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Award Number: W81XWH-04-1-0444

TITLE: Pharmacological and Behavioral Enhancement of Neuroplasticity in the MPTP-Lesioned Mouse and Nonhuman Primate

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REPORT DATE: May 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2008	Annual	1 MAY 2007 - 30 APR 2008

4. TITLE AND SUBTITLE

Pharmacological and Behavioral Enhancement of Neuroplasticity in the MPTP-Lesioned Mouse and Nonhuman Primate

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0444

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California
Los Angeles, CA, 90033

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this proposal is to investigate the mechanisms involving pharmacological and behavioral enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. This proposal has two components. Using the MPTP C57BL/6 mouse Component One will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate Component Two will test the hypothesis that the D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses.

15. SUBJECT TERMS
MPTP, neuroplasticity, models of injury, repair, basal ganglia

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	142	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The primary focus of this research proposal is to determine the underlying mechanisms responsible for neuroplasticity in the injured adult basal ganglia. For these studies we utilize the neurotoxicant MPTP that selectively destroys nigrostriatal dopaminergic neurons and leads to the depletion of striatal dopamine as well as the development of parkinsonian features. In the squirrel monkey these features include slowness of movement, balance impairment and diminished hand dexterity. In our laboratory we utilize both the MPTP-lesioned C57BL6 mouse and the MPTP-lesioned squirrel monkey. Both models show intrinsic plasticity through either striatal dopamine return (mouse) and/or behavioral recovery (squirrel monkey). In this proposal we were particularly interested in understanding whether exercise (mouse) or dopamine replacement therapy (monkey) might enhance intrinsic neuroplasticity of the injured basal ganglia. For this purpose, the proposal was divided into two components, a mouse exercise study and a squirrel monkey dopamine replacement study. These studies were designed to be complementary in that both non-pharmacological and pharmacological effects of neuroplasticity are being investigated.

In the following sections are included the abstract, introduction and specific aims from the original proposal. This is followed by the accomplishments and research outcomes from year one. This annual report also includes manuscripts in the form of appendices.

Abstract (From the Original Application)

The purpose of this proposal is to investigate the molecular mechanisms involving pharmacological and behavioral (exercise) enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. The following proposal has two complementary components using two animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we hope to identify novel therapeutic targets for the treatment of brain injury and neurotoxic insult. Since military personnel are at risk for a wide range of brain injuries including head trauma, neurotoxic exposure (from pesticides, hostile enemy poisoning, viral and biological weapon based agents) it is imperative that medical strategies be made available to reverse the debilitating neurological deficits.

D: STATEMENT OF WORK (From the original Application)

The brain's capacity for recovery from damage is far greater than previously recognized. It is now understood that neuroplasticity can be modulated through activity-dependent processes including exercise and environmental enrichment, and through pharmacological manipulation. Most of our understanding of exercise and pharmacological enhanced neuroplasticity is derived from studies in the cortex and the hippocampus, but there is mounting evidence that the same phenomenon occurs in the injured basal ganglia. The molecular mechanisms for this phenomenon are not well understood. Using two animal models of injury induced

neuroplasticity in the basal ganglia (the MPTP-lesioned mouse and MPTP-lesioned non-human primate) we propose to examine two modes of intervention to enhance neuroplasticity. These include exercise in the MPTP-lesioned mouse model and pharmacological intervention in the MPTP-lesioned non-human primate. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions.

The following proposal has two complementary components using both animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we will identify new therapeutic targets for the treatment of traumatic brain injury and neurotoxic insult, two high-risk morbidities that are common to military personnel.

<u>Component One</u>: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Component One will utilize the following 4 treatment groups for **Study1** through **Study 4**:

(1) Saline-injected;
(2) MPTP-injected;
(3) Saline-injected + exercise;
(4) MPTP-injected + exercise.

Study 5 will utilize the following glutamate antagonists: AMPA antagonist (GYKI-52466) and the NMDA antagonist (MK-801) in the following 8 treatment groups:

(1) Saline-injected + GYKI-52466;
(2) MPTP-injected + GYKI-52466;
(3) Saline-injected + exercise + GYKI-52466;
(4) MPTP-injected + exercise + GYKI-52466;
(5) Saline-injected + MK801;
(6) MPTP-injected + MK801;
(7) Saline-injected + exercise + MK801;
(8) MPTP-injected + exercise +MK801.

Exercise will be performed on a motorized rodent treadmill. Brain tissue will be collected after 30 days of running.

Study 1: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

Study 2: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, *in situ* hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

Study 3: The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.

Study 4: The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and *in situ* hybridization histochemistry.

Study 5: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist*. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

Component Two: To test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-lesioned non-human primate through its effect on dopamine (biosynthesis, uptake, and receptor expression) and glutamatergic synapses.

Component Two will utilize the following treatment groups (n = 4 per group):

(1) Saline-injected harvested at 6 weeks after the last injection;
(2) Saline-injected harvested at 16 weeks after the last injection;
(3) MPTP-injected harvested at 6 weeks after the last injection;
(4) MPTP-injected harvested at 16 weeks after the last injection;
(5) Saline-injected + pramipexole harvested at 6 weeks after the last injection;
(6) Saline-injected + pramipexole harvested at 16 weeks after the last injection;
(7) MPTP-injected + pramipexole harvested at 6 weeks after the last injection;
(8) MPTP-injected + pramipexole harvested at 16 weeks after the last injection.

Study 1: The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.

Study 2: The pattern of expression of proteins and mRNA transcripts important for dopaminergic function, (including TH, DAT, VMAT2) at the level of the SNpc and CPu will be determined. Preliminary data supports our ability to carry out western immunoblotting, immunocytochemistry and *in situ* hybridization in the MPTP-lesioned non-human primate.

Study 3: The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using *in situ* hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.

Study 4: The effect of pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.

At the conclusion of these studies we will have a better understanding on the role of exercise and dopamine agonist (pramipexole) treatment in enhancing neuroplasticity of the injured basal ganglia in the mouse and the non-human primate. This may then identify important therapeutic targets (through glutamate and dopamine) for the treatment of brain injury.

Table 1: Timeline of Experimental Design for Component One (Exercise in the MPTP-Lesioned Mouse Model).

Component One: Exercise in the MPTP-lesioned Mouse				
	Year 1	**Year 2**	**Year 3**	**Year 4**
Study 1: Analysis of Dopamine and its metabolites	HPLC			
Study 2: Analysis of TH, DAT, CREB, and DARPP-32	Immunocytochemistry, In Situ Hybridization, Western Immunoblotting			
Study 3: Analysis of striatal glutamate synapses		Immuno-electron microscopy		
Study 4: Analysis of NMDA and AMPA receptor subtypes		Immunocytochemistry, In Situ Hybridization, Western Immunoblotting		
Study 5: Attenuate neuroplasticity with NMDA and AMPA receptor antagonists			Immunocytochemistry, In Situ Hybridization, Western Immunoblotting, Immuno-electron microscopy	

<u>Component 2</u>: <u>Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.</u>

Time Line:

Table 2: Timeline of Experimental Design for Component Two (MPTP-Lesioned Primate Model).

Specific Aim	Year 1	Year 2	Year 3	Year 4
	Lesion animals and administer Pramipexole			
Study 1: Behavior	Behavioral analysis			
Study 2: Molecular		TH, DAT, VMAT mRNA and protein using WIB, ICC and ISH		
Study 3: Dopamine Receptors		Dopamine Receptor D1, D2, and D3 using WIB, ICC, and ISH		
Study 4: Glutamate		Analysis of glutamatergic synapses using immuno-EM		

Key Research Accomplishments for Years One Through Three

Component One: Enhancement of neuroplasticity in the MPTP-lesioned mouse

(i) Intensive treadmill exercise leads to improved motor performance of both MPTP-lesioned and saline treated mice. Specifically exercised animals run faster and for a longer duration. This is published in Fisher et al 2004 and Petzinger et al 2007.

(ii) Analysis of behavior using the accelerating rotarod as a second measure shows that both saline and MPTP-lesioned mice demonstrate that these mice display increased level of motor learning compared to animals that have not gone through intensive treadmill exercise. This is published in Petzinger et al 2007.

(iii) Intensive treadmill exercise suppresses the intrinsic return of striatal dopamine transporter (DAT) protein. On further analysis tyrosine hydroxylase protein does not appear to be significantly altered by exercise in the MPTP-lesioned mouse. This is published in Fisher et al 2004 and Petzinger et al 2007.

(iv) Intensive treadmill exercise suppresses the expression of dopamine transporter mRNA transcripts in both saline + exercise and MPTP + exercise mice. On further analysis there does not appear to be a significant reduction for the tyrosine hydroxylase mRNA transcript after exercise.

(v) Intensive treadmill exercise causes a normalization of synaptic glutamate to levels seen in non-lesioned mice without exercise. This is published in Fisher et al 2004.

(vi) The administration of AMPA and NMDA receptor antagonists altered the pattern of expression of tyrosine hydroxylase and dopamine transporter mRNA transcription in nigrostriatal dopaminergic neurons as well as the pattern of expression of striatal tyrosine hydroxylase.

(vii) Electrophysiological analysis of dopamine release using fast-cyclic voltammetry indicates increased release of dopamine in the MPTP-lesioned mouse undergoing intensive treadmill exercise compared to MPTP-lesioned or saline. This is published in Petzinger et al 2007.

(vii) Intensive treadmill running increases the D2 receptor mRNA transcript expression but does not alter the expression of D1 receptor subtype. This is published in Fisher et al 2004.

(viii) There is no alteration in the number of SNpc neurons with exercise in either MPTP-lesioned or saline treated mice. This is published in Petzinger et al 2007.

(ix) Immunohistochemical staining with antibody probes against GluR1 and GluR2 and their phosphorylated state shows exercise dependent alterations in the degree and pattern of expression. Results are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled "Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury".

(x) Analysis of mRNA transcripts for the AMPA receptors GluR1 through GluR4 (including their flip and flop isoforms) and the NMDA receptors NR1, NR2A through 2D has been carried out. Results indicate differential expression of some subunits in response to exercise or MPTP-lesioning. Some of these data are presented in this report and are also are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled "Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury".

(xi) Electrophysiological analysis of the relative contribution of AMPA and NMDA receptors in the conductance of currents within the striatum show that there are changes that are exercise dependent. Alterations in LTP and

LTD and the ratio of AMPA to NMDA receptor expression with exercise in the MPTP-lesioned mouse model will be completed in year 4 of this proposal.

(xii) Golgi staining shows some differences in the density of dendritic spines but we are unable to differentiate between the direct population of striatal projection neurons (dopamine receptor D1 containing) and the indirect population (expressing the dopamine receptor D2) using this technique. We have initiated an alternative approach to delineate these populations and analyze the density of dendritic spines with exercise in both saline and MPTP-lesioned mice.

Component Two: Enhancement of neuroplasticity in the MPTP-lesioned nonhuman primate.

(i) The administration of the dopamine agonist Pramipexole induces dyskinesia. This occurs to a lesser degree than that observed with Sinemet.

(ii) Pramipexole and Sinemet increases dopamine levels in both the MPTP-lesioned mouse and MPTP-lesioned squirrel monkey. These changes are most evident in the ventral striatum.

(ii) The addition of microdialysis studies supports the neurochemical HPLC analysis. Specifically treated animals that have undergone repeated microdialysis studies demonstrate an increase in amphetamine induced dopamine release after termination of either Sinemet or Pramipexole treatment.

(iv) Western blot analysis demonstrates a slight increase in TH and DAT protein expression in the dorsal caudate in Pramipexole-treated animals, but no change in DAT expression. Studies examining subregions of the striatum for changes in TH, DAT, DARPP-32, and VMAT protein expression both by western and immunocytochemistry techniques show altered patterns of expression in animals receiving dopamine replacement therapy.

(v) Neurophysiological studies show no difference in dopamine release using fast-scan cyclic voltammetry. Methodological differences between microdialysis and voltammetry may explain why no increase in dopamine was observed in the drug treated groups using voltammetry.

(vi) Neurophysiological studies have demonstrated a low AMPA to NMDA ratio in normal medium spiny neurons of the nonhuman primates. After MPTP lesioning this ratio appears to diverge into two distinct AMPA/NMDA ratio characteristics. In general the population of medium spiny cells appear to diverge either to increase AMPA to NMDA ratio and another medium spiny cell type decreases AMPA to NMDA ration. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery.Also, that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. These neurophysiological studies have been added as an important and informative deviation from the original proposal outline.

(vii) MPTP-lesioned animals demonstrate increased glutamate terminal density relative to saline treated animals. After treatment with either Sinemet or Pramipexole, there is reduced glutamate terminal density occupancy. This finding would support but not confirm that drug treatment facilitates the release of glutamate within corticostriatal terminals. Given the presence of dyskinesia in these treated animals this finding may also support the hypothesis that increased dyskinesia may be in part related to glutamate release.

Key Research Accomplishments for Year Three

One of the key aims of this proposal is to elucidate the mechanisms responsible for improved motor behavior in the MPTP-lesioned mouse model of basal ganglia injury. In our previous report (Fisher et al 2004) studies using immuno-electron microscopy showed that there were alterations in the synaptic occupancy of glutamate such that the MPTP-induced increase is normalized by intensive treadmill exercise. In addition, ongoing electrophysiological studies examining the changes in glutamate receptor expression through pharmacological analysis in *in vitro* slice culture have indicated alterations in the ration of AMPA to NMDA receptor composition of medium spiny neurons as well as a shift in the subunit specific makeup of receptors. To examine the basis of this shift in subunit composition we have carried out an analysis of the pattern of expression of two key AMPA receptor subunits GluR1 and GluR2. The analysis of these subunits is also influenced by our recent findings showing that there is a shift between long-term potentiation and long-term depression with exercise in the MPTP-lesioned mouse undergoing intensive treadmill exercise. The following section describes alterations in the pattern of expression of AMPA receptor subunits GluR1 and GluR2 mRNA transcripts and their flip and flop isoforms using quantitative real-time PCR as well as their protein expression patterns including phosphorylated states using immunohistochemical immuno-staining. These analyses focused on the dorsolateral striatum, a region responsible for motor control.

Results

Treadmill Running Behavior

The time course of improvement in running velocity of both the saline+exercise and MPTP+exercise groups over the 6 weeks (28 days) of treadmill running is shown in Figure 1. Saline+exercise mice in the first week of treadmill running started at a velocity of 14 ± 1.4 m/min that further increased to 22.6 ± 0.3 m/min by the final week. The MPTP+exercise group had a running velocity of 9.2 ± 1.1 m/min during the first week that further increased to 20.5 ± 0.7 m/min in the last week. As shown in our previous papers, there was a significant difference in velocity at week 1 between the saline+exercise and MPTP+exercise groups and this difference was not significant at the completion of the treadmill running regimen [Fisher, 2004; Petzinger 2007).

HPLC Analysis of Striatal Dopamine

HPLC analysis was used to determine levels of striatal dopamine, its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and the metabolites turnover ratio, defined as [(DOPAC + HVA)/dopamine]. These data are shown in Table 2. To determine the degree of dopamine depletion by MPTP-lesioning, a subset of non-exercised mice from the saline and MPTP-lesioned groups was analyzed 10 days post-lesioning. At the 10-day time point, the MPTP (48.0 ± 8.4 ng dopamine/mg protein) mice showed significantly lower levels of striatal dopamine compared to the saline group (269.5 ± 24.9 ng dopamine/mg protein) ($p < 0.05$), which represented an 83% depletion. Analysis of dopamine turnover ratio showed a significant elevation in the MPTP group (turnover ratio = 2.3), at the 10-day time point, compared to the saline group (turnover ratio = 0.3) ($p < 0.05$).

HPLC analysis of striatal dopamine at the completion of the 28 days of treadmill running (42 days post-MPTP lesioning) showed that dopamine levels remained significantly depleted in MPTP-lesioned mice compared to saline controls ($F = 229.3$, $p < 0.0001$). There was no significant difference in striatal dopamine levels comparing MPTP+exercise with MPTP sedentary mice. There was a significant effect of exercise on the saline treated group, where saline+exercise mice had a higher level of striatal dopamine compared to saline mice ($F = 7.78$, $p = 0.015$). There were no significant effects of MPTP or exercise, or interaction between these two factors on turnover ratio, with the ratios of MPTP = 0.36, MPTP+exercise = 0.34, saline = 0.26, and saline+exercise group = 0.34.

Analysis of AMPA Receptor Subunits GluR1 and GluR2 Expression

The pattern of expression of mRNA transcripts and proteins for the AMPA receptor subunits GluR1 and GluR2 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) and

immunohistochemical staining, respectively. The design of primer sets for qRT-PCR also allowed for the analysis of the common isoforms of both GluR1 and GluR2 due to alternative transcript splicing, termed flip and flop. In addition, immunohistochemical staining for AMPA-R subunits was determined using antibodies that recognized either the pan or phosphorylated forms of GluR1 and GluR2.

Receptor Subunit GluR1

Immunohistochemical staining of GluR1 subunit expression using a pan-specific antibody shown in Figure 2 showed that the total number of immunoreactive-positive cells decreased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice, but did not quite reach statistical significance ($F = 4.852$; $p = 0.0587$). There was no significant effect with exercise ($F = 0.04$; $p = 0.846$) and no interaction ($F = 0.063$; $p = 0.809$); specifically, there were no significant differences between saline and saline+exercise groups or the MPTP and MPTP+exercise groups. Analysis of GluR1-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.599$; $p = 0.242$) and between the exercise and non-exercise groups ($F = 1.247$; $p = 0.297$), and there was no interaction ($F = 0.905$; $p = 0.369$).

Immunohistochemical staining for the phosphorylated form of GluR1 was carried out using an antibody that recognizes GuR1~phospho-Ser845 (see Figure 3). There were no significant differences seen in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 0.918$; $p = 0.352$) nor between the exercise and non-exercise groups ($F = 1.493$; $p = 0.239$), and there was no interaction ($F = 0.113$; $p = 0.742$). Analysis of GuR1~phospho-Ser845 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.0$; $p = 0.329$) and between the exercise and non-exercise groups ($F = 0.999$; $p = 0.329$), and there was no interaction ($F = 1.0$; $p = 0.329$).

There was an apparent difference in the intensity of cell body immuno-staining between the different treatment groups within the dorsolateral striatum. Optical density measurements of cell body immunoreactivity captured at high magnification showed no significant difference between MPTP-lesioned mice compared to saline mice ($F = 0.029$; $p = 0.866$), and no significant difference between exercise and non-exercise mice ($F = 1.564$; $p = 0.226$). There was a significant interaction between treatment and exercise ($F = 6.728$; $p = 0.017$), such that exercise led to decreased expression of GluR1~phospho-Ser 845 immunoreactivity within cell bodies of saline treated mice and an increase in expression in cell bodies of MPTP-lesioned mice.

Analysis of mRNA transcript for the pan-GluR1 within the dorsolateral striatum (see Figure 4A) showed that there was a significant decrease in the expression of GluR1 in MPTP-lesioned compared with saline treated mice ($F = 444.0$; $p < 0.0001$) and a significant decrease in the expression of GluR1 in exercise compared to non-exercise mice ($F = 159.0$; $p < 0.0001$). In addition there was a significant interaction between treatment and exercise ($F = 135.9$; $p < 0.0001$), such that exercise led to a decrease expression of GluR1 in the saline group.

Analysis of mRNA transcript for the flip (see Figure 4B) and flop (see Figure 4C) isoforms of GluR1 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR1-flip in the exercise compared to non-exercise groups ($F = 52.05$; $p < 0.0001$). There was no significant effect of MPTP-lesioning on GluR1-flip expression ($F = 0.640$; $p = 0.447$) and no interaction between exercise and MPTP-lesioning ($F = 0.0002$; $p = 0.989$). With GluR1-flop there was a significant decrease in the MPTP-lesioned group compared to saline ($F = 103.3$; $p < 0.0001$). There was no significant effect of exercise on GluR1-flop expression ($F = 3.646$; $p = 0.093$). There was a slight trend for an interaction between exercise and MPTP-lesioning ($F = 3.979$, $p = 0.081$), due to an increased expression in GluR1-flop with exercise in the saline group.

Receptor Subunit GluR2

Immunohistochemical staining of GluR2 subunit expression using a pan-specific antibody shown in Figure 5 showed that the total number of immunoreactive-positive cells increased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice ($F = 10.370$; $p = 0.012$). There was no significant effect with exercise ($F = 1.133$; $p = 0.318$). There was a trend towards significance in the interaction between

exercise and MPTP-lesioning ($F = 4.083$; $p = 0.078$). Specifically, we observed an increase in the expression of GluR2-immuno-reactivity in the MPTP+exercise mice compared to the MPTP non-exercise mice. Analysis of GluR2-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 0.358$; $p = 0.566$) and between the exercise and non-exercise groups ($F = 0.178$; $p = 0.684$), and there was no interaction ($F = 0.564$; $p = 0.474$).

In addition to an increase in the number of immunoreactive cells, we also observed morphological changes (degree of arborization) in cells expressing GluR2 (See Figure 5). Specifically, there was a decrease in the arborization of GluR2 immunoreactive cell bodies in MPTP-lesioned compared with saline mice. Interestingly, we observed a dramatic increase in arborization in saline + exercise compared with saline non-exercise mice.

Immunohistochemical staining for the phosphorylated form of GluR2 was carried out using an antibody that recognizes GuR2~phospho-Ser880 (see Figure 6). There was no significant difference in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 2.136$; $p = 0.182$). There was a significant effect with exercise ($F = 19.22$; $p < 0.002$) and there was a significant interaction between exercise and MPTP-lesioning ($F = 5.805$; $p < 0.043$). Specifically, we observed an increase in the expression of immuno-reactivity in the saline+exercise mice compared to the saline non-exercise mice. Analysis of GuR2~phospho-Ser880 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 2.345$; $p = 0.164$) and between the exercise and non-exercise groups ($F = 0.461$; $p = 0.516$), and there was no interaction ($F = 2.245$; $p = 0.172$). In addition to an increase in the number of immunoreactive cells, we also observed changes in the pattern of immunoreactivity within cell bodies expressing GuR2~phospho-Ser880 between the exercise and non-exercise groups (See Figure 6). The non-exercise group showed more homogeneous staining and the exercise group showed predominant staining in the perimeter of the cell body.

Analysis of mRNA transcript for the pan-GluR2 within the dorsolateral striatum (see Figure 7A) showed that there was a significant decrease in the expression of pan-GluR2 in MPTP-lesioned compared with saline treated mice ($F = 14.83$; $p < 0.005$) and a significant decrease in the expression of pan-GluR2 in exercise compared to non-exercise mice ($F = 9.180$; $p < 0.016$). There was no significant interaction between treatment and exercise ($F = 1.224$; $p = 0.301$).

Analysis of mRNA transcript for the GluR2-flip (see Figure 7B) and GluR2-flop (see Figure 7C) isoforms of GluR2 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR2-flip in the exercise compared to non-exercise groups ($F = 35.90$; $p < 0.0003$). There was also a significant decrease in the expression of GluR2-flip in the MPTP-lesioned group compared to the saline group ($F = 22.47$; $p < 0.0015$). There was no interaction between exercise and MPTP-lesioning ($F = 1.542$; $p = 0.25$). With GluR2-flop there was a significant increase in the MPTP-lesioned group compared to saline ($F = 55.71$; $p < 0.0001$). There was no significant effect of exercise on GluR2-flop expression ($F = 0.04$; $p = 0.843$) and no interaction between exercise and MPTP-lesioning ($F = 2.935$; $p = 0.125$).

DARPP-32

Immunohistochemical staining of DARPP-32 expression was carried out using antibodies that recognize either the pan-specific isoforms (see Figure 8) or the phospho-Thr75 isoforms (see Figure 9). Analysis of DARPP-32 with a pan-specific antibody demonstrated that there was no significant change in the number of immuno-positive cells in MPTP-lesioned group compared to the saline group ($F = 1.334$; $p = .286$). There was a decrease in animals that underwent intensive treadmill exercise compared to non-exercised animals (see Figure 8). This change, however, did not reach statistical significance ($F = 2.47$; $p = 0.16$). There was no significant interaction between exercise and MPTP-lesioning ($F = 0.002$; $p = 0.97$). Further analysis using the antibody specific for the phospho-Thr75 isoform of DARPP-32 demonstrated no significant change in the number of immuno-positive cells in the MPTP-lesioned group compared to saline animals ($F = 0.315$; $p = 0.590$), no significant change in the exercise compared to the non-exercise group ($F = 0.167$; $p = 0.693$), and no significant interaction ($F = 1.599$; $p = 0.242$).

Key Research Accomplishments for Year Four

Overview:

The overall goal of this NETRP grant is to understand to role of experience (exercise) and dopamine replacement therapy on neuroplasticity of the injured basal ganglia utilizing the MPTP-lesioned mouse and MPTP-lesioned nonhuman primate models of PD. Examining the effects of exercise on the MPTP-lesioned mouse, we have focused on investigating the role of AMPA-R subtype of glutamate receptors in experience dependent plasticity of the injured basal ganglia. The AMPA-R is responsible for fast excitatory neurotransmission in the CNS and is critically involved in leaning and encoding such as occurs with long-term depression (LTD) and long-term potentiation (LTP) New electrophysiological data support alterations in the AMPA-R, and specifically an increase in GluR2 expression with exercise. In addition, we are investigating whether changes in AMPA-R are specific to certain cells of the striatum. Using a transgenic mouse cell line that aids us to identify medium spiny neurons within the striatum that are primarily projecting to the direct pathway (D1 dopamine receptor expressing) or to the indirect pathway (D2 dopamine receptor expressing), we are beginning molecular and neurophysiological studies to explore whether changes in AMPA are specific to either pathway. We are also beginning to explore downstream effector molecules that may be modulated by both dopamine and glutamate through either of their respective receptors. In addition, we have also begun to validate our findings of an exercise -induced increase of the dopamine D2 receptor by utilizing PET imaging and a novel D2 receptor ligand, called F18-Fallypride. We have begun to translate our exercise findings in the MPTP-lesioned mouse to a study examining the effects of high intensity treadmill exercise in individuals with PD. This has already led to the publication of one manuscript showing that intense exercise can improve motor function in individuals with PD and lead to changes in cortical excitability. We are currently undergoing a PET study using F18-Fallypride to examine whether high intensity treadmill exercise leads to an increase in dopamine D2 receptor expression in individuals with PD. Exercise induced increase in dopamine D2 receptor may allow for compensation in the presence of low levels of dopamine, as occurs in PD. Given the link between neuroplasticity, the glutamatergic system, and morphology, we have also initiated a study to examine exercise-induced alterations in dendritic spine density. Additional studies carried out this year was a study comparing voluntary to forced exercise in the MPTP-lesioned mouse to begin to examine the question of stress and exercise, and finally a study beginning to examine mood and cognitive features with neurochemical correlates in the MPTP-lesioned mouse. The later study will allow us to begin to address the role of exercise in mood and cognition in the MPTP mouse model of basal ganglia injury.

In the MPTP-lesioned nonhuman primate our focus has been to understand the role of dopamine replacement therapy on the neuroplasticity of the striatum, and to examine for regional differences in both dorsal and ventral striatum. Our data suggests that dopamine replacement therapy leads to a modest increase in dopamine levels (HPLC and microdialysis) and proteins important for dopamine biosynthesis and storage. These subtle changes appear to be most evident in the ventral striatum where dopamine terminals are characteristically less affected by the MPTP-induced injury. Behaviorally these animals, as predicted, develop pronounced dyskinesia with either dopamine agonists or L-dopa, and so this modest benefit in restoring dopamine within the ventral striatum must be weighed against this disabling drug-induced motor complication. While the majority of the western blotting has been completed to examine these proteins of interest, our justification for the no-cost extension is to allow us to complete their analysis and prepare a manuscript for publication. Given our findings that the AMPA-R subtype of glutamate appears to be important in experience dependent plasticity of the injured basal ganglia, we are examining alterations in the AMPA-R after dopamine replacement in the MPTP-lesioned nonhuman primate. Our previous EM studies support changes in glutamatergic neurotransmission, as measured by glutamate terminal immunolabeling, in the MPTP-lesioned NHP treated with dopamine replacement therapy. Most of the westerns examining changes in AMPA-R subunits are completed but the additional no-cost extension will allow us to complete the analysis of the study. Our previous year three and during year four neurophysiological studies in the MPTP-lesioned nonhuman primate have supported changes in the AMPA-R and GABA during the intrinsic recovery process of the striatum. This neurophysiological study has provided new insight regarding long-term alterations in glutamatergic neurotransmission during the intrinsic recovery period of the injured basal ganglia.

Details: MPTP-lesioned mouse

(1) **Additional supportive evidence for the AMPA receptor (especially the GluR2 subunit) in experience-dependent plasticity specifically the role of intensive treadmill exercise in the injured basal ganglia**: In year three we reported that high intensity treadmill exercise in the MPTP-lesioned mouse led to an increase in GluR2, and GluR2 phosphoserine882 expression in the MSNs of the striatum using immunocytochemistry. No exercise -induced detectable changes were noted in the GluR1 subunit. In year 4 we have conducted neurophysiological studies within the MSNs of the striatum to support our molecular findings. Specifically, neurophysiological studies using whole cell voltage clamp demonstrated that treadmill exercise leads to both a reduction of the size of the Excitatory Post-Synaptic Currents (EPSCs) in the medium spiny neurons (input-output curves) and a loss of polyamine sensitive inward rectification, both supportive of a GluR2 increase. Within the final phase of this grant we are also preparing to examine the exercise-induced alterations in the relative contribution of AMPA and NMDA in the MSNs of the striatum. ($p < 0.004$) In the top Panel, A: Example of EPSCs evoked from dorsolateral corticostriatal synapses in brain slices take from MPTP alone and MPTP + exercise mice. EPSCs were produced by the stimulation intensities shown in the x-axis of B. B: plot of input (stimulation intensity)- output (EPSC amplitude) for corticostriatal synapses from MPTP treated mice. C: Bar graph of slope calculated for the input-output relationship for corticostriatal synapses as shown in B. In the lower panel, A: Example of EPSCs evoked in dorsolateral striatal neurons at holding potentials of -80 and +60 mV from MPTP alone and MPTP + exercise mice (stimulus intensity =80uA). B: Current-voltage plots of synaptic currents evoked for the cells illustrated in A. Synaptic currents were normalized to the peak synaptic current evoked at -80mV for ease of presentation. C: Measurement of rectification in current-voltage relationship, or the rectification index(RI) (ratio of synaptic conductance at +60mV vs -80 mV). Synapses from MPTP mice demonstrated a significantly lower RI (more rectification) versus MPTP + Exercise mice. ($p < 0.03$)

(2) Development of technique for the preparation of enrichment for synaptic proteins called synaptoneurosomes:

Analysis of the pattern of protein expression of the AMPA receptor subunits GluR1, GluR2, and their phosphorylated states was carried out using immunohistochemistry approaches in fixed tissue sections. Our analysis was able to report relative differences between treatment groups (i) saline, (ii) saline + exercise, (iii) MPTP, and (iv) MPTP + exercise. Many of these findings were reported in the Year 3 Annual Report as well as the manuscript from our lab that is included as a pdf in the Appendix of this Year 4 Annual Report.

While we examined protein changes in cell bodies expression, the primary role of AMPA receptors in regulating the glutamatergic properties of a cell is at the level of the synapse. Therefore our next series of experiments will entail examining the expression of these proteins within synaptic contacts of the striatum. The technique we employed involves the enrichment of synapses, termed synaptoneurosomes, in which homogenized tissue of interest is passed through a series of micro-grids thus removing cell bodies and their cytoplasm. A Figure outlining this methodology is presented. Proteins prepared for synaptoneurosomes were subjected to western immunoblots to determine the relative degree of expression of proteins of interest. At the time of the writing of this report we are engaged in examining the relative degree of expression of the AMPA receptor subunits GluR1, GluR2 and their phosphorylated states to determine if changes observed using ICC are also seen with synaptoneurosome preparations. Differences between ICC and western immunoblotting may reflect site of occupancy of these proteins (receptors) to either the synapses or cytoplasm.

