Sains Malaysiana 53(1)(2024): 49-61
http://doi.org/10.17576/jsm-2024-5301-05

MPTP- Induced BALB/C Mice Recapitulate Compensatory Parkinson's-Like Motor Features

(MPTP- Mencit Aruhan BALB/C Merupakapitulasikan Pampasan Ciri Motor Seperti Parkinson)

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Received: 2 June 2023/Accepted: 2 January 2024

ABSTRACT

This study aimed to assess motor responses and associated pathological changes caused by MPTP neurotoxicity in Balb/c mice. Male mice (13 weeks old, 25-30 g) were divided into four groups and received intraperitoneal injections of normal saline or different doses of MPTP-HCl for five consecutive days. Body weight was monitored, and behavioral tests were conducted. Histological examination with H&E staining was performed on the striatum and substantia nigra. Contrary to expectations, MPTP-treated mice showed increased locomotor activity in the open field test, covering a greater distance and exhibiting more rearing compared to control mice ($p<0.05$). The catalepsy test also showed lower catalepsy scores in the MPTP-treated group ($p<0.05$). However, the pole test did not indicate the presence of MPTP-induced bradykinesia ($p>0.05$). Similarly, the traction and hang tests showed no significant effects of MPTP on motor balance or muscle strength ($p>0.05$). Among the MPTP-treated groups, the 30 mg/kg MPTP-HCl group displayed the most severe pathological changes, including reactive gliosis, as observed in histological examination. In conclusion, the subacute MPTP mouse model used in this study did not exhibit noticeable motor deficits or significant weight loss in Balb/c mice, possibly due to subthreshold dopamine depletion compensatory mechanisms. This model could provide valuable insights into the compensatory mechanisms involved in Parkinson's disease.

Keywords: Balb/c mice; MPTP; Parkinson's-like symptoms

ABSTRAK

Penyelidikan ini bertujuan untuk menilai respons motor dan perubahan patologi yang berkaitan disebabkan oleh neurotoksin MPTP pada model tikus Balb/c untuk penyakit Parkinson. Mencit jantan Balb/c berumur 13 minggu dan berat antara 25-30 g telah dibahagikan secara rawak kepada empat kumpulan. Mereka menerima suntikan intraperitoneal dengan larutan garam fisiologi 0.9% atau dos berbeza MPTP-HCl selama lima hari berturut-turut. Berat badan tikus dipantau dan ujian tingkah laku dilakukan. Setelah itu, pemeriksaan histologi menggunakan pewarnaan H&E dilakukan pada striatum dan substantia nigra. Berbeza dengan jangkaan, mencit yang diberikan MPTP menunjukkan aktiviti lokomotor yang meningkat dalam ujian lapangan terbuka, menempuh jarak yang lebih jauh dan menunjukkan lebih banyak gerakan memanjat berbanding dengan mencit kawalan ($p<0.05$). Ujian katalepsi juga menunjukkan skor katalepsi yang lebih rendah pada kumpulan yang diberikan MPTP ($p<0.05$). Walau bagaimanapun, ujian tiang tidak menunjukkan kehadiran bradikinesia yang disebabkan oleh MPTP ($p>0.05$). Begitu juga, ujian daya tarikan dan gantungan tidak menunjukkan kesan yang signifikan MPTP terhadap keseimbangan motor atau kekuatan otot mencit ($p>0.05$). Antara kumpulan yang diberikan MPTP, kumpulan MPTP-HCl dengan dos 30 mg/kg menunjukkan perubahan patologi yang paling teruk, termasuk hipokromasia dan gliosis yang teruk, seperti yang dilihat dalam pemeriksaan histologi. Secara kesimpulannya, model mencit MPTP subakut yang digunakan dalam kajian ini tidak menunjukkan ketidakupayaan motor yang ketara atau kehilangan berat badan yang signifikan pada mencit Balb/c. Ini mungkin disebabkan oleh mekanisme pampasan yang mengatasi penurunan dopamin melalui pemulihan dan mekanisme kelebihan. Model ini dapat memberikan pandangan yang berharga mengenai mekanisme pampasan yang terlibat dalam penyakit Parkinson.

Kata kunci: Gejala seperti Parkinson; Mencit Balb/c; MPTP

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that primarily affects the elderly population, and its exact cause is still unclear. However, it is believed that a complex interplay between genetic and environmental factors contributes to the pathogenic mechanisms leading to selective neuronal loss (Garza-Ulloa 2019; Obergasteiger et al. 2018). Animal models play a crucial role in improving our understanding of the etiology, pathogenesis, and molecular mechanisms underlying PD (Zeng, Geng & Jia 2018). These models aim to replicate the major pathological features observed in human PD, such as selective damage to dopaminergic neurons, depletion of striatal dopamine, and the formation of neuronal inclusion bodies known as Lewy bodies (Jo et al. 2019). In addition, the animal models should exhibit a Parkinsonian Syndrome from a behavioral standpoint, characterized by bradykinesia, muscular rigidity, tremor, and postural instability (Bhaduri, Abhilash & Alladi 2018). Although animal models have become valuable tools in PD research, there is no consensus on the most accurate rodent model of the disease. However, the Balb/c mouse has shown promise as a model species for biomedical research, including neurotoxicology and screening of neurotherapeutic drugs (Brooks 2011).

For decades, the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity has been extensively used to induce behavioral, biochemical, and cellular changes similar to those observed in human PD in experimental mice (Rai & Singh 2020). Compared to other toxin models of PD, the MPTP-induced mouse model offers advantages in terms of simplicity, affordability, practicality, better clinical correlation, and fewer ethical considerations (Mustapha & Mat Taib 2021). MPTP administration leads to a range of behavioral impairments, striatal dopamine loss, and nigral cell loss, depending on the specific protocol used (Kozina et al. 2011). To date, three different MPTP dose regimens have been employed in mice to induce Parkinson-like symptoms: Acute, subacute, and chronic (Blesa & Przedborski 2014). Among these, the subacute MPTP model has gained popularity due to its relatively short treatment duration, lower animal stress levels, and closer resemblance to PD pathogenesis (Zhang et al. 2017). However, there is a lack of consistency and specificity in reporting the behavioral outcomes and associated pathological changes in the subacute MPTP model, as well as the optimal MPTP dose for a reproducible Balb/c mouse model of PD. Establishing an accurate and predictive PD model is essential for advancing our understanding of the condition and developing new therapeutic approaches. Moreover, it aligns with Sustainable Development Goal 3, which aims to ensure healthy lives and promote well-being for all individuals by supporting research on non-communicable diseases, including PD. The present study is part of an ongoing project focused on establishing a suitable PD model. It aimed to assess and report changes in body weight, behavioral motor responses, and associated pathological changes induced by MPTP neurotoxicity in Balb/c mice.

Materials and Methods

Ethical Statement

The experiment followed ethical guidelines for the care and use of laboratory animals, as outlined by the National Institutes of Health. The Animal Research: Reporting of *in vivo* Experiments (ARRIVE) protocols for reporting *in vivo* experiments were also observed. The study protocols were approved by the Institutional Animal Care and Use Committee at Universiti Putra Malaysia, with reference number UPM/IACUC/AUP-R027/2020. The researchers made efforts to minimize animal suffering, reduce the number of animals used, and explore alternative methods when possible.

Animals and Reagents

The Balb/c male mice (13 weeks, 25–30 g) and Hematoxylin and Eosin (H&E) stain used for the experiments were procured from Interscience Sdn. Bhd., Selangor, Malaysia. The MPTP-HCL used was purchased from Sigma Corporation (Sigma-Aldrich, St. Louis, MO, USA). The mice were acclimatized at a temperature of 22±1 °C, with a 12-h light/dark cycle, and were allowed free access to food and water during the experiment. The mice were randomly assigned into four groups, with n = 4 per group.

