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Annual report: Year 1 of a no-cost extension phase (9/15/20- 9/14/21).

14. ABSTRACT

An increasingly common problem in Parkinson's disease (PD) and its progression is cognitive impairment, yet it is rarely addressed with currently accepted therapeutics and is difficult to treat. Recent findings support the hypothesis that exercise, and particularly exercise that incorporates both skill and aerobic components (SAE), is a viable and effective treatment option for cognitive impairment in PD. Using a rodent model of Parkinsonism (striatal 6-hydroxydopamine model), the current project has applied methods of animal behavior, immunohistochemistry, molecular biology, functional brain mapping, and micro-neuroanatomy, to the question of exercise-related restoration of cognitive function and the role of corticostriatal circuits. Understanding the impact of exercise in the basal ganglia and its related circuitry may represent a new frontier in understanding mechanisms of neuroplasticity and repair and, thus lead to novel therapeutic targets for PD. It provides a framework for guiding future human trials aimed at optimizing specific, cost-effective rehabilitation strategies and reducing the burden of disease, not only for PD patients, but also for persons with a broad range of neurologic disabilities.

15. SUBJECT TERMS
Parkinson's Disease, exercise, skilled training, cognition, learning, executive function, dopamine, plasticity, metabolic, prefrontal, striatum, nigrostriatal, animal models, operant, brain mapping

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1. INTRODUCTION:

Parkinson's disease (PD) is the 2nd most frequent neurodegenerative disorder at old age and diminishes the quality of life in over 630,000 people in the USA, with numbers projected to double by the year 2040. An increasingly common problem in PD and its progression is cognitive impairment, yet it is rarely addressed with currently accepted therapeutics and is difficult to treat. Importantly, cognitive impairment leads to challenges in daily function, as well as significant social and psychological burdens. A wide range of exercise modalities have been examined in the motor rehabilitation of PD patients. However, investigation on the relationship between exercise and cognitive function in PD remains a major gap in knowledge. Recent findings support the hypothesis that exercise, and particularly exercise that incorporates both skill and aerobic components (SAE), is a viable and effective treatment option for cognitive impairment in PD. Animal studies have been critical for providing evidence for exercise-induced neuroplasticity of corticostriatal circuits that are profoundly affected in PD. Work by our laboratory in a rat model of basal ganglia injury early on provided preliminary evidence that SAE compared to simple aerobic exercise (AE) results in a differential enhancement of prefrontal cortex-mediated control of motor function, though cognition had not been examined. The evaluation of the effects of different forms of exercise on cognitive function in the PD rat model has been to focus of the current project

Using a rat model (striatal 6-hydroxydopamine model), the current study applied methods of animal behavior, immunohistochemistry, molecular biology, functional brain mapping, and micro-neuroanatomy, to the question of exercise-related restoration of cognitive function and the role of frontostriatal circuits. During the Covid pandemic labs were closed starting 3/15/20 in alignment with the city's and university's lockdown and 'stay-at-home' orders. All animal colonies were mandated to be euthanized to minimize need for vivarium staff to care for them. This included animals in the pipeline for our long-term, ongoing studies to evaluate the behavioral, molecular, imaging, electrophysiologic outcomes of chronic exercise using both skilled and nonskilled training. Labs were permitted to reopen at 50% capacity on 8/31/2020, and at 100% occupancy by 3/29/21. The delay resulting from the pandemic initiated the request for the 1st no-cost extension (9/12/20-9/14/21), as well as the 2nd no-cost extension (9/15/21 – 9/14/22).

In this first year of the no-cost extension this project, we have continued our data collection and analysis (behavioral, molecular, immunohistochemical, neuroanatomic). Analysis of data collected to date has shown that dorsomedial bilateral lesions of the striatum, while they do not alter general motor function or appetitive behavior, clearly impair learning of two separate matching-to-sample tasks (3-Choice serial reaction time task, T-maze task), with additional impairment noted during rule reversal. Further analysis of the operant serial reaction time task shows impairment in attention, processing speed, working memory, mental flexibility, as well as impulsivity – all components of executive function. Skilled exercise training results in a significant, gradual and progressive, improvement in executive function. To our surprise, there were no significant differences between skilled and nonskilled training and between skilled exercise and high intensity aerobic training.

Results of a molecular analysis suggest a differential and dynamic effect of exercise across the striatal subsectors, with changes in dopaminergic, synaptic and metabolic markers confined to the dorsal subsectors. The lack of changes in ventral subsectors suggests regional specificity. During the past year, we have completed the data collection on a brain mapping study that seeks to examine the effects of lesioning and of exercise on motor and cognitive circuits. Preliminary analysis suggests that exercise has profound effects on normalizing brain glucose uptake and functional connectvity, not only in the cortico-striatal circuit but also in the hippocampus. These results suggest that moderate exercise normalizes lesion-induced

metabolic alterations of the brain, and does so with circuit specificity, with largest changes noted not only in cortico-striatal circuit, but also in cognitive areas including the hippocampus. As part of the ongoing 2nd year of the no-cost extension, we are currently undertaking a deep analysis of the imaging data, to look at exercise-related changes in functional connectivity in cognitive regions of the brain.

Understanding the impact of exercise in the basal ganglia and its related circuitry may represent a new frontier in understanding mechanisms of neuroplasticity and repair and, thus lead to novel therapeutic targets for PD. It provides a framework for guiding future human trials aimed at optimizing specific, cost-effective rehabilitation strategies and reducing the burden of disease, not only for PD patients, but also for persons with a broad range of neurologic disabilities.

2. KEYWORDS:

Parkinson's Disease, exercise, skilled training, cognition, learning, executive function, dopamine, plasticity, metabolic, prefrontal, striatum, nigrostriatal, animal models, operant, brain mapping

3. ACCOMPLISHMENTS:

What were the major goals of the project? In year 1 of this no-cost extension the following goals were pursued:

MAJOR GOALS PROJECT 2

TASK 3: Evaluate Effects of Skill-based v. Aerobic Exercise on Executive Function
Subtask 2: Performance of operant training (set-shifting task)
Subtask 3: Assessment of lesion size (TH staining)

TASK 4: Brain Imaging
Subtask 1: Perfusion autoradiography
Subtask 2: Assessment of lesion size (TH staining)

TASK 5: Bench Research
Subtask 1: Spine counts, dendritic branching
Subtask 2: Electrophysiology
Subtask 3: HPLC
Subtask 4: qRT-PCR
Subtask 5: Western Blots

TASK 6: Data Analysis

What was accomplished under these goals?

TASKS 3 & 6: Effects of exercise on cognition / Lesion Size

3-choice serial reaction time task

Results in the 3-choice serial reaction time task show that exercise in lesioned rats elicits a significant, modest improvement in reversal learning, as well as a robust improvement in inhibitory aptitude.

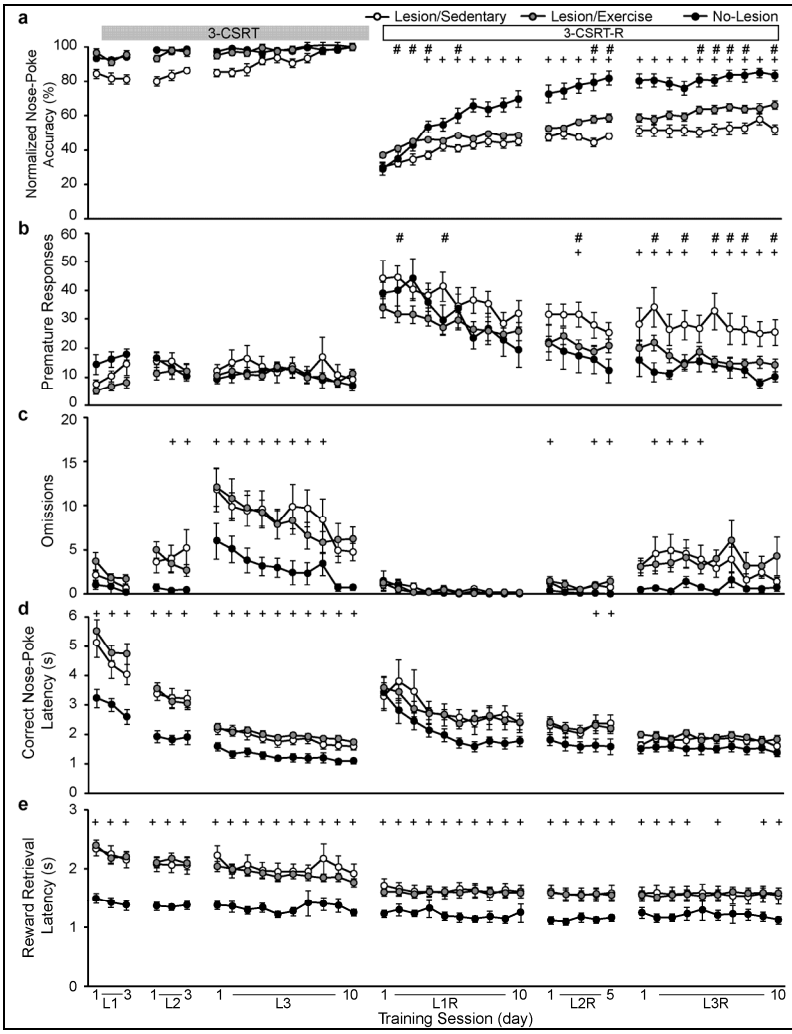


Figure 1. Effects of exercise and lesion on the 3-choice serial reaction time task (3-CSRT) acquisition and reversal learning (3-CSRT-R). Shown are group mean ± SEM of **(a)** normalized nose-poke accuracy, **(b)** premature responses, **(c)** omissions, **(d)** correct nose-poke latency, and **(e)** reward retrieval latency for No-Lesion (*n* = 12), Lesion/Sedentary (*n* = 12) and Lesion/Exercise group (*n* = 22). #: $p < 0.05$ Lesion/Exercise vs. Lesion/Sedentary; +: $p < 0.05$ Lesion/Sedentary vs. No-Lesion, Fisher's LSD multiple comparisons test. Data were also analyzed with two-way ANOVA with repeated measures (results not shown).

Results above are for the pooled exercise groups (**Fig. 1**). Comparison of 3 different types of *forced* exercise showed no significant difference between different training modalities (**Fig. 2**).

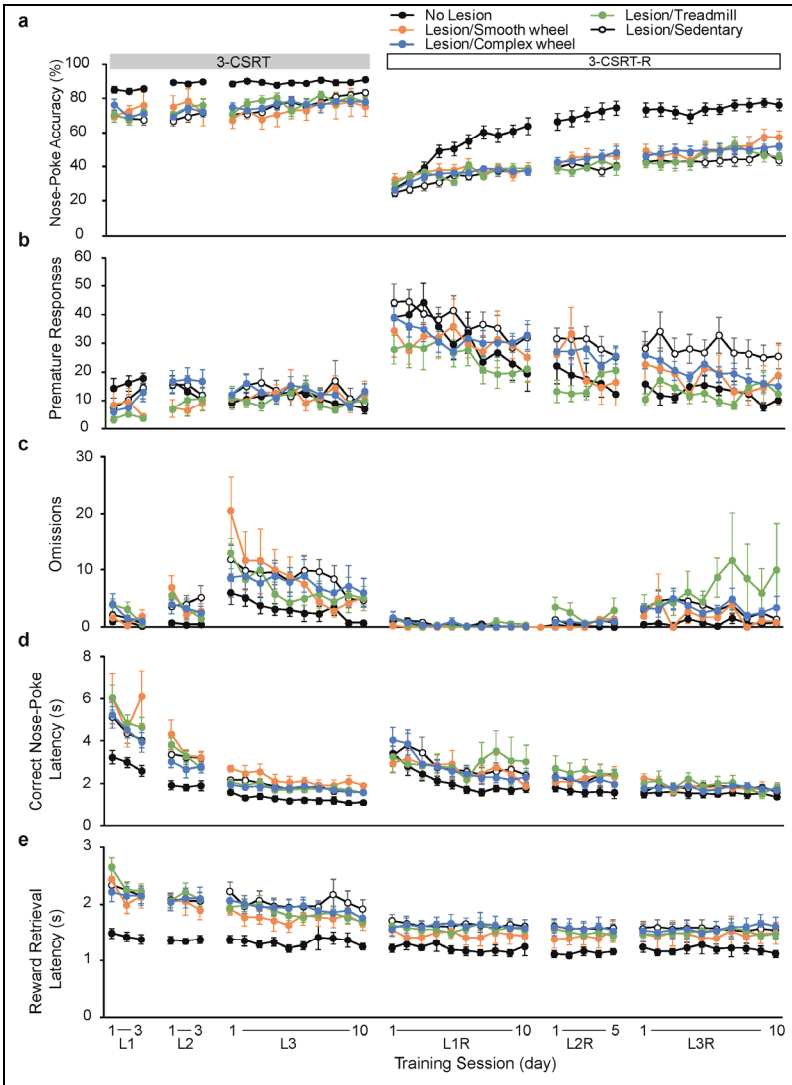


Figure 2. 3-Choice serial reaction time task with reversal learning (3-CSRT, 3-CSRT-R) in individual groups. Data are presented as mean ± S.E.M. Exercise effect was assessed using a two-way ANOVA with repeated measure among Lesion/Sedentary (*n* = 12), Lesion/Complex wheel (*n* = 12), Lesion/Smooth wheel (*n* = 4), and Lesion/Treadmill (*n* = 6) groups. There was no statistically significant exercise effect in (**a**) nose-poke accuracy ($p > 0.35$), (**b**) premature responses ($p > 0.077$), (**c**) omissions ($p > 0.13$), (**d**) correct nose-poke latency ($p > 0.39$), and (**e**) reward retrieval latency ($p > 0.73$). Fisher's LSD multiple comparisons test showed significant difference in premature responses between the Lesion/Sedentary and Lesion/Treadmill group during the 3rd phase of reversal learning (L3R, $p = 0.015$). Due to institutional and local restrictions in response to the Covid-19 pandemic, we were not able to complete the Lesion/Smooth wheel and Lesion/Treadmill group, or to increase sample size. We combined the 3 exercise group into a single Lesion/Exercise group (*n* = 22) in this report.

In addition, we examined lesioned animals undergoing *voluntary* aerobic running in running wheels integrated into the home cages. The voluntary exercise group was added to explore a group that was free of the stress of forced exercise training. Comparison of the 4 different types of exercise on the last days of reversal training after undergoing *forced* exercise (nonskilled and skilled wheel running, horizontal treadmill) or *voluntary* wheel running is shown below (**Fig. 3**). Results show no significant difference in percent accuracy in the 3-CSRT-R paradigm between different training modalities.

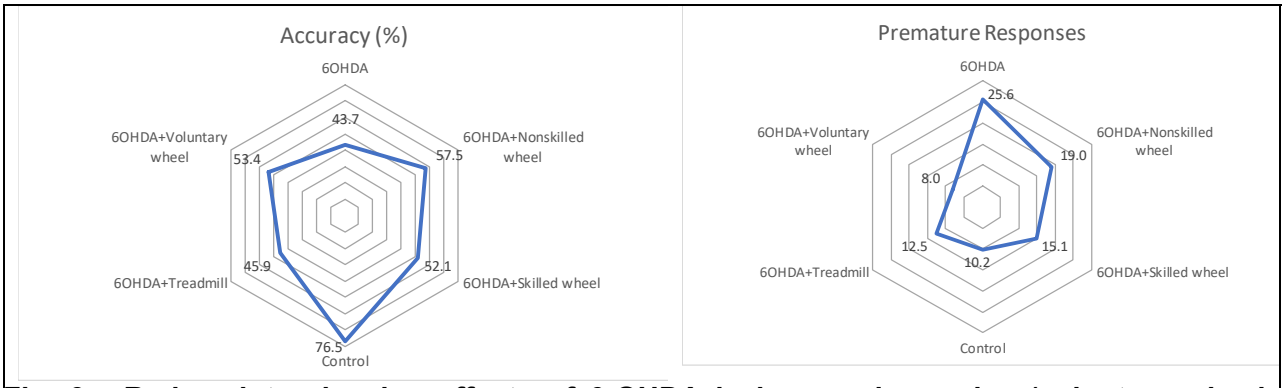


Fig. 3: Radar plots showing effects of 6-OHDA lesions and exercise (voluntary wheel running, forced horizontal treadmill running, forced skilled wheel running, forced nonskilled wheel running) on accuracy and premature responses in an operant task on the final day of reversal training.

T-Maze learning

Exercise improves response accuracy in the T-maze, shortens time until achieving the learning criterion, while diminishing perseverative errors following rule reversal (Win-Shift phase)(**Fig. 4**).

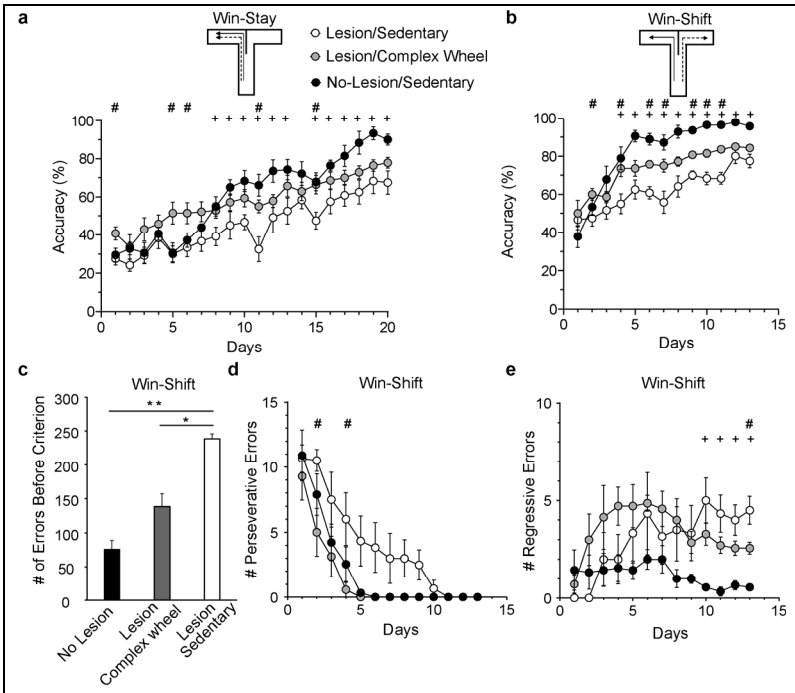


Fig. 4: Effects of lesion and exercise on T-maze learning of rewarded matching-to-sample (Win-Stay) followed by reversal (Win-Shift). (**a**) Rats were trained in a Win-Stay strategy (solid line arrow = sample trial; dashed line arrow = choice trial) for 20 days, followed by training in **(b)** Win-Shift strategy for an additional 13 days. Response accuracy (percentage of correct responses) is shown for No-Lesion/Sedentary (n = 9), Lesion/Complex wheel (n = 7) and Lesion/Sedentary (n = 6). **(c)** Total number of incorrect trials performed until criterion (9 correct responses in 10 consecutive trials) was reached during the Win-Shift phase. **(d)** Perseverative errors during the Win-Shift phase. **(e)** Regressive errors during the Win-Shift phase. Mean ± SEM. #: $p < 0.05$ Lesion/Complex wheel vs. Lesion/Sedentary; +: $p < 0.05$ Lesion/Sedentary vs. No-Lesion/Sedentary, Fisher's LSD multiple comparisons test. *: $p < 0.0002$, **: $p < 0.001$ (Student's t-test).

Conclusion: Exercised/lesioned rats showed a significant, modest improvement in response accuracy in the 3-CSRT-R and T-maze, as well as a robust improvement in inhibitory aptitude in the 3-CSRT-R. Our prospective study demonstrates that following dopaminergic

deafferentation, moderate exercise is able to provide improvements in cognitive flexibility and inhibitory aptitude.

We have continued our characterization of the brain lesions using tyrosine hydroxylase immunohistochemistry and HPLC for dopamine (**Fig. 5**)

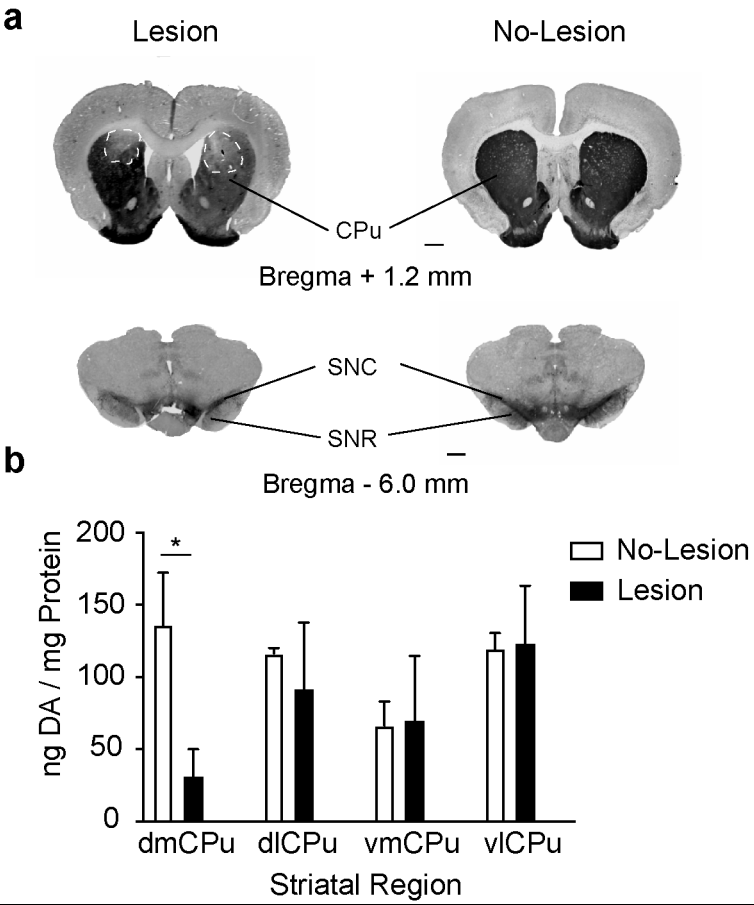


Fig. 5. Immunostaining for tyrosine hydroxylase and HPLC analysis of regional dopamine levels to determine the degree and anatomical site of lesion.
(a) Representative images of coronal sections reveal bilateral loss in tyrosine hydroxylase immunoreactivity in the dorsomedial striatum (bregma AP +1.20 mm) and midbrain showing immunostaining of the substantia nigra pars compacta (bregma -6.0 mm). Scale bar = 0.5 mm.
(b) HPLC analysis of striatal dopamine from tissues collected from coronal slice (bregma AP +2.00 to 0.00 mm) from non-lesioned and lesioned rats in striatal tissue.

