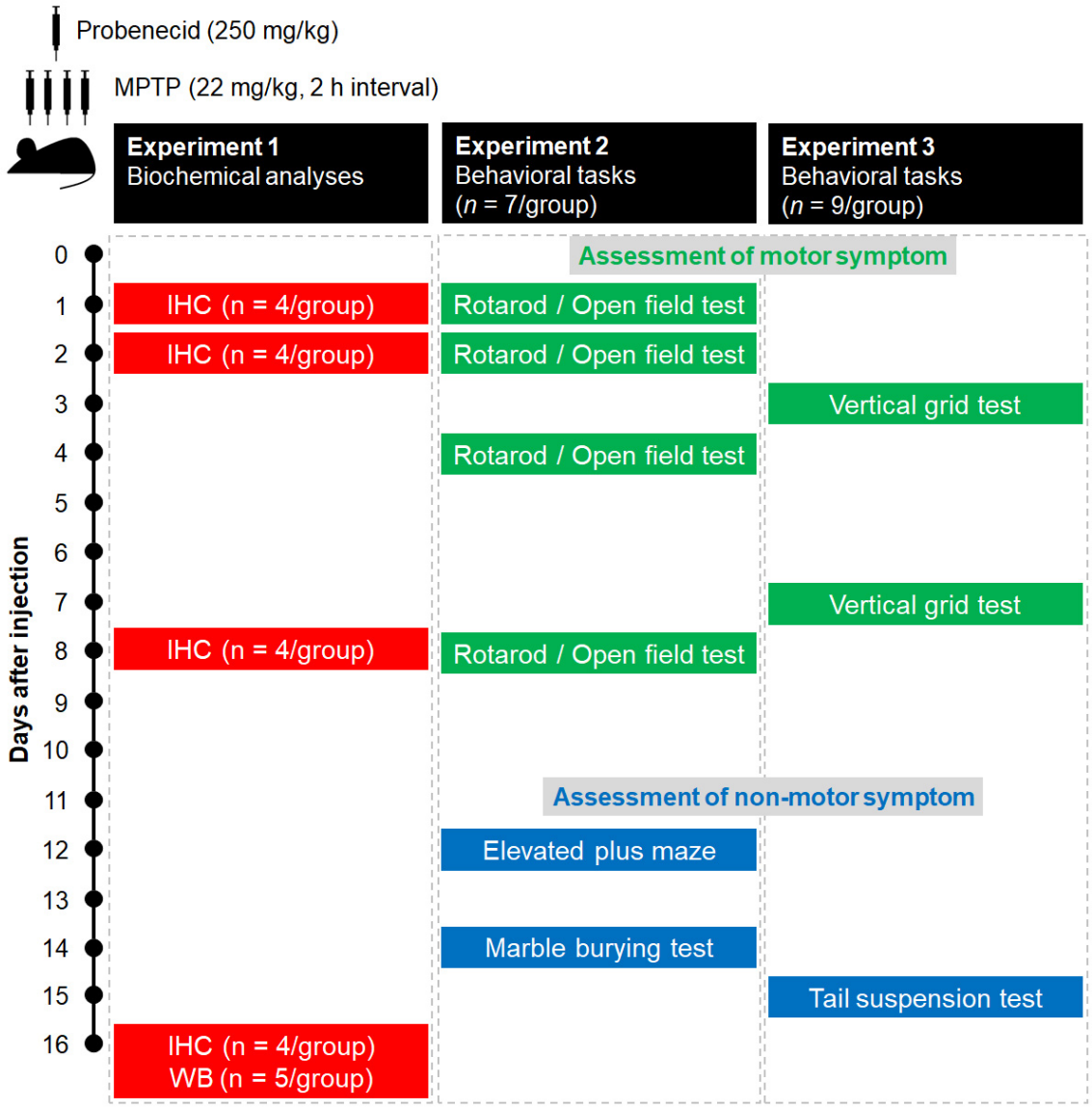

Fig. 1. Schematic diagram of the experimental procedure. On day 0, C57BL/6N mice in the control group received intraperitoneal injections of saline, and those in the MPTP/p-lesioned group received multiple injections of MPTP (22 mg/kg; four times at 2-h intervals) 30 min after a single dose of probenecid (250 mg/kg). In experiment 1, the mice were sacrificed for the sampling of brain tissues at the indicated time points to be used for IHC (n = 4/group) and WB (n = 5/group) analyses. In experiment 2, the open field and rotarod tests (for evaluating motor symptoms) along with the elevated plus-maze and marble burying tests (for evaluating non-motor symptoms) were performed at the indicated time points (n = 7/group). In experiment 3, the vertical grid (for evaluating motor symptoms) and tail suspension tests (for evaluating non-motor symptoms) were performed at the indicated time points (n = 9/group). IHC, immunohistochemistry; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; WB, western blot.

peroxidase complex (Vector ABC Elite Kit) for 1 h at RT. The peroxidase reaction was performed after washing using a diaminobenzidine (DAB) substrate prepared in accordance with the manufacturer's instructions (DAB kit; Vector Laboratories). A few brain sections were not exposed to primary antibodies in each experiment (data not shown) as negative controls.

Images of immunohistochemically stained slices were observed with a Leica microscope and captured using the Leica Caption Suite software (v4.12.0; Leica Microsystems CMS GmbH). The images were converted to grayscale to measure TH immunoreactivities, and the threshold in each tissue was comparably modified using background subtraction. For further correction, the tissue sections from the negative controls (without primary antibodies) were used to identify a single threshold for all slides. The mean gray value (256 gray levels) was calculated using ImageJ software (NIH, Bethesda, MD, USA) for each area chosen. The substantia nigra (approximately 3.64 mm caudally from the bregma) and striatal sections (approximately 0.14 mm rostrally from the bregma) were selected from each brain. The intensities were measured in the substantia nigra and striatum. The relative

changes in intensity levels were expressed as relative TH optical densities (ODs) after setting the mean intensity of the control to 1. Moreover, the number of DCX-positive neuroblasts in the dentate gyrus (DG) subregion of hippocampal sections (approximately 1.94 mm caudally from the bregma) were counted as previously described [35].