MPTP Injection

The MPTP used in the study was purchased as MPTP-HCL, which has a molecular weight (MW) of 209.7. The HCL component, with a molecular weight of 35.4, accounts for 17% of the MPTP-HCL MW. To calculate the equivalent dose of the free base MPTP, the MPTP-HCL doses were multiplied by a factor of 1.17. Therefore, for the doses of 15 mg/kg, 30 mg/kg, and 45 mg/kg, the equivalent doses of the free base MPTP were 17.55 mg/

kg (15 mg/kg × 1.17), 35.1 mg/kg (30 mg/kg × 1.17), and 52.7 mg/kg (45 mg/kg × 1.17), respectively. This conversion was based on the protocol described by Jackson-Lewis and Przedborski (2007). The MPTP doses selected were adopted from previous studies (Bhaduri, Abhilash & Alladi 2018; Campolo et al. 2017; Luchtman, Shao & Song 2009). To ensure an adequate supply of MPTP for the study and to account for any potential losses due to the syringe's dead space, a 10% additional solution was prepared. The total volume of MPTP required for the study was calculated by adding the weight of all mice injected with MPTP in each model group, taking into account the additional 10% solution.

EXPERIMENTAL PROCEDURES

The study employed the following experimental procedures as illustrated in Figure 1. The mice underwent a one-week acclimatization period, followed by behavioral habituation testing spanning days 0 to 4. Subsequently, the mice were randomly assigned to one of four groups (n = 4/group): the normal control group (treated with 0.01 mL/g 0.9% normal saline) and the 15 mg/kg, 30 mg/kg and 45 mg/kg MPTP-induced PD model groups. Over five consecutive days (days 5 to 9), intraperitoneal injections of free MPTP base and normal saline were administered (Tatton & Kish 1997). During this period, the mice's body weights were documented daily. On day 15, 24-h after the last behavioral test, euthanasia was performed via cervical dislocation.

BEHAVIOURAL EXPERIMENTS

The behavioral tests were performed 24 h after the final MPTP injection. Prior to the experiment, the animals were acclimated to the experimental room in the animal house for at least one hour. The test sessions were scheduled between 9 a.m. and 12 p.m to align with circadian rhythms, which results in enhanced alertness and cognitive performance during this time (Brooks 2011). Each day, only one behavioral test was conducted to ensure that the animals had enough time to fully recover from any potential stress induced by the sequence of trials and handling. It is known that training and previous experiences can significantly impact and modify the animal's performance in subsequent tests (Voikar, Vasar & Rauvala 2004).

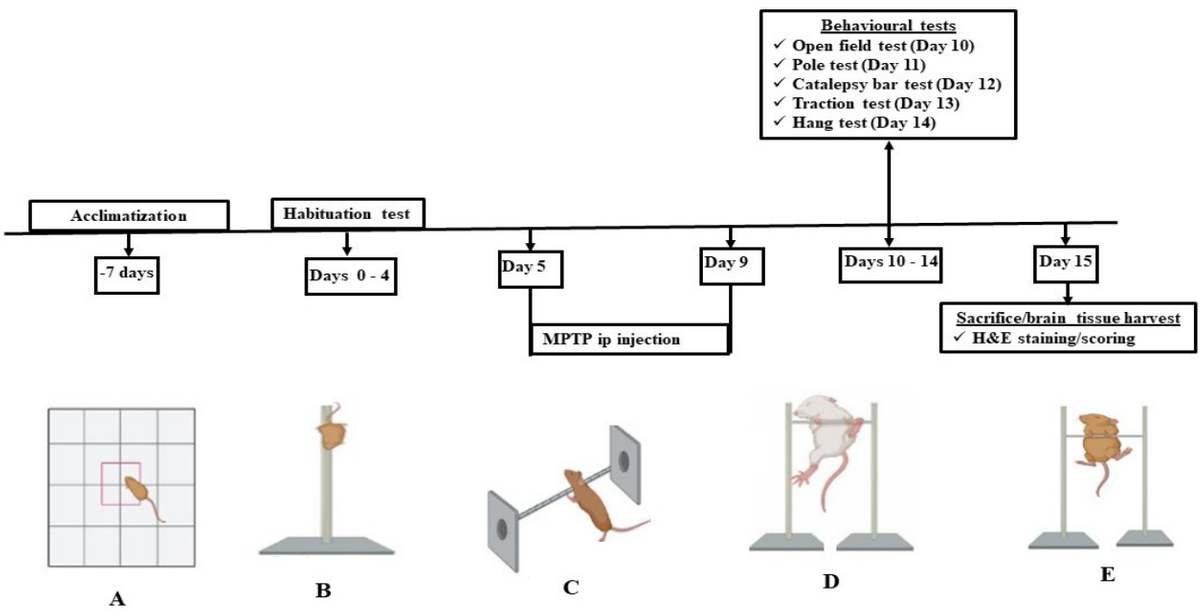

FIGURE 1. Experimental procedures. A: OFT apparatus; B: Pole test apparatus; C: Catalepsy bar test apparatus; D: Traction test apparatus; E: Hang test apparatus (Created with Biorender.com)

Open field test

The open-field test (OFT) was conducted on day 10. OFT is a technique used to assess spontaneous motor performance and exploratory behavior in rodents. The method was derived from a protocol initially described by Hall (1934) and later refined by Nagarajan et al. (2015) and Liu et al. (2019). To conduct the test, mice were placed in the center of a plexiglass square arena measuring 45 cm in length, 45 cm in width, and 40 cm in height. They were allowed to freely explore the arena for a duration of 5 min while their movements were recorded. After the 5-min session, the mice were returned to their home cages, and the open-field test equipment was cleaned with 70% ethanol to prevent odor cues from influencing subsequent mice. The camera and video tracking system software (ANY-Maze, version 7) were utilized to measure and analyze two parameters: the total distance traveled by the mice and the frequency of rearing behavior (i.e., the number of times the mice stood on their hind limbs).

Pole test

The pole test is a behavioral test utilized to evaluate bradykinesia in rodent models of PD. It was conducted on day 11. The protocol was adopted from the original description by Ogawa et al. (1985) with minor adjustments (Huang & Jiang 2019; Shi et al. 2021). In this test, a metallic pole measuring 50 cm in height and 0.5 cm in diameter was wrapped in cotton gauze to prevent slippage. The base of the pole, measuring 20 cm × 12.5 cm, was placed in the animal's home cage. A rubber ball with a diameter of 2.5 cm was attached to the top of the pole to discourage animals from sitting on it and facilitate their positioning on the pole (by sliding the forepaws over the ball while holding the animal by the tail). The mice were positioned head downward at the upper end of the vertically oriented, rough-surfaced pole. The scoring of the test begins when the mouse initiates downward movement. The latency to descend, referred to as the time it takes for the mouse to reach the ground (T descend), is recorded. If a mouse fails to descend within 60 s, it is gently guided to do so. In the event that a mouse falls immediately, the longest T descend observed within that particular group is assigned as the maximum time for the experiment. A successful run requires the mouse to descend without pausing; if it pauses during the trial, the trial is discarded and must be repeated. Prior to receiving MPTP injections, the mice were trained, and each mouse completed three consecutive trials with a 5-min intertrial interval during both the pre- and post-MPTP sessions. The average of the three trials was calculated for statistical analysis. Following each trial, the pole was wiped clean with 70% alcohol.

Catalepsy bar test

The catalepsy bar test is used to assess muscular rigidity in mice. It was done on day 12. The behavioral assay was conducted following a previously described protocol (Ali & Rajini 2016; Sanberg et al. 1988). The mouse's forepaws were positioned on a metallic horizontal bar, measuring 30 cm in length and 0.2 cm in diameter, which was fixed at a height of 4 cm above the work area. Three trials were conducted to determine catalepsy at a specific time point, with a one-minute rest period between each trial. By summing or averaging the results of these trials, the variability in data induced by stress during handling is reduced compared to relying on a single trial for determination. The duration of catalepsy was measured in each trial, starting from when the mice were initially placed on the bar until they removed both forepaws or climbed over the bar using their hind limbs. The maximum duration allowed for each trial was set at 120 s. Following each trial, the catalepsy bar was cleaned with 70% alcohol.