TASKS 4 & 6: Brain mapping / Data Analysis
We have shown that the Parkinsonian animal (bilateral dorsomedial striatal 6-OHDA lesions) compared to normal controls shows a diminished glucose uptake in the basal ganglia-thalamo-cortical circuit and increased glucose uptake in the hippocampus (Lesion Effect). See **Fig. 6: (A)** striatum, CPu; globus pallidus, GP; substantia nigra, SN; subthalamic nucleus STh, lateral dorsal/posterior thalamus LD/LP; primary motor cortex, M1), HPC hippocampus. These changes in cerebral metabolism in the lesioned animal are dramatically reversed by long-term exercise training **(B)**.

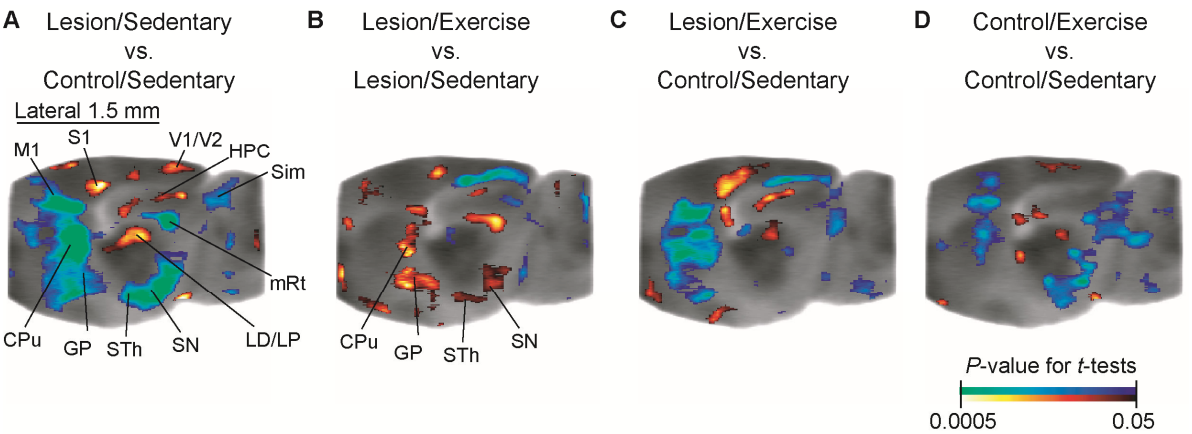


Fig. 6: Lesion and exercise effects on glucose uptake in the basal ganglia-thalamocortical circuit. Shown are statistically significant difference (P<0.05, >100 contiguous voxels) in the uptake of [^{14}C]-2-deoxyglucose during a motor learning task. Statistical parametric mapping of 3-D reconstructed whole brain autoradiograms: **(A)** lesioned animals compared to normal controls show bilateral hypometabolism of the striatum, CPu; globus pallidus, GP; substantia nigra, SN; subthalamic nucleus STh, lateral dorsal/posterior thalamus LD/LP; and primary motor cortex, M1, as well as increased metabolism in the hippocampus, HPC; **(B)** after 6 weeks exercise training lesioned animals compared to their sedentary counterparts (n=10 per group) show a reversal of these changes in metabolism, with red/blue color indicating significant increased/decreased glucose uptake (P<0.05 for > 100 contiguous significant voxels).

In the current quarter, we have extended the analysis to show that exercise in the lesioned animal increases metabolic functional connectivity of the dorsal striatum to primary motor cortex (M1) (**Fig. 7**).

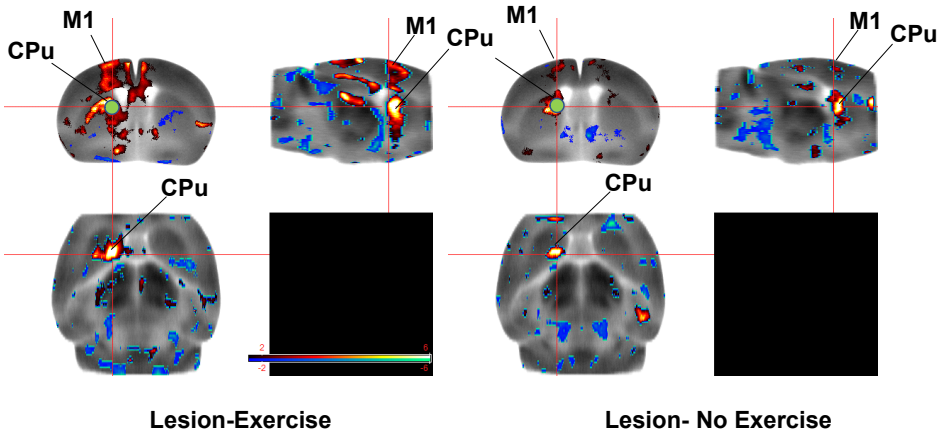


Fig. 7: 6 weeks of treadmill training increases functional metabolic connectivity between dorsomedial striatum and primary motor cortex (M1) that was diminished in the lesioned Parkinsonian animal in the absence of exercise. Shown are coronal, sagittal, and transverse views of functional connectivity of a single seed region (green circle) placed into the left dorsomedial striatum of the lesioned rodent. Red/blue colors denote significant positive/negative correlations between metabolic activity of these brain regions and the seed (P<0.05 for > 100 contiguous significant voxels).

Conclusion: Preliminary analysis of our imaging results shows that moderate exercise training in the Parkinsonian animal normalizes lesion-induced metabolic alterations of the striatum and hippocampus, while reestablishing functional connectivity within the cortico-striatal circuit.

TASKS 5 & 6: Bench Research / Molecular Data Analysis

Exercise elicited increased expression of the dopamine receptors (Drd1, Drd3, Drd4), and the synaptic markers synaptophysin and Dlg4 (also known as PSD95) in the dorsal (associative and sensorimotor) striatal regions.

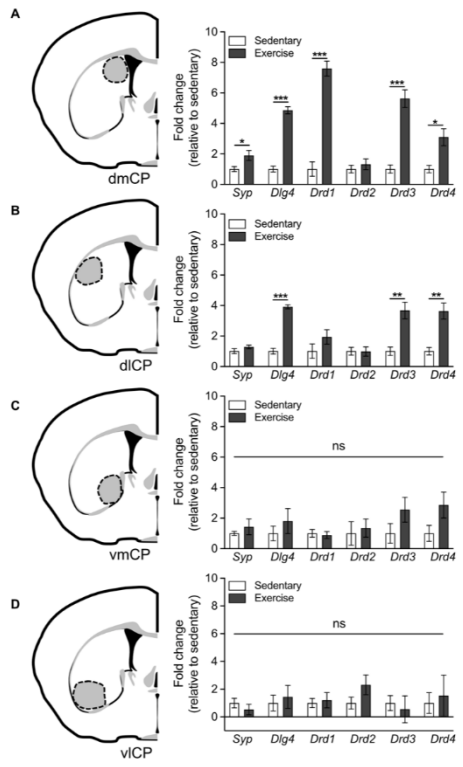
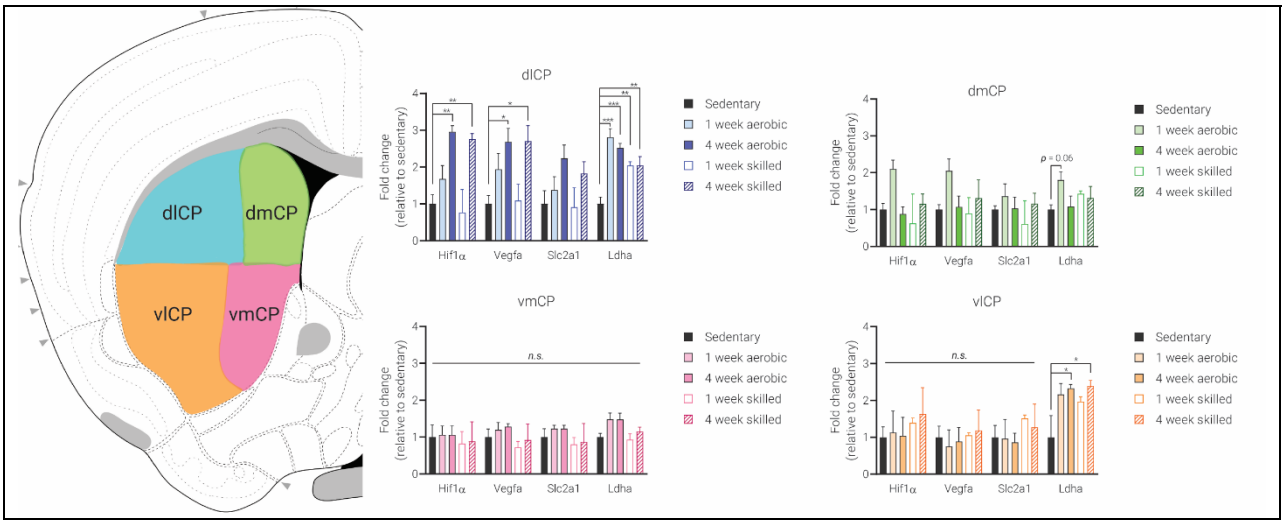


Fig. 8. Dopaminergic signaling and synaptogenic gene expression changes across caudate putamen quadrants following exercise (4 weeks) in 6-hydroxydopamine lesioned rats. Rat caudate putamen was divided into four quadrants for transcript analysis: **(A)** dorsomedial (dmCPu), **(B)** dorsolateral (dlCPu), **(C)** ventromedial (vmCPu), and **(D)** ventrolateral (vlCPu). Corresponding gene expression changes for four dopamine receptor (*Drd1, Drd2, Drd3, Drd4*) and two synaptic (*Syp, Dlg4*) genes in the exercise group (pooled complex and smooth wheel running, n = 12) compared to sedentary controls (n = 6). Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to sedentary control (Student's *t*-test).

Fig. 9: Metabolic gene expression differentially changes across caudate-putamen quadrants, exercise duration, and exercise type.
(Left) Rat caudate putamen color coded into dorsomedial (dmCP), dorsolateral (dlCP), ventromedial (vmCP), and ventrolateral (vlCP) quadrants. (Right) Corresponding gene expression changes for four metabolism-related genes (*Hif1a, Vegfa, Slc2a1, Ldha*) in each of the CP quadrants. n = 6 rats per group; mean ± SEM. One-way ANOVA with Dunnett's multiple comparisons for each gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to sedentary control.



Conclusion: Overall these results suggest a differential effect of exercise across the striatal subsectors. Four weeks of exercise changed dopamine receptor/synaptic markers and metabolic markers in the dorsomedial and dorsolateral striatum, with no significant changes noted in the ventromedial and ventrolateral striatum. Significant changes in the markers of cerebral metabolism (Hif1, Vegfa, Slc2a1, Ldha) were noted in the dorsolateral and dorsomedial striatum after 1 week of exercise, but only dorsolaterally after 4 weeks. In the ventral striatum, of the metabolic genes tested only lactate dehydrogenase (Ldha) showed significant increases in the ventrolateral subsector after 4 weeks of exercise. Our prospective study demonstrates that following dopaminergic deafferentation, moderate exercise is able to provide improvements in cognitive flexibility and inhibitory aptitude, while eliciting increased expression of Drd1, Drd3, Drd4, synaptophysin, and PSD-95 largely in the associative and sensorimotor dorsal regions of the striatum.

What opportunities for training and professional development has the project provided?

This project has provided the following opportunities for training and development.

- Research electives for 3 undergraduate students
- Components of this project and data collection will be part of the doctoral thesis work of 3 USC doctoral students in the USC Neuroscience graduate program (A Lundquist, I Flores, E Donahue)

How were the results disseminated to communities of interest?

- Lundquist AJ, Petzinger GM, Jakowec MW, "Astrocytic lactate modulates striatal dopamine to enhance behavioral performance", Virtual Glia Symposium Gordon Research Conference in March 2021
- Kishi SH, Lundquist AJ, Llewellyn GN, Jakowec NA, Cannon PM, Petzinger GM, Jakowec MW, “The role of lactate shuttling in mediating synaptogenesis and learning in motor cortex”, Virtual Glia Symposium Gordon Research Conference in March 2021
- Published manuscripts (see below)

What do you plan to do during the next reporting period to accomplish the goals?

In the ongoing 2nd year of the no-cost extension, we plan to:

(a) Complete the functional brain mapping analysis of the effects of lesions and exercise. Specifically, to apply functional connectivity analyses to explore the effects of dopaminergic deafferentation and exercise on the prefrontal-striatal and prefrontal-hippocampal-striatal circuit.

(b) Continue Golgi analysis of spine counts, dendritic branching studies using our upgraded analysis system.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

- Work from this project provided the impetus for Drs. Holschneider, Jakowec and Petzinger to apply for a grant from the Dept. of Defense, FY21 Neurotoxin Exposure Treatment Parkinson's (NETP, Synergistic Idea award, submitted 9/29/2021). "Impact of diet and physical activity on cognitive and mitochondrial dysfunction in Parkinson's disease". Dr. Holschneider is the PI ("partnering PI") of the preclinical project, while Dr. Petzinger (USC Dept. of Neurology) is the PI of the clinical project for this synergistic dual grant proposal.
- Work from this project provided the impetus for Drs. Holschneider, Jakowec and Petzinger to apply for a NINDS Morris K. Udall Centers of Excellence for Parkinson's Disease Research (P50 grant submitted 9/21/21) entitled "Mechanisms of Cognitive Impairment in Parkinson's Disease". The application proposes a multi-institution, translational, collaboration between the University of Southern California (USC), University of California, Los Angeles (UCLA), University of California, San Diego (UCSD), and University of Virginia (UVA). Dr. Holschneider will be co-Director with Dr. Petzinger (USC Dept. of Neurology) and also share the preclinical project with Dr. Jakowec (USC Dept. of Neurology). The topic will concern the role of astrocytes, glymphatic function and cognition in models of Parkinson's Disease.

What was the impact on other disciplines?

Nothing to report

What was the impact on society beyond science and technology?

Results from this project are likely to provide evidence for the benefits of exercise in the cognitive neurorehabilitation of Parkinson's patients. Work by our extended Parkinson's Research group is aiding through community lectures to raise awareness of the benefits of daily exercise training in the management and treatment of not only the motor deficits, but also cognitive impairment characteristic of Parkinson's Disease.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

None

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Impact of COVID-19: During the height of the pandemic the laboratory was shut-down as per the university's mandate. All animal colonies were mandated to be euthanized to minimize need for vivarium staff to care for them. This included animals in the pipeline for our long-term, ongoing studies to evaluate the behavioral, molecular, imaging, electrophysiologic outcomes of chronic exercise using both skilled and nonskilled training. While a portion of brain tissue was harvested according to our prescribed experimental protocols, other had to be prematurely harvested, or could not be used (e.g. rats intended for functional brain mapping or awaiting lesioning). Labs were permitted to reopen at 50% capacity on 8/31/2020, and at 100% occupancy by 3/29/21. The delay resulting from the pandemic initiated the request for the 1st no-cost extension (9/12/20-9/14/21), as well as the 2nd no-cost extension (9/15/21 – 9/14/22).

Changes that had a significant impact on expenditures

The slow-down of laboratory work caused by the pandemic, resulted in a shift of percent effort into the no-cost extension period. Some of this effort at this time during the resumption of work is being covered by nonDoD funds.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

- Wang Z, Flores I, Donahue E, Lundquist A, Guo Y, Jakowec MW, Holschneider DP
  "Cognitive Flexibility Deficits in Rats with Dorsomedial Striatal 6-OHDA Lesions Tested Using a 3-Choice Serial Reaction Time Task with Reversal Learning", *NeuroReport, 31(15):1055-1064, 2020.*
- Caldwell CC, Petzinger GM, Jakowec MW, Cadenas E,
  "Treadmill exercise rescues mitochondrial function and motor behavior in the CAG140 knock-in mouse model of Huntington's disease", *Chemico-Biological* Interactions, 315 108907, 2020, doi: 10.1016/j.cbi.2019.108907
- Petkus AJ, Filoteo JV, Schiehser DM, Gomez ME, Hui JS, Jarrahi B, McEwen S, Jakowec MW, Petzinger GM.
  "Mild cognitive impairment, psychiatric symptoms, and executive functioning in patients with Parkinson's disease."
  Int J Geriatr Psychiatry. 2020 Apr;35(4):396-404. doi: 10.1002/gps.5255. Epub 2020 Jan 23.
- Sanford MT, Yeh JC, Mao JJ, Guo Y, Wang Z, Zhang R, Holschneider DP, Rodriguez LV
  "Voluntary exercise improves voiding function and bladder hyperalgesia in an animal model of stress-induced visceral hypersensitivity: a multidisciplinary approach to the study of urologic chronic pelvic pain syndrome (MAPP) research network study", *Neurourology & Urodynamics, 2020, Jan 13. doi: 10.1002/nau.24270*
- Donahue EK, Murdos A, Jakowec MW, Sheikh-Bahaei N, Toga AW, Petzinger GM, Sepehrband F.
  "Global and Regional Changes in Perivascular Space in Idiopathic and Familial Parkinson's Disease", *Mov Disord*. 2021 May;36(5):1126-1136. doi: 10.1002/mds.28473. Epub 2021 Jan 20. PMID: 33470460
- Chung YC, Fisher BE, Finley JM, Kim A, Petkus AJ, Schiehser DM, Jakowec MW, Petzinger GM.
  "Cognition and motor learning in a Parkinson's disease cohort: importance of recall in episodic memory", *Neuroreport*. 2021 Oct 6;32(14):1153-1160. doi: 10.1097/WNR.0000000000001707. PMID: 34334776
- Petkus AJ, Jarrahi B, Holschneider DP, Gomez ME, Filoteo JV, Schiehser DM, Fisher BE, Van Horn JD, Jakowec MW, McEwen SC, Petzinger G.
  "Thalamic volume mediates associations between cardiorespiratory fitness (VO2max) and cognition in Parkinson's disease", *Parkinsonism Relat Disord*. 2021 May;86:19-26. doi: 10.1016/j.parkreldis.2021.03.019. Epub 2021 Mar 29. PMID: 33819900

- Lundquist AJ, Gallagher TJ, Petzinger GM, Jakowec MW.
  "Exogenous l-lactate promotes astrocyte plasticity but is not sufficient for enhancing striatal synaptogenesis or motor behavior in mice", *J Neurosci Res.* 2021 May;99(5):1433-1447. doi: 10.1002/jnr.24804. Epub 2021 Feb 25. PMID: 33629362
- Jarrahi, B., S. McEwen, D. P. Holschneider, D. Schiehser, A. Petkus, J. D. van Horn, V. Filoteo, Jakowec MW, Petzinger GM,
  "The Effects of Cardiorespiratory and Motor Skill Fitness on Intrinsic Functional Connectivity of Neural Networks in Individuals with Parkinson's Disease", *Brain Plasticity*, 2021, DOI: 10.3233/BPL-200115.
- Lundquist, A. J., S. H. Kishi, G. N. Llewellyn, N. A. Jakowec, P. M. Cannon, G. M. Petzinger, and M. W. Jakowec
  "Knockdown of astrocyte-specific monocarboxylate transporter-4 in the mouse striatum results in increased striatal dopamine levels and elevated sensitivity to the dopamine depleting neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine", *Molecular Neurobiology*, 2021, In Press.

Manuscripts in review

- Wang Z, Lundquist AJ, Donahue E, Guo Y, Phillips D, Petzinger GM, Jakowec MW, Holschneider DP
  "A mind in motion: Exercise improves cognitive flexibility, impulsivity and alters dopamine receptor gene expression in a Parkinsonian rat model", in review.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

Website(s) or other Internet site(s)

Currently we are developing a "Translational Research in Parkinson's Disease" website for our research group to more widely be able to disseminate our work, resources and community event announcements.

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Daniel P. Holschneider, MD
Project Role: partnering PI (with Dr. Giselle Petzinger, award # W81XWH-18-1-0665)
Research Identifier: N/A
Nearest person month worked: 2.26 mo (of this 2.13 mo were covered by nonDoD funding)
Contribution to the project: No change. Project design, project management, directing functional brain mapping studies, data analysis.

Name: Michael Jakowec, Ph.D.
Project Role: co-I
Research Identifier: N/A
Nearest person month worked: 1.2 mo (of this 1.2 mo were covered by nonDoD funding)
Contribution to the project: No change. Project design, directing molecular, electrophysiologic, and neuroanatomic studies.

Name: Zhuo Wang, Ph.D.
Project Role: co-I
Research Identifier: N/A
Nearest person month worked: 3.6 mo
Contribution to the project: No change. Stereotaxic lesioning, directing operant studies, functional brain mapping, data analysis.

