AWARD NUMBER: W81XWH-19-1-0757

TITLE: **Increasing Nigral Tyrosine Hydroxylase Expression as a Mechanism of Exercise-Mediated Recovery: Evaluation in Toxin and Rat Parkinson's Disease Genetic Models**

PRINCIPAL INVESTIGATOR: Michael F. Salvatore, Ph.D., Professor

CONTRACTING ORGANIZATION: University of North Texas Health Science Center-Fort Worth

REPORT DATE: **October 2021**

TYPE OF REPORT: **Annual**

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE:	**2. REPORT TYPE**	**3. DATES COVERED**
October 2021	Annual	Sept 1, 2020 - Aug 31, 2021

4. TITLE AND SUBTITLE: Increasing Nigral Tyrosine Hydroxylase Expression as a Mechanism of Exercise-Mediated Recovery: Evaluation in Toxin and Rat Parkinson's Disease Genetic Models

5a. CONTRACT NUMBER
W81XWH-19-1-0757

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S):
Michael F. Salvatore, Ph.D

E-Mail: Michael.Salvatore@unthsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Texas Health Science Center-Fort Worth
3500 Camp Bowie Blvd
Fort Worth, Texas 76107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The health benefits of regular exercise are documented in hundreds of studies. More recently, clinical studies indicate exercise may alleviate motor symptoms of Parkinson's disease (PD). Understanding how exercise affects the CNS to produce these patient benefits is critical for identifying highly-translatable targets that would enable pharmacological or genetic approaches to improve motor function in PD patients that can no longer exercise. Despite much work in rodent PD models, the mechanisms of exercise-related motor benefits are not established. A critical guideline for identifying translatable exercise-responsive targets in human PD is to conduct exercise in PD rodent models that tethers the exercise intensity and frequency within the capabilities of the PD patient. Our goal is to utilize two different rat PD models (toxin- or genetic-based) to evaluate the exercise impact on motor function and nigrostriatal mechanisms in both the striatum and substantia nigra (SN). Based upon previous work from others and our lab, we are testing the hypothesis that exercise-related motor benefits are not dependent upon augmenting dopamine (DA) function in the striatum, but within the SN. Evidence to support a role for nigral DA function in exercise-related motor benefits will guide research for genetic and pharmacological strategies to augment DA function in the SN for optimal relief of motor disability in the PD patient.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	15	

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

TABLE OF CONTENTS

1. Introduction:

Exercise in rat Parkinson's models: rationale for optimal translation to human Parkinson's disease

Determining therapeutic approaches to halt the progressive nature of motor disabilities in Parkinson's disease (PD) is a critical research priority. While restorative therapies for the PD patient have yet to be established, emerging evidence supports that aerobic exercise can mitigate progression of motor symptoms. These effects strongly suggest that exercise induces CNS responses that mitigate or improve components of dopamine (DA) signaling in nigrostriatal neurons lost due to PD pathology. However, the mechanisms by which exercise imparts these beneficial motor effects are still yet to be elucidated. Identifying these exercise-responsive CNS mechanisms in rodent PD models can identify the optimal genetic- or pharmacologically-based targets to mitigate or halt progressive motor disability for the PD patient. Our experiments are designed to evaluate the nigrostriatal neuron dopamine (DA) signaling components that respond to exercise using two established rat models of PD, 6-OHDA lesion or genetic-based Pink1 knockout. Maintaining fidelity of realistic exercise capabilities of the PD patient into rodent PD exercise studies is an often-neglected priority in preclinical studies. To address this need for translation into the PD patient, our exercise protocol is designed to reflect the exercise capabilities of the early-stage PD patient, by tethering exercise intensity, frequency, and duration to the capabilities reported in PD patient studies. Moreover, to further optimize translation of our studies, exercise is initiated after locomotor impairment is established. Therefore, these experimental design guidelines arguably will yield insight into the CNS mechanisms of exercise for optimal translation potential to the PD patient. In this annual report, we will present results evaluating CNS mechanistic findings of the nigrostriatal pathway during lesion, and response to exercise. Highlighted results will include the differences in striatal and nigral DA signaling following nigrostriatal lesion. These differences form the basis of the rationale for timing of exercise intervention and to evaluate its impact in conjunction with motor function. We will include an accounting of the cellular impact on TH cell counts from the laboratory of Dr. Jason Richardson. We will also present DA signaling changes from contemporaneous evaluation of striatal and nigral extracellular DA following 6-OHDA lesion from our collaboration with Dr. Bishop. Finally, we also have new insight into upstream growth factor-related mechanisms that can influence DA signaling. We will therefore report the approach of imaging used in Dr. Richardson's laboratory to evaluate expression of the GDNF receptor, GFR-α1, in astrocytes and in nigrostriatal neurons. This is a timely advance to further our investigation into potential involvement of astrocytes to mediate exercise effects. GDNF signal transduction is hypothesized to be a key mediator of exercise impact. We have new results to show both GFR-α1 and RET show progressive decline in the striatum as time past nigrostriatal lesion increases, with the progressive loss of TH protein in the SN. Finally, we have new insights in exercise impact on not only GDNF signaling, but BDNF as well. This includes full assessment of their respective receptors, in addition to striatal TH and DA measures. From our other PD rat model, we also have new results from our longitudinal assessment of motor and cognitive function from the Pink1 knockout rat, currently at a year old.

2. Keywords:

Parkinson's disease, exercise, glutamate transporter, tyrosine hydroxylase, dopamine, GDNF, GFR-alpha 1, RET, Pink1, mitochondria, BDNF, TrkB

3. Accomplishments:

Major Goals (as described in updated and approved SOW, June 4, 2019) and current status

Our major goal is to *delineate the neurobiological mechanisms by which exercise may prevent or restore loss of motor function in rat models of Parkinson's disease (PD).* The progressive nature PD and barriers to exercise faced by the PD patient necessitates identifying neurobiological mechanisms that are driven by exercise intervention that promote motor improvement. These exercise-related changes in the CNS reveal optimal neurobiological targets for the PD patient to mitigate motor impairment and slow progression.

1) **Protocol submission and approval:** The PI prepared and submitted institutional ACUC protocol and ACURO animal protocol renewals in March and April 2021. Approvals were obtained from the institutional ACUC on March 31, 2021 and ACURO on May 14, 2021

2) Experiments: update on results

Timeline of nigrostriatal lesion impact in the 6-OHDA lesion model: 95% complete.

Rationale for evaluating dopamine signaling in the substantia nigra

Our major hypothesis is that exercise-responsive CNS mechanisms that mitigate motor impairment in our rat models will augment DA signaling in the substantia nigra (SN), rather than the striatum. It is well-established that nigrostriatal DA neuron loss occurs in PD, and >80% loss of DA markers in the striatum must occur prior to onset of motor symptoms. However, many exercise studies in rodent PD models have shown little or no change in striatal DA signaling despite evidence of improved motor function, which argues against the premise that restoration of components that regulate striatal DA signaling is a primary mechanism of exercise impact. What is often neglected with respect to changes that occur, in addition to those in striatum, at the time of locomotor impairment is loss of DA markers in the SN. We argue that this level of loss in the SN, which is ~40% (nearly half of that in striatum), provides much wider window for interventions, like exercise, to preserve or restore remaining DA markers. We also contend that preserving nigral DA markers by exercise has functional impact on motor function. Our recent work shows that nigral DA signaling can be experimentally modulated, autonomous from any influence on DA signaling within the striatum, and this modulation affects locomotor activity. Thus, when DA signaling is decreased, locomotor activity decreases (PMID 30056575).