2.4 Western blot analysis

Western blot analysis was performed as described previously [32]. Briefly, samples of the medial prefrontal cortex (mPFC), hippocampus, and striatum were separately sonicated in buffer H (50 mM β-glycerophosphate, 10 μg/mL aprotinin, 1.5 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 2 μg/mL pepstatin, 10 μg/mL leupeptin, 0.1 mM Na_3VO_4, and 1 mM phenylmethanesulfonyl fluoride; pH 7.4) for 8 s. Samples were made by adding sodium dodecyl sulfate (SDS) sample buffer (4×) to sample homogenates followed by heating to 100 °C for 10 min. Proteins were separated by 7–15% SDS polyacrylamide gel electrophoresis. Separated proteins were then transferred to polyvinylidene difluoride membranes. Thereafter, the membranes were blocked with 1% (v/v) BSA (Sigma-Aldrich) with 2% (v/v) NGS (Vector Laboratories) in PBS containing 0.1% (v/v) Tween 20 (PBS-T; pH 7.4) for 1 h at RT. Subsequently, the membranes were incubated overnight at 4 °C with primary rabbit anti-noradrenalin transporter (NET) antibody (1 : 1000; Abcam, Cambridge, UK). After three washes with PBS-T, the membranes were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody overnight at 4 °C, and signals were then visualized using a chemiluminescence kit (SuperSignal West Pico; Thermo Fisher Scientific). After stripping, membranes were reprobed with mouse anti-β-actin antibody (1 : 5000; Sigma-Aldrich, St. Louis, MO, USA) for 2 h at RT. The OD of each band was determined using a C-DiGit Blot Scanner 3600 (Li-Cor, Lincoln, NE, USA). The ratio of the density of each band relative to that of β-actin was estimated using ImageJ software. A value of 1 was assigned to the mean intensity of the control, and the relative OD was defined as the change in intensity level relative to that of the control.

2.5 Behavioral analysis of motor symptoms

2.5.1 Open field test

To evaluate the activity of the mice, an open field test was performed as per previous studies' protocols [32, 36]. For each test, a mouse was first placed in the center of an open field, and various parameters, including the ambulatory move time (s) and ambulatory distance (cm), were determined over a 30-min period using a TruScan Photo Beam Activity System (Coulbourn Instruments, Whitehall, PA, USA).

2.5.2 Rotarod test

According to previous reports with some modifications, the motor endurance of the mice was evaluated using a rotarod test performed according to previous reports [37]. Briefly, the rotarod apparatus (Mouse Rota-Rod; Ugo Basile, Varese, Italy) was programmed to rotate with linearly increasing speed from 5 rpm to 40 rpm in 300 s. When a mouse fell off the rod, the time (s) and speed attained (rpm) were automatically recorded; a total of three trials were performed with a 20-min interval between trials. The results were expressed as the average of the three successive trials. The mice were pre-trained for 2 days before MPTP/p treatment. During pre-training, the testing day protocol was followed except for the rotation speed, which was kept at a constant speed of 5 rpm for a period of 300 s.

2.5.3 Vertical grid test

A vertical grid test, which is employed to measure deficits in fine motor skills, was performed in accordance with that described in previous work [38]. Briefly, the mice were placed 3 cm from the top of the apparatus (W × D × H: 5 × 8 × 55 cm), facing upward, and were allowed to spontaneously turn around and climb down. The following parameters were measured: total time (s), the time needed to turn (s), the time needed to climb down (s), and a total number of successful hind limb steps needed to reach the bottom of the apparatus. The cut-off time was set at 60 s. The mice were pre-trained 1 day before the testing day.

2.6 Behavioral analysis of non-motor symptoms

2.6.1 Elevated plus maze

An EPM test was used to evaluate the levels of anxiety in MPTP/p-lesioned mice. The protocol used has been described previously [39]. Briefly, a maze comprising a central square platform (10 × 10 cm) and two open arms perpendicular to two closed arms are enclosed by 40-cm high transparent walls with the resulting four arms (50 × 10 cm) converging the central platform was used. The maze was elevated 50 cm above the floor. The mice were positioned in the central area facing one open arm and were allowed to explore the maze for 10 min under 70-lux lighting conditions. The maze was thoroughly cleaned with 70% ethanol between trials. The percentage of entries and time spent (s) in the open, closed, and central arms were automatically recorded using SmartScan (Panlab, Barcelona, Spain).

2.6.2 Marble burying test

To further confirm the results of the EPM test performed by MPTP/p-lesioned mice, we conducted a marble-burying test. It is a defensive behavior test for mice, and a more significant number of buried marbles indicates higher anxiety levels. The protocol was adapted from previous work [40]. Briefly, each mouse was introduced to a new cage (W × D × H: 255 × 400 × 170 cm) with a 5-cm thick layer of pine bedding (Aspen Bedding, Damul Science, Seoul, Korea) containing 20 small glass marbles (15 mm in diameter) arranged into four evenly spaced rows of five marbles each. Care was taken to ensure that the marbles were entirely on the surface of the bedding. Each mouse was returned to its home cage after 30 min in the cage used for the test, and the number of buried marbles was recorded. A marble was considered 'buried' if at least 2/3rd of the marble was covered with bedding.

Table 1. Two-way ANOVA tests for the effects of treatment and time on acute MPTP/p-induced changes in each dependent variable.

