AWARD NUMBER: W81XWH-21-1-0777

TITLE: Dopamine D3 Agonists: Developing Treatments for Sexual Dysfunction in Chronic Spinal Cord-Injured Male Rats

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CONTRACTING ORGANIZATION: Kent State University, Kent, OH

REPORT DATE: September 2023

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2023	Annual	15Aug2022-14Aug2023

4. TITLE AND SUBTITLE

Dopamine D3 Agonists: Developing Treatments for Sexual Dysfunction in Chronic Spinal Cord-Injured Male Rats

5a. CONTRACT NUMBER
W81XWH-21-1-0777

5b. GRANT NUMBER
SC200285

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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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

Spinal cord injury (SCI) and resulting dysfunction is a major health concern for military men and has immense impact on quality of life for the patients and their families. Genitourinary dysfunction is extremely common among men with SCI. This dysfunction greatly hinders quality of life and emotional well-being and is thus of great concern to military men. Surveys of men with SCI have demonstrated that regaining sexual function is an important goal, even surpassing that for recovery of walking among paraplegic men. This is of particular relevance to the military as combat-related spinal trauma is the highest in American military history and the average age of spinal casualties is 26-27 years, hence issues impacting quality of life are of great importance. Thus, the development of novel, innovative interventions is of significant relevance for patients in both the military and civilian populations. Despite the identified need and desire to improve sexual function among SCI men, we have a poor understanding of the mechanisms by which chronic injury so radically influences the spinal ejaculation generator. The current proposal aims to fill that gap: studies detailed in this proposal will test the functional benefits of a novel treatment approach for ejaculatory dysfunction. Secondly, studies are proposed to move towards an improved understanding of the effects of chronic spinal injury on the overlapping neural circuits that regulate the ejaculation reflex. Together, these studies will be beneficial in developing treatments to restore sexual function and fertility to SCI men and importantly improve their overall emotional-well-being and quality of life. Since military men suffer SCI at a relatively young age, potential for treatments of these secondary dysfunctions and thus improving quality of life is highly relevant and impactful.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

Spinal cord injury (SCI) is a devastating clinical injury with extreme cost and burden on patients and their families. Following initial stabilization after spinal cord injury, quality of life issues become increasingly important to patients. In particular, genitourinary dysfunctions are extremely common among SCI patients, including sexual dysfunction. Surveys of men with SCI have demonstrated that regaining sexual function is an important goal, even surpassing that for recovery of walking among paraplegic men. Unfortunately, treatment strategies to overcome genitourinary dysfunctions in SCI men are limited and invasive, and development of non-invasive treatments has been impaired due to poor understanding of the mechanisms by which chronic injury so radically influences the spinal reflex pathways. Moreover, ejaculatory dysfunction is of particular relevance to military patients, as combat-related spinal trauma is the highest in American military history and military men suffer SCI at a relatively young age. Hence, potential for treatments of these secondary dysfunctions and thus improving quality of life is highly relevant and impactful. Of particular relevance to the patient population and to this proposal is that preclinical and clinical studies that determine benefits of a potential treatment for sexual function simultaneously are sparse. The current proposal aims to fill that gap: studies detailed in this proposal will test the functional benefits of a novel treatment approach for ejaculatory dysfunction, using an established preclinical model and a pharmacological treatment that is already well-established for other clinical use. Secondly, studies are proposed to move towards an improved understanding of the impacts of chronic spinal injury on the neural circuits that regulate the ejaculation reflex. Such studies are needed to further advance the proposed treatment options in preclinical models towards clinical treatments.

2. Keywords

Spinal cord injury, sexual dysfunction, anejaculation, dopamine, contusion injury

3. Accomplishments

Major Goals of the Project:
Major Task 1: Study Regulatory Set-up
Major Task 2 (Specific Aim 1A): To test the hypothesis that acute treatment with D3 agonist pramipexole restores ejaculatory function following SCI.
Major Task 3 (Specific Aim 1b): To test the hypothesis that chronic treatment with D3 agonist pramipexole restores ejaculatory function following SCI.
Major Task 4 (Specific Aim 2): To examine localization of D3 receptors in and dopaminergic innervation of neurons controlling ejaculatory reflexes, and effects of SCI on dopaminergic transmission.