Results: TH and dopamine regulation between striatum and SN after nigrostriatal lesion

We evaluated changes in striatal and nigral DA markers at two time periods post-lesion, at day 7 and day 28. The rationale was to establish a timepoint for exercise intervention that would capture what changes were in force to regulate DA signaling in striatum and the SN, when locomotor impairment would be established prior to exercise intervention. In the previous annual report, initial results showed tyrosine hydroxylase (TH) had a more rapid rate of loss in striatum compared to the SN, being near 95% by day 7, with no greater loss at day 28. In contrast TH loss in the SN was significant at day 7, ~60%, but was greater at day 28, near ~80%. New updated results with inclusion of a second cohort confirms these findings (Fig. 1). Another significant and novel was a gradual loss of TH also occurred in the SN, contralateral to the lesioned side. In both instances, this progressive pattern of TH loss reflects nigrostriatal pathology seen in human PD, in that DA marker loss in striatum precedes loss in the SN, with a more rapid rate of loss in striatum seen early after PD diagnosis.

We now have completed evaluation of DA tissue content with the complete assessment of TH protein. Figure 1 depicts the differences between striatum and SN in TH protein expression vs. DA tissue content. Evidence for compensatory increases in DA biosynthesis are indicated in the SN, where despite TH loss by day 7, there is no decrease in DA tissue content in the SN ipsilateral to 6-OHDA lesion. Moreover, despite the decrease in TH protein in SN contralateral to lesion by day 28, DA tissue content is unaffected at that time.

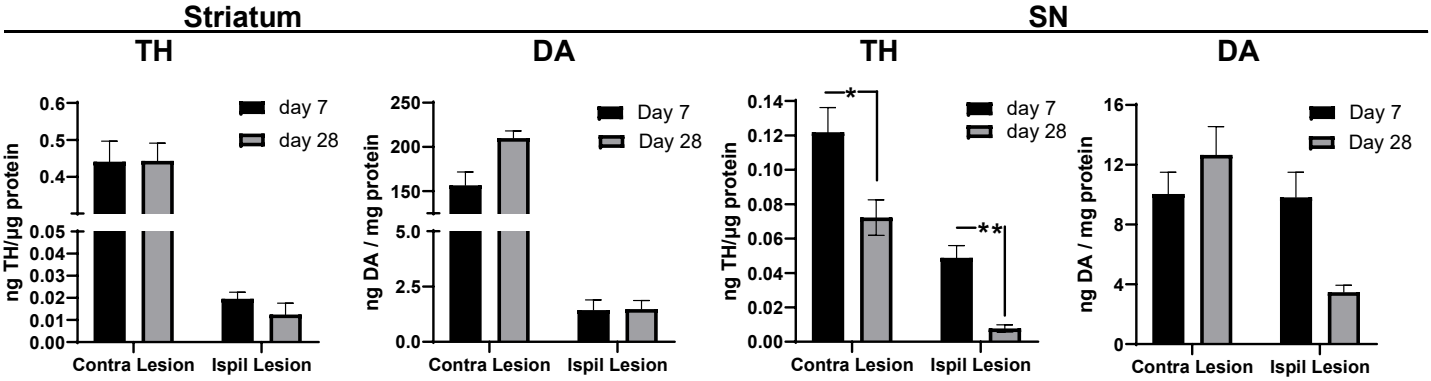


Figure 1. Timeline of nigrostriatal 6-OHDA lesion impact on tyrosine hydroxylase (TH) protein expression and DA tissue content in striatum (left) and substantia nigra (SN) (right). Striatum. TH protein. Lesion decreased TH protein expression. (Two way ANOVA repeated measures: Lesion ($F_{(1,16)}$ =129.6, p<0.0001); time after lesion ($F_{(1,16)}$ =0.004, ns); lesion X time after lesion ($F_{(1,16)}$ =0.015, ns); *Ipsi lesion.* Day 7 vs. day 28 (t=0.62 ns). *Contra lesion.* Day 7 vs. day 28 (t=0.09 ns). **DA tissue content**. Lesion decreased DA tissue content. Lesion ($F_{(1,15)}$ =418, p<0.0001); time after lesion ($F_{(1,31)}$ =7.93, p=0.01); lesion X time after lesion ($F_{(1,15)}$ =7.5, p=0.015); *Ipsi lesion.* Day 7 vs. day 28 (t=0.08 ns). *Contra lesion.* Day 28 vs. day 7 (t=3.1, p=0.007). **Substantia nigra (SN). TH protein** Lesion decreased TH protein expression. Lesion ($F_{(1,15)}$ =58.9, p<0.0001); time after lesion ($F_{(1,16)}$ =17.7, p=0.0007); lesion X time after lesion ($F_{(1,15)}$ =0.27.ns); *Ipsi lesion.* Day 28 vs. day 7 (t=5.24 p<0.0001). *Contra lesion.* Day 28 vs. day 7 (t=2.82 p=0.01). **DA tissue content**. Lesion decreased DA tissue content on day 28, but not day 7. Lesion ($F_{(1,16)}$ =12.88, p=0.003); time after lesion ($F_{(1,16)}$ =1.3, ns); lesion X time after lesion ($F_{(1,16)}$ =11.66, p=0.004); *Ipsi lesion.* Day 28 vs. day 7 (t=3.62, p=0.002). *Contra lesion.* Day 7 vs. day 28 (t=1.1, ns).

Our laboratory has established evidence that ser31 TH phosphorylation plays a critical role in regulating DA biosynthesis *in vivo* (22242182), and may play a key role in compensating for TH protein loss during nigrostriatal lesion progression to increase DA (PMID 24410633). In light of our new findings of DA regulation against TH loss (Fig. 1), we have new results indicating increased ser31 phosphorylation is offsetting progressive TH loss in the SN, but not striatum , wherein lesion produces a significant decrease (Fig. 2).

