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TITLE: Investigating Exercise-Induced Neuroplasticity and its Mechanisms in Parkinson's Disease: Targeting Executive Function and Brain Circuitry

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13. SUPPLEMENTARY NOTES
Annual report: Year 2 of 2. Because of the slow-down engendered by the COVID-19 pandemic, the project is currently in 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 rat model of PD (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 frontostriatal 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 has provided evidence that SAE compared to simple aerobic exercise (AE) results in a differential enhancement of prefrontal cortex-mediated control of motor and possibly cognitive function. Using a rat model (striatal 6-hydroxydopamine model), the current study applies 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. In the second year of 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. There were no significant differences between skilled and nonskilled training and between skilled exercise and high intensity aerobic training. The COVID pandemic had kept the lab closed for 3 months, and required euthanasia of a number of experimental animals in long-term protocols. The lab reopened thereafter at 25% capacity, now at 50% capacity. We are in the process of initiating a series of brain mapping studies to examine if the effects of lesioning and of exercise, respectively impair and improve engagement of the prefrontal-striatal circuit that underlies executive function during performance of the operant task. Results of the molecular analysis suggest a differential and dynamic effect of exercise across the striatal subsectors. Thus, while 4 weeks of exercise elicits significant changes in dopaminergic markers, plasticity markers, and metabolic markers in the dorsolateral striatum, changes in the dorsomedial striatum are largely in dopaminergic and synaptic markers, suggesting regional specificity. In the ongoing no-cost extension of this project, we plan to (a) continue to add animals, (b) reinitiate the functional brain mapping of the effects of lesions and exercise on the prefrontal-striatal circuit, and (c) to add an additional group of animals undergoing voluntary exercise to evaluate if the stress of training may have attenuated exercise effects on cognition. 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?

MAJOR GOALS PROJECT 2 (preclinical project from SOW months 13-24)

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

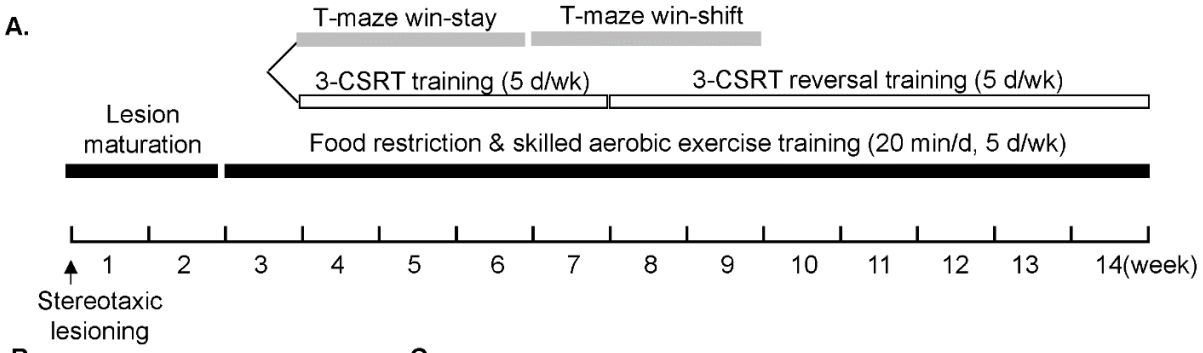
TASK 6: Data Analysis
Subtask 1: Coordinate with Data Core for monitoring data

What was accomplished under these goals?

TASKS 3 / 6: Evaluate Effects of Skill-based v. Aerobic Exercise on Executive Function/Data Analysis

Experimental protocol: Rats were lesioned. 2 weeks after lesion maturation exercise training was initiated and cognition was examined using either the operant 3-choice serial reaction time task (3-CSRT) or a T-maze task (matching-to-sample and reversal).
3-Choice serial reaction time task with reversal learning (3-CSRT-R): Behavior was shaped and learning tested (4-weeks 3-CSRT) in a fixed ratio 1 schedule response-reward task in which nose poke to a lit aperture resulted in a reward. During the reversal phase of training (7-weeks, 3-CSRT-R), the stimulus was switched from being rewarded for a nose poke into a lit aperture among dark apertures to being rewarded for a nose poke into a dark aperture among lit apertures.

Figure 1 *(see also Appendix): Experimental protocol for operant training.* A. Timeline of experiment. B. 3-CSRT-reversal task. C. Progressive training schedule.