Accomplishments under these goals:
Major Task 1: Study Regulatory Set-up:
Subtask 1.1: Submit documents to the Kent State University IACUC and obtain approval.
Progress: Documents were submitted, and approval was received in February 2023. Complete,

Subtask 1.2: Submit animal protocol to DoD's ACURO and make revisions as necessary.
Progress: Documents were submitted in March 2023 (not received due to email server issues), and again in June 2023. ACURO approval was received July 24, 2023. Complete.

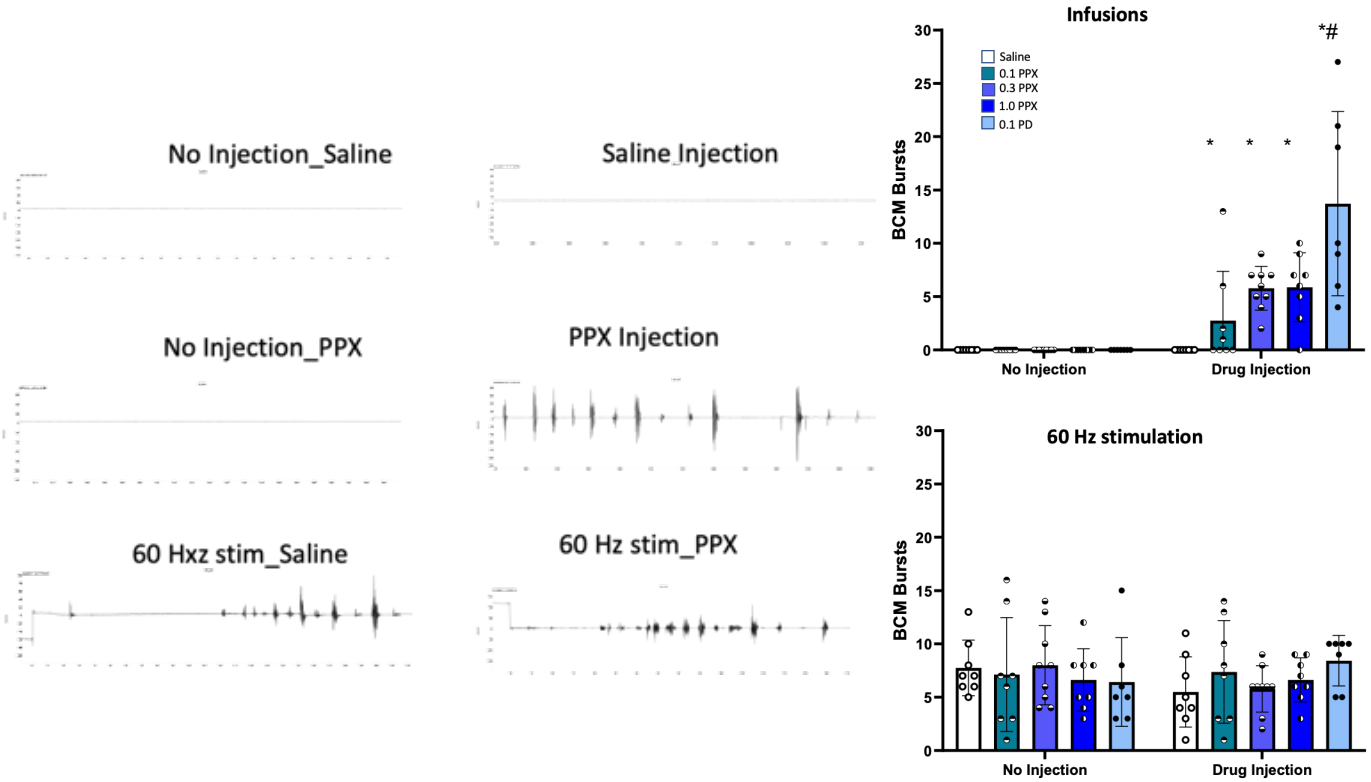
Subtask 1.3: Hire and train study personnel on RNAscope, immunofluorescent staining techniques, confocal microscopy, fluorescent microscopy, animal surgeries, pharmacological and physiological procedures
Progress: No new personnel was hired during the reporting period. Complete.