	Figure	Treatment	Time	Interaction
Immunohistochemical analysis of TH expression				
Relative OD in the substantia nigra	Fig. 2A	F(1, 6) = 93.06 $P < 0.0001$	F(3, 18) = 1.205 $P = 0.3362$	F(3, 18) = 2.276 $P = 0.1144$
Relative OD in the striatum	Fig. 2B	F(1, 6) = 255.6 $P < 0.0001$	F(3, 18) = 9.971 $P = 0.0004$	F(3, 18) = 5.639 $P = 0.0066$
Behavioral analysis using the open field test				
Ambulatory move time	Fig. 3A	F(1, 12) = 28.31 $P = 0.0002$	F(3, 36) = 16.18 $P < 0.0001$	F(3, 36) = 23.92 $P < 0.0001$
Ambulatory distance	Fig. 3A	F(1, 12) = 29.52 $P = 0.0002$	F(3, 36) = 8.111 $P = 0.0003$	F(3, 36) = 7.551 $P = 0.0005$
Behavioral analysis using the rotarod test				
Rotation speed attained	Fig. 3B	F(1, 12) = 13.02 $P = 0.0036$	F(3, 36) = 10.76 $P < 0.0001$	F(3, 36) = 4.966 $P = 0.0055$
Latency to fall	Fig. 3B	F(1, 12) = 11.57 $P = 0.0053$	F(3, 36) = 16.92 $P < 0.0001$	F(3, 36) = 5.942 $P = 0.0021$
Behavioral analysis using the vertical grid test				
Total time	Fig. 3C	F(1, 8) = 5.366 $P = 0.0492$	F(1, 8) = 0.00335 $P = 0.9552$	F(1, 8) = 2.335 $P = 0.1650$
Time to climb down	Fig. 3C	F(1, 8) = 2.090 $P = 0.1863$	F(1, 8) = 0.09613 $P = 0.7644$	F(1, 8) = 0.4998 $P = 0.4997$
Time to turn	Fig. 3C	F(1, 8) = 4.306 $P = 0.0717$	F(1, 8) = 0.04870 $P = 0.8309$	F(1, 8) = 3.441 $P = 0.1007$
Successful hindlimb steps	Fig. 3C	F(1, 8) = 15.87 $P = 0.0040$	F(1, 8) = 0.7146 $P = 0.4225$	F(1, 8) = 1.399 $P = 0.2709$

ANOVA, analysis of variance; MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid; OD, optical density; TH, tyrosine hydroxylase.

2.6.3 Tail suspension test

To assess other non-motor symptoms, such as depression, we performed a TST. This behavior test was performed as reported previously [41]. Briefly, the mice were suspended from their tails at the height of 50 cm above the surface with adhesive tape wrapped around the tail 2 cm from the tip. Immobility—defined as the absence of all limb or body movements, except those due to breathing or gravity—was measured for 6 min.

2.7 Statistical analysis

All statistical analyses were performed using Prism (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (ANOVA) [42] tests were used to test for the main effects of the treatment and time and their interactions on the immunohistochemical analysis of TH expression and the behavioral analysis of motor symptoms. When required according to significant omnibus F-statistic, a Šidák's posthoc test was used to test for multiple comparisons. The results of the ANOVA tests are presented in Table 1, and the results of the multiple comparison tests are presented in the corresponding figures mentioned. Unpaired Student's *t*-tests were used for all other analyses. For all statistical tests, a *P*-value < 0.05 was considered statistically significant.

3. Results

3.1 Acute MPTP/p treatment significantly decreased DA signaling of the nigrostriatal region in C57BL/6N mice

There is consensus that the change in DA signaling in the nigrostriatal region is the most common outcome in PD models. Therefore, to confirm successful MPTP/p lesioning, DA loss was evaluated using immunohistochemistry for TH expression in both the substantia nigra and striatum at different time points (n = 4 mice/group at 1, 2, 8, and 16 days after acute MPTP/p treatment). The semi-quantitative results revealed that TH intensities (relative OD) in both the substantia nigra (Fig. 2A) and striatum (Fig. 2B) in the MPTP/p-lesioned group were significantly lower than those in the CON group at all time points (all $P < 0.0001$). Interestingly, the TH immunoreactivity in the striatum of MPTP/p-lesioned brains was substantially increased; the intensity on day 16 was approximately 44.49%, 44.24%, and 28.01% higher on days 1, 2, and 8, respectively (all $P < 0.05$). However, there was still a statistically significant difference in TH intensity between CON and MPTP/p-lesioned groups at each time point.

3.2 Acute MPTP/p-lesioned mice demonstrated spontaneous recovery from motor dysfunctions

In MPTP/p-lesioned mice, DA loss in the nigrostriatal pathway often correlates with motor dysfunctions [43, 44].

