Executive Summary - MPTP "Acute" Model
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GENERAL OVERVIEW OF EXPERIMENTAL DESIGN

C57BL/6 male mice, 8-10 weeks of age (25-30 g), were purchased from Charles River Laboratories (Raleigh, NC). Upon arrival, all mice were quarantined and acclimated to the animal facility for approximately 1 week prior to MPTP administration. Only investigators trained in the handling of MPTP performed the dose administrations. Mice were injected intraperitoneally (ip) with a single MPTP injection (20 mg/kg calculated to the freebase) every 2 hours for a total of 4 injections over the course of 1 day (total of 80 mg/kg/day), or equivalent injections of sterile isotonic saline. A total of 50 animals were assigned to either the vehicle control or MPTP group. Of the 25 animals per group, 10 were scheduled for euthanasia at 1-week postdose (i.e., 5 for neurochemical evaluations and 5 for immunohistochemistry). The remaining 15 animals per group continued through the study for behavioral assessments, and 5 per group were selected for neurochemical and another 5 per group for immunohistochemical evaluations at study termination. The following table is a summary of the experimental design.

Table 1. Experimental Design for "Acute" MPTP Study

"Acute" MPTP Mouse Model (20 mg/kg X 4 injections at 2-hr intervals on 1 day)	
Animals	C57BL/6 males (8–10 weeks, 25–30 g on receipt).
Quarantine and Acclimation	Animals received and quarantined for at least 6 days prior to study activities. Clinical observations for general health.
Assignment to Treatment Groups	Twenty-five mice/group X 2 groups (saline or MPTP) = 50 mice.
Assignment to Experimental Endpoints within Groups	5 mice for neurochemistry and 5 mice for immunohistochemistry terminated at 1-week post-exposure 15 mice for behavioral assays, including 5 mice for neurochemistry and 5 mice for immunohistochemistry at study termination
MPTP Administration	20 mg/kg MPTP administered ip every 2 hours for a total of 4 injections (total dose 80 mg/kg/day for 1 day). Dose volume per administration was 5 ml/kg for MPTP-treated and saline controls.
Clinical Observations	Twice daily mortality/moribundity checks. Clinical observations performed within 1 hour following the completion of dose administration and daily thereafter through study completion.

"Acute" MPTP Mouse Model (20 mg/kg X 4 injections at 2-hr intervals on 1 day)	
Body Weight and Feed Consumption	Body weight and feed consumption determinations daily during first 7 days following MPTP exposure and once weekly thereafter.
Functional Observational Battery (FOB) Test Days	Quarantine (5 days after arrival) Postdose: 24 hours, 48 hours, 1 week, 2 weeks, 3 weeks, 4 weeks (7 time points total).
Rotarod and Motor Activity Test Days	Postdose: 72 hours, 1 week, 2 weeks, 3 weeks, 4 weeks (5 time points total).
Necropsy Neurochemistry (HPLC analysis of DA, DOPAC and HVA); Immunohistochemistry (TH-positive cell count and volume)	Time after final MPTP dose: 1 week; 6 weeks (behavioral animals).

Neurobehavioral Tests

Functional Observational Battery

The Functional Observational Battery (FOB) is a noninvasive testing battery used to detect gross functional deficits. FOB evaluations were conducted at 24 and 48 hours, 1, 2, 3, and 4 weeks post MPTP exposure. The procedures are conducted rapidly to assess autonomic, sensory, and motor integrity of rodents (5-10 minutes per animal), and all data are recorded manually. The temporal sequence of tests is such that the least interactive assessments occur first, and the most manipulative procedures performed last. Thus, FOB testing is always performed prior to other behavioral testing. In brief, the following are examples of FOB evaluations:

- **Brief Home-Cage Assessments:** Activity.
- **Handling Observations:** Ease of removal and handling, lacrimation, salivation, piloerection, muscle tone, exophthalmus, righting reflex, and forelimb placing.
- **Open-Field Observations:** Akinesia, rearing, convulsions, tremors, posture, gait, total gait score, alertness, activity, tail elevation, defecation, urination, writhing, bizarre behaviors, stereotypic behaviors, circling, and vocalizations (2 minutes of open-field observation).
- **Sensory and Neuromuscular Observations:** Catalepsy, approach response, startle response, and grip strength. (Note: *Hindlimb grip strength was not conducted during the high-risk period for MPTP exposure in order to decrease the chances of a bite.)*
- **Body temperature:** °C

Automated Motor Activity

For motor activity assessments, animals were placed in specially equipped residential (home-cage environment) cages that detect movement when the animal breaks photocell beams; only one beam can be broken at a time (Polycarbonate Cage Photobeam Activity System, San Diego Instruments, Inc., San Diego, CA). Motor activity assessments were conducted at 72 hours, 1, 2, 3, and 4 weeks post MPTP exposure. Each animal was tested in a 4-hour session, divided into 24 intervals of 10 minutes each. Motor activity was monitored by an automated recording device, and ambulation and fine movements over the 4-hour session were recorded on the computer. Dividing each session into 24 data collection intervals allowed for the evaluation of within-session habituation.

Rotarod

The rotarod is a test for assessing motor coordination in mice. Rotarod assessments were conducted following motor activity at 72 hours, 1, 2, 3, and 4 weeks post MPTP exposure. A mouse was placed on a rotating rod (Rotamex Rotarod, Columbus Instruments, Columbus, OH) at a continuous speed of 7 rotations per minute (rpms), and the latency to fall off the rotating rod was recorded. Latency to fall was recorded with a stopwatch, with a maximum latency of 30 seconds. The test subject was immediately given 2 more trials, and mean latencies were subject to statistical analysis.