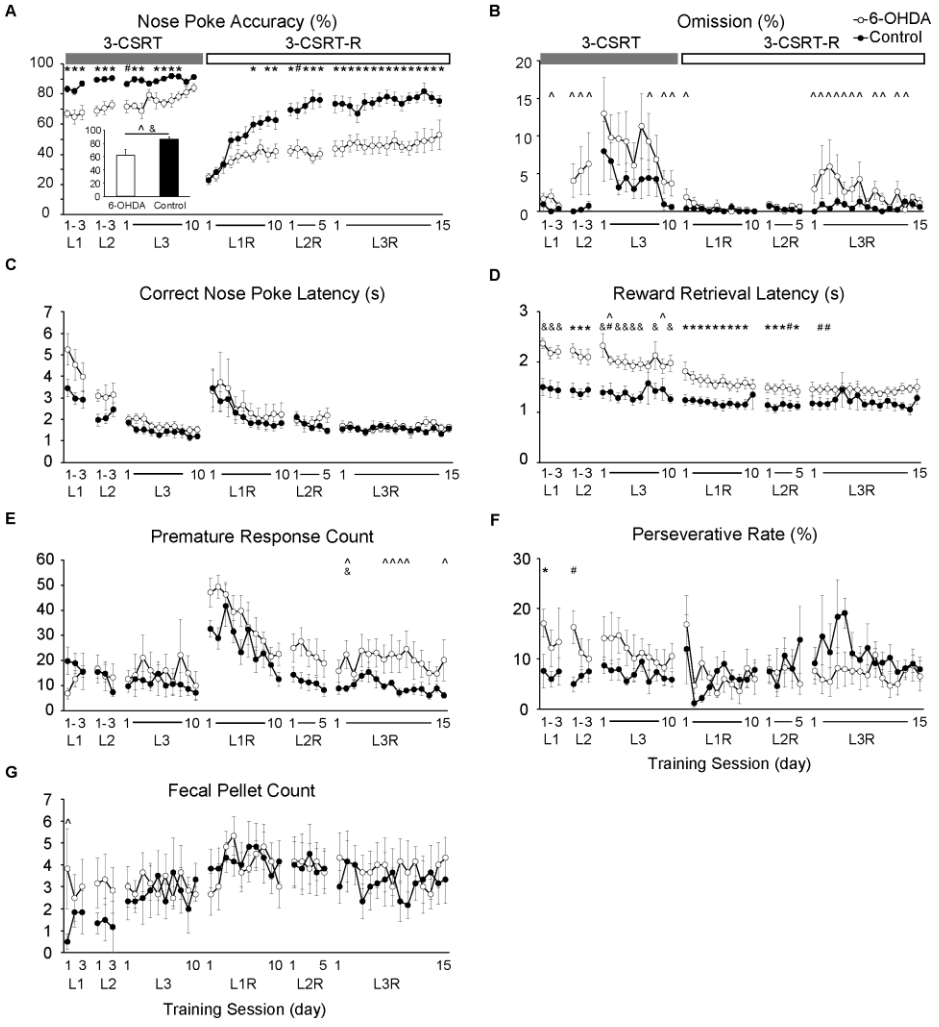


C.

3-CSRT	Stim Dur (s)	Limited Hold (s)	ITI (s)	TO (s)	Days
Stage 1a,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 Level 2	15	15	2	2	3
Stage 3 Level 3	5	10	2	2	10
3-CSRT Reversal					
Stage 1aR	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

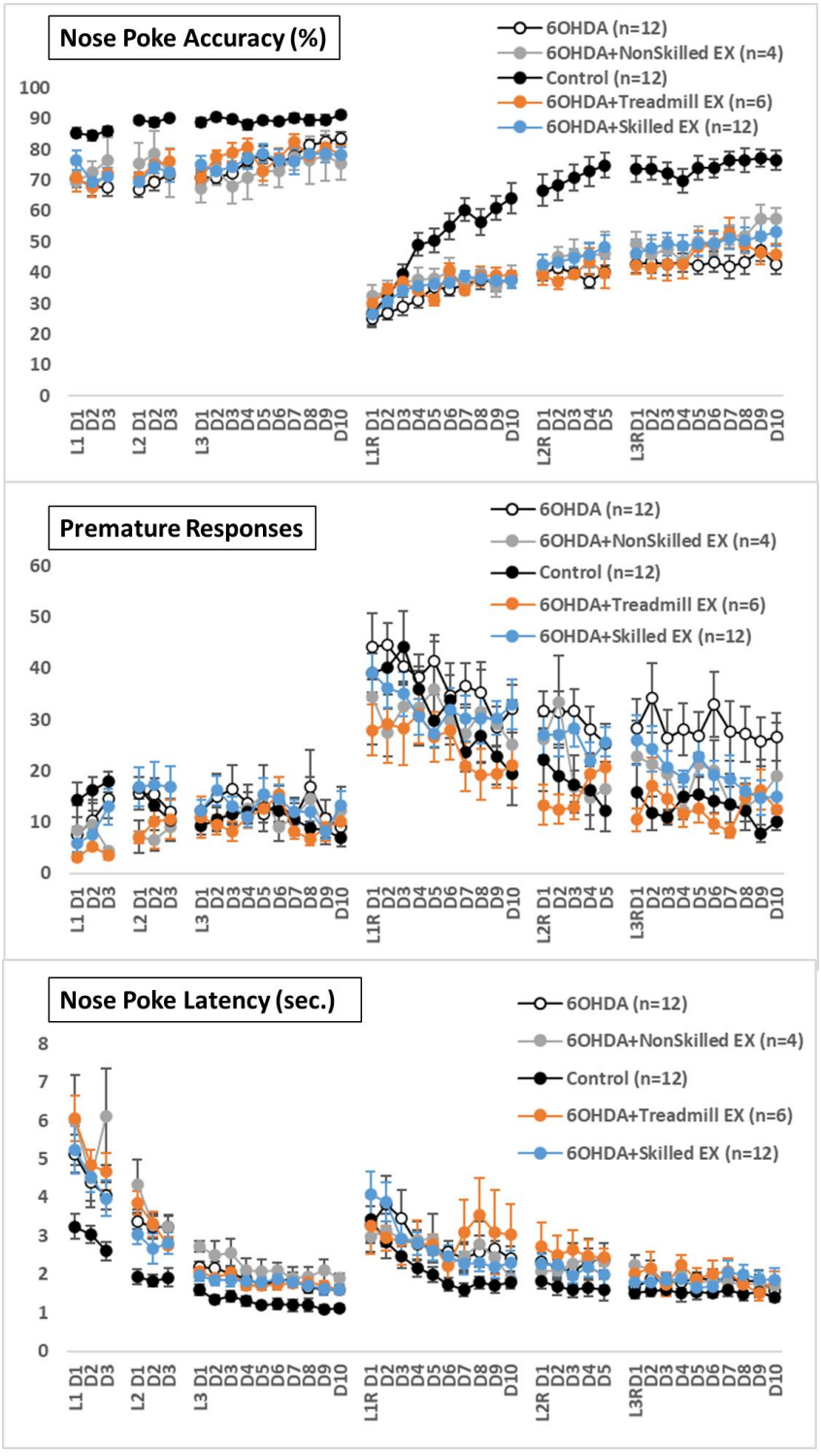
Lesion effect (See also Appendix): Although the rate of acquisition in 3-CSRT was slower in lesioned compared to control rats, lesioned animals were able to acquire a level of accuracy comparable to that of control animals following 4 weeks of initial training. In contrast, substantial deficits were apparent during the reversal learning phase which persisted during the 7 weeks of reversal training. These were most notable for decreases in nose poke accuracy and increases in perseverative responses. 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.