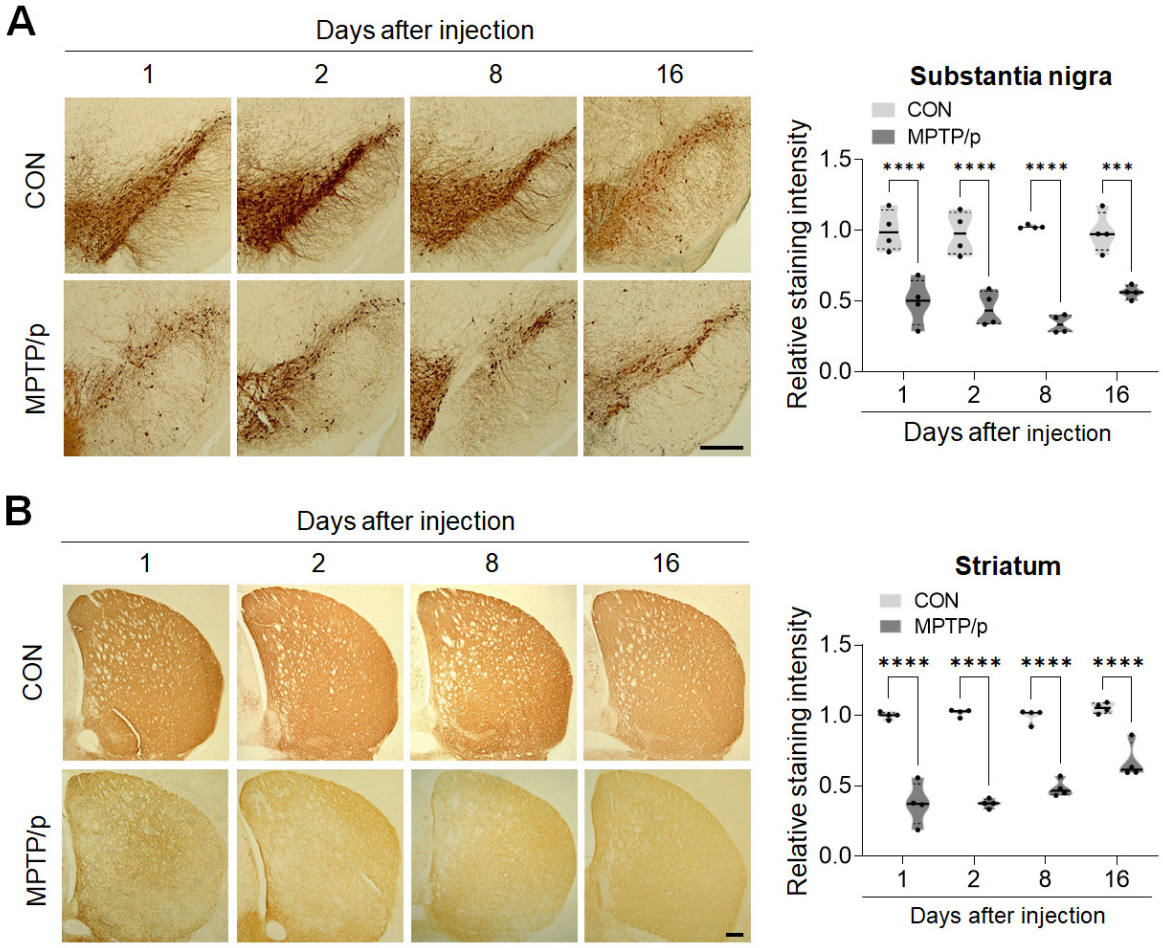

Fig. 2. Acute MPTP/p treatment persistently decreased TH immunoreactivity in the nigrostriatal dopaminergic pathway. (Left panels in A and B) Representative photomicrographs of TH protein expression in the substantia nigra (A) and striatum (B). Scale bars represent 200 μm. (Violin plots in A and B) Relative ODs of TH expression were examined in the substantia nigra and striatum at different time points (1, 2, 8, and 16 days) following acute MPTP/p treatment (n = 4 mice/group). Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. ****$P < 0.0001$ vs. CON. CON, control (vehicle-treated group); MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group; OD, optical density; TH, tyrosine hydroxylase.

Motor functions in the mice were assessed using the open field, rotarod, and vertical grid tests (Fig. 3). In the open field test (n = 7 mice/group), the ambulatory movement time was significantly decreased in MPTP/p-lesioned mice at 1 day (mean ± standard deviation; CON: 855.6 ± 92.79 s; MPTP/p: 93.00 ± 29.25 s; $P < 0.0001$) and 2 days (CON: 787.1 ± 144.1 s; MPTP/p: 499.4 ± 133.8 s; $P < 0.01$) after the final injection compared to that of CON mice (Fig. 3A, left violin plot). The ambulatory distance of MPTP/p-lesioned mice was significantly lower at 1 day (CON: 2809 ± 540.5 cm; MPTP/p: 85.83 ± 59.88 cm; $P < 0.0001$), 2 days (CON: 2713 ± 800.8 cm; MPTP/p: 762.8 ± 399.8 cm; $P < 0.001$), and 4 days (CON: 2577 ± 615.3 cm; MPTP/p: 1319 ± 892.1 cm; $P < 0.05$) compared to those of the CON group (Fig. 3A, right violin plot).

Motor endurance was evaluated using the rotarod test simultaneously as those of the open field test (n = 7 mice/group). Rotation speeds attained (Fig. 3B, left violin plot) in the MPTP/p-lesioned group were lower than those in the CON group at 1 day (CON: 31.38 ± 5.328 rpm; MPTP/p: 14.10 ± 4.413 rpm; $P < 0.001$) and 2 days (CON: 34.48 ± 4.064 rpm; MPTP/p: 20.33 ± 9.828 rpm; $P < 0.01$) after treatment with MPTP/p. The MPTP/p-lesioned group exhibited a significantly lower retention time on the rod in the rotarod test (Fig. 3B, right violin plot) than that exhibited by the CON group at 1 day (CON: 221.6 ± 47.38 s; MPTP/p: 71.57 ± 35.63 s; $P < 0.001$) and 2 days (CON: 246.4 ± 37.26 s; MPTP/p: 127.6 ± 82.27 s; $P < 0.01$) post-injection. However, there were no significant differences in locomotor functions between the CON and MPTP/p-lesioned groups in both the open field and rotarod tests 8 days after acute MPTP/p treatment.

We performed the vertical grid test at 3 and 7 days after acute MPTP/p treatment (n = 9 mice/group). The total time (Fig. 3C, left violin plot; CON: 27.89 ± 17.81 s; MPTP/p: 71.55 ± 42.60 s; $P < 0.05$) and time to turn (Fig. 3C, middle

