NeuroDetective® International

a contract research network in the neurosciences

Example Report

Behavioral and Morphological Evaluation of Neuroprotection by Three Company Compounds in MPTP-receiving Mice

(DATE)

This study was conducted under the terms of a Services Agreement between NeuroDetective Inc. and Company, dated

1. Executive Summary

Purpose:	This study (1) examined the effects of 3 different compounds supplied by Company on behavior in an MPTP mouse model of Parkinson's disease; and (2) compared the ability of the same three compounds to preserve dopamine function in the nigro-striatal pathway of the same animals used in the behavioral study.
Design:	In the behavioral study, five groups of adult male C57B1/B6 mice received implants of osmotic minipumps containing a 14-day supply of either vehicle or one of three compounds supplied by Company (designated 1, 2, and 3). Three days later the animals received two behavioral assays of striatal function (Open Field test, Tail Hanging). Immediately following these tests the animals received systemic injections of a toxin known to kill dopamine neurons in the substantia nigra (MPTP). The same behavior tests were then administered 10-12 days post-MPTP (i.e., while the pumps were still active) and then again 30-40 days post-MPTP (when the pumps were inactive). These tests (Open Field, Tail Hanging) were also administered a fourth time, 50-55 days post-MPTP. A group of unoperated, untreated (normal) mice served as controls. There were 10 mice in each group. In the study of dopamine function, the brains of the same animals tested behaviorally were removed for histological examination. The brains were sectioned and stained with a tyrosine hydroxylase immunohistochemical (TH) stain. The stained sections were mounted on microscope slides and the cells stained for TH in the substantia nigra (SN) and ventral tegmental area (VTA) were counted. Numbers of TH-positive neurons in these two structures are a measure of the number of functioning dopaminergic neurons.

Results:	• In the most commonly used behavioral measure in this model (locomotor activity), both 1 and 2 but not 3 tended to attenuate MPTP-induced hyperactivity, with 1's improvement reaching statistical significance while it was still being delivered (10 Days post-MPTP). • In the behavioral measure most sensitive to loss of nigro-striatal dopamine (unsupported rearing, i.e. the ability to balance on the hindlimbs without forelimb support), 1 but not 2 or 3 significantly reduced the chronic MPTP-induced impairment in this balancing task (60 Days post-MPTP). Since this result occurred a month after the drug ceased being delivered, it implies a long-lasting protective effect. • 1 but not 2 or 3 significantly attenuated MPTP-induced impairment of forelimb extension while the compound was being administered (10 Days post-MPTP). At 30 Days and 60 Days post-MPTP, mice that had received either of the three compounds tended to be less impaired, though this chronic improvement reached statistical significance only with 1 at 30 Days post-MPTP. • There were more TH-positive neurons in the substantia nigra (SN) of animals treated with 1, 2, or 3 than in vehicle treated animals. The numbers of these dopaminergic neurons were not different among the three test compounds. All groups of animals with MPTP lesions had significantly fewer TH-positive SN neurons than unlesioned controls. • Only animals receiving 1 had significantly more TH-positive neurons in the ventral tegmental area (VTA), compared to lesioned vehicle animals. All groups of lesioned animals had significantly fewer TH-positive neurons in their VTA compared to unlesioned controls.

2. Introduction

Mice that receive systemic injections of the pyridine toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively lose significant numbers of dopaminergic neurons in two midbrain structures, the substantia nigra (SN) and the ventral tegmental area (VTA). Loss of dopamine cells in SN mimics the clinical condition in Parkinson's disease, and leads to motor dysfunction. The dopaminergic loss in mouse VTA is of unknown relevance to Parkinson's disease, but may contribute to the cognitive deficits of Parkinson's disease, because of these neurons' projections to the frontal cortex.

In this study we tested the ability of two Company compounds, 1 and 2 as well as a comparison compound, 3, to prevent the loss of dopaminergic neurons in this MPTP mouse model of Parkinson's disease, as well as to prevent the motor deficits that occur in this model.