Traction test

To evaluate balance in rodents, the traction test was employed (Sun et al. 2018) and it was conducted on day 13. The apparatus used in this study consisted of a metallic wire measuring 2.5 mm in diameter and 50 cm in length, securely clamped 30 cm from the base between two retort stands. A foam bedding was placed at the bottom of the setup to cushion any potential falls. To prevent the mouse from traversing along the wire and reaching the posts, a small piece of cardboard was positioned on the wire. The mouse's forepaws were positioned at the center of the wire, and the placement of its hind limbs was assessed using a scoring system ranging from 1 to 3, with a lower score indicating a more severe impairment. The scoring criteria were as follows: A score of 3 was assigned if both hind limbs gripped the rope, a score of 2 if one hind limb grasped the rope, and a score of 1 if neither hind limb held onto the rope (Hu et al. 2018). If a mouse received a score of 3 in the initial trial, the test was discontinued. However, if the mouse did not achieve the maximum score, two additional attempts were conducted after a 5-min intertrial rest period, and an average score was calculated. After each trial, the wire was cleaned

with 70% alcohol to maintain cleanliness and eliminate potential confounding factors.

Hang test

Muscle strength was assessed using the hang test (Oliván et al. 2015), performed with the traction test set-up (Hu et al. 2018). It was conducted on day 14. To prevent hindlimb grip on the set-up during the trial, a nontoxic adhesive paper was gently applied to the hind paws of the mouse. The mouse was then gently grasped by the tail and allowed to grip the wire at the center firmly. The latency to fall was recorded, with a maximum duration of 60 s for each hanging attempt. If a mouse successfully completed the allotted hanging time in the first trial, the test was discontinued. However, if the mouse did not reach the maximum duration, two additional attempts were conducted after a 5-minutes intertrial rest period, and an average latency was calculated. After each trial, the wire was cleaned with 70% alcohol to maintain cleanliness and eliminate any potential confounding factors.

H & E STAINING

Twenty-four hours after the last behavioral test (day 15), the mice in each group were euthanized via cervical dislocation. The whole brains were then isolated, washed in ice-cold phosphate-buffered saline for 1-2 min, and transferred into 10% neutral buffered formalin for fixation and hardening for forty-eight hours before further processing. To expose specific brain regions, each brain was coronally cut anteriorly to show the striatum (STR) and dorsally at the level of the inferior colliculus, after carefully removing the cerebellum to expose the midbrain containing the substantia nigra, following the mouse brain's stereotaxic atlas (Paxinos & Franklin 2019). The dissected brain tissues, approximately 300-500 µm in thickness, were placed in tissue cassettes and dehydrated using an automated tissue processor. Subsequently, the processed brain tissues were embedded in paraffin blocks using an embedding machine, and coronal sections of the tissue blocks were cut at 5 µm thickness using a microtome to generate a continuous ribbon of tissue sections. The tissue sections were then de-paraffinized at 70 °C for 120 min in an oven, stained with H&E using an automated staining machine, mounted with dibutyl phthalate xylene mounting fluid, and covered with a cover slip. High-resolution imaging of the sections was performed using a pathologist-grade Olympus BX51TRF-CCD compound microscope equipped with a Dino eye (Dino lite, Dunwell Tech Inc., Torrance, Los Angeles, CA, USA).

In collaboration with an independent pathologist, at least 4 to 5 non-overlapping spots were randomly selected from the entire STR/substantia nigra pars compacta (SNPc) region. These spots were then evaluated for the severity of pathological features using a semi-quantitative 4-point rating scale: 0 = no pathology; 1 = mild pathology (1-5 eosinophilic neurons); 2 = moderate pathology (5-10 eosinophilic neurons, Rosenthal fibers covering one-third to one-half of the tissue); 3 = severe pathology (>10 eosinophilic neurons, Rosenthal fibers surrounding more than one-half of the tissue) (Hu, Cui & Zhang 2021). The evaluation was conducted using the ImageJ software provided by the National Institutes of Health (rsb.info.nih.gov/ij). The regions of interest in different groups were carefully chosen to have the same STR/SNPc shape, verified by cross-referencing with atlases such as Paxinos and Watson (2019) (Figure 5(A)). Three slices from each animal were used for statistical analysis.

STATISTICAL ANALYSIS

To determine the normality of the data, the Shapiro-Wilk test was conducted. The data were reported as mean (SEM). One-way or two-way analysis of variance (ANOVA) was used to compare means, followed by the Tukey post hoc test. Kaplan-Meier survival analysis was performed to calculate the probability of mortality from MPTP neurotoxicity. A p-value < 0.05 was considered statistically significant. The analysis was conducted using GraphPad Prism software (version 9.0.0.2) from ISI, USA.

RESULTS

GENERAL OBSERVATION

The MPTP-HCL treated group exhibited evident signs of acute neurotoxicity in a dose-dependent manner, including straub tail, piloerection, tremors, head twitches, teeth chatter, hyperpnea, and increased locomotor activity shortly after drug administration. In the 45 mg/kg MPTP-HCL group, all mice experienced mortality (100%) on the first day of drug administration, presumably due to the systemic effects of MPTP on the cardiovascular system (Jackson-Lewis & Przedborski 2007). Consequently, this dose was deemed lethal and excluded from the study. Group 1 (control), group 2 (15 mg/kg MPTP-HCL), and group 3 (30 mg/kg MPTP-HCL) exhibited 100% survival and were utilized for further analysis. However, groups 2 and 3 continued to display hyperactivity after treatment, although other signs of acute neurotoxicity disappeared

within 1 to 2 h post-MPTP administration. Kaplan-Meier analysis indicated a higher unadjusted mortality probability in group 4 (100%) compared to groups 1, 2, and 3 (0.0%). The log-rank test (Mantel-Cox) confirmed that this difference was statistically significant ($\chi 2$ (3) = 19, p = 0.0003).

IMPACT OF MPTP ON MOUSE WEIGHT

In the present study, MPTP exhibited a significant interaction with mouse weight, accounting for 15.2% of the total variance {F (30) = 14.62, p = 0.006}, as determined by a two-way ANOVA (Figure 2(A)). The mean body weight in group 2 mice (30.00 ± 0.080 g) was significantly higher than that in group 1 mice (27.00 ± 0.039 g) {F (12) = 34, p = 0.0001}, with a mean difference (SEM) of 3.0 (0.4) and a 95% confidence interval (CI) of -3.0 to -1.4. However, there was no significant difference in mean body weights between group 3 (28.00 ± 0.04 g) and group 1 (27.00 ± 0.039 g) mice {F (12) = 0.94, p = 0.958}, with a mean difference (SEM) of 1.0 (0.001) and a 95% CI of 0.83 to 0.69 (Figure 2(A)). Additionally, there were no significant mean differences in the percentage of body weight change between the groups {F (2, 9) = 1.4, p = 0.295} (Figure 2(B)).

MPTP ENHANCES LOCOMOTION AND EXPLORATORY BEHAVIOUR IN THE OFT

The mean total distance traveled in group 2 mice (0.040±0.003 m) was significantly higher than that in group 1 mice (0.023 ± 0.005 m) {F (9) = 8.4, p = 0.018}, with a mean difference (SEM) of 0.017 (0.005) and a 95% confidence interval (CI) of -0.030 to -0.003. Similarly, the total distance traveled in group 3 mice (0.041±0.002 m) was significantly higher than that in group 1 mice (0.023 ± 0.005 m) {F (9) = 8.4, p = 0.013}, with a mean difference (SEM) of 0.018 (0.0049) and a 95% CI of -0.031 to -0.0042 (Figure 3(A): i-iii & 3(B)).

Furthermore, group 3 mice exhibited a significantly higher mean rearing frequency per 5 minutes (8.0±0.041) compared to group 1 mice (6.0±0.41) {F (9) = 4.3, p = 0.004}, with a mean difference (SEM) of 2.0 (0.71) and a 95% CI of -4.0 to -0.026. However, the mean differences in rearing frequency between groups 1 (6.0±0.41) and 2 (6.5±0.65) did not reach a level of statistical significance {F (9) = 4.3, p = 0.766}, with a mean difference (SEM) of -0.50 (0.71) and a 95% CI of -2.5 to 1.5 (Figure 3(C)). Finally, the 15 and 30 mg/kg MPTP-HCL doses did not exhibit any dose-response relationship with the total distance traveled or rearing frequency (p>0.05).