Figure 2 *(see also Appendix)****:*** *Differences in 3-CSRT acquisition (L1-L3) and reversal learning (3-CSRT-R, L1R-L3R) in 6-OHDA lesioned animals compared to controls.* (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 five days of L3R (level 3, reversal) normalized by the mean of last five 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.



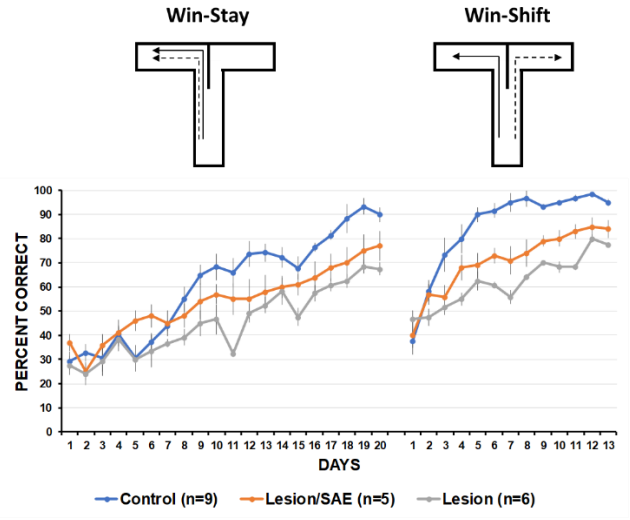
Exercise Effects: Ongoing experiments examined the effect of skilled aerobic exercise on reversal learning deficit in 6-OHDA lesioned animals. **Fig. 3** shows trend of increased nose poke accuracy and decreased premature responses in exercised compared to non-exercised lesioned animals in reversal learning phase. Nose poke latency remained largely unchanged suggesting the absence of lesion-related motor deficits.

Figure 3: Effect of lesion and 3 different types of exercise on nose poke accuracy, premature responses and nose poke latency in 3-CSRT acquisition (L1-L3) and reversal learning (L1R-L3R). Comparison is between 6-OHDA-lesioned rats that were sedentary and those receiving either skilled exercise training, nonskilled exercise training, or high intensity aerobic training ('Treadmill').



T-maze with rewarded matching-to-sample and reversal was used to assess cognition. *Methods:* Rats were lesioned and 2 weeks after lesion maturation cognition was examined using the T-maze. Rats were food restricted to 85% of their free-feeding weight, thereafter they were habituated to the maze and then trained with for 3 days, 10 trials (5 sec. intertrial interval) in the morning and again in the afternoon using a sucrose pellet reward. Thereafter, the rat was trained in a 'Win-Win' paradigm (10 sample run→choice trials twice per day, 5 seconds intertrial interval, x 13days), 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, 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 (10 sample run→choice trials twice per day, 5 seconds intertrial interval, x 13 days). Number of correct entries into the bated choice arm were recorded for each trial. Performance was expressed as a percentage of correct choices made in each session. *Results*: T-maze detected a clear significant lesion effect ($P < 0.05$), both during the Win-Stay strategy and its reversal as Win-Shift. Data collection is ongoing to increase the number of animals in the Control-exercise and Lesion-exercise groups.