3. Methods and Experimental Design

3.1. Design

Fifty adult male C57Bl6 mice were randomly divided into 5 groups. All were tested in the Open-Field and Tail-Hanging tests of striatal function. The Open Field test is the most frequently used behavioral assay in the MPTP mouse model, and is thought to be sensitive to loss of dopaminergic input to the striatum from the substantia nigra. The Tail Hang test is known to be sensitive to direct striatal damage.

Four of the 5 groups of mice received an infusion of 1 of the 3 test compounds or an infusion of a vehicle substance containing no compound. The infusions were administered via subcutaneously implanted osmotic mini-pumps over a 14 day period (Alzet #1002; 0.25 ul/hr flow rate). Three days following implantation of the pumps the mice were given the Open Field and Tail Hang tests, as was the group of unoperated (control) mice. Immediately following the behavioral tests, the pump-containing mice

received a 40 mg/kg injection of MPTP subcutaneously. All groups were then given the Open Field and Tail Hang tests, at 10-12 days and 30-40 days post-MPTP. The Open Field and Tail Hang tests were also given again at 50-55 days post-MPTP.

For the Open Field test, total distance traveled and total number of vertical movements (i.e. rears) were recorded, except that only number of rears was recorded at 50-55 days post-MPTP. The number of unsupported rears (i.e., using the hindlimbs only with no support from the forelimbs along the walls of the test enclosure) is the most reliable measure of dopamine neuron loss in this test, and is most evident at long post-lesion times.

For the Tail Hang test, the degree to which the forelimbs and hindlimbs were extended when the animal was suspended by the tail was rated on a 0-3 scale. The rater was blind to the experimental conditions.

3.2. Subjects

The mice were C57B1/B6 type and were obtained from Jackson Laboratories. They were 8-12 weeks old upon arrival at the NeuroDetective animal colony. They were allowed to acclimatize for 10-14 days prior to any experimental manipulations. All mice were housed in groups of 3-4 per cage and were provided with ad-libitum access to food and water. They were maintained on a 12 hr:12hr light/dark cycle. The animals were randomly assigned to five groups (3 groups received MPTP plus one of three compounds, a 4^{th} group received MPTP plus the vehicle, and the 5^{th} group were controls and did not receive either a compound or MPTP treatment).

There were initially 10 mice in each group. Following MPTP injections 9 mice died: 3 from the Vehicle group, 1 from the 1 group, 2 from the 2 group, and 3 from the 3 group. A Chi-Square analysis showed no significant difference in the mortality rates for the different groups of lesioned animals.

Through the course of behavioral testing post MPTP injection, 1 mouse from the Vehicle group and 1 mouse from the Control group died. During histological processing the tissue from some brains did not respond sufficiently to

the staining to permit accurate cell counting. Thus, the final set of neuron counting data was from 6 Vehicle, 8 1, 8 2, 6 3, and 7 Control brains.

3.3. Procedures

After the mice had been acclimated they were randomly assigned to one of the 5 groups (see above). Each mouse was then given an initial Open-Field test and a Tail Hanging test (described below). Following these tests each mouse in 4 of the groups was implanted with a mini-osmotic pump. The implants were performed under isoflurane anesthetic. The mini-pumps contained one of three compounds: 2 (3 mg/kg/day in DMSO vehicle), 1 (3 mg/kg/day in DMSO vehicle), or 3 (1 mg/kg/day); or vehicle only. The infusions were given at the rate of 0.25 ul/hr for 14 days. Three days following implantation of the pumps, and following behavioral testing (see Design), the mice received a subcutaneous injection of 40 mg/kg MPTP. Ten-twelve days following MPTP injections each mouse was tested again in the Open-Field and Tail Hanging tests (see below). These tests was repeated at 30-40 days post-MPTP, and again at 50-55 days post MPTP injections.