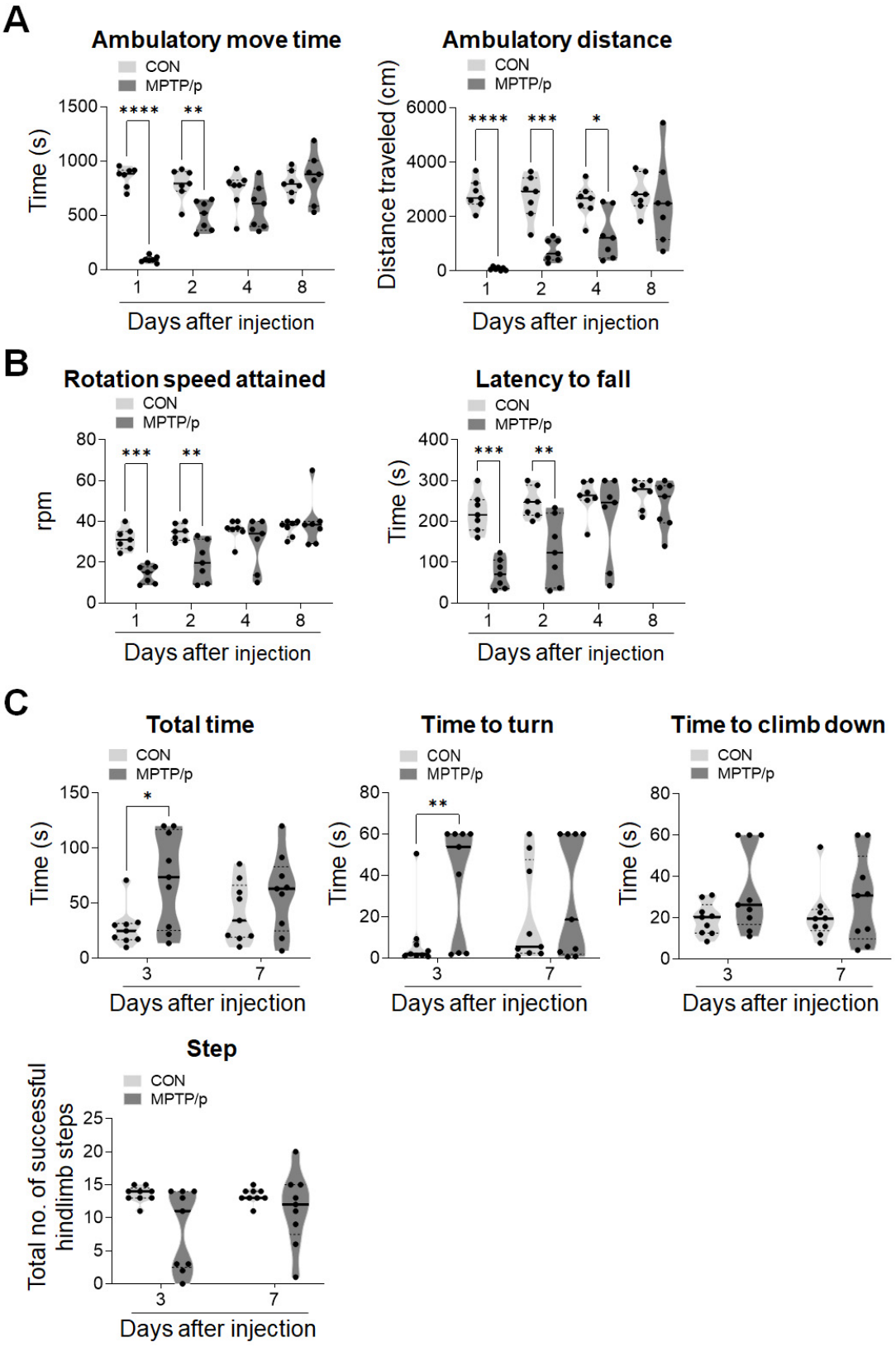

Fig. 3. C57BL/6N mice spontaneously recovered from motor symptoms induced by acute MPTP/p treatment. Motor symptoms were evaluated using open field (A), rotarod (B), and vertical grid (C) tests. Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. The data are reported from two separate experiments for the open field and rotarod tests (n = 7 mice/group) and the vertical grid test (n = 9 mice/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. CON. CON, control (vehicle-treated group); MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group.

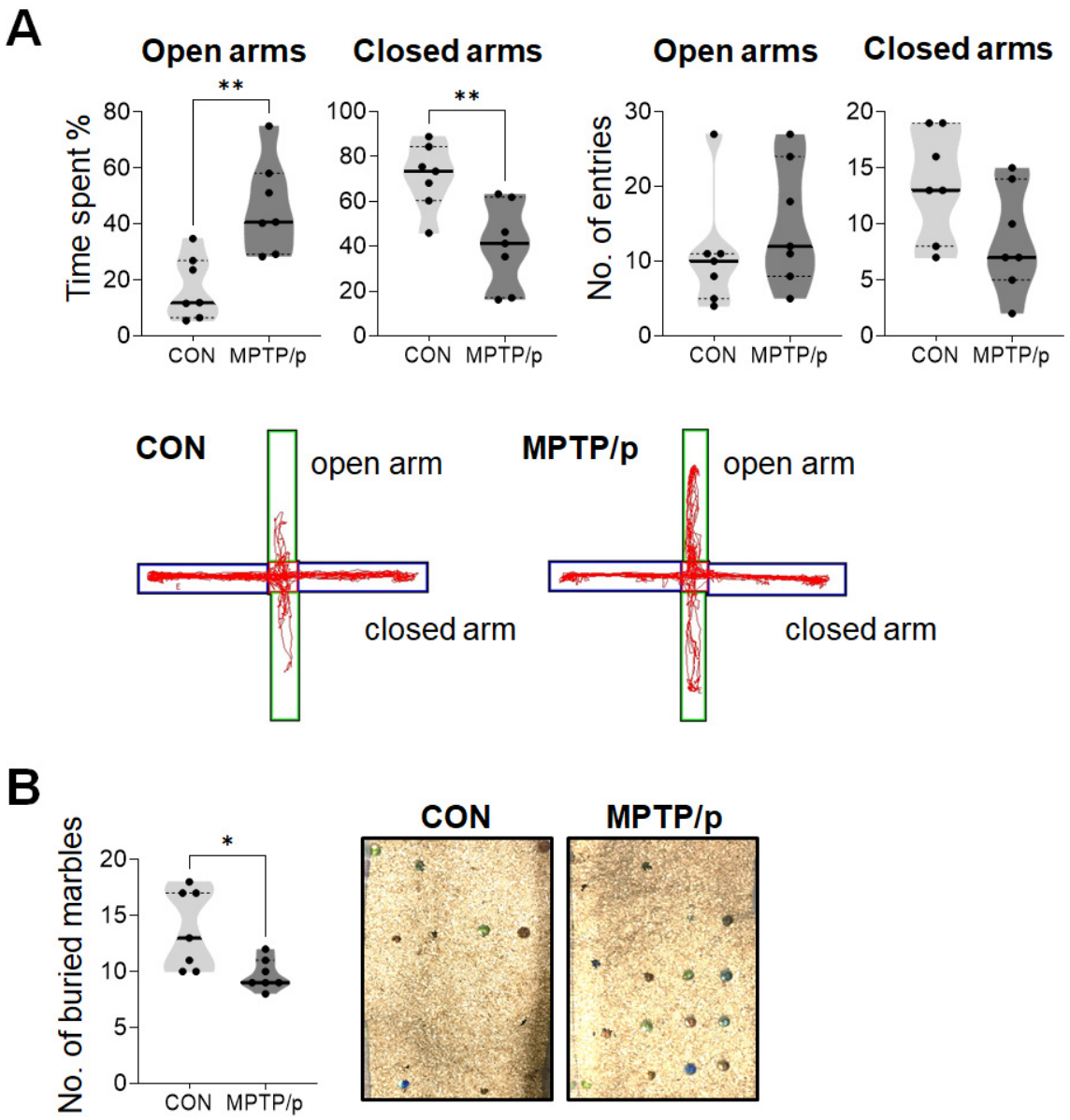

Fig. 4. MPTP/p-lesioned C57BL/6N mice showed anxiolytic behaviors after recovery from motor symptoms. Anxiolytic behaviors were evaluated using EPM (A) and marble burying (B) tests. Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. The data are reported from two separate experiments for the EPM and marble burying tests (n = 7 mice/group). *$P < 0.05$, **$P < 0.01$ vs. CON. CON, control (vehicle-treated group); EPM, elevated plus maze; MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group.