Figure 4: *T-maze with rewarded matching-to-sample and reversal:* Rats were trained in a Win-Stay strategy (solid arrow/dashed arrow) for 20 days, followed by training in a Win-Shift (solid arrow/dashed arrow) strategy for an additional 13 days. Results are shown for controls (n=9) and lesioned rats exposed to skilled exercise training (SE, orange color, n=5) or no exercise (grey color, n=6). Shown are group means of the percent correct responses (group mean ± standard error).



Conclusion: The T-maze is sensitive to detecting executive function deficits related to rule reversal. Data collection to detect exercise effects is ongoing. Results suggest an absence of exercise effect in the control animals.

TASK 4: Brain Imaging: We are restarting these studies that were halted when our animals had to be euthanized during the March lockdown of the COVID pandemic.

TASKS 5 / 6: Bench Research / Data Analysis

qRT-PCR: Regions of interest (mPFC, dm/dl/vm/vlCP), were rapidly and unilaterally microdissected and submerged in an RNA stabilization solution (pH 5.2) at 4°C, Gene expression changes were measured with quantitative RT-PCR (qRT-PCR) as previously described [1, 2]. Briefly, qRT-PCR was run with 2 µl of cDNA and qPCRBIO SyGreen master mix on an Eppendorf Mastercycler Ep Realplex 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. Gene expression changes were examined for four metabolism-related genes (*Hif1a, Vegfa, Slc2a1, Ldha*), as well as three dopamine receptor (*Drd1, Drd2, Drd4*) and two synaptic (*Syp, Dlg4*) genes. Standard ΔΔCT analysis [3] was used to quantify fold changes in gene expression in experimental groups normalized to controls, with *Actb* serving as a housekeeping gene.

Results: see figure below.

Figure 5: *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.

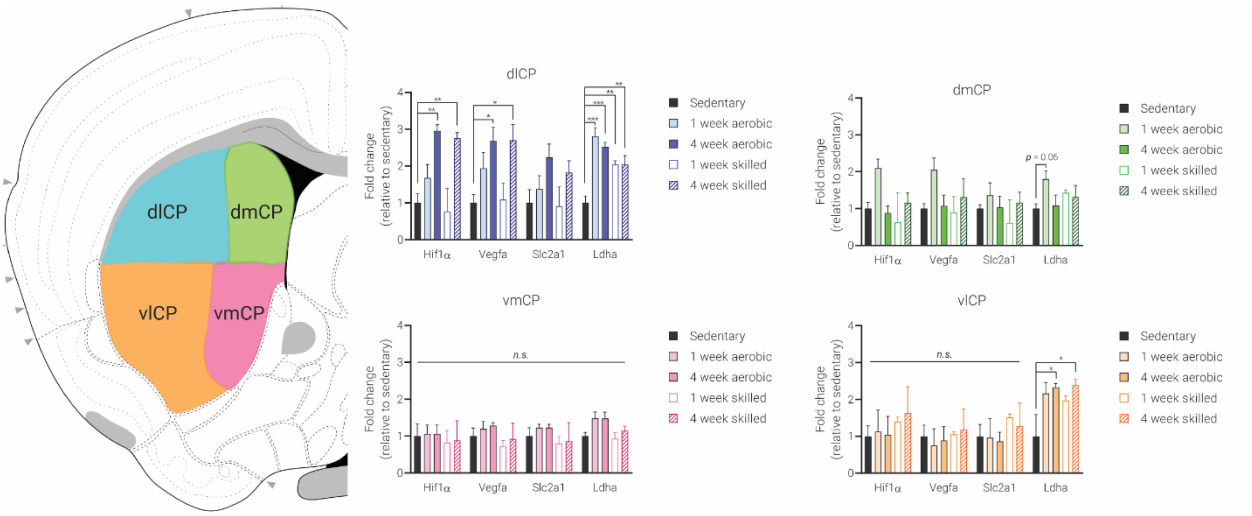
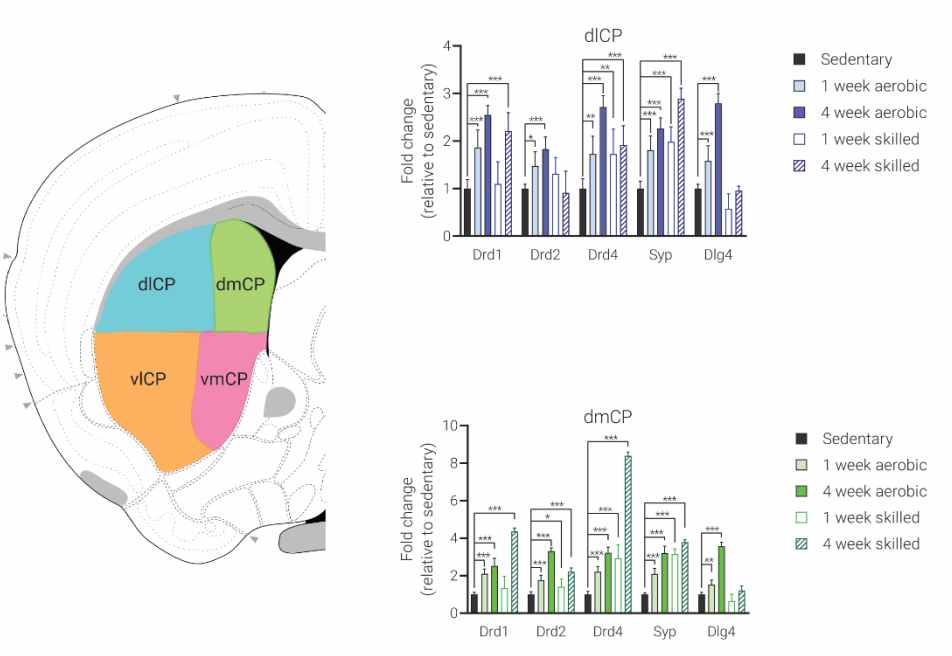