BEHAVIORAL TESTING:

(1) Open-Field test

Each animal is placed in a clear plastic open-field box (36cm x 36cm) with two rows of photo-beams mounted on the sides to detect and distinguish between horizontal movements (from which distance traveled is calculated) and vertical movements, i.e. rears (Digiscan Activity Meter, Omnitech Electronics). Ambient conditions include low noise and dim lighting. The animal's movements within the first 5 minutes of being placed in this box are calculated from automated recordings of the crossing of these photobeams ("beam-breaks"). On the last of the four Open Field tests, only rears were counted, and these were divided into "supported" and "unsupported" rears. In supported rears, the mouse places at least one of its forelimbs upon the side wall of the box while a rear is

recorded. In unsupported rears, there is no placement of the forelimbs on the wall and the mouse is balanced solely on its hindlimbs while a rear is recorded. Use of the forelimbs is determined from videorecordings of the test.

(2) Tail-Hanging

The mice are hung by their tails 3 times each, for approximately 10 sec each time. Each mouse is hung by the base of the tail about 30 cm above the surface of a table until the mouse turns either to the left or to the right. A left turn is given a score of 0, a right turn receives a score of 1.

While hanging during the turns test, forelimb placement is analyzed. The limbs are scored on a 4-point scale. Limbs which are properly extended out and above the head are given a score of 0. Limbs which are clasped or held against the body are given a score of 3. Scores of 1 or 2 are assigned for relative stages between the two extremes.

The hind-limbs are also analyzed while hanging in the turns test. A scale of 0 to 3 is used as per the forelimb rating test.

For all behavioral tests, the results on each measure were first analyzed using a repeated measures ANOVA followed by tests of the between groups effect using Fisher's HSD method.

NEURON COUNTING:

Between 55 and 60 days post MPTP injections, the mice were sacrificed by an overdose of Sodium Nembutal. Their brains were perfused with phosphate buffered saline followed by Lana's fixative (paraformaldehyde and picric acid). The brains were removed and placed in Lana's fixative for 7 – 10 days. The brains were then cut coronally at 50 µm using a vibratome, and the sections stained with an antibody to tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis which is also

highly concentrated in the cell body and its processes. The specific protocol was according to the Vectastain PK-6102 Mouse IgG staining kits and Vector SK-4100 DAB kit (Sigma-Aldrich). Once stained and cover-slipped the tissue was examined under a microscope at 100X magnification. Two tissue slices (−2.9 mm and −3.6 mm posterior to Bregma) were selected for cell counting in the SN and the VTA. These sections are at the midpoint of the rostral half and the midpoint of the caudal half of SN respectively. Each TH-labeled cell that was clearly visible and had between 2 and 6 neurites was considered a neuron and counted. An overall average count for each animal was calculated from the four sections (rostral and caudal, left and right). An average was obtained for the four sections. Separate analyses were performed on the neuron counts from SNR and VTA. These counts were analyzed using a repeated measures ANOVA followed by tests of the between groups effect using Fisher's LSD method (p's < .05 for statistical significance).

4. Results

4.1. Open Field test

Prior to MPTP lesioning there was no significant difference among the 5 groups in the open-field activity test in distance traveled or number of rears, although the 3 group tended to be the least active and Company compound groups the most active in overall locomotor activity (p. A1). These tendencies could reflect slight differences in overall responsiveness to the different drugs after 3 days, absent any lesion effect.

Locomotion: Following MPTP lesioning, the vehicle group was significantly more active than controls at 10 days post-MPTP ($p<0.02$) (pp. A5, A6), and this effect persisted at 30 days post-MPTP ($p<0.02$) (pp. A7, A8). Overall ANOVA, first test: $F(4,36) = 3.51$, $p<.02$; second test: $F(4,34) = 2.84$, $p<.04$. 3 treatment did not show the slightest suggestion of altering the MPTP-induced greater activity at either post-lesion time point. However Company 196923

did significantly reduce activity compared to vehicle ($p<0.008$) at 10 days post-MPTP (pp. A5, A6), to the point of not being different from control, and 2 showed a slight trend in the same direction ($p=0.17$). Neither Company compound was significantly different from vehicle at 30 days post-MPTP, though 2 tended in that direction ($p=0.13$) and in fact was not significantly different from normal (pp. A7, A8).