Neurochemistry

For the animals assigned to neurochemical evaluations, the striatum was removed for analysis of dopamine (DA) and its metabolites, 3, 4-dihydroxyphenylacetic acid (DOPAC) levels and homovanillic acid (HVA). Neurochemical analyses were performed by high performance liquid chromatography using electrochemical detection (HPLC-EC).

Immunohistochemistry

Animals assigned to immunohistochemical evaluations were perfused. Whole brains were fixed in paraformaldehyde and shipped to a subcontractor (FD Neurotechnologies, Ellicot City, MD) for immunohistochemical staining. The prepared slides will be shipped to Stereology Resource Center (SRC, Chester, MD) for quantification of total number and mean neuron volume of tyrosine hydroxylase (TH) immunopositive neurons in the substantia nigra using computerized stereology analysis.

RESULTS

Functional Observational Battery (FOB):

FOB endpoints can be binary, ordinal, or continuous. Binary outcomes were analyzed using Fisher's Exact Test. For ordinal outcomes, standard nonparametric tests were conducted, and for continuous data, analysis of variance (ANOVA) was used. All data were analyzed using SAS® (Release 9, SAS Institute, Cary, NC), and the level of significance was $p \leq 0.05$. There were no significant alterations in homecage or handling observations. At 24 hours postdose, the MPTP-exposed animals showed depressed arousal, depressed activity, and decreased rearing in the open field. At 1 week postdose, parameters of activity had returned to normal. For hindlimb grip strength (kg), there was no statistically significant difference between groups at 48 hours, 1, 2, or 3 weeks postdose, but at 4 weeks postdose the hindlimb grip strength was increased among the MPTP-exposed animals relative to controls.

Automated Motor Activity

Motor activity data were analyzed using repeated measures ANOVA. Total activity counts over the 4-hour session were analyzed as well as habituation within a trial. All data were analyzed using SAS® (Release 9, SAS Institute, Cary, NC), and the level of significance was $p \leq 0.05$. For motor activity outcomes, ambulation (i.e., walking) and fine movements were recorded over the 4-hour test period. In addition to total activity counts, habituation was evaluated by analyzing the 24 10-minute intervals. Total counts (ambulation + fine movements), ambulation alone, and fine movements alone were evaluated. Overall, there were no significant treatment effects on 4-hour activity (ambulation, fine movement, or total). At each time point (72 hours, 1, 2, 3, and 4 weeks following MPTP exposure), there was either a trend toward or a statistically significant increase during the first 10-minute interval (i.e., upon introduction to a novel environment). This increase in activity (both ambulation and fine movement) was marginally significant at 72 hours (P=0.07), but statistically significant for the remaining time points, and in some cases extended beyond the first interval. Additionally during each session, there was an overall effect of time, indicating that both the control and the MPTP-exposed animals habituated to the chambers over the 4-hour test. There were no dose-by-time interactions indicating that both groups habituated at the same rate.

Rotarod

Mean latencies over the 3 consecutive trials were analyzed using repeated measures ANOVA. All data were analyzed using SAS® (Release 9, SAS Institute, Cary, NC), and the level of significance was $p \leq 0.05$. With respect to rotarod performance, there was a significant postdose time effect, demonstrating that both groups improved over time. However, there were no significant interactions, indicating that the control and MPTP-exposed animals did not differ in their ability to remain on the rotarod when tested at 72 hours, 1, 2, 3, or 4 weeks postdose.

Neurochemistry and Immunohistochemistry

The subset of animals sacrificed at 1-week postdose showed an 86% decrease in striatal dopamine and a 77% and 57% percent decrease in DOPAC and HVA levels relative to the controls, respectively. By 6 weeks postdose, there was some evidence of slight recovery, with a 79% decrease in striatal dopamine levels relative to the control value, and DOPAC and HVA levels were reduced 68% and 50% respectively. Immunohistochemistry is in progress.

SUMMARY AND CONCLUSIONS

Of the behavioral endpoints assessed (FOB, motor activity, and rotarod) at each time point, the single persistent behavioral alteration was detected in the automated motor activity assay. With respect to both ambulation and fine movements, there was a significant increase in motor activity counts (i.e., beam breaks) during initial placement into the activity chamber. With respect to FOB findings, there were no consistent observations following MPTP exposure. The findings at 24-hours postdose were transient and likely indicative of general malaise. Rotarod testing at a continuous speed of 7 rpms did not yield successful results in terms of differentiating control and MPTP-exposed animals. With regards to neurochemical outcomes, the "acute" dosing paradigm did result in substantial dopamine depletion, consistent with the published literature. Immunohistochemical analyses of TH-positive neurons in the substantia nigra are expected in the near future (3-4 months) and will hopefully confirm the neurochemical findings.

For future MPTP-based studies, and for screening potential neuroprotective compounds, the above findings lead us to conclude that the FOB and current rotarod paradigm should be removed from the battery of tests. The motor activity paradigm should remain the same; however, the test session could potentially be reduced to 2 hours as animals had habituated by 120 minutes. Other behavioral assays that require further discussion are the use of an accelerating rotarod paradigm and possibly a stride length assay. With respect to neurochemical and immunohistochemical endpoints, no changes to the current protocol are recommended.