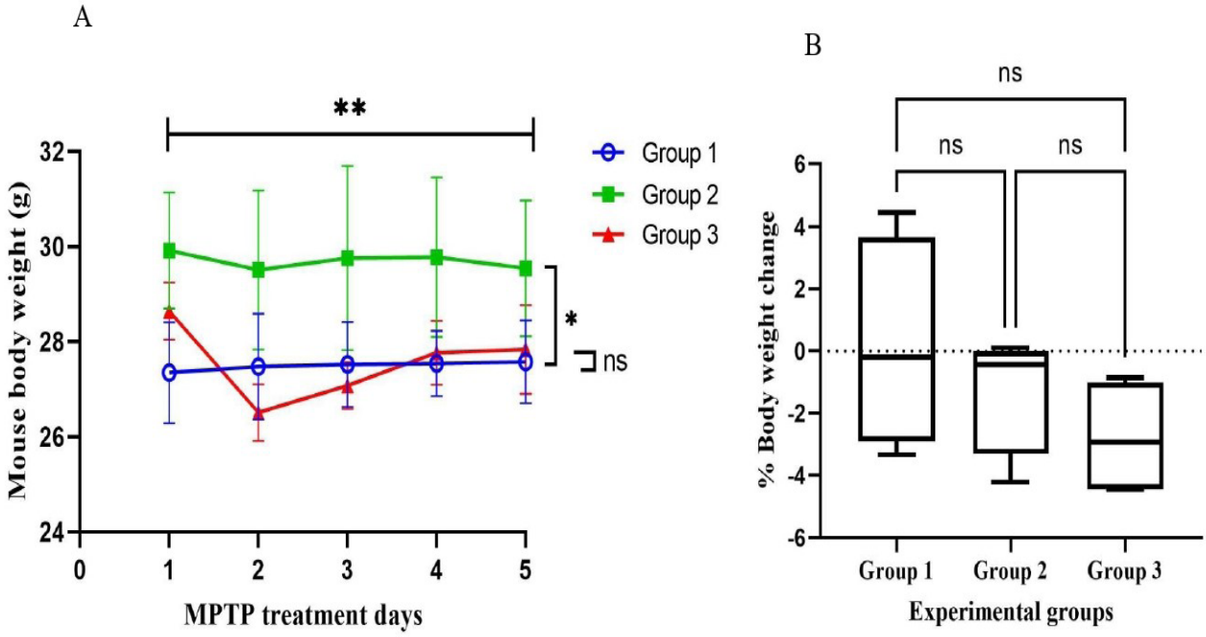

FIGURE 2. Effect of MPTP on mouse body weight (A) and Mean % body weight change difference (B). Values are expressed as mean±SEM; Group 1: 0.01 mL/g 0.9% N/saline; Group 2: MPTP-HCL@15 mg/kg; Group 3: MPTP-HCL@30 mg/kg (n = 4/group). **p<0.001; * p<0.05; ^{ns} p>0.05, using one-way ANOVA followed by Tukey's multiple comparison test

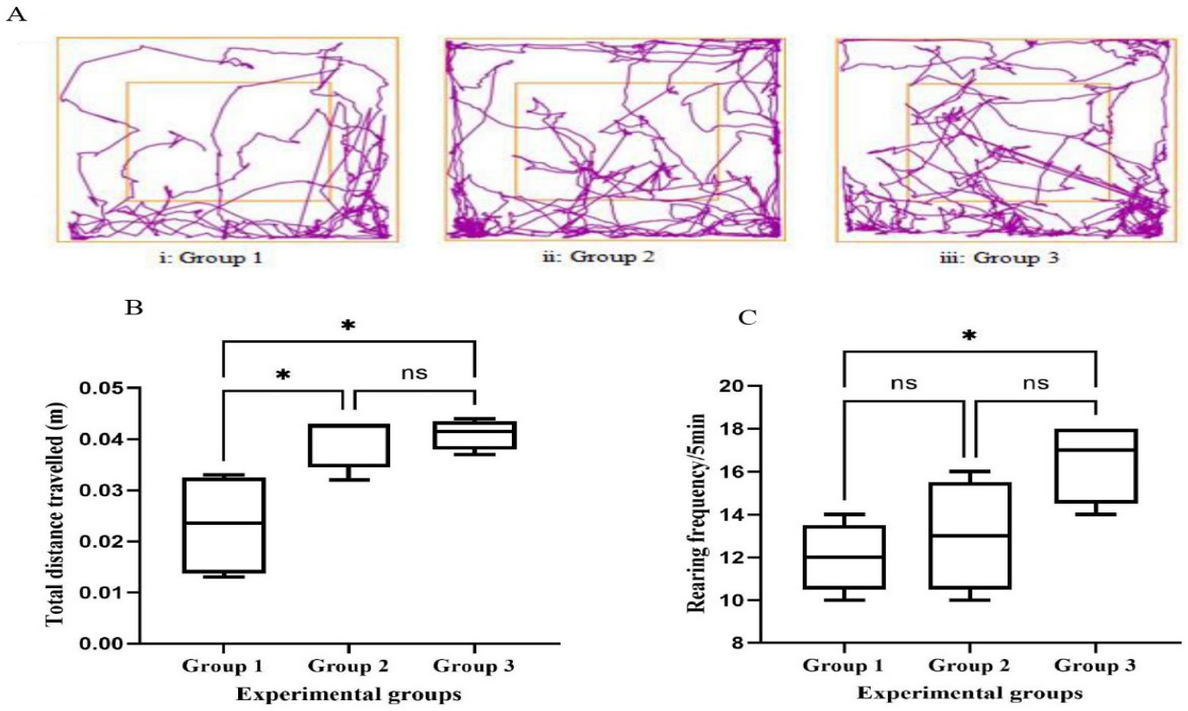

FIGURE 3. Trajectory plot (A), Mean difference in total distance traveled (B), and Mean difference in rearing frequency per 5 min (C). Values are expressed as mean±SEM; Group 1: 0.01 mL/g 0.9% N/saline; Group 2: MPTP-HCL@15 mg/kg; Group 3: MPTP-HCL@30 mg/kg) (n = 4/group). *p<0.05; ^{ns} p>0.05, using one-way ANOVA followed by Tukey's multiple comparison test

MPTP DID NOT INDUCE CATALEPSY, BRADYKINESIA, OR AFFECT MOTOR BALANCE AND MUSCLE STRENGTH IN THE MICE

In Figure 4(A), a significant reduction in the mean catalepsy score was observed in MPTP-treated mice when comparing group 1 (5.20±1.10 s) and group 2 (2.50±0.30 s) mice {F (9) = 10, p = 0.02}, with a mean difference (SEM) of 3.2 (0.96) and a 95% confidence interval (CI) of 0.53 to 5.90. Additionally, a significant difference was noted in the mean catalepsy score between group 1 (5.20±1.10 s) and group 3 (1.50±0.07 s) mice {F (9) = 10, p = 0.004}, with a mean difference (SEM) of 4.1 (0.96) and a 95% CI of 1.50 to 6.80. However, no significant dose-response relationship was observed between the 15 mg/kg and 30 mg/kg MPTP-HCL doses and the catalepsy score (p > 0.05). Similarly, there were no significant mean differences in the latency to descend in the pole test between group 1 (9.60±0.71 sec) and group 2 (11.00±1.10 s) mice {F (9) = 0.35, p = 0.710}, with a mean difference (SEM) of -1.2 (1.7) and a 95% CI of -5.90 to 3.50. Furthermore, there were no significant mean differences in the latency to descend between group 1 (9.60±0.71 s) and group 3 (9.60±0.91 s) mice {F (9) = 0.35, p = 0.990}, with a mean difference (SEM) of 0.04 (1.7) and a 95% CI of -4.70 to 4.80 (Figure 4(B)).