Figure 6: *Dopaminergic signaling and synaptogenic gene expression changes across dorsal caudate putamen across exercise duration and types.*

(Left) Rat caudate putamen color coded into dorsomedial (dmCP), dorsolateral (dlCP), ventromedial (vmCP), and ventrolateral (vlCP) quadrants. (Right) Corresponding gene expression changes for three dopamine receptor (*Drd1, Drd2, Drd4*) and two synaptic (*Syp, Dlg4*) genes for the two dorsal 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. While exercise changed dopamine receptor/synaptic markers and metabolic markers to the greatest degree in the dlCPu, robust changes, particularly in dopamine receptor/synaptic markers, were noted also in the dmCPu, with no significant changes noted in the vmCP, and only Lhda showing significant exercise effects in the vlCP. An 'exercise dose' effect (i.e. 4 weeks > 1 week) was noted for the dopamine receptor/synaptic markers and metabolic markers in the dlCPu, but only for select plasticity markers in the dmCPu. Increases after skilled training typically were slightly larger than those after aerobic training, with the largest significant increase noted in Drd4 expression within the dmCPU after 4 weeks of skilled exercise compared to the sedentary state (>8-fold increase). Thus, while exercise elicits in the dlCPu both significant plasticity and metabolic changes, in the dmCPu the changes are largely in dopamine receptor/synaptic markers. These results are broadly consistent with findings that acute treadmill walking in the rat, both in the skilled and simple wheels [4], as well as on the rotarod [5] shows greatest changes in rCBF in the dlCPu, though functional connectivity to medial prefrontal cortex increases particularly in the dorsomedial striatum [4].

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)
- Work from this project provided the impetus for Drs. Jakowec and Petzinger to apply and receiving university funds for a conference entitled "Metaplasticity and Megaplasticity: Changing the Brain from Synapse to Community" which was held at Lake Arrowhead, CA 12/6-8/2019. Participants included faculty, doctoral and undergraduate students, from USC, other California universities, as well as the East Coast.

How were the results disseminated to communities of interest?

- Results were presented at the above-mentioned meeting.

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

In the ongoing 2nd year of this project, we plan to (a) continue to add animals, (b) complete the functional brain mapping of the effects of lesions and exercise on the prefrontal-striatal circuit, (c) and to address the question of a possible differential effect of skilled versus simple aerobic exercise on cognitive, molecular and physiologic (including electrophysiologic) outcomes.

4. IMPACT:

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

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

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

The effect of exercise on improving cognitive flexibility was demonstrated in two independent paradigms and data sets (Operant training, T-maze). Given that the effects were significant but modest in magnitude, we have added two extra groups to examine whether
(a) exercise of greater intensity (horizontal treadmill running at high speed), or
(b) exercising with minimal stress (homecage voluntary wheels)
can elicit greater cognitive improvement in lesioned rats. Preliminary data (see above) from the the horizontal treadmill task suggests that no additional benefit on cognitive flexibility was noted on the operant task when high intensity exercise was undertaken. We are currently evaluating the effects of the voluntary, low stress wheel running on cognitive flexibility as a way of examining if stress may have attenuated the magnitude of the exercise effect in paradigms of forced training. This question is of central importance in optimizing the effects of exercise.

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: With the shut-down of Los Angeles by Mayor Garcetti 3/15/20, and the university's mandate to halt all laboratory work, we did so. Initially students were dismissed, followed by staff. All animal colonies were mandated by the university 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). The lab closed 3/27/20, with no access of faculty, staff or students to either the lab or the vivarium. The labs reopened at 30% occupancy on 6/29/20, and with a limited cap on allowed animal number of 12 rats total. Undergraduate students remain barred from hands-on laboratory work. While teleworking additionally been ongoing on aspects of the project, this has been limited, given that much of the research requires hands-on data collection. Weekly lab meeting continued via Zoom, as well as the preparation of manuscript drafts. The lab was granted 50% occupancy on 8/31/2020 and has continued in this fashion. The current project started a no-cost extension phase 9/15/20 to complete the original aims.