Name: Yumei Guo, MS
Project Role: Staff
Research Identifier: N/A
Nearest person month worked: 2.7 mo
Contribution to the project: No change. Skilled and nonskilled exercising of animals, immunohistochemical staining (tyrosine hydroxylase).

Name: Adam Lundquist, BS
Project Role: Graduate Student
Research Identifier: N/A
Nearest person month worked: 6.0 mo (nonDoD funding)
Contribution to the project: Western blotting, qRT-PCR, brain dissection, data analysis

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

There are no updates to the active other support of the PD/PI or senior/key personnel since the last reporting period 9/15/2019 – 9/14/2020

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS: See attached

9. APPENDICES: Publications

- Wang Z, Flores I, Donahue E, Lundquist A, Guo Y, Jakowec MW, Holschneider DP, "Cognitive Flexibility Deficits in Rats with Dorsomedial Striatal 6-OHDA Lesions Tested Using a 3-Choice Serial Reaction Time Task with Reversal Learning", *NeuroReport, 31(15):1055-1064, 2020.*
- Wang Z, Lundquist AJ, Donahue E, Guo Y, Phillips D, Petzinger GM, Jakowec MW, Holschneider DP, "A mind in motion: Exercise improves cognitive flexibility, impulsivity and alters dopamine receptor gene expression in a Parkinsonian rat model" in review.

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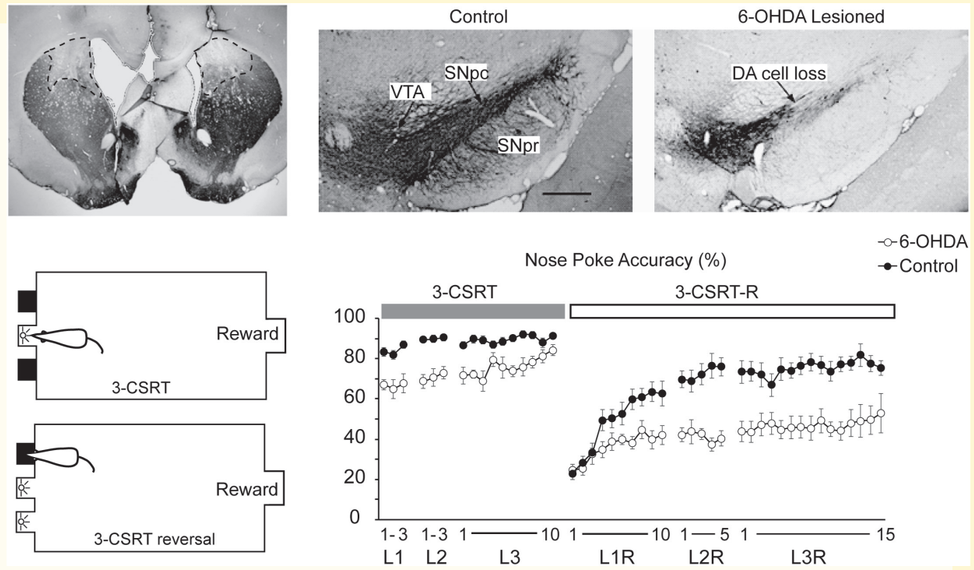

Unmasking deficits in cognitive flexibility after dopaminergic deafferentation.
see inside back cover

Wolters Kluwer

On the cover of this issue:

An early feature of Parkinson's disease (PD) is deficits in cognitive flexibility, an aspect of executive functions, which involve cognitive processes of set-shifting, working memory, and information processing. This can lead, even in early phases of the illness, to difficulty in multitasking, initiating new tasks, and switching tasks. In a rat model that reproduces features of PD, 6-hydroxydopaminergic lesioning of the dorsomedial striatum (top row, left-most figure) results in retrograde dopaminergic deafferentation at the level of the substantia nigra (SN, top row right-most figure) and cognitive deficits in a nose poke 3-choice serial reaction time task (3-CSRT, lower row) that is accentuated during reversal learning (3-CSRT-R). We propose that use of 3-CSRT-R testing in rats with bilateral dorsomedial striatal lesions may be a useful animal model for the future evaluation of treatments aimed at improving the executive dysfunction that is seen in PD.

Reproduced with kind permission from Daniel P. Holschneider and taken from the article Cognitive flexibility deficits in rats with dorsomedial striatal 6-hydroxydopamine lesions tested using a three-choice serial reaction time task with reversal learning. The paper appears on pages 1055–1064 of this issue.

Cognitive flexibility deficits in rats with dorsomedial striatal 6-hydroxydopamine lesions tested using a three-choice serial reaction time task with reversal learning

Zhuo Wang[a], Ilse Flores[b], Erin K. Donahue[b], Adam J. Lundquist[b], Yumei Guo[a], Giselle M. Petzinger[b,c], Michael W. Jakowec[b,c] and Daniel P. Holschneider[a,b,c,d]

Lesions of the dorsomedial striatum elicit deficits in cognitive flexibility that are an early feature of Parkinson's disease (PD), and presumably reflect alterations in frontostriatal processing. The current study aimed to examine deficits in cognitive flexibility in rats with bilateral 6-hydroxydopamine lesions in the dorsomedial striatum. While deficits in cognitive flexibility have previously been examined in rodent PD models using the cross-maze, T-maze, and a food-digging task, the current study is the first to examine such deficits using a 3-choice serial reaction time task (3-CSRT) with reversal learning (3-CSRT-R). Although the rate of acquisition in 3-CSRT was slower in lesioned compared to control rats, lesioned animals were able to acquire a level of accuracy comparable to that of control animals following 4 weeks of training. In contrast, substantial and persistent deficits were apparent during the reversal learning phase. Our results demonstrate that deficits in cognitive flexibility can be robustly unmasked by reversal learning in the 3-CSRT-R paradigm, which can be a useful test for evaluating effects of dorsomedial striatal deafferentation and interventions. ***NeuroReport*** **31: 1055–1064**

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Keywords: animal model, cognition, executive function, frontostriatal, mild cognitive impairment, operant learning, Parkinson's disease, striatum

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Introduction

Deficits in cognition, ranging from mild cognitive impairment (MCI) to dementia, are debilitating nonmotor symptoms in Parkinson's disease (PD) [1]. Prior even to the appearance of motor symptoms, patients may manifest an impairment of executive function. Executive dysfunction in PD can elicit deficits in attentional control, cognitive inhibition, inhibitory control, working memory, and cognitive flexibility, all of which can impair a patient's ability to plan, organize, initiate, and regulate goal-directed behavior. This can lead, even in early phases of the illness, to difficulty in multitasking, initiating new tasks, and switching tasks. While the etiology remains to be fully understood, the frontostriatal circuits, dopaminergic and cholinergic systems have been implicated in executive dysfunction [2].

Early diagnosis and intervention at the stage of MCI are believed to be critical for treatment. Animal models and behavioral tests that allow investigation of PD-related cognitive deficits are key to mechanistic research and preclinical testing of new treatments. However, studies in this area have been relatively few compared to research on motor deficits. A challenge is that cognitive tests in animals often rely on motor functions and motor impairment in many animal models of PD can therefore be a confound. Recent preclinical PD research using an animal model has explored the dorsomedial aspect of the striatum, a brain region associated with behavioral flexibility and cognitive switching [3]. Cognitive tests using touch screens that presumably require only limited locomotor activities have also been utilized with PD animal models [4].

The five-choice serial reaction time task (5-CSRT), modeled after clinical tests, has been broadly used to study operant learning, impulse control, and visual attention in rodents [5–7]. Tsutsui-Kimura *et al.* [8] reported a three-choice variation (3-CSRT) to shorten the training time for rats to reach learning criteria. The current study applied 3-CSRT with reversal learning (3-CSRT-R) to test cognitive flexibility in rats with 6-hydroxydopamine (6-OHDA) lesion to the bilateral dorsomedial striatum. Whereas past research has generally used lever press in the 5-CSRT, it has been shown that nose poke is an easier response to learn in rats [9]. We therefore chose nose poke, which has been used in more recent 5-CSRT studies [6,7]. In addition, sufficient time was allowed (stimulus duration) for completion of the nose poke choice. These choices were made to lower the task difficulty so

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that lesioned animals could rapidly learn the task to a similar level as controls prior to the initiation of reversal learning in which a rule change required cognitive switching. Thereby, cognitive flexibility could be investigated largely in separation from other possible cognitive deficits in the initial ability to acquire operant learning, attention, and impulse control.

Methods

Animals

Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Southern California, an institution approved by the Association for Assessment and Accreditation of Laboratory Animal Care, as well as by the Animal Use and Care Review Office of the US Department of the Army, and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats were purchased from Envigo (Placentia, California, USA) at age 8–9 weeks. Animals were housed under standard vivaria conditions in pairs on a 12 h light/12 h dark cycle (dark cycle 6 p.m. to 6 a.m.). A total of 38 rats were used. The main cohort of animals underwent 3-CSRT-R operant training as shown in Fig. 1a (lesioned rats, n = 6; controls, n = 6). Some lesioned animals in this cohort were also evaluated in overnight locomotor activities (n = 4), rotarod running (n = 2), sucrose preference (n = 2), and tyrosine-hydroxylase (TH) immunostaining (n = 4). In addition, 26 animals (lesioned n = 15, controls n = 11) from a pilot study were used to examine the effects of lesion on overnight locomotor activity (n = 9 lesioned), rotarod running (n = 6 lesioned, n = 8 controls), sucrose preference (n = 6 lesioned, n = 6 controls), and TH staining (n = 2 lesioned).

Animal model and stereotaxic surgical procedure

The 6-OHDA basal ganglia lesion model is a widely accepted model of dopaminergic deafferentation, and parallels many pathophysiologic features of the human disorder [10]. Animals were about 10 weeks old at the time of surgery. The procedure was as described before with changes in the injection sites [11]. To prevent any noradrenergic effects of the toxin, animals received desipramine (25 mg/kg in 2 ml/kg bodyweight in saline, i. p.; Sigma-Aldrich Co., St. Louis, Missouri, USA) before the start of surgery. They were then placed under isoflurane anesthesia (1.5% in 30% oxygen and 70% nitrous oxide) in a stereotaxic apparatus (David KOPF Instruments, Tujunga, California, USA) and received injection of 6-OHDA (Sigma-Aldrich Co.) at four sites targeting the dorsomedial striatum bilaterally (AP: +1.5, ML: ±2.2, DV: –5.2 mm, and AP: +0.3, ML: ±2.8, DV: –5.0 mm, relative to the bregma), which is the primary striatal sector targeted by the medial prefrontal cortex (anterior cingulate, prelimbic area) [12]. Injection of 10 µg of 6-OHDA dissolved in 2 µl of 0.1% L-ascorbic acid/saline was made at each site through a 10 µl Hamilton 1701 microsyringe (Hamilton Company, Reno, Nevada, USA) fitted with a

Fig. 1

3-CSRT	Stim Dur (s)	Limited Hold (s)	ITI (s)	TO (s)	Days
Pretraining a,b	n/a	n/a	n/a	n/a	1 (twice)
Pretraining c	∞	∞	2	n/a	2
Training Level 1	30	30	2	2	3
Training Level 2	15	15	2	2	3
Training Level 3	5	10	2	2	10
3-CSRT Reversal					
Pretraining aR	n/a	n/a	n/a	n/a	1 (twice)
Pretraining cR	∞	∞	2	n/a	5
Training Level 1R	30	30	2	2	10
Training Level 2R	15	15	2	2	5
Training Level 3R	5	10	2	2	15

Experimental protocol for operant training. (a) Timeline of experiment. (b) 3-CSRT and reversal learning. (c) Progressive training schedule. 3-CSRT, 3-choice serial reaction time task.

30-gauge, blunted needle, at 0.4 µl/min controlled by a Micro4 microsyringe pump controller (World Precision Instruments, Sarasota, Florida, USA). After injection, the needle was left in place for 5 minutes before being slowly retracted (1 mm/min). To allow comparison to normal 3-CSRT learning, naïve rats were used for controls. Carprofen (2 mg in 5 g tablet, p. o.; Bio-Serv, Flemington, New Jersey, USA) was administered for 1 day preoperatively and for 2 days postoperatively for analgesia. Following lesioning, animals were left to recover for 2 weeks prior to initiating the 3-CSRT training.

Tyrosine hydroxylase immunostaining

TH immunostaining data were collected from n = 6 lesioned animals about 16 weeks after 6-OHDA lesioning. Rats were humanely anesthetized and subjected to transcardial perfusion with 100 ml of ice-cold saline followed by 250 ml of ice-cold 4% PFA/PBS. Brains were removed, transferred to the same fixative for 24 h, and then immersed in 20% sucrose for 48 h. After sinking, brains were flash-frozen, mounted and cut at 25 µm thickness on a cryostat microtome in the coronal orientation throughout the entire anterior-posterior extent of the brain. Selective sections representing levels of the brain spanning the site of 6-OHDA in both the striatum and midbrain were subjected to TH-immunostaining. Sections were rinsed with tris-buffered saline (TBS) at room temperature (RT) for 30 minutes, and quenched with 3% H_2O_2 for 10 minutes at RT. After rinsing in TBS, slides were washed in TBS +0.2% Triton-X 100 (TBST-0.2%) for 60 minutes at room temperature, then blocked with 4% NHS/TBST-0.2% and incubated overnight at 4°C in primary antibody solution (1:2500 anti-tyrosine hydroxylase, clone LNC1; Millipore, Billerica, Massachusetts, USA) in 2% NHS/TBST-0.2%. Sections were visualized using secondary antibody solution (biotinylated anti-mouse IgG, Vectastain Elite ABC kit) 1:1000 in 2% NHS/TBST-0.05%, 60 minutes at RT. After rinsing with TBS, sections were placed in ABC Reagent for 60 min at RT. Staining was developed with 3,3′-diaminobenzidine (Vector Labs DAB Peroxidase Substrate Kit, Burlingame, California, USA), until optimal contrast on sections was achieved. Sections were then mounted, dried overnight, and dehydrated before being coverslipped.

Images of tissues were made using an Olympus BX-50 microscope at low magnification, and the lesion size of the striatum was estimated bilaterally in the digitized, thresholded images of each rat by manual tracing using ImageJ 1.52k (Wayne Rasband, National Institutes of Health). Certain anatomical landmarks were used in the selection of striatal sections to demarcate rostral (bregma +2.28 to +1.28 mm, representative level +1.80 mm, genu of the corpus callosum with lateral appearance of the anterior commissures), mid (bregma +1.28 to +0.36 mm, representative level 0.72 mm, medially located anterior commissure), and caudal levels (bregma +0.24 to −0.48 mm, representative level 0.00 mm, posterior tail of the anterior commissure). Lesioned striatal areas were evaluated as a percentage of bilateral total striatal area.

Spontaneous overnight locomotor activity

Overnight locomotor activity of the animals (n = 13) was recorded prior to 6-OHDA lesioning and 2 weeks thereafter. Recordings were performed in the vivarium during the dark cycle (6 p.m. to 6 a.m.). Animals were individually placed into clear plastic filtertop cages (46 cm length × 25 cm width × 22 cm height) with fresh direct bedding. During the recording period, animals were given a water gel cup for fluid intake but no food chow. Activity counts of each rats were recorded in the horizontal and vertical planes in time bins of 15 minutes by an infrared beam break system (Opto-M3, 160 Hz beam scan rate, 2.5 cm sensor spacing, 16 × 16 sensor grid; Columbus Instruments, Columbus, Ohio, USA) mounted around their cages.

Accelerating rotarod test

The effects of lesioning on coordination, balance and strength were evaluated on rotarod, a rotating cylinder treadmill with a diameter of 7.3 cm [11]. Data were collected from animals (n = 8 lesioned, n = 8 controls) after the completion of operant training and with ad-lib food access. Rats were familiarized with the rotarod (Columbus Instruments) at 2.3 m/min for 3 minutes twice the day before testing. Rats were run using an acceleration paradigm (initial speed: 5 rpm = 1.15 m/min, acceleration rate: 6 rotations/min^2 = 1.38 m/min^2, two trials/day, 30-minute intertrial interval for 2 days) until they fell onto a padded surface or reached the 5 minutes cutoff time (maximum speed: 35 rpm = 8.02 m/min). The outcome variable was the latency to fall averaged over four trials.

Sucrose preference test

We examined the effects of lesioning on sucrose preference to investigate anhedonia, which could emerge in toxin-induced models and therefore impact the motivation for reward in 3-CSRT. Data were collected from animals (n = 8 lesioned, n = 6 controls) after the completion of operant training and with ad-lib access to food. The protocol used was similar to those we have previously published [13]. To minimize neophobia, rats were exposed to the sucrose solution overnight for 2 days. The water bottle in each home-cage was replaced with two 50-ml bottles fitted with ball-point drinking spouts containing 2% sucrose. On the day of testing, rats were water-deprived for approximately 9 h. At the onset of the dark phase, rats were individually housed overnight with access to two bottles, one containing 2% sucrose and the other water. Each filled bottle was weighed before and after the sucrose preference test, with fluid consumption measured by the difference. The location of the sucrose bottle (to the right or left side of the cage) was alternated to minimize side

preferences. Sucrose preference was calculated as a percentage of total fluid intake, that is, 100 × volume sucrose intake/(volume of sucrose + volume water).

Food restriction

Food restriction was started 2 weeks after lesion surgery and maintained throughout the experiment. Animals were brought to 85% of their baseline bodyweight in 1 week and were allowed to gain 5 g in body weight per week thereafter, with ad libitum access to water. Animals were fed after behavioral training. Body weights were recorded Monday–Friday, with meal size (Rodent Diet #5001; LabDiet, St. Louis, Missouri, USA) individually adjusted on a daily basis.

Three-choice serial reaction time task with reversal learning

We modified the well-established 5-CSRT protocol and its 3-CSRT variation (Tsutsui-Kimura *et al.*, 2009) (Fig. 1a). (1) Animals were trained through three difficulty levels with progressively shortened stimulus durations. While most 5-CSRT protocols train the animal at each difficulty level for a variable number of days until the animal reaches certain performance criteria, we chose to control the number of training days for each level across animals to facilitate between-group comparison. (2) The final stimulus duration was set at 5 seconds, reflecting a moderate level of difficulty. This was selected based on pilot data showing that 6-OHDA lesioned animals can reach a performance level comparable to that of control animals at this difficulty level. Differences in reversal learning can thus be interpreted as differences in cognitive flexibility, rather than as differences in operant learning per se. (3) During the reversal phase of training, the rule was switched from rewarding nose poke into a lit aperture to rewarding nose poke into a dark aperture.

Training was started 1 week following the initiation of food restriction and was always performed between 8 a.m. and 1 p.m. Each operant cage (MedAssociates, St. Albans, Vermont, USA) consisted of a sound-attenuating cubicle (63 cm width, 46 cm depth, 61 cm height) with a fan which was always turned on during testing, modular test chamber (33 cm width, 25 cm depth, 33 cm height) with grid floor, house light, three-bay nose poke wall, pellet dispenser on the wall opposite the nose poke bays, pellet trough receptacle, receptacle light, head entry detector, smart controller, and infrared camera (Birdhouse Spy Cam, West Linn, Oregon, USA) for real-time viewing of animals on a TV monitor. Cages were operated by MED-PC software using a personal computer. Pellet dispensers were loaded with dustless sucrose pellets (45 mg/pellet, #F0025; Bio-Serv). Behavioral training was implemented with a fixed ratio FR1 schedule response-reward task (up to 90 trials or 30 min/day, 5 days/week). The walls, nose poke apertures, food receptacle, and grid floor were wiped with 70% isopropyl alcohol between animals.

Habituation and shaping of behavior

Rats were familiarized with the test chamber and sugar pellets prior to training. Nose poke and reward retrieval behavior were shaped in pretraining. In pretraining '*a*', 10 sugar pellets were put in each nose poke aperture and pellet receptacle, and the animal was allowed to explore the test chamber and retrieve the pellets for 15 minutes. In pretraining '*b*', the animal was kept in the test chamber for 10 minutes, while a sugar pellet was dispensed into the receptacle every 20 seconds with the receptacle light turned on. The receptacle light was turned off 2 seconds after detection of a head entry (reward retrieval). Pretraining '*a*' and '*b*' were repeated once during the same day. In pretraining '*c*', the animal was trained to associate nose poking into a lit aperture with receiving a single sugar pellet reward into the receptacle. Each daily session lasted 30 minutes or until the animal received 90 rewards. For each trial, the light in a pseudorandomly chosen nose poke aperture was turned on (stimulus). When a nose poke was detected in the lit aperture (correct nose poke), the aperture light was turned off, and the light in the pellet receptacle was turned on with a sugar pellet dispensed (reward). The receptacle light was turned off 2 seconds after detection of a head entry. After a 2-second inter-trial interval (ITI), the next trial was started.

Three-choice serial reaction time task training

During the regular phase of 3-CSRT training, the animal was trained following a progressive schedule (Fig. 1b and c). The animal was trained to make a correct nose poke in response to a relatively short stimulus duration. Each single daily session lasted 30 minutes or until 90 trials were reached. At the start of each trial, the chamber light was turned on and a randomly selected stimulus was started. The stimulus stayed on for a set duration or until a nose poke (correct or incorrect) was detected. The animal received a food reward following a correct nose poke within the set limited hold duration, which was set to be the same as the stimulus duration or slightly longer for short stimulus durations. Following reward retrieval and ITI, the next trial was started. If an incorrect nose poke was detected, the animal was punished with a time out (TO), during which the chamber light was turned off for 2 seconds. If no nose poke was detected within the limited hold duration, an omission was recorded, and the animal punished with a TO. After each TO, the chamber light was turned on, and after an ITI, the next trial was started. If a nose poke was detected during the ITI, a premature response was recorded without incrementing the trial number, and the animal punished with a TO. Any nose pokes following a correct response and before reward retrieval were recorded as perseverative responses.