Synaptoneurosome Preparations from Striatal Tissue Method: Synaptoneurosomes are prepared from by the method of Johnson et al. 1997 and also used by others (Banko et al. 2004,Villasana et al. 2006) with slight modifications. This procedure is rapid and gentle requiring about 40 minutes. Brain tissue is homogenized with

a Teflon-homogenizer (4 strokes at 1000 rpm) in buffer (1/10wt/vol), containing 0.35M sucrose pH7.4, 10mM HEPES, 1mM EDTA, 0.25mM dithiothreitol, 30U/ml RNAse inhibitor and a protease inhibitor cocktail (Roche, Inc). Cell debris and nuclei are removed by centrifugation at 1000g for 10 min at 4°C yielding pellet P1 and supernatant S1. The S1 fraction is passed through a series of 4 nylon mesh screens with decreasing pore size finishing with passage through a 5-micron screen. The final filtrate is resuspended in 3 volumes of buffer without sucrose for mRNA extraction, or appropriate buffer for subsequent assays, and centrifuged at 2000g, for 15 minutes at 4°C. For detection of nuclear contamination, smears of synaptoneurosome pellets are air-dried onto microscope slides then fixed in ice-cold acetone for 5 minutes. The pellet is assayed for protein content and either snap-frozen or suspended in incubation buffer. These preps are then utilized in the western immunoblot and co-IP techniques to detect proteins of interest.

(3) Analysis of the pattern of expression of the dopamine D2 receptor using in vivo PET-imaging:

Using a novel D2 receptor ligand, called F18-Fallypride we have been able to examine exercise-induced alterations in the dopamine D2 receptor. Preliminary studies in PET suggest that exercise leads to an increase in the D2 receptor, and that these exercise effects appear to be specific to the MPTP-lesioned mouse as these changes are not observed in exercise + saline animals.

(4) Proof of principle for analysis of dopamine D2 receptor binding potential in patients with Parkinson's disease:
We are beginning to translate findings from the MPTP + exercise study to a human study examining exercise induced alterations in the dopamine D2 receptor in individuals with PD. The impact of this translational study is enormous because it allows us to test the hypothesis that exercise leads to compensatory changes in the dopamine signal in patients with PD, and thus may facilitate a form of disease modification.

(5) Analysis of the transcription factor CREB:

Within medium spiny neurons the transcription factor CREB and its activated state phospho-CREB can act as mediators of glutamatergic and dopaminergic neurotransmission. This factor is able to regulated a wide spectrum of target genes and may be one means by which experience-dependent plasticity influence the recovery of motor behavior seen in our model. To examine the pattern of expression of CREB and phospho-CREB in our model of basal ganglia injury and exercise we examined the expression of these proteins in the dorsolateral striatum of mice from all groups including (i) saline, (ii) saline + exercise, (iii) MPTP-lesioned, and (iv) MPTP + exercise. After 30 days of intensive treadmill exercise, mice were perfusion fixed and brain tissue sections through the mid-striatum prepared for immunohistochemical analysis. Our analysis of the number of medium spiny neurons and the intensity of protein expression indicate elevated expression of phospho-CREB in the striatum of MPTP + exercise mice. Studies will be completed in the last phase of this study using western immunoblot analysis to determine the relative pattern of expression between the four groups of mice. This Figure is a representative analysis of expression of the phosphorylated form of CREB highlighting the dorsal striatum. The bottom panels are a higher magnification of images from MPTP-lesioned (left bottom panel) and MPTP + exercise (right bottom panel) showing increased expression in large neurons within the dorsolateral striatum.

(6) Acquisition of the BAC-D2-eGFP protein:

The changes in the dopamine D2 receptor leads us to investigate whether the changes in this receptor are localized to either the direct (predominantly D1 receptor containing) or indirect (predominantly D2 receptor containing) pathways of the striatum. To address this issue we have obtained a transgenic mouse strain in

which the enhanced green fluorescent protein (eGFP) is under the control of the D2 promoter. This transgenic strain, termed BAC-D2-eGFP, allows us to directly perform electrophysiological studies as we have conducted as outlined in this report. This strain was obtained from the laboratory of Michael Levine (UCLA) during the 4th year of this grant. At the writing of this report we have begun to breed these transgenic mice in order to have sufficient numbers for studies, have characterized PCR-based screening methods for genotyping mice in our colony, have verified the these mice express eGFP and therefore display green florescence in striatal neurons that can then be subjected to electrophysiological studies, and we have administered MPTP using our standard regimen of 4 injections of MPTP i.p. of 20 mg/kg free-base. These mice are susceptible to MPTP as assessed through measurement of striatal dopamine, TH-immunoreactivity, and cell loss in the substantia nigra pars compact based on unbiased stereological counting techniques. The aim of studies is to now use this strain for molecular, morphological, and electrophysiological studies to localize the changes we observe in both dopamine D2 receptor and the GluR2 AMPA receptor to the indirect pathway. It is possible that changes in these receptors may occur on other anatomical sites within the striatum including the direct pathway, pre-synaptic sites (nigrostriatal or corticostriatal), or interneurons. The Figure illustrates some of these points the left panels highlight the striatal pathways and below the cartoon the BAC-D2-eGFP (indirect pathway) and BAC-D1-eGFP (direct pathways) are illustrated (images from Gerfen 2008). The right top panel shows GFP cells in a striatum from a mouse in our colony and the bottom panel is an image depicting a striatal neuron and the juxtaposition of electrode for recordings.

(7) Analysis of dendritic spine density:

Our findings with elevated expression of both the dopamine D2 receptor and GluR2 AMPA receptor suggests that such changes may be accompanied by changes in the morphology of dendritic spines in medium spiny neurons. To investigate this potential relationship between intensive treadmill exercise and recovery of motor behavior we are examining the density and morphology of dendritic spines on striatal neurons. Brain tissues from mice in groups from saline and MPTP with and without exercise were prepared for Golgi impregnation. In conjunction with computer assisted image analysis using the program Bioquant and an Olympus BX-51 microscope with motorized stage we are now carrying out an extensive analysis of medium spiny neuron morphology. While we are able to detect some differences between MPTP-lesioning and exercise we anticipate completion of this phase during the no-cost extension period of this grant by examining more mice from all groups and the analysis of the type of spine based on its morphology. As an alternative approach we are planning to investigate whether the BAC-D2-eGFP mice, where expression of GFP in striatal neurons of the indirect pathway may be utilized with confocal microscopy to analyze spine density. This possibility will be explored in the last phase of this grant.

(8) Examination of affective behavior in the MPTP-lesioned mouse model:

A key question in the studies in this grant has been the effects of intensive treadmill exercise on the recovery of motor behavior. While our exercise regimen leads to enhanced recovery of motor features we though an important corollary of these studies was to determine if MPTP-lesioning and exercise have effects on non-motor features including affective behaviors (anxiety and depression), associative memory, and fear conditioning. This is important in light of the fact that patients suffering from PD also display affective behavioral changes, which in themselves can be very debilitating, and that these features are thought to be due to dysfunction in both dopamine and serotonin neurotransmission. Therefore, a major question is whether the MPTP-lesioned mouse model of basal ganglia injury also displays non-motor behavioral features that can be detected. For these studies, mice were administered saline or MPTP (4 injections of 20 mg/kg free-base, 2

hour intervals) and subjected to a battery of behavioral tests at both 7 and 30 days after MPTP-lesioning that included associative memory (social transmission of food preference), fear-conditioning, anxiety (light-dark preference, and hole board), and depression (tail suspension and sucrose preference). Our overall conclusion is that the MPTP-lesioning regimen that we use in 8 to 10 week old C57BL/6 mice manifests deficits in impairment of associative memory, increased extinction of fear-conditioning, but no detectable increase in anxiety and depression. While this model serves as an excellent means to study dopamine depletion and basal ganglia function the precise parameters underlying some of the non-motor features as seen in PD have yet to be established.

A copy of this manuscript in pdf form is included in the Appendix of this report. This manuscript is the re-submission in reply to the initial review in the journal *Neurobiology of Disease*.

(9) Comparison of forced (intensive treadmill) exercise with voluntary home-cage wheel running:

Studies in this grant have focused on intensive treadmill exercise as a means to enhance the recovery of motor behavior features. While we have not examined the precise role of stress in our exercise paradigm that employs running on a motorized treadmill the question arises as to whether voluntary running on a running-wheel in the home cage is different from the results we observe. Initial results indicate based on thymus weighs as an indicator of stress that there is no difference in stress levels between forced treadmill exercise and voluntary running-wheel at the completion of a 30 day exercise program. CORT levels are now being analyzed to verify this finding.

Details: MPTP-lesioned Nonhuman Primate

Conclusions

1. Vesicular dopamine release, measured via cyclic voltammetry, demonstrates that striatal dopamine signal is significantly decreased in the MPTP treated squirrel monkey compared to saline control even at one year. Animals display full behavioral recovery at one year despite the low dopamine levels within the putamen. (figure 2)

2. Parkinsonian-like squirrel monkeys display excessive corticostriatal excitation at 8 weeks post MPTP treatment as demonstrated by input/output curves (fig. 3). However, cortical drive (as determined by input/output data) and behavior returns to normal by one year post MPTP.

3. Medial to lateral differences in short and long-term plasticity exist at corticoputamen synapses of the squirrel monkey. One year post-MPTP lateral synapses show enhanced LTD in spite of the lack of return of dopamine. This property may reflect an increase in the sensitivity and expression of D2 receptors laterally.(figures 4, and 5)

4.Increased $GABA_A$ receptor mediated inhibition is seen in squirrel monkeys treated with MPTP one year earlier as compared to animals treated with MPTP 8 weeks earlier (figure 6)

5. No differences were seen between control, saline injected, 8 week post MPTP and one year post MPTP synapses in the $EPSP_{NMDA}/EPSC_{AMPA}$ ratio. (figure 7). There was a trend toward greater NR2B subunit expression in animals treated with MPTP 8 weeks earlier.

6. MPTP treated animals showed greater sensitivity to GYKI than saline treated animals. This increased sensitivity may be due to either an increase in the absolute number of AMPA receptors and/or alterations in AMPA-R subunit composition. (figure 8)

(1) Electrophysiological Studies in the nonhuman Primate

Fig. 5. Time course of tetanus-induced plasticity at corticoputamen synapses obtained from individual neurons from the squirrel monkey. The data illustrates the medial trend of LTP and the lateral trend of LTD. Interestingly, block of D2 receptors with sulpiride enables the expression of LTP in the lateral putamen in spite of the marked decline in putamen dopamine observed one year after MPTP (see Fig. 2). A possible explanation for this effect of D2 receptor block in the MPTP treated animals is a compensatory increase in the activity of putamen D2 receptors when dopamine is depleted

Reportable Outcomes

Component 1: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.

Study 1*: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.*

This Aim has been completed. The findings are described in our recent publication Petzinger et al 2007 in *Journal of Neuroscience*. This manuscript is included in the Appendix of this report. Briefly, with exercise there is no enhanced return of striatal dopamine in the MPTP-lesioned mouse but rather we observed increased release of dopamine in surviving nigrostriatal dopaminergic projections with exercise. This analysis was made using fast-scan cyclic voltammetry.

There are no deviations from Study 1. However, to strengthen and further understand this phenomenon of alterations in dopamine with exercise, we have added two important studies: (i) fast-cyclic voltammetry; and (ii) accelerated mouse rotarod. The fast-scan cyclic voltammetry allowed us to discover that with exercise there is enhanced dopamine release in the MPTP-lesioned mouse model. These findings are described in Petzinger et al 2007, included in the Appendix of this report. The addition of the rotarod analysis of motor behavior allowed us to determine that there is in fact enhanced motor learning in the MPTP-lesioned mouse subjected to intensive treadmill exercise. These findings are also described in Petzinger et al 2007.

As a complement to extend these studies we have been developing a means to examine changes in dopamine neurotransmission in our model of exercise-enhanced motor behavior recovery. Since we observed changes in the dopamine D2 receptor using molecular techniques we wanted to know if similar changes could be detected in vivo in the mouse brain. Using PET-imaging with a novel dopamine D2-specific ligand 18F-fallypride we have shown that mice undergoing intensive treadmill exercise do in fact display increased expression of D2 as determined by increased binding potential for this PET imaging ligand.

Study 2*: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, in situ hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice*.

This aim is near completion. We have completed the analysis of TH and DAT proteins using western immunoblotting and immunohistochemical staining, and the analysis of their mRNA transcripts using in situ hybridization histochemistry. These findings are described in Fisher et al 2004 and Petzinger et al 2007, both included in the Appendix of this report. The analysis of DARPP-32 and its phosphorylated isoform at Thr75 is completed has been presented in abstract form and will be included in a manuscript to be prepared in the upcoming period. CREB and phospho-CREB have been partially completed in Year 4 and will be completed in the no-cost extension period.

There were no deviations from this aim.

Study 3: *The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy*.

This aim has been completed. Results from this study using immuno-electron microscopy with an antibody against glutamate are presented in the published manuscript Fisher et al 2004 included as an Appendix in this report.

There were no deviations from this aim.

Study 4: *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry*.

The first phase of this aim, analyzing the pattern of expression of the AMPA receptor subunits GluR1 and GluR2 using qRT-PCR and immunohistochemical staining within the dorsolateral striatum, has been completed. This analysis also included determination of the pattern of expression of the phosphorylated forms of these protein subunits as well as the expression of the flip and flop mRNA isoforms due to alternative splicing. These data are included in this report and are the basis of a manuscript vanLeeuwen et al 2008, included in the Appendix of this Report. We have also used qRT-PCR for the analysis of AMPA receptor subunits GluR2 and GluR3 as well as the NMDA receptor subunits NR1, NR2A through NR2D. We anticipate these findings to be included in a manuscript in the near future.

We have deviated from this aim with the addition of Golgi staining to determine potential alterations in the pattern of dendritic spine density. Our initial analysis included quantification of dendritic spine density within the dorsolateral striatum of mice from all four groups including (i) saline control, (ii) saline+exercise, (iii) MPTP-lesioned, and (iv) MPTP+exercise. The rationale for this approach is the fact that dendritic spine density is influenced by glutamatergic neurotransmission. We do observe MPTP-dependent changes comparing saline and MPTP-lesioned groups. We suspect that there are differences in the pattern of expression on D1-containing (direct path) and D2-containing (indirect path) medium spiny neurons but their discovery are masked by our inability to delineate between these striatal projection pathways using this approach since Golgi stain occurs in a subset of all neurons. Since we observe changes in dopamine receptor D2 with exercise (as outlined in Fisher et al 2004) we hypothesize that changes in dendritic spine density may be localized to this pathway. Therefore, we have obtained and have been breeding a transgenic strain of mice expressing green fluorescent protein in or D2-dependent striatal projection populations. This transgenic strain will play an important role in addressing the issue of morphological changes in specific striatal neuron populations.

We have deviated from this aim with the addition of quantitative real-time PCR using an Eppendorf realplex thermocycler recently acquired by our lab. This approach will allow for the analysis of mRNA transcripts to complement in situ studies and protein expression in our lesioning and exercise paradigm. Findings from this approach are included in this report and will be part of an upcoming manuscript to be submitted for publication. An advantage of this approach is that smaller amounts of starting tissue are required and the relative concentration in expression can be determined with high precision.

Findings that examine changes in the pattern of expression of AMPA receptor subunits GluR1 and GluR2 and their phosphorylated states have been submitted as a manuscript for publication. A copy in pdf form is included as an Appendix to this report.

Study 5*: <u>We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist</u>. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning*.

The initial phase of this aim has been completed. We have administered both an AMPA receptor antagonist (GYKI-52466) and a NMDA receptor antagonist (MK-801) to mice administered either saline or MPTP. Findings suggest that the administration of glutamate antagonist attenuates the recovery of the

nigrostriatal dopaminergic system following MPTP-lesioning. The next phase is to subject mice to these same glutamate antagonists while they are undergoing intensive treadmill exercise. At this point we intend to deviate from the initial experimental design in our approach but should reach the same scientific conclusions. In collaboration with Dr. John Wash (USC Andrus Gerontology Center) we have pursued the analysis of the glutamatergic contribution to exercise-enhanced neuroplasticity in striatal slice cultures in our model of lesioning and exercise. Findings using fast-scan cyclic voltammetry show altered dopamine release in MPTP-lesioned mice subjected to intensive treadmill exercise have been included as an important part of our manuscript Petzinger et al 2007 in the Journal of Neuroscience (included in the Appendix). Studies currently underway include the examination of changes in the ration of AMPA to NMDA currents within the dorsolateral striatum, analysis of long-tern potentiation and long-term depression with exercise, as well as pharmacological examination of glutamatergic currents using subunit specific antagonists to delineate changes in channel composition in both NMDA and AMPA receptors. Studies examining changes in the AMPA to NMDA receptor currents with exercise are to be included in an upcoming manuscript.

Studies have been carried out using immuno-electron microscopy with an antibody against glutamate showing no significant alteration in glutamate synaptic occupancy in mice administered the AMPA receptor antagonists GYKI-52466 or the NMDA receptor antagonists MK-801.

An objective of this Study is to better understand the relative contribution of the AMPA and NMDA receptors in the striatum following MPTP-lesioning and to determine if intensive treadmill exercise leads to meaningful changes that may underlie recovery of motor behavior. As a slight deviation from this Aim, which still addresses the core issues, has been the utilization of electrophysiological approaches to examine alterations in glutamate neurotransmission. As included in our manuscripts (Petzinger et al, 2007; vanLeeuwen et al, 2008) electrophysiological studies have supported our molecular findings that intensive treadmill exercise leads to increased expression of GluR2 (a subunit that leads to reduced calcium influx when incorporated into heteromeric glutamate channels) and changes in rectification consistent with this. Overall, the electrophysiological approaches we have employed allow us to better understand meaningful changes in the composition of glutamate receptors within the basal ganglia in our model of experience-dependent neuroplasticity. While pharmacological approaches selectively blocking AMPA or NMDA channels are one means to investigate glutamate receptor changes we have found that an approach using electrophysiological techniques has provide additional information that links molecular and physiological changes that may underlie the motor behavior recovery in our model.

Additional studies to be completed in the final phase of this grant include:

(i) Quantification of dendritic spine density in the BAC-D2-eGFP transgenic mouse.

(ii) Electrophysiological studies to determine changes in the ratio of AMPA to NMDA channels.

Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.

Study 1: *The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of Pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.*

An additional phase of this aim was to characterize the behavioral, morphological, and neurochemical neuroplasticity (recovery) in the MPTP-lesioned nonhuman primate without pharmacological treatment. The analysis of TH, DAT, midbrain dopaminergic neurons, motor behavior, and levels of CPu dopamine were evaluated in squirrel monkeys administered MPTP in a series of 6 or 2 injections (subcutaneous, 2 mg/kg free-base, 2 weeks between injections) over several months. Six injections corresponded to a moderate parkinsonian state and two injections to a mild parkinsonian state. These findings are described in detail in a recently published manuscript Petzinger et al, 2006, which is included in the appendix of this report. One interesting finding of this study was that the return of normal motor behavior at 9 months in MPTP-lesioned squirrel monkeys rendered moderately parkinsonian, was accompanied by an incomplete return of striatal dopamine. Specifically we observed a greater than 90% level of total dopamine depletion in the dorsolateral putamen at 9 months when animals were fully recovered. While it has been reported that pre-synaptic adaptations in remaining dopaminergic neurons and terminals are thought to lead to normalization of extracellular levels of dopamine especially in animals with lesions resulting in less than 80% depletion, it has also been shown that animals with greater than 80% depletion show only partial normalization of extracellular striatal dopamine (Castaneda 1990). We carried out voltammetry studies in a subset of moderately parkinsonian animals at 9 months post-MPTP lesioning, to examine changes within the nigrostriatal terminals. We observed that the dopaminergic terminals in the dorsolateral striatum remained substantially depleted (> 90%) (see Study 4 below) along with the total levels of striatal dopamine. As other investigators have reported, however, we observed was a greater degree of total dopamine return in the ventral striatum and a normalization of dopamine turnover. Tyrosine hydroxylase and DAT expression was increased in late stage recovery even in dopamine depleted regions and supports a role for sprouting. We also observed an increase in DARPP-32 expression within medium spiny neurons of recovered animals, which supports the role of post-synaptic compensatory changes as an underlying mechanism of this recovery.

The main component of aim 1 was to investigate the long-term effect of dopamine replacement therapy on the behavioral recovery of the MPTP-lesioned squirrel monkey. For study 1 we treated MPTP-nonhuman primates with either Pramipexole or L-DOPA/ Carbidopa for 4 weeks (3 days/week, twice daily, Tues-Thurs) and then washed out for 3 weeks. A baseline evaluation was performed on week 1, animals were treated with dopamine replacement therapy from week 2-5, and then animals were washed out weeks 6-8. We added a Sinemet (L-DOPA/ Carbidopa) group for comparison with Pramipexole based on the scientific rationale that L-dopa, unlike Pramipexole, is metabolized and stored by dopaminergic terminals and therefore may have a more direct effect on the regulation of endogenous dopamine production and behavioral recovery and offers an interesting comparison to a compound that is not taken up by terminals.

One important outcome in Study 1 was the unexpected induction of dyskinesia in the MPTP-lesioned animals administered Pramipexole. This new finding has not been reported in the literature by other investigators and we are preparing a manuscript reporting this novel finding (Figure 8). This may indicate that Sinemet and Pramipexole may both induce dyskinesias through similar mechanisms.

The behavioral assessment in this first group of animals carried out up to 8 weeks after the last injection of MPTP showed a slight enhancement of behavioral recovery in both the Sinemet and Pramipexole groups versus the saline treated group (Figure 9).

Nonhuman primates that were initially to be used for the long-term behavioral studies, were used for (1) adding a L-dopa group (2) conducting microdialysis studies to extend our findings in Study 2 (neurochemistry); and (3) to examine alterations in electrophysiolgical properties of corticostriatal neurons and voltammetry. See Sections below.

Study 2*: Analysis of brain tissue from MPTP-lesioned squirrel monkeys administered Pramipexole or L-dopa/carbidopa or saline. This analysis included neurochemistry and molecular studies that examined the pattern of expression of proteins and mRNA transcripts important for dopaminergic function (including TH, DAT, VMAT2) at the level of the SNpc and CPu.*

Studies under this aim included (1) HPLC analysis of dopamine and its metabolites in the dorsal and ventral caudate nucleus and putamen, (2) Microdialysis of dopamine in the putamen, and (3) western immunoblot analysis of CPu proteins including TH, DAT, VMAT-2, DARPP-32, DARPP-32~phosphoThr34, and DARPP-32~phosphoThr75. Immunohistochemical staining for these same proteins are currently underway and their analysis is not yet completed. The following section highlights our current findings using these techniques for Study 2.

(1) Total dopamine levels and metabolites were analyzed from all groups of animals, using HPLC. Brains were removed from all groups at completion of 4 weeks of drug or saline treatment, followed by 3 weeks of drug washout. Microdialysis of the putamen was added to this study to complement and support findings from out HPLC analysis of tissue. We found there was a slight increase in dopamine levels in the ventral caudate and putamen of animals receiving Pramipexole or Sinemet. These results are shown in Figure 10.

Figure 10: Analysis of dopamine and its metabolites in the MPTP-lesioned squirrel monkey. Squirrel monkeys were MPTP-lesioned and then treated one week after the last injection of MPTP with either Sinemet (10 mg/kg twice daily), or Pramipexole (1 mg/kg twice daily). Animals were treated for four weeks. On each week animals received drug for three days (Tue, Wed, Thurs)) and then saline for four days (Fri, Sat, Sun, Mon). Animals were rated each day for parkinsonian features and for dyskinesia. Drug was washed out for 3 weeks and then animals were euthanized. Brain tissue was collected and striatal tissue dissected 8 weeks (1 week monitoring + 4 weeks drug treatment + 3 weeks washout) after MPTP. HPLC analysis showed that Pramipexole and Sinemet (L-dopa + carbidopa) treated animals had a slight increase in striatal dopamine, especially in the ventral putamen, compared to MPTP + saline treated nonhuman primates.

(2) Microdialysis was carried out to determine in vivo levels of dopamine within the putamen. Briefly, microdialysis was carried out on 3 squirrel monkeys prior to MPTP-lesioning (as baseline), immediately following MPTP lesioning, during treatment with either pramipexole or Sinemet, and again after a 3 week washout. The primary finding was that pramipexole treated animals displayed elevated amphetamine-evoked dopamine release compared to MPTP untreated animals. Sinemet animals had a level of dopamine release intermediate to these two groups. The adjacent Figure shows the timeline of microdialysis studies. Figure 12 shows HPLC analysis of dopamine levels from microdialysis.

Figure to the left shows a representative microdialysis experiment with the same animal used as its own control and undergoing repeated microdialysis studies. Our studies show that Pramipexole or Sinemet treated animals have greater amphetamine-induced dopamine release.

(3) Western immunoblot analysis of proteins for TH, DAT, VMAT-2, and DARPP32 were carried out on tissues derived from either the ventral or dorsal caudate nucleus or ventral or dorsal putamen. Figure 13 displays a subset of the analysis of the western immunoblot data. To summarize, we found that within the dorsal caudate and putamen there is a slight elevation of TH and DAT expression in MPTP-lesioned animals treated with either Sinemet or pramipexole. There was no apparent change in VMAT-2 expression between all lesioned groups. There appears to be elevated DARPP-32 expression in MPTP-lesioned animals treated with Sinemet. There was also an increase in the phosphorylated forms of DARPP-32 (~phosphoThr34, and ~phosphoThr75) in Sinemet treated animals that was not observed in Pramipexole treated animals. We are currently analyzing the remaining western immunoblots and determining if our findings are consistent amongst the groups. We are also finishing the western blot analysis of the ventral caudate and putamen. These analyses plus the determination of the profiles of expression of the dopamine receptors will be completed in year 4 of this proposal. These studies will be complemented with immunohistochemical staining of tissues sections with antibody probes against TH, DAT, DARPP-32, and VMAT-2. Staining of sections has just been completed and are currently under analysis.

Much of the preliminary data generated in this Aim using western immunoblotting approaches were presented in the previous Annual Report. The purpose of the no-cost extension is to permit completion of these studies. We anticipate that this Aim will be completed in the final phase of this grant.

Study 3: *The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using in situ hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate*.

This aim will be carried out in the final phase of this grant as part of the request for a no-cost extension.

Study 4: *The effect of Pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.*

In collaboration with Dr. Charles Meshul (Oregon Health Sciences University, Portland, OR) perfusion fixed brain tissues were harvested from a nonhuman primate from each group for analysis using immuno-electron microscopy with an antibody against glutamate. These results are summarized in the Figure below. Following MPTP-lesioning there is an increase in the relative density of striatal glutamate immunolabeling (second bar) within corticostriatal terminals. After treating MPTP-lesioned animals with Pramipexole or Sinemet the relative density of glutamate immunolabeling is reduced. Increased density of striatal glutamate within the terminal is thought to reflect decreased glutamate release. Our study would suggest that Sinemet increases glutamate release to a slightly greater extent than Pramipexole. This increased glutamate release may be one means by which dyskinesia is elicited to a greater extent in Sinemet treated animals than Pramipexole treated animals.

The following studies have been added to this proposal with the aim of understanding the impact of alterations in the expression of glutamate receptors on the electrophysiological properties of striatal neurons.

Electrophysiological Studies of Basal Ganglia Function the Nonhuman Primate Model of PD

An important aspect of the nonhuman primate is the anatomical similarity of basal ganglia structure and function to that of humans, thereby providing an important tool for investigating basal ganglia function, such as neurophysiological properties in the normal and disease state, and thus serves as the foundation for identifying new therapeutic treatments. For example neurophysiological studies have implicated over-activity at corticostriatal synapses as one underlying mechanism for the development of motor impairment in PD (Konitsiotis *et al.*, 2000; Soares *et al.*, 2004; Wichmann and DeLong, 2003). Electrophysiological studies in our labs, using the MPTP-lesioned squirrel monkey, have shown changes in AMPA and GABA mediated synaptic neurotransmission that may account for excessive excitatory corticostriatal drive. For these studies, we administered MPTP in a series of 6 subcutaneous injections of 2.0 mg/kg (free-base) every 2 weeks for a total of 12 mg/kg. Whole brains were harvested at either 6 weeks (when animals are parkinsonian) or 9 months (when animals are motorically recovered) after the last injection of MPTP and striatal synaptic function was examined in coronal *in vitro* brain slices. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. The relative strength of $GABA_A$ versus AMPA receptor mediated synaptic responses was calculated as the I_{GABA-} changes in $_A$ / I_{AMPA} ratio. Interestingly, we also found a reduced I_{GABA-A}/I_{AMPA} ratio 6 weeks after MPTP. These GABAergic inhibition that we and others have observed may play an important role in facilitating the synchrony and oscillatory patterns of discharge found throughout the basal ganglia motor circuit in MPTP-treated akinetic primates (Goldberg *et al.*, 2002; Raz *et al.*, 1996; Raz *et al.*, 2001). Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Specifically we found the input/output ratio for AMPA receptor-mediated synaptic responses and the I_{GABA-A} / I_{AMPA} ratio returned back to control levels (Figure 3). These observations are in agreement with the view that excessive glutamatergic corticostriatal synaptic function may be a contributing factor to the behavioral pathology of PD (Konitsiotis *et al.*, 2000; Muriel *et al.*, 2001). Future studies will exam whether changes in glutamatergic drive in fully recovered animals differentially impacts corticostriatal synapses in direct versus indirect basal ganglia pathways, as has been reported in the parkinsonian state (Day *et al.*, 2006; Wichmann and DeLong, 2003).

Dopamine denervation in animal models of PD is also associated with changes in the molecular composition of AMPA and NMDA receptors in the striatum (Betarbet *et al.*, 2004; Betarbet *et al.*, 2000; Hallett *et al.*, 2005; Hurley *et al.*, 2005; Nash *et al.*, 2004). We also found evidence for changes in the pharmacological profile of AMPA and NMDA receptors, which are consistent with these molecular studies. For example as shown in Figure 3, in animals examined 6 weeks post MPTP-lesioning, we found; (i) a decrease in the I_{NMDA}/I_{AMPA} ratio; (ii) an alteration in the NMDA receptor subunit composition as indicated by increased sensitivity to the selective NR2B antagonist CP-101,606; and (iii) an alteration in AMPA receptor mediated synaptic responses, as indicated by changes in the sensitivity to the selective AMPA receptor antagonist,

GYKI-52466 compared to saline control animals (Nash *et al*., 2004; Ruel *et al.*, 2002). Again, with behavioral recovery at 9 months post-MPTP-lesioning, we observed the trend of a return of NMDA and AMPA receptor function to match that seen in saline injected squirrel monkeys (Figure 14).

The glutamatergic corticostriatal and the dopaminergic nigrostriatal system are important mediators of synaptic plasticity, termed long-term depression (LTD) and long-term potentiation (LTP), within the basal ganglia (Centonze *et al.*, 2001; Mahon *et al.*, 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Electrophysiological studies in our lab, using saline control squirrel monkeys, have shown that the induction of long-term synaptic plasticity at corticostriatal synapses is region specific, with LTP being induced in more medial regions and LTD in more lateral regions. These findings agree with previous reports from the rodent model of PD (Partridge *et al.*, 2000; Smith *et al.*, 2001). Studies in the rat have shown a loss of synaptic plasticity after 6-OHDA administration, which we have observed in the MPTP-lesioned mouse model, 1 to 2 weeks after neurotoxicant exposure (Calabresi *et al.*, 1992; Centonze *et al.*, 1999; Kreitzer and Malenka, 2007). Presently, there is little known regarding alterations in synaptic plasticity immediately following MPTP-lesioning in the nonhuman primate.