Changes that had a significant impact on expenditures

Nothing to report

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

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

- Halliday, M.R., D. Abeydeera, A.J. Lundquist, G.M. Petzinger, and M.W. Jakowec, Intensive treadmill exercise increases expression of hypoxia-inducible factor 1alpha and its downstream transcript targets: a potential role in neuroplasticity. *Neuroreport, 2019. 30(9): p. 619-627.*
- Holschneider DP, Wang, Z, Guo Y, Sanford MT, Yeh JC, Mao JJ, Zhang R, Rodriguez LV "Exercise modulates neuronal activation in the micturition circuit of chronically stressed rats: A multidisciplinary approach to the study of urologic chronic pelvic pain syndrome (MAPP) research network study", *Physiology & Behavior, 2019, Dec 27;215:112796. doi: 10.1016/j.physbeh.2019.112796.*
- Lundquist AJ, Parizher J, Petzinger GM, Jakowec MW. "Exercise induces region-specific remodeling of astrocyte morphology and reactive astrocyte gene expression patterns in male mice." *J Neurosci Res. 2019 Sep;97(9):1081-1094. doi: 10.1002/jnr.24430. Epub 2019 Jun 7.*
- 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*
- 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
- 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.*
- 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.*

Abstracts

- Lundquist, A.J., Gallagher, T.G., Petzinger, G.M., Jakowec, M.W., "Lactate administration recapitulates the astrocyte-specific neuroplastic effects of exercise", Abstract #204.12; Annual meeting of the Society for Neuroscience, Chicago, IL, 10/19/2019

Manuscripts in review

- Jarrahi B, McEwen S, Holschneider DP, Schiehser D, Petkus A, van Horne JD, Filoteo V, Jakowec MW, Petzinger GM
  "The Effects of Cardiovascular and Motor Skill Fitness on Intrinsic Functional Connectivity of Neural Networks in Patients with Parkinson's disease", *in review*
- Petkus AJ, Jarrahi B, Gomez ME, Filoteo V, Schiehser DM, Fisher BE, Jakowec MW, Holschneider DP, van Horn JD, McEwen S, Petzinger GM
  "Thalamic volume mediates links between cardiorespiratory fitness and cognition in Parkinson's Disease", *in review*

Manuscripts in preparation:

- "Functional remodeling of corticostriatal function with exercise"
- "Skilled exercise training differentially regulates gene expression in subsectors of the rat striatum"

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)

Not applicable.

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: 2.0 mo
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: 1.0 mo
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: 4.0 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: 6.0 mo
Contribution to the project: No change. Skilled and nonskilled exercising of animals, immunohistochemical staining (tyrosine hydroxylase).

Name: Adam Lundquist, BS (replaces former doctoral student Matthew Halliday)
Project Role: Graduate Student
Research Identifier: N/A
Nearest person month worked: 6.0 mo
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?

Updates to the active support of members of the research team (Holschneider, Jakowec, Wang) are outlined below.

1. Grant #W81XWH18-1-0666/ PD170037P1 (Dept. of the Defense, Dept. of the Army), 9/15/2018-9/14/2020, no-cost extension 9/15/2020 – current, total direct cost
PI: Holschneider (Effort: 15%)
"Investigating Exercise-Induced Neuroplasticity and its Mechanisms in Parkinson's disease: Targeting Executive Function & Brain Circuitry."

2. GRANT # W81XH19/ PD180100 (Dept. of Defense), total direct cost
9/1/19- 8/31/22
PI: Jakowec, Co-Investigators: D. Holschneider, Z. Wang
"The Role of Astrocytes and Microglia in Exercise-Induced Neuroplasticity in Parkinson's Disease"
Major Goals: To investigate the role of the immune system in regulating exercise induced synaptogenesis and behavioral recovery in rodent models of PD using imaging and molecular biology approaches.

3. 5RO1 DK118402 (NIDDK), total direct cost 07/1/18-06/30/22
PI: Kanoski, Co-investigators: D. Holschneider, Z. Wang
"Control of feeding behavior by melanin-concentrating hormone"
Major Goal: Evaluation of neural circuits underlying feeding behavior

4. 1K01DK118000, NIDDK, total direct cost 3/26/19 -12/31/23
PI: Noble, Role: D. Holschneider is a co-Mentor with Dr. Kanoski
"Melanin-Concentrating Hormone and the Neural Regulation of Feeding"
Major Goals: To study the neural systems that lead to excessive feeding behavior and food impulsivity. To identify the mechanisms through which the neuropeptide, melanin-concentrating hormone, promotes excessive food intake.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *See attached*