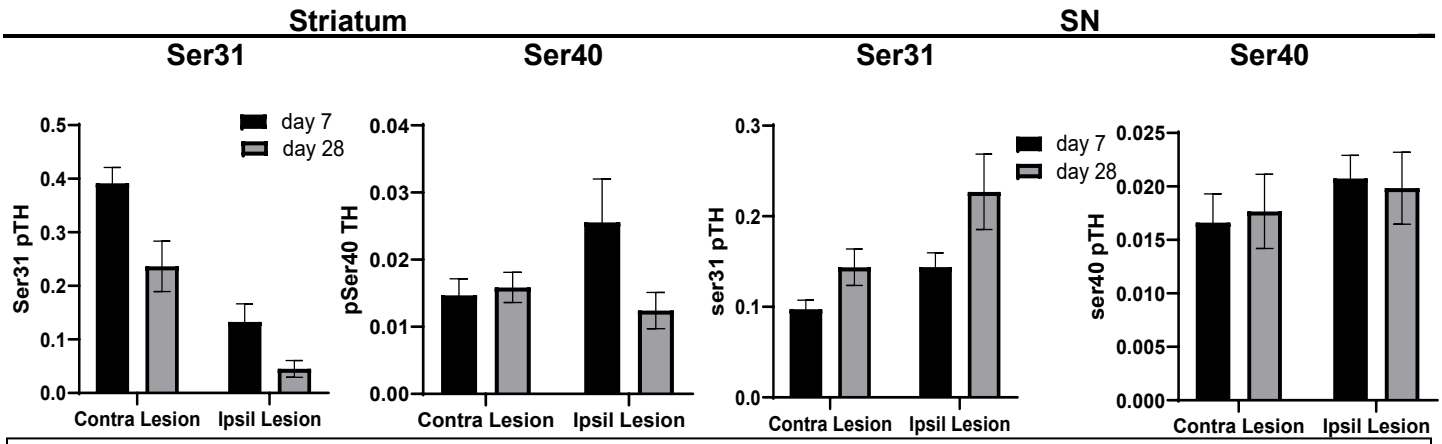


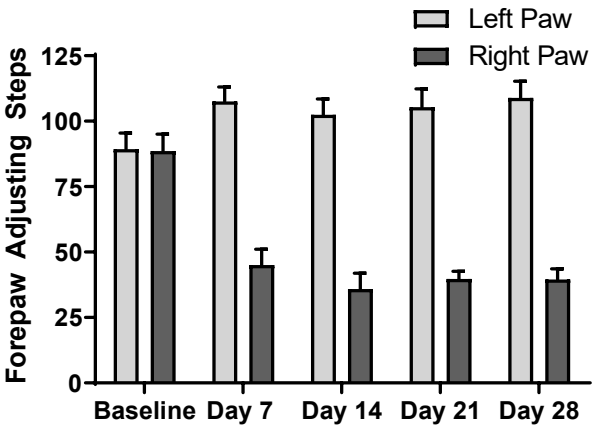
Figure 2. Timeline of nigrostriatal 6-OHDA lesion impact on TH phosphorylation stoichiometry (pTH) at Ser 31 and Ser40 striatum (left) and substantia nigra (SN) (right). Striatum. Ser31. Lesion decreased ser31 pTH. (Two way ANOVA repeated measures: Lesion ($F_{(1,11)}$ =104.8, p<0.0001); time after lesion ($F_{(1,12)}$ =8.97, p=0.01); lesion X time after lesion ($F_{(1,11)}$ =2.00, ns); *Ipsi lesion.* Day 7 vs. day 28 (t=2.22, p<0.049 ns). *Contra lesion.* Day 7 vs. day 28 (t=2.94, p=0.01). **Ser40.** Lesion had no effect on ser40 pTH. Lesion ($F_{(1,14)}$ =0.82, ns); time after lesion ($F_{(1,14)}$ =1.87, ns); lesion X time after lesion ($F_{(1,14)}$ =3.0, ns). **Substantia nigra (SN). Ser31.** Lesion increased ser31 pTH. Lesion ($F_{(1,15)}$ =11.2, p=0.005); time after lesion ($F_{(1,15)}$ =5.51, p=0.03); lesion X time after lesion ($F_{(1,15)}$ =0.91, ns); *Ipsi lesion.* Day 7 vs. day 28 (t=1.964 , p=0.07) . *Contra lesion.* Day 7 vs. day 28 (t=2.14 p<0.05). **Ser40** Lesion had no effect on ser40 pTH. Lesion ($F_{(1,12)}$ =1.89, ns); time after lesion ($F_{(1,12)}$ =0.00, ns); lesion X time after lesion ($F_{(1,12)}$ =0.18, ns).

The TH phosphorylation results give insight into another example that DA regulation within the nigrostriatal pathway is distinctly different between the striatum and SN during the progression of nigrostriatal neuron loss. In striatum, compensatory increases in DA levels against loss of TH are non-existent, as evidenced by a no change in ser40, and a decrease in ser31 phosphorylation that is progressive during the course of lesion progression. Moreover, there is a precipitous decrease in DA content (<99%) within 7 days after lesion that does not change. In contrast, ser31 phosphorylation in the SN increases after lesion, and also increases in the contralateral side by day 28. Notably, despite TH loss by day 7 in the SN of nearly 60%, DA tissue content is unchanged. Furthermore, despite loss of TH by day 28 in SN contralateral to lesion, there is no decrease DA tissue content. With no evidence of any change in ser40 TH phosphorylation, these results argue that ser31 TH phosphorylation in the SN is a compensatory mechanism to offset TH loss. We plan to evaluate ser31 TH phosphorylation further in our exercise studies to determine if this mechanism may be further increased above lesion levels following exercise.

Results: Motor function during nigrostriatal lesion progression

There are two main locomotor evaluations used in the 6-OHDA lesion studies, the forepaw adjustment step test (FAS) and open-field locomotor activity. Both evaluations are used to determine lesion impact on motor function and exercise-related effects on motor function and recovery. They are translational to human PD motor impairment from the perspective of impaired movement initiation and continuation, which are measured by the timed up and go (TUG) and six minute walk test.

Our results from the lesion timeline study show rapid loss of use of the forepaw associated with lesioned side (the paw contralateral to the lesioned side) by day 7 after nigrostriatal lesion. The impairment is sustained out to at least 28 days (Fig. 3). Of note, in our 6-OHDA model, there is also a small (~20%) but significant increase in the use of the forepaw associated with the unlesioned side that is also sustained out to at least day 28 (Fig. 3). In contrast to the rapid and sustained onset of impaired forelimb use following nigrostriatal lesion, locomotor activity in the open-field declines but in a more protracted timeline than the decline in forepaw use, reaching nearly a 30% decrease from baseline levels by 28 days (Fig.4).



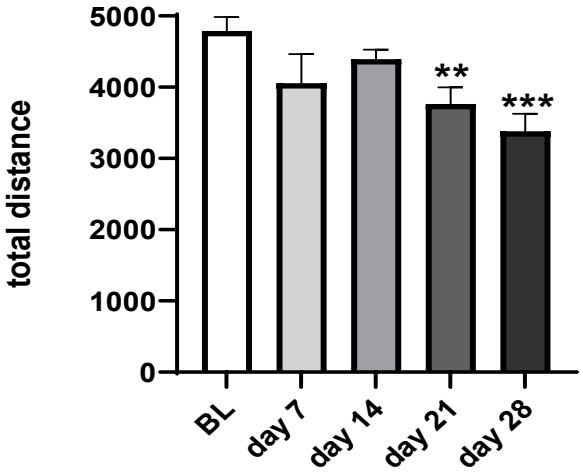
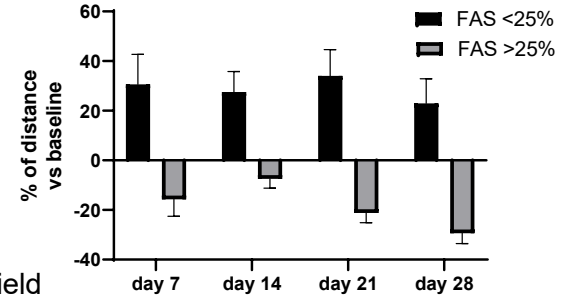


Figure 3 (left). Decreased forepaw use associated with unilateral 6-OHDA lesion and increased forepaw use associated with non-lesioned side. Lesion ($F_{(1,16)}$ =50.7, p<0.0001); time after lesion ($F_{(4,64)}$ =15.5, p<0.0001) lesion X time after lesion ($F_{(4,64)}$ =57.8, p<0.0001). Dunnet's post-doc test showed highly significant differences for both paws at each time point (day 7,14,21,28) versus baseline values.
Figure 4 (right). Decreased locomotor activity following 6-OHDA is protracted. Repeated measures one-way ANOVA evaluating distance traveled (one hour session) post-leson. Lesion ($F_{(4,28)}$ =6.59, p=0.0007). Dunnett's multiple comparison test. BL v day 7 (q=2.44, p=0.07); BL v day 14 (q=1.32, ns); BL v day 21 (q=3.41, **p=0.007); DL v day 28 (q=4.694, ***p=0.00003)

Despite the more protracted rate of decline in locomotor activity compared to rapid loss of forepaw use by day 7, there is a relationship between the two readouts. Qualifying a nigrostriatal lesion is done on day 7 by FAS evaluation. If there is less than 25% reduction in forepaw use in the lesioned side, the use will not further decline by day 28. Moreover, the open-field activity will not decline (Fig. 5).