As illustrated in Figure 4(C), there were no statistically significant differences in the traction scores obtained from the traction test between group 1 (3.00±0.00) and group 2 (2.30±0.48) mice {F (9) = 1.5, p = 0.260}. The mean difference (SEM) was 0.7 (0.44), with a 95% confidence interval (CI) of -0.48 to 2.00. Similarly, no significant differences were observed between group 1 (3.00±0.00) and group 3 (2.30±0.25) mice {F (9) = 1.5, p = 0.260}, with a mean difference (SEM) of 0.7 (0.44) and a 95% CI of -0.48 to 2.00 (Figure 4(C)). Likewise, there were no significant mean differences in the hanging time as assessed by the hang test between group 1 (60.00±0.00 s) and group

2 (53.00±4.50 s) mice {F (9) = 2.5, p = 0.165}. The mean difference (SEM) was 7.0 (3.7), with a 95% CI of -2.90 to 18.00. Additionally, there were no significant mean differences in the hanging time between group 1 (60.00±0.00 s) and group 3 (59.00±0.75 s) mice {F (9) = 2.5, p = 0.980}. The mean difference (SEM) was 1.0 (3.7), with a 95% CI of -9.60 to 11.00 (Figure 4(D)).

HISTOPATHOLOGICAL CHANGES IN THE STRIATUM AND SUBSTANTIA NIGRA

The H & E staining in Figure 5(B) showed that the control group exhibited a higher density of viable striatal neurons with a round or oval-shaped nuclear structure (indicated by white arrowheads) (Figure 5(B)-i). In contrast, the MPTP-HCL@15 mg/kg and 30 mg/kg groups showed moderate and severe reactive gliosis, respectively, characterized by the presence of enlarged and vesicular nuclei with prominent nucleoli (indicated by black arrowhead) and eosinophilic Rosenthal fibers (indicated by yellow arrowhead) (Figure 5(B)-ii & 5(B)-iii). Histological semi-quantitative assessment demonstrated dose-dependent pathological changes compared to the control group ($p<0.05$) (Figure 5C-STR).

Similarly, the H & E staining showed that the control group had a higher density of viable neurons in the SNPc with an elliptical nuclear structure (indicated by white arrowhead) (Figure 5(B)-iv). In contrast, the MPTP-HCL@15 mg/kg group showed a reduced number of viable SNPc neurons with a few enlarged and pale eosinophilic nuclei (indicated by black arrowheads)

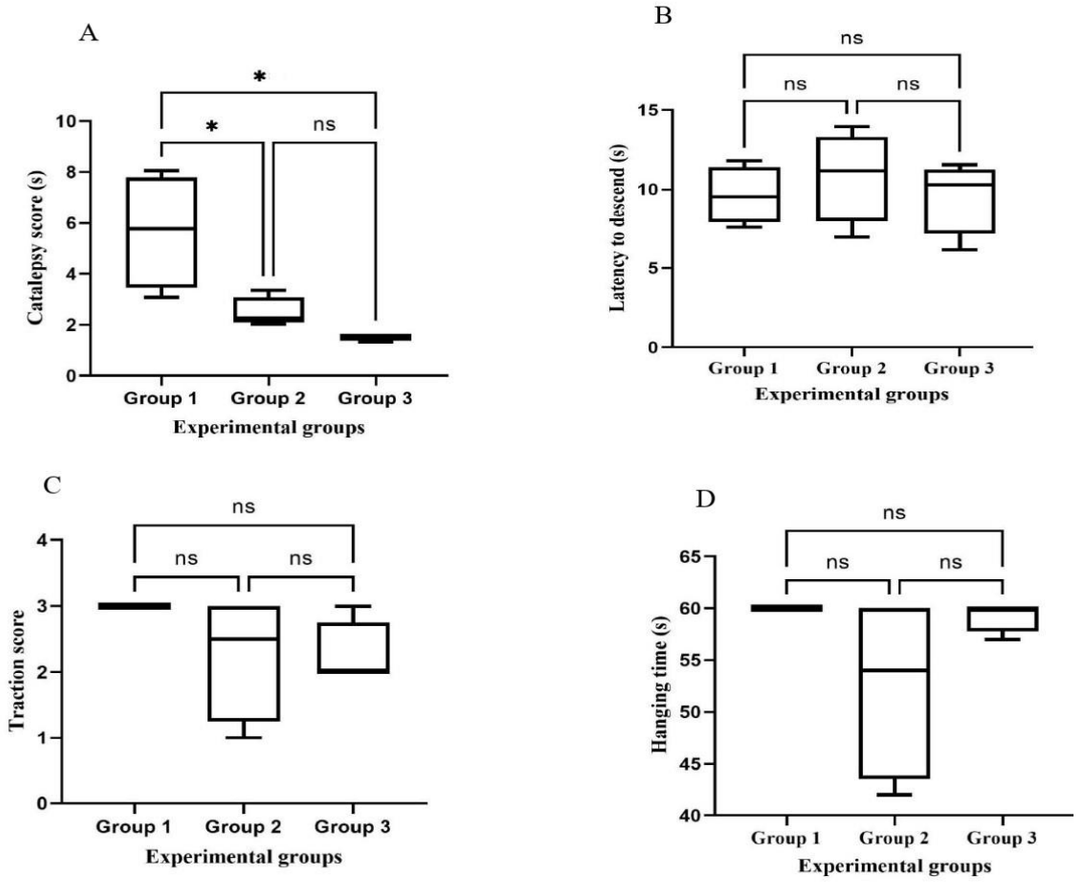

FIGURE 4. The mean difference in catalepsy score (A), latency to descend (B), traction score (C), and hanging time (D). Values are expressed as mean±SEM; Group 1: 0.01 mL/g 0.9% N/saline; Group 2: MPTP-HCL@15 mg/kg; Group 3: MPTP-HCL@30 mg/kg (n = 4/group). *$p<0.05$; ns $p>0.05$, using one-way ANOVA followed by Tukey's multiple comparison test

(Figure 5(B)-v). Additionally, the MPTP-HCL@30 mg/kg group exhibited mild reactive gliosis, characterized by the presence of enlarged and vesicular nuclei with prominent nucleoli (indicated by black arrowhead) and eosinophilic Rosenthal fibers (indicated by yellow arrowhead) in the SNPc (Figure 5(B)-vi). Histological semi-quantitative assessment demonstrated dose-dependent pathological changes compared to the control group ($p<0.05$) (Figure 5(C)-SNPc).

DISCUSSION

Behaviour refers to the net sensorimotor and integrative processes that occur in the nervous system. Behaviour can serve as a sensitive indicator of exposure, as it reflects the coordinated function of a substantial component of the brain network. In PD models, behavioural tests can be used to assess the extent of a lesion or explore potential treatment methods. While MPTP-induced lesions can vary, these tests can identify animals at high risk