Analysis of the expression of synaptic plasticity in the squirrel monkey 9 months after MPTP-lesioning has shown that LTD and LTP expression is evident. In the same animals used for analysis of glutamate neurotransmission above, we observed a dramatic and permanent decrease in dopamine release as measured by fast-scan cyclic voltammetry (Cragg, 2003) (Figure 4). This finding is in agreement with previous reports examining dopamine function in the squirrel monkey using HPLC (Petzinger *et al*., 2006). The expression of dopamine-dependent forms of LTP we observed in the dopamine depleted squirrel monkey suggest an adaptation may occur in the expression and/or sensitivity of both D1 and D2 receptors (Centonze *et al*., 2001; Mahon *et al*., 2004; Picconi *et al*., 2005; Reynolds and Wickens, 2002). Preliminary studies in our lab have shown that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent, since this effect is blocked by the D2 receptor antagonist *l*-sulpiride. In addition, use of *l*-sulpiride results in the unexpected expression of LTP in lateral synapses (Figure 4). Our findings are consistent with the literature, where dopamine receptors D1 and D2 have been shown to play an important role in LTP and LTD, respectively (Calabresi *et al.,* 1992; Centonze *et al*., 1999; Wang *et al.,* 2006). Taken together, these data suggest behavioral recovery from MPTP exposure in the squirrel monkey may be due at least in part to compensatory increases in the sensitivity of dopamine receptors, which enables the normal and expected expression of long-term plasticity at corticostriatal synapses.

Reportable Outcomes For Years One to Four

The following sections outline the reportable outcomes including Abstracts, Manuscripts, and Presentations. The manuscripts are included as pdf attachments.

Abstracts:

(1) Society for Neuroscience Annual Meeting, San Diego, 2004 ABSTRACT #1

Behavioral recovery in the MPTP-lesioned nonhuman primate: Altered dopamine biosynthesis and storage.

Hogg, E, M. W. Jakowec, K. L. Nixon, A. T. Abernathy, P. Arevalo, B. E. Fisher, M. Liker, and G. M. Petzinger.

(2) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #1

Exercise induced behavioral recovery and plasticity in the MPTP-mouse model of Parkinson's disease.

Jakowec, M. W., P. Arevalo, M. Vuckovic, P. Turnquist, E. Hogg, J. Walsh[#], G. Akopian[#], C. Meshul*, A. Abernathy, M. Ramirez, B. Fisher and G. M. Petzinger.

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The adult brain possesses a tremendous capacity for activity-dependent neuroplasticity. Following injury to the brain, physical therapy plays an important role in promoting recovery. In neurodegenerative disorders such as Parkinson's disease, physical activity improves motor function and may lead to alterations in disease progression. To better understand the role of activity-dependent plasticity in brain repair we are investigating the application of intensive treadmill exercise training in the MPTP mouse model of basal ganglia injury and dopamine depletion. Mice were administered MPTP (4 20 mg/kg each) and subjected to intensive treadmill running for 30 days starting 4 days after the last injection of MPTP (when cell death is complete) at a speed up to 20 meters/minute for 1 hour. During the exercise paradigm, mice were investigated for improvement in behavioral motor features and learning. Harvested brain tissues were analyzed by HPLC for levels of dopamine and its metabolites, and glutamate and the pattern of expression of genes and proteins for tyrosine hydroxylase, dopamine transporter, dopamine receptors D1 and D2, and AMPA and NMDA glutamate receptors using western immunoblotting, immuno histochemistry, and in situ hybridization histochemistry. Electrophysiological analysis of dopamine release was determined using fast cyclic voltammetry on brain slices. Our findings indicated that there was an enhancement of both motor behavior recovery and rotarod learning in exercised mice despite no change in the number of SNpc dopaminergic neurons and the striatal levels of dopamine. Molecular analysis showed down-regulation of DAT and TH, and significant changes in the pattern of expression of ionotropic glutamate receptors in the cortex and striatum. In addition, exercise resulted in an increase in dopamine release compared to MPTP-lesioned mice without exercise. These findings demonstrate that intensive exercise can induce dramatic neuroplasticity in an animal model of basal ganglia injury and provides a valuable framework for supporting exercise in patients with Parkinson's disease.
Supported by grants to J. Walsh (RO1 AG21937), M. Jakowec (RO1 NS44327) and G. Petzinger (US Army NETRP W81XWH-04-1-0444).

(3) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #2

Changes in dopamine and glutamate electrophysiology in the MPTP-treated non-human primate and the exercised MPTP-treated mouse. J Walsh*, G Akopian*, M, Jakowec[§], G, Petzinger[§]. USC Neuroscience Program, USC Davis School of Gerontology*, Department of Neurology - USC Keck School of Medicine[§].

We tested the hypotheses that dopamine (DA) and glutamate physiology are altered in the MPTP-treated squirrel monkey using electrophysiological methods. Fast cyclic voltammetry analysis of the monkey putamen revealed that MPTP treatment (6 weeks earlier) resulted in a dramatic loss in DA released in response to intra-putamen stimulation (bipolar tungsten wire electrode, 0.1 msec 100-50 μA stimulus). Saline injected monkeys showed greater DA release in the lateral versus medial putamen. To determine if excitatory amino acid receptor-mediated physiology is altered in the MPTP-treated monkey putamen we applied whole cell voltage clamp techniques and examined the relative contribution of AMPA and NMDA receptors to corticostriatal synaptic events. Saline injected monkeys showed a relatively uniform NMDA/AMPA receptor ratio, while data from MPTP-treated monkeys suggested that two new populations emerged; one with a reduced NMDA/AMPA ratio and another with an enhanced NMDA/AMPA ratio.

We applied a similar strategy to examine the impact of MPTP toxicity on DA and glutamate physiology in the mouse and, more importantly, to determine if changes striatal DA or glutamate physiology tracked the behavioral recovery induced by exercise in the MPTP-treated mouse. Cyclic voltammetry revealed a dramatic reduction in evoked DA release in the striatum of mice treated a month earlier with MPTP. A parallel group of mice were treated with MPTP and exercised daily on a treadmill. MPTP treated mice were significantly compromised in treadmill performance initially but achieved the same performance as saline injected mice by the end of one month of training. The exercise-mediated enhancement of motor skills transferred to a rotarod task. Prior work demonstrated exercise-induced suppression in striatal DAT immunocytochemistry in the MPTP-treated mouse (Fisher et al, 2004, J Neur Res 77:378), but voltammetry revealed a significant exercise-induced increase in DA release in the MPTP treated mouse.

These data demonstrate emergent dopaminergic and glutamatergic plasticity created in the striatum following exposure to the neurotoxin MPTP. We hypothesize these forms of synaptic plasticity underlie both behavioral deficits created early as well as recovery seen later in the MPTP model.

This research is supported by grants to J Walsh (RO1 AG21937), M Jakowec (RO1 NS44327) and G Petzinger (US Army NETRP W81XWH-04-1-0444), and the Zumberge Foundation.

(4) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #1

Dopamine treatment effects on neuroplasticity in the MPTP-lesioned Squirrel Monkey (*Saimiri sciureus*).

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The administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the Squirrel monkey (*Saimiri sciureus*) leads to the onset of parkinsonian symptoms due to the loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine.

Animals were administered a series of 3 s.c. injections of MPTP (2.0 mg/kg, free-base, 2 wks between injections) or saline as control. Starting 4 wks after the last injection of MPTP, when the time course of cell death is complete, saline and MPTP-lesioned animals were administered either saline, levodopa plus carbidopa (7.5 mg/kg), or the dopamine agonist pramipexole (0.1 mg/kg) 5 days per wk for 4 wks followed by a 3 wks washout. A subset of animals underwent a series of microdialysis studies in conjunction with amphetamine challenge at pre-MPTP-lesioning, post-lesioning but before dopamine treatment, and 4 weeks after completion of dopamine therapy. During drug treatment all animals were subjected to a clinical rating scale to evaluate parkinsonian motor features. At the completion of the washout period brain tissues was collected from all animals and used for analysis of striatal dopamine levels using HPLC, perfusion fixed for immuno-EM, slice culture for electrophysiological studies, and proteins of interest including tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter-2 (VMAT-2), and the effector molecule DARPP-32 using western immunoblot and immunohistochemical staining focusing on the caudate nucleus and putamen.

Our findings show that MPTP-lesioned animals treated with Pramipexole or Sinemet showed (1) enhanced amphetamine evoked dopamine release; (2) normalization of Corticostriatal drive; (3) normalization of corticostriatal terminal glutamate density; (4) increased protein expression of TH, DAT, and VMAT-2 and (5) an increase in the phosphorylated forms of DARPP-32.

Our data indicate that in addition to symptomatic treatment of parkinsonian motor features, both dopamine replacement therapy in the form of levodopa or dopamine agonists may lead to enhanced neuroplasticity in the MPTP-lesioned basal ganglia as indicated by the up-regulation of proteins important for dopamine biosynthesis, storage and transmission. The precise mechanism is currently unknown but may involve either direct neurotrophic benefit via dopamine receptor stimulation or enhanced engagement of animals with their environment due to dopamine replacement therapy. This finding raises the issue that starting dopamine replacement therapy early in the course of Parkinson's disease may have additional benefit.

This research is supported by grants to G. M. Petzinger (US Army NETRP W81XWH-04-1-0444), J. Walsh (RO1 AG21937), and M.W. Jakowec (NIH RO1 NS44327-1).

(5) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #2

The role of brain-derived neurotrophic factor (BDNF) over-expression in basal ganglia function and response to exercise in the MPTP-lesioned mouse model.

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Our previous work has shown that intensive treadmill exercise leads to improved motor performance in the MPTP-lesioned mouse model of basal ganglia injury. In addition, we have observed alterations in components of both the dopaminergic and glutamatergic neurotransmitter systems including altered patterns of expression of genes and proteins encoding receptor subunits as well as increased dopamine release with exercise in MPTP-lesioned mice subjected to intensive treadmill exercise. While the precise link between glutamate and dopamine neurotransmission with exercise is currently unknown, based on reports in the literature, we hypothesize that the neurotrophic factor BDNF may play a role. This factor is central to a number of important aspects of basal ganglia function including synaptogenesis, plasticity, and response to MPTP exposure. For these studies we utilized a transgenic mouse that over-expresses BDNF throughout the brain. BDNF-tg or C57BL/6 mice were administered MPTP in a series of 4 injections (20 mg/kg, i.p., 2 hours apart). A subset of mice was harvested 7 days post-lesioning for analysis of the degree of lesioning by examining protein expression of tyrosine hydroxylase and counting nigrostriatal dopaminergic neurons. Another subset of both BDNF-tg and C57 BL/6 mice were assigned to different groups including (1) saline injected, (2) saline + exercise, (3) MPTP injected, and (4) MPTP + exercise. The exercise regimen was initiated 5 days after the last injection of MPTP, when cell death is completed, and continued for 28 days (5 days/wk) achieving a rate of approximately 20 m/min for 60 minutes each session. Fast-scan cyclic voltammetry (FSCV) was used to examine electrically evoked dopamine release in striatal coronal brain slices. Using this method, dopamine release was sampled in five anatomically distinct sites that varied in dorsal to ventral and medial to lateral dimensions. Analysis of evoked dopamine release showed no release in non-Tg lesioned mice. However, BDNF-tg mice showed small amounts of dopamine release indicating a potential protective role by BDNF. We also examined long-term plasticity at corticostriatal synapses. Control mice demonstrated LTP in medial and LTD at lateral corticostriatal synapses. However, post-MPTP lesioning neither LTP nor LTD could not be evoked as has been reported for the 6-OHDA treated rat (Calabresi et al, 1992; Kreitzer and Malenka, 2007). Our data suggests BDNF over-expression accelerates the recovery of dopamine release and normal expression of long-term plasticity at corticostriatal synapses following exposure to MPTP.

This research is supported by grants to J. Walsh (RO1 AG21937), M.W. Jakowec (NIH RO1 NS44327-1) and G. M. Petzinger (US Army NETRP W81XWH-04-1-0444).

(6) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #3

Altered AMPA receptor expression with exercise in the MPTP-lesioned mouse model of Parkinson's disease.

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We have previously demonstrated that intensive treadmill running leads to motor improvement in the 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP)-lesioned mouse model of Parkinson's Disease. In this study, we investigated changes in the pattern of expression of glutamate receptors and postsynaptic effector molecules to elucidate the molecular modifications that influence the improvement seen in MPTP-lesioned mice after intensive exercise. Four groups of animals were used to examine these changes: (i) Saline; (ii) Saline + Ex; (iii) MPTP; (iv) MPTP + Ex. C57 BL/6 mice were administered four i.p. injections of MPTP (20mg/kg free-base, 2 hours apart) which yields 90% dopamine depletion in the striatum. Exercise was started 5 days (a time point when cell death is complete) after MPTP lesioning and continued for 28 days (5 days a week) using a motorized treadmill. At completion of the exercise regimen, tissue was harvested and the expression of mRNA transcript and protein for the α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) subtype of the glutamate receptor superfamily, as well as the effector molecule DARPP-32, was analyzed within the dorsolateral striatum. The changes in mRNA transcript expression of AMPA receptor subunits, including their alternative splice isoforms, flip and flop, were determined using qRT-PCR analysis. Using immunohistochemical staining, we examined the expression of AMPA receptor subunits, including their phosphorylated states, and the effector molecule DARPP-32. Our results indicate that exercise causes a downregulation of mRNA transcript in the pan forms of GluR1 and GluR2, and in the Flip isoform of the GluR2 subunit. Mice lesioned with MPTP also displayed decreased mRNA for GluR1 and GluR2. Immunostaining revealed changes not accounted for by transcript expression. No significant changes occurred in the expression of the GluR1 protein. We found an upregulation of the GluR2 protein subunit and its phosphorylated state (serine 880) in MPTP + Ex mice. The expression of the effector molecule DARPP-32, was downregulated in exercised mice. These studies showed that exercise influences the pattern of expression of the AMPA receptors within the striatum but that this phenomenon is not explained by mRNA transcript expression, suggesting that alternative mechanisms are involved in this process, such as protein interactions or localization in relation to the synapse. Findings from this study indicate that changes in AMPA receptor subunits may play a key role in putative molecular adaptations that are necessary for activity dependent synaptic plasticity in the dopamine depleted striatum, as is found in the Parkinsonian state.

This research is supported by grants to J. Walsh (RO1 AG21937), M.W. Jakowec (NIH RO1 NS44327-1) and G. M. Petzinger (US Army NETRP W81XWH-04-1-0444).

(7) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #4

Memory impairment and affective behavior in the MPTP-lesioned mouse model of basal ganglia injury.

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Abstract: Depression, anxiety and dementia are common in patients with Parkinson's disease (PD). Molecular mechanisms connecting the loss of dopamine (DA) with mood and memory disorders are not well understood. The present study investigated whether the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesion of basal ganglia in mice can be used as an animal model of memory and affective dysfunction associated with PD. Established mouse behavior tests were used to compare control and MPTP-lesion mice. Depression was measured by tail suspension and sucrose preference. Anxiety was tested using light-dark preference and hole board, and fear was assessed with fear conditioning. Olfactory memory was tested by social transmission of food preference. Separate groups of adult male C57BL/6 mice were evaluated 7 and 30 days after MPTP lesion. Lesioning consisted of 4 i.p. injections of 20 mg/kg MPTP at 2 h intervals. This regimen has been shown to produce severe DA loss in the striatum (up to 90% loss) and 50-70% cell loss in the substantia nigra pars compacta. Control mice received 4 i.p. injections of saline. In the social transmission of food preference test, mice acquired information about novel flavor from a conspecific demonstrator. Subsequently, when presented with two unfamiliar flavors, control mice showed a strong preference (79.0 ± 3.3%) for the flavor consumed by the demonstrator. This preference was significantly decreased in mice 30 days after MPTP (58.7 ± 6.3%, $p< 0.05$), but not 7 days post-lesion (79.1 ± 3.3%). Fear conditioning at 7 and 30 days post-MPTP showed faster extinction of the freezing response to a tone compared to control mice. After 6 minutes of continuous tone exposure, control mice spent significantly more time freezing (43.8 ± 6.0%, $p<0.05$) compared to mice at 7 days (9.6 ± 3.2%) or 30 days post-MPTP (16.5 ± 7.3%). The tail suspension test showed a significant increase in percent of time spent in immobility 30 days compared to 7 days post-lesion (44.2 ± 3.2%, 29.3 ± 3.3%, $p<0.005$), but there was no difference between these two groups compared to control mice (36.6 ± 3.1%). There was no change in sucrose consumption between lesioned and control mice. There was no increase in time spent in the dark compartment in the light-dark preference test between the groups. Similarly, there was no difference in the number of nose pokes in the hole board test between lesioned and control mice. Overall, these data suggest that the MPTP-lesioned mouse has potential to be used as an animal model of memory impairments associated with PD. On the other hand, acute treatment with MPTP does not induce significant changes in affective behavior in C57BL/6 mice.

(8) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #1

Molecular mechanisms of glutamate neurotransmission after high intensity exercise in the MPTP mouse model of basal ganglia injury.

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Studies in our labs have shown that intensive physical exercise is beneficial for both patients with Parkinson's disease (PD) and animal models of basal ganglia injury (Fisher et al , 2004 and 2008; Petzinger et al 2007). However, the underlying molecular mechanisms are poorly understood. In this study we used the MPTP-lesioned mouse model of basal ganglia injury to investigate molecular mechanisms responsible for the beneficial effects of high intensity treadmill exercise. For this purpose, 8-10 week old male C57BL/6 mice were lesioned with 4 intraperitoneal (i.p.) injections of 20 mg/kg MPTP (free-base) or saline at 2 hr intervals. MPTP-lesioned and control mice were split into four experimental groups: (1) saline, (2) saline + exercise, (3) MPTP, and (4) MPTP + exercise. Treadmill exercise was initiated 5 days after MPTP-lesioning. Mice were habituated to run on a motorized mouse treadmill for 1hr daily and gradually trained to reach speed of 18m/min. Exercise was conducted 5 days per week for a total of 30 days of running. Electrophysiological studies showed exercise-induced reduction in excitatory post-synaptic currents of the medium spiny neurons in MPTP-lesioned mice, and decrease rectification in AMPA receptor conductance. Molecular analysis showed changes in the AMPA receptor subunit GluR2 and its phosphorylated state at Ser-880 supporting our electrophysiological findings. Using other techniques including qRT-PCR and western immunoblot analysis of striatal synaptoneurosomes (enriched for post-synaptic density complexes) we have documented changes in both mRNA transcripts and proteins important for striatal neurotransmission including PSD-95, synapsin I, and several glutamate receptor accessory proteins. Additionally, we have correlated these findings to changes in the morphology of medium spiny neurons in the dorsolateral striatum using the method of Golgi impregnation. Our hypothesis is that in MPTP-lesioned mice, high intensity treadmill exercise modulates the expression of glutamate receptors (especially the AMPA subtype) and normalizes glutamate neurotransmission in medium spiny neurons leading to decreased cortico-striatal hyper-excitability. These changes in glutamate receptors along with the changes we observe in the dopamine D2 receptor (see abstract from M. Vuckovic et al 2008) indicate that the experience-dependent neuroplasticity through intensive exercise has a dramatic influence on the injured basal ganglia and may represent a novel therapeutic target for modifying disease progression.

(9) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #2

Neuroplasticity in the mptp-lesioned squirrel monkey (saimiri sciureus)

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Abstract:
The purpose of this study was to examine molecular and neurophysiological correlates of striatal plasticity in the MPTP-lesioned nonhuman primate. In addition, we examined these same parameters in the context of dopamine replacement therapy using either L-dopa or dopamine agonist (pramipexole) administration. The administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the Squirrel monkey (*Saimiri sciureus*) leads to the onset of parkinsonian symptoms due to the loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine. Animals were administered a series of 3 s.c. injections of MPTP (2.0 mg/kg, free-base, 2 wks between injections) or saline as control. Animals were harvested at either 2 or 12 months. Animals at 12 months demonstrated full behavioral recovery. In a subset of animals drug administration consisted of either levodopa plus carbidopa (7.5 mg/kg), or the dopamine agonist pramipexole (0.1 mg/kg) 5 days per wk for 4 wks followed by a 3 wks washout. Microdialysis studies were conducted in a subset of treated animals to assess alterations in dopamine storage. Brain tissues analysis was carried out for striatal dopamine and glutamate levels using HPLC, slice culture for electrophysiological studies, and proteins of interest including tyrosine hydroxylase (TH), dopamine transporter (DAT), Dopamine Receptors D2 and D1, and AMPA-R and NMDA-R subunits using western immunoblot and immunohistochemical staining focusing on the striatum. Animals were examined for alterations in excitatory amino acid receptor-mediated physiology using whole cell voltage clamp techniques and examined for alterations in the input-output relationship between the intensity of stimulus delivered at the corpus callosum (input) and the size of the excitatory synaptic response (EPSC). Electrophysiological studies also examined the relative contribution of NMDA/AMPA receptor ratio. Our findings show that MPTP-lesioned animals either during behavioral recovery (12 month) or during drug treatment showed (1) normalization of Corticostriatal drive; (2) normalization of glutamate levels (4) alterations in dopamine storage and (5) alterations in AMPA and NMDA Receptor subunit expression. Glutamatergic and dopaminergic neuroplastic changes may be similar in both recovery and dopamine treated animals.

(9) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #3

High Intensity Treadmill Exercise Normalizes Dopamine Neurotransmission in the MPTP Mouse Model of Basal Ganglia Injury

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Keywords: Parkinson's disease, neuroplasticity, positron electron transmission, dopamine receptor D2, dorsal striatum

Physical exercise has beneficial effects on patients with Parkinson's disease (PD), but the underlying molecular mechanisms are poorly understood. Our previous work showed that high intensity treadmill exercise increases dopamine D2 receptor mRNA expression in the dorsal striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of basal ganglia injury. The present study used *in vivo* positron electron transmission (PET) imaging to investigate effects of high intensity treadmill running on dopamine receptor D2 function in MPTP-lesioned mouse. For this purpose, 8-10 week old male C57BL/6 mice were lesioned with 4 intraperitoneal (i.p.) injections of 20 mg/kg MPTP (free base) at 2 h intervals producing up to 90% dopamine loss in the striatum and 50-70% cell loss in the substantia nigra pars compacta. Control mice received 4 i.p. injections of saline. Lesioned and control mice were split into four experimental groups as follow: (1) saline, (2) saline + exercise, (3) MPTP, and (4) MPTP + exercise. Treadmill exercise was initiated 5 days after MPTP-lesioning. Mice were habituated to run on a motorized mouse treadmill for 1h daily and gradually trained to reach speed of 18m/min. Exercise was conducted 5 days per week for a total of 30 days of running. Three or four mice from each group were randomly selected for PET imaging with a high affinity D2 receptor ligand [^{18}F]-fallypride. Radioactive ligand was administrated to anesthetized mice via the tail vein. A 20 min transmission scan was collected immediately following ligand administration. Dynamic scans were collected over a time window of 90 min after the transmission scan. Our preliminary results show a significant increase in striatal D2 receptor binding potential (BP) in the MPTP-lesioned mice + high intensity treadmill exercise (BP = 7.8 ± 1.0) compared to MPTP-lesioned mice with no exercise (BP = 4.4 ± 0.2). Ongoing studies are investigating whether the increase in D2 binding potential in MPTP-lesioned mice is preserved for 6 weeks after the end of daily treadmill running. Overall, these data suggest that high intensity treadmill exercise has an effect in normalizing dopaminergic neurotransmission in injured basal ganglia. Findings from this study support a disease modifying role of treadmill exercise and can be used to help design rehabilitation and physical therapy programs for patients with PD.

(10) Society for Neuroscience Annual Meeting, Washington DC, 2008, ABSTRACT #4

Affective and Motor Behavior in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Mouse Model of Basal Ganglia Injury

Lori M. Gorton, Marta G. Vucković, Nina V. Vertelkina, Giselle M. Petzinger, Michael W. Jakowec, Ruth I. Wood

Though largely classified as a progressive movement disorder, Parkinson's disease (PD) patients may exhibit a wide variety of non-motor, neuropsychiatric symptoms such as anxiety and depression. Exercise has been shown to improve psychological health in depressed populations but the mental health benefits of exercise in PD have not been thoroughly assessed. To investigate the relationship between dopaminergic nigrostriatal pathway degeneration and mood disorders, we lesioned C57Bl/6 mice with MPTP (4 X 20mg/kg free base, 2 h intervals) or gave saline vehicle (n=24/group). Mice were exercised 5 day/week for 6 weeks on a treadmill (8/group) for 1 hr. To control for the potential stress of forced exercise, an additional group of mice (n=8) was allowed to run voluntarily on a wheel for the same time period. Motor behavior was determined by daily total distances and average speeds, and overall rotorod performance after week 6. Depression and anxiety-like symptoms were assessed using established tests (sucrose preference, tail suspension, elevated plus maze, and marble-burying). **Results**: Wheel running mice ran significantly more than mice undergoing forced treadmill exercise, (30d avg =1.1 ± 0.05km vs. 0.44 ± 0.02km). MPTP lesion slighty reduced (1505 ± 97 vs. 1734 ± 41) while exercise increased overall Rotarod performance (ORP; 1660 ± 67 vs. 1481 ± 85.98). MPTP-lesioned mice buried more marbles vs. controls (62 ± 3.8% vs. 45 ± 6.6%); sedentary MPTP-lesioned mice buried the most marbles (74 ± 5.2%). The number of open and closed arm entries into the elevated plus maze was not differerent in any group. Neither MPTP lesion nor exercise had an effect on behavioral tests of depression. **Conclusion:** MPTP mice display subtle motor deficits 44 days post-lesion. MPTP mice may be more anxious than NaCl controls but show no indication of despair or anhedonia. Despite differences in the amount of running, neither forced nor voluntary exercise significantly improved measures of mood.

(11) Plasticity and Repair in Neurodegenerative Disorders, May 15-18, 2008, Lake Arrowhead, CA.

Molecular Mechanisms of Normalized Dopamine and Glutamate Neurotransmission After High Intensity Exercise in the MPTP Mouse Model of Basal Ganglia Injury.

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We are using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of basal ganglia injury to investigate molecular mechanisms responsible for the beneficial effects of high intensity exercise on the dopaminergic and glutamatergic systems. We hypothesize that in MPTP-injured mice, high intensity treadmill exercise induces molecular adaptations in the basal ganglia which normalize dopamine and glutamate neurotransmission. These changes may work synergistically to decrease cortico-striatal hyperexcitability in medium spiny neurons and underlie the beneficial effects of exercise. These studies support a disease modifying role of exercise.

Within the basal ganglia, cortico-striatal plasticity is proposed to be important for the control of motor function and for learning of motor skills. Previous studies suggested that dopamine receptor isoform D2, and glutamate receptors NMDA and AMPA could be key molecular candidates involved in cortico-striatal plasticity in medium spiny neurons of mouse striatum. Recent studies shown that high intensity daily exercise does not change the total DA levels in striatum, but increase stimulus-evoked DA release in stiatal brain slices and D2 receptor mRNA in dorsolateral striatum.

Positron Electron Transmission (PET) imaging will was used to quantify D2 receptor function *in vivo* at the level of the dorsal striatum in MPTP-lesioned and saline-treated mice after 30 days of high intensity treadmill exercise or no exercise. Three or four mice from each group were selected for imaging. For this study, a high affinity D2 receptor ligand [^{18}F]-fallypride was used. Ligand was administrated to anesthetized mice via the tail vein. Following a 30 min transmission scan, dynamic scans were collected over a time window of 90 min immediately following ligand administration. Our results show increased striatal D2 receptor binding potential in the MPTP mouse and is consistent with an increase in D2 protein expression.

Exercise increased expression of GluR2 and phosphoGluR2-Serine880 in the MPTP mouse. Electrophysiological studies show exercise-induced reduction in excitatory post-synaptic currents of the medium spiny neurons in MPTP mice, and decrease rectification in AMPA receptor conductance. Findings support molecular analysis of GluR2 and an exercise induced decrease in excitability of medium spiny neurons in the MPTP mouse.

Ongoing studies are examining the expression of D2 receptor at the level of protein and mRNA in dorsolateral and ventral striatum using western blot, and rtPCR methods.

(14) Plasticity and Repair in Neurodegenerative Disorders, May 15-18, 2008, Lake Arrowhead, CA.

Voluntary and Forced Running Effects on Affective and Motor Behavior in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Mouse Model of Basal Ganglia Injury

Lori M. Gorton, Marta G. Vucković, Nina V. Vertelkina, Giselle M. Petzinger, Michael W. Jakowec.

Though largely classified as a progressive movement disorder, Parkinson's disease (PD) patients present with a wide variety of non-motor, neuropsychiatric symptoms such as anxiety and depression. Exercise has been shown to improve psychological health in depressed populations but the mental health benefits of exercise in patients with PD comorbidity have not been thoroughly assessed. To investigate the relationship between dopaminergic nigrostriatal pathway degeneration and mood disorders, we lesioned C57Bl/6 mice with MPTP (4 X 20mg/kg free base, 2 h intervals) or administered saline vehicle (n=24/group). One week later, mice were randomly assigned to a 5 day/week exercise regimen for six weeks, or to sedentary control groups. Exercised mice were run on a treadmill (8/group) with increases in time and distance so that a 6-week goal of two 30-minute sessions at a max speed of 18m/minute could be achieved To control for the potential stress imposed by forced exercise, an additional group of mice (n=8) was allowed to run voluntarily on a running wheel for the same time period. Motor behavior was compared by calculating daily total distances (expressed as average m/min) and overall rotorod performance after week 6. Depression and anxiety-like symptoms were assessed endophenotypically using established tests (sucrose preference, tail suspension, elevated plus maze with fecal boli quantitation, and marble-burying). **Results**: At the end of three weeks, exercised saline-treated animals (forced treadmill and voluntary wheel) showed an increased preference for 2% sucrose solution from baseline ($p<0.05$). No change in sucrose preference was observed in any of the MPTP-lesioned animals or in the saline sedentary group. Mice subject to forced treadmill exercise ran an average of 8 m/min for 50 minutes with an imposed max speed of 10m/min. By contrast, mice exercising voluntarily on running wheels ran significantly more (21.01 ± 1.3m/min for 50 minutes), and there were no differences between MPTP and control wheel-running groups. **Conclusion:** Preliminary results suggest that the motor deficits of MPTP-lesioned animals are not associated with decreased running distances. Also, After 3 weeks, we find no evidence of anhedonia in any group measured.

(15) Movement Disorders Society, Chicago, IL, June 2008.

Altered glutamate (AMPA) and dopamine (D2) receptor expression with treadmill exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury

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Objective: To determine the effects of high-intensity treadmill exercise (HITE) on striatal AMPA subunit (GluR1/GluR2) and D2 receptor expression using molecular analysis and PET imaging in the MPTP mouse model of Parkinson's Disease (PD).

Background: Exercise is beneficial for patients with PD. However, the underlying mechanisms and potential for disease modification are unknown. Alterations in dopaminergic and glutamatergic neurotransmission, induced by activity dependent (exercise) processes, may mitigate the cortically driven hyper-excitability in the basal ganglia normally observed in the parkinsonian state.

Methods: Mouse groups: saline; saline + exercise; MPTP; MPTP + exercise. Saline and MPTP mice were treadmill exercised (1hr/day; 28d). At exercise completion, dynamic PET scans were performed using ^{18}F-Fallypride (SA>6000Ci/mmole, 280uCi) and a microPET R4 scanner (90min) and images reconstructed using MAP algorithm. Binding potential (BP) was calculated using a dynamic model. Striatal tissue analysis included western blot analysis (D2) and qRT-PCR and immnuohistochemical staining (GluR1, GluR2, phosphorylated states). Whole cell voltage clamp methodology examined (i) input (stimulus strength)/output (excitatory post-synaptic current) relationship to evaluate synaptic strength (ii) rectification index to delineate AMPA-R subunit composition.

Results: HITE increased striatal D2 receptor BP in the MPTP mouse and is consistent with an increase in D2 protein expression. Exercise increased expression of GluR2 and phosphoGluR2-Serine880 in the MPTP mouse. Electrophysiological studies show exercise-induced reduction in EPSCs of MSNs in MPTP mice, and decrease rectification in AMPA receptor conductance. Findings support molecular analysis of GluR2 and an exercise induced decrease in excitability of medium spiny neurons (MSN) in the MPTP mouse.