Figure 5. Relationship of percent decline in FAS performance at day 7 vs decline in distance (locomotor activity). Successful nigrostriatal lesion is defined by at least 25% reduction in lesioned paw use by day 7. With >25% reduction in paw use, locomotor activity (Distance traveled in 1 hr) declines significantly by day 21. FAS threshold ($F_{(3,33)}$ =39.5, p<0.0001). Noeffect of time past-lesion ($F_{(3,33)}$ =2.22, ns) or interaction ($F_{(3,33)}$ =1.33, ns

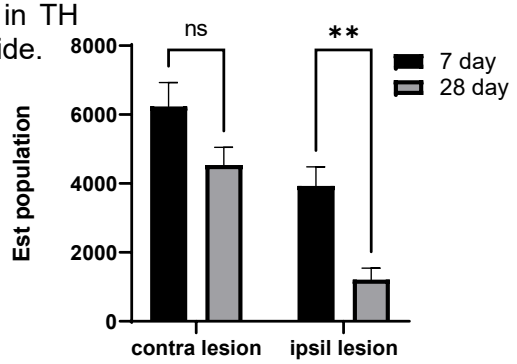


In summary, the locomotor assessments of FAS and open-field locomotor activity both illustrate motor deficits akin to those seen in human PD from the perspective of hypokinesia, or difficulty initiating movement. Although deficits in the open-field occur ~2 weeks following a FAS deficit, a reduction of forepaw use of >25% will predict eventual decline in locomotor activity.

Results: TH cell counts vs. Nissl Stain (Richardson Lab)

The 6-OHDA lesion timeline study indicates progressive loss of TH protein in the SN, with greater loss of TH on the lesioned side at day 28 vs. day 7, and significant loss contralateral to lesion by day 28. Curiously, no loss of TH is seen in the striatum contralateral to lesion at day 28. To ascertain the source of TH loss in the SN, the same timeline study was conducted with stereological assessment of TH cell number and compared with cell number by Nissl staining. The results follow the same differences in TH expression in the SN in terms of progressive loss on the lesioned side. There was a trend toward loss in the SN contralateral to lesion (Fig. 6).

Figure 6. Estimated population of TH cells in the SN following nigrostriatal lesion. Progressive loss of TH cells was observed in the SN ipsilateral to lesion and contralateral to lesion. Lesion ($F_{(1,11)}$ =43.5, p<0.0001); time after lesion ($F_{(1,11)}$ =13.1, p=0.004); lesion X time after lesion ($F_{(1,11)}$ =1.44, ns).



Following confirmation of Nissl staining quantification for total cell number in these midbrain sections, the current results suggest that TH protein loss on the contralateral side to lesion may be due to loss of protein itself, rather than cell number. As such, this would suggest acceleration of TH protein loss precedes cell loss. This is the apparent case as seen at day 7 in the SN on the ipsilateral to lesion (Fig. 1 vs. Fig 6). Figure 7 shows representative images, with comparison to sham-op groups.

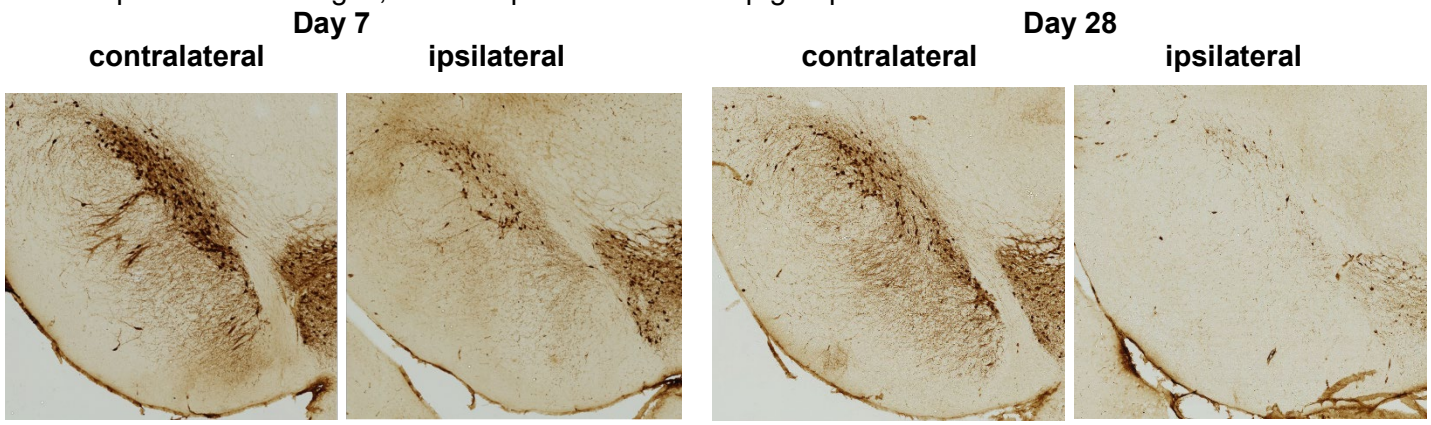
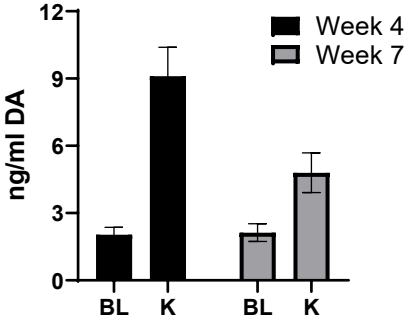


Figure 7. Representative images of TH cell number. Visual depiction of the SN at day 7 and day 28 past unilateral 6-OHDA nigrostriatal lesion, comparing ipsilateral and contralateral sides to lesion. Progressive loss of TH cell number is clearly visible between day 7 and 28 ipsilateral to lesion.

Results: sample collection at 4 and 7 weeks and evaluation of extracellular DA in striatum and SN (Bishop Lab) (100%)

A primary goal of our microdialysis-based evaluation of extracellular DA in a planned pilot study was to determine if DA could be reliably determined from at least two sample times during a study. The value of this approach is two-fold; 1) reduce the number of rats needed to complete a study, 2) have a paired comparison of two results from the same test subject. In this case, we plan to evaluate exercise impact by comparing post-exercise sample results to pre-exercise sample results to determine if exercise could augment DA signaling in the SN or striatum. We are able to show that collection at two time points is possible (Fig. 8), and demonstrate basal and K+-stimulated differences in DA release in the striatum and SN (data not shown).

Figure 8. Striatal extracellular DA evaluation at 4 and 7 weeks post-surgery. K+-stimulation produced a highly-significant effect on DA release at both 4 and 7 weeks. ($F_{(1,14)}$ =45.3.7, p<0.0001). There was an interaction between K+-stimulation and week of collection ($F_{(1,14)}$ =9.7, p<0.008).