Reversal training

During the reversal phase of 3-CSRT-R training (Fig. 1b and c), the stimulus was switched from a lit aperture

among dark apertures to a dark aperture among lit apertures. The animal was trained progressively to learn to nose poke the dark aperture to receive reward. The current task, while not a classical reversal task [14,15] that usually involves two stimuli and two locations, incorporated essential elements of reversal learning [16], with the addition of a third location that aided in avoiding solving the discrimination using simple configural learning strategies [16,17]. Pretraining cR (Fig. 1c), in which the animal was rewarded until it made a correct nose poke (into a dark hole) without any timeout punishment, was critical to initial acquisition of the reversal task. This modification was necessitated by an increase in the task difficulty and to avoid diminishing the animal's motivation to complete the task.

Analysis of the operant behavior included [7]:

(1) nose poke accuracy = (number of correct responses)/(number of correct + number of incorrect responses) × 100%, a primary measure of operant learning;
(2) omissions rate = (number of omissions)/(number of trials completed), a measure of attention;
(3) premature responses, a measure of impulsivity;
(4) perseverative rate = (number of perseverative responses)/(number of correct responses), a measure of compulsive behavior;
(5) correct nose poke latency = average time from onset of stimulus to a correct response, a measure of attention and cognitive processing speed;
(6) reward retrieval latency = average time from correct response to retrieval of sugar pellet, a measure of motivation.

It is important to note that sensorimotor functions contribute critically to the operant training performance. Therefore, interpretation of the above variables should take into consideration possible lesion-induced sensorimotor dysfunctions.

Statistical analysis

Data are presented as the mean ± SEM and analyzed using GraphPad Prism (version 8.3.0; GraphPad Software, San Diego, California, USA). All data were subjected to the Shapiro–Wilk test for normality. The following data transformations were applied to improve normality and homogeneity of variance: arcsine for nose poke accuracy, logarithm for nose poke latency, reciprocal for reward latency, square root for premature responses and perseverative rate. 3-CSRT data were analyzed using a two-way analysis of variance (ANOVA) with repeated measures for each training level, with lesion and time as the two factors, and with Holm–Sidak's post-hoc multiple comparisons test. Data for individual days that failed the normality test were excluded from ANOVA and analyzed separately using the Mann–Whitney test. Data were subjected to Bartlett's test for homogeneity of variance for each training level. Data that failed the Bartlett's test were analyzed using unpaired Student's or Welch's *t*-test (based on Levene's test for equal variance) to compare 6-OHDA lesioned and control group on individual days instead of ANOVA. Overnight activity data were analyzed using paired Student's *t*-test. Accelerating rotarod and sucrose preference data were analyzed using unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Lesion verification

TH immunostaining confirmed that the dopamine-depletion lesion was mainly limited to the dorsomedial aspect of the striatum, a region of the basal ganglia central to cognitive processing. Figure 2a shows representative brain slices at three bregma levels designated as rostral, mid, and caudal with reduced TH immunoreactivity in the dorsomedial striatum. Lesioned area was quantified as a percentage of total striatal area bilaterally: rostral (26.21 ± 6.23%), mid (32.89 ± 3.18%), and caudal (34.92 ± 2.43%) (Fig. 2b). There was also loss of TH-immunoreactive cells in the substantia nigra pars compacta (Fig. 2d compared to Fig. 2c).

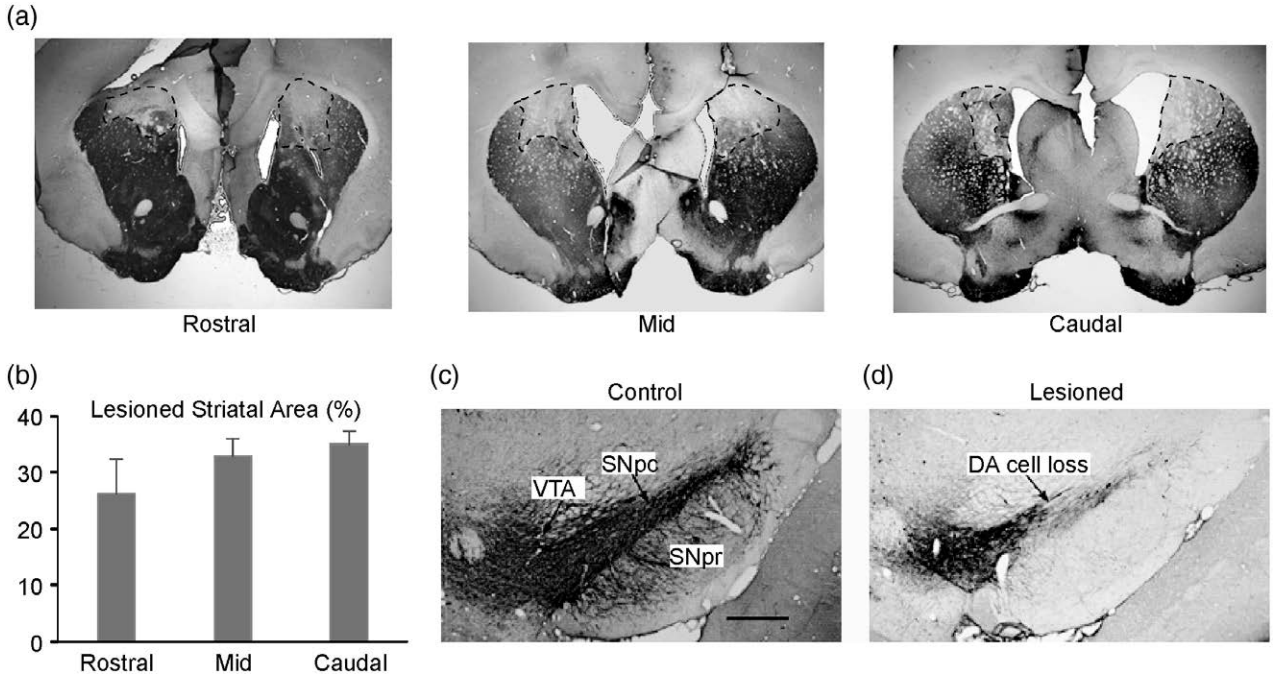
Lesion spared motor functions and sucrose preference

Overnight activity measurement in home cage showed no significant differences in horizontal activity counts 2 weeks after 6-OHDA lesioning (15 077 ± 1296 counts, $n = 13$) compared to baseline (18 223 ± 1815 counts, $P = 0.074$, paired Student's *t*-test), and in vertical activity counts (4870 ± 1129 counts) compared to baseline (6033 ± 1531 counts, $P = 0.52$; Fig. 3a). There was also no significant lesioning effect on the maximum velocity during any 15-minute intervals (data not shown). In the accelerating rotarod test, no significant differences were evident in latency to fall between control (169 ± 15 seconds, $n = 8$) and lesioned animals (161 ± 22 seconds, $n = 8$, $P = 0.77$, unpaired Student's *t*-test; Fig. 3b). Analysis of sucrose preference (Fig. 3c) revealed that 6-OHDA lesioned animals (74.76 ± 4.60%, $n = 8$) did not differ from controls (78.76 ± 6.06%, $n = 6$, $P = 0.20$, unpaired Student's *t*-test).

Lesion induced deficits in cognitive flexibility

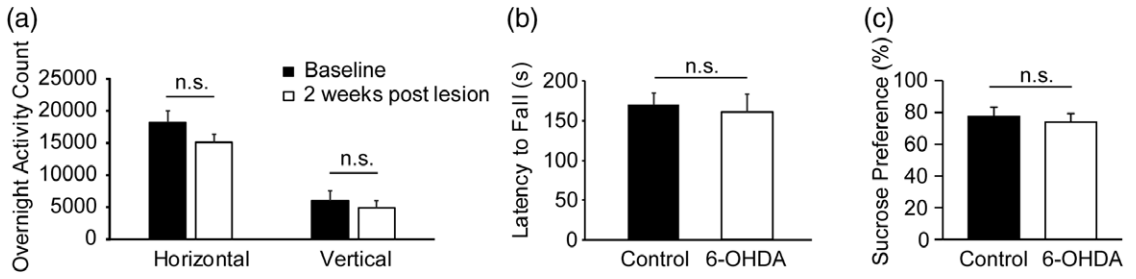
During the acquisition phase of 3-CSRT (levels L1, L2, and L3), both lesioned and control rats showed improvement in nose poke accuracy and shortening of correct nose poke latency (Fig. 4). Lesioned rats compared to controls showed: (1) statistically significant lower nose poke accuracy (L1: $F_{1,10} = 26.39$, $P = 0.0004$; L2: $F_{1,10} = 40.68$, $P < 0.0001$; L3: $F_{1,10} = 23.47$, $P = 0.0007$. Main lesion effect, two-way ANOVA repeated measure) that diminished towards the end of L3 ($P = 0.16$ for day 9 and day 10 of L3, Holm–Sidak post-hoc test; Fig. 4a); (2) no significant differences in omission rate ($P > 0.05$, Mann–Whitney test), but statistically significant differences in variance (Levene's test; Fig. 4b); (3) no significant differences in nose poke latency (L1: $F_{1,10} = 4.121$, $P = 0.070$; L2: $F_{1,10} = 3.58$, $P = 0.088$; L3: $F_{1,10} = 1.942$, $P = 0.19$. Main

Fig. 2



Immunostaining for tyrosine hydroxylase to determine the degree and anatomical site of lesion. (a) Representative images of coronal sections reveal bilateral loss in TH immunoreactivity in the dorsomedial striatum (rostral: bregma + 1.80 mm, mid: +0.72 mm, caudal: +0.00 mm). (b) Lesioned striatal areas were quantified as percent of bilateral striatal area at rostral, mid, and caudal levels ($n = 6$). (c and d) Representative images showing lesion-induced loss in TH immunoreactivity in the substantia nigra pars compacta (bregma − 5.28 mm). Scale bar = 0.5 mm. TH, tyrosine-hydroxylase.

Fig. 3

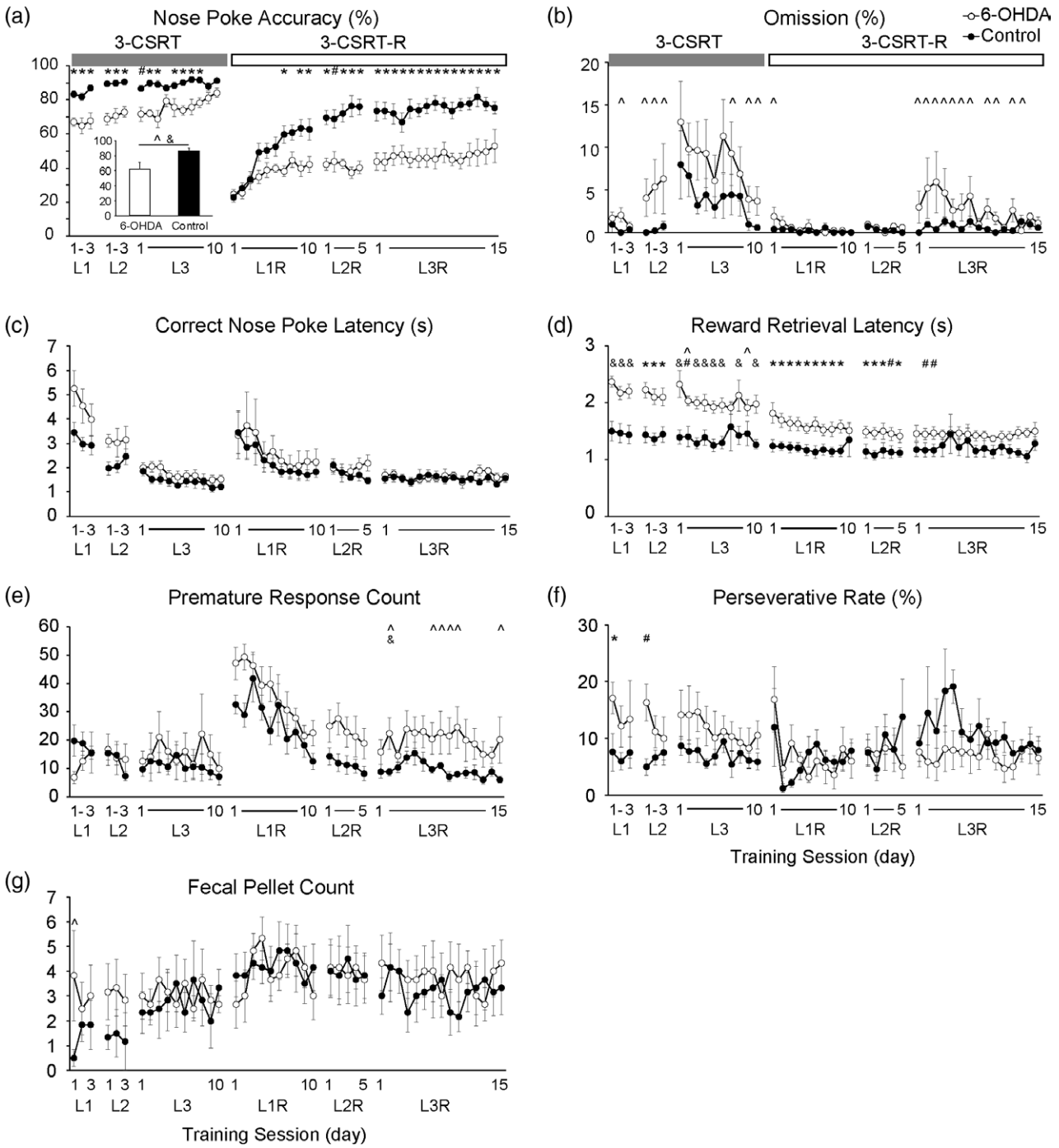


Lesion largely spared motor functions and sucrose preference. (a) Overnight locomotor activity before and 2 weeks after 6-OHDA lesioning ($n = 13$, horizontal $P = 0.074$, vertical $P = 0.52$, unpaired Student's t-test). (b) Accelerating rotarod test showed no significant differences in mean latency to fall between lesioned ($n = 8$) and control rats ($n = 8$, $P = 0.77$). (c) Analysis of sucrose preference revealed no significant differences between lesioned ($n = 8$) and control rats ($n = 6$, $P = 0.20$, unpaired Student's t-test). 6-OHDA, 6-hydroxydopamine.

lesion effect, ANOVA; Fig. 4c); (4) statistically significant greater reward retrieval latency (L1: $P < 0.005$, Student's t-test; L2: $F_{1,10} = 17.71$, $P = 0.0018$, ANOVA; L3: $P < 0.05$, t-test and Mann–Whitney test; Fig. 4d); (5) no differences in premature responses (Fig. 4e) and fecal pellet count (Fig. 4g); and (6) statistically higher perseverative rate in L1 ($F_{1,10} = 17.71$, $P = 0.033$, main lesion effect, ANOVA) and day 1 of L2 ($P = 0.043$, Mann–Whitney) that diminished in L3 ($F_{1,10} = 2.024$, $P = 0.19$, main lesion effect, ANOVA; Fig. 4f).

At the start of the 3-CSRT-R, when the rule for correct (rewarded) response was switched from nose poking a lit aperture to nose poking a dark aperture, both lesioned and control rats showed a sudden drop in performance with decreased nose poke accuracy to the same extent, increased correct nose poke latency, and increased premature responses. Both groups showed improvement in these parameters with continued training. Control rats improved nose poke accuracy to a plateau of about 75%, while lesioned animals only improved accuracy modestly

Fig. 4



Differences in 3-CSRT acquisition and reversal learning (3-CSRT-R) in 6-OHDA lesioned animals ($n = 6$) compared to controls ($n = 6$). (a) While 6-OHDA animals were moderately impaired in 3-CSRT acquisition with lower nose poke accuracy, profound deficits were noted during the reversal phase compared to controls. Inset shows average nose poke accuracy over the last 5 days of L3R (level 3, reversal) normalized by the mean of last 5 days of L3 (level 3). The normalize accuracy was significantly lower in 6-OHDA animals ($P < 0.05$, Welch's *t*-test). (b) Omission rate. (c) Correct nose poke latency. (d) Reward retrieval latency was longer in 6-OHDA animals compared to controls. (e) Premature responses. (f) Perseverative rate. (g) Fecal pellet count. $P < 0.05$, 6-OHDA vs. control groups: *Holm–Sidak post-hoc test, two-way ANOVA repeated measure; #Mann–Whitney test; &Student's or Welch's *t*-test; and ^Levene's test for homogeneity of variance. 3-CSRT, 3-choice serial reaction time task; 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance.

to a plateau of about 50%. There were statistically significant differences between the two groups (Fig. 4a, L1R: $F_{1,10}$ = 6.451, P = 0.029; L2R: $F_{1,10}$ = 29.26, P = 0.0003; L3R: $F_{1,10}$ = 16.28, P = 0.0024; main lesion effect, ANOVA). Figure 4a inset shows average nose poke accuracy over the last 5 days of L3R normalized by the mean of the last 5 days of L3. The normalized accuracy was significantly lower in 6-OHDA animals (P = 0.041, Welch's t-test). Lesioned compared to control rats continued to demonstrate significantly greater reward retrieval latency up to day 3 of L3R (Fig. 4d, L1R: $F_{1,10}$ = 11.16, P = 0.0075; L2R: $F_{1,10}$ = 6.69, P = 0.027; L3R: $F_{1,10}$ = 4.843, P = 0.052; main lesion effect, ANOVA; days 2 and 3 of L3R, $P < 0.05$, Mann–Whitney). There were no significant differences in premature responses (Fig. 4e, L1R: $F_{1,10}$ = 3.241, P = 0.10; L2R: $F_{1,10}$ = 4.602, P = 0.058, main lesion effect, ANOVA), except on day 2 of L3R (P = 0.037, Welch's t-test), but significant differences in variance (L3R, Levene's test). Also noted were significant differences in variance in omission rate between the lesioned and control rats (Fig. 4b, Levene's test). No between-group differences were noted in correct nose poke latency, perseverative rate, and fecal pellet count.

Discussion

We modified the well-established 5-CSRT paradigm to a 3-CSRT task with nose poke and reversal learning to test cognitive flexibility in animals with 6-OHDA lesion to the dorsomedial striatum. Lesioned animals compared to controls showed robust and persistent deficits in reversal learning, despite having previously learned the task to an equivalent extent, and in the absence of anhedonia and general deficits in motor functions.

Acquisition of three-choice serial reaction time task

The CSRT paradigm is an operant learning task widely used to study attention and impulse control in rodents [6]. The task requires a consecutive series of information processing, decision making, and actions, including waiting for the stimulus and inhibition of premature responses during the inter-trial interval → attention to the stimulus → recall of prior successful responses → choice of nose poke response → nose poke → recall of reward retrieval → decision to initiate reward retrieval → reward retrieval. Control rats quickly learned the 3-CSRT task and reached a plateau of about 90% accuracy. Lesioned rats, while showing deficits in the initial phase of training, were able to reach a comparable level of accuracy (84.24 ± 2.64%) after 4 weeks of training. During the acquisition of the 3-CSRT, there were no significant group differences in premature responses. This suggests that during acquisition there was little evidence for a group difference in impulsive behavior. It is important to note that the animals were not challenged with longer ITIs to test impulsivity more vigorously. The lesioned animals compared to controls did not show significant differences in means, but did show statistically significant differences in variance during the later portion of 3-CSRT-R, suggesting a mild lesion-induced deficit in attention.

Lesioned compared to control rats demonstrated a significantly greater reaction time for reward retrieval. Although motor deficits could in principle contribute to group differences seen in reward retrieval latency and omission rate, several lines of evidence argued against general motor impairment. Lesion did not induce significant differences in spontaneous locomotor activity or in general motor strength, balance and coordination as measured using the accelerating rotarod test. Of importance, the correct nose poke latency following the first week of initial learning was almost identical between the two groups. This suggests that after the first week, lesioned compared to control animals showed comparable levels of attention, speed for information processing and decision making, and speed to nose poke action. Likewise, no lesion effect was noted in the appetitive preference for sucrose reward using the sucrose preference test. This suggests that differences in reward retrieval latency (or omissions rate) likely reflect a slowing of cognitive processing of reward expectation and mildly impaired attention, rather than general motor dysfunction, lack of motivation, or severe attention deficit. The number of fecal pellets counted during the learning phase showed no group differences, suggesting no lesion effect on anxiety-like behavior during the cognitive challenges in 3-CSRT. Our study did not assess possible lesion effects on the somatosensory perception which if present chronically could have modulated our behavioral responses.

Reversal learning in three-choice serial reaction time task

The reversal learning phase was initiated at a time point when lesioned and control animals had reached similar levels of accuracy. During the initial stage of reversal learning, both lesioned and control rats showed a sudden drop in nose poke accuracy to the same extent. However, with continued training of only a few sessions, control rats rapidly improved their performance, reaching a plateau of about 80% accuracy, while lesioned animals only improved modestly and reached a plateau of about 50% accuracy. We further normalized the average accuracy over the last 5 days of reversal learning (L3R) by the mean of accuracy over the last 5 days of regular training (L3), to control any possible lesion-related deficits in motor and cognitive functions. The normalized 3-CSRT-R accuracy remained significant lower in lesioned (61.83 ± 8.78%) compared to control animals (86.00 ± 4.28%) (Fig. 4a, inset). Thus, the 3-CSRT-R task unmasked lesion-induced deficits in cognitive flexibility.