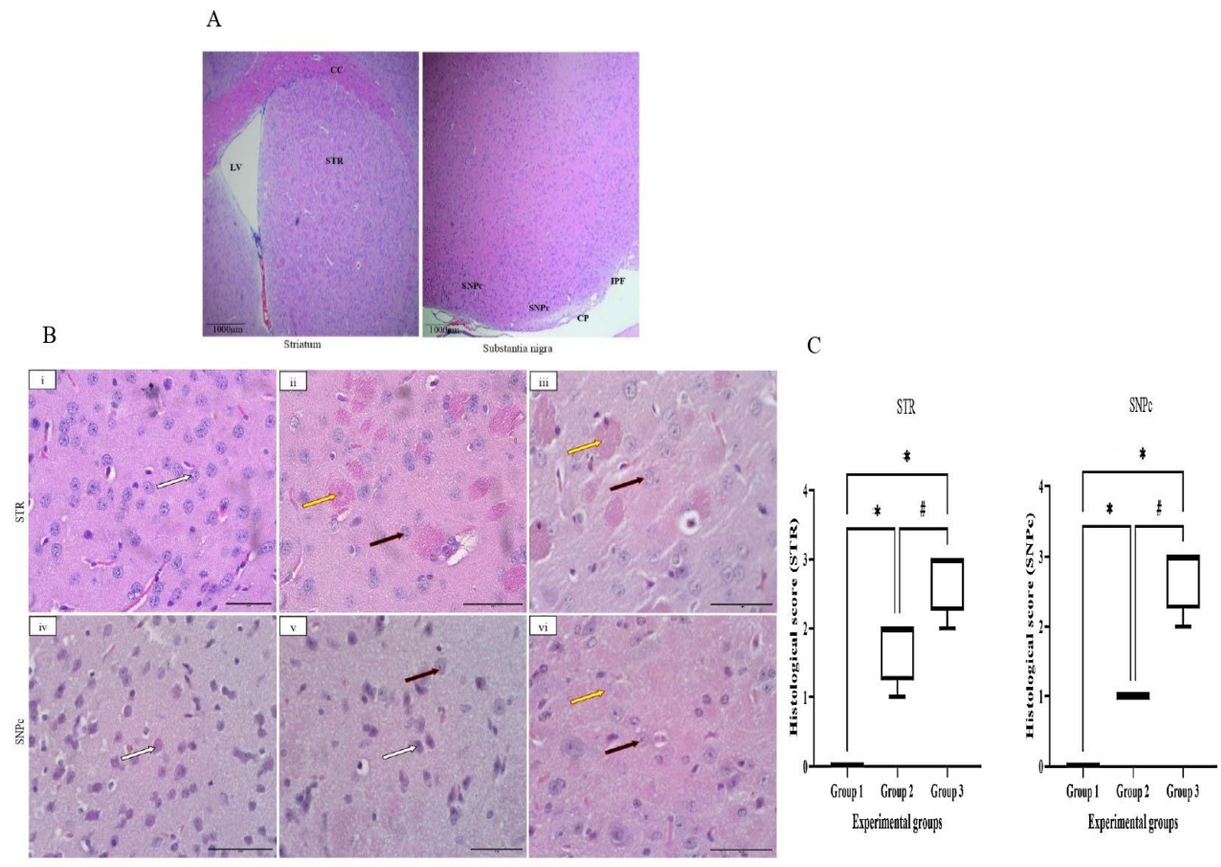

FIGURE 5. (A) Photomicrograph of the coronal section of the regional boundaries of the striatum and substantia nigra (Scale bar 1000 µm = 40× magnification) as determined by cross-referencing with atlases of Paxinos and Watson (2019). Abbreviations: STR: Striatum, CC: corpus callosum; LV: lateral ventricle; SNPc: substantia nigra pars compacta; SNPr: substantia nigra pars reticularis; CP: cerebral peduncle; IPF: interpeduncular fossa (B) Representative photomicrographs of the structural changes in Striatum and Substantia nigra pars compacta (Scale bar 100 µm = 400x magnification); i & iv: 0.001 mL/g 0.9% N/saline (Group 1) (shows normal cytoarchitecture); ii & v: MPTP-HCL@15 mg/kg (Group 2) (shows mild to moderate reactive gliosis); iii & vi: MPTP-HCL@30 mg/kg (Group 3) (shows severe reactive gliosis); White arrow: viable cell; Black arrow: non-viable cell; Yellow arrow: reactive gliosis. (C) Mean difference in the histological score (STR) and (SNPc). Values are expressed as mean±SEM; Group 1: 0.01 mL/g of 0.9% N/saline; Group 2: MPTP-HCL@15 mg/kg; Group 3: MPTP-HCL@30 mg/kg (n = 4/group). *$p<0.05$ and #$p<0.05$, using one-way ANOVA followed by Tukey's multiple comparison test

of dopaminergic neuronal degeneration and associated behavioural consequences (Brooks 2011). The present study aimed to explore the neurotoxic potential of MPTP in Balb/c mice using a subacute dosing regimen with different MPTP dosages.

Interestingly, in this study, the use of larger doses of MPTP (45 mg/kg) resulted in 100% mortality in group 4. Death following MPTP administration is likely due to the systemic actions of MPTP on the cardiovascular system (Zhang et al. 2017). However, some studies have reported substantially higher doses of MPTP (>40 mg/kg) without mentioning mortality (Campolo et al. 2017). The choice of Balb/c mouse strain, route of MPTP administration, and MPTP dosing regimen could account for the discrepancies observed in this study. Based on our findings, the lethal threshold dose for Balb/c mice in the subacute MPTP regimen is approximately 45 mg/kg MPTP, although further validation is required. Since MPTP can cross the blood-brain barrier, it is expected to cause weight loss in treated animals through various mechanisms (Jackson-Lewis & Przedborski 2007). Some of these mechanisms though, reported in human PD include impairing the homeostatic regulation of energy metabolism, damaging orexin and ghrelin-producing neurons in the lateral hypothalamus, selectively damaging dopaminergic neurons in the enteric nervous system, leading to gastrointestinal dysfunction, and disrupting hand-to-mouth coordination. Additionally, increased energy expenditure from tremors, dyskinesia, and muscular rigidity in PD can contribute to weight loss (Kistner, Lhommée & Krack 2014). This pathophysiology can be extrapolated to animals. Several studies have observed weight loss in MPTP-treated mice compared to controls (Chen et al. 2008; Zhang et al. 2017). However, in the present study, MPTP had a significant relationship with mouse weight ($p<0.05$), resulting in higher mean weight values in the MPTP-treated group compared to the control group ($p<0.05$). This finding is atypical, and possible theories to explain this observation include MPTP-induced homeostatic dysregulation of the mesolimbic dopaminergic pathway, compensatory overshoot following dopamine depletion, and altered eating behavior (Figure 6(A)). However, these findings require further validation, as no studies with similar results have been reported. The weight variation observed in other studies may be influenced by factors such as mouse strain, weight and age, MPTP dosing regimen, and calculation protocols based on free MPTP.

Motor activity, assessed through tests such as the open field, pole, catalepsy, traction, and hang tests, is a standard endpoint in evaluating MPTP neurotoxicity (Gibrat et al. 2009). The OFT is commonly used to assess general locomotor and exploratory activities. In this study, MPTP-treated mice exhibited improved locomotion and exploratory behavior compared to control mice ($p<0.05$). This finding is atypical and inconsistent with previous literature (Zhang et al. 2017). The increased total distance traveled, and rearing frequency observed in the MPTP-treated mice indicate hyperactivity, which may be associated with a probable subthreshold dopamine depletion (<60 – 70%) after SNPc lesion (Figure 6(B)) (Mann & Chesselet 2015; Zhang et al. 2017). This hyperactivity reflects the compensatory mechanisms activated during the pre-symptomatic and premotor phases of PD, which aim to postpone the emergence of clinically debilitating symptoms (Blesa et al. 2017). The hyperactivity is attributed to dysfunctional and aberrant basal ganglia output resulting from the hyperactive globus pallidus externa-subthalamic nucleus-globus pallidus interna (GPe-STN-GPi) network (Figure 6(C)). Compensatory changes in the STR, including increased dopamine production, release, and turnover, as well as changes in dopamine receptor activity, contribute to these compensatory mechanisms (Blesa et al. 2017). While the dose-response relationship between 15 mg/kg and 30 mg/kg MPTP-HCL did not show significant differences phenotypically ($p>0.05$), histological analysis showed the most profound pathological changes in the striatum and SNPc at 30 mg/kg (Figure 5).

Catalepsy, characterized by muscle rigidity and the inability to change position, is clinically significant as it resembles PD behavior. In this study, MPTP-treated mice exhibited significantly lower catalepsy scores compared to controls ($p<0.05$). The shortened cataleptic time can be attributed to compensatory hyperfunctioning of the nigrostriatal dopaminergic system (Wile et al. 2017). Notably, the pole test did not show significant bradykinesia in the treated mice ($p>0.05$). Previous studies have shown that MPTP-treated mice recover faster than saline-treated mice, possibly due to overcompensation by the striatal dopaminergic system (recovery overshoot) (Luchtman, Shao & Song 2009). The absence of bradykinesia in the pole test could be due to compensatory mechanisms 'masking' the effects of MPTP, rather than a lack of effect. The traction and hang tests, which assess balance and muscle strength, respectively, did not show significant impacts

of MPTP on motor balance and muscle strength in this study ($p>0.05$). This could be attributed to compensatory recovery overshoot resulting from MPTP-induced striatal dopaminergic lesion.