Progress update: Nigrostriatal lesion timeline study: impact of extracellular DA (Bishop Lab) (50%)

The Bishop Lab has completed the first leg of work to evaluate changes in extracellular DA under the basal and K+-stimulated conditions at 7 and 28 days post-lesion. Cannula were placed into both striatum and SN either in the contralateral side to lesion or side ipsilateral to lesion for contemporaneous collection of dialysate. Our goal is to characterize the changes in extracellular DA at basal conditions in the striatum and SN, and K+-stimulated DA levels between sham-operated and lesioned rats. Of the 6-OHDA treated rats, the grouping and number of rats from which sample collection was conducted is as follows, relative to lesion:
1) contra/7 day (6 female; 2 male); **2)** contra/28 day (5 male, 1 female); **3)** ipsil/7 day (5 female; 2 male); **4)** ipsil/28 day (4 female, 3 male). Of the sham-operated rats, the number of rats per group is as follows: **5)** contra/7 day (3 female; 3 male); **6)** contra/28 day (2 male, 4 female); **7)** ipsil/7 day (3 female; 3 male); **8)** ipsil/28 day (3 female, 3 male). The samples were shipped to the PIs lab in August. New research associates have been trained in HPLC sample preparation, machine maintenance, sample analysis, and peak analysis. They have also conducted this work independently after training. Sample analysis will commence in October 2021. We will report the results to Dr. Bishop and his graduate student and discuss if additional rats are needed to obtain sufficient statistical power. One goal is to verify that there is consistent DA signal in the sham operated groups, particularly in the contralateral side collections. This would verify that the double collection

approach is sound and no technical issues would influence interpretation of results coming from the 6-OHDA group, wherein changes in DA levels are expected as the lesion progresses.

Impact of treadmill exercise on motor recovery, striatal DA regulation and growth factor signaling

A rationale for timing of exercise intervention versus nigrostriatal lesion progression and motor impairment

In the first two years, we have established the rationale for the timing of exercise intervention against the changes to motor and nigrostriatal DA markers that are present before and after exercise intervention. First, using the 6-OHDA rat PD model, we have characterized the differences in DA and TH loss between striatum and the SN in a timeline study, evaluating differences in DA markers at two time points following nigrostriatal lesion. Therefore, our exercise protocol begins when striatal TH loss is >80% and nigral TH loss is ~60%. This dichotomy of loss within the nigrostriatal neuron is reflective of human PD pathology, wherein it has been shown that DA marker loss in striatum is much greater than nigral loss at the time of locomotor impairment. With these differences in loss in place at the time of exercise intervention, it is possible to ask two questions;

1) does exercise initiation restore DA marker loss in striatum and the SN, or
2) does exercise initiation prevent further DA marker loss in the SN, with no effect in the striatum.

For this study, there are four treatment groups; **1)** Sham-op, exercise, **2)** Sham-op non-exercise, **3)** 6-OHDA lesion, exercise, **4)** 6-OHDA lesion, non-exercise. In collaboration with Dr. Jason Richardson, the results of this study will be evaluated for loss of TH+ cells, as explicated in detail in experiment #4 in the proposal. Exercise will be implemented for 3 weeks on day 8-10 post 6-OHDA. Motor assessments were done at the baseline (prior to lesion induction), day 7 (to confirm lesion and its extent on motor function by the FAS test), and then 3x per week on a treadmill at 10-11 m/min. Motor function is assessed at the end of each week of exercise, the first day after exercise, and then at the end of the study at 28 days post-lesion. The tissues will be collected promptly after assessment of open-field activity and FAS within 24 hr of the final exercise session.

Motor function:

The exercise paradigm, featuring a three times per week 40 min per session regimen, improved movement initiation (Fig. 9) and locomotor activity (Fig 10) over the course of the three week long regimen. Recovery of movement initiation was observed first, with gradual increases in forepaw use compared to the pre-exercise deficit. Locomotor activity declined and then recovered in the exercise group by the end of the third week of exercise.

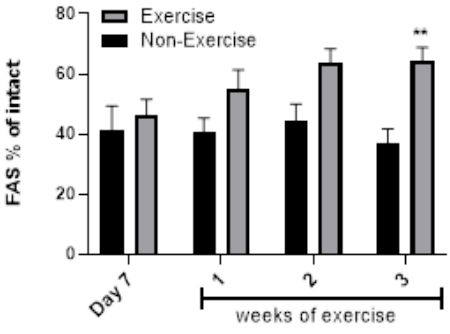
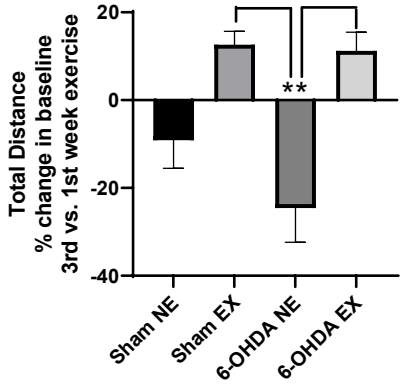


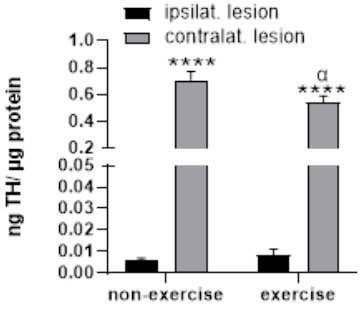
Figure 9 (left). FAS recovery following exercise in 6-OHDA lesioned rats. The exercise regimen increased FAS by 50%. 2 way ANOVA repeated measures: Exercise round ($F_{(2,34)}$= 4.14, **p*=0.02), exercise ($F_{(1,16)}$= 5.49, **p*=0.03), exercise X exercise round ($F_{(3,47)}$= 5.53, **p*=0.003). 3rd week (t=4.28, ***p*=0.002). **Figure 10 (right). Recovery of locomotor activity.** Locomotor activity increased by 3rd week of exercise in 6-OHDA lesioned group. One-way ANOVA $F_{(3,28)}$=9.00, *p*=0.0002. 6-OHDA NE vs 6-OHDA EX (t=4.34,**p*<0.001)

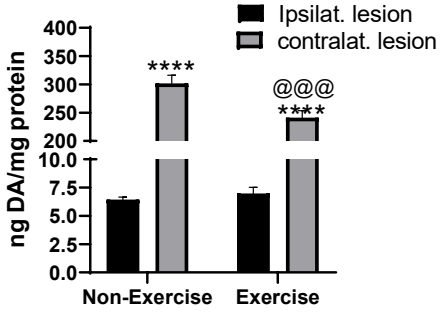


Striatal TH and DA regulation

The locomotor recovery associated with the exercise regimen did not affect the magnitude of loss of TH protein (Fig. 11) nor DA tissue content in striatum (Fig. 12). In fact, DA levels decreases in the striatum contralateral to lesion in the exercise group.

Figure 11 (left panel). Striatal TH expression is unaffected by exercise in 6-OHDA lesioned rats. Exercise (F(1,17)= 4.99, *p=0.03). EX vs. NE, contralat to lesion (t=3.21, α *p*<0.01).
Figure 12 (right panel). Striatal DA tissue content is unaffected by exercise in lesioned side and decreased contralateral to lesion. Exercise (F(1,26)= 10.1, *p=0.004). EX vs. NE, contralat to lesion (t=4.5, @@@*p*<0.001).