Deficits in reversal learning can be impacted by 'perseverant' responses, that is, the inappropriate maintenance of responses previously associated with either reward

(learned-reward response) and/or with non-reward (learned-nonreward responses) [18]. The exact contribution of persistent learned-reward or learned-nonreward responses in the lesioned animals, and the role these might play in inhibiting new learning of the reversal task is unclear. There was a nonsignificant trend of greater premature responses and omission rate in lesioned compared to control animals, as well as significant differences in variance, suggesting mild impairment in impulse control and attention.

Our findings suggest that learning is substantially more rapid in the 3-CSRT and 3-CSRT-R nose-poke tasks than has been typically reported with either the 5-CSRT and 5-CSRT-R lever-press task [19,20], the 5-CSRT touchscreen task [17], or the 5-CSRT nose-poke task [6,7]. In part, such difference may be related to the fact that nose-poke responses occur at a higher baseline rate compared to those of lever pressing or touchscreen responses. Significant lesion effect in nose poke accuracy was achieved with a relatively small number of animals (n = 6/group), possibly a reflection of smaller variability in a behavior well within the natural repertoire of the animal.

Of note, De Bruin *et al.* [21] previously applied a variant of the 5-CSRT lever as a 2-CSRT lever-pressing task, a paradigm later adapted by Homberg et al. to a two-choice nose-poke paradigm (2-CSRT) [22]. The latter, using a fixed-ratio FR3 schedule of reinforcement in nonlesioned rats, demonstrated learning acquisition in 25 training sessions of 50 trials per session, and reversal-learning to criterion performance in three sessions. This shortened duration for reversal training is consistent with the notion that reversal learning decreases in difficulty as the number of holes available for nose poke decreases. While the shortening of training time is desirable, the lower level of task difficulty may decrease the sensitivity to detect deficits in executive function. Therefore, experimental design, and the choice of 5-CSRT or 3-CSRT should be based upon the anticipated magnitude of the deficit.

Dorsomedial striatal lesions

An early feature of PD is deficits in cognitive flexibility, an aspect of executive functions, which involve cognitive processes of set-shifting, working memory, and information processing. Dopamine loss in PD patients is predominant in the posterior putamen, a region associated with the control of habitual behavior. It has been proposed that executive dysfunction, may result as patients become overly reliant on the goal-directed mode of action control that is mediated by comparatively preserved processing in the rostromedial striatum [23]. While not identical to PD, the 6-OHDA lesioning of dopaminergic neurons of the nigrostriatal system reproduces many of its features [10]. Dopaminergic lesions in the dorsomedial striatum of rodents are critical for successfully observing impaired reversal learning [3,24,25]. Past work has shown that the formation of the critical action-outcome associations mediating goal-directed learning are localized to the dorsomedial striatum, whereas the sensorimotor connections that control the performance of habitual actions or procedural learning are localized to the dorsolateral striatum [26]. In patients and in the animal models, such deficits presumably reflect alterations in frontostriatal processing [12]. Our TH immunostaining results showed that lesions were primarily localized in the mid-caudal levels of the striatum, and appropriately limited to the dorsomedial quadrant of the striatum. Retrograde dopaminergic cell losses were also apparent bilaterally in the substantia nigra. While in 6-OHDA rodent model deficits in cognitive flexibility have previously been examined using the cross-maze [3], T-maze [27], and a food-digging task [28], the current study is the first to examine such deficits during the reversal phase of a choice serial reaction time task. Our results demonstrate that dramatic and persistent deficits in cognitive flexibility can be robustly detected in the 3-CSRT-R nose-poke paradigm 2 weeks after initiation of the change of rule. While differences in experimental design and criteria for determining 'learning', as well as rodent strains may affect the final duration of experimentation, our findings underscore the practicality of using a 3-CSRT-R nose poke paradigm in evaluating cognitive flexibility. We propose that use of the 3-CSRT-R in rats with bilateral dorsomedial striatal lesions may be a useful model for future testing of treatments aimed at improving executive dysfunction in PD.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Current Research in Neurobiology

A mind in motion: Exercise improves cognitive flexibility, impulsivity and alters dopamine receptor gene expression in a Parkinsonian rat model

--Manuscript Draft--

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Manuscript Classifications:	Executive function and thought; Learning and memory; Nervous System Disorder; Neurobiology of Learning; Neurological Disease Treatment; Neurological Systems of Learning
Abstract:	Cognitive impairment, including deficits in executive functions (EF) is common in Parkinson's disease (PD) but inadequately addressed with current therapeutics. Here we examine the hypothesis that exercise improves cognitive flexibility, an important component of EF, in a rodent model of PD. Rats received 6-hydroxydopamine lesions of the bilateral, dorsomedial striatum, a cerebral region central to set shifting and reversal learning. Animals were exercised on motorized running wheels or horizontal treadmills for 6-12 weeks. Cognitive flexibility was evaluated using an operant 3-choice serial reaction time task (3-CSRT) with rule reversal (3-CSRT-R), and a T-maze task with reversal. Changes in striatal transcript expression of dopamine receptors (Drd1-4) and synaptic proteins (Synaptophysin, PSD-95) were separately examined following 4 weeks of training. Exercised/lesioned rats showed a significant, modest improvement in response accuracy in the 3-CSRT-R and T-maze, as well as a robust improvement in inhibitory aptitude in the 3-CSRT-R. Exercise also elicited increased expression of Drd1 , Drd3 , Drd4, synaptophysin, and PSD-95 in the dorsal (associative and sensorimotor) striatal regions. Our results underscore the observation that exercise, in addition to its accepted role in improving motor function, can also improve EF and dopaminergic function at the level of the striatum in animal models of PD.
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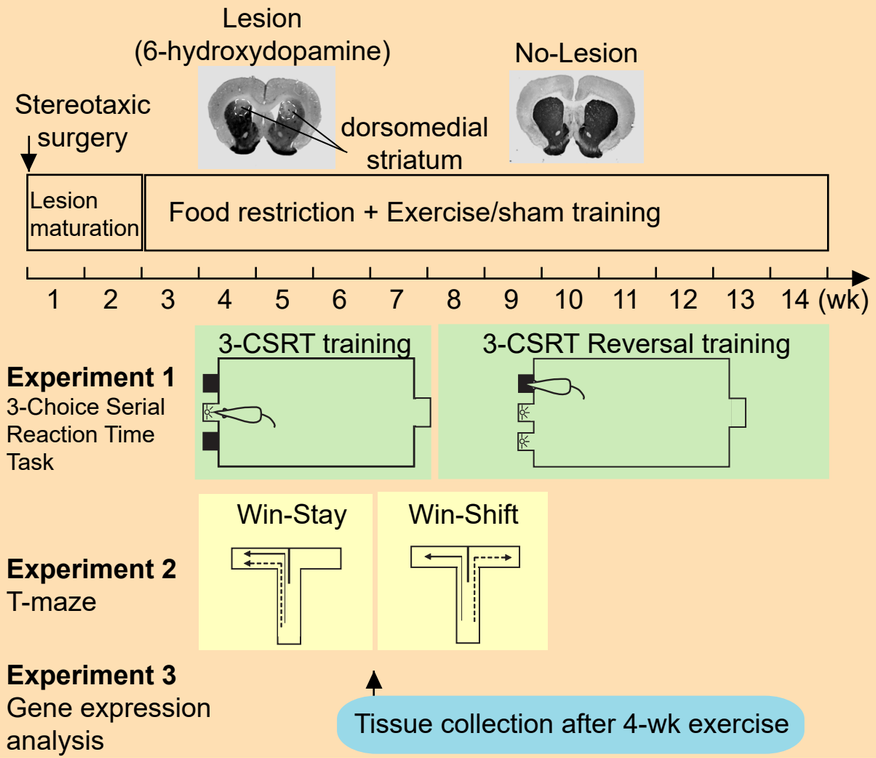
	Phillip M Baker, Ph.D. Assistant Professor, Seattle Pacific University bakerp5@spu.edu Expertise in neural basis of behavior, rodent models. Author of paper of relevance: Gena M. Grospe, Phillip M. Baker, Michael E. Ragozzino. (2018). Cognitive flexibility deficits following 6-OHDA lesions of the rat dorsomedial striatum. Neuroscience 374, 80-90.
	Jeff Beeler, Ph.D. Professor, CUNY Queens College: Queens College jbeeler@qc.cuny.edu Expertise in information processing in the basal ganglia and its role in learning and motivation, with relevance to Parkinson's disease

Authors' Public and Media Statement

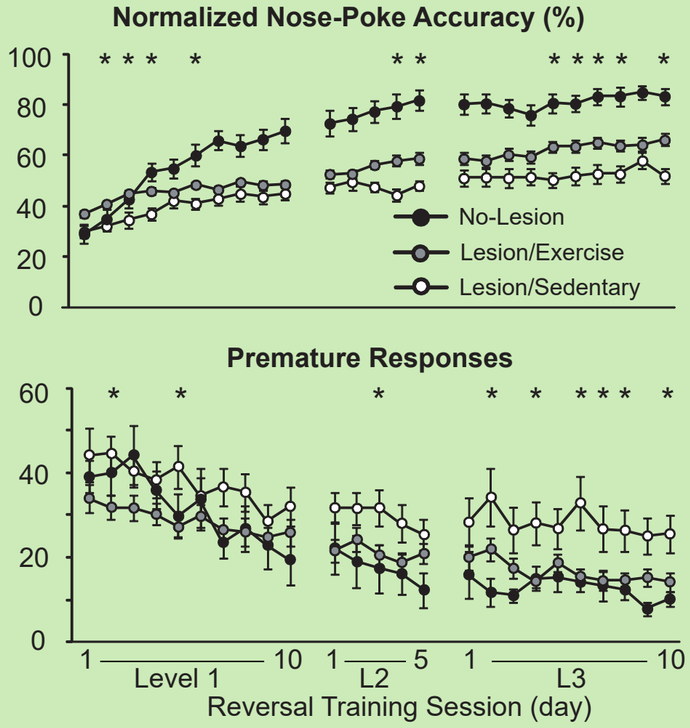
Parkinson's disease (PD) affects more than 6 million people worldwide, making PD the second most common neurodegenerative disease. As a result of the aging of the population and by-products of industrialization, PD is now the most rapidly growing neurological disorder in the world, with the numbers of Parkinson's patients expected to rise exponentially from 6.9 million in 2015 to 14.2 million in 2040. While motor symptoms are common in Parkinson's Disease, cognitive decline is an underappreciated and a poorly understood consequence of chronic PD, which typically transitions to dementia, fall risk, and poor Quality of Life. Currently, there is no effective treatment for cognitive deficits. A public health priority is to identify cost-effective therapies to combat progressive cognitive impairment in PD. There is significant evidence from short-term clinical studies that different types of physical exercise may help motor rehabilitation in PD. Fewer studies have examined the long-term relationship between exercise and long-term cognitive function in PD patients. The current study examined the effects of physical exercise on cognition in an animal model of PD. Animals were exercised on motorized running wheels or horizontal treadmills for 6-12 weeks. Cognitive flexibility was evaluated using tasks in which animals learned a rule to obtain a food reward, a rule which subsequently was reversed to obtain the same reward. Exercise in the Parkinsonian rats showed a significant, modest improvement in response accuracy as well as a robust improvement in inhibitory aptitude in the cognitive tasks. In addition, exercise elicited increased expression of dopamine receptors typically altered in PD, as well as markers of brain plasticity, all suggesting the beneficial cognitive effects of physical exercise in this disorder.

A mind in motion: Exercise improves cognitive flexibility, impulsivity and alters dopamine receptor gene expression in a Parkinsonian rat model

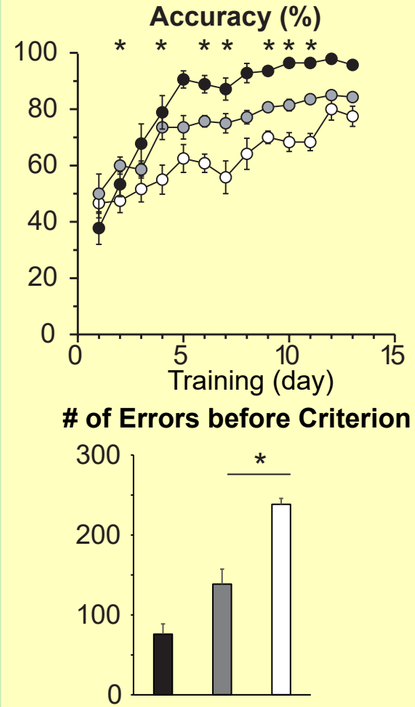
Experiment Design



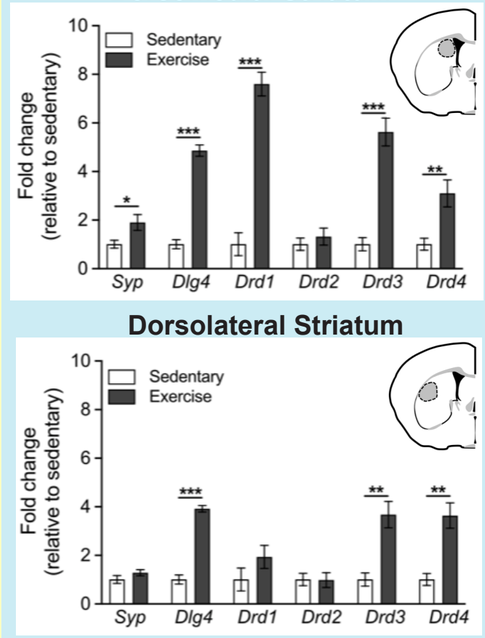
Experiment 1 Exercise improved lesion-induce deficits in reversal learning and impulsivity



Experiment 2 Exercise improved lesion-induce deficits in learning of the Win-Shift task



Experiment 3 Exercise increased expression of genes for dopaminergic receptor (DrD1, DrD3, DrD4) and synaptic plasticity (Syp, Dlg4)



CONCLUSIONS Our findings add to the expanding research reports showing the beneficial cognitive effects of physical exercise. Following dopaminergic deafferentation, moderate exercise improved cognitive flexibility and impulsivity, while eliciting increased expression of Drd1, Drd3, Drd4, synaptophysin, and PSD-95 in the associative and sensorimotor dorsal regions of the striatum.

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HIGHLIGHTS

- Cognitive impairment is common in Parkinson’s disease (PD) but inadequately treated
- In a PD rat model, exercise improved cognitive flexibility and inhibitory aptitude
- Exercise also elicited increase in gene expression of striatal dopamine receptors

A mind in motion: Exercise improves cognitive flexibility, impulsivity and alters dopamine receptor gene expression in a Parkinsonian rat model

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Author Contributions: ZW, AJL, EKD, YG, GMP, MWJ, DPH contributed to the conception and design of the study; ZW, AJL, EKD, YG, DP, GMP, MWJ, DPH contributed to data acquisition; ZW, AJL, EKD, YG, GMP, MWJ, DPH were responsible for data analysis; ZW, AJL, DPH were responsible for statistical analyses; ZW, AJL, EKD, GMP, MWJ, DPH were responsible for writing and editing the manuscript; GMP, MWJ, and DPH were responsible for acquisition of funding support.

Declarations of interest: None

ABSTRACT

Cognitive impairment, including deficits in executive functions (EF) is common in Parkinson's disease (PD) but inadequately addressed with current therapeutics. Here we examine the hypothesis that exercise improves cognitive flexibility, an important component of EF, in a rodent model of PD. Rats received 6-hydroxydopamine lesions of the bilateral, dorsomedial striatum, a cerebral region central to set shifting and reversal learning. Animals were exercised on motorized running wheels or horizontal treadmills for 6-12 weeks. Cognitive flexibility was evaluated using an operant 3-choice serial reaction time task (3-CSRT) with rule reversal (3-CSRT-R), and a T-maze task with reversal. Changes in striatal transcript expression of dopamine receptors (*Drd1-4*) and synaptic proteins (*Synaptophysin, PSD-95)* were separately examined following 4 weeks of training. Exercised/lesioned rats showed a significant, modest improvement in response accuracy in the 3-CSRT-R and T-maze, as well as a robust improvement in inhibitory aptitude in the 3-CSRT-R. Exercise also elicited increased expression of *Drd1*, *Drd3*, *Drd4, synaptophysin, and PSD-95* in the dorsal (associative and sensorimotor) striatal regions. Our results underscore the observation that exercise, in addition to its accepted role in improving motor function, can also improve EF and dopaminergic function at the level of the striatum in animal models of PD.

1. INTRODUCTION

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder that diminishes the quality of life in over 630,000 people in the USA, which is projected to double by year 2040 as our population ages (Dorsey *et al.*, 2013; Kowal *et al.*, 2013). An early non-motor feature of PD is deficits in attention and executive functions (EF), including cognitive processes of set-shifting, inhibitory control, and decision-making (Dirnberger and Jahanshahi, 2013; Parker *et al.*, 2013). In PD, deficits in the fronto-striatal circuit represent a common pathophysiology of executive function (EF) (Robbins and Cools, 2014). The importance of the striatum in EF, also called the basal ganglia (BG) in humans, is based on a large animal and human literature (see review(Macdonald and Monchi, 2011)). For example, in individuals with PD, resting state fMRI as well as positron emission tomographic neuroimaging has demonstrated hypo-activation in a number of cortical and sub-cortical (BG) regions impacting the EF network (Boord *et al.*, 2017; Dong *et al.*, 2020; Hirano *et al.*, 2012; Lozza *et al.*, 2004).

Animal studies have specifically implicated the role of the dorsomedial striatum in EF, and its importance in set shifting and reversal learning, that involves selecting among various stimuli and competing responses, particularly when selection requires discounting more salient stimuli (Cools *et al.*, 2006; Thoma *et al.*, 2008; van Schouwenburg *et al.*, 2010; Yehene *et al.*, 2008). Although the pathophysiological changes in the dorsomedial striatum that underlie EF changes in PD are complex, loss of synaptic integrity, including synaptophysin and PSD95, and dopamine neurotransmission, including dopamine loss and altered DA receptor expression, are reported to contribute (Salame *et al.*, 2016). Dopamine (DA) receptors, comprised of DA receptor 1-like (DAR-D1 and DAR-D5), D2-like (DAR-D2, D3, and D4) and play a central role in learning and EF related cognitive flexibility (Sala-Bayo *et al.*, 2020; Wang *et al.*, 2019).

A public health priority is to identify cost-effective therapies to combat progressive cognitive impairment in PD, which is not addressed by current therapies (Burn *et al.*, 2014). There is significant evidence utilizing short-term clinical studies that different types of exercise may help motor rehabilitation in PD, including aerobic exercise (treadmill walking, cycling), resistance training, balance training, and multifaceted exercise (Tai Chi, dancing) (Alberts and Rosenfeldt, 2020; Intzandt *et al.*, 2018). Fewer studies have examined the long-term relationship between exercise and long-term cognitive function in PD patients.

Even less is known regarding the molecular underpinnings of exercise related benefits in PD-related EF impairment. Indeed, the long-term effects of chronic exercise on cognition remains controversial (Brown *et al.*, 2021; Sanders *et al.*, 2020; Schootemeijer *et al.*, 2020). There is a need for research into the causality of the relationship between physical activity, cognitive performance and insights regarding exercise-induced repair mechanisms. Using the 6-hydroxydopamine lesioned model of PD, targeting specifically the dorsomedial striatum, this study sought to test the hypothesis that daily exercise training improves EF-related cognitive flexibility and restores synaptic integrity and DA neurotransmission.

2. METHODS

2.1. Animals

Male Wistar rats 8 to 9 weeks of age were purchased from Envigo Corporation (Placentia, CA, USA). Housing was under standard vivarium conditions in pairs on a 12-hr light/12-hr dark cycle (dark cycle 6 p.m. to 6 a.m.). All cages included a plastic pipe (10cm diameter, 15cm length) as an enrichment object. Animals had ad libitum access to food and water, except during food restriction as described below. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California, an institution approved by the Association for Assessment and Accreditation of Laboratory Animal Care, as well as by the Animal Use and Care Review Office of the US Department of the Army, and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th Edition, 2011.

2.2. Food Restriction

Food restriction was started 2 weeks after surgery and maintained throughout the 3-CSRT (Experiment 1) and T-maze (Experiment 2) behavioral studies. Animals were brought to 85% of their baseline bodyweight in one week and allowed to gain 5 g in bodyweight per week thereafter, with ad libitum access to water. Animals were fed between behavioral testing that took place in the morning and exercise training that took place in the afternoon. Body weights were recorded Monday-Friday, with meal size individually adjusted on a daily basis, including weekends.

2.3. *Rat Model of Bilateral, Dorsomedial Striatal Dopamine-depletion* (Wang *et al.*, 2020)

We targeted the dorsomedial quadrant of the striatum (dmCPu) as past work has shown that this region in rodents is critical for reversal learning (Baker and Ragozzino, 2014; Grospe *et al.*, 2018; O'Neill and Brown, 2007), with alterations in the rostromedial striatum presumably resulting in deficits in frontostriatal processing (Voorn *et al.*, 2004). In brief, rats received stereotaxic injection of 6-OHDA at 4 striatal injection sites (2 in each hemisphere)(Sigma-Aldrich Co., St. Louis, MO, USA, 10 µg/site dissolved in 2 µL of 0.1% L-ascorbic acid/saline, 0.4 µL/min) targeting the bilateral dmCPu (AP: + 1.5mm, ML: ± 2.2mm, DV: - 5.2mm, and AP: + 0.3mm, ML: ± 2.8mm, DV: - 5.0mm, relative to bregma), which is the primary striatal sector targeted by the medial prefrontal cortex (Voorn *et al.*, 2004) and critical for flexible shifting responses (Baker and Ragozzino, 2014; Grospe *et al.*, 2018; O'Neill and Brown, 2007; Tait *et al.*, 2017). After injection, the needle was left in place for 5 min before being slowly retracted (1 mm/min). Naïve rats were used for controls. To prevent noradrenergic effects of the toxin, rats received desipramine (Sigma Aldrich, 25 mg/kg i.p.) before surgery (Roberts *et al.*, 1975). Carprofen (2 mg in 5 g tablet, p. o., Bio-Serv, Flemington, NJ, USA) was administered for one day preoperatively and for two days postoperatively for analgesia. Exercise was initiated 2 weeks thereafter when lesion maturation was complete (Sauer and Oertel, 1994; Yuan *et al.*, 2005). To verify the striatal target of 6-OHDA lesion, brains from a subset of rats were collected for immunohistochemical staining for tyrosine hydroxylase protein and HPLC analysis of dopamine.