Total Rearing: MPTP tended to reduce the total number of times the mice made vertical rearing movements at 10 days post-lesion, but this difference (Vehicle vs. Control) just missed statistical significance ($p<0.08$) (pp. A9, A10). Overall ANOVA: $F(4,36) = 4.76$, $p < .004$. At 30 days post-lesion there was no suggestion of an MPTP effect on overall rearing (p. A11). Both Company compounds were not significantly different from Vehicle at either post-lesion time, although at 10 days post-MPTP mice receiving either Company compound did have significantly reduced overall rearing compared to Control ($p<0.0009$ for 1 and $p<0.004$ for 2), pp. A9, A10. Since there was no significant MPTP effect, these results are difficult to interpret.

Unsupported Rearing: At 50-60 days post-MPTP, which is 40-50 days after the compounds ceased being delivered, mice that had received 2 made significantly more unsupported rears than mice that had received Vehicle ($p<0.05$), which in turn made significantly fewer unsupported rears than normals ($p<0.0001$) (p. A13). Overall ANOVA: $F(4,34) = 7.75$, $p < .0003$. That is, 2 significantly attenuated the MPTP-induced reduction in unsupported rearing.

There was no significant effect of either MPTP or any treatment on supported rearing (p. A14).

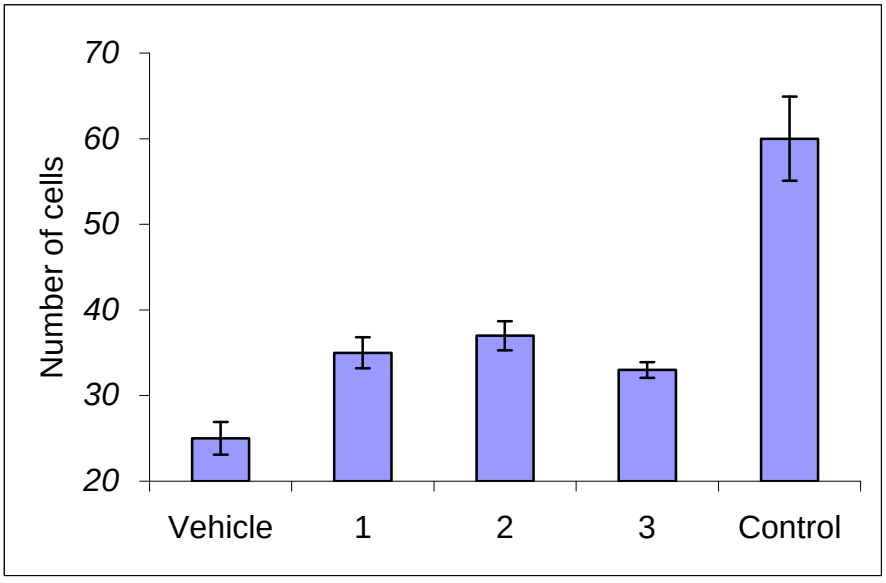
4.2. Tail-Hanging

No significant group differences were observed in the tail hang test prior to MPTP treatment, and none were observed post-MPTP in the hindlimbs. However MPTP did significantly impair forelimb extension at all three post-lesion time points: $p<0.0001$ at 10, 30 and 60 days (p. A17,

pp. A19 and A20, and p. A23 respectively). Thus there was no recovery of this impairment over time. Overall ANOVAs: $F(4,35) = 13.8$, $p < .0001$ (10 days), $F(4,34) = 16.0$, $p < .0001$ (30 days), and $F(4,35) = 16.0$, $p < .0001$ (60 days). 2 significantly reduced this impairment while the compound was being administered (10 Days post-MPTP), $p<0.05$ (p. A17), while 1 and 3 showed no such trend. However all three compounds tended to reduce the impairment when measured post-dosing, a tendency that achieved statistical significance for 1 at 30 Days post-MPTP ($p<0.05$; pp. A19, A20). It should be noted that the measure used in this test is a truncated scale (4 points) and therefore real but partial effects can be difficult to detect with smaller sample sizes.