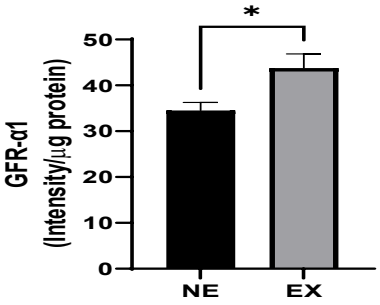


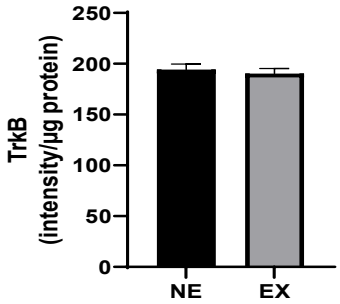
Growth factor signaling: GDNF and BDNF role in exercise impact

In addition to recovery of motor functions, the exercise regimen increased expression of GFR-α1 (Fig. 13) and RET (data not shown) in the striatum. No effect on TrkB, the receptor for BDNF, was observed (Fig.14).

Figure 13 (left) GFR expression increases in striatum with exercise. (t=2.53, *p<0.05, df=15). Unpaired two-tailed t-test.

Figure 14 TrkB expression is unaffected by exercise. (t=0.61, ns, df=17). Unpaired two-tailed t-test.





To be completed:

GFR-α1 expression in astrocytes vs.nigrostriatal neurons.

One of the major goals of the project is to evaluate if increased expression of GFR-α1, one of two receptors for GDNF (the other being RET) mediates exercise-related changes in DA signaling to preserve or restore locomotor activity. We are also interested to determine the cellular source of GFR-α1 expression that would mediate this effect. Astrocytes and nigrostriatal neurons are known to express these receptors. In collaboration with Dr. Richardson, his lab has developed a triple-staining Approach to evaluate expression of GFR-α1 in GFAP+ (astrocytes) and TH+ (nigrostriatal) neurons.

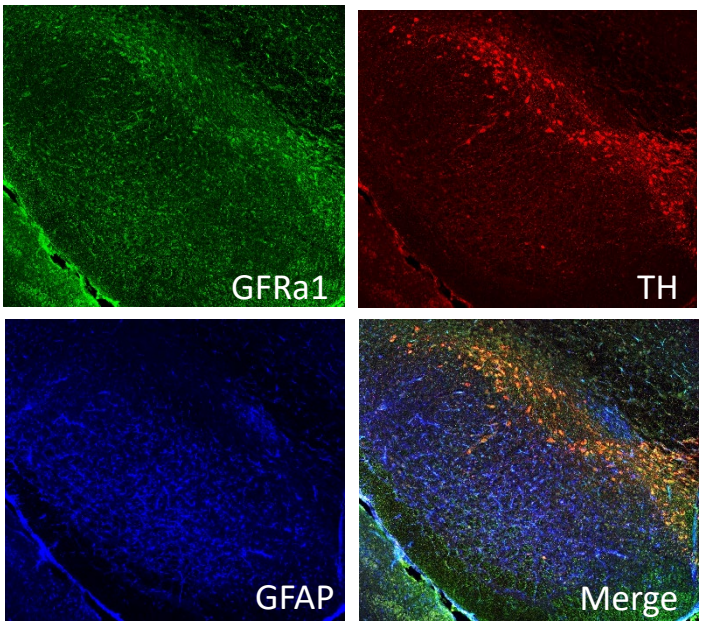


Figure 15. GFR-α1, TH, GFAP staining in the SN. Images of the GDNF receptor GFR-α1, TH, GFAP, and a merge of the images. In both astrocytes (GFAP+) and nigrostriatal neurons (TH+), there is evidence of co-labeling. This approach will permit us to evaluate how nigrostriatal lesion affects GFR-α1 expression in these cells, and if exercise will offset any lesion effect.

Progress update: Exercise impact on extracellular DA in striatum and SN (Bishop Lab)

The evaluation of exercise impact on extracellular DA regulation began in July 2021. The lab received guidance from the PIs lab to learn how to exercise the rats for optimal compliance. The PI and Dr. Bishop discussed the experimental design and agreed upon a pre- and post-exercise collection of dialysate. Successes have been seen with cannula patency, as all rats had intact cannula at end of the exercise study. Re-probing for dialysate collection at day 28 has been successful with no issues to note. Flow rates and sample volumes from both striatum and nigral sites have been consistent.

Some difficulties have been noted with recovery of rats that receive the 6-OHDA lesion. Extra post-surgical care has typically been required for these rats. It has also been noted that although compliance is high pre-surgery, the 6-OHDA rats struggle early. Accommodations have been made to pull the rats through the three times per week regimen by slowing down the treadmill speed to from 10 to 8 m/min.

- Cohort 1 (N=9 of 16 made it through), all male: 6-OHDA-NE (n=2), 6-OHDA-Ex (n=3), Sham-NE (n=2), Sham-Ex (n=3)
- Cohort 2 (N=9 of 16 have made it through D7 microdialysis), all female: 6-OHDA-NE (n=2), 6-OHDA-Ex (n=3), Sham-NE (n=1), Sham-Ex (n=3)
- Morbidity/mortality and drop-outs remain high (>40%), to reach our goal number of 16/group (8M/8F), if these rates continue, we will need to run 7 cohorts of 16 instead of 4 cohorts (assuming everything went perfectly). Thus, our final cohort will be run starting in **mid-Feb 2022 and end in mid-March 2022**.

Pink 1 knockout rat: Longitudinal assessment of motor and cognitive function

We are approaching the conclusion of a 12 month long longitudinal evaluation of locomotor and cognitive function in the Pink1 knockout rat. Consistent with the approach we have taken with the timing of exercise intervention in the 6-OHDA model, we are determining the timing of exercise intervention in the Pink1 knockout rat. The goal is to intervene with exercise after motor impairment has begun to determine if exercise will restore decreased locomotor activity in the knockout, and if there is an increase in nigral DA commensurate with this improvement. Of note, we were encouraged by the reviewers to evaluate non-motor symptoms of PD, if possible. Thus, we have also incorporated evaluation of cognitive function in these rats using the novel object recognition test (data not shown). Evaluations have been done on a monthly basis.

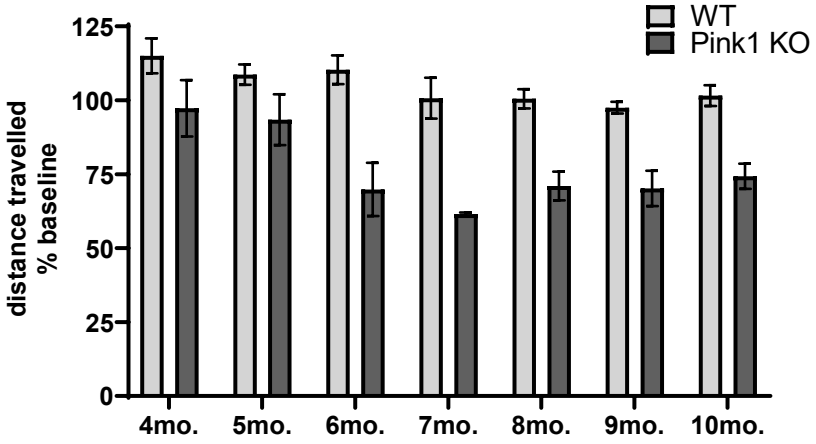


Figure 15. Progressive decrease in locomotor activity in the Pink1 KO rat. Beginnng at 6 mos old, there is a significantly greater decrease in locomotor activity in the Pink1 KO rat. Two way repeated measures ANOVA results: Genotype, $\mathbf{F}_{(1,12)}$ =14.9, p=0.002); age $\mathbf{F}_{(6,61)}$ =7.78, p<0.0001); age X genotype $\mathbf{F}_{(6,61)}$ =2.45, p=0.035).

Training and professional development opportunities: At the PI's institution, UNT Health Science Center, the following trainees have gained professional development opportunities.