violin plot; CON: 8.47 ± 16.02 s; MPTP/p: 37.86 ± 27.48 s; $P < 0.01$) in the MPTP/p-lesioned group were significantly lower compared to those in the CON group at 3 days post-treatment. However, at 7 days post-treatment, there was no significant difference between the CON and MPTP/p-lesioned groups in this parameter. Furthermore, there were no significant differences in the time to climb down (Fig. 3C, right violin plot) and the total number of successful hind limb steps (Fig. 3C, bottom violin plot) at all time points tested.

3.3 Acute MPTP/p-lesioned mice exhibited anxiolytic and antidepressive behaviors after recovery from motor deficits

To investigate the occurrence of non-motor symptoms after recovery from motor symptoms, anxiety-like behaviors were evaluated using the EPM and marble burying tests on days 12 and 14 after MPTP treatment, respectively. In the EPM test, MPTP/p-lesioned mice (n = 7) spent more time in the open arm areas compared to that spent by mice of the CON group (open arms, CON: 17.30 ± 11.23% [of time spent], MPTP/p: 46.16 ± 16.68%, t(12) = 3.796, P = 0.0025; closed arms, CON: 70.95 ± 14.58%, MPTP/p: 40.19

± 19.07%, t(12) = 3.390, $P = 0.0054$; Fig. 4A, left violin plots). However, there was no significant difference in the number of entries into open and closed arms between the two groups (open arms, CON: 10.86 ± 7.64, MPTP/p: 15 ± 8.24, t(12) = 0.9746, $P = 0.3490$; closed arms, CON: 13.57 ± 4.82, MPTP/p: 8.57 ± 4.72, t(12) = 1.96, $P = 0.073$; Fig. 4A, right violin plots). In the marble burying test, MPTP/p-lesioned mice (n = 7) tended to leave a significantly higher number of glass marbles unburied compared to that left by CON mice (CON: 13.71 ± 3.54, MPTP/p: 9.71 ± 1.38, t(12) = 2.782, $P = 0.0166$; Fig. 4B).

As depression is another major non-motor feature of PD that often accompanies anxiety, depression-like behavior was evaluated using the TST on day 15 after MPTP/p treatment. MPTP/p-lesioned mice (n = 9 mice/group) presented significantly lower immobility time than that seen with CON mice (CON: 151.8 ± 21.35 s, MPTP: 109.5 ± 53.89 s, t(16) = 2.191, $P = 0.0436$) in the TST (Fig. 5).

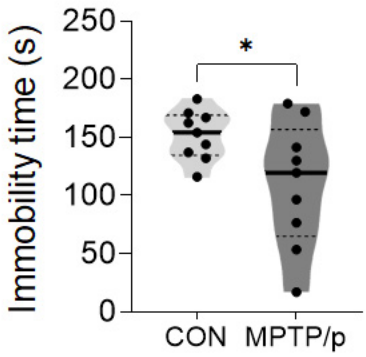

Fig. 5. MPTP/p-lesioned C57BL/6N mice showed antidepressive behaviors after recovery from motor symptoms. Antidepressive behaviors were evaluated using the TST. Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. The data are reported from two separate TST experiments (n = 9 mice/group). *$P < 0.05$ vs. CON. CON, control (vehicle-treated group); MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group; TST, tail suspension test.

3.4 Increased levels of NET expression in the mPFC, hippocampus, and striatum of acute MPTP/p-lesioned mice

To identify the possible mechanism underlying the anxiolytic and antidepressive behaviors and the recovery of motor functions in acute MPTP/p-lesioned mice, NET expression levels in the mPFC, hippocampus, and striatum at 16 days post-treatment (n = 5) were evaluated by western blot analysis (Fig. 6). The NET expression levels (relative OD) in the mPFC (CON: 1.0 ± 0.227, MPTP/p: 1.441 ± 0.226, t(8) = 3.084, $P = 0.015$) and hippocampus (CON: 1.072 ± 0.164, MPTP/p: 1.674 ± 0.4521, t(8) = 2.797, $P = 0.023$) of acute MPTP/p-lesioned mice were significantly higher than those of the CON mice. Similarly, in the striatum, NET expression was significantly higher in MPTP/p-lesioned group than in CON group following acute MPTP/p treatment (CON: 0.887 ± 0.210, MPTP/p: 2.420 ± 0.4918, t(8) = 6.408, $P = 0.0002$).

3.5 Acute MPTP/p treatment significantly increased the rate of adult neurogenesis in the hippocampus

Immunohistochemical analyses showed considerable changes in the number of neuroblasts in the hippocampus of acute MPTP/p-lesioned mice at 16 days post-treatment. The number of DCX-positive neuroblasts in the hippocampal DG of MPTP/p-lesioned mice was significantly higher than in the control mice (CON: 86.0 ± 25.23, MPTP/p: 158.8 ± 50.23, t(6) = 2.588, $P = 0.0413$; Fig. 7).

4. Discussion

We assessed the practicability of using C57BL/6N mice and adjunct probenecid treatment with an acute MPTP regimen for establishing PD models. We found that our treatment protocol induced DA neurodegeneration and elicited both motor and non-motor behavioral alterations. As predicted, the motor impairment was pronounced and transient, but unexpected non-motor behaviors were observed upon recovery from the motor impairments.