Conclusions: HITE leads to alterations in dopaminergic and glutamatergic signaling within the injured basal ganglia. These changes may work synergistically to mitigate corticostriatal hyperexcitability in MSNs and underlie the anitparkinsonian effects of exercise. These studies support a disease modifying role of exercise.

Tuesday, June 24, 2008 12:30 PM

(16) Movement Disorders Society, Chicago, IL., June 2008

Sex differences in the MPTP mouse model of Parkinson's disease

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Objective: To investigate sex differences in anatomical and behavioral impairments in the MPTP mouse model of Parkinson's disease.

Background: Parkinson's Disease (PD) is a motor disorder resulting from the progressive loss of dopaminergic neurons of the substantia nigra pars compacta (SNC). Sex differences in PD have been reported in humans and in rodent models, with males being more impaired than females. Gonadal steroid hormones are thought, in part, to underlie this sex difference. The current study examined anatomical and behavioral sex differences in the MPTP mouse model of PD. We hypothesized that MPTP lesioning would produce more neuronal death in SNC of male mice, resulting in greater motor deficits relative to females. **Methods:** Male and female mice were gonadectomized and received physiologic replacement with testosterone or estrogen to ensure constant hormone levels. Mice were injected with MPTP (10 mg/kg BW ip) or saline daily for 5 days. One week after the last injection, motor function was measured using the gait, pole, and rotarod tests. Immediately afterwards, animals were sacrificed. Caudal brain blocks containing SNC were immunostained for tyrosine hydroxylase (TH) and counterstained for Nissl.

Results: In unlesioned mice, males outperformed females on all three motor tests. Male mice had longer strides, descended the pole apparatus faster, and stayed on the rotarod longer than females. MPTP lesioning impaired overall rotarod performance (ORP) in both sexes. After MPTP treatment, ORP was equivalent in males and females (695 ±64 vs. 608 ±91, n.s.). Compared with unlesioned controls, MPTP-lesioned male mice had a more severe motor deficit than females (43% vs. 37%). MPTP treatment did not deplete TH neurons in SNC and there was no sex difference post-lesion.

Conclusions: MPTP lesioning produced a larger motor deficit in male mice than in females. These results support neurochemical studies in rodents showing more severe striatal dopamine depletion in males after MPTP. Furthermore, they support human gender studies that report a higher incidence and more severe PD phenotype in men. Interestingly motor dysfunction after MPTP was not accompanied by a parallel depletion of dopaminergic SNC neurons. This suggests that significant dopaminergic cell loss in SNC is not required to elicit behavioral deficits in motor performance.

(17) Society for Nuclear Medicine

Nacca, A., Q. Li, M. Vuckovic, R. Leahy, P. Conti, B. Fisher, **M. W. Jakowec**, and G. M. Petzinger (2008) The Effects of intensive treadmill exercise on striatal D2 dopamine receptors binding in the MPTP-lesioned mouse model of Parkinson's disease. Society for Nuclear Medicine Annual Meeting, New Orleans.

Publications:

(1) Jakowec M.W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. 76 (4) 539-550.

(2) Fisher, B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and M. W. Jakowec (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research 77: 378-390.

(3) Jakowec, M.W., and G.M. Petzinger (2004) The MPTP-Lesioned Model of Parkinson's Disease with Emphasis on Mice and Nonhuman Primates. Comparative Medicine 54 (5) 497-513.

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(6) Petzinger, G. M., and M. W. Jakowec (2007) Animal Model of Parkinson's Disease. In: Handbook of Parkinson's Disease, eds. R. Pawha and K. Lyons, Marcel Dekker, Inc., NY, NY.

(7) Petzinger, G. M., D. M. Togasaki, G. Akopian, J. P. Walsh, and M. W. Jakowec (2007) Nonhuman primate models of Parkinson's disease and experimental therapeutics. In: Parkinson's Disease: Pathogenic an Therapeutic Insights from Toxin and Genetic Models. Ed. By R. Nass and S. Przedborski, Elsevier Co., San Diego, CA.

(8) Petzinger, G. M., J. Walsh, G. Akopian, E. Hogg, A. Abernathy, P. Arevalo, P. Turnquist, B. E. Fisher, D. Togasaki, and M. W. Jakowec (2007) Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-lesioned mouse model of basal ganglia injury. Journal of Neuroscience 27 (20) 5291-5300.

(9) Manuscript in Preparation: Jon VanLeeuwen, Giselle M. Petzinger, Marta Vuckovic, Maria Ramirez, Pablo Arevalo, and Michael W. Jakowec. Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury.

Selected Presentations:

(1) Petzinger, Giselle "Neuroplasticity in the MPTP-lesioned Nonhuman Primate", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(2) Jakowec, Michael " The Role of Exercise in Enhancing Neuroplasticity in the MPTP-lesioned mouse", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb 19-22, 2004.

(3) Petzinger, Giselle "Enhancing Neuroplasticity in models of Basal Ganglia Injury", Van Der Muelen Symposium, University of Southern California, Keck School of Medicine, April 1, 2005.

(4) Petzinger, Giselle “Neuroplasticity and behavioral recovery in the MPTP-lesioned nonhuman primate” Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, May 11-14, 2006.

(5) Petzinger, Giselle “Exercise-enhanced motor recovery in the MPTP-lesioned mouse model of Parkinson’s disease”. Parkinson Study Group Annual Meeting, Chicago, IL. October 2006.

(6) Petzinger, Giselle “Exercise, neuroplasticity, and animal models of Parkinson’s disease”. Parkinson Disease Foundation 50th Anniversary Research Symposium, New York City, October 2007.

(7) Jakowec, Michael “Exercise and Parkinson’s Disease”, National Parkinson’s Disease Research Symposium, San Diego, CA, November 2007.

(8) Jakowec, NPF

Jakowec Grand Rounds, University of Pittsburgh

Jakowec, Grand Rounds, Robert Wood Johnson Medical School

Petzinger Gait Amsterdam, February 3, 2008

Jakowec and Petzinger, Winter Conference on Brain Research February 1, 2008

Plasticity and Repair in Neurodegenerative Disorders May 15-18, 2008, Lake Arrowhead, CA
Symposium: Exercise, Plasticity, and parkinsonism.
Michael Jakowec, USC (moderator); Giselle Petzinger, USC; Diana Neely, Vanderbilt University; Daniel Holschneider, USC; Marjorie Ariano, Rosalind Franklin University.

Winter Conference on Brain Research, Snowbird, UT January 26 to February 2, 2008
Session 80
Title: Exercise Enhanced Neuroplasticity in Parkinson’s Disease and its Animal Models
Participants: Michael Jakowec, PhD (Chair) Univ. So. Cal; Giselle Petzinger, MD: Univ. So. Cal.; Charles Meshul, PhD: VA Medical Center/Oregon Health & Sciences University; Richard Smeyne, PhD: St. Jude, Memphis; Beth Fisher, PT/PhD: Univ. So. Cal

Conclusions:

The MPTP-lesioned mouse and squirrel monkey are valuable models for investigating neuroplasticity of the injured basal ganglia. These models can serve as valuable tool to investigate the molecular mechanisms by which extrinsic factors can be applied to enhance recovery. In mice, studies in this proposal are designed to determine the role of intensive treadmill exercise in enhancing motor recovery. Meanwhile the nonhuman primate, with its exquisite parkinsonian features and similarity of anatomical features to the human condition, serves as an excellent means to examine the role of pharmacological replacement therapy targeting the dopaminergic system and the potential role in influencing recovery.

Studies in the MPTP-lesioned mouse model and exercise from the first component of this proposal indicate that intensive treadmill exercise can enhance motor behavioral recovery employing mechanisms that are different from those seen with intrinsic neuroplasticity. Our results indicate that intensive treadmill exercise in MPTP-lesioned mice leads to (i) increased motor recovery and enhanced motor learning (ii) suppression of striatal DAT and TH proteins, (iii) increased stimulus evoked dopamine release as seen in fast-scan cyclic voltammetry, (iv) differential expression of DAT and TH mRNA transcripts, (v) altered expression of specific subunits of the AMPA and NMDA receptor subtypes, and (vi) altered expression of the dopamine receptor D2.

Studies in the second component of this proposal utilizing the MPTP-lesioned nonhuman primate show that the pharmacological application through dopamine replacement therapy with Sinemet (levodopa plus carbidopa) or the D2/D3 agonist pramipexole leads to enhancement of the intrinsic neuroplasticity we observe. For example, treated MPTP-lesioned animals show (i) increased levels of striatal dopamine, (ii) increased amphetamine-evoked dopamine release using microdialysis, (iii) elevated levels of TH and DAT protein in the caudate and putamen, (iv) differential expression of DARPP-32 and its phosphorylated forms in the caudate-putamen. Electrophysiological studies have shown a shift in the AMPA/NMDA ratio, altered corticostriatal drive, shifts in the subunit composition of glutamate channels, and that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. One unexpected finding was the development of dyskinesia during treatment with Pramipexole, a behavioral characteristic not yet reported in the scientific literature.

Appendices: Attached.

(1) Manuscript: Jakowec M.W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger. (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. **76** (4) 539-550.

(2) Manuscript: Fisher, B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and M. W. Jakowec. (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research **77**: 378-390.

(3) Manuscript: Shakil, S. S., H. K. Homer, C. Moore, A. T. Abernathy, M. W. Jakowec, G. M. Petzinger, and C. K. Meshul (2005) High and low responders to novelty show differential effects in striatal glutamate. Synapse **58** (3) 200-207.

(4) Manuscript: Hughes-Davis, E. J., J. P. Cogen, M. W. Jakowec, H. W. Cheng, G. Grenningloh, C. K. Meshul, and T. H. McNeill. (2005) Differential regulation of the growth-associated proteins GAP-43 and superior cervical ganglion-10 in response to lesions of the cortex and substantia nigra in the adult rat. Neuroscience. **135** (4) 1231-1239.

(5) Manuscript: Petzinger, G.M., K. Nixon, B. E. Fisher, A. Abernathy, and M. W. Jakowec. (2006) Behavioral Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Squirrel Monkey (*Saimiri sciureus*): Analysis of Striatal Dopamine and the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins. J. Neurosci. Res. 83: 332--347.

(6) Manuscript: Petzinger, G. M., D. M. Togasaki, G. Akopian, J. P. Walsh, and M. W. Jakowec (2008) Nonhuman primate models of Parkinson's disease and experimental therapeutics. In: Parkinson's Disease: Pathogenic and Therapeutic Insights from Toxin and Genetic Models. Ed. By R. Nass and S. Przedborski, Elsevier Co., San Diego, CA.

(7) Manuscript: Petzinger, G. M., J. Walsh, G. Akopian, E. Hogg, A. Abernathy, P. Arevalo, P. Turnquist, B. E. Fisher, D. Togasaki, and M. W. Jakowec (2007) Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-lesioned mouse model of basal ganglia injury. Journal of Neuroscience 27 (20) 5291-5300.

(8) Manuscript: Petzinger, G. M., Movement Disorders.

(9) Manuscript: VanLeeuwen, J.,

(10) Manuscript: Vuckovic, M.,

(11) Manuscript: Akopian

Elsevier Editorial System(tm) for Neurobiology of Disease
Manuscript Draft

Manuscript Number: NBD-08-173R1

Title: Memory, Mood, Dopamine, and Serotonin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury

Article Type: Regular Article

Section/Category:

Keywords: affective behavior; MPTP; Parkinson's disease; anxiety; depression; conditioned fear; associative memory

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Manuscript Region of Origin:

Abstract: The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse serves as a model of basal ganglia injury and Parkinson's disease. The present study investigated the effects of MPTP-induced lesioning on associative memory, conditioned fear, and affective behavior. Male C57BL/6 mice were administered saline or MPTP and separate groups were evaluated at either 7 or 30 days post-lesioning. In the social transmission of food preference test, mice showed a significant decrease in preference for familiar food 30 days post-MPTP compared to controls. Mice at both 7 and 30 days post-MPTP-lesioning had increased fear extinction compared to controls. HPLC analysis of tissues homogenates showed dopamine and serotonin were depleted in the striatum, frontal cortex, and amygdala. No changes in anxiety or depression were detected by the tail suspension, sucrose preference, light-dark preference, or hole-board

tests. In conclusion, acute MPTP-lesioning regimen in mice causes impairments in associative memory and conditioned fear, no mood changes, and depletion of dopamine and serotonin throughout the brain.

July 6, 2008

Dear Dr. Greenamyre,

We would like to thank the reviewers of our manuscript “Memory, Mood, Dopamine and Serotonin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury” for their insightful comments and suggestions. In this letter we articulate the precise response to the points brought up by each reviewer and indicate where in the manuscript these changes are located. We have incorporated all the suggested changes made by the reviewers into this revised manuscript. We feel that these changes have greatly improved the quality of our manuscript and hope they satisfy the concerns of the reviewers.

We look forward to hearing from you very soon.

Sincerely,

Michael Jakowec, Ph.D.

July 6, 2008

Dear Dr. Greenamyre,

We would like to thank the reviewers of our manuscript “Memory, Mood, Dopamine and Serotonin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury” for their insightful comments and suggestions. In this letter we articulate the precise response to the points brought up by each reviewer and indicate where in the manuscript these changes are located. We have incorporated all the suggested changes made by the reviewers into this revised manuscript. We feel that these changes have greatly improved the quality of our manuscript and hope they satisfy the concerns of the reviewers.

We look forward to hearing from you very soon.

Sincerely,

Michael Jakowec, Ph.D.

In the following sections we have re-stated the comment/suggestions of the reviewers from the critique verbatim in their order. Our response follows the statement and is written in *italics*. We also indicate the location of changes made to the text of the manuscript and when appropriate indicate the precise text changes made.

Response to Reviewer #1

(1) The behavioral data are the most relevant part of the manuscript. Unfortunately, significant discrepancies arise from the description in the methods section, the results reported and the time schedule in fig.1. Authors should specify the progression of the behavioral assessment consistently with the timeline and report the results in the proper order.

We have corrected the order of the behavioral testing presentation in the Results and Discussion sections such that it reflects the order presented in Figure 1. For example, in the Results section we present the findings from each of the behavioral tests in the order by which they were carried out. These changes are reflected in the second, third and the fourth paragraphs of the Results section of the manuscript. Also, the order of figures has been revised to reflect the order of data presentation.

(2) There are also relevant inconsistencies regarding the number of animals used in the different tests. In most of the tasks authors reported 20 mice in the saline group, 10 mice in the 7 days post-MPTP group and 12 mice in 30 days post-MPTP group but in the auditory fear conditioning they used only 5 animals as control group, 6 in 7 days post-MPTP group and 8 mice in 30 days post-MPTP group. Authors should explain why 75% of control mice were excluded from this test and then a total of 12 brains from the same group were collected for tissue preparation (immunohistochemistry and HPLC). Same kind of inconsistency should be explained for the other two experimental groups: 8 brains collected from the 7 days post-MPTP but only 6 evaluated in the auditory fear conditioning and 10 brains collected from the 30 days post-MPTP but only 8 evaluated in the auditory fear conditioning.

We have clarified the number of animals used in each behavioral test. A total of 42 mice were used in these studies (Methods section, paragraph 1). Division into group assignments was as follows: saline n = 20, MPTP-7-days n = 10, and MPTP-30-days n = 12. All mice went through the behavioral tests STFP, L-D preference, sucrose preference, hole-board, conditioned odor aversion, and tail suspension. In a couple of cases mice were excluded from the test results if they failed the test. For example, two mice in the saline group did climb up their tails during the tail suspension test and were not included in the final analysis; therefore n = 18). Two mice in the saline group spilled the test liquid in the sucrose preference and were not included; therefore n = 18. We have multiple setups for each of these tests and are able to test large numbers of mice in a reasonable period of time. This is in contrast where a subset of animals was used for the fear condition testing since this test is more time consuming requiring at least 15 minutes per animal (setup, testing, cleaning) all performed with a single apparatus in an isolated room. To do all 42 mice would require a 12-14 hour period. We were able to gather meaningful data from saline n = 5, MPTP-7-days n = 6, and MPTP-30-days n = 8. It is these data that we report in this manuscript.

We have also clarified any inconsistency between the number of mice available for behavioral testing and the number of mice from which brains were collected for immunohistochemistry (ICC) and

HPLC. In the previous version of this manuscript we presented this information in what was a little confusing since 7 mice were collected for ICC from the saline group but only 3 used for final analysis (the other 3 mice collected for ICC were never used since we had obtained meaningful data from the initial 4 mice). Therefore, in this revised manuscript to eliminate any potential confusion with the number of mice used we state that 5 from each group were processed for HPLC and 4 from each processed for ICC (which included stereological counting of SNpc neurons to verify the degree of lesioning).

These clarifications are stated in the text of the Material and Methods section, paragraph 1 "Animals, Treatment Groups and MPTP Administration", and in the same section, page 9, paragraph 1 "Tissue Preparation" and page 10, paragraph "Immunohistochemical Staining" and paragraph "Unbiased Stereological Counting of Dopaminergic Neurons".

(3) In the light-dark exploration test results from latency to first enter the dark chamber are missing.

These data have been included and are presented in the Results section, page 12, paragraph "Light-Dark Preference and Hole-Board".

(4) In fig 6 B the authors claimed a statistical difference in the amygdala after 30 days post-MPTP but the asterisk is missing.

The asterisk has been added to the Figure6B.

(5) There are minor typographical errors throughout the manuscript. For example, in the Methods, test number 5 is mentioned as conditioned taste aversion p.5, whereas in the same section at p.7 conditioned odor aversion is reported. In the legend of figure 6, frontal cortex is abbreviated as Fr.Ctx whereas in fig 6 fCtx is reported.

We have made corrections to the manuscript for the typos indicated by the reviewer. On the page 5, paragraph 3, the text "conditioned taste aversion" is replaced with "conditioned odor aversion". In the legend of figure 6, frontal cortex is now abbreviated as fCtx. We have also carefully gone through the manuscript to correct other typos missed in the previous version.

Response to Reviewer #3 (there was no Reviewer #2):

(1) Page 5: methods: behavioral testing: the tests were conducted from the least to the most stressful. First, how was this actually determined (this should be commented on in this section) and second, did the authors try changing the order or randomizing the tests to see if, in fact, it made a difference? The fact that the order of the tests was always the same may result in bias being created which may have affected the results of the tests. For example, if conditioned fear was tested first in some animals, would the results have been the same compared to this test always being administered last? If the authors carried out a pilot study to determine that the order of the tests did make a difference in terms of the outcome, those results should be reported.

The reviewer points out a potentially important issue for which we would like to clarify our statement regarding the order of behavioral tests in this study. We revised the sentence on page 5, paragraph "Behavioral Testing" in the Methods section to read: "Tests were conducted over 6 days with the following order: (1) STFP, (2) light-dark exploration, (3) sucrose preference, (4) hole-board, (5) conditioned odor aversion, (6) tail suspension, and (7) conditioned fear (Fig 1). Tests that induce acute stress response (tail suspension and fear conditioning) were conducted at the end. The order of tests was not randomized." While we did not determine corticosteroid or ACTH levels in mice after each behavioral test, it is well documented uncontrollable and inescapable situations (such as tail suspension

and fear conditioning) induce a stress response in mice (Brown et al., 1984; Liu et al., 2003; Pugh et al., 1997). During the light-dark preference and the hole-board tests mice are placed in novel environments which causes acute elevation of adrenocorticotropic hormone (ACTH) and corticosterone (Frederic et al., 1997; 2006). To prevent carry-over stress effects, these two tests were administrated on different days in our study. When testing mice for multiple behaviors, it is important to order tests by increased stress levels (Crawley, 2008). Additionally, in our study, all mice were tested at the same time and the potential carry–over effects are the same in all three groups. These statements and references are now included in the revised manuscript,

(2a) Page 9: tissue preparation: since it appears that the brain areas were dissected by hand vs tissue punches from frozen sections in order to be more precise anatomically, there is some concern about the interpretation of the neurochemical data based on this more crude dissection. For example, considering the extent of dopamine tissue loss from the striatum, it was quite surprising to see the relative lack of change in dopamine levels in the ventral mid-brain. This could be explained by the fact that the VTA is included as part of the mesencephalon and most of the time, dopamine levels in the VTA are not affected by MPTP. Regardless, the authors need to comment about this concern.

We agree with the reviewer that any changes in dopamine within the SNpc may be masked by dopamine in the VTA, which often show less depletion sing MPTP. As a first approach in our studies we decided not to delineate these two regions since there an overlap and both can be altered by MPTP-lesioning. In our lesioning regimen typically we see about 60 to 70% loss in the SNpc and about 30% loss in the VTA (Jackson-Lewis et al., 1995). To better communicate this concern, we have included a comment on this in the Methods section, page 9, paragraph "Tissue Preparation" and in the Results section, page 15, paragraph 2 (In the amygdala….).

(2b) Although the authors have reported in previous studies that there is about 60% cell loss in the substantia nigra pars compacta (SN-PC) after such MPTP treatment, the lack of dopamine loss in this brain region suggests that perhaps there wasn't a 60% cell loss in the current study. The authors need to comment about this concern in the discussion section and why TH cell counts were not done. It is the opinion of this reviewer that such cell counts or at least optical density measurements of TH immunolabeling of the SN-PC in a comparable series of animals to show that the extent of cell loss was similar to that previously reported by this group in a comparable series of animals.

The Reviewer brings up an important issue for which we now include supportive data. We have in fact performed analyses on the same mice from these studies to evaluate the degree of MPTP-lesioning. Using unbiased stereological counting techniques in our lab we found that the degree of TH-immunoreactive neurons in the midbrain is 68% in mice at 7 days post-MPTP and 66% in mice at 30 days post-MPTP and that there is no statistically significant difference between the two lesioned groups. The additional data is presented in the Results section, page 16, paragraph "TH-Immunoreactivity in Dorsal Striatum and Dopaminergic Cell Loss in SNpc". In addition, we have also re-evaluated the degree of TH-immunoreactive fibers in the striatum. Rather than using conventional immunohistochemical staining with DAB processing we have included our analysis using fluorescent-labeled antibody and captured images using the LI-COR Odyssey infrared imaging system. This allows more precise quantification of the relative differences between striatal TH immunolabeling in the different groups since this system has a much higher degree of sensitivity and is linear over 5 logs. Accordingly, the Methods section is updated to reflect these changes, on pages 10 and 11, paragraphs "Immunohistochemical Staining" and "Unbiased Stereological Counting of SNpc Dopaminergic Neurons" The revised data is presented in the Results section, page 16, paragraph "TH-Immunoreactivity in Dorsal Striatum and Dopaminergic Cell Loss in SNpc". For clarity, we have also modified Figure 6 such that it includes only the HPLC analysis of DA and serotonin. We felt, and hope the Reviewers agree, that the degree of lesioning determined by unbiased stereological counting and striatal TH-ir with ICC is sufficient to report in the Results section but does not necessitate its own figure.

(2c) The same concern is with regards to the loss of 5-HT in the striatum or frontal cortex, while there was no change in levels in the raphe nucleus. Considering how small the raphe nucleus is in the mouse, it is difficult to understand how the authors could claim that they accurately dissected the dorsal and medial parts.

As pointed out by the Reviewer we would like to clarify that samples form the raphe nucleus do contain both the dorsal and medial part and that we did not attempt to dissect by hand these regions. Similar to analysis of the VME we designed these studies as a first pass to detect any significant changes in neurotransmitter levels. Our interpretation of HPLC results is that the raphe nucleus is the locus of serotonergic cell bodies and serotonin levels measured there were smaller compared to the striatum and the frontal cortex where serotonergic terminals are located, similar to the nigrostriatal dopaminergic system. We did detect changes in 5-HT and turnover in this region, while potentially biologically important did not reach statistical significance due to variability in HPLC analysis at these small concentrations. In both the Methods and Results section of this revised manuscript we point out the our dissections of the VME contain the VTA and SN, and our dissections of the raphe nucleus contain both the dorsal and medial aspects.

(2d) In terms of the frontal cortex, did the authors dissection include the motor cortex, which does extend quite a bit rostrally? If so, then the loss of dopamine in this region may be more related to the motor vs non-motor affects and this could affect the interpretation of their data in terms of effects on associative memory. The authors need to comment about what was included in the dissection of the frontal cortex and if indeed it included parts of the motor cortex.

We did not intentionally include any motor cortex into the analysis. Our dissections focused on the most rostral aspect of the cortex (rostral to Bregma +2.50). It is however possible that some minute amount of motor cortex may have been captured in the collection and analysis, but such a small amount should not increase the volume of tissue significantly and hence affect our data. We mention this potential confounder in the Methods section, page 9, paragraph "Tissue Preparation" The revised sentence now reads:" Frontal cortex (rostral to Bregma +2.50), dorsal and ventral regions of mid-striatum (including the nucleus accumbens), amygdala, ventral mesencephalon (containing substantia nigra and VTA) and the raphe nucleus (dorsal and medial part) were rapidly dissected, immediately frozen in isopentane on dry ice and stored at -80°C."

(2e) For the ventral striatum dissection, the assumption of this reviewer is that it included both the ventral part of the striatum itself and the nucleus accumbens. Considering that the nucleus accumbens receives a significant input from the amygdala, it would have made for a stronger paper if the nucleus accumbens could have been separately dissected compared to the rest of the ventral striatum.

The dissections of the ventral striatum did include some the nucleus accumbens. We were able to capture serotonin and dopamine loss as a consequence of MPTP-lesioning in our ventral striatum dissections using HPLC. We intend to more precisely delineate the anatomical site of these changes in future work (i.e. delineating ventral striatum and different regions of the nucleus accumbens such as the core and the shell). To address this issue, we have revised paragraph "Tissue Preparation" in the Methods section, page 9. The revised sentence now reads:" Frontal cortex (rostral to Bregma +2.50), dorsal and ventral regions of mid-striatum (including the nucleus accumbens), amygdala, ventral mesencephalon (containing substantia nigra and VTA) and the raphe nucleus (dorsal and medial part) were rapidly dissected, immediately frozen in isopentane on dry ice and stored at -80°C." We have also included this comment in the Discussion section page 20, end of first paragraph.

(3) Page 11 (Conditioned fear), Figure 3: Although the ANOVA showed a significant difference in

freezing between the saline and MPTP groups, the only difference was at two time points out of 8. Therefore, the entire argument that MPTP affected the conditioned fear response was truly based on only these two time points being significantly different. Although this reviewer is aware that statistically there was a difference between the control and treatment groups, the authors need to explain how the difference in just two time points is relevant.

In order to better communicate the results of this experiment, we revised the Figure 5. The percent of time spent in immobility in now presented as bar graph. The revised figure better communicates our data that MPTP-lesioned mice are not different in their freezing behavior at the baseline (min 1 and 2) and at the onset of conditional stimulus (min 3 and 4). The difference in the behavior is evident only at the end of the test, during the habituation period (min 5-8). Both MPTP-lesioned groups returned to the baseline levels of freezing at the end of the test while saline-injected mice spend significantly more time freezing at the same time point.

(4) Page 15, Figure 6: TH analysis: It is very curious that the relative optical density level of striatal TH immunolabeling after 30 days was nearly 80% of the control value while dopamine tissue values were about 20% of the control value. The authors should offer an explanation for this obvious discrepancy between dopamine levels and TH immunolabeling. This also makes the behavioral data more difficult to interpret in terms of suggesting the importance of dopamine.

As indicated in point 2b above we have evaluated the degree of TH-immunoreactivity using a more sensitive and reliable method of detection. We now report that the degree of TH-immunoreactivity is 42% and 55% of saline at 7 and 30 days post-MPTP, respectively. We have also included this comment in the Methods section and have reduced the complexity of Figure 6 to only focus on dopamine and serotonin levels, the gold standard of basal ganglia integrity.

(5) Page 17: discussion, line 14: it is stated that although dopamine loss in the amygdala was not different in the two groups, "dopaminergic projections form other brain regions to the amygdala …." What other dopamine brain regions project to the amygdala besides the midbrain? Please clarify.

We have changed this sentence to read: "Although dopamine loss in the amygdala did not reach statistical significance in our MPTP-lesioned mice, altered cortical input to the amygdala could be responsible for observed behavioral response."

Article type: Research Article

Title: Memory, Mood, Dopamine, and Serotonin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury

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ABSTRACT

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse serves as a model of basal ganglia injury and Parkinson's disease. The present study investigated the effects of MPTP-induced lesioning on associative memory, conditioned fear, and affective behavior. Male C57BL/6 mice were administered saline or MPTP and separate groups were evaluated at either 7 or 30 days post-lesioning. In the social transmission of food preference test, mice showed a significant decrease in preference for familiar food 30 days post-MPTP compared to controls. Mice at both 7 and 30 days post-MPTP-lesioning had increased fear extinction compared to controls. HPLC analysis of tissues homogenates showed dopamine and serotonin were depleted in the striatum, frontal cortex, and amygdala. No changes in anxiety or depression were detected by the tail suspension, sucrose preference, light-dark preference, or hole-board tests. In conclusion, acute MPTP-lesioning regimen in mice causes impairments in associative memory and conditioned fear, no mood changes, and depletion of dopamine and serotonin throughout the brain.

Keywords: affective behavior; MPTP; Parkinson's disease; anxiety; depression; conditioned fear

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor impairment including slowness of movement, rigidity, balance dysfunction, and resting tremor. However, disabling non-motor symptoms are seen in 30 to 60% of patients and include semantic and episodic memory loss, impairment of executive function, depression, and anxiety (Cummings, 1992; Hornykiewicz, 1963; Pillon et al., 1989a; Walsh and Bennett, 2001). A number of brain regions have been implicated in influencing non-motor behavioral symptoms including the basolateral amygdala, nucleus accumbens, frontal cortex, and the raphe nucleus (Ressler and Nemeroff, 2000) (Walsh and Bennett, 2001). Together with dopamine, serotonin from the dorsal and medial raphe nuclei is thought to play a central role in regulating affective behavior. Perturbation of serotonin neurotransmission in normal individuals can lead to depression, anxiety, and memory impairment (Mann and Yates, 1986; Mann, 1999; Pillon et al., 1989b). Patients with PD develop central serotonergic dysfunction, such as low cortical serotonin levels and degeneration of the dorsal raphe nucleus (Agid et al., 1989; Cummings, 1992; Gotham et al., 1986; McCance-Katz et al., 1992; Scatton et al., 1983). 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) neurotoxicity in the substantia nigra pars compacta (SNpc) produces severe dopamine depletion in mice and nonhuman primates, and causes significant decrease of serotonin across multiple brain regions. For example, chronic MPTP-treatment in monkeys decreases levels of serotonin in the caudate nucleus, putamen, nucleus accumbens, hypothalamus, and cortical areas (Frechilla et al., 2001; Perez-Otano et al., 1991; Pifl et al., 1991; Russ et al., 1991). In mice, acute administration of MPTP leads to serotonin loss in the striatum and frontal cortex one week after lesioning (Rousselet et al., 2003).

The purpose of the current study was to evaluate the effects of MPTP-lesioning on associative memory, conditioned fear, depression, and anxiety, since this neurotoxic injury leads to depletion of dopamine and serotonin in brain regions important for these behaviors. After acute MPTP-lesioning, mice were tested at 7 days (greatest dopamine depletion) and 30 days (partial recovery of striatal dopamine). We used established mouse tests for associative memory (social transmission of food preference), fear conditioning, anxiety (light-dark preference, hole board), and depression (tail suspension, sucrose preference). Brain regions involved in control of affective behavior (frontal cortex, amygdala, and the raphe nucleus), as well as the basal ganglia

(ventral mesencephalon, striatum) were examined for levels of dopamine, serotonin and their metabolites. We observed impairment in associative memory in mice at 30 days post-MPTP-lesioning and increased fear extinction at both 7 and 30 days post-MPTP. Despite significant depletion of both dopamine and serotonin at these time points, there was no significant increase in depression and anxiety compared to control mice. Overall, these results indicate that the acute MPTP-lesioned mouse model manifests some but not all non-motor behaviors seen in patients with PD.