1) **Dr. Ella Kasanga, Ph.D.** Dr. Kasanga received her doctoral degree in May 2021. She played a major role in the conduct of all experiments described as a graduate student under the mentorship of the PI. During her time in the PI's Lab, she went to collaborator Dr. Jason Richardson's lab for seven weeks (May 28 – July 23 2021) to learn advanced microscopy and stereology techniques on experimental samples obtained from our collaboration. Travel and living expenses were covered by her Visiting Scholar Award from the Parkinson's Foundation. She then returned to the PI's lab to finish her work on the timeline study and analyses of samples from the exercise study until September 3, 2021. She has now accepted and begun a postdoctoral fellowship at the Van Andel Institute under the mentorship of Dr. Patrik Brundin.
2) **Caleb Parry, medical student at Texas College of Osteopathic Medicine (TCOM).** Mr. Parry approached the PI during the first year of the grant (May 2020) to pursue neuroscience-based research in movement disorders. He was very active in the lab, learning western blot techniques for verifying nigrostriatal lesion efficacy. He took on a specific project in Fall 2020 pertaining to our first exercise study by examining BDNF signaling; a very important aspect to examine if this growth factor pathway was also affected by exercise. His work evaluated expression of pro and active forms of BDNF, and later during a 4th year rotation elective in September 2021, the receptors, TrkB and p75. He presented his BDNF work at UNT Health Science Center Research Appreciation Day in March 2021 and has completed analysis of the BDNF receptors, the results of which are presented in this annual report.
3) **Joshia John, medical student, TCOM.** Ms. John approached the PI during the second year of the grant to pursue a research interest in movement disorders. She decided to focus upon the role of mitochondrial protein regulation in the Pink1 knockout rat and in the 6-OHDA lesion model. She began her work in Summer 2021 and is continuing to work in the PI's lab as time between her studies in year 2 of medical school permits. Thus far she has produced results that have established an assay to confirm Pink1 expression.
4) **Isabel Soto, doctoral student** Ms. Soto joined the PI's lab in Fall 2020 and is co-mentored by Consultant, Dr. Vicki Nejtek, and the PI. Her role in the project is investigating the utility of the Pink1 knockout rat in evaluating the efficacy of exercise. She currently is directing a project involving the full longitudinal characterization of the Pink 1 knockout rat model on locomotor and cognitive function. Furthermore, she will be evaluating serum biomarkers in this study, comparing the pre- and post-motor and cognitive impairments against changes in these serum biomarkers, with the goal to establish a peripheral biomarker matrix that can portend these impairments.

5) **Ashley Centner, doctoral student**. Ms. Centner is a doctoral student in the Bishop Lab. She has taken the lead role in conducting surgeries for lesion and microdialysis cannula implants, locomotor assessments, and exercise. She has provided detailed accounting of successes and challenges in doing this work.

Dissemination of Results: Nothing to report at this time.

Plans for next reporting period:

1) **Complete assessment of the impact of gender on lesion impact**
2) **Complete second exercise study, with evaluation of nigral DA levels, TH, and TH phosphorylation in comparison with striatal changes**
3) **Complete evaluation of TH cell number in first exercise study (Richardson)**
4) **Complete evaluation of GFRα1 expression in GFAP and TH+ cells from first exercise study**
5) **Complete exercise study to evaluate impact on DA levels in striatum and SN (Bishop)**
6) **Complete analysis of DA levels in striatum and SN in lesion timeline study (Bishop)**
7) **Complete longitudinal assessment of Pink1 KO rats, evaluate striatal and nigral DA markers**

4. Impact

Potential impact on the Parkinson's disease research field:

Aerobic-based exercise is showing significant promise in helping people with PD stave off the debilitating and progressive nature of motor impairment. Understanding how exercise affects neurochemistry and circuit function in the brain would reveal Mother Nature's recommended course of action for treatment. This is because the changes in neurochemistry are endogenous responses within the brain. As such, targeting these changes in brain by well-designed drugs or genetic-based approaches would conceivably prove an optimal treatment strategy. However, in order to achieve such a goal, it is critical to use established rodent PD models wherein the relationship between neurochemistry and motor function can be revealed before and after exercise. Moreover, the implementation of exercise in these models should reflect the physical capabilities of the person who has PD.

Our work has several of these important elements embedded in both our PD models, as well as the implementation of exercise. First, with regard to the nigrostriatal toxin model employed, our method replicates the well-established pattern of nigrostriatal dopamine neuron loss in which the axon terminals are the first to be lost in dopamine markers and function. Following this loss is loss of these markers in the substantia nigra. Moreover, we are learning the adaptive responses in this neuronal pathway that occur during progression of neuron loss, such as changes in dopamine biosynthesis. With this human relevant neurobiological platform in place, we are using an exercise regimen that is similar in intensity, frequency, and duration shown in humans. With this regimen in place, we are showing evidence of motor recovery by this exercise regimen. Notably, this recovery occurs after motor impairment is established before exercise begins. As such, we are poised to advance our understanding of the neurobiological changes in response to exercise within the physical capabilities of a person with PD. We have already identified several important responses to exercise in the brain, and also, potentially ruled out other possible mechanisms for their involvement in motor recovery.

What was the impact on other disciplines? Our exercise paradigm may be applicable for use in other neurodegenerative disease models, given the success of the regimen to promote motor recovery.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Our exercise work has the potential to motivate those with Parkinson's disease to increase their practice of exercise. This will be particularly true when our findings have been published and have potential news outlets. The basis for this possibility is that because to our knowledge this is the first exercise study to be done in rodent PD models with translation at the level of human PD patient physical capabilities (which is no more than three times per week at 40 min sessions each) and that exercise began *after* establishment of motor impairment. Our main result was that there was motor recovery from this exercise regimen. This means that the neuro-biological changes that we will identify in brain may also occur in human PD.

5. Changes/Problems

Changes in approach: Nothing to report with regard to a change in objective or scope of work

Actual or anticipated problems or delays and actions or plans to resolve them:
Research assistant McElroy left position as research assistant abruptly in April 2021. PI worked with UNTHSC HR to post position on several websites (ie. Indeed.com) to immediately recruit. This process took three months before the first hire, Robert McManus. Research assistant Shifflet gave one month notice to advise that she was accepted into a graduate program at Oklahoma State. Fortunately, another high-ranking candidate was already interviewed, and was offered the position. Thus, there was ~a three and one-half month shortage (April-mid July) of full research assistant expertise.

Changes that had a significant impact on expenditures: Three months of research assistant salary was saved by the unexpected departure of a research assistant and the time needed to interview and vet the ideal candidates. Tuition was also covered by the project for final (Spring 2021) semester for Kasanga. Savings were made in this regard however as she won a fellowship from the institution, thus defraying expenses for her stipend.

Significant changes in use or care of vertebrate animals: New protocol submitted and approved by institution and ACURO in Spring 2021. New methods introduced for blood collection from tail vein and cognitive assessment (novel object recognition).

In the Bishop Lab, a particularly high mortality or drop-out rate (40%) of 6-OHDA lesioned rats that are double-cannulated has been documented. The laboratory personnel have adopted proactive measures to maintain rat welfare, and greater success for viability has been noted by hand feeding the rats 3x per day, inclusion of gel cups, electrolytes, and saline injections.

Significant changes in use of biohazards and/or select agents: Nothing to report.