The majority of previous studies have used the ability of the MPTP toxin to cause a depletion of DA levels in the nigrostriatal region to replicate motor function impairments seen in patients with PD [45]. We found a substantial decrease in motor activity directly after MPTP/p injection, owing to an acute decline of about 50% in nigrostriatal DA signaling within 24 h after MPTP/p lesioning. The lesioned mice recovered spontaneously starting from 4 days post-treatment and attained full recovery by 8 days post-injection. The observed motor dysfunctions agree with previous studies that employed the acute MPTP mouse model [46, 47]. Interestingly, this spontaneous recovery of motor function was not accompanied by a significant DA rescue in the substantia nigra. Instead, the TH-positive DA signaling remained depleted in the lesioned animals. Thus, it is highly likely that other compensatory mechanisms had developed for the nigrostriatal and/or non-dopaminergic neurotransmission [48, 49]. We observed the following: (1) a small but gradually significant recovery of DA signaling in the striatum and (2) a significant increase in NET expression levels in the striatum of acute MPTP/p-lesioned brains, both of which coincided with the full recovery of motor functions at 16 days post-treatment. The slight improvement in striatal DA signaling has also been reported previously by Mitsumoto *et al.* [50]; they found that acute MPTP-lesioned C57BL/6 mice established without probenecid treatment spontaneously regenerated their striatal axons independent of degenerative changes in TH-positive cell bodies of the substantia nigra at 3 days post-injection.

Additionally, [51] suggested that NET-mediated reuptake of extracellular DA plays a role in regulating the recovery of DA signaling in lesioned mice. Therefore, we suggest that the eventual recovery of motor function observed

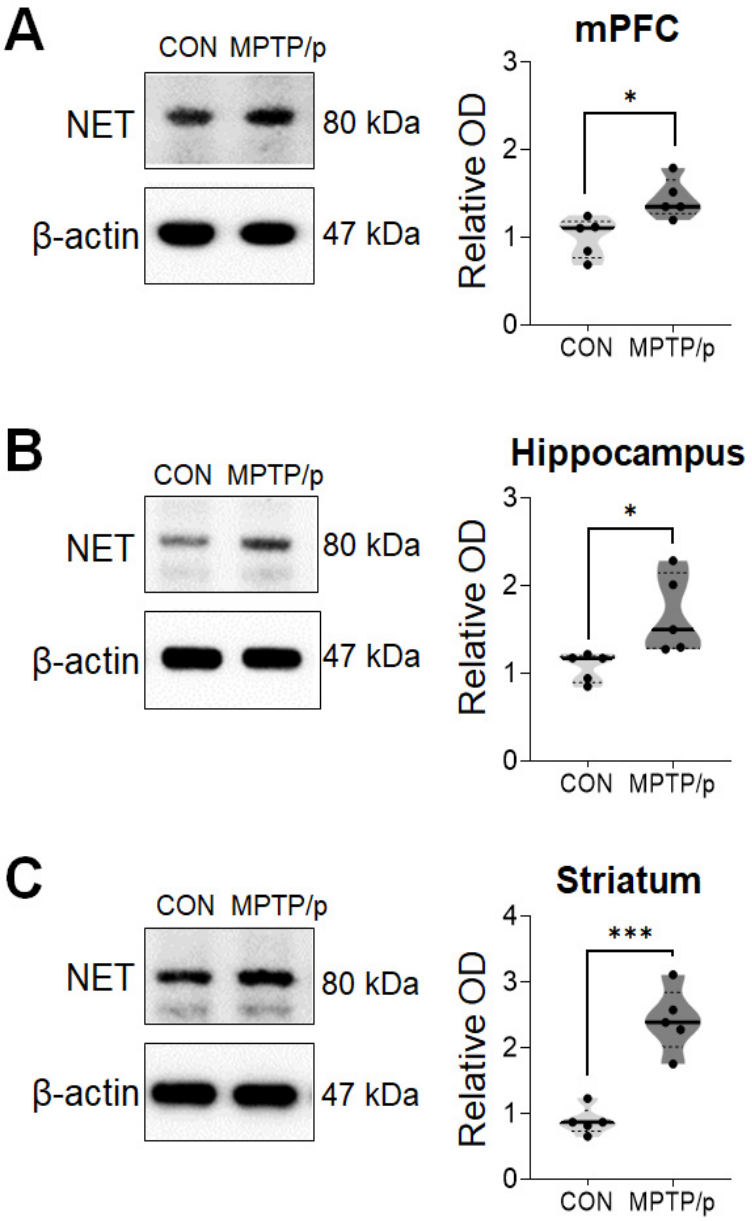

Fig. 6. Acute MPTP/p treatment significantly increased the expression levels of NET in the mPFC, hippocampus, and striatum. (Left panels in A–C) Representative immunoblot images of NET expression in the mPFC (A), hippocampus (B), and striatum (C) at 16 days post-treatment. (Right panels in A–C) Violin plots showing semi-quantitative data analyses (relative OD, n = 5 mice/group). Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. The relative NET expression levels were calculated after normalization to that of the β-actin band for each sample. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. CON. CON, control (vehicle-treated group); mPFC, medial prefrontal cortex; MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group; NET, norepinephrine transporter; OD, optical density.

may be attributed partly to the gradual improvement of DA levels in the striatum through nigral cell axonal sprouting and the regulation of aberrant DA neurotransmission increased NET-dependent re-uptake of DA in MPTP/p-lesioned mice. However, further studies are warranted to confirm these hypotheses and identify other mechanisms possibly involved in the recovery of motor functions.

Upon recovery from motor deficits, MPTP/p-lesioned mice were evaluated for non-motor symptoms to eliminate possible artifacts due to locomotor deficits [52]. We evaluated anxiety-related behavior in the post-motor impairment period of acute MPTP/p treatment using the EPM and marble burying tests. Interestingly, the current model showed anxiolytic behavior instead of the usual anxiogenic effect of acute MPTP administration in other models [10, 53–57]. Anxiolytic behaviors in the current model are mainly related to (1) reduced passive aversion of possible danger (as evaluated using the EPM test) and (2) decreased defensive burying in response to a distinct danger (as evaluated using the marble burying test) [10]. As anxiety almost always concomitantly