MATERIALS AND METHODS

Animals, Treatment Groups and MPTP Administration

Male C57BL/6 mice, 8 to 10 weeks of age (Charles River Laboratories, Wilmington, MA) and weighing between 25 and 30g were group-housed in a temperature-controlled room under a 12h light/12h dark cycle with free access to water and standard rodent food. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Southern California. A total of 42 mice were used in this study. For lesioning, mice received 4 i.p. injections of 20 mg/kg MPTP (free-base; Sigma-Aldrich, St. Louis, MO) in saline at 2h intervals or 4 injections of 0.1 ml 0.9 % NaCl as control. Mice were tested either at (i) 7 days post-saline (n=20), (ii) 7 days post-MPTP (n=10) or (iii) 30 days post-MPTP (n=12). The degree of lesioning was determined at both 7 and 30 days post-lesioning using unbiased stereological counting of the remaining dopaminergic neurons in the SNpc as well as analysis of the immunoreactivity for tyrosine hydroxylase protein within the striatum. These methods are outlined below and the degree of lesioning was in agreement with previous reports using the same MPTP-lesioning regimen (Jackson-Lewis et al., 1995; Jakowec et al., 2001; Petzinger et al., 2001; Petzinger et al., 2007; Przedborski et al., 2001).

Behavioral Testing

After MPTP-lesioning, mice were subjected to a series of behavioral tests for anxiety (light-dark exploration, hole-board), depression (sucrose preference, tail suspension), associative memory (social transmission of food preference [STFP]), and conditioned fear. Tests were conducted over 6 days with the following order: (1) STFP, (2) light-dark exploration, (3) sucrose preference, (4) hole-board, (5) conditioned odor aversion, (6) tail suspension, and (7) conditioned fear (Fig 1). The design of the behavior testing battery took into account starting with the least stressful test and progressing to the most stressful (Crawley, 2008). The tests which are known to induce the most acute stress response (tail suspension and fear conditioning) (Brown et al., 1984; Liu et al., 2003; Pugh et al., 1997) were conducted at the end of the battery. The order of tests was not randomized. Any potential carry-over effects were equal between the groups as all mice were tested in the same order. Tests occurring on the same day were conducted at least 3h apart. Each behavioral

test was administered once to each mouse. All tests were performed in a darkened room with dim red lights and animals were allowed to habituate to the testing room for 1h prior to each test. Details for each test are presented in the following sections.

Social transmission of food preferences (STFP) for olfactory memory was conducted as described previously (Holmes et al., 2002; Kogan et al., 1997; Wrenn et al., 2003). Briefly, a demonstrator mouse was randomly chosen from each home cage prior to MPTP administration. Initially, all mice were habituated for 18h to powdered chow presented in two 125 g food jar assemblies (Dyets, Inc., Bethlehem, PA) in the opposite corners of the home cage. During this time, standard food pellets were unavailable. One day later, demonstrator mice were removed from their home cages, individually housed, and food-deprived overnight with free access to water. The next day, each demonstrator mouse received powdered chow mixed with either 1% cinnamon or 2% cocoa (w/w) for 1h, or until at least 0.2 g of powdered food was consumed. To avoid a bias in the cued flavor, half of the demonstrators randomly received cinnamon- and the other half cocoa-flavored food. Immediately afterwards, demonstrator mice were returned to their home cages to interact with observer mice for 30 min. At the end of the interaction period, demonstrator mice were removed. Testing of food preference in observer mice took place 24h later, following overnight food deprivation with free access to water. During the test, observer mice were caged individually and were given a free choice of food flavored with 1% cinnamon or 2% cocoa. To control for possible place preference, the position of the food jar assemblies with the cued flavor was balanced between cages. Observer mice were allowed to eat for 1h and food consumption from each jar was determined by weight. The percent of total food intake consumed as the cued flavor was determined.

The **light-dark exploration** test for anxiety was conducted as previously described (Holmes et al., 2002). The test uses the ethological conflict between the tendencies of mice to explore a novel environment and to avoid a brightly lit open area. This test has been shown to be sensitive to changes in serotonergic tone (Holmes et al., 2002). A standard polypropylene mouse cage (30 x 19 x 13 cm) was divided with an opaque partition containing a small opening at the bottom (8 x 5 cm) into a larger light chamber and a smaller dark

chamber. The light chamber (20 x 19 x 13 cm) was transparent and brightly illuminated by a 60 watt bulb placed 40 cm above the cage top. The dark chamber (10 x 19 x 13 cm) was black and closed at the top with a black Plexiglas lid. The test was conducted in a soundproof room and the apparatus was cleaned with warm water and 70% ethanol between each mouse. Each mouse was placed in the lighted chamber facing away from the entrance to the dark chamber, and its behavior was recorded on video for 5 min. Measurements were obtained for: (i) latency to first enter the dark chamber, (ii) time spent in the dark, and (iii) number of transitions between the two compartments. A transition was considered only when a mouse entered into a compartment with 3 or more paws.

The **sucrose preference test** for depression was performed as a modification of the 2-bottle preference test for mice (Strekalova et al., 2004). Mice were deprived of food and water overnight and placed in separate cages 1h before the start of testing. Mice were offered solutions of 1% sucrose (Sigma-Aldrich, MO) or tap water for 1h. Fluid consumption was determined by weight and expressed as percent of total fluid intake consumed from the sucrose solution. The positions of the water and sucrose bottles were alternated to control for side preferences.

The **hole-board test** for anxiety based on exploratory behavior was performed as previously described (Boissier and Simon, 1962; do-Rego et al., 2006). This test is frequently used as an indicator of directed exploratory behavior in rodents (Crawley, 1985). The testing apparatus consisted of a 2 cm-thick square plastic board, 40 × 40cm, with 16 holes (2 cm diameter) regularly spaced 7cm apart over the surface and 3.5 cm from the edges (Ugo Basile, Italy). The board was positioned 50 cm above floor level. Each animal was placed at the corner of the board and allowed to freely explore for 5 min. The number and location of head dips was recorded using a video camera and videotapes were scored by a trained observer blinded to the treatment group. A head dip was considered when a mouse placed its head into a whole up to the neck. Between testing of each mouse, the board surface was cleaned with water and 70% ethanol.

The **conditioned odor aversion with isoamyl-acetate** was used as a rapid assessment of odor detection in mice. The one bottle test was performed as previously described (Passe and Walker, 1985; Wright and Harding, 1982) with minor modifications. Mice were deprived of food and water overnight and housed separately 1h before the start of testing. Testing consisted of four 10 min trials. Breaks between the trials lasted 30 min. During the first two trials, mice were allowed to drink water containing both 0.1% (v/v) isoamyl acetate (artificial banana aroma) and 0.5% (w/v) quinine hydrochloride (bitter taste) (Sigma-Aldrich, MO). During the third trial, mice were tested for avoidance of isomayl acetate odorized water without quinine hydrochloride. The last trial consisted of tap water. Fluid consumption was determined by weight. Preference ratio for isoamyl acetate was determined from the last two trials as follows: odorized water (g) / odorized water + tap water (g). Preference ratio of below 0.5 indicates aversion, and therefore detection of the odorant.

The **tail suspension test** for depression was preformed as previously described (Steru et al., 1985). This test relies on immobility as a measure of “behavioral despair” once the mouse perceives that the escape from the apparatus is impossible. Mice were individually suspended by their tails at a height of 20cm using a piece of adhesive tape wrapped around the tail, 2 cm from the tip. Behavior was videotaped for 6 min. The duration of immobility was measured using a stopwatch. Mice were considered immobile only when hanging completely motionless. Mice that climbed up their tails were excluded from analysis. Results were expressed as percent of time spent immobile.

Auditory conditioned fear response was assessed as previously described (LeDoux, 2000). The test consisted of an 8 min acquisition phase on the first day and an 8 min extinction phase on the next day. Training was conducted in a soundproof room with dim red light and background noise level of 50dB. Each mouse was placed in the middle of a testing chamber (23 × 20 × 20 cm) with an electrified metal rod floor (2 mm diameter, 6 mm separation). The chamber was cleaned with water and 70% ethanol before testing each mouse. The fear acquisition consisted of a 3 min acclimation period, 3 pairings of tone/foot shock separated with 1 min quiet intervals, and 1 min quiet consolidation period at the end of testing. For each pairing of tone/foot shock, mice were presented tone (30 s of 80dB, 1000Hz/8000Hz continuous alternating sequence of

250 ms pulses) generated using LabView 7.1 software (National Instruments Corporation, Austin, TX) and delivered through speakers on the top of the testing chamber. Each tone was immediately followed by a mild foot shock (2 s, 0.6 mA). Freezing behavior (no visible movement except for respiration) was recorded on videotape during the test. The extinction phase was conducted the next day in a different room illuminated with blue indirect light. Each mouse was placed in a cylindrical Plexiglas observation chamber (diameter 28 cm) with a smooth Plexiglas floor. Following an initial 2 min acclimation period, recall and extinction of freezing in response to the tone (presented continuously for 6 min) was monitored in the absence of foot shock. The duration of the freezing response in seconds was measured within each 1-minute interval of both acquisition and extinction phases. Freezing behavior was manually scored using Observer XT version 6.1.35 software (Noldus Information Technology, San Diego, CA).

Tissue Preparation

Brains were collected 24h after the last behavior test. For immunohistochemistry, a subset of mice (n = 4 per group), were sacrificed by pentobarbital overdose and transcardially perfused with 4% paraformaldehyde, post-fixed in the perfusion fixative for 48h at 4℃, cryoprotected in 20% sucrose for 24h, frozen in isopentane on dry ice, and stored at -80℃. For HPLC analysis, another subset of mice (n = 5 per group) were killed by cervical dislocation. Brains were quickly removed and regions of interest identified using a standard mouse brain atlas (Paxinos and Franklin, 2001). Frontal cortex (rostral to Bregma +2.50), dorsal and ventral regions of mid-striatum (including the nucleus accumbens), amygdala, ventral mesencephalon (containing substantia nigra and VTA) and the raphe nucleus (dorsal and medial part) were rapidly dissected, immediately frozen in isopentane on dry ice and stored at -80℃.

Neurochemical Analysis

Neurotransmitter concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, and 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC with electrochemical detection as previously described (Irwin et al., 1992; Kilpatrick et al., 1986; Petzinger et al., 2007). The system consisted of an ESA auto-sampler (ESA Inc., Chelmsford, MA) equipped with a 150 x 3.2

mm reverse phase C-18 column (3µm diameter) regulated at 28℃ and a CoulArray 5600A (ESA Inc, Chelmsford, MA), equipped with a 4-channel analytical cell with potentials set at -100 mV, -30 mV, 220 mV and 350 mV. The HPLC was integrated with a DellGX-280 computer with CoulArray analytical program for Windows (ESA Inc, Chelmsford, MA). Mobile phase consisted of acetonitrile in phosphate buffer and an ion-pairing agent and was delivered at a rate of 0.6ml/min. Fresh frozen tissue was homogenized in 0.4 M $HClO_4$, and centrifuged to separate precipitated protein. The pellet was resuspended in 0.5 M NaOH and used to determine total protein concentration with the CoomassiePlus protein assay (Pierce, Rockford, IL) and microplate reader ELx800 (BioTek Instruments Inc., Winooski, VT) equipped with KCjunior software.

Immunohistochemical Staining

Analysis of relative expression of striatal tyrosine hydroxylase (TH) immunoreactivity was carried out as previously described (Jakowec et al., 2004; Petzinger et al., 2006; Petzinger et al., 2007). Briefly, coronal brain sections were cut at 25µm thickness through the mid-striatum and collected in phosphate-buffered saline (PBS, pH 7.2). Sections were exposed to rabbit polyclonal anti-tyrosine hydroxylase antibody (1:5000, Chemicon, Temecula, CA) for 24h at 4℃ followed by 2h incubation in IRDye700 conjugated goat anti-rabbit IgG (1:2500, Molecular Probes, Eugene, OR). Following extensive washing, sections were mounted on gelatin-coated slides and scanned using LI-COR Odyssey near infrared imaging platform (LI-COR Biotechnology, Lincoln, NE). Multiple brain sections at the mid-striatum (4 to 5 sections per mouse) from 4 mice per group were prepared and analyzed in parallel. Fluorescence intensity within an oval shaped region of interest ($1mm^2$) in dorsal striatum was measured and corrected for background by subtracting the adjacent corpus callosum. Values for treatment groups were normalized to saline animals prior to statistical analysis.

Unbiased Stereological Counting of SNpc Dopaminergic Neurons

The degree of MPTP-lesioning at 7 and 30 days post-MPTP lesioning was determined by unbiased stereological counting of dopaminergic neurons in the SNpc. For this purpose, coronal sections were collected starting rostral to the substantia nigra at Bregma –2.50 mm before the closure of the third ventricle through to the prominence of the pontine nuclei at Bregma –4.24 mm according to the stereotaxic atlas of the mouse brain

(Paxinos and Franklin, 2001). Every sixth section from 4 mice per group was included in the analysis. Sections were exposed to rabbit polyclonal anti-tyrosine hydroxylase antibody (1:5000, Chemicon, Temecula, CA) for 24h at 4℃ followed by and avidin-biotin complex (AB C elite Kit, Vector Labs, Burlingame, CA). Staining was visualized by exposure to 3,3'diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL), after which sections were mounted on gelatin-coated slides, and cover-slipped. Cell nuclei were visualized by cresyl-violet staining. The SNpc was delineated from the rest of the brain based on TH-ir. Sections were examined using an Olympus BX-50 microscope (Olympus Optical, Tokyo, Japan) equipped with a motorized stage and digital Retiga cooled CCD camera (Q-Imaging, Burnaby, British Columbia, Canada). Each stained ventral mesencephalon section was viewed at low magnification (4x) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at higher magnification (40x) and counted if they displayed TH-ir and had a clearly defined nucleus, cytoplasm, and nucleolus. Analysis was performed with the computer-imaging program BioQuant Nova Prime (BioQuant Imaging, Nashville, TN). The total number of SNpc dopaminergic neurons was determined based on the method of Gundersen and Jensen (1987).

Statistical Analysis

With the exception of fear-conditioning, all results were evaluated by a one-way analysis of variance (ANOVA) and Bonferroni multiple comparison test when appropriate, or by one-way ANOVA on ranks followed by Dunn's post-hoc test when the normality test or equal variances test failed. Software used for statistical analysis was Prism5 for Windows (Graph Pad Software Inc., San Diego, CA). A repeated measures ANOVA was used to analyze freezing behavior from the fear-conditioning test. Data from all experiments are presented as mean ± SEM and $p<0.05$ was considered significant.

RESULTS

Social Transmission of Food Preference

Associative olfactory memory was assessed using the STFP test (Fig. 2). When presented with 2 unfamiliar flavors of powdered food, control mice strongly preferred the flavor previously consumed by the demonstrator mouse (79.0 ± 3.7% of total food intake). Lesioned mice tested 7 days after MPTP showed a similar preference (79.0 ± 3.3%). However, preference for the demonstrated flavor declined significantly in mice tested 30 days post-MPTP (58.7 ± 6.3%) ($F_{(2,41)}$=5.614; $p<0.05$). As the STFP test relays on the ability of mice to discriminate odors, it was important to test if all mice had similar olfactory function. For this purpose, the conditioned odor aversion test with isoamyl acetate was used. Mice from all three groups avoided water odorized with isoamyl acetate (preference ratio for saline: 0.4 ± 0.1; 7 days post-MPTP: 0.2 ± 0.1; 30 days post-MPTP: 0.3 ± 0.1). Preference ratio below 0.5 indicates the ability to discriminate odors. Based on these results, mice in this study did not display detectable impairment in olfactory function.

Light-Dark Preference and Hole-Board

Light-dark preference and the hole-board tests were used to determine levels of anxiety in mice 7 and 30 days post-MPTP-lesioning (Fig. 3). Mice from all three groups showed a similar preference for the dark compartment ($F_{(2,39)}$ = 1.428; $p>0.05$) during the light-dark exploration test (Figure 3A). Saline-treated mice spent 66.1 ± 3.7% of the time in the dark. Likewise, mice at 7 days post-MPTP-lesioning spent 74.5 ± 3.6% of time in the dark and those tested at 30 days post-MPTP-lesioning spent 65.4 ± 3.3% of the time in the dark. The average number of transitions between the light and dark compartments was similar in all three groups (13.1 ± 1.6 for saline mice; 12.6 ± 1.3, and 16.0 ± 1.9 for mice 7 days and 30 days post-MPTP-lesioning, respectively; $F_{(2,39)}$ = 1.246; $p>0.05$). There was no significant difference between the latency to first enter the dark compartment between groups (26.7 ± 5.3 s for saline mice; 15.9 ± 4.2 s and 25.6 ± 5.6 s for mice 7 days and 30 days post-MPTP-lesioning, respectively; $F_{(2,39)}$ = 0.9846; $p>0.05$). Exploratory behavior was measured in the hole-board test as a second test of anxiety (Fig. 3B). As measured by head dipping, saline treated mice visited 24.8 ± 2.2 holes during the 5 min test, similar to mice at 7 days post-MPTP-lesioning (24.5 ± 3.0 holes). Mice tested at 30 days post-MPTP-lesioning had 33.0 ± 3.1 head dips. The difference in the total number of head dips between groups was not statistically significant ($F_{(2,33)}$ = 3.053; $p>0.05$).

Sucrose Preference and Tail Suspension

Sucrose preference and the tail suspension tests were used to determine levels of depression in mice after MPTP-lesioning (Fig. 4). In particular, the sucrose preference test measures anhedonia following overnight water deprivation (Strekalova et al., 2004). Mice from all three groups had high preference for a 1% sucrose solution (80-85%) compared to tap water (15-20%) (Fig. 4A). There was no significant difference in the amount of sucrose consumed ($F_{(2,38)} = 0.968$; $p>0.05$). Furthermore, all mice had similar fluid intake during the 1h testing period (saline mice: 0.9 ± 0.1g; 7 days post-MPTP-lesioning: 0.8 ± 0.1g; and 30 days post-MPTP-lesioning: 1.1 ± 0.1g; $F_{(2,37)} = 1.268$; $p>0.05$). The tail suspension test measures behavioral despair (Steru et al., 1985). Saline-treated mice spent 36.6 ± 3.1% of total time in passive immobility (Fig. 4B). Similarly, mice at 7 days post-MPTP-lesioning spent 29.3 ± 3.3% of total time in immobility. Interestingly, mice tested at 30 days post-MPTP-lesioning spent significantly more time immobile (44.3 ± 3.2%) compared to the 7 days post-MPTP group ($F_{(2,39)} = 4.372$; $p<0.05$), however, this was not statistically different compared to saline-treated mice. Only one mouse in the control group was excluded form the test because it climbed up its tail. None of the MPTP-lesioned mice were excluded from the test.

Conditioned Fear

Acquisition and extinction of conditioned fear response was measured using the auditory fear conditioning test. All mice showed little or no freezing during the baseline period of acquisition session (percent time freezing for saline: 1.4 ± 0.4%; for 7 days post-MPTP: 0.2 ± 0.1%; and for 30 days post-MPTP: 0.7 ± 0.4%). During subsequent pairings of tone and foot shock, all mice showed increased freezing behavior. Mice in all three groups had similar levels of freezing after the third foot shock (saline: 54.9 ± 10.7%; 7 days post-MPTP: 46.2 ± 10.5%; and 30 days post-MPTP: 49.4 ± 4.1%; $F_{(2,16)} = 0.249$; $p>0.05$). The next day, (Fig. 5), the baseline freezing response of all mice was similar (percent freezing for saline: 23.7 ± 6.2%; 7 days post-MPTP: 9.6 ± 2.4%; and 30 days post-MPTP: 18.5 ± 8.3%). At the onset of auditory stimulus (without foot shock), all mice showed a robust increase in the freezing response (saline: 68.8 ± 3.1%; 7 days post-MPTP: 56.8 ± 6.7%; and 30 days post-MPTP: 51.0 ± 6.2%). However, after 6 minutes of continuous tone exposure, both groups of MPTP-lesioned mice spent significantly less time freezing (7 days post-MPTP: 9.5 ± 3.2%; and 30 days post-

MPTP: 17.5 ± 8.2%) compared to saline controls (43.7 ± 6.0%). Repeated measures ANOVA followed by Bonferroni post-test showed a significant difference in freezing response over time between saline-treated and MPTP-lesioned mice at both 7 and 30 days ($F_{(2,17)}$ = 23.08; $p<0.05$).

HPLC Analysis of Dopamine, Serotonin, and their Metabolites

HPLC analysis was used in tissue homogenates to determine the levels of dopamine and its metabolites (DOPAC and HVA), as well as serotonin and its metabolite 5-HIAA. The turnover ratio for dopamine was determined as ([DOPAC] + [HVA]) / [dopamine] and for serotonin as [5-HIAA] / [serotonin]. Six brain regions were analyzed including the frontal cortex (rostral to the motor cortex), dorsal striatum, ventral striatum (including the nucleus accumbens), ventral mesencephalon (VME) (including the substantia nigra and VTA), amygdala, and the raphe nucleus (including the dorsal and medial raphe) (Table 1 and Fig. 6). Taken together, MPTP-lesioned mice had severe dopamine depletion in the dorsal and ventral striatum and frontal cortex, and no significant loss in the amygdala, VME, or the raphe nucleus. The greatest depletion of dopamine was measured at 7 days post-MPTP. Serotonin was significantly depleted in the dorsal and ventral striatum, frontal cortex, and amygdala. The greatest loss of serotonin was measured at 30 days post-MPTP-lesioning. There was no significant change in the level of the serotonin metabolite 5-HIAA in any of the examined brain regions.

Among all six regions investigated, the dorsal striatum of saline-treated mice contained the highest concentration of dopamine (141.3 ± 12.4 ng dopamine/mg protein). Here, acute MPTP-lesioning caused a significant loss of dopamine that persisted for at least 30 days ($F_{(2,14)}$ = 89.00; $p<0.05$). There was 95% depletion in mice at 7 days post-MPTP (7.5 ± 1.6 ng dopamine/mg protein) and 86% depletion at 30 days post-MPTP-lesioning (19.9 ± 5.2 ng dopamine/mg protein). The change in dopamine turnover ratio did not reach significance at 7 days (1.1 ± 0.4) nor at 30 days post-MPTP-lesioning (1.4 ± 0.3) compared to controls (0.2 ± 0.0) ($F_{(2,14)}$ = 3.654; $p>0.05$). The ventral striatum of saline-treated mice contained the second highest concentration of dopamine (91.0 ± 7.7 ng dopamine/mg protein). In this region MPTP caused an 86% depletion at 7 days post-MPTP-lesioning (13.0 ± 1.3 ng dopamine/mg protein) and 77% depletion at 30 days post-MPTP

(21.6 ± 6.1 ng dopamine/mg of protein) ($F_{(2,14)}$ = 56.63; $p<0.05$). However, differences in dopamine turnover ratio were not statistically significant ($F_{(2,14)}$ = 3.654; $p>0.05$), similar to dopamine turnover in the dorsal striatum.

In the frontal cortex of control mice the dopamine concentration (4.0 ± 1.5 ng dopamine/mg protein) was low compared to the striatum. Nonetheless, MPTP-lesioning caused significant dopamine loss (88% depletion) at 7 days after MPTP (0.5 ± 0.1 ng dopamine/mg protein) and at 30 days post-MPTP (0.6 ± 0.2 ng dopamine/mg protein; 86% depletion) ($F_{(2,14)}$ = 5.43; $p<0.05$). Furthermore, dopamine turnover ratio was significantly increased at 7 days (1.4 ± 0.2) and 30 days (4.2 ± 1.3) post-MPTP-lesioning, compared to controls (0.8 ± 0.2) ($F_{(2,14)}$= 5.863; $p < 0.05$).

In the amygdala of saline-treated mice dopamine levels (9.2 ± 2.5 ng dopamine/mg protein) were about one-tenth of those in the striatum. MPTP-induced dopamine loss in amygdala did not reach statistical significance ($F_{(2,14)}$ = 3.132; $p>0.05$), and dopamine turnover ratio remained similar between the groups (Table 1). Cell bodies of dopamine and serotonin-producing neurons are located in VME and the raphe nucleus, respectively. These two regions had low detectable levels of dopamine in control mice (Table 1) but MPTP-lesioning did not cause statistically significant dopamine loss in the VME nor the raphe nucleus (Fig.6A). The fact that the VME also contained the VTA, which is less affected by MPTP-lesioning, accounts for the fact changes in dopamine within this region were not statistically different from saline control at 7 days post-lesioning but did show a non-statistical difference at 30 days post-lesioning.

HPLC analysis of serotonin in tissue homogenates showed that loss of this neurotransmitter was modest compared to dopamine loss following MPTP-lesioning. In the dorsal striatum of saline control mice, serotonin was low (7.1 ± 0.8 ng serotonin/mg protein) but nonetheless, was significantly depleted at 7 days (3.7 ± 0.5 ng serotonin/mg protein, 48% loss) and at 30 days post-MPTP-lesioning (3.3 ± 0.4 ng serotonin/mg protein, 64% loss) ($F_{(2,14)}$ = 13.10; $p<0.05$). The ventral striatum of control mice contained twice as much serotonin compared to the dorsal striatum (16.4 ± 1.8 ng serotonin/mg protein). Here, the serotonin loss was

moderate but still significant: 10.0 ± 0.9 ng/mg protein or 39% depletion at 7 days and 9.1 ± 1.6 ng serotonin/mg protein or 44% depletion at 30 days post-MPTP-lesioning respectively ($F_{(2,14)}$ = 6.80; $p<0.05$). The serotonin turnover ratio in both the dorsal and ventral striatum did not change significantly following MPTP-lesioning (Table 1).

Serotonin concentrations in the frontal cortex (12.8 ± 1.1 ng serotonin/mg protein) and amygdala (11.9 ± 0.7 ng serotonin/mg protein) were similar in control mice. MPTP-lesioning caused a 48% depletion in the frontal cortex at 7 days (to 6.7 ± 1.5 ng serotonin/mg protein) and 60% depletion at 30 days post MPTP-lesioning (to 5.1 ± 1.0 ng serotonin/mg protein). This decrease was statistically significant compared to controls ($F_{(2,14)}$ = 11.28; $p<0.05$). There was no change in serotonin turnover ratio in the frontal cortex (Table 1). In the amygdala, serotonin remained unchanged at 7 days post-MPTP (8.5 ± 1.6 ng serotonin/mg protein); however, it was significantly depleted at 30 days post-MPTP-lesioning (6.9 ± 1.3 ng serotonin/mg protein) compared to control ($F_{(2,14)}$ = 4.03; $p<0.05$).

The raphe nucleus (containing both the dorsal and medial aspects) and VME (containing the substantia nigra and VTA) had high average serotonin concentrations in all mice (Table 1) and MPTP-lesioning did not cause a significant depletion of serotonin in these regions.

TH-Immunoreactivity in Dorsal Striatum and Dopaminergic Cell Loss in SNpc

TH immunoreactivity is often used as a marker of the integrity of dopaminergic axons in the striatum (Jakowec et al., 2004; Petzinger et al., 2007). There was significant reduction of TH-immunireactivity (TH-ir) in dorsal striatum of mice 7 and 30 days post-MPTP-lesioning ($F_{(2,9)}$ = 45.41; $p<0.05$). MPTP caused a 58% reduction of TH-ir in the dorsal striatum examined at 7 days post-lesioning and 45% loss at 30 days post-MPTP-lesioning.

The number of surviving TH-positive neurons in the SNpc was used as an additional measure of the integrity of midbrain dopaminergic system 7 and 30 days post-MPTP-lesioning. MPTP-lesioning caused 68% loss of TH-ir SNpc neurons in 7 days post-MPTP and 66% loss at 30 days post-MPTP.

DISCUSSION

The MPTP-lesioned mouse serves as a model of basal ganglia injury and Parkinson's disease. While the majority of studies focus specifically on motor deficits, few studies have addressed the non-motor features including affective behavior. The purpose of this study was to examine non-motor behaviors (associative memory, conditioned fear, anxiety, and depression) in the C57BL/6 mouse following an standardized acute lesioning regimen with MPTP (Jackson-Lewis et al., 1995). We report associative memory impairment measured by STFP evident only at 30 days post-MPTP. In addition, mice had increased fear extinction at both 7 days and 30 days post-MPTP. In contrast, there were no significant changes in anxiety (measured by the hole-board and light-dark preference tests), or depression (measured by the sucrose preference and tail-suspension tests). Dopamine and serotonin levels in brain homogenates were depleted in the striatum, frontal cortex, and amygdala.

Dopaminergic neurotransmission within the basal ganglia has been implicated in cognitive processes, and specifically, in associative learning (Alcaro et al., 2007). However, only a few studies have examined memory function after basal ganglia injury in rodent models. A previous study reported that MPTP-lesioned CD-1 mice had impaired social memory and recognition behavior (Dluzen and Kreutzberg, 1993). In our study, the STFP test revealed impaired associative memory in mice only at 30 days post-MPTP-lesion compared to saline-treated mice. At this time point, there was still an 86% depletion of dopamine and a 60% depletion of serotonin within the frontal cortex. This is consistent with other studies using 6-OHDA-lesioning in rats where performance in the STFP test depends on an intact frontal cortex (Ross et al., 2005) and basal forebrain (Berger-Sweeney et al., 2000). Taken together, these data support the importance of the frontal cortex and the role of dopamine and serotonin in the associative learning processes.

The fear conditioning response is mediated by the basolateral amygdala, hippocampus, medial prefrontal cortex, and nucleus accumbens (Davis and Whalen, 2001; Helmstetter, 1992; LeDoux, 2000; Maren and Quirk, 2004). The extinction of conditioned fear is a progressive decrease of the fear response generated by repeated presentation of the conditioned stimulus (tone) without any unconditioned stimulus (foot-shock). Changes in the extinction of conditioned fear can be influenced by either glutamate or dopamine neurotransmission particularly in the frontal cortex and amygdala (Falls et al., 1992; Guarraci et al., 1999; Ledgerwood et al., 2003; Walker et al., 2002). For example, dopamine D1 and D2 receptor antagonists targeting the amygdala can lead to potentiated extinction of fear response (Greba et al., 2001; Greba and Kokkinidis, 2000; Nader and LeDoux, 1999; Ponnusamy et al., 2005). In our study, MPTP-lesioning had no effect on the acquisition of the fear response; instead we observed increased fear extinction at both early and late time points after MPTP-lesioning. Interestingly, a recent study in patients with PD reported decrease of the startle response to aversive stimuli and this behavior was linked to dopamine dysfunction in the amygdala and frontal cortex (Bowers et al., 2006). Although dopamine loss in the amygdala did not reach statistical significance in our MPTP-lesioned mice, altered cortical input to the amygdala could be responsible for observed behavioral responses. It is possible that significant loss of dopamine in the frontal cortex in mice at 7 and 30 days post-MPTP could be involved in this process. It is know that GABAergic interneurons surrounding the basolateral amygdala receive cortical and mesolimbic dopaminergic input (Marowsky et al., 2005). Modulation of dopamine gate in these neurons may modulate stress-induced behavioral responses (Bowers et al., 2006; Marowsky et al., 2005). Dopamine loss in the frontal cortex, which we observed in MPTP-lesioned mice, could cause disinhibition of these interneurons and block the output of the amygdala in response to aversive stimuli, thus preventing the normal fear induced freezing response.

Dopamine, serotonin, and other neurotransmitter system perturbations are involved in anxiety disorders and may account for the clinical anxiety seen in more than 40% of patients with PD (Walsh and Bennett, 2001; Wood and Toth, 2001). In our study, using light-dark exploration and the hole-board tests we found no increase in anxiety at either 7 or 30 days post-MPTP-lesioning. These results are in agreement with others

who also showed no difference in anxiety using the light-dark preference test 7 days after MPTP-lesioning in the mouse (Rousselet et al., 2003). Depression is the most common co-morbid disorder affecting up to 45% of patients with PD (Slaughter et al., 2001). We examined depression in our model using both the tail suspension and sucrose preference tests, since both may be influenced by serotonin dysfunction (Jones and Lucki, 2005; Lira et al., 2003; Mayorga et al., 2001; Steru et al., 1985). We did not observe increased depression in MPTP-lesioned mice using these tests. Interestingly, a recent study using the bilateral 6-OHDA-lesioned rat reported an increase in depression-like behavior that was measured using the forced swim test, with no changes in serotonin levels (Branchi et al., 2008). It is possible that the neurotoxicant lesioning paradigm, time after lesion, and species used could underlie differences in observed behaviors in these two animal models.