4.4 Neuron Counts

Substantia nigra. The graph below shows the average count of tyrosine hydroxylase (TH) positive neurons in the SN for each group.

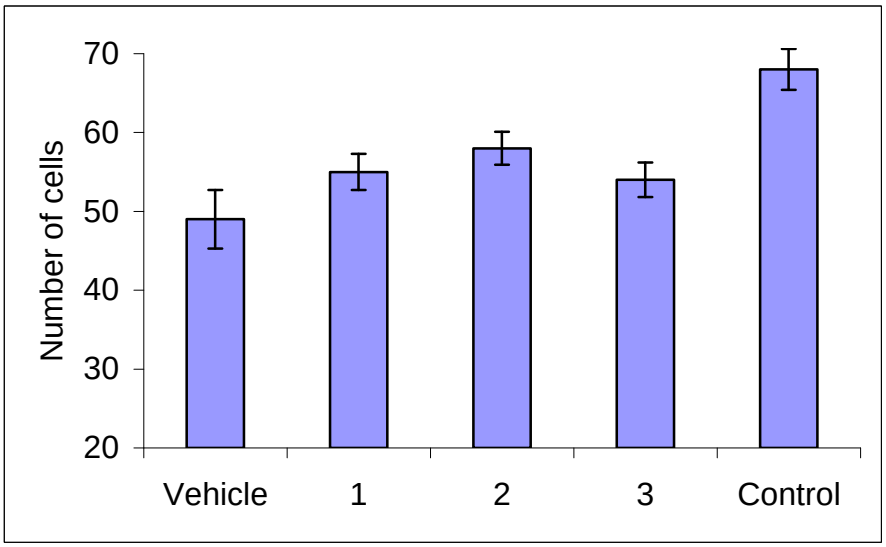


On average, groups that received systemic MPTP injections had 58% fewer TH neurons than unlesioned control mice. However lesioned mice that received one of the 3 test compounds showed significantly less of a loss, averaging a 42% reduction in TH labeled neurons. Overall

ANOVA: $F(4,29) = 24.652$, $p < 0.0001$. Post-hoc Fisher's tests showed that each test compound produced significant sparing of TH positive neurons, compared to Vehicle animals, but that the test compounds were not different from each other in this effect (see table below).

	Diff.	P-value
Vehicle vs. 3	7.950	.0411*
Vehicle vs. 1	10.229	.0064*
Vehicle vs. 2	11.317	.0029*
Vehicle vs. Control	34.783	.0001*
3 vs. 1	2.279	.5176
3 vs. 2	3.367	.3413
3 vs. Control	26.833	.0001*
1 vs. 2	1.088	.7381
1 vs. Control	25.554	.0001*
2 vs. Control	23.467	.0001*

Ventral Tegmental Area. The following graph shows the average number of TH labeled neurons found in the VTA for each group.



MPTP injections resulted in an average of 28% fewer TH neurons in the VTA (Vehicle vs. Control), a difference that

was significant (see below). The test compounds reduced this loss by only about 10%, on average. Statistical analysis of these data using repeated measures ANOVA showed an overall significant effect ($F(4,29) = 6.278$, $p < 0.0010$), but post-hoc Fisher's tests showed that only 2 treatment led to significantly less loss of TH neurons compared to vehicle (see table below).

	Diff.	P-value
Vehicle vs. 3	4.283	.2783
Vehicle vs. 1	6.083	.1042
Vehicle vs. 2	8.371	.0283*
Vehicle vs. Control	18.383	.0001*
3 vs. 1	1.800	.6233
3 vs. 2	4.087	.2689
3 vs. Control	14.100	.0011*
1 vs. 2	2.288	.5010
1 vs. Control	12.300	.0020*
2 vs. Control	10.012	.0099*