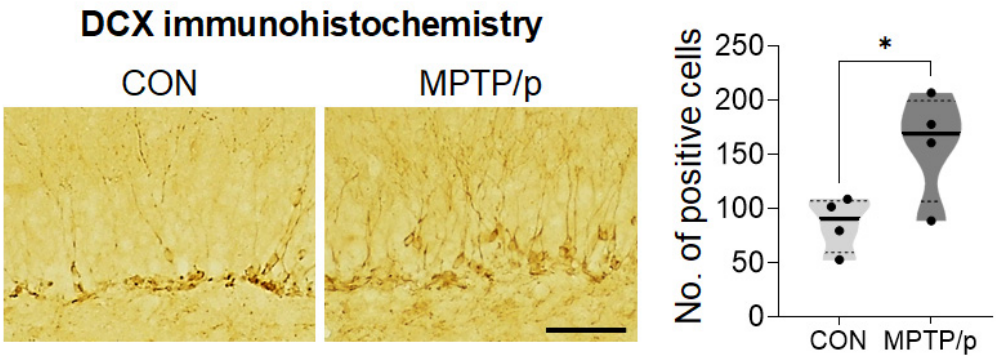

Fig. 7. Increased rate of adult neurogenesis in the hippocampus of mice treated with acute MPTP/p protocol. (Left panel) Representative images of immunohistochemical stainings for DCX, a marker for neuroblasts, in the hippocampal DG at 16 days post-treatment. The scale bar represents 50 μm. (Right panel) Violin plots show the semi-quantitative analyses of the data (No. of DCX-positive cells, n = 5 mice/group). Upper and lower dashed lines signify the upper and lower quartiles, respectively, and the median is represented by a solid black line within a violin plot. *$P < 0.05$ vs. CON. CON, control (vehicle-treated group); DCX, doublecortin; DG, dentate gyrus; MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid-treated group.

occurs with depression in clinical cases of PD [58, 59], we also investigated any possible depression-like behavior in the post-motor impairment period of our acute MPTP/p model using the TST paradigm. Together with signs of anxiolytic activity, we observed antidepressive behaviors in the current model. Again, this contrasts with previous reports that evaluated depression-like symptoms in acute MPTP-treated mice established without probenecid administration [60–65]. Although the exact mechanisms behind the non-motor behavioral features exhibited by the models remain unclear, the involvement of extranigral pathways is highly probable [66, 67]. Our current model found molecular alterations in the mPFC-hippocampal-striatal region to increase NET expression levels in the mPFC, hippocampus, and striatum after acute MPTP/p treatment. Although the reason for this increase in NET levels is unknown, its possible involvement in the altered emotional behavior of the current model cannot be ruled out. We hypothesize that since NETs are the primary target of antidepressants and psychostimulants [68, 69], any aberrations in their expression may also translate to abnormal depression-related emotional dysregulation. Moreover, as anomalies in DA neurotransmission are also correlated with anxiety [70] and depression-related behaviors [71], a possible NET-induced alteration in the DA control of emotional processes should be considered.

Additionally, we found that acute MPTP/p treatment enhanced the rate of hippocampal neurogenesis as the number of DCX-positive neuroblasts increased upon recovery from motor dysfunction. Furthermore, acute MPTP treatment is known to induce neurogenesis in the DG, striatum, and rostral subventricular zone to contribute to the functional replacement within the nigrostriatal circuitry [72, 73]. In our current model, the behavioral changes following acute MPTP/p treatment are consistent with the evidence of hippocampal neurogenesis-induced anxiolytic and antidepressive behaviors reported by previous studies [74–77]. Moreover, several classes of antidepressant drugs are known to increase hippocampal neurogenesis [78–80]. Thus, we further hypothesize that hippocampal neurogenesis-related factors may be involved in an additional mechanism by which abnormal anxiolytic and antidepressive behaviors were induced in the acute MPTP/p model.

The acute MPTP/p model utilized varies from the original protocol regarding probenecid supplementation and the strain of mice used. Probenecid administration and MPTP mainly serve to increase the degree of and prolong the progression of DA damage. We found that in the current set-up, probenecid treatment did not seem to offer any advantage in eliciting motor nor non-motor symptoms of PD, as compared with previous studies using acute MPTP treatment without probenecid treatment [26, 46, 53, 54]. Nevertheless, a possible effect of an interplay between probenecid and C57BL/6N substrain, within the frame of the acute MPTP protocol, on the development of unexpected emotional abnormalities cannot be discounted. Further research is warranted to clarify other specific factors, such as age, sex, and/or protocol of behavioral tests, which may also be responsible for the behavioral aberrations detected in our research. Additionally, more specific mechanistic gain- or loss-of-function studies exploring the underlying molecular processes involved in developing aberrant behaviors will further substantiate our findings.

5. Conclusions

The supplementation of probenecid and C57BL/6N mice for an acute MPTP PD model could evoke robust nigrostriatal DA degeneration and motor symptoms. We also report the unexpected manifestation of non-motor symptoms in this acute MPTP/p model, wherein anxiolytic and antidepressive behaviors were observed. These findings open the door to in-depth investigations on the various animal models currently used in the research on PD. Our results challenge the current evidence available in support for the use of acute MPTP/p-lesioned mice for modeling emotional dysfunction seen in PD.

Abbreviations

BSA, bovine serum albumin; CON, control; DA, dopaminergic; DAB, diaminobenzidine; DG, dentate gyrus; DCX, doublecortin; EPM, elevated plus maze; mPFC, medial prefrontal cortex; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPTP/p, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine + probenecid; NET, noradrenaline transporter; NGS, normal goat serum; OD, optical density; PD, Parkinson's disease; PBS, phosphate-buffered saline; RT, room temperature; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; TST, tail suspension test.

Author contributions

CM conceived and designed the experiments; MW, MJA, PDEW-M, S-HK, J-CK, TS and CM designed the methodology; MW, MJA and PDEW-M performed the experiments; MW, MJA, PDEW-M and CM did the formal analysis; CM provided the resources; MW, MJA, PDEW-M and CM wrote the original draft; MJA, PDEW-M and CM edited and reviewed the draft; CM supervised the work, CM was responsible for funding acquisition.

Ethics approval and consent to participate

The procedures and protocols followed were approved by the Institutional Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2019-22). Animal care was in accordance with the National Institute of Health (NIH) guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978). Care was taken to reduce the suffering of the animals throughout the experiments.

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Conflict of interest

The authors declare no conflict of interest.

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