Lesioning of the dopaminergic system, using 6-OHDA or MPTP, also leads to perturbations of the serotonergic system. However, the extent and nature of these perturbations depends on a number of factors including animal age, toxin used, lesioning regimen, and lesion severity. For example, rats and mice lesioned with 6-OHDA in early postnatal life develop serotonergic hyper-innervation within the striatum and frontal cortex and elevated levels of serotonin (Avale et al., 2004; Berger et al., 1985; Snyder et al., 1986; Yamazoe et al., 2001). This is in contrast to lesioning in adults where serotonin levels remain unchanged (Branchi et al., 2008; Snyder et al., 1986; Stachowiak et al., 1986). On the other hand, MPTP-lesioning in adult animals causes a significant decrease of striatal and extra-striatal serotonin levels. For example, in the nonhuman primate, MPTP-lesioning causes decreased levels of serotonin in multiple brain regions including the caudate nucleus, putamen, nucleus accumbens, hypothalamus, and cerebral cortex (Frechilla et al., 2001; Perez-Otano et al., 1991; Pifl et al., 1991; Russ et al., 1991). In our studies, we found significant depletion of serotonin following MPTP-lesioning in the mouse, a similar effect reported by others (Rousselet et al., 2003) but not all (Sedelis et al., 2000). Despite serotonin depletion in brain regions important for affective behavior, our MPTP-lesioned mouse did not show significant changes in anxiety and depression. The lack of behavioral effect could be explained by (i) the level of serotonin depletion may not be severe enough to manifest elevated anxiety, or (ii) the affected neurotransmitter systems may compensate to overcome perturbation. The serotonergic system

following MPTP-lesioning may compensate in an analogous fashion to that of the dopaminergic system where studies in our lab have shown recovery of dopamine function due to increased evoked release of dopamine and altered dopamine receptor expression (Petzinger et al., 2007). On the other hand, studies in PD patients have shown neuronal loss in the dorsal raphe nucleus which could cause mood disorders (Agid and Blin, 1987; Paulus and Jellinger, 1991; Scatton et al., 1983). However, these abnormalities in humans are thought to develop at later stages of the disease, following dysfunction of dopaminergic system (Dauer and Przedborski, 2003). With this in mind, there is a possibility that behavioral changes in our acute MPTP-lesioned mouse may develop at later time points after treatment. Future studies could be designed to examine different lesioning regimens (acute versus chronic), different time points after lesioning (early versus late), and could include pharmacological challenges to the serotonergic system to reveal dysfunction in this neurotransmitter system. In addition, our initial analysis of neurotransmitter changes involved tissue sections such as the ventral striatum that did not delineate potentially interesting anatomical sites such as the core and shell of the nucleus accumbens. Future studies using higher resolution techniques such as molecular imaging can be designed to examine altered innervation and synaptogenesis within these regions thought to influence non-motor behaviors.

The MPTP-lesioned mouse is commonly used as an animal model of PD. Specific motor functions such as skilled forepaw use, balance, and coordination have been consistently reported to be impaired following MPTP-lesioning (Meredith and Kang, 2006; Meredith et al., 2008; Rozas et al., 1998; Sedelis et al., 2000; Tillerson et al., 2002; Tillerson and Miller, 2003). The behavioral tests used in our study do not rely on any of these specific motor skills. For example, saline control and MPTP-lesioned mice showed similar levels of ambulatory activity in the hole-board and light-dark preference tests. We therefore conclude that MPTP-lesioned mice in the present study did not exhibit obvious impairment of spontaneous locomotor activity that could influence their performance in affective behavior tests used in these studies.

In conclusion, our data showed impairment in associative memory at 30 days and increased fear extinction at both 7 and 30 days post-MPTP-lesioning, but no significant increase in depression or anxiety. Impairments in memory and fear conditioning were accompanied by severe depletion in dopamine and serotonin levels in the amygdala, frontal cortex, and striatum. It is possible that the emergence of depression and anxiety in this mouse model depends upon greater serotonin loss in critical brain regions. The findings from this study suggest that mood disorders in patients with PD with may not develop before extensive damage in multiple brain regions occurs.

Acknowledgements: The authors would like to acknowledge the generous support of Team Parkinson, The Parkinson Alliance, and the Whittier PD Education Group. This study was funded by NINDS RO1 NS44327-1, NIDCD K18 DC009125, and U.S. Army NETRP (Grant # W81XWH-04-1-0444). We are thankful to Friends of the USC Parkinson's Disease Research Group including George and MaryLou Boone and Walter and Susan Doniger for their generous support. Authors would like to thank Susan Geffen for help with statistical analysis.

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Figure and Table Legends:

Fig. 1: Diagram representing the timeline and order of behavioral tests following acute MPTP-lesioning (4 x 20 mg/kg, 2h apart) or saline injections (4 x 0.1 ml, 2h apart). The order of behavioral tests was designed according to increased stress. Separate groups of mice were tested at 7 or 30 days post-MPTP-lesioning.

Fig. 2: Associative memory impairment in MPTP-lesioned mice measured by the social transmission of food preference test. The preference for familiar food was tested in control (n = 20) and lesioned mice at 7 days (n = 10) and 30 days post-MPTP (n = 12). Data are presented as mean ± SEM of percent preference for familiar food. The symbols " * " and " # " represent statistically significant differences compared to the saline control and the 7 days post-MPTP groups, respectively ($p < 0.05$).

Fig. 3: Anxiety in mice 7 and 30 days post-MPTP-lesioning measured in light-dark preference and the hole-board tests. Data are presented as mean ± SEM from control (n = 20), 7 days (n = 10) and 30 days post-MPTP (n = 12). (A) Time spent in the dark compartment during the 5 min light-dark preference test (in percent of total time). (B) The total number of head dips during the 5 min hole-board test.

Fig. 4: The effect of acute MPTP-lesioning on depression in mice 7 and 30 days post-treatment measured using sucrose preference and tail suspension tests. Data are presented as mean ± SEM of (A) preference for 1% sucrose and (B) time spent in immobility (in percent of total time) for control (n = 18), 7 days (n = 10) and 30 days post-MPTP (n = 12). The symbol " # " represents statistically significant difference compared to the 7 days post-MPTP group ($p < 0.05$).

Fig. 5: Acquisition and extinction of fear response in MPTP-lesioned mice measured in the fear conditioning test. Fear-induced immobility was measured in control (n = 5), 7 days (n = 6), and 30 days post-MPTP mice (n

= 8). Data are presented as mean ± SEM of the freezing response (percent of 2 or 4 min periods). Following 2 min baseline, continuous tone was played for 6 min without foot shock (tone onset: min 3-4; tone continuation: min: 5-8). The symbol " * " indicates statistically significant difference compared to the saline control group ($p < 0.05$).

Fig. 6: Dopamine and serotonin levels in MPTP-lesioned mice. Twenty four hours following behavioral testing, the dorsal striatum (dStr), ventral striatum (vStr), frontal cortex (fCtx), amygdala, ventral mesencephalon (VME), and the raphe nucleus (RN) tissue homogenates were analyzed for monoamine concentrations (n= 5/group) using HPLC. Data are presented as mean ± SEM. (A) Dopamine and (B) serotonin loss relative to control mice in brain regions regulating associative memory and affective behavior at 7 and 30 days post-MPTP-lesioning. The symbol " * " indicates statistically significant differences compared to the saline control group ($p < 0.05$).

Table 1: HPLC analysis of dopamine, serotonin, and their calculated turnover ratios in the dorsal striatum, ventral striatum, frontal cortex, amygdala, ventral mesencephalon (VME), and raphe nucleus from control, 7 days, and 30 days post-MPTP-lesioned mice (n = 5/group). Data are presented as mean ± SEM of monoamine concentrations (in ng/mg of protein). Abbreviations: DA – dopamine; 5-HT – serotonin. Dopamine turnover ratio was calculated as: ([DOPAC] + [HVA]) / [DA] and serotonin turnover ratio as: [5-HIAA] / [5-HT]. The symbol "*" indicates statistically significant differences compared to the saline control group ($p < 0.05$).

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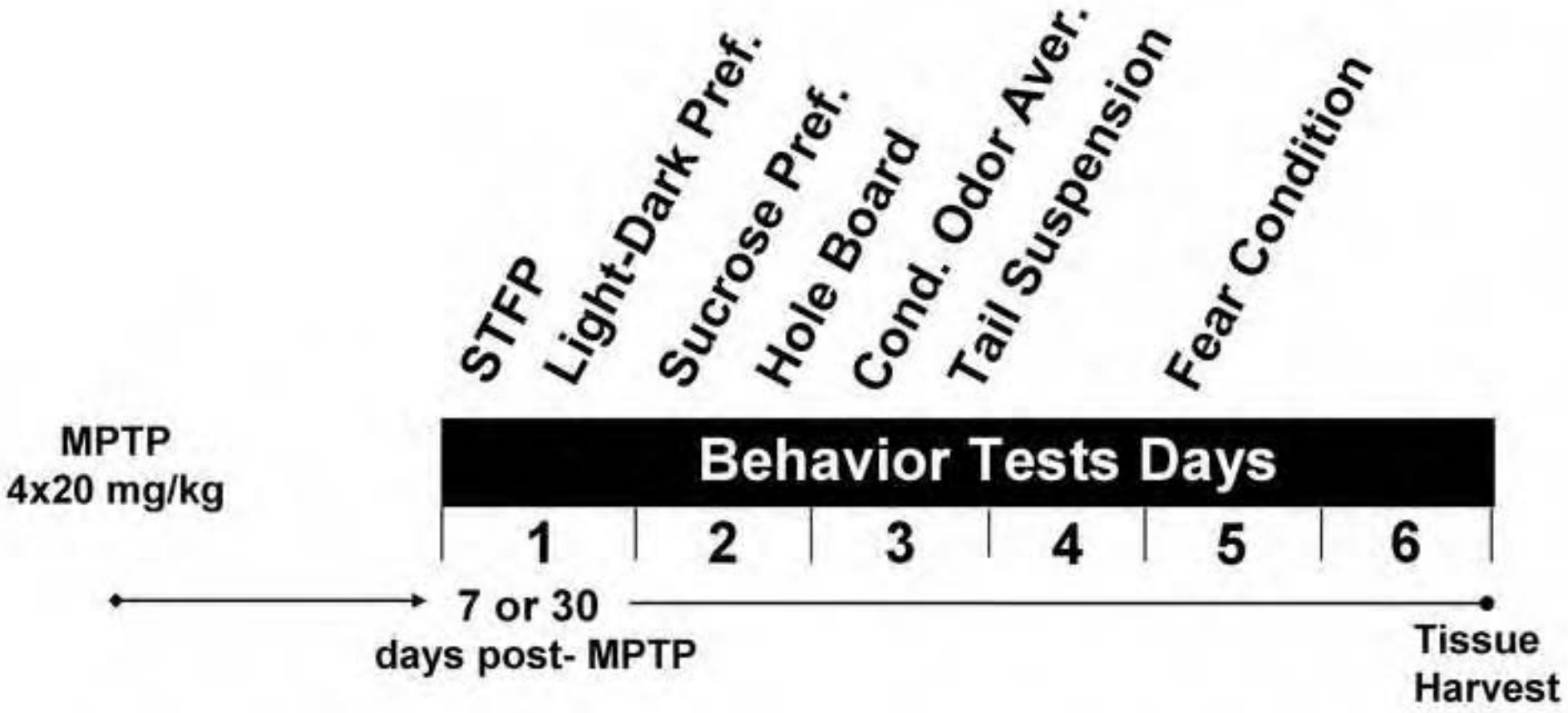

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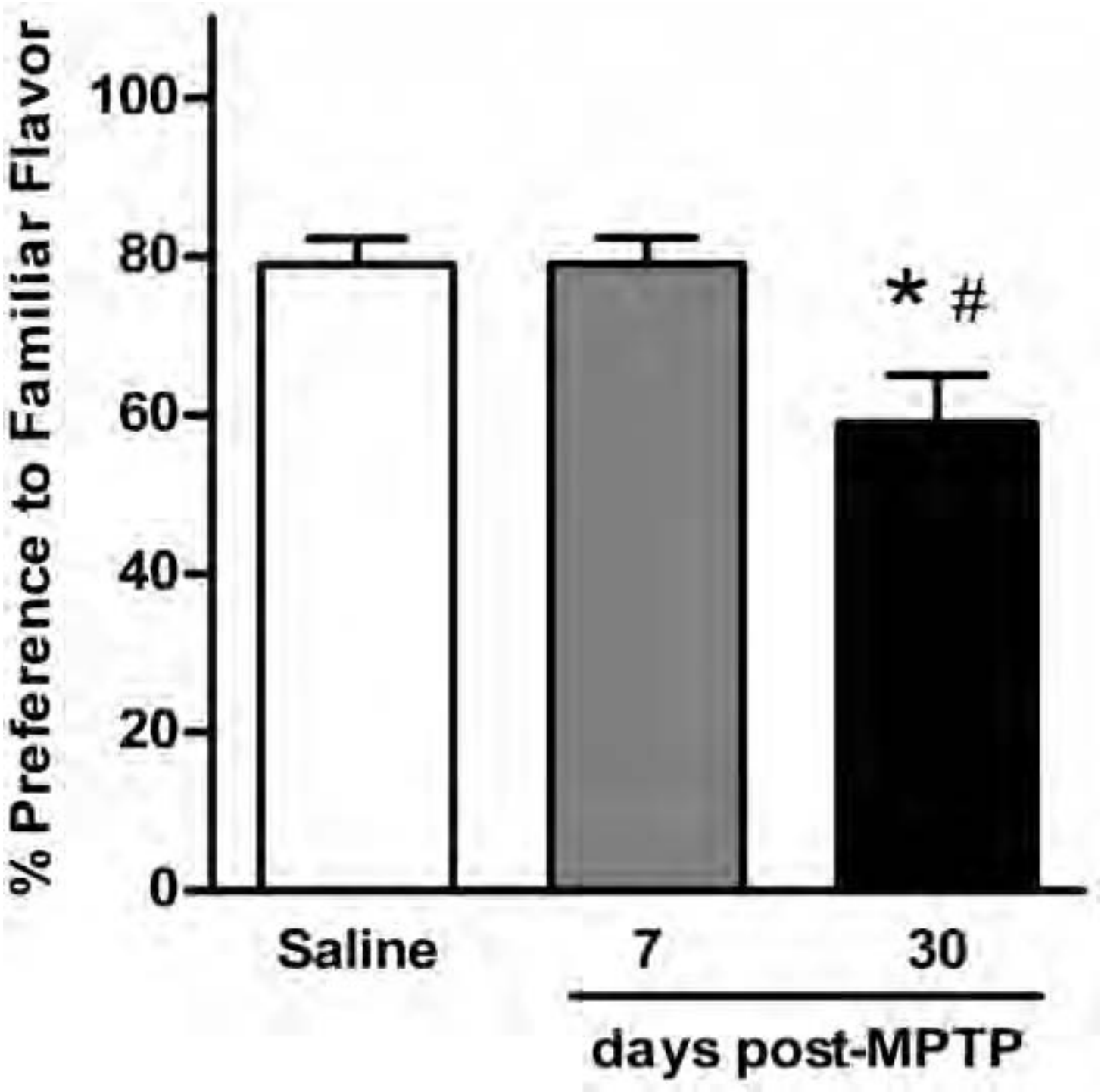

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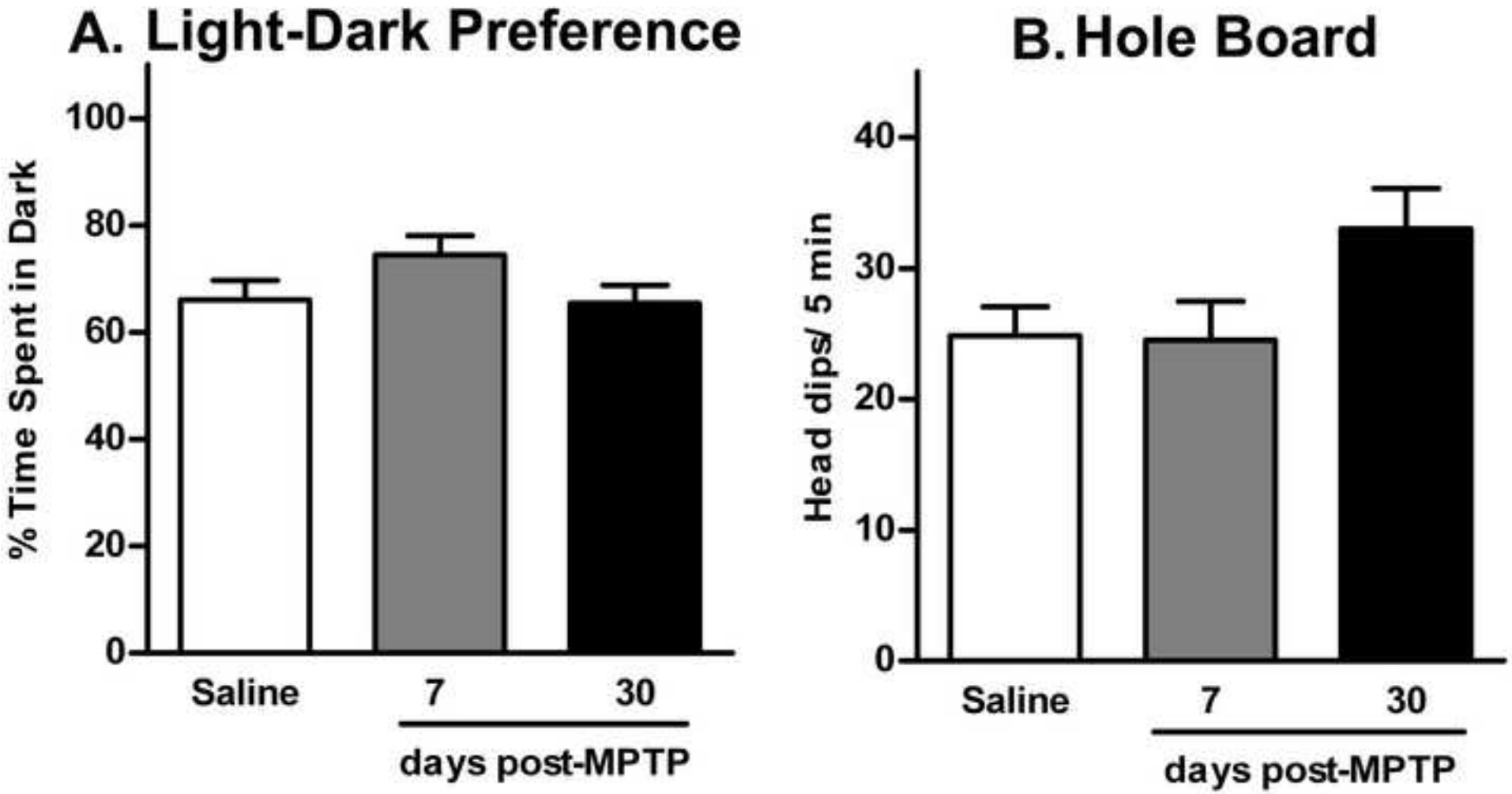

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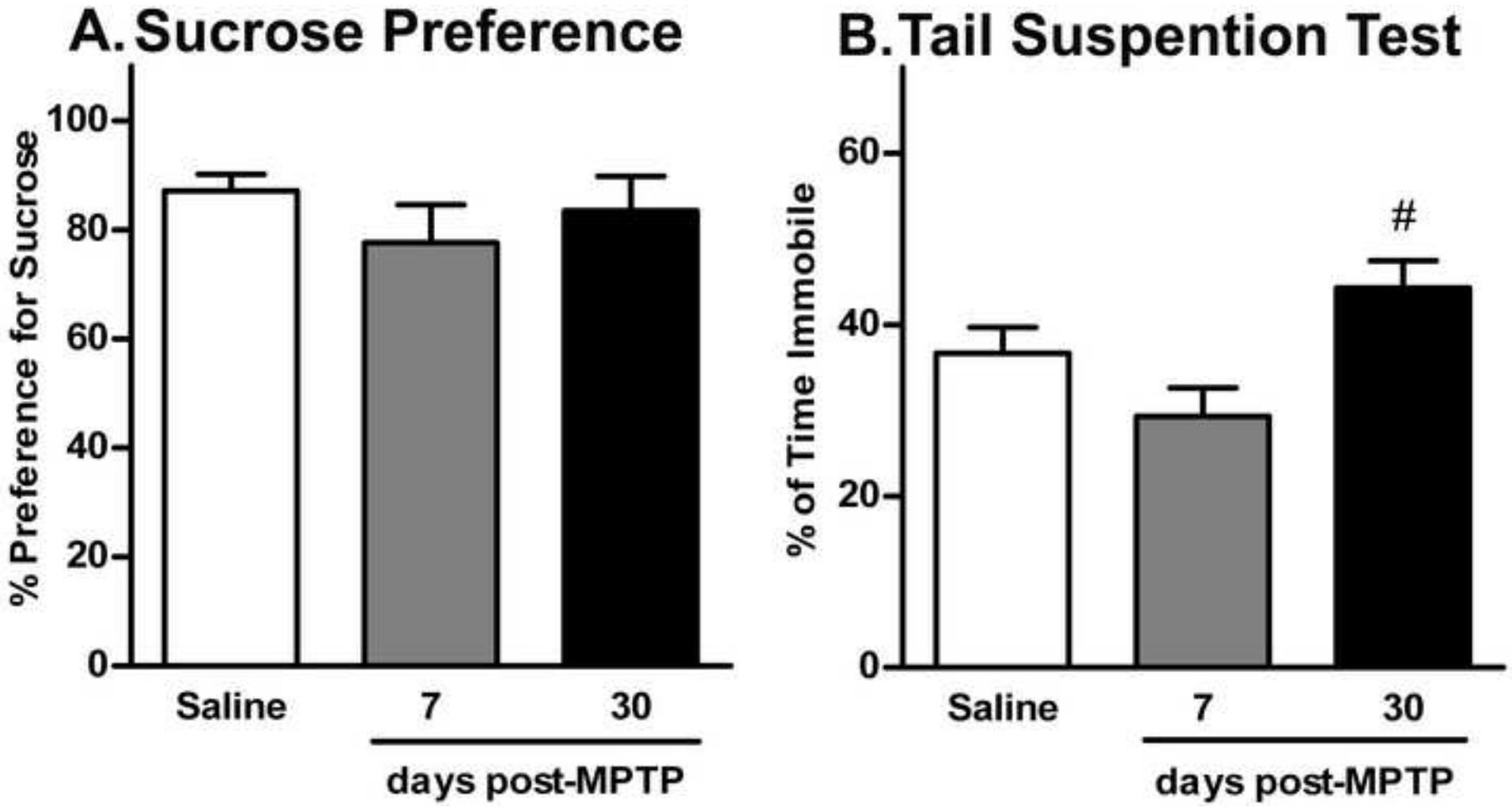

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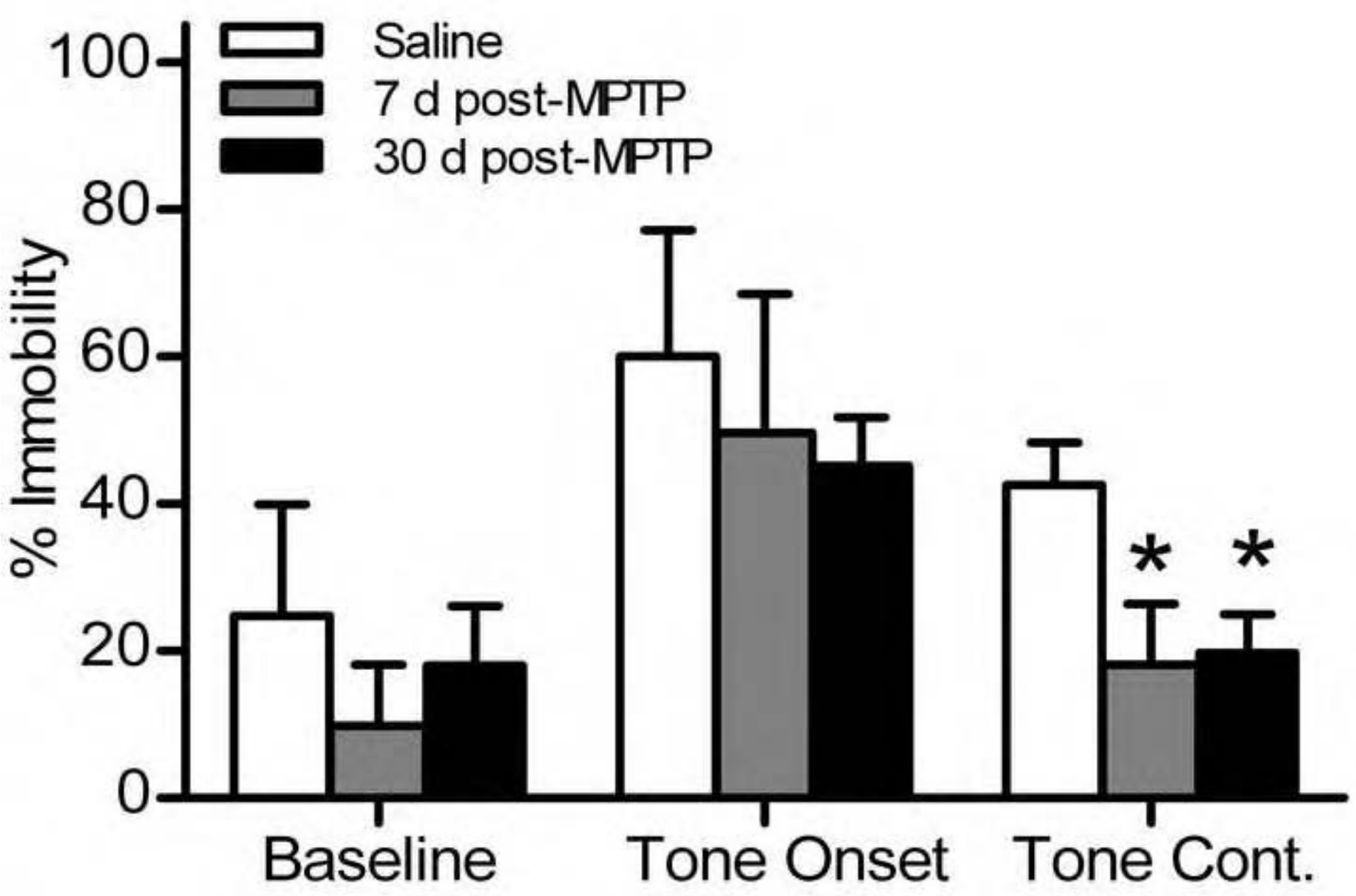

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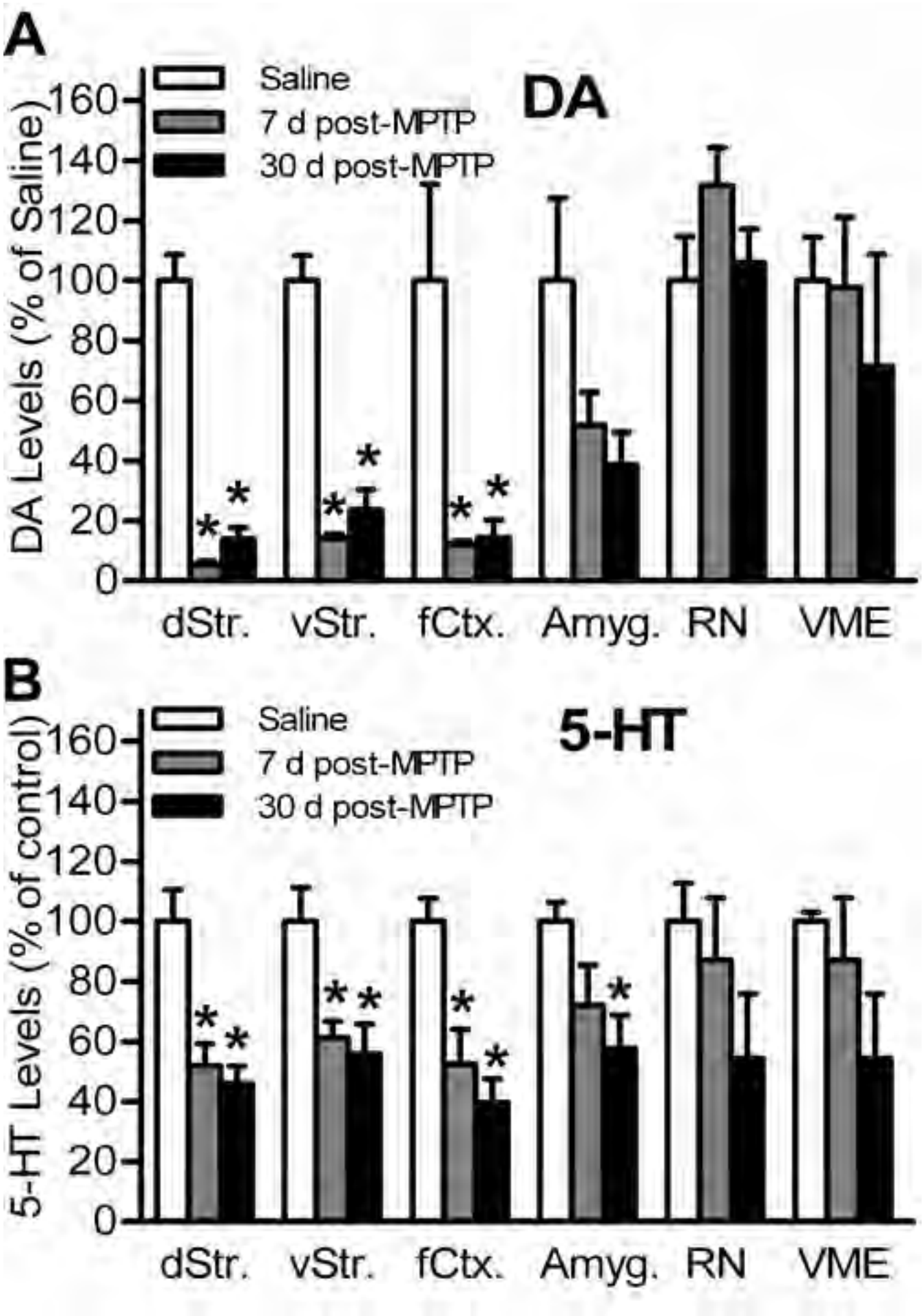

4-Table

Dopamine and serotonin levels measured by HPLC and their calculated turnover ratios in six brain regions of the MPTP-lesioned and control mice.