2.3.1. Tyrosine hydroxylase (TH) immunostaining

TH immunostaining data were collected as previously reported and described quantitatively (Wang *et al.*, 2020). Rats were anesthetized, subjected to transcardial perfusion with ice-cold saline followed by ice-cold 4% PFA/PBS. Brains were removed, transferred to the same fixative for 24 hours, immersed in 20% sucrose for 48 hours, and cryo-sectioned at 25 µm thickness throughout the entire anterior-posterior extent of the brain. Selective sections spanning the 6-OHDA lesion in both the striatum and midbrain were subjected to TH-immunostaining using a primary antibody solution (1:2500 anti-tyrosine hydroxylase, Cat #AB152, Millipore-Sigma, Billerica, MA, USA), then visualized using a secondary antibody solution (1:5000 IRDye

800CW Goat anti-mouse Cat #926-3221, LI-COR, Lincoln, NE, USA). Images of tissues at the levels of the striatum representing the site of the 6-OHDA lesioning targeting the dmCPu and mid-ventral mesencephalon showing the midbrain dopaminergic neurons were obtained using a LI-COR Odyssey CLx.

2.3.2. *HPLC analysis of striatal dopamine*

Neurotransmitter concentrations were determined according to an adaptation of Mayer and Shoup 1983 (Mayer and Shoup, 1983). Tissue sections from striatal quadrants were dissected (dlCPu, dmCPu, vlCPu and vmCPu) from a coronal section spanning bregma AP +2.00 to 0.00 mm, and immediately frozen on dry ice and stored at –80°C until analysis. Tissues were homogenized in 0.4 N perchloric acid, proteins were separated by centrifugation, and the supernatant used for HPLC analysis by electrochemical detection on an ESA HPLC system (ESA, Chelmsford, MA, USA) consisting of an ESA Model 582 pump, ESA Model 542 autosampler, ESA Model 5600 Detector and separation column (MD-150x3.2mm). Data analysis employed the CoulArray for Window Application program (ESA Biosciences, Chelmsford, MA, USA). The protein pellet was resuspended in 0.5 N NaOH and total protein concentration determined using the BCA detection method (Pierce, Rockford, IL, USA). Striatal dopamine was expressed as nanograms dopamine per milligram protein.

2.4. *Experimental Design Overview*

Animal groups included 6-OHDA lesioned or non-lesioned rats. Rats were subjected to running in motorized running wheels (complex or smooth), on a horizontal treadmill, or remained sedentary. The following section describes the experimental approaches, including the cognitive testing (**Fig. 1**) using the operant 3-CSRT with rule reversal (Experiment 1) and T-maze with rewarded matching-to-sample (win-stay) and reversal (win-shift) (Experiment 2) to evaluate executive function, as well as a striatal transcript analysis for dopamine receptors D1 through D4 and the synaptic genes PSD-95 and synaptophysin (Experiment 3).

2.5. Exercise Training on Running Wheels and Treadmill

2.5.1. Exercise training on complex or smooth running wheels

Rats assigned to the skilled exercise group were trained in enclosed, motorized, running wheels (35.6 cm diameter, Lafayette Instrument, Lafayette, IN, USA) with irregularly spaced rungs, termed 'complex' running wheel, which demand the constant adaptation of stride length. A pseudo random pattern of rung spacing was achieved by repeating a pattern OOOOXOX, where O indicates a rung, and X a missing rung, resulting in inter-rung distance of either 1.3 or 2.6 cm.). Training was as per our prior methods (Wang *et al.*, 2015) and lasted 30 min/day (4 sessions, 5 min/session, 2-min inter-session interval), 5 consecutive days/week (Mon - Fri). Rats were subjected to 1 week of individually adjusted, performance-based speed adaptation to reach a plateau speed of 5 m/min, a speed achievable by most 6-OHDA lesioned rats in the complex wheel following 1 week of training (Wang *et al.*, 2015; Wang *et al.*, 2013). Titration of speed has been described in detail in our prior publication (Wang *et al.*, 2013). No-exercise rats were left in a stationary running wheel for 30 min/day. Running speeds for the nonlesioned, control animals were normed to speeds achievable by lesioned rats in the complex wheel (5m/min maximum). An additional group of lesioned rats was trained in motorized running wheels identical to those described above, except that these wheels had regularly placed rungs and an inner plastic 'smooth' floor covering the metal rungs and were termed 'smooth' running wheel as previously reported (Wang *et al.*, 2015; Wang *et al.*, 2013). The modification made foot placement easier for lesioned rats, and therefore minimized the 'motor skill' factor. Running speeds for the smooth running wheels were matched to those of the complex running wheels.

2.5.2. Exercise training on horizontal treadmill

Rats were trained on a motorized, 10-lane horizontal treadmill (lane width 10 cm, length 94 cm, wall height 20 cm, custom made) for 65 min a day, 5 days per week (Mon - Fri) starting two weeks after stereotaxic surgery. Each training session consisted of a 15-min warm-up, 15-min running, 5-min break, 15-min running, and 15-min cool-down. The warm-up and cool-down speed started at 4 m/min and went up to 10 m/min in 2 weeks, while the running speed started at 6 m/min and went up to 30 m/min in 4 weeks. The animals were trained at 10 and 30 m/min for the rest of the experiment. A researcher prompted the rats to stay on the treadmill and run by lightly brushing the rear end of any rat that fell back with a brush. After several days of such training, rats typically will stay on the treadmill running.

2.6. Operant Training

2.6.1. Groups

In experiment 1, rats with bilateral, dmCPu 6-OHDA lesions were exposed to exercise for 12 weeks in either: (1) motorized, complex running wheel (Lesion/Complex Wheel, *n* = 12), (2) motorized smooth running wheel (Lesion/Smooth Wheel, *n* = 4), or (3) motorized horizontal treadmill (Lesion/Treadmill, *n* = 6). Control rats included 6-OHDA lesioned, sedentary rats (Lesion/Sedentary, *n* = 12) or non-lesioned (No-Lesion, *n* = 12). The non-Lesion group consisted of both 6 sedentary (n = 6) and complex running wheel (n = 6) and since they showed no significant differences in operant training outcomes they were pooled (**Supplementary Fig. S1**).

Whereas in our original design sample size was balanced across the groups to access modality-specific effects of exercise, we were not able to complete all experiments due to local restrictions and university-mandated euthanasia of animals during the height of the Covid-19 pandemic. These animals were excluded from analysis. Because preliminary analysis showed the exercise effects to be modest (**Supplementary Fig. S2**), we pooled the three exercise groups to form a single Lesion/Exercise group (*n* = 22) in this report.

2.6.2. Three-Choice serial reaction time nose-poke task with rule reversal (Fig. 1)

We chose a 3-CSRT paradigm because deficits in cognitive flexibility can be robustly unmasked after a rule reversal. Detailed methods are provided in our prior publication (Wang *et al.*, 2020). In brief, rats were food restricted and randomized to receive complex wheel exercise, simple wheel exercise, treadmill exercise, or no exercise, 1 week prior to operant training, during which time they were handled on a daily basis and given 5 sucrose pellets per day in their home cage (45 mg/pellet, chocolate flavor, #F0025, Bio-Serv, Frenchtown, NJ, USA). Each modular test chamber (MedAssociates, St. Albans, VT, USA) was housed in a sound attenuating cubicle, and consisted of grid floor, house light, 3-bay nose-poke wall, pellet trough receptacle, receptacle light, head entry detector, and a PC-controlled smart controller. Animals were trained to associate nose poking into a lit aperture with receiving a single sucrose pellet reward. Rats were

familiarized with the test chamber and nose-poke and reward retrieval behavior were shaped in pretraining (Wang *et al.*, 2020). Thereafter, during the regular phase of 3-CSRT training, the animal was trained following a progressive schedule with a fixed ratio 1 schedule response-reward task (up to 90 trials or 30 min each session per day, 5 days/week, 16 sessions). The walls, nose-poke apertures, food receptacle, and grid floor were wiped with 70% isopropyl alcohol between animals.

For each trial, the light stimulus was turned on in a pseudo-randomly chosen nose-poke aperture (**Fig. 1b**). The stimulus stayed on for a set duration or until a nose-poke (correct or incorrect) was detected. The animal received a food reward following a correct nose-poke within the set limited hold duration, which was set to be the same as the stimulus duration or slightly longer for short stimulus durations. Following reward retrieval and a 2-s intertrial interval (ITI), the next trial was started. If an incorrect nose-poke was detected, the animal was punished with a 2-s time out (TO), during which the chamber light was turned off. If no nose-poke was detected within the limited hold duration, an omission was recorded, and the animal punished with a 2-s TO. After each TO, the chamber light was turned on, and after a 2-s ITI, the next trial was started. If a nose-poke was detected during the ITI, a premature response was recorded without incrementing the trial number, and the animal punished with a 2-s TO. Any nose-pokes following a correct response and before reward retrieval were recorded as repetitive responses. Animals were trained through 3 difficulty levels with progressively shortened stimulus durations. We chose to control the number of training days for each level across animals to facilitate between-group comparison. This was selected based on our prior work showing that lesioned animals can reach a performance level comparable to that of control animals at this difficulty level (Wang *et al.*, 2020). Differences in reversal learning can thus be interpreted as differences in cognitive flexibility, rather than differences in operant learning per se. The final stimulus duration was set at 5 s, reflecting a moderate level of difficulty.

During the rule-reversal phase of 3-CSRT-R training (**Fig. 1c**), the stimulus was switched from a lit aperture among dark apertures to a dark aperture among lit apertures. The animal was trained progressively to learn to nose-poke the dark aperture to receive reward (25 sessions).

For initial learning and reversal learning the following behaviors were captured (Asinof and Paine, 2014): (a) nose-poke accuracy = (number of correct responses)/(number of correct + number of incorrect responses) * 100%, a primary measure of operant learning; to account for modest differences in 3-CSRT acquisition, we normalized nose-poke accuracy during the reversal phase by accuracy at the end of acquisition (L3, Day 10, **Fig. 3**); (b) number of omissions, a measure of attention; (c) premature responses, a measure of impulsivity and response inhibition, (d) correct nose-poke latency = average time from onset of stimulus to a correct response, a measure of attention and cognitive processing speed; (e) reward retrieval latency = average time from correct response to retrieval of sugar pellet, a measure of motivation.

2.7. T-maze

2.7.1 Groups

In experiment 2, rats (n = 7) with 6-OHDA lesions were exercised for 6 ½ weeks in the complex wheel. Controls included lesioned, sedentary rats (Lesion/Sedentary, *n* = 6), and non-lesioned, sedentary controls (Non-Lesion/Sedentary, *n* = 9).

2.7.2. *T-maze with rewarded matching-to-sample and reversal*

Cognition testing of executive function in rats was adapted from methods from our work (Stefanko *et al.*, 2017) and that of others (Deacon and Rawlins, 2006). Rats were food restricted and randomized to receive exercise in the complex wheel or no exercise, 1 week prior to T-maze training, during which time they were handled on a daily basis and given 5 dustless, chocolate-flavored sucrose pellets per day in their home cage (45 mg/pellet, #F0025, Bio-Serv, Frenchtown, MJ, USA). The T-maze was constructed as a cross maze of black, opaque Plexiglas. Arms (15.2cm width, 50.8 cm length, 35.2 cm height) could be sealed off by guillotine doors (15.2 cm width x 35.2 cm height) to prevent entry to an enclosed central platform (15.2 cm width, 15.2 cm length, 35.2 cm height). Two opposing arms were designated as the branch arms, with one of the remaining two arms randomized to be designated the stem arm with its counterpart sealed during the 'T-maze' testing. A partition extended across the central platform and 6.4 cm into the chosen stem arm, allowing entry into either of the open branch arms. Arm entry was defined as having all four paws in the arm. If an

animal failed to run within 90 s, it was removed from the maze, to be exposed again 10 min. later. Uneaten sucrose pellets and fecal pellets were removed from the maze between trials, and the maze wiped with 70% isopropyl alcohol solution.

T-Maze acclimatization occurred over 3 days during which time animals were allowed to explore the maze. Initially the floor of the maze was baited with individual sucrose pellets, followed by baiting of both maze arms, followed by baiting of both food cups. Rats were trained for 3 days in a forced trial paradigm, in which food reward was available only in one arm (randomized), with the other branch arm blocked. Rats were trained for 3 days, 10 trials (5-s intertrial interval) in the morning and again in the afternoon. Thereafter, they were trained in a 'Win-Stay' paradigm (sample run→choice trial, 10 sequences twice per day, 5-s intertrial interval, x 20 days), in which animals had to choose the same arm during a choice trial (both arms open) that had previously been rewarded on the preceding sample trial (one arm closed). Sample trials were randomized across both arms. Thereafter, during implementation of a rule reversal, rats were exposed to a 'Win-Shift' strategy, in which the rat was only rewarded in the choice run if it entered the branch arm opposite the one chosen in the sample run (sample run→choice trial, 10 sequences twice per day, 5-s intertrial interval, x 13 days). The number of correct entries into the bated choice arm were recorded for each trial.

2.8. Quantitative RT-PCR for Striatal Dopamine Receptor and Synaptic Gene Expression

In experiment 3, two additional groups of 6-OHDA-lesioned rats were exercised in the complex running wheel (Lesion/Complex Wheel, n = 6) or smooth running wheel (Lesion/Smooth Wheel, n = 6) for 4 weeks before being euthanized for molecular assay. Controls included 6-OHDA-lesioned, sedentary rats (Lesion/Sedentary, n = 6). Since there were no statistically significant differences in gene expression between the Smooth wheel and the Complex wheel groups, the gene expression data from the two exercise modalities were pooled to form a single composite Exercise group (n=12), in parallel with the approach in Experiment 1. We examined the pattern of expression of several genes including *Syp* (synaptophysin, Gene ID 24804), *Dlg4* (discs large MAGUK scaffold protein 4, also known as PSD95, Gene ID 29495), *Drd1* (dopamine receptor D1, Gene ID 24316), *Drd2* (dopamine receptor D2, Gene ID 24318), *Drd3* (dopamine receptor D3, Gene ID 29238), and *Drd4* (dopamine receptor D4, Gene ID 25432). Immediately after the final exercise session, each

exercise group (sedentary, smooth wheel, and complex wheel, *n* = 6 rats per group) were sacrificed via decapitation and whole brains were extracted. Fresh tissue was rapidly micro-dissected in blocks from the striatum (caudate-putamen, CPu; Bregma +1.4 to 0.0 mm A.P., including tissue bordered ventrally by the anterior commissure, dorsally by the corpus callosum, medially by the lateral ventricle, and ± 5.0 mm laterally from the midline) (Kintz *et al.*, 2013; Lundquist *et al.*, 2019). Striatal blocks were further sub-dissected to four quadrants, using the dorsal-ventral and medial-lateral divisions detailed previously (Voorn *et al.*, 2004) to collect tissue from the dorsomedial striatum, dorsolateral striatum, ventromedial, and ventrolateral quadrants (dmCPu, dlCPu, vmCPu, vlCPu). Tissues were submerged in an RNA stabilization solution (pH 5.2) at 4°C, containing in mM: 3.53 ammonium sulfate, 16.66 sodium citrate, and 13.33 EDTA (ethylenediaminetetraacetic acid), transferred to a sterile tube containing 300 µl TRI-reagent (Cat. No. 11-330T, Genesee Scientific, San Diego, CA, USA), and homogenized with a mechanical pestle before centrifuging at 13,000 x *g* for 3 minutes. Supernatant was removed to a new tube and 250 µl of chloroform was added and tubes vigorously shaken twice for 10 seconds, followed by 3 minutes of rest on ice and centrifugation at 13,000 x *g* for 18 minutes at 4°C. The upper aqueous layer was carefully removed to a new tube, an equal volume of 100% ethanol was added, and the sample was thoroughly mixed before RNA purification using the Zymo Direct-zol RNA Miniprep (Cat. No. 11-330, Genesee Scientific) according to the manufacturer's instructions. RNA was eluted in 35 µl of DNAse/RNAse free water before spectrophotometric analysis of RNA purity and concentration. Complementary DNA (cDNA) was synthesized from 1µg isolated RNA using the qPCRBIO cDNA Synthesis Kit (Cat. No. PB30.11-10, PCR Biosystems, Wayne, PA, USA) following manufacturer's guidelines before being diluted 1:5 in DNAse/RNAse free water and stored at -20°C. Gene expression changes were measured with quantitative RT-PCR (qRT-PCR) as previously described (Lundquist *et al.*, 2021; Lundquist *et al.*, 2019). Briefly, qRT-PCR was run with 2 µl of cDNA and qPCRBIO SyGreen master mix (Cat. No. PB20.11-01, PCR Biosystems) on an Eppendorf Mastercycler Ep Realplex (Eppendorf, Hauppauge, NY, USA) using a program of 15 min at 95°C, followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. Data was collected and normalized on Eppendorf Realplex ep software. Standard delta-CT analysis (Livak and Schmittgen, 2001) was used to quantify fold changes in gene expression in experimental groups

normalized to controls, with Actb serving as a housekeeping gene. A complete list of primer pairs can be found in **Supplementary Table S8**.

2.9. Statistical analysis

Data are presented as mean ± S.E.M. and analyzed using GraphPad Prism (version 8.3.0, GraphPad Software, San Diego, CA, USA).

2.9.1. 3-CSRT/3-CSRT-R

3-CSRT data were analyzed using two-way ANOVA with repeated measures for each training level, with lesion and time, or exercise and time as the factors, and with Fisher's LSD multiple comparisons test comparing groups for individual training session. In addition, to control for any possible subtle lesion-related deficits in motor and cognitive functions at the end of the 3-CSRT acquisition phase, we also examined a normalized nose-poke accuracy in which the group average of accuracies during the 3-CSRT-R were normalized by the group mean accuracy on the final day of regular training (L3 of 3-CSRT). All statistical test results for main effect of lesion and exercise are included in **Supplementary Table S1**.

2.9.2. T-maze with reversal

The lesion effect was analyzed by a mixed model with repeated measures in 'time' and main effect being ‘lesion’ ($p < 0.05$). Accuracy in the Win-Stay and Win-Shift paradigms was separately analyzed by a two-way ANOVA with repeated measures in ‘time’ and main effect being ‘exercise’ ($p < 0.05$). Performance was evaluated as: (a) percent of correct responses per session during initial learning (Win-Stay) and reversal learning (Win-Shift); (b) the number of trials an individual rat required to reach the learning criterion during the reversal phase, defined as 9 out of 10 correct choices in consecutive trials; and (c) perseverative or regressive errors made during the reversal learning phase. Perseverative and regressive errors were defined, respectively, as the number of incorrect choices made until or after the rat chose the correct arm in 5 consecutive runs. Perseverative and regressive errors during the Win-Shift phase were separately analyzed

by a two-way ANOVA with repeated measures in 'time', and main effect either being 'lesion' or 'exercise' ($p < 0.05$). Fisher's LSD multiple comparisons tests were used to compare groups for individual days.

2.9.3. Transcript and HPLC analysis

All statistical tests were carried out and graphs made in Prism 91 (GraphPad, San Diego, CA, USA) with statistical significance set at $p < 0.05$. No sample size calculations were performed prior to the start of the study but are based on previous publications. Unpaired, two-tailed T-tests were used for all qRT-PCR analysis between control and pooled exercise groups (smooth wheel, complex wheel). One-way ANOVA with Tukey's multiple comparisons was used for analysis of gene expression across exercise groups (sedentary, exercise). Statistical analysis for HPLC data was carried out by using a one-way ANOVA with Dunnett's posttest comparing saline (control) treatment with 6-OHDA-lesioned groups. All statistical test results are included in **Supplementary Table S3-7**.

3. RESULTS

3.1. Assessment of 6-OHDA-Lesioning on Dopamine Levels and Tyrosine Hydroxylase Expression

Analysis of TH immunoreactivity demonstrated a significant reduction in TH immunostaining in the dmCPU in lesioned animals compared to non-lesioned animals (**Fig. 2a**). There were no significant differences in the dlCPu, vmCPu, and vlCPu of lesioned compared to non-lesioned control animals. Examination of TH immunostaining in the midbrain showed reduced staining of the substantia nigra from 6-OHDA-lesioned rats compared to non-lesioned rats. Analysis of dopamine levels by HPLC showed a significant difference in only the dmCPu quadrant, the region targeted for stereotaxic delivery of 6-OHDA (136.1 ± 36.1 vs. 31.2 ± 18.7 ng DA/mg Protein, $p < 0.05$). All other quadrants of striatal tissues did not show a statistically significant difference comparing non-lesioned and lesioned tissue sections (**Fig. 2b)**. These results show that lesions were limited to the dorsomedial quadrant of the striatum, with retrograde bilateral dopaminergic cell losses also apparent at the level of the substantia nigra.