9. APPENDICES:

- 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.*

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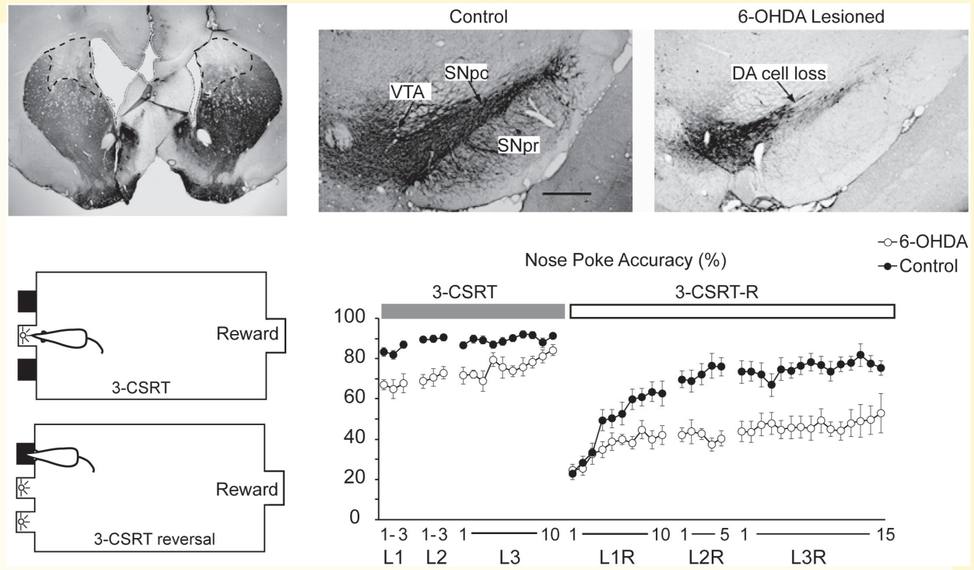

Unmasking deficits in cognitive flexibility after dopaminergic deafferentation.
see inside back cover

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).

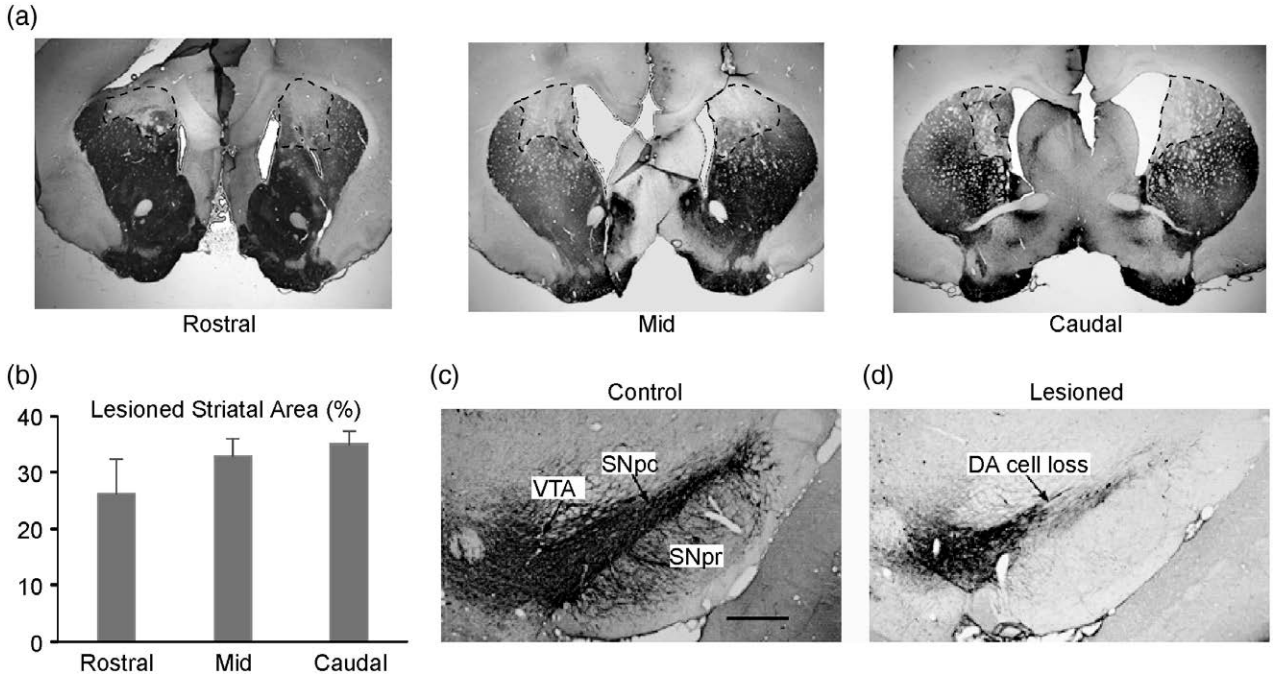
Lesion spared motor functions and sucrose preference