	Treatment	DA	DA Turnover	5-HT	5-HT Turnover
Dorsal Striatum	Control	141.3 ± 12.4	0.2 ± 0.0	7.1 ± 0.8	1.0 ± 0.1
	7 d post-MPTP	7.5 ± 1.6*	1.1 ± 0.5	3.7 ± 0.5*	2.4 ± 1.0
	30 d post-MPTP	19.9 ± 5.2*	1.4 ± 0.3	3.3 ± 0.4*	2.9 ± 0.5
Ventral Striatum	Control	91.1 ± 7.7	0.2 ± 0.0	16.4 ± 1.8	0.7 ± 0.1
	7 d post-MPTP	13.0 ± 1.3*	0.6 ± 0.2	10.0 ± 0.9*	1.1 ± 0.4
	30 d post-MPTP	21.6 ± 6.1*	1.2 ± 0.4	9.1 ± 1.6*	1.9 ± 0.6
Frontal Cortex	Control	4.0 ± 1.5	0.8 ± 0.2	12.8 ± 1.1	0.5 ± 0.0
	7 d post-MPTP	0.5 ± 0.1*	1.4 ± 0.2	6.7 ± 1.5*	0.8 ± 0.3
	30 d post-MPTP	0.6 ± 0.2*	4.2 ± 1.3*	5.1 ± 1.0*	2.5 ± 1.0
Amygdala	Control	9.2 ± 2.5	0.7 ± 0.1	11.9 ± 0.8	1.2 ± 0.2
	7 d post-MPTP	4.8 ± 1.0	0.8 ± 0.3	8.6 ± 1.6	1.9 ± 0.8
	30 d post-MPTP	3.5 ± 1.0	2.1 ± 1.0	6.8 ± 1.3*	3.2 ± 1.2
VME	Control	5.2 ± 0.8	1.1 ± 0.0	23.7 ± 0.7	1.1 ± 0.0
	7 d post-MPTP	5.1 ± 1.2	1.8 ± 0.6	20.7 ± 4.9	2.8 ± 1.2
	30 d post-MPTP	3.8 ± 2.0	4.6 ± 1.4*	12.9 ± 5.2	6.6 ± 2.4
Raphe Nucleus	Control	1.4 ± 0.2	1.0 ± 0.0	15.8 ± 2.0	1.6 ± 0.4
	7 d post-MPTP	1.9 ± 0.2	0.8 ± 0.1	12.5 ± 3.5	3.2 ± 1.5
	30 d post-MPTP	1.5 ± 0.2	1.2 ± 0.3	11.5 ± 2.9	3.3 ± 1.1

Concentrations are expressed as ng/mg of protein. Data are presented as mean ± SEM.
Abberviations: DA: dopamine; 5-HT: serotonin; VME: ventral mesencephalon.
DA turnover: ([DOPAC] + [HVA]) / [DA]; 5-HT turnover: [5-HIAA] / [5-HT]
* $p < 0.05$ compared to the saline group.

Altered AMPA-Receptor Expression with Intensive Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury.

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Running Title: Treadmill exercise in MPTP mice.

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Number of Figures:
Number of Tables:
Number of Pages:

Abstract (150 words)

Keywords: immunohistochemistry, MPTP, DARPP-32, glutamate, neuroplasticity, Parkinson's

Introduction (500 word limit)

The glutamatergic system is the major excitatory neurotransmitter system in the brain and plays an impotant role in motor function and neuroplasticity (REF, Starr). In the striatum, the primary glutamatergic pathway is the corticostriatal input to the medium spiny neurons (REF, Starr). Within the striatum, the α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) glutamate receptor subtype is highly expressed, with GluR1 and GluR2 being the most abundantly occurring AMPA-R subunits. While the function of these two receptor subunits within the hippocampus and cerebellum, inlcuding their role in AMPA receptor trafficking, and calcium conductance, as well as their role in regulating NMDA receptor function has been well defined, their specific role in striatal neuroplasticity is not well understood (REF).

The primary interest of our lab is to investigate neuroplasticity in the injured basal ganglia, using the MPTP-lesioned mouse model, and the modulatory role of intensive treadmill exercise. Our previous studies have shown that intensive treadmill exercise regimen initiated 5 days after lesioning, a time point when cell death is complete, leads to motor improvement and that this functional improvement is accompanied by glutamatergic changes. Using immuno-electron microscopy we found that treadmill exercise reversed the MPTP- induced increase level of expression of synpatic glutamate immunolabeling within the striatal nerve terminals (REF). To further delineate the potenital role of the glutamatergic system in activity dependent neuroplasticity in our exercise model, we examined alterations in the pattern of expression of the AMPA-R subunits, GluR1 and GluR2. We also investigated changes in DARPP-32, an effector molecule important in neuroplasticity and in mediating glutamatergic and dopaminergic neurotransmission (REF). For these studies, we used four groups of mice: (1) Saline; (2) Saline + Exercise; (3) MPTP; (4) MPTP + Exercise. C57 BL/6 mice were administered four i.p. injections of MPTP (20mg/kg free-base, 2 h apart). Intensive treadmill exercise was started 5 d after MPTP lesioning (a time point when cell death is complete) and continued for 28 d (5 d/wk). At completion of the exercise regimen, striatal tissue was harvested. Using qRT-PCR,

GluR1 and GluR2 mRNA transcript and their alternative splice isoforms, flip and flop, were analyzed. Using antibody specific probes in intact tissue, the immunohistochemical staining pattern of expression of GluR1, GluR2 and DARPP-32, including their phosphorylated states, was examined.

Our results indicate that exercise leads to alterations in the pattern of expression of GluR1 and GluR2 in mRNA transcript expresssion and protein expression, including their phosphorylated forms. Interestingly, changes in transcript level was not necessarily reflected in the pattern of protein expression. To a lesser extent, the effector molecule DARPP-32 was altered by exercise. These findings may indicate that the glutamatergic system may play a role in activity dependent plasticity which may be enhanced by exercise within the normal and injured basal ganglia.

Our results indicate that there are increased levels of expression of the GluR2 subunit and its phosphoryklated form at serine 880 within th edorsoalteral striatum of mice undergoing intensive treadmill exercise. With exercise there is a decrease in mRNA transcript expression using eith the pan-specific ot flip-specific probes. We observed no change in the expression of DARPP-32 or its phosphorylated form at Thr-75. The changes in GkluR2 are supported by electrophysiological findings that exercise reduced the size of the EPSCs in MSNs in MPTP-lesioend mice.

Materials and Methods

Animals

Mice used for these studies were young adult (8 to 10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory, Inc. (Bar Harbor, Maine). There were 4 treatment groups including: (i) saline, (ii) saline+exercise, (iii) MPTP, and (iv) MPTP+exercise. Animals were housed 5 to a cage and acclimated to a 12-hour shift in light/dark cycle so that the exercise occurred during the animals normal wake period. A total of 72 mice were used in these studies and the number for each procedure

indicated in each relevant methods section. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC).

MPTP-Lesioning

MPTP (Sigma, Inc, St. Louis, MO) was administered in a series of 4 intraperitoneal injections of 20 mg/kg (free-base) at 2-hour intervals for a total administration of 80 mg/kg. This regimen leads to approximately 60% loss of nigrostriatal neurons (as determined by unbiased stereological techniques for both TH staining and Nissl substance and an 80-90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995; Jakowec et al., 2004). Nigrostriatal cell loss is complete by day 3 after MPTP administration and as determined by counting remaining nigrostriatal tyrosine hydroxylase immuno-reactive cells and reduced silver staining for degenerating neurons (Jackson-Lewis et al., 1995; Jakowec et al., 2004). For these studies a total of 48 mice were used with n=12 per group.

Treadmill Exercise

One week before the start of the treadmill exercise paradigm, sixty mice that could maintain a forward position on the 45-cm treadmill belt for 5 minutes at 5.0 m/min were randomly assigned to the 4 groups to insure that all animals performed similarly on the treadmill task prior to MPTP lesioning. The treadmill used in these studies was a Model EXER-6M Treadmill manufactured by Columbus Instruments (Columbus, Ohio). A non-noxious stimulus (metal beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. As a result, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 5 days following saline or MPTP-lesioning when cell death is complete. Mice from each of the two exercise groups (saline + exercise and MPTP + exercise) were run at the same time in the 6-lane treadmill. Exercise duration was incrementally increased starting with 30 minutes on day 1 to reach the goal duration of 2 sessions of 30 minutes each (for a total of 60 minutes), 5 days/week (with a 5 minute warm-up period)

for a total of 30 days of exercise (corresponding to a final of 45 days after MPTP-lesioning). Treadmill speed and exercise duration for each group was increased when all mice within each group maintained a forward position on the treadmill belt, for 75% of the running period. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) non-exercised groups were placed on the top of the treadmill apparatus for a time period equivalent to exercise training (Fukai et al., 2000; Kojda et al., 2001).

Tissue Collection

Brain tissue from all groups of mice was collected on the last day of exercise (day 28 of exercise corresponding to 42 days after MPTP-lesioning). Striatal brain tissue was also collected from a subset of animals from each experimental group at 5 days of exercise, 10-days post-lesioning, to determine the degree of dopamine depletion at an earlier exercise and MPTP time point. Mice were sacrificed by cervical dislocation for fresh tissues (for HPLC or qRT-PCR) or by pentobarbital followed by transcardial perfusion with fixative (for immunohistochemistry). Striatal tissues for HPLC analysis were collected fresh en block corresponding to anatomical regions Bregma 1.20 to 0.60, with borders dorsal tot the anterior commissure, ventral to the corpus callosum, medial to the lateral ventricle, and lateral 2.5 mm from midline and frozen until In addition, to evaluate the initial degree of MPTP-mediated striatal dopamine depletion, brain tissue for HPLC analysis was collected at 10 days post-MPTP-lesioning from a subset of non-exercise mice from both the saline and MPTP groups.

HPLC Analysis of Dopamine and its Metabolites

Neurotransmitter concentrations were determined according to an adaptation of Irwin et al (1992) of the method of Kilpatrick and colleagues (1986) (Kilpatrick et al., 1986; Irwin et al., 1992). Tissues for analysis, n = 6 per group at the 10-day and 42-day time points, were homogenized in 0.4 N perchloric acid and centrifuged at 12,000-x g to separate precipitated protein. The protein pellet was re-suspended in 0.5N NaOH and the total protein concentration determined using the Coomassie Plus protein assay system (Pierce, Inc) using a Biotek Model Elx800 microplate reader and the software

KCjunior. The concentrations of dopamine, DOPAC (3,4-dihydroxyphenylacetic), HVA (homovanillic acid), were assayed by high performance liquid chromatography (HPLC) with electrochemical detection (ECD). Samples were injected with an ESA autosampler. Dopamine and its metabolites were separated by a 150 mm X 3.2 mm reverse phase 3 um diameter C-18 column (ESA, Chelmsford, MA) regulated at 28^{o}C. The mobile phase MD-TM (from ESA) consisted of acetylnitrile in phosphate buffer and an ion-pairing agent delivered at a rate of 0.6 ml/minute. The electrochemical detector was an ESA model Coularray 5600A with a 4-channel analytical cell with three set potentials at –100 mV, 50 mV, and 220 mV. The HPLC was integrated with a Dell GX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (San Diego, CA).

Immunohistochemical Staining

All proteins were visualized using an immuno-peroxidase labeling method on fixed mouse tissue. Mice (n = 3 to 5 for each treatment group) were administered pentobarbital (160 mg/kg, i.p.), perfused transcardially with cold saline, followed by 4% paraformaldehyde/phosphate-buffered saline, pH 7.2 (called 4% PFA/PBS). After perfusion, brains were expeditiously extracted, immersion fixed in 4% PFA/PBS at 4ºC for 48 hrs., then cryoprotected in 20% sucrose until they sank then quickly frozen in 2-methylbutane on dry ice, stored at -80ºC. Tissue was cut into 25-µm-thick sections, placed in PBS, pH 7.2 for immediate use. Sections were rinsed in TBS three times, quenched in 10% methanol/ 10% H_20_2/ 50mM Tris, pH 7.2, blocked for one hour in 4% normal serum, and exposed to monoclonal or polyclonal primary antibody (concentration of 1:1000) for 24 hours at 4º C. Polyclonal probes made in rabbit included GluR1, GluR1 phosphorylated at Serine 845, GluR2 phosphorylated at Serine 880 and TH; monoclonal antibodies made in mouse included GluR2 and TH. Sections were then rinsed three times in TBS, incubated in secondary antibody (concentration 1:500) made against the species of the primary antibody for 1 h. and then in avidin-biotin complex (using the ABC elite kit; Vector, Burlingame, CA). Sections were visualized in 0.1% 3,3'-diaminobenzadine tetrahydrochloride and 0.1% H_20_2. Antibody specificity was validated by subjecting sections to the same experimental

conditions but without the addition of primary and or secondary antibody, thereby eliminating staining. After staining, sections were mounted on gelatin subbed slides, dried, cleaned and then cover-slipped.

Quantification of Tissue Immuno-staining

Immuno-stained sections were quantified for the relative expression of (i) total number of stained cell bodies; and (ii) the intensity of neuropil staining focusing on the dorsolateral region of the mid-striatum (Bregma 0.74 to 0.38 mm). In tissue sections where differences in cell body staining were noticeable, the relative degree of immunostaining in cell bodies was determined. All analysis was performed on an Olympus BX-51 light microscope, interfaced with a Retiga 3000 camera. Digitized images (captured as tif format) were analyzed using the computer assisted image analysis program Bioquant Nova Prime (Bioquant Imaging, Nashville, TN).

The number of immuno-stained cell bodies within the dorsolateral striatum was determined in representative non-adjacent tissue sections captured at 400X magnification. The region of interest (ROI) consisted of a .15 square mm field within the dorsolateral striatum. For each treatment group (saline, saline+exercise, MPTP, and MPTP+exercise), 3 to 6 mice were used for analysis. The number of stained cells was determined in at least 2 non-adjacent tissue sections per animal at a comparable Bregma level. A threshold based on three-color channels, which uses 24-bit color discrimination, was created in order to automatically differentiate cell bodies from background or other artifacts. Using the intensity established by thresholding, cell bodies were then manually selected based on size (greater than 15 microns), morphology (appearance of dendritic arbor, large soma, and intact nucleus), location (within the perimeter of the dorsolateral striatum), and automatically counted. For each animal, in each treatment group, the average number of immuno-stained cells per ROI was calculated for analysis.

The relative optical density (OD) of the neuropil within stained tissue sections was determined at high magnification (1000X). A typical ROI (.023 square mm) was selected that did not include stained cell bodies or artifacts. Images were captured as TIFF format and analyzed using Bioquant. Measurements produced values ranging from 0 (black) to 255 (white). Each value was subtracted from 255 such that a larger value corresponded to greater immunostaining. To control for non-specific staining, measurements from the corpus callosum of the same tissue section were used as background and subtracted from total neuropil staining. For analysis all treatment groups were normalized against saline.

The relative OD of the immuno-staining within cell bodies was determined at high magnification (1000X) within the dorsolateral striatum. For each animal, 1 to 2 representative tissues sections were analyzed and two ROIs (.023 square mm) per section were selected (2 to 4 ROIs per animal). A total of 3 to 6 animals were evaluated per group. For each ROI, a minimum of five representative cells were randomly selected that satisfied the selection criteria based on size (greater than 15 microns), morphology (appearance of dendritic arbor, large soma, and intact nucleus), location (within the perimeter of the dorsolateral striatum) and the relative OD captured for each cell. The relative OD was then averaged across all cells in a ROI, and averaged across all ROIs to provide a mean cell body OD per animal.

Quantitative Real-time PCR

Total RNA was isolated from n= 4 mice per group using the RNeasy Lipid Tissue Mini Kit and RNeasy Mini Spin Column rom Qiagen (Valencia, CA) following the manufacturers instructions. RNA was quantified by measuring absorbency at 260 nm. Reverse-transcription to generate first strand complementary DNA (cDNA) was achieved by mixing 5µg of total RNA with 2µL Oligo $(dT)_{20}$ Primer, 1µL of Omniscript Reverse Transcriptase, 2µL of dNTP mix, 2µL of 10X Buffer and 1µL of RNase inhibitor (Omniscript RT Kit, Valencia, CA) and incubated for 60 min. at 37°C, followed by 5 min at 93°C. The reverse-transcribed cDNA was diluted with RNase-free water to a final concentration of

1:200 and used as the template for analysis. Gene expression of AMPA receptor (AMPA-R) subunits within the striatum was determined with mouse primers sets that based on the GeneBank sequences with the accession numbers X57497 (GluR1) and L32204 (GluR2) and the flip and flop splice variants were analyzed using the primers sets outlined in Table 1. Quantitative real-time PCR (qRT-PCR) analysis was performed with the Mastercycler ep realplex^2 PCR system (Eppendorf, Inc) using 20x SYBR Solution RealMasterMix (Eppendorf, Inc). In the qRT-PCR protocol, the samples were subjected to de-naturation at 95°C for 2 min, followed by 45 cycles of amplification and quantification at 95°C for 15 s and 58°C for 15 s, respectively, with one cycle of the finishing program (68°C for 20 s). The qRT-PCR efficiency was validated by melting curve analysis. To accurately assess the amount of RNA expression, each sample was normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Electrophysiological Studies

Mice from the MPTP and MPTP+exercise groups (n = 2 per group) were anesthetized in a desiccator containing halothane vapors, killed by decapitation, and brains removed. Tissue was blocked in cold low-sodium sucrose-substituted saline 90 mM saline with 105 mM sucrose), striatal coronal sections were cut at 200 µm thickness in ice-cold low-sodium sucrose-substituted saline (using a Camden vibroslicer, WPI). Slices were stored in artificial cerebral spinal fluid (aCSF consisting of 124 mM NaCl, 1.3 mM $MgSO_4$, 3 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 10 mM glucose) at room temperature (23^o C) for at least one hour prior to recording. All solutions were continuously oxygenated with 95% O_2, 5% CO_2. Slices were then transferred to a submerged brain slice-recording chamber perfused with oxygenated aCSF kept at a recording temperature of 32^o C (as outlined in Akopian and Walsh, 2007). All experiments were performed with 30µM bicuculline methiodide (BIC-aCSF). Picrotoxin was used to block gamma-amino butyric acid-A ($GABA_A$) receptor mediated inhibition in an attempt isolate excitatory synaptic events.

Whole cell voltage clamp methods were used to examine corticostriatal synaptic input to reduce possible activation of postsynaptic conductance's, which can contribute to changes in synaptic strength under current clamp conditions (Akopian and Walsh, 2002). Whole cell recordings from visualized neurons were obtained using a fixed stage microscope and water immersion lenses (Zeiss Axioscop, Germany). Patch electrodes were pulled on a Flaming/Brown P-87 Micropipette Puller (Sutter Instruments) and backfilled with internal solution that consisted of (in mM): 120 Cs-gluconate; 2 $MgCl_2$; 0.5 EGTA; 10 HEPES; 10 TEA; 3 QX-314; 3 Na-ATP; pH 7.2; (270-280 mOsm). Electrode resistance was constantly monitored in voltage clamp mode using the Clampex data acquisition software and an Axopatch-1D patch clamp amplifier (Axon Instruments). Series resistance was monitored throughout the experiment by measuring the instantaneous current response to a -10 mV voltage step from -70mV. A gravity-fed array of inflow tubes of ~100 μm inner diameter and an outflow tube attached to a vacuum reservoir provided solution flow. The ground electrode consisted of a salt bridge constructed from glass electrode filled with agar. Large tipped whole cell electrodes filled with 140 mM NaCl were positioned 100-500 μm from the recording electrode at the border between the dorsolateral striatum and the overlying corpus callosum. All recordings were taken from the dorsolateral aspect of the striatum, in coronal sections. Constant current stimuli (10-100 μA) were delivered using durations less than 0.1 msec through the second channel of the Axoclamp-2A. Input (stimulation intensity) – output (synaptic response) relationships were determined for corticostriatal synapses by applying a standard ascending sequence of stimulus intensities to the corpus callosum and recording excitatory postsynaptic currents (EPSCs).

Statistical Analysis of Data

Statistical analysis was performed using SPSS version 14.0 for Windows (SPSS, Inc., Chicago, IL) or GraphPad Prism 4 (San Diego, CA). Differences in behavioral tests between groups were analyzed using repeated-measures analysis of variance (ANOVA) with the between-subjects factors being lesion (saline or MPTP) and the within subject factor being time. For HPLC analysis and immunocytochemistry staining, a Student's t-test or a two-way ANOVA was performed to compare the

different groups and examine for significant interactions. Post-hoc contrasts with Bonferroni correction were performed to determine the locus of any significant differences. For physiological studies, the slope of the input output relationship was determined for each cell and compared using unpaired Student t-tests For all analyses, a significance level of $p < 0.05$ was used.

Results

Treadmill Running Behavior

The time course of improvement in running velocity of both the saline+exercise and MPTP+exercise groups over the 6 weeks (28 days) of treadmill running is shown in Figure 1. Saline+exercise mice in the first week of treadmill running started at a velocity of 14 ± 1.4 m/min that further increased to 22.6 ± 0.3 m/min by the final week. The MPTP+exercise group had a running velocity of 9.2 ± 1.1 m/min during the first week that further increased to 20.5 ± 0.7 m/min in the last week. As shown in our previous papers, there was a significant difference in velocity at week 1 between the saline+exercise and MPTP+exercise groups and this difference was not significant at the completion of the treadmill running regimen [Fisher, 2004 #7021][Petzinger, 2007 #13756].

HPLC Analysis of Striatal Dopamine

HPLC analysis was used to determine levels of striatal dopamine, its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and the metabolites turnover ratio, defined as [(DOPAC + HVA)/dopamine]. These data are shown in Table 2. To determine the degree of dopamine depletion by MPTP-lesioning, a subset of non-exercised mice from the saline and MPTP-lesioned groups was analyzed 10 days post-lesioning. At the 10-day time point, the MPTP (48.0 ± 8.4 ng dopamine/mg protein) mice showed significantly lower levels of striatal dopamine compared to the saline group (269.5 ± 24.9 ng dopamine/mg protein) ($p < 0.05$), which represented an 83% depletion.

Analysis of dopamine turnover ratio showed a significant elevation in the MPTP group (turnover ratio = 2.3), at the 10-day time point, compared to the saline group (turnover ratio = 0.3) ($p < 0.05$).

HPLC analysis of striatal dopamine at the completion of the 28 days of treadmill running (42 days post-MPTP lesioning) showed that dopamine levels remained significantly depleted in MPTP-lesioned mice compared to saline controls ($F = 229.3$, $p < 0.0001$). There was no significant difference in striatal dopamine levels comparing MPTP+exercise with MPTP sedentary mice. There was a significant effect of exercise on the saline treated group, where saline+exercise mice had a higher level of striatal dopamine compared to saline mice ($F = 7.78$, $p = 0.015$). There were no significant effects of MPTP or exercise, or interaction between these two factors on turnover ratio, with the ratios of MPTP = 0.36, MPTP+exercise = 0.34, saline = 0.26, and saline+exercise group = 0.34.

Analysis of AMPA Receptor Subunits GluR1 and GluR2 Expression

The pattern of expression of mRNA transcripts and proteins for the AMPA receptor subunits GluR1 and GluR2 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining, respectively. The design of primer sets for qRT-PCR also allowed for the analysis of the common isoforms of both GluR1 and GluR2 due to alternative transcript splicing, termed flip and flop (REF). In addition, immunohistochemical staining for AMPA-R subunits was determined using antibodies that recognized either the pan or phosphorylated forms of GluR1 and GluR2.

Receptor Subunit GluR1

Immunohistochemical staining of GluR1 subunit expression using a pan-specific antibody shown in Figure 2 showed that the total number of immunoreactive-positive cells decreased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice, but did not quite reach statistical significance ($F = 4.852$; $p = 0.0587$). There was no significant effect with exercise ($F = 0.04$; $p = 0.846$) and no interaction ($F = 0.063$; $p = 0.809$); specifically, there were no significant differences

between saline and saline+exercise groups or the MPTP and MPTP+exercise groups. Analysis of GluR1-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.599$; $p = 0.242$) and between the exercise and non-exercise groups ($F = 1.247$; $p = 0.297$), and there was no interaction ($F = 0.905$; $p = 0.369$).

Immunohistochemical staining for the phosphorylated form of GluR1 was carried out using an antibody that recognizes GuR1~phospho-Ser845 (see Figure 3). There were no significant differences seen in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 0.918$; $p = 0.352$) nor between the exercise and non-exercise groups ($F = 1.493$; $p = 0.239$), and there was no interaction ($F = 0.113$; $p = 0.742$). Analysis of GuR1~phospho-Ser845 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.0$; $p = 0.329$) and between the exercise and non-exercise groups ($F = 0.999$; $p = 0.329$), and there was no interaction ($F = 1.0$; $p = 0.329$).

There was an apparent difference in the intensity of cell body immuno-staining between the different treatment groups within the dorsolateral striatum. Optical density measurements of cell body immunoreactivity captured at high magnification showed no significant difference between MPTP-lesioned mice compared to saline mice ($F = 0.029$; $p = 0.866$), and no significant difference between exercise and non-exercise mice ($F = 1.564$; $p = 0.226$). There was a significant interaction between treatment and exercise ($F = 6.728$; $p = 0.017$), such that exercise led to decreased expression of GluR1~phospho-Ser 845 immunoreactivity within cell bodies of saline treated mice and an increase in expression in cell bodies of MPTP-lesioned mice.

Analysis of mRNA transcript for the pan-GluR1 within the dorsolateral striatum (see Figure 4A) showed that there was a significant decrease in the expression of GluR1 in MPTP-lesioned compared with saline treated mice ($F = 444.0$; $p < 0.0001$) and a significant decrease in the expression of GluR1

in exercise compared to non-exercise mice ($F = 159.0$; $p < 0.0001$). In addition there was a significant interaction between treatment and exercise ($F = 135.9$; $p < 0.0001$), such that exercise led to a decrease expression of GluR1 in the saline group.

Analysis of mRNA transcript for the flip (see Figure 4B) and flop (see Figure 4C) isoforms of GluR1 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR1-flip in the exercise compared to non-exercise groups ($F = 52.05$; $p < 0.0001$). There was no significant effect of MPTP-lesioning on GluR1-flip expression ($F = 0.640$; $p = 0.447$) and no interaction between exercise and MPTP-lesioning ($F = 0.0002$; $p = 0.989$). With GluR1-flop there was a significant decrease in the MPTP-lesioned group compared to saline ($F = 103.3$; $p < 0.0001$). There was no significant effect of exercise on GluR1-flop expression ($F = 3.646$; $p = 0.093$). There was a slight trend for an interaction between exercise and MPTP-lesioning ($F = 3.979$, $p = 0.081$), due to an increased expression in GluR1-flop with exercise in the saline group.

Receptor Subunit GluR2

Immunohistochemical staining of GluR2 subunit expression using a pan-specific antibody shown in Figure 5 showed that the total number of immunoreactive-positive cells increased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice ($F = 10.370$; $p = 0.012$). There was no significant effect with exercise ($F = 1.133$; $p = 0.318$). There was a trend towards significance in the interaction between exercise and MPTP-lesioning ($F = 4.083$; $p = 0.078$). Specifically, we observed an increase in the expression of GluR2-immuno-reactivity in the MPTP+exercise mice compared to the MPTP non-exercise mice. Analysis of GluR2-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 0.358$; $p = 0.566$) and between the exercise and non-exercise groups ($F = 0.178$; $p = 0.684$), and there was no interaction ($F = 0.564$; $p = 0.474$).

In addition to an increase in the number of immunoreactive cells, we also observed morphological changes (degree of arborization) in cells expressing GluR2 (See Figure 5). Specifically, there was a decrease in the arborization of GluR2 immunoreactive cell bodies in MPTP-lesioned compared with saline mice. Interestingly, we observed a dramatic increase in arborization in saline + exercise compared with saline non-exercise mice.

Immunohistochemical staining for the phosphorylated form of GluR2 was carried out using an antibody that recognizes GuR2~phospho-Ser880 (see Figure 6). There was no significant difference in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 2.136$; $p = 0.182$). There was a significant effect with exercise ($F = 19.22$; $p < 0.002$) and there was a significant interaction between exercise and MPTP-lesioning ($F = 5.805$; $p < 0.043$). Specifically, we observed an increase in the expression of immuno-reactivity in the saline+exercise mice compared to the saline non-exercise mice. Analysis of GuR2~phospho-Ser880 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 2.345$; $p = 0.164$) and between the exercise and non-exercise groups ($F = 0.461$; $p = 0.516$), and there was no interaction ($F = 2.245$; $p = 0.172$). In addition to an increase in the number of immunoreactive cells, we also observed changes in the pattern of immunoreactivity within cell bodies expressing GuR2~phospho-Ser880 between the exercise and non-exercise groups (See Figure 6). The non-exercise group showed more homogeneous staining and the exercise group showed predominant staining in the perimeter of the cell body.

Analysis of mRNA transcript for the pan-GluR2 within the dorsolateral striatum (see Figure 7A) showed that there was a significant decrease in the expression of pan-GluR2 in MPTP-lesioned compared with saline treated mice ($F = 14.83$; $p < 0.005$) and a significant decrease in the expression of pan-GluR2 in exercise compared to non-exercise mice ($F = 9.180$; $p < 0.016$). There was no significant interaction between treatment and exercise ($F = 1.224$; $p = 0.301$).

Analysis of mRNA transcript for the GluR2-flip (see Figure 7B) and GluR2-flop (see Figure 7C) isoforms of GluR2 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR2-flip in the exercise compared to non-exercise groups ($F = 35.90$; $p < 0.0003$). There was also a significant decrease in the expression of GluR2-flip in the MPTP-lesioned group compared to the saline group ($F = 22.47$; $p < 0.0015$). There was no interaction between exercise and MPTP-lesioning ($F = 1.542$; $p = 0.25$). With GluR2-flop there was a significant increase in the MPTP-lesioned group compared to saline ($F = 55.71$; $p < 0.0001$). There was no significant effect of exercise on GluR2-flop expression ($F = 0.04$; $p = 0.843$) and no interaction between exercise and MPTP-lesioning ($F = 2.935$; $p = 0.125$).

DARPP-32

Immunohistochemical staining of DARPP-32 expression was carried out using antibodies that recognize either the pan-specific isoforms (see Figure 8) or the phospho-Thr75 isoforms (see Figure 9). Analysis of DARPP-32 with a pan-specific antibody demonstrated that there was no significant change in the number of immuno-positive cells in MPTP-lesioned group compared to the saline group ($F = 1.334$; $p = .286$). There was a decrease in animals that underwent intensive treadmill exercise compared to non-exercised animals (see Figure 8). This change, however, did not reach statistical significance ($F = 2.47$; $p = 0.16$). There was no significant interaction between exercise and MPTP-lesioning ($F = 0.002$; $p = 0.97$). Further analysis using the antibody specific for the phospho-Thr75 isoform of DARPP-32 demonstrated no significant change in the number of immuno-positive cells in the MPTP-lesioned group compared to saline animals ($F = 0.315$; $p = 0.590$), no significant change in the exercise compared to the non-exercise group ($F = 0.167$; $p = 0.693$), and no significant interaction ($F = 1.599$; $p = 0.242$).

Physiological Studies using Whose-Cell Voltage Clamping: Treadmill exercise reduces the input-output relationship at corticostriatal synapse of MPTP treated mice. Systematic increases in the intensity of stimulation delivered to the corpus callosum overlying the dorsolateral striatum produced a

steady increase in the amplitude of corticostriatal EPSCs. The dorsolateral striatum was selected since our previous work has shown this region is targeted for exercise-induced changes in its physiology (Petzinger et al, 2007). The resulting input-output relationship for the synapses was used to evaluate the strength of corticostriatal connections in MPTP treated mice. We found treadmill exercise reduced the size of EPSCs evoked by equal intensities of stimulation in MPTP treated mice. The slope of the EPSC amplitude versus the intensity of stimulation used to evoke the EPSC was determined in MPTP alone and MPTP + exercise synapses and exercise was found to reduce the slope of this relationship (Fig. XX)($p<0.004$). The slope of the input-output relationship for the MPTP alone group was 19.9 ± 1.8 (n=2) and the slope of the input output relationship for the MPTP + exercise group was 7.0 ± 0.6 (n=2).