3.2. Experiment 1: Effect of lesion and of exercise on operant training (3-CSRT/3-CSRT-R)

3.2.1. Lesion effects

During the acquisition phase of 3-CSRT (Levels L1, L2, L3), Lesion/Sedentary rats compared to No-Lesion controls showed: 1) statistically significant lower nose-poke accuracy (**Supplementary Fig. S2a**. $p < 0.0001$, two-way ANOVA repeated measures. See **Supplementary Table S1** for *F* and *p* values.) that diminished towards the end of L3 (L3 Day 10: Lesion/Sedentary 83.77 ± 2.05% vs. No-Lesion 91.41 ± 1.10%); 2) no differences in the number of premature responses ($p > 0.05$. **Fig. 3b**); 3) significantly higher number of omissions in L2 and L3 ($p < 0.05$. **Fig. 3c**); 4) significantly greater correct nose-poke latency ($p < 0.005$. **Fig. 3d**); and 5) significantly greater reward retrieval latency ($p < 0.001$. **Fig. 3e**).

A reversal was introduced to evaluate cognitive flexibility. At the start of 3-CSRT-R, when the rule for correct (rewarded) response was switched from nose-poking a lit aperture to nose-poking a dark aperture, both Lesion/Sedentary and No-Lesion/Sedentary rats showed a sudden drop in performance with decreased nose-poke accuracy to the same extent, increased correct nose-poke latency, and increased premature responses. Both groups showed improvement in these parameters with continued training. No-Lesion/Sedentary controls improved nose-poke accuracy rapidly to a plateau of about 75% (L3R Day 10, 76.55 ± 3.21%), while Lesion/Sedentary animals only improved accuracy modestly to a plateau of about 40% (L3R Day 10, 43.69 ± 2.95%). Lesion/Sedentary rats compared to No-Lesion controls showed: (1) statistically significant lower nose-poke accuracy ($p < 0.001$. **Supplementary Fig. S2a**); (2) significantly higher number of premature responses in L3R ($p = 0.0088$. **Fig. 3b**); (3) significantly higher number of omissions in phases L2R and L3R ($p < 0.05$. **Fig. 3c**); (4) no differences in correct nose-poke latency ($p > 0.05$. **Fig. 3d**); and (5) significantly greater reward retrieval latency ($p < 0.05$. **Fig. 3e**).

3.2.2. Exercise effects

In general, Lesion/Complex Wheel, Lesion/Smooth Wheel, and Lesion/Treadmill animals showed similar outcomes in the 3-CSRT task (**Supplementary Fig. S2**). Whereas in our original design sample size was balanced across the groups to access modality-specific effects of exercise, we were not able to complete all experiments due to local restrictions in response to the Covid-19 pandemic. As a result, we pooled these

three groups to form a Lesion/Exercise group (n = 22). Lesion/Exercise compared to Lesion/Sedentary rats showed a statistically significant, lower number of premature responses during reversal phase L3R (p = 0.018. **Fig. 3b**). To account for modest differences in 3-CSRT acquisition, we normalized nose-poke accuracy during the reversal phase by accuracy at the end of acquisition (L3, Day 10). Lesion/Exercise rats showed significantly higher normalized nose-poke accuracy in all reversal levels ($p < 0.05$. **Fig. 3a**). There were no differences in omission, correct nose-poke latency, and reward retrieval latency between the two groups (**Fig. 3c-e**).

3.3. Experiment 2: Effect of Exercise on T-maze Task with Reversal

To examine the effect of dmCPu 6-OHDA Lesion and Exercise on cognitive flexibility, behavior in the T-Maze with reversal was tested. The groups examiend were Lesion/Sedentary, No-Lesion/Sedentary, and Lesion/Complex Wheel. During the Win-Stay phase, there was a significant effect of lesion ($F_{1,13}$ = 13.47, p = 0.0028) and a lesion x time interaction ($F_{19,217}$ = 3.34, $p < 0.0001$) in response accuracy (**Fig. 4a, Supplementary Table S2**). Exercise improved accuracy in lesioned animals ($F_{1,11}$ = 8.063, p = 0.016), without exercise x time interaction. During the rule reversal Win-Shift phase, there were significant effects of lesion ($F_{1,13}$ = 41.83, $p < 0.0001$) and lesion x time interaction ($F_{12,142}$ = 4.76, $p < 0.0001$. **Fig. 4b**). No-Lesion/Sedentary compared to Lesion/Sedentary animals reached the learning criterion significantly earlier ($p < 0.00001$. **Fig. 4c**) and showed fewer perseverative errors ($F_{1,13}$ = 4.69, $p < 0.05$. **Fig. 4d**) and fewer regressive errors ($F_{1,13}$ = 6.76, $p < 0.05$. **Fig. 4e**). During the Win-Shift phase, there was a significant effect of exercise ($F_{1, 11}$ = 15.57, p = 0.0023. **Fig. 4b**), but no significant exercise x time interaction. Lesion/Complex wheel compared to Lesion/Sedentary rats reached the learning criterion significantly earlier ($p < 0.002$. **Fig. 4c**) and showed fewer perseverative errors ($F_{1,11}$ = 7.29, $p < 0.02$. **Fig. 4d**), with no significant differences in regressive errors (**Fig. 4e**). In summary, the T-maze detected a clear significant lesion effect during in the Win-Stay paradigm, a significant difference that was accentuated during the Win-Shift phase. Lesioned animals that underwent complex wheel exercise showed a greater number of correct responses compared with sedentary animals. This effect showed a significant difference during the Win-Stay phase and was accentuated during the Win-Shift phase of training.

3.4. Transcript analysis

Following 6-OHDA lesioning of the dorsomedial striatum, the effects of 4 weeks of different exercise modalities – aerobic running in a smooth wheel, or skilled running in a complex wheel with irregularly spaced rungs – on synaptic plasticity (*Syp, Dlg4*) and dopamine receptor gene (*Drd1, Drd2, Drd3, Drd4*) expression were examined in four quadrants of the striatum using qRT-PCR. There were no statistically significant differences in gene expression between exercise in the Smooth or Complex Running Wheel groups (**Supplementary Fig. S3, Tables S3-S6**). Therefore, gene expression data from the two exercise modalities were pooled to form a composite Exercise group, in parallel with the approach in Experiment 1. In the dmCPu, exercise caused a significant increase in the expression of *Syp* (synaptophysin, a pre-synaptic marker; $p = 0.033$) and *Dlg4* ($p < 0.001$). Additionally, exercised compared to sedentary animals increased the expression of three of the four dopamine receptors examined, including *Drd1* ($p < 0.001$), *Drd3* ($p < 0.001$), and *Drd4* ($p = 0.006$) (**Fig. 5a**). In the dlCPu, exercise caused a significant increase in the expression of *Dlg4* ($p < 0.001$) and two dopamine receptors, including *Drd3* ($p = 0.001$) and *Drd4* ($p = 0.001$) (**Fig. 5b**). In the ventral quadrants of the striatum (vmCPu, vlCPu), there was no change in gene expression following exercise (**Fig. 5c, d**). A complete list of *t*-test results may be found in **Supplementary Table S7**.

4. DISCUSSION

4.1. Effects of Lesions and Exercise on Cognitive Outcomes

Although the rate of acquisition in the 3-CSRT was slower in lesioned compared to control rats, lesioned animals were able to acquire a level of accuracy (84%) comparable to that of controls (90%) following 16 days of training. There were also no significant group differences in premature responses, a measure of impulsive behavior, after 16 days of learning the 3-CSRT. Lesioned rats compared to controls showed a trend of higher omission rate and a statistically significant greater standard deviation of omissions, suggesting a mild deficit in attention. Consistent with prior work (Hauber and Schmidt, 1994), lesioned compared to control rats had a small but significantly longer reaction time (<1 sec.) for reward retrieval. This difference was unlikely due to motor deficits as dmCPu lesions do not alter spontaneous locomotor activity,

rotarod performance (Wang *et al.*, 2020) or forelimb motor function (Chang *et al.*, 1999). Furthermore, dmCPu lesions do not significantly alter appetitive preference (Wang *et al.*, 2020). Thus, differences in reward retrieval latency (or omissions rate) likely reflect a slowing of cognitive processing and mildly impaired attention, rather than a general motor dysfunction or a lack of motivation.

In contrast to the 3-CSRT, substantial and persistent deficits were unmasked during the rule-shift phase. During the initial day of the 3-CSRT-R, both lesioned and control rats showed an equivalent drop in nose-poke accuracy. With continued training, controls rapidly improved (~80% accuracy), while lesioned animals reached a plateau (~50% accuracy) (**Fig. 3**). Lesions resulted also in significant and persistent increased premature responses. Thus, the 3-CSRT-R task unmasked lesion-induced deficits in cognitive flexibility and response inhibition.

Qualitatively, the lesion effect on T-maze learning mirrored those in the operant task. Lesioned rats were slow to learn the Win-Stay task, and never achieved correct response rates above 70%, even after 20 days of training, while controls achieved a 90% correct response rate (**Fig. 4**). Rule reversal (Win-Shift) widened these differences, with the lesioned animals significantly delayed in achieving the learning criterion, and never achieving more than 78% correct after 13 days of training, at a time when controls showed 95% correct responses. Perseverative and regressive errors during the reversal phase were significantly greater in lesioned rats than in controls as has previously been reported (Grospe et al. 2018).

Exercise in both the operant task and T-maze improved performance in lesioned animals. Results are consistent with earlier work in a rat stroke model showing that running exercise facilitates learning of a subsequent skilled forelimb task (Ploughman *et al.*, 2007). Exercise improved nose-poke accuracy during initial learning of the 3-CSRT task. However, as noted above, lesioned/sedentary rats were able to achieve levels of accuracy equivalent to those of the controls and lesioned/exercised rats by days 14-16. The effect of exercise on cognitive improvement was most apparent during the rule shift phase (3-CSRT-R). Here, improvements in accuracy in lesioned rats undergoing exercise were progressive, with differences relative to lesioned/sedentary animals preserved after 25 training sessions. Cognitive gains, however, were modest and significant only when exercise of all types were pooled. A greater effect of exercise in lesioned rats was seen on decreases in premature responses, a measure of behavioral impulsivity.

In the T-maze, the effect of exercise on cognitive improvement was also most apparent during rule reversal (Win-Shift). However, unlike results in the 3-CSRT-R operant task, lesioned/sedentary rats were able to match the performance of lesioned/exercised rats in the T-maze by day 12. The reason likely was that the Win-Shift T-maze task was easier to learn than the 3-CSRT-R operant task as judged by the fewer number of sessions to reach plateau levels in the former. This suggests that exercise can accelerate learning, but for simpler cognitive challenges, lesioned/sedentary rats can 'catch up' to the performance of exercised/lesioned rats. Whereas, for more challenging tasks such as the 3-CSRT-R, exercise provided a prolonged performance advantage that extended across 25 test days. Future studies would have to evaluate this claim at longer follow-up periods.

4.2. Changes in Dopaminergic Receptors and Synaptic Markers with Lesion and Exercise

Effects of exercise on dopamine receptor subtypes has been reported largely in healthy subjects; in the lesioned brain they remain mostly unstudied. Our results in lesioned rats showed exercise increased mRNA expression of Drd1, and the D2-like family of receptors Drd3 and Drd4, but without discrete increases in expression of Drd2 itself. Increases in Drd1, Drd3 and Drd4 were seen in the dmCPu, with increases in Drd3 and Drd4 in dlCPu, and with no significant increases ventrally (**Fig. 5**). There was a nonsignificant trend for larger increases after complex wheel running compared to smooth wheel running (**Supplementary Fig. S3**). These results suggest topographic differences in the effects of exercise on different quadrants of the CPu, with no significant effect of the type of exercise. Different than our results in the 6-OHDA model, past work in nonlesioned rodents has shown that exercise consistently increase Drd2 mRNA in the dorsal and ventral portions of the striatum, with variable changes in Drd1 (Clark *et al.*, 2014; Foley and Fleshner, 2008; Robison *et al.*, 2018). Expression of different dopamine receptor subtypes are likely altered by dopaminergic deafferentation, and by lesion type, exercise type and brain region.

The role dopamine receptor subtypes play in cognition remains an area of active research, without clear consensus. In general, activity in the D1-expressing direct, striatonigral ''GO'' pathway favors disinhibition of cortical activity and facilitates behavioral throughput. Activity in the D2-expressing, indirect striatopallidal ''NOGO'' pathway, in contrast, favors inhibition of cortical activity and inhibits behavioral

throughput (Kravitz *et al.*, 2010; Obeso *et al.*, 2004). Our findings of exercise-related increases in Drd1 mRNA expression suggest that in the lesioned rat, exercise may modulate dopaminergic-related functional changes to a greater extent in the direct "GO" pathway. Furthermore, an exercise-dependent increase in Drd1 but not Drd2 mRNA, alongside an exercise-dependent improvement in cognitive flexibility, is consistent with recent reports showing that D1 receptors on medium spiny neurons in the dmCPu play an important role in reversal tasks (Wang *et al.*, 2019)

D3 receptors have also been associated with cognitive functioning in animal models, in healthy humans and those with neuropsychiatric disorders (Nakajima *et al.*, 2013). Of related interest is a report that exercise in human subjects increases D2/D3 binding potential in the striatum (Robertson *et al.*, 2016). The exercise-related increases in our study in Drd4 have not been previously reported. They are intriguing given the association of the D4 receptor with cognition (Browman *et al.*, 2005; Woolley *et al.*, 2008; Zhang *et al.*, 2004) in general, and in particular with its possible associations with cue salience (Connolly and Gomez-Serrano, 2014) and inhibitory control (Mulligan *et al.*, 2014).

Exercise in lesioned rats increased the mRNA expression of synaptic markers synaptophysin and PSD95 in a sub-region-specific manner. Increases in postsynaptic PSD95 were seen in the dmCPu and dlCPu, while synaptophysin expression increased in the dmCPu—both without a significant effect of the type of training (**Supplementary Fig. S3**). Previous work in non-human primates and mice has demonstrated a significant reduction in dendritic spine density in the striatum of monkeys and mice following dopaminergic deafferentation (Villalba *et al* 2009, Toy *et al* 2014), with exercise eliciting a significant restoration of spine density, along with increases in synaptophysin and PSD95 expression (Toy *et al* 2014). The mechanisms by which exercise may restore synapse-related mRNA expression in our model remains unknown. Prior work in the 6-OHDA model has suggested a role for Arc (activity-regulated cytoskeleton-associated protein) (Garcia *et al*, 2017), a modulator of dendritic spine formation and experience-dependent modification (Peebles *et al*, 2010).

4.3. Translational Aspects

Behavioral studies have shown that despite their slower learning-rates, PD subjects retain more or less intact motor learning (Nieuwboer *et al.*, 2009). However, 'task-switching deficits' makes it difficult to translate learning acquired in a rehabilitation session to a real-world situation where responses must be adapted to context (Onla-or and Winstein, 2008). This inflexibility of thought and associated increased cognitive retention rates leads to errors of repetition when transferring between new categories of learning (Steinke and Kopp, 2020). We made a similar observation in our animal model. While the rate of acquisition of the 3-CSRT was delayed, lesioned animals were able to acquire a level of accuracy comparable to that of sham animals within 16 days of training (though not in the T-maze task). However, dramatic and persistent deficits were apparent in both the operant and T-maze tasks following the rule shift, such that lesioned animals never improved their performance much above chance levels, even after an extended period of training.

4.3.1. Effects of exercise on cognitive flexibility

Our findings demonstrated small but significant exercise-related improvements to deficits in cognitive flexibility. Results were consistent with those of a recent meta-analysis with similar findings in healthy human subjects (Ludyga *et al.*, 2020). Improved cognitive flexibility may reflect underlying functional adaptation in cerebral regions of the cortico-striatal-thalamo-cortical and cortical-thalamo-hippocampal circuits (Wang *et al.*, 2013), important in executive function and working memory. While in the current study, exercise effects on nose-poke accuracy of lesioned rats were modest, greater effects were noted for response inhibition. The findings mirror a report in PD patients where six weeks of intermittent aerobic walking elicits significant improvement in cognitive inhibition (Flanker test) but not on set shifting (Wisconsin card sort, Trail Making tests) (Uc *et al.*, 2014). Others have observed improvements in inhibitory aptitude (Stroop test) but not in cognitive flexibility (Trail making test) in PD patients following 3 months of intermittent aerobic cycling (Duchesne *et al.*, 2015).

It has been suggested that motor rehabilitation programs for PD patients should include a relatively high cognitive demand, such that by engaging patients to practice task-switching, they might be able to overcome their context-dependency (Onla-or and Winstein, 2008; Petzinger *et al.*, 2013). Surprisingly, we did

not see a significant difference in cognitive outcomes in comparing different exercise modalities in lesioned rats (see **Supplementary Fig. S2**). This observation was valid even when exercise was undertaken for two different skill levels at comparable speeds and durations using the same exercise modality (complex versus smooth wheel running, **Fig. S2**). This was contrary to our expectation which, based on greater functional connectivity of the medial prefrontal-striatal circuit during acute walking in the skilled compared to the smooth wheel, had anticipated a differential cognitive effect of these different exercise modalities (Guo *et al.*, 2017). Our findings differed from those reported in a recent meta-analysis in healthy human subjects, where there appears to be higher benefits after coordinative exercise compared to endurance, resistance and mixed exercise (Ludyga *et al.*, 2020). However, a recent systematic review of randomized controlled trials of physical exercise programs on cognitive function in PD reported that exercise-related improvements in global cognitive function, processing speed, sustained attention and mental flexibility (da Silva *et al.*, 2018) showed the largest effect for intense treadmill training, not for skilled training (tango or cognitive training associated with motor training). A head-to-head comparison of different exercise modalities on cognitive function has to date not been done in PD patients. In our study, though behavioral outcomes of nose poke accuracy closely tracked across different exercise modalities, our study was insufficiently powered to detect small differences. Furthermore, we did not explore a full range of exercise durations and intensities, variables that in prior studies have shown differential efficacy across different exercise training modalities (Coetsee and Terblanche, 2017), though this itself remains controversial (Brown *et al.*, 2021; Sanders *et al.*, 2020). Future studies may wish to explore the effects of a broader range of exercise intensities and durations across different exercise modalities.

5. CONCLUSION

In summary, our data adds to the expanding research reports showing the beneficial cognitive effects of physical exercise. Our prospective study demonstrates that following dopaminergic deafferentation, moderate exercise is able to provide improvements in cognitive flexibility and inhibitory aptitude, while eliciting increased expression of Drd1, Drd3, Drd4, synaptophysin, and PSD-95 in the associative and sensorimotor dorsal regions of the striatum.

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Figure Legends

Figure 1. Experimental protocol for operant training, T-maze, and exercise. (a) Timeline of experiments 1 and 2. (**b)** Protocol for the 3-choice serial reaction time task (3-CSRT) and reversal learning (3-CSRT-R). Gray shaded cells depict choices made by the animal. Black shaded cells depict consequences of those choices. Adapted from Asinof and Paine (2014) (Asinof and Paine, 2014)**. (c)** Acquisition of the 3-CSRT and 3-CSRT-R. **(d)** Progressive training schedule.

Figure 2. Immunostaining for tyrosine hydroxylase to determine the degree and anatomical site of lesion. (a) Representative images of coronal sections reveal bilateral loss in tyrosine hydroxylase immunoreactivity in the dorsomedial striatum (bregma AP +1.20 mm) and midbrain showing immunostaining of the substantia nigra pars compacta (bregma -6.0 mm). Scale bar = 0.5 mm. (b) HPLC analysis of striatal dopamine from tissues collected from coronal slice (bregma AP +2.00 to 0.00 mm) from non-lesioned and lesioned rats in striatal tissue quadrants (dorsomedial dmCPu, dorsolateral dlCPu, ventromedial vmCPu, and ventrolateral vlCPu).

Figure 3. Effects of exercise and lesion on the 3-choice serial reaction time task (3-CSRT) acquisition and reversal learning (3-CSRT-R). Shown are group mean ± SEM of **(a)** normalized nose-poke accuracy, **(b)** premature responses, **(c)** omissions, **(d)** correct nose-poke latency, and **(e)** reward retrieval latency for No-Lesion (n = 12), Lesion/Sedentary (n = 12) and Lesion/Exercise group (n = 22). #: $p < 0.05$ Lesion/Exercise vs. Lesion/Sedentary; +: $p < 0.05$ Lesion/Sedentary vs. No-Lesion, Fisher's LSD multiple comparisons test. Data were also analyzed with two-way ANOVA with repeated measures (results listed in **Supplementary Table S1**).

Figure 4. Effects of lesion and exercise on T-maze learning of rewarded matching-to-sample (Win-Stay) followed by reversal (Win-Shift). (**a)** Rats were trained in a Win-Stay strategy (solid line arrow = sample trial; dashed line arrow = choice trial) for 20 days, followed by training in **(b)** Win-Shift strategy for an

additional 13 days. Response accuracy (percentage of correct responses) is shown for No-Lesion/Sedentary (n = 9), Lesion/Complex wheel (n = 7) and Lesion/Sedentary (n = 6). **(c)** Total number of incorrect trials performed until criterion (9 correct responses in 10 consecutive trials) was reached during the Win-Shift phase. **(d)** Perseverative errors during the Win-Shift phase. **(e)** Regressive errors during the Win-Shift phase. Mean ± SEM. #: $p < 0.05$ Lesion/Complex wheel vs. Lesion/Sedentary; +: $p < 0.05$ Lesion/Sedentary vs. No-Lesion/Sedentary, Fisher's LSD multiple comparisons test. *: $p < 0.0002$, **: $p < 0.001$ (Student's t-test).