Histological examination using H&E staining showed a dose-response relationship in pathological changes, which is challenging to quantitatively correlate with perturbations in striatal dopamine levels. While dopamine, the principal pathognomonic sign of PD, was not assessed in this study, it could be inferred that the compensatory dopamine overshoot physiology following subthreshold dopamine depletion and the resultant behavioral outcomes observed in this study provides an inkling to the likely subthreshold dopamine depletion in the pathogenesis of MPTP neurotoxicity in Balb/c mice. However, the non-assessment of dopamine and other related biomarkers that may further give credence to this study's findings is acknowledged as one of the limitations of the study. It is imperative to reiterate that the present exciting findings reported in this manuscript is a part of an ongoing research to develop and optimize a suitable PD mouse model. Subsequent paper will look into some of the inadequacies of the present one. Despite the shortcomings in this paper, it has provided a hint on the likely role of Balb/c mice as a good model for studying MPTP-induced compensatory Parkinson's-like motor features.

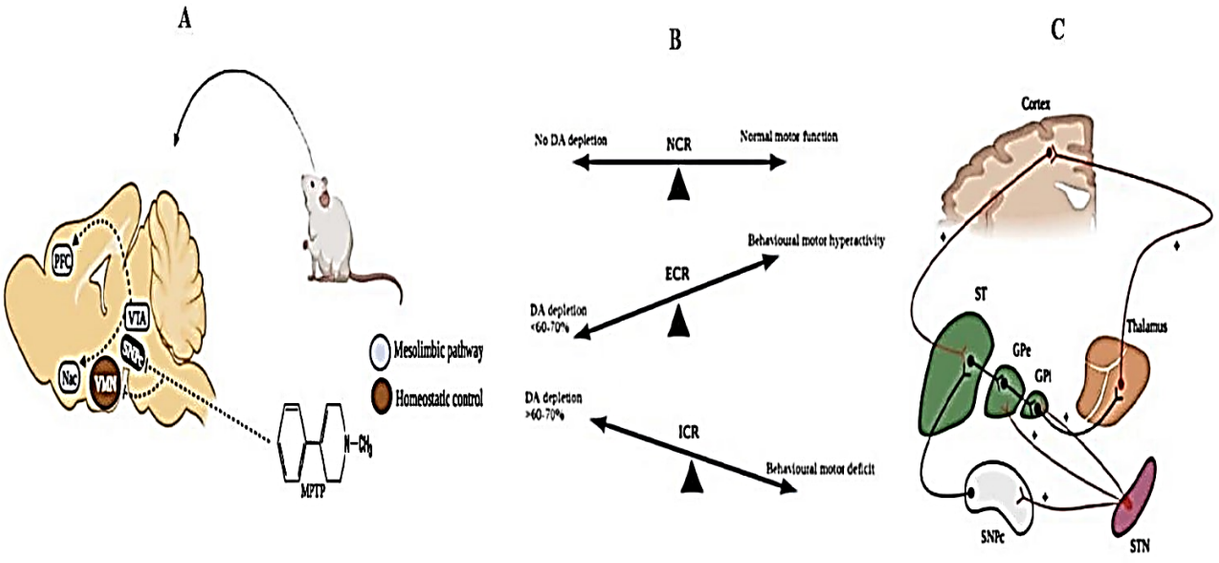

FIGURE 6. (A) Mesolimbic and homeostatic mechanisms of weight gain in MPTP mice. MPTP damages the VMN leading to hyperphagia/weight gain (homeostatic control). MPTP selectively damages the SNPc, leading to decreased striatal dopamine. As a compensatory response, dopaminergic neurons in VTA release dopamine into the Nac; subsequently, the dopamine activates the limbic system's cortical and subcortical structures, resulting in aberrant motivation to eat and weight gain (mesolimbic pathway). (B) The balance between DA depletion and compensatory reaction. (C) Compensatory striatal mechanism. Striatal dopamine loss <60-70% causes compensatory hyperactivity of STN, leading to hyperexcitation of GPe and subsequently increased inhibition of GPi, maintaining the motor circuit's output to hyperactivity. Abbreviations: SNPc: Substantia nigra pars compacta; VTA: Ventral tegmental nuclei; VMN: Ventromedial nucleus of the hypothalamus; Nac: Nucleus accumbens of the ventral striatum; PFC: Prefrontal cortex; NCR: no compensatory response; ECR: excessive compensatory response; ICR: insufficient compensatory response; STN: Subthalamic nucleus; SNPc: Substantia nigra pars compacta; ST: Striatum; GPe: Globus pallidus externa; GPi: Globus pallidus interna; +: Activate; red lines: hyperexcitation

Conclusions

The present study investigated the motor responses and associated pathological alterations induced by MPTP neurotoxicity in Balb/c mice. Surprisingly, the MPTP-treated mice demonstrated increased locomotor activity and reduced catalepsy scores, contrary to initial expectations, while other motor tests did not reveal significant deficits. The histological findings, particularly in the 30 mg/kg MPTP-HCl group, highlighted notable pathological changes, suggesting that compensatory mechanisms may play a role in mitigating motor deficits in this subacute MPTP mouse model. These findings provide valuable insights into potential compensatory mechanisms at play in PD.

Acknowledgements

We acknowledge the support provided by the Ministry of Higher Education, Malaysia through the Fundamental Research Grant Scheme (FRGS) under the project code FRGS/1/2019/STG03/UPM/02/15. This research was carried out at Universiti Putra Malaysia with the reference number 01-01-19-2117FR.

References

Ali, S.J. & Rajini, P.S. 2016. Effect of monocrotophos, an organophosphorus insecticide, on the striatal dopaminergic system in a mouse model of Parkinson's disease. *Toxicology and Industrial Health* 32(7): 1153-1165. doi.org/10.1177/0748233714547733

Bhaduri, B., Abhilash, P.L. & Alladi, P.A. 2018. Baseline striatal and nigral interneuronal protein levels in two distinct mice strains differ in accordance with their MPTP susceptibility. *Journal of Chemical Neuroanatomy* 91: 46-54. doi.org/10.1016/j.jchemneu.2018.04.005

Blesa, J. & Przedborski, S. 2014. Parkinson's disease: Animal models and dopaminergic cell vulnerability. *Frontiers in Neuroanatomy* 8: 155. doi.org/10.3389/fnana.2014.00155

Blesa, J., Trigo-Damas, I., Dileone, M., Del Rey, N.L., Hernandez, L.F. & Obeso, J.A. 2017. Compensatory mechanisms in Parkinson's disease: Circuits adaptations and role in disease modification. *Experimental Neurology* 298: 148-161. doi.org/10.1016/j.expneurol.2017.10.002

Brooks, S.P. 2011. Neurological evaluation of movement disorders in mice. In *Animal Models of Movement Disorders,* Neuromethods, vol. 61, edited by Lane, E. & Dunnett, S. New Jersey: Humana Press. pp. 65-86. doi.org/10.1007/978-1-61779-298-4_5

Campolo, M., Casili, G., Biundo, F., Crupi, R., Cordaro, M., Cuzzocrea, S. & Esposito, E. 2017. The neuroprotective effect of dimethyl fumarate in an MPTP-mouse model of Parkinson's disease: Involvement of reactive oxygen species/nuclear factor-κB/nuclear transcription factor related to NF-E2. *Antioxidants and Redox Signaling* 27(8): 453-471. doi.org/10.1089/ars.2016.6800

Chen, L., Ding, Y., Cagniard, B., Van Laar, A.D., Mortimer, A., Chi, W., Hastings, T.G., Kang, U.J. & Zhuang, X. 2008. Unregulated cytosolic dopamine causes neurodegeneration associated with oxidative stress in mice. *Journal of Neuroscience* 28(2): 425-433. doi.org/10.1523/JNEUROSCI.3602-07.2008

Garza-Ulloa, J. 2019. Update on Parkinson's disease. *American Journal of Biomedical Science and Research* 2(6): 229-236. doi.org/10.34297/AJBSR.2019.02.000614

Gibrat, C., Saint-Pierre, M., Bousquet, M., Lévesque, D., Rouillard, C. & Cicchetti, F. 2009 Differences between subacute and chronic MPTP mice models: Investigation of dopaminergic neuronal degeneration and α-synuclein inclusions. *Journal of Neurochemistry* 109(5): 1469-1482. doi.org/10.1111/j.1471-4159.2009.06072.x