Discussion (total 1500 words)

- our major findings

- what are the effects of these changes we observe

- what have others found

- how does this fit into what we are doing with electrophysiology

- what could these changes mean

- what are the potential impacts of these findings

- where are we going

The AMPA subtype of ionotrophic glutamate receptors are responsible for mediating fast excitatory neurotransmission (REF). In conjunction with NMDA receptors they mediate calcium flux in many neuronal populations including projection neurons within the striatum (REF). The AMPA-R subtype consists of 4 major subunits including the GluR1 through GluR4. These subunits are also translated into alternative isoforms due to either alternative exon splicing (termed flip or flop) or post-transcriptional modification by deaminase at the Q/R site (REF). Within the striatum, the majority of

AMPA-R are composed of heteromeric subunits in either the GluR1/GluR2 or GluR2/GluR3 stoichiometry (REF). Therefore, dependeing on the subunit composition and isoform For this study, we focused on the pattern of expression of mRNA transcript and protein of subunits GluR1 and GluR2. Immunohistochemical staining using antibody probes

Our results indicate that exercise leads to alterations in the pattern of expression of causes a downregulation of mRNA transcript in the pan forms of GluR1 and GluR2, and in the flip isoform of the GluR2 subunit. Assessment of AMPA-R protein expression in exercised mice, however, revealed changes not accounted for by transcript expression. Exercise had no significant impact on GluR1 protein expression in the dorsolateral striatum. We found that phosphorylation at serine 880 of the GluR2 subunit increased in exercised animals, irrespective of their lesioned state, and that MPTP-lesioned mice experienced an upregulation in total GluR2 expression. Lastly, the effector molecule DARPP-32 was differentially expressed in exercised mice. Our studies show that exercise alters the pattern of expression of AMPA-Rs within the striatum but that this phenomenon is not explained by changes in mRNA transcript, suggesting that alternative mechanisms are involved, including protein-protein interactions, turnover and synaptic localization/trafficking.

A major role of da in the striatum is to modulate glutamatergic receptor states and therefore function. Modulation of glutamatergic activity has

The dopaminergic system has been shown to be important in motor execution and motor learning. The glutamatergic system, which has a strong interaction with dopamine within the striatum, is also known to play an important role in motor function (learning and execution) and synaptic plasticity throughout the brain, including within the basal ganglia. both of which can undergo modification that significantly affects neural transmission by influencing receptor properties such as conductance, protein interactions, and localization (synaptic incorporation/trafficking). Our findings suggest that

alterations in the pattern of expression of AMPA-Rs may be a contributing factor to the motor improvement seen in intensely exercised MPTP-lesioned mice.

Findings from this study indicate that changes in AMPA-Rs may play a key role in putative molecular adaptations necessary for activity dependent synaptic plasticity in the

dopamine depleted striatum, as found in PD.

Notes:

Other studies have shown a slight increase of GluR1 in the striatum of MPTP-lesioned primates (Betarbet, R., 2000. Journal of Neurochemistry); however, others have reported little to no change in primates and mice (Silverdale, 2002. Experimental Neurology and Wullner, 1993. Experimental Neurology).

A. GluR1 by itself produces a larger response to glutamate than GluR1+GluR2 (Heinemann 1)
B. GluR2 decreases Calcium permeability of AMPA channels (Heinemann 2)

Flip/flop
A. Flip variants have larger synaptic currents and the show less desensitization (they stay active longer) than flop variants (Glur1 or Glur2) (Koike et al, 2000; Partin et al, 1996 - Meyer lab; Sommer and Seeburg - TIPS 1992).

DAARP ?
cAMP-dependent protein kinase increases the conductance of GluR1 (Banke et al, 2000 - Traynelis lab)

We see a smaller response in MPTP + exercise, why?
1) exercise shows less expression of flip in both GluR1 and GluR2
2) GluR2 increased expression would also reduce responses to glutamate (Heinemann 1)
3) reduced DAARP suggest PKA is reduced, which should reduce phosphorylation of GluR1 (less conductive)

PAPERS FOR JON- GLUT RECEP CHANGES AFTER DOPA DENNERVATION/ Neurophys to support:

Alterations in the phosphorylation state of glutamate receptor subunits and alterations in subunit composition may contribute to altered corticostriatal synaptic plasticity in PD and its animal models(Oh et al., 1998; Dunah et al., 2000).

Supported: Neurophysiological analysis has demonstrated that long-term synaptic depression (LTD) is the preferred form of synaptic plasticity evoked at corticostriatal synapses from

normal animals (Calabresi et al., 1992; Walsh, 1993; Choi and Lovinger, 1997; Villar and Walsh, 1999).

Supported: For example, in the 6-OHDA-lesioned rat, neurophysiological studies have shown absence of striatal LTD and in some cases, increased expression of LTP (Centonze et al., 1999; Calabresi et al., 2000; Picconi et al., 2003; Picconi et al., 2004).
disease.

Acknowledgements: The authors would like to acknowledge the generous support of the Parkinson's Disease Foundation, Team Parkinson LA, Parkinson Alliance, and the Whittier PD Education Group, NINDS RO1 NS44327-1, U.S. Army NETRP (Grant # W81XWH-04-1-0444). A special thanks to Friends of the USC Parkinson's Disease Research Group including George and Mary Lou Boone, and Walter and Susan Doniger. Special Thanks to our colleagues for helpful discussions including Dan Togasaki, Elizabeth Hogg, and Charles Meshul.

Table and Figure Legends

Table 1: Primer sets used for quantitative real-time PCR (qRT-PCR). Tissue from the dorsal striatum was used for analysis of mRNA transcripts of GluR1 and its flip and flop alternative splice variants and GluR2 and its flip and flop alternative variants. GAPDH was used as house keeping control.

Table 2: HPLC Analysis of striatal dopamine. Total levels of dopamine and its metabolites DOPAC and HVA, expressed as ng per mg protein for each, were determined in whole tissues dissected from the dorsolateral striatum at the completion of the exercise regimen corresponding to 42 days post-MPTP-lesioning. Dopamine was also determined at the 10-day time point to determine the degree of MPTP-lesioning. The turnover ratio was expressed as (DOPAC + HVA)/Dopamine.

Figure 1: Comparison of the treadmill running velocity of MPTP and saline mice. Mice were run on the motorized treadmill 5 days per week for 6 weeks. The average weekly running velocity for saline (open bars) and MPTP-lesioned (black bars) mice is shown. The asterisks indicate statistically significant difference in velocity between the two groups in weeks 1 through 4. There was no significant difference between the two groups at weeks 5 and 6.

Figure 2: Immunohistochemical staining against GluR1.

Figure 3: Immunohistochemical staining against GluR1~phosphoSer$_{845}$.

Figure 4: qRT-PCR Analysis of GluR1.

Figure5: Immunohistochemical staining against GluR2.

Figure 6: Immunohistochemical staining against GluR2~phosphoSer$_{880}$. .

Figure 7: qRT-PCR Analysis of GluR2.

Figure 8: Immunohistochemical staining against DARPP-32.

Figure 9: Immunohistochemical staining against DARPP-32~phosphoThr$_{75}$.

Figure 10: Exercise reduces the input-output relationship for corticostriatal synapse in MPTP treated mice. A: Example of EPSCs evoked from dorsolateral corticostriatal synapses in brain slices taken from MPTP alone and MPTP + exercise mice. EPSCs were produced by the stimulation intensities shown in the x-axis of B. B: Plot of input (stimulation intensity) – output (EPSC amplitude) for corticostriatal synapse from MPTP treated mice. C: Bar graph of slope calculated for the input-output relationship for corticostriatal synapses as shown in B (* = $p<0.004$).

Table 3: Summary of results.

References

The following papers are to be considered for the discussion:

(Adesnik and Nicoll, 2007)

(Anwyl, 2006)

(Betarbet and Greenamyre, 1999)

(Betarbet et al., 2000)

(Betarbet et al., 2004)

(Barry and Ziff, 2002)

(Banke et al., 2000)

(Bats et al., 2007)

(Bibbiani et al., 2003)

(Biggs and Starr, 1997)

(Brorson et al., 2004)

(Brown et al., 2005)

(Carlson et al., 2000)

(Chung et al., 2000)

(Cragg and Rice, 2004)

(Cull-Candy et al., 2006)

(Dietrich et al., 2005)

(Derkach et al., 2007)

(Deng et al., 2007)

(Dunah et al., 2000)

(Elias and Nicoll, 2007)

(Emmi et al., 1996)

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(Matsuda et al., 2000)

(Oh and Derkach, 2005)

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(Olsen et al., 1987)

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(Perez et al., 2001)

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(Quirk et al., 2004)

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Figure 1

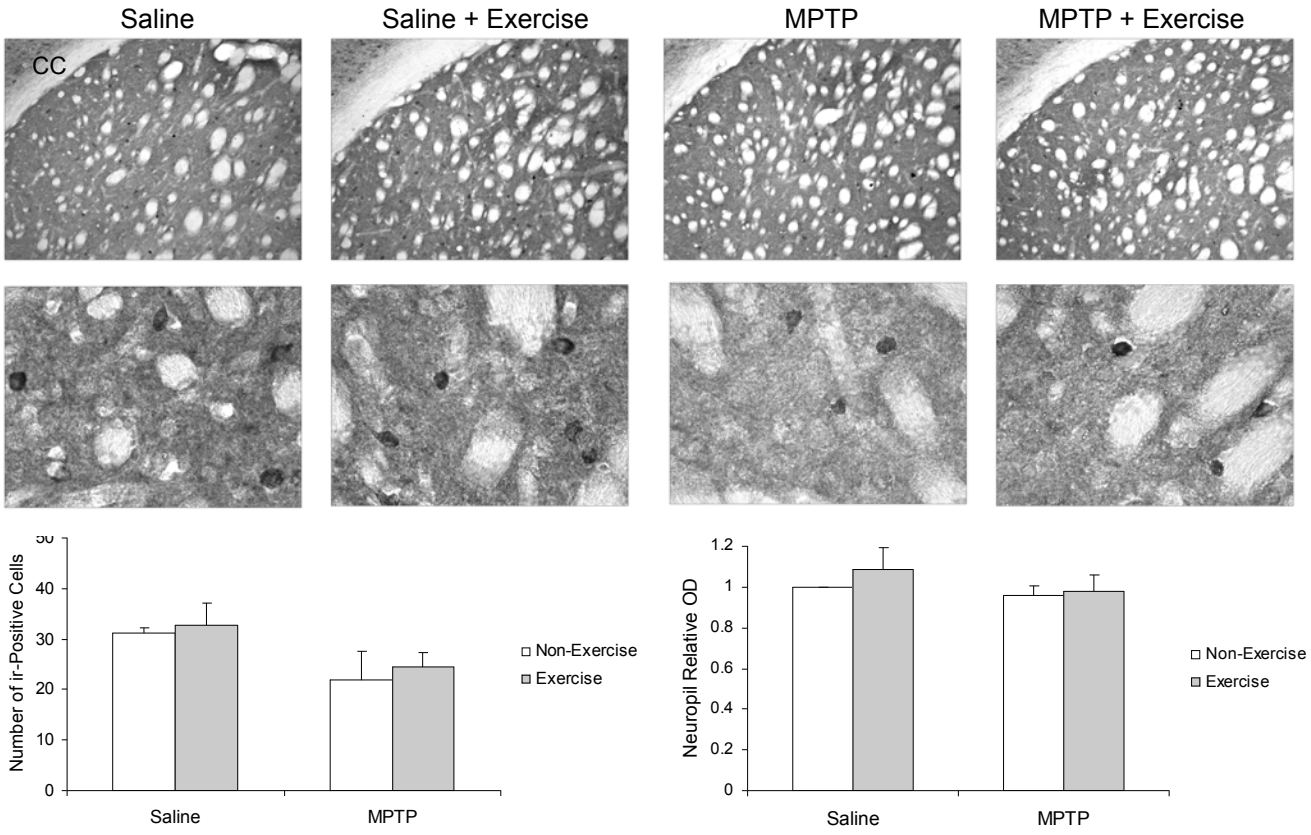


Figure 2: Immunohistochemical staining for GluR1

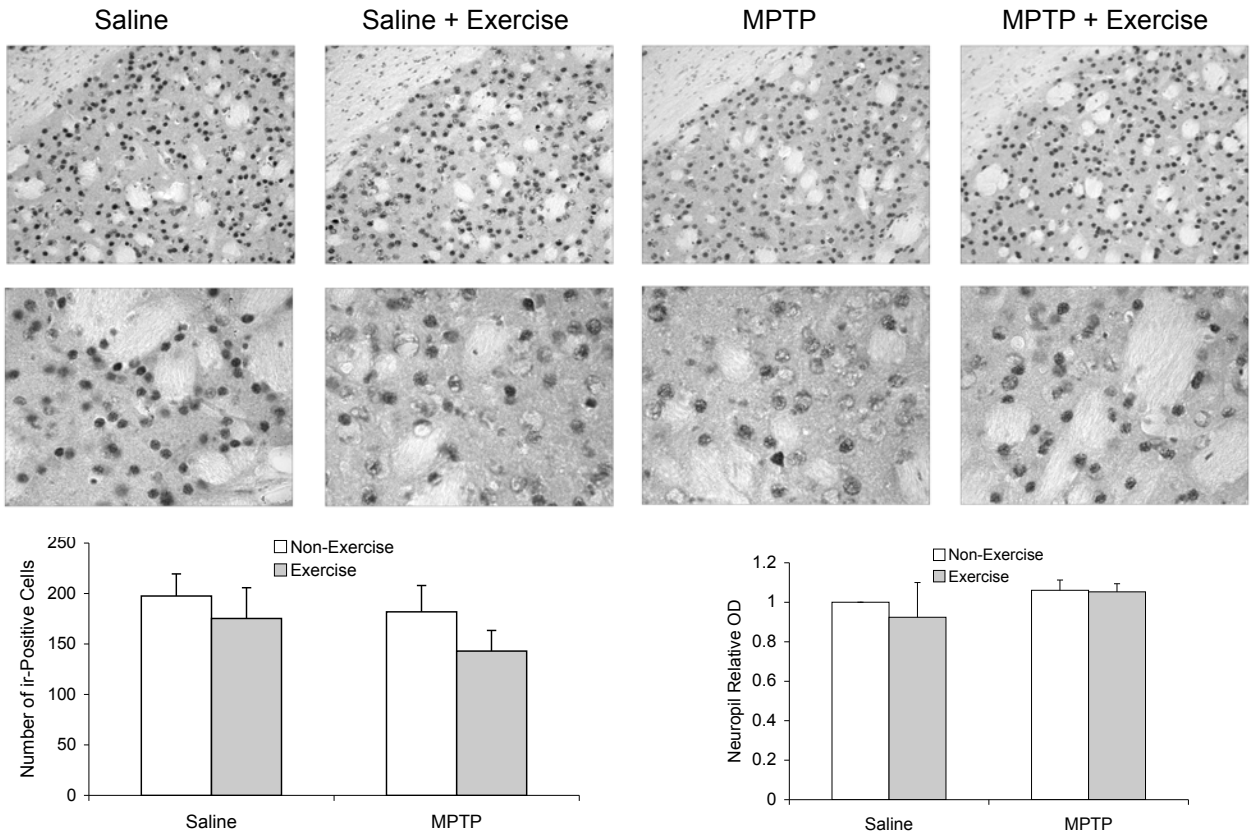


Figure 3: Immunohistochemical Staining for GluR1-Ser845

Figure 4: qRT-PCR GluR1

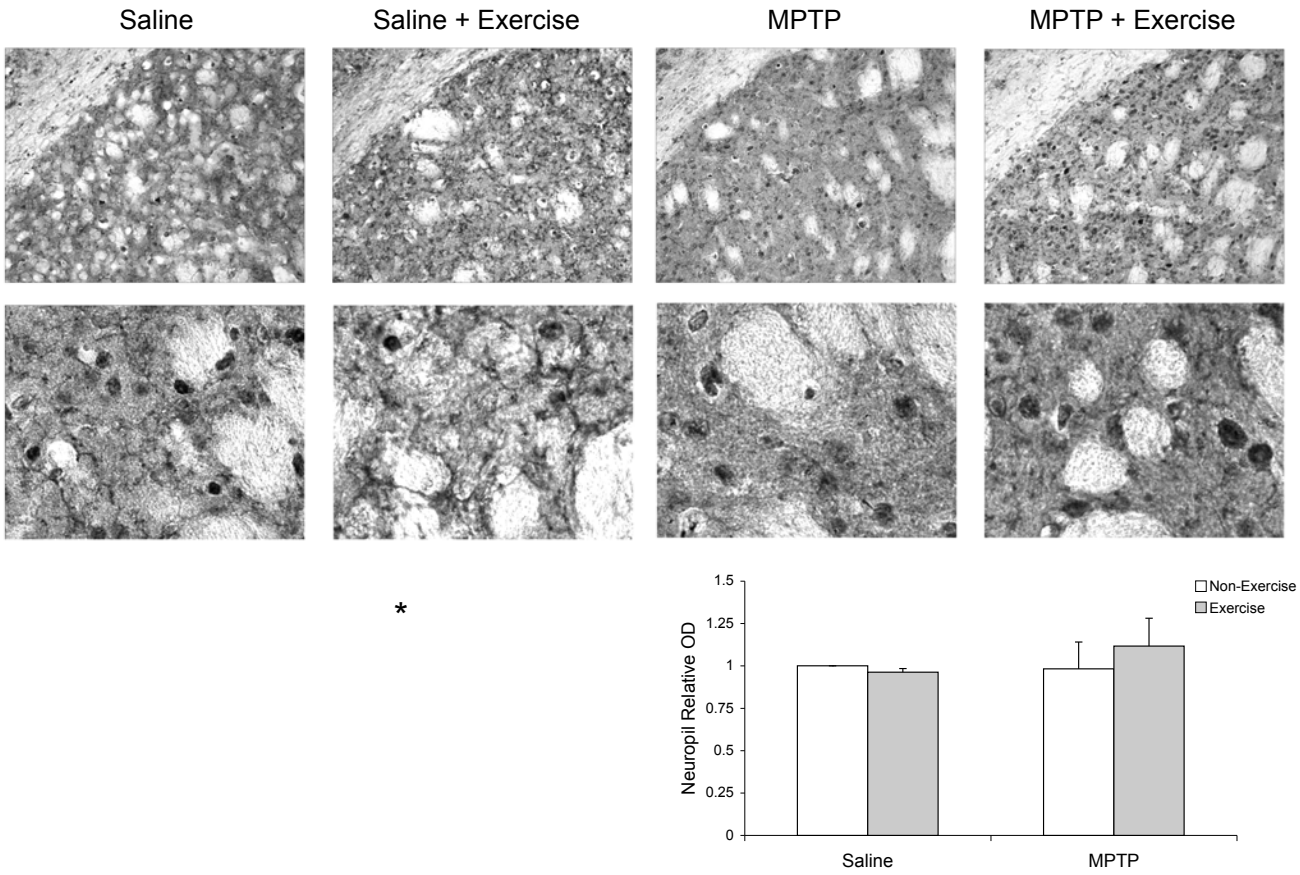
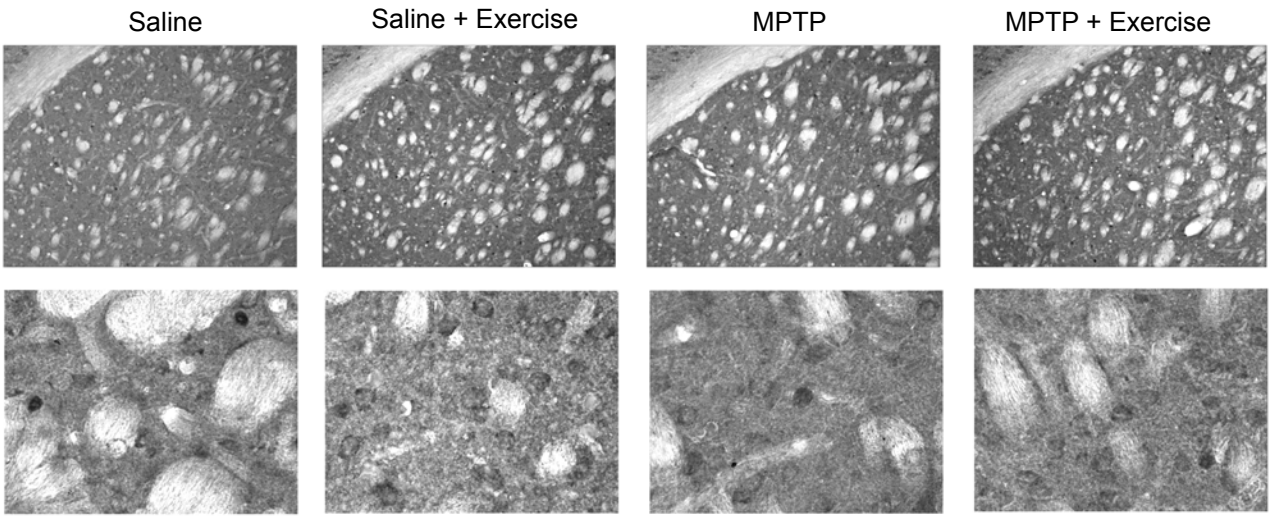


Figure 5: Immunohistochemical staining for GluR2


Saline
Saline + Exercise
MPTP
MPTP + Exercise

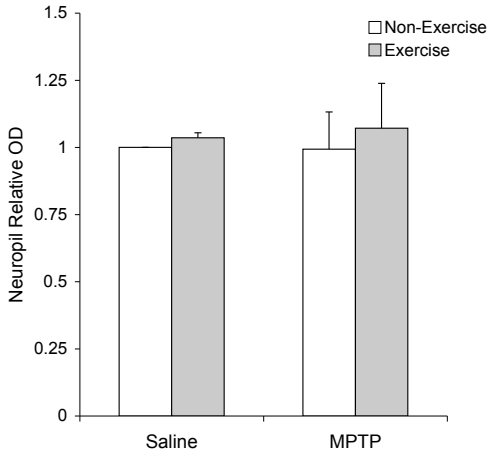

Non-Exercise
Exercise
Neuropil Relative OD
1.5
1.25
1
0.75
0.5
0.25
0
Saline
MPTP

Figure 7: qRT-PCR GluR2

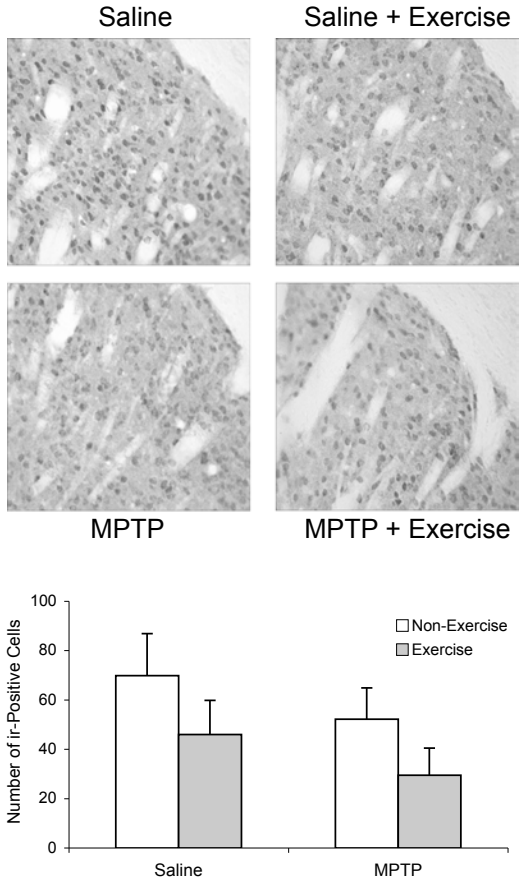


Figure 8: Immunohistochemical staining for DARPP-32

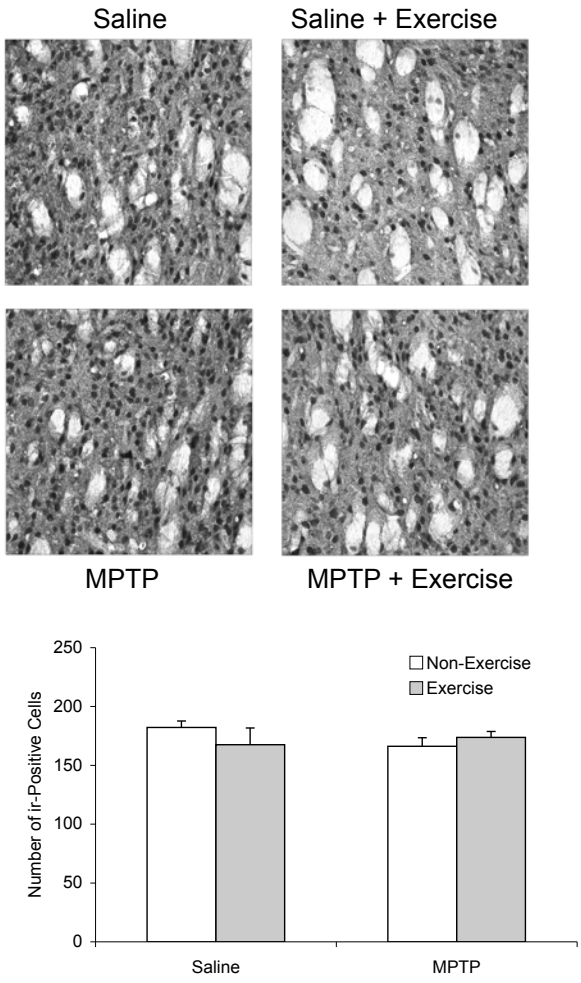


Figure 9: Immunohistochemical staining for DARPP-32 Thr75

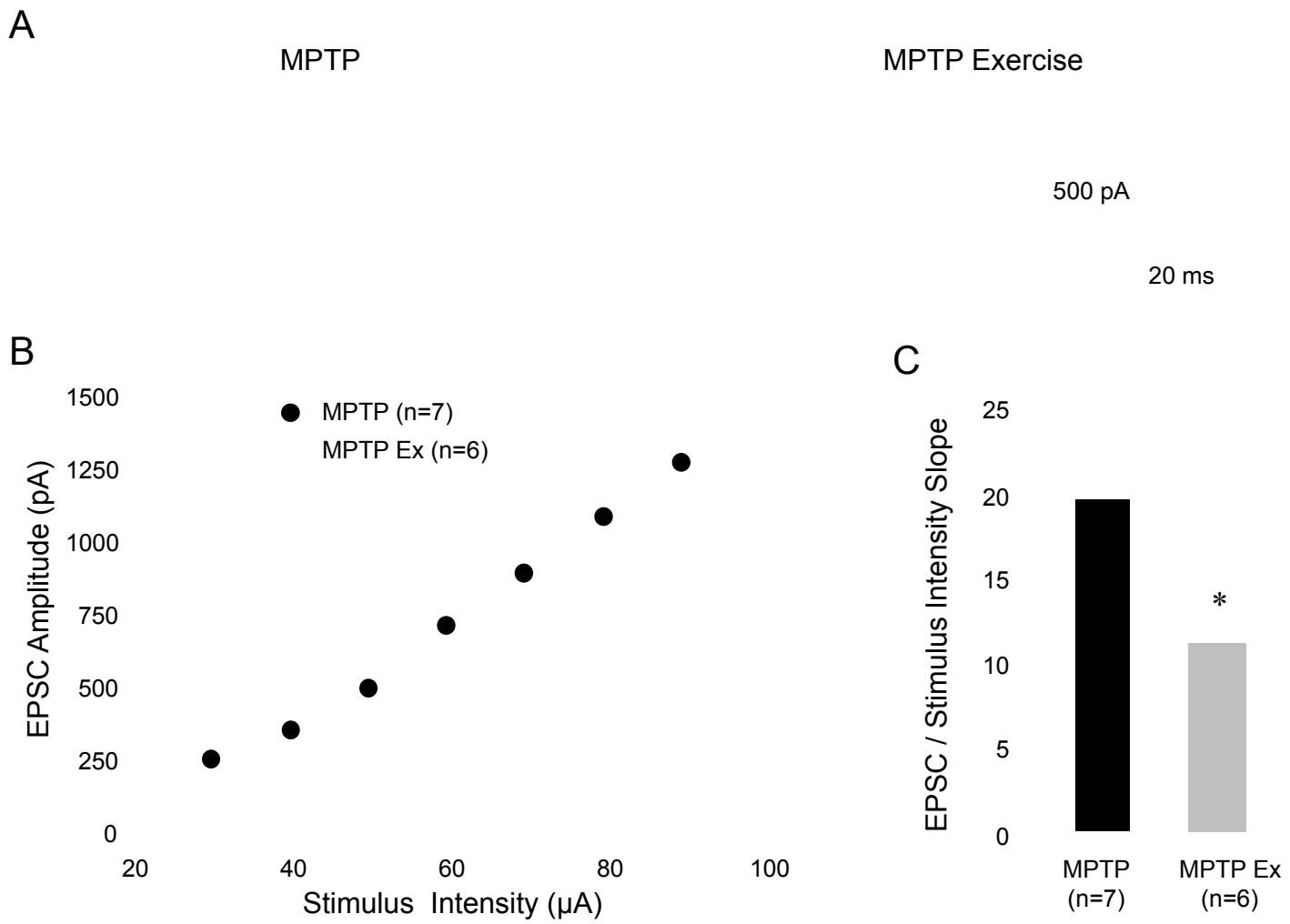

A
MPTP
MPTP Exercise
500 pA
20 ms
B
1500
1250
1000
750
500
250
0
EPSC Amplitude (pA)
MPTP (n=7)
MPTP Ex (n=6)
20
40
60
80
100
Stimulus Intensity (μA)
C
25
20
15
10
5
0
EPSC / Stimulus Intensity Slope
*
MPTP
(n=7)
MPTP Ex
(n=6)

Table 1: PCR Primer sets

Primer Sequences for AMPA-R Subunits and alternative spice isoforms		
Subunit	***Species***	***Nucleotide sequence (5'-3')***
GluR1 (F)	Mouse	ACA CCA TGA AAG TGG GAG GTA ACT
GluR1-flip (R)		ACT GGT CTT GTC CTT ACT TCC GGA
GluR1-flop (R)		ACT GGT CTT GTC CTT GGA GTC ACC
GluR2 (F)	Mouse	ACA CCA TGA AAG TGG GCG GCA ACC
GluR2-flip (R)		ACT GGT CTT TTC CTT ACT TCC CGA
GluR2-flop (R)		ACT GGT CTT TTC CTT GGA ATC ACC
Control	Mouse	
GAPDH (F)		TGC ACC ACC AAC TGC TTA G
GAPDH (R)		GGA TGC AGG GAT GAT GTT GTTC

Table 2:

			Dopamine	DOPAC	HVA	Turnover
Day 10 Post-MPTP	No Exercise	Saline	269.5 ± 24.9	33.4 ± 4.5	33.1 ± 3.2	0.3 ± 0.08
		MPTP	48.0 ± 8.4*	11.7 ± 1.9*	87.2 ± 9.6*	2.3 ± 0.28*
Day 42 Post-MPTP	No Exercise	Saline	246.9 ± 19.8	36.5 ± 4.5	27.4 ± 1.7	0.3 ± 0.01
		MPTP	77.9 ± 12.0*	14.7 ± 2.4*	13.5 ± 1.9*	0.4 ± 0.01
	Exercise (28 days)	Saline	315.2 ± 9.0^{+}	39.3 ± 2.8	25.9 ± 1.3	0.2 ± 0.01
		MPTP	69.8 ± 11.7*	11.3 ± 1.5*	13.5 ± 1.2*	0.4 ± 0.05

Probe	MPTP	Exercise	Interaction
GluR1-pan protein			
cell counts	↓ T	-	-
neuropil	-	-	-
GluR1~Ser845 protein			
cell counts	-	-	-
neuropil	-	-	-
soma density	-	-	+ 1
GluR1-pan mRNA	↓	↓	+ 2
GluR1-flip mRNA	-	↓	-
GluR1-flop mRNA	↓	-	-
GluR2-pan protein			
cell counts	↑	-	+ 3T
neuropil	-	-	-
arbor	↓	-	↑ 4
GluR2~Ser880 protein			
cell counts	-	↑	-
neuropil	-	-	-
soma density	-	↓ 5	
GluR2-pan mRNA	↓	↓	-
GluR2-flip mRNA	↓	↓	-
GluR2-flop mRNA	↑	-	-
DARPP-32 protein	-	↓ T	-
DARPP-32~PhosphoThr$_{75}$	-	-	-

T: Denotes trend
1: Increase in MPTP+exercise vs MPTP no exercise;
decrease in saline+exercise vs saline no exercise.
2: Decrease saline+exercise vs saline no exercise.
3: Increase in MPTP+exercise vs MPTP no exercise (trend).
4: Increase in saline+exercise vs saline no exercise.
5: Decreased uniform staining in saline group with exercise.

Table 3: Summary