Figure 5. Dopaminergic signaling and synaptogenic gene expression changes across caudate putamen quadrants following exercise. Rat caudate putamen was divided into four quadrants for transcript analysis: **(a)** dorsomedial (dmCPu), **(b)** dorsolateral (dlCPu), **(c)** ventromedial (vmCPu), and **(d)** ventrolateral (vlCPu). Corresponding gene expression changes for four dopamine receptor (*Drd1, Drd2, Drd3, Drd4*) and two synaptic (*Syp, Dlg4*) genes in the exercise group (pooled complex and smooth wheel running, n = 12) compared to sedentary controls (n = 6). Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to sedentary control (Student's t-test).

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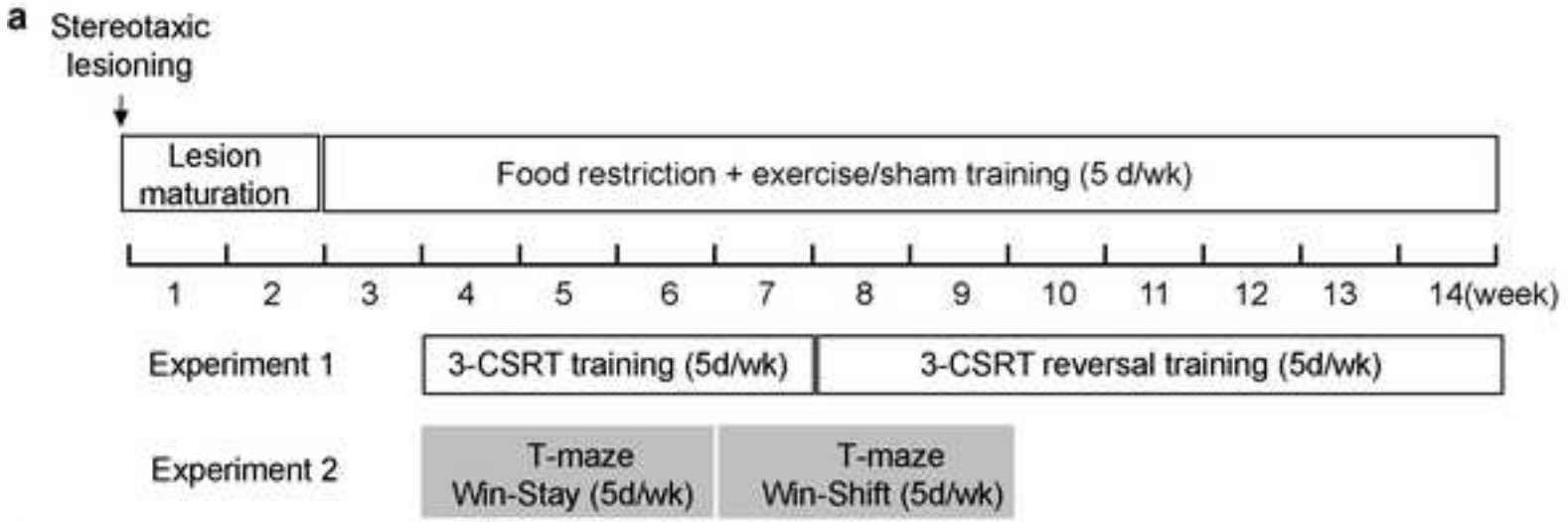
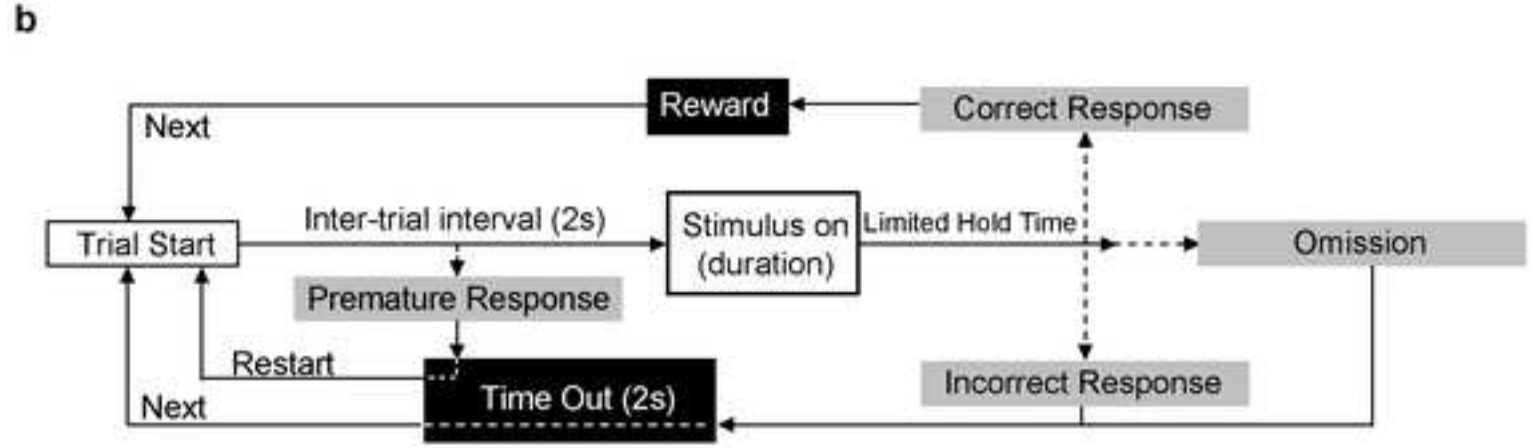
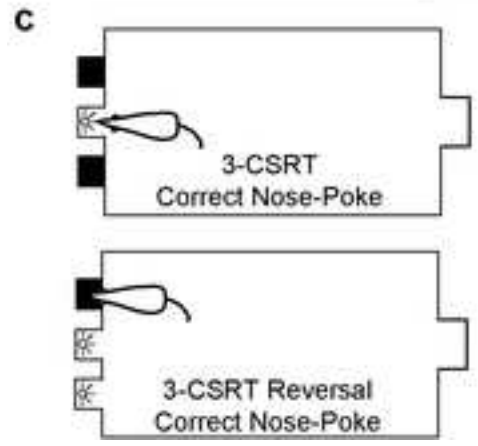
Figure 1







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3-CSRT	Stim Dur(s)	Limited Hold(s)	ITI (s)	TO (s)	Days
Stage 1 a, b	n/a	n/a	n/a	n/a	1 (twice)
Stage 2	∞	∞	2	n/a	2
Stage 3 Level 1	30	30	2	2	3
Stage 3 Lavel 2	15	15	2	2	3
Stage 3 Level 3	5	10	2	2	10
3-CSRT Reversal					
Stage 1 aR	n/a	n/a	n/a	n/a	1 (twice)
Stage 2R	∞	∞	2	n/a	5
Stage 3 Level 1R	30	30	2	2	10
Stage 3 Level 2R	15	15	2	2	5
Stage 3 Level 3R	5	10	2	2	15

Figure 2

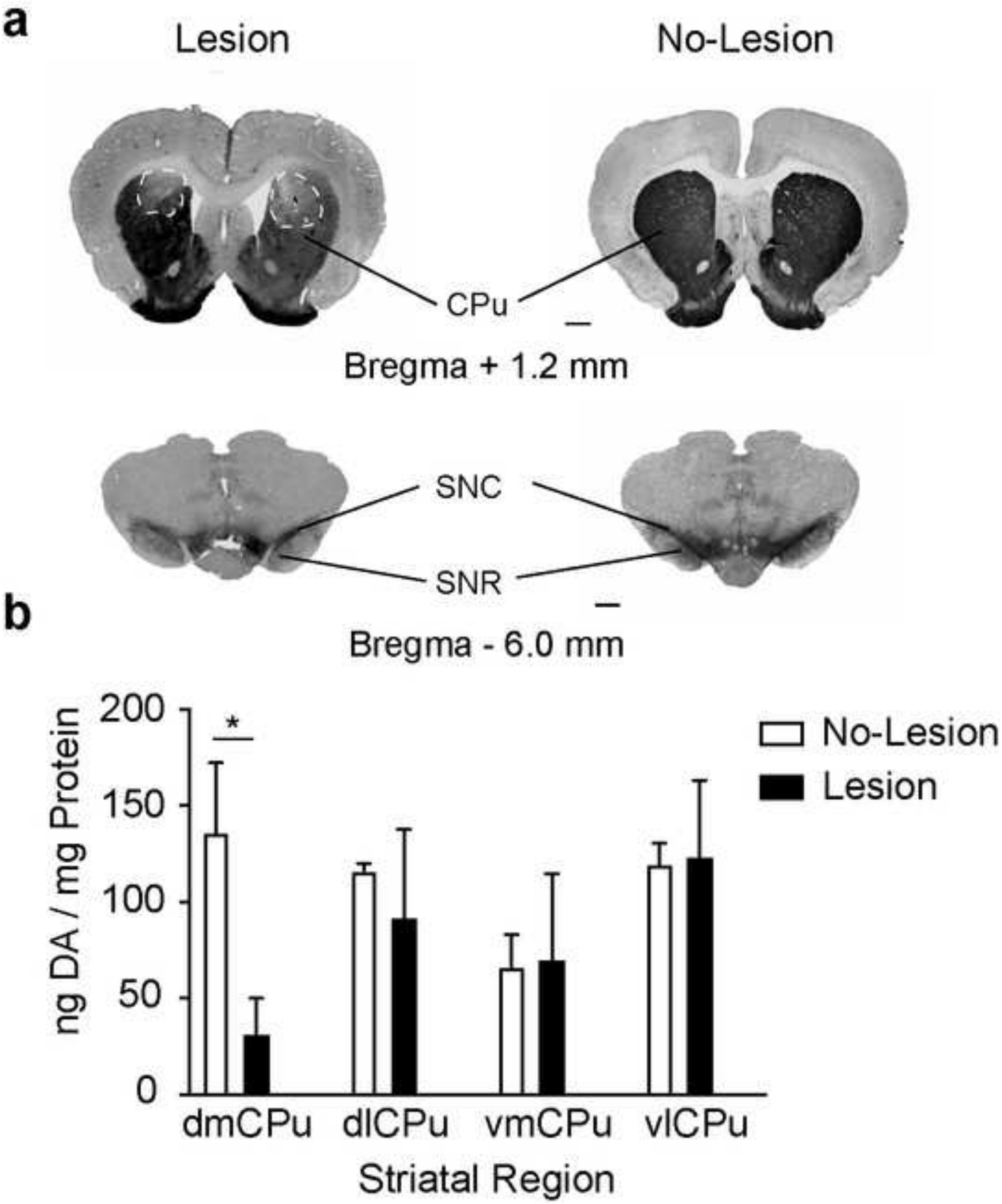
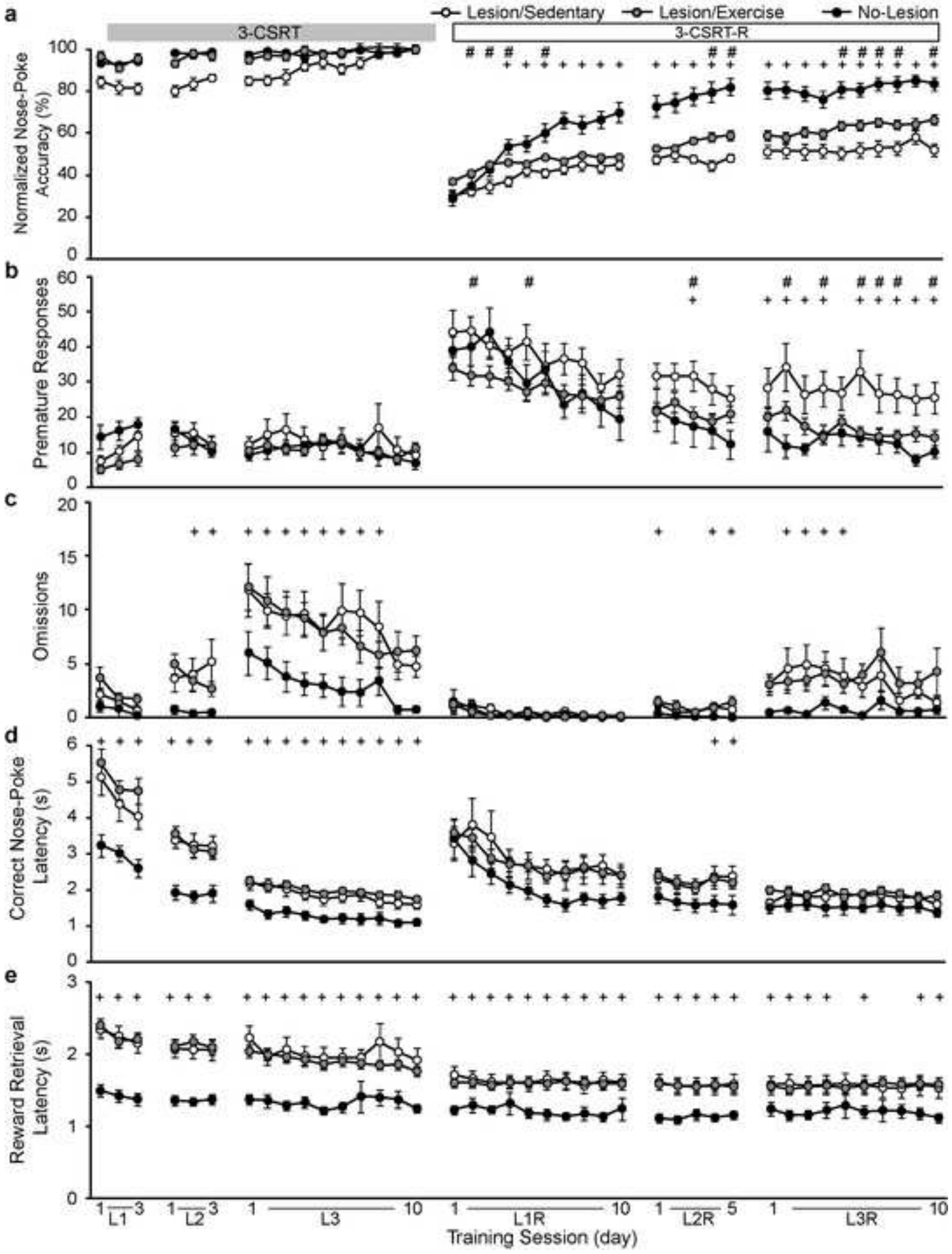


Figure 3



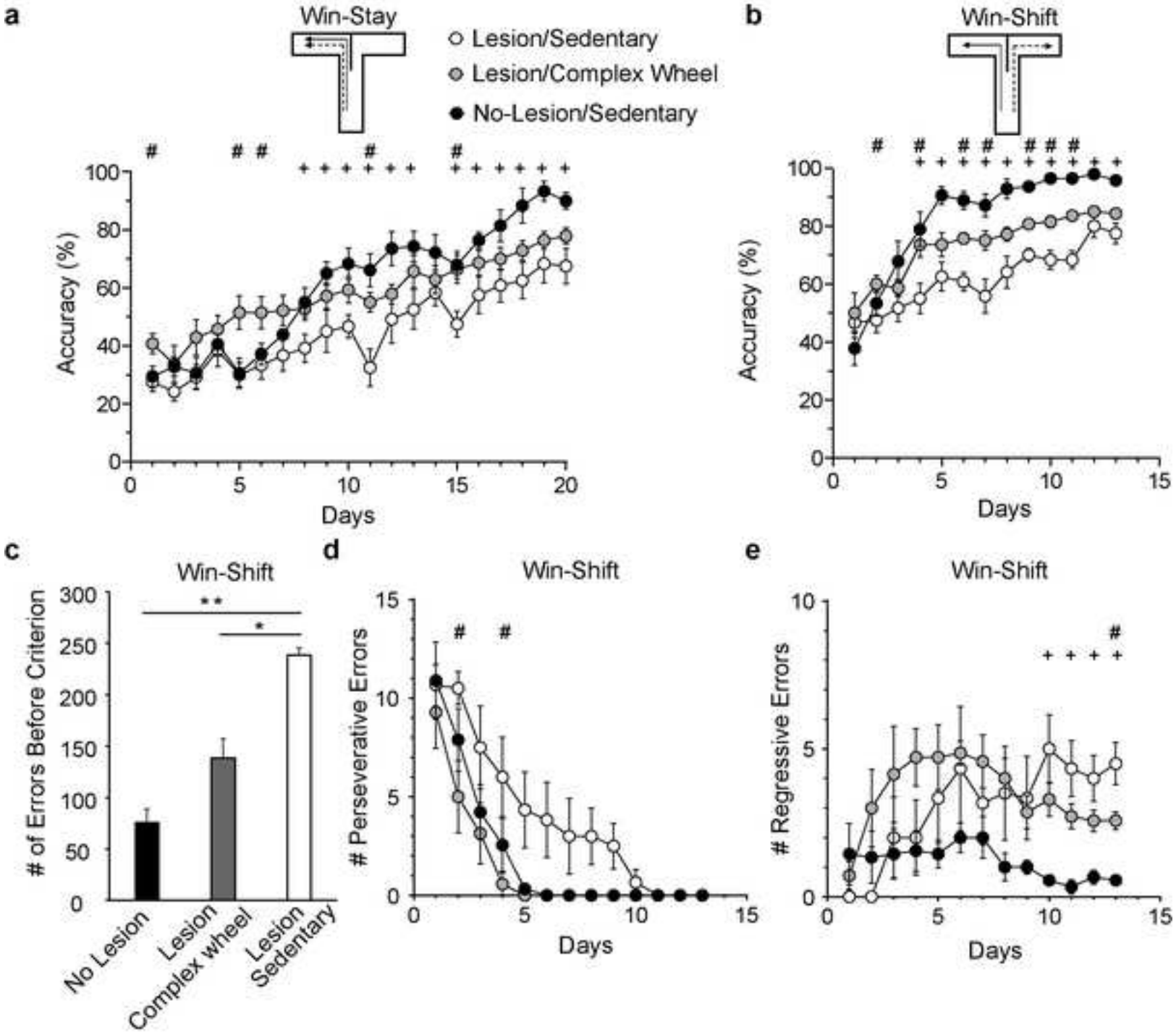
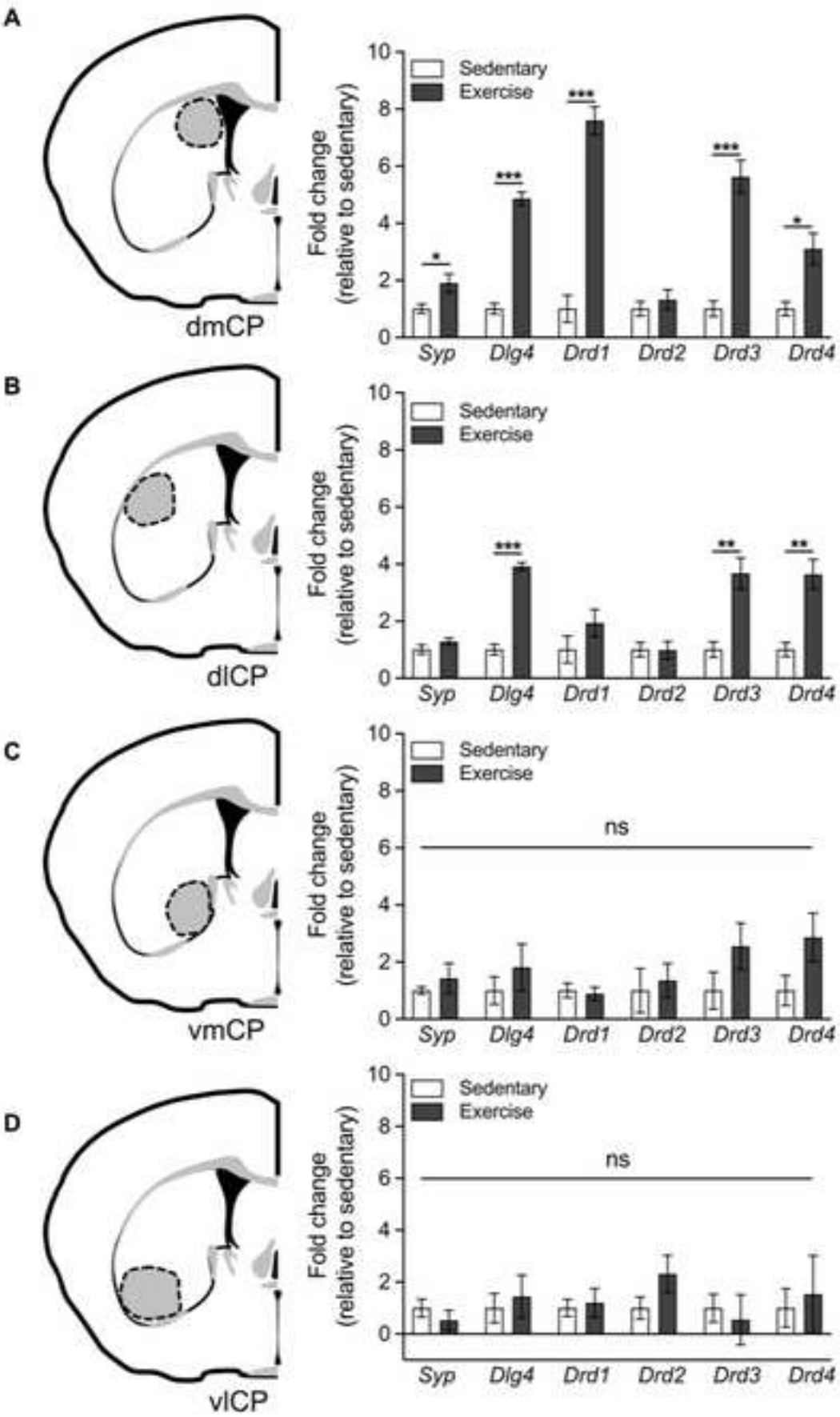


Figure 5



Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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