Hall, C.S. 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *Journal of Comparative Psychology* 18(3): 385-403. doi.org/10.1037/h0071444

Huang, J. & Jiang, Q. 2019. Dexmedetomidine protects against neurological dysfunction in a mouse intracerebral hemorrhage model by inhibiting mitochondrial dysfunction-derived oxidative stress. *Journal of Stroke and Cerebrovascular Diseases* 28(5): 1281-1289. doi.org/10.1016/j.jstrokecerebrovasdis.2019.01.016

Hu, M., Li, F. & Wang, W. 2018. Vitexin protects dopaminergic neurons in MPTP-induced Parkinson's disease through PI3K/Akt signaling pathway. *Drug Design, Development and Therapy* 12: 565-573. doi.org/10.2147/DDDT.S156920

Hu, D., Cui, Y. & Zhang, J. 2021. Nervonic acid amends motor disorder in a mouse model of Parkinson's disease. *Translational Neuroscience* 12(1): 237-246. doi.org/10.1515/tnsci-2020-0171

Jackson-Lewis, V. & Przedborski, S. 2007. Protocol for the MPTP mouse model of Parkinson's disease. *Nature Protocols* 2(1): 141-151. doi.org/10.1038/nprot.2006.342

Jo, M.G., Ikram, M., Jo, M.H., Yoo, L., Chung, K.C., Nah, S.Y., Hwang, H., Rhim, H. & Kim, M.O. 2019. Gintonin mitigates MPTP-induced loss of nigrostriatal dopaminergic neurons and accumulation of α-synuclein via the Nrf2/HO-1 pathway. *Molecular Neurobiology* 56(1): 39-55. doi.org/10.1007/s12035-018-1020-1

Kistner, A., Lhommée, E. & Krack, P. 2014. Mechanisms of body weight fluctuations in Parkinson's disease. *Frontiers in Neurology* 5: 84. doi.org/10.3389/fneur.2014.00084

Kozina, E.A., Khaindrava, V.G., Kudrin, V.S., Kucheryanu, V.G., Klodt, P.D., Bocharov, E.V., Raevskii, K.S., Kryzhanovskii, G.N. & Ugryumov, M.V. 2011. Experimental modeling of functional deficiency of the nigrostriatal dopaminergic system in mice. *Neuroscience and Behavioral Physiology* 41(7): 671-679. doi.org/10.1007/s11055-011-9471-0

Liu, Q., Zhu, D., Jiang, P., Tang, X., Lang, Q., Yu, Q., Zhang, S., Che, Y. & Feng, X. 2019. Resveratrol synergizes with low doses of L-DOPA to improve MPTP-induced Parkinson disease in mice. *Behavioural Brain Research* 367: 10-18. doi.org/10.1016/j.bbr.2019.03.043

Luchtman, D.W., Shao, D.I. & Song, C. 2009. Behavior, neurotransmitters and inflammation in three regimens of the MPTP mouse model of Parkinson's disease. *Physiology and Behavior* 98(1-2): 130-138. doi.org/10.1016/j.physbeh.2009.04.021

Mann, A. & Chesselet, M.F. 2015. Techniques for motor assessment in rodents. In *Movement Disorders: Genetics and Models.* 2nd ed., edited by LeDoux, M.S. Massachusetts: Academic Press. pp. 139-157. doi.org/10.1016/B978-0-12-405195-9.00008-1

Mustapha, M. & Mat Taib, C.N. 2021. MPTP-induced mouse model of Parkinson's disease: A promising direction for therapeutic strategies. *Bosnian Journal of Basic Medical Sciences* 21(4): 422-433. doi.org/10.17305/bjbms.2020.5181

Nagarajan, S., Chellappan, D.R., Chinnaswamy, P. & Thulasingam, S. 2015. Ferulic acid pretreatment mitigates MPTP-induced motor impairment and histopathological alterations in C57BL/6 mice. *Pharmaceutical Biology* 53(11): 1591-1601. doi.org/10.3109/13880209.2014.993041

Obergasteiger, J., Frapporti, G., Pramstaller, P.P., Hicks, A.A. & Volta, M. 2018. A new hypothesis for Parkinson's disease pathogenesis: GTPase-p38 MAPK signaling and autophagy as convergence points of etiology and genomics. *Molecular Neurodegeneration* 13(1): 40. doi.org/10.1186/s13024-018-0273-5

Ogawa, N., Hirose, Y., Ohara, S., Ono, T. & Watanabe, Y. 1985. A simple quantitative bradykinesia test in MPTP-treated mice. *Research Communications in Chemical Pathology and Pharmacology* 50(3): 435-441.

Oliván, S., Calvo, A.C., Gasco, S., Muñoz, M.J., Zaragoza, P. & Osta, R. 2015. Time-point dependent activation of autophagy and the UPS in SOD1G93A mice skeletal muscle. *PLoS ONE* 10(8): e0134830. doi.org/10.1371/journal.pone.0134830

Paxinos, G. & Franklin, K.B. 2019. *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates*. 5th ed. Massachusetts: Academic Press.

Rai, S.N. & Singh, P. 2020. Advancement in the modelling and therapeutics of Parkinson's disease. *Journal of Chemical Neuroanatomy* 104: 101752. doi.org/10.1016/j.jchemneu.2020.101752

Sanberg, P.R., Bunsey, M.D., Giordano, M. & Norman, A.B. 1988. The catalepsy test: its ups and downs. *Behavioral Neuroscience* 102(5): 748-759. doi.org/10.1037/0735-7044.102.5.748

Shi, X., Bai, H., Wang, J., Wang, J., Huang, L., He, M., Zheng, X., Duan, Z., Chen, D., Zhang, J., Chen, X. & Wang, J. 2021. Behavioral assessment of sensory, motor, emotion, and cognition in rodent models of intracerebral hemorrhage. *Frontiers in Neurology* 12: 667221. doi.org/10.3389/fneur.2021.667511

Sun, M.F., Zhu, Y.L., Zhou, Z.L., Jia, X.B., Xu, Y.D., Yang, Q., Cui, C. & Shen, Y.Q. 2018. Neuroprotective effects of fecal microbiota transplantation on MPTP-induced Parkinson 's disease mice: Gut microbiota, glial reaction and TLR4/TNF-α signaling pathway. *Brain, Behavior, and Immunity* 70: 48-60. doi.org/10.1016/j.bbi.2018.02.005

Tatton, N.A. & Kish, S.J. 1997. *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* 77(4): 1037-1048. doi.org/10.1016/S0306-4522(96)00545-3

Voikar, V., Vasar, E. & Rauvala, H. 2004. Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: Implications for phenotyping screens. *Genes, Brain and Behavior* 3(1): 27-38. doi.org/10.1046/j.1601-183X.2003.0044.x

Wile, D.J., Agarwal, P.A., Schulzer, M., Mak, E., Dinelle, K., Shahinfard, E., Vafai, N., Hasegawa, K., Zhang, J., McKenzie, J., Neilson, N., Strongosky, A., Uitti, R.J., Guttman, M., Zabetian, C.P., Ding, Y.S., Adam, M., Aasly, J., Wszolek, Z.K., Farrer, M., Sossi, V. & Stoessl, A.J. 2017. Serotonin and dopamine transporter PET changes in the premotor phase of LRRK2 parkinsonism: Cross-sectional studies. *The Lancet Neurology* 16(5): 351-359. doi.org/10.1016/S1474-4422(17)30056-X

Zeng, X.S., Geng, W.S. & Jia, J.J. 2018. Neurotoxin-induced animal models of Parkinson disease: Pathogenic mechanism and assessment. *ASN Neuro* 10: 1759091418777438. doi.org/10.1177/1759091418777438

Zhang, Q.S., Heng, Y., Mou, Z., Huang, J.Y., Yuan, Y.H. & Chen, N.H. 2017. Reassessment of subacute MPTP-treated mice as animal model of Parkinson's disease. *Acta Pharmacologica Sinica* 38(10): 1317-1328. doi.org/10.1038/aps.2017.49

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