6. Products

Conference papers and presentations:
Three presentations scheduled at Society for Neuroscience Annual Meeting 2021

“Exercise-related motor recovery in the rat 6-OHDA hemi-parkinson’s model without increased striatal dopamine biosynthesis: a glimpse into exercise impact in human Parkinson’s disease?”
E. A. KASANGA1, M. SHIFFLET2, *M. SALVATORE2;
1Inst. of Healthy Aging, Univ. of North Texas Hlth. Sci. Ctr., Fort Worth, TX; 2Univ. of North Texas Hlth. Sci. Ctr., Ft Worth, TX
Session Number: P264
Session Title: Neuroprotective Mechanisms II

“Glial cell line-derived neurotrophic factor signaling: a new mechanistic perspective in exercise-mediated recovery of motor impairment in a rat hemi-parkinson's model”
*E. A. KASANGA1, M. K. SHIFFLET1, V. A. NEJTEK1, M. F. SALVATORE2;
Univ. of North Texas Hlth. Sci. Ctr., Fort Worth, TX; 2Inst. for Healthy Aging, Univ. of North Texas Hlth. Sci. Ctr., Ft Worth, TX
Session Number: P263
Session Title Neuroprotective Mechanisms I

"Is the Pink1 knockout rat a translatable animal model to evaluate prodromal cognitive and motor symptoms of Parkinson’s Disease?”
I. Soto, V.A. Nejtek, E. Kasanga, M.F. Salvatore
1Inst. of Healthy Aging, Univ. of North Texas Hlth. Sci. Ctr., Fort Worth, TX; 2Univ. of North Texas Hlth. Sci. Ctr., Ft Worth, TX
Session Number P262
Session Title: Rat and mouse Toxins and Behavioral Models

Websites:

We will post publications and conference papers on our website: www.salvatorelab.net

Other products:

The comparative sparing of nigral, but not striatal, TH during lesion progression following 6-OHDA lesion induction to the nigrostriatal pathway represents an opportunity to potentially stave off further loss of the DA neuron phenotype. In this project, we will investigate exercise as one possible approach

7. Participants & Other Collaborating Organizations

Graduate Assistant:

Ella A. Kasanga. 12 person months, Salvatore (PI and mentor) Through April 30, 2021.
Contribution to project: study planning and execution, locomotor assessment, animal surgery, exercise schedule and execution, neurochemical analysis, analysis and reporting of results.

Isabel Soto. 6 person months. Salvatore (PI and co-mentor with Dr. Vicki Nejtek)
Contribution to project: study planning and execution, locomotor assessment, cognitive assessment, neurochemical analysis, analysis and reporting of results.

Post-doctoral fellow: Dr. Ella A. Kasanga, PhD. May 17, 2021-August 31, 2021 Contribution to project: study planning and execution. Provided training for locomotor assessment, animal surgery, neurochemical analysis, analysis and reporting of results.

Research Assistants: 12 person months each (except Doshier (3 person months)): Marla Shifflet (through August 31, 2021), Christopher McElroy (through April 1, 2021), Robert McManus (Start date July 19, 2021-current), Walter Navarette (Start date August 2, 2021-current), Kirby Doshier (part-time, start date July 2021-current).

Medical Students: Caleb Parry, Joshia John

Contributions to project: Shifflet, McElroy, McManus, Navarette, Doshier: HPLC, RT-PCR, animal exercise and surgery, locomotor assessment, neurochemical analysis

Consultant: Dr. Vicki Nejtek. Human Parkinson's disease relevant observations to motor and cognitive function similarities in rat models.

Changes in active support of PI or key personnel: Nothing to report

Partner organizations:

Collaborators:

1) Binghamton University
Binghamton, New York

Dr. Christopher Bishop, Co-investigator, 1 month

Ashley Centner, Graduate Assistant, 12 months

2) Florida International University
Miami, Florida

Dr. Jason Richardson, Co-Investigator, 1 month
Yoonhee Han, postdoctoral fellow, 1 month

8. Quad chart attached with report.

Increasing nigral tyrosine hydroxylase expression as a mechanism of exercise-mediated recovery: Evaluation in toxin and rat Parkinson's disease genetic models
Log number: PD180098
Award number: W81XWH1910757

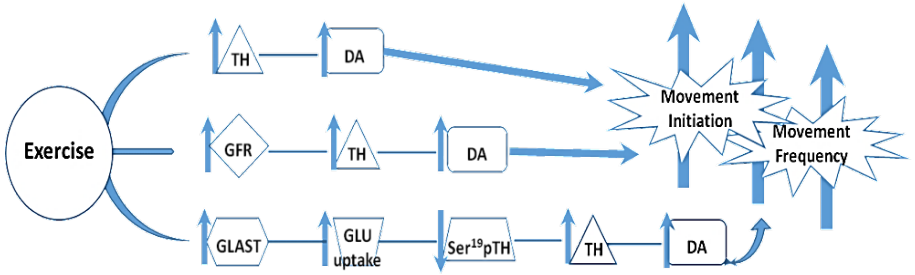
PI: Michael F. Salvatore **Org:** University of North Texas Health Science Center **Award Amount:** 1,491,860

Study/Product Aim(s)

- **Aim 1. Nigral TH expression affects exercise efficacy to attenuate or restore motor impairment.**
- **Aim 2. Increased GFR α1 increases TH expression and motor recovery in the SN following exercise.**
- **Aim 3. Increased GLAST expression following exercise prevents TH loss to increase exercise-related motor recovery.**

Approach

Completion of these studies will demonstrate that TH expression, specifically in the SN, affects DA neurotransmission locally therein to play a role in locomotor function in response to exercise. Such results would indicate that preservation of remaining TH in the SN in the PD patient could be targeted to improve motor function. Identification of GFR α1 and GLAST as upstream regulators of TH expression would provide new insight into understand how TH is regulated during nigrostriatal neuron loss, how exercise affects TH expression, and identify new targets for TH preservation or possible restoration in PD patients.



Accomplishment: Using an exercise regimen tethered to the physical capabilities shown by human PD patients that exercise, our new results show that this regimen exercise produced a highly significant recovery from nigrostriatal neuron loss-related motor deficits in an established rat Parkinson's disease model.

Timeline and Cost

Activities CY	19	20	21	22
Nigral TH targeting and DA analysis of exercise impact in 6-OHDA rat				
Nigral TH targeting and DA analysis of exercise impact in Pink 1 KO rat				
Targeting GFR role in exercise impact				
Targeting GLAST role in exercise impact)				
Estimated Budget ($K)	**$445K**	**$455**	**$455**	

Updated: October 1, 2021

CY19-21 Milestones – Establish exercise impact using human Parkinson's patient exercise frequency (3 times/week) as metric.

1) Collaborative exercise studies with Bishop and Richardson Labs are underway to measure extracellular DA contemporaneously in substantia nigra and striatum and assess TH cell loss vs astrocytic markers in the nigra.

2) Established rationale for timing of exercise intervention following experimental outcomes using the 6-OHDA and Pink1 knockout models

3) Established Pink1 knockout rat does exercise with compliance

CY21-22 Goals – Continue collaborative exercise studies with Bishop and Richardson Labs. Evaluate impact of exercise on lesioned and contralateral to lesion SN and striatum on TH cell loss, astrocytic markers, GFR and GLAST expression, DA and DA turnover, and TH phosphorylation in 6-OHDA and Pink1 knockout rats..

- Start mechanistic studies to target substantia nigra as center of motor impact by modulation of DA signaling within nigra

Comments/Challenges/Issues/Concerns: Lab shutdown for 3 months due to COVID.

Budget Expenditure to Date: August 31, 2021
Projected Expenditure: $994,573
Actual Expenditure: $953,317