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

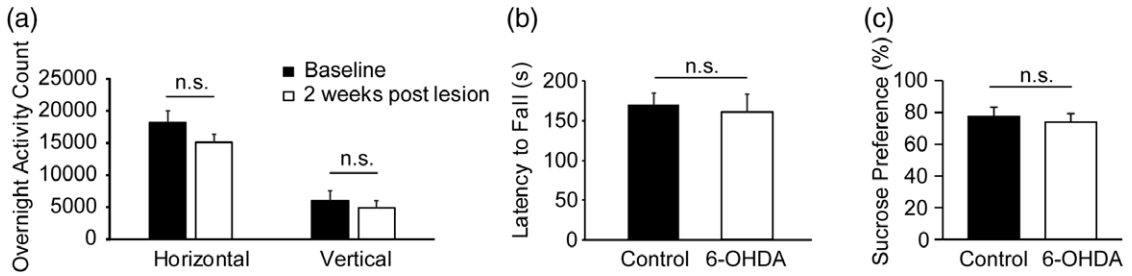
During the acquisition phase of 3-CSRT (levels L1, L2, and L3), both lesioned and control rats showed improvement in nose poke accuracy and shortening of correct nose poke latency (Fig. 4). Lesioned rats compared to controls showed: (1) statistically significant lower nose poke accuracy (L1: $F_{1,10} = 26.39$, $P = 0.0004$; 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

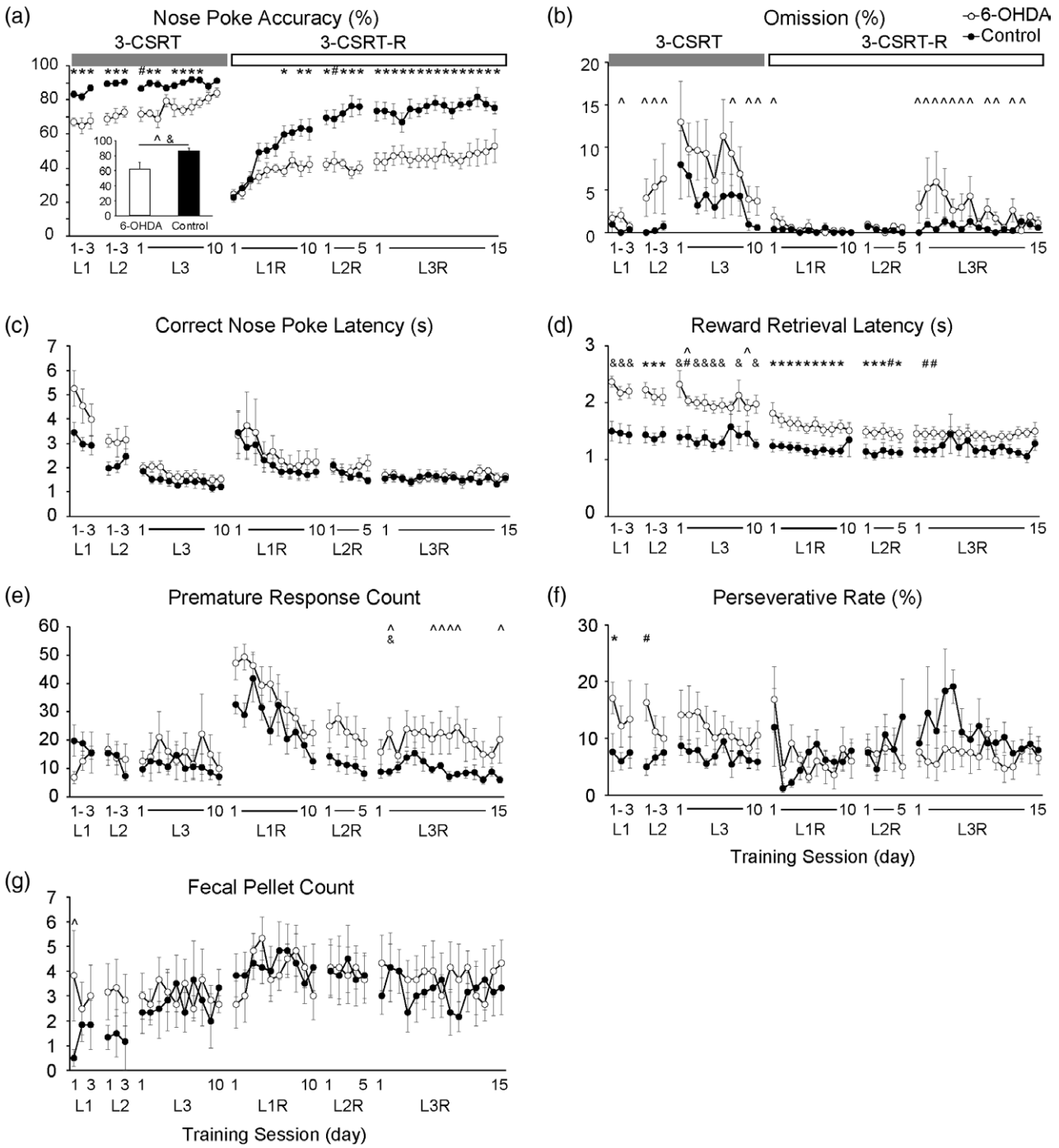


Lesion largely spared motor functions and sucrose preference. (a) Overnight locomotor activity before and 2 weeks after 6-OHDA lesioning ($n = 13$, horizontal $P = 0.074$, vertical $P = 0.52$, unpaired Student's t-test). (b) Accelerating rotarod test showed no significant differences in mean latency 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.

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Conflicts of interest

There are no conflicts of interest.

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