Major Task 2 (Specific Aim 1A): To test the hypothesis that acute treatment with D3 agonist pramipexole restores ejaculatory function following SCI.
Subtask 2.1: Complete sham and SCI surgeries, measure locomotor activity, and administer the D3 receptor agonist pramipexole at one of three dosages (0.1, 0.3 or 1 mg/kg) or saline vehicle at 2 weeks post-surgery. Sexual reflexes will be examined. This will include 8 groups (12 rats/group) of male Sprague Dawley rats for a total N = 96.
Subtask 2.2: Complete sham and SCI surgeries, measure locomotor activity, and administer the D3 receptor agonist pramipexole at one of three dosages (0.1, 0.3 or 1 mg/kg) or saline vehicle at 6 weeks post-surgery. Sexual reflexes will be examined. This will include 8 groups (12 rats/group) of male Sprague Dawley rats for a total N = 96.
Subtask 2.3: Complete sham and SCI surgeries, measure locomotor activity and administer the D3 receptor agonist pramipexole at one of three dosages (0.1, 0.3 or 1 mg/kg) or saline vehicle at 12 weeks post-surgery. Sexual reflexes will be examined. This will include 8 groups (12 rats/group) of male Sprague Dawley rats for a total N = 96.

Progress: ACURO approval was received July 24, 2023. One replication experiment was conducted in the reporting period that we determined was essential before starting Subtasks 2.1-3: we tested the effects of a single PPX injection in sham control animals. The main purpose of the study was to replicate our previously published study using D2/3 agonist 7-OH-DPAT but using Pramipexole (PPX). Thus, even though this replication study was not described in the Subtasks, it was instrumental to start Major Task 2.

Hypothesis was tested that PPX can facilitate ejaculatory reflexes in sham control animals (n=41) after isolation of the spinal ejaculation generator (SEG) from any remaining supraspinal influences by an acute and complete spinal transection immediately prior to testing. All animals were anesthetized using urethane (1.5 g/kg). All animals were then subjected to two testing Trials. Trial 1: Baseline/No drug trial; BCM EMG activity recorded for 15 minutes in all animals. 5 minutes later, dorsal penile nerve (DPN) was stimulated at 30 and 60 Hz, frequency that triggers ejaculation in sham control males after isolation of the SEG. Trial 2: Drug trial; Animals were divided into 5 groups (n=7-9) and received either saline, one of three dosages of Pramipexole (PPX; D3 agonist with minimal D2 action; 1, 0.3, 0.1 mg/kg s.c.) or PD 128907 (D2/3 agonist; 0.1 mg/kg, s.c.) and BCM EMG activity were recorded. Subsequently, DPN stimulations were again administered and BCM EMG recorded BCM EMG activity were analyzed for numbers of EMG bursts, spikes, and latency to first burst. Data were analyzed using LabChart 8 and statistical comparisons were conducted using Prism 9 for 2-way ANOVA and Holms Sidak pairwise comparisons.

Results (See Figure) showed that PPX infusions triggered BCM bursting (indicator of ejaculation) in a majority of male rats, compared to saline-negative control, and with the highest responses in animals receiving 0.3 or 1 mg/kg dosages. Saline had no effect as expected (negative control) and PD 128907 infusions triggered an even greater response (significantly higher than all PPX groups), suggesting involvement of both D3 and D2 receptors in SEG function. PPX nor PD 128907 had effects on BCM bursting triggered by the subsequent DPN stimulation (60 Hz stimulation) as all groups responded equally. This study thus replicated our previous findings using 7-OH-DPAT (Kozyrev et al, J. Neurotrauma 2016) and forms the basis for the studies in Subtasks 2.1-2.3. See Figure next page.



Currently in Progress: Subtask 2-2 was started during the reporting period and completed during the first 2 months of the third reporting period. Detailed report of results will be included in the Annual report for the third reporting year, but I will add here that we saw significant improvement of ejaculatory reflexes with 1.0 mg/kg PPX in SCI rats tested at 6 weeks after injury, and without any further removal of remaining supraspinal inputs to the SEG. Major Task 2 is expected to be completed during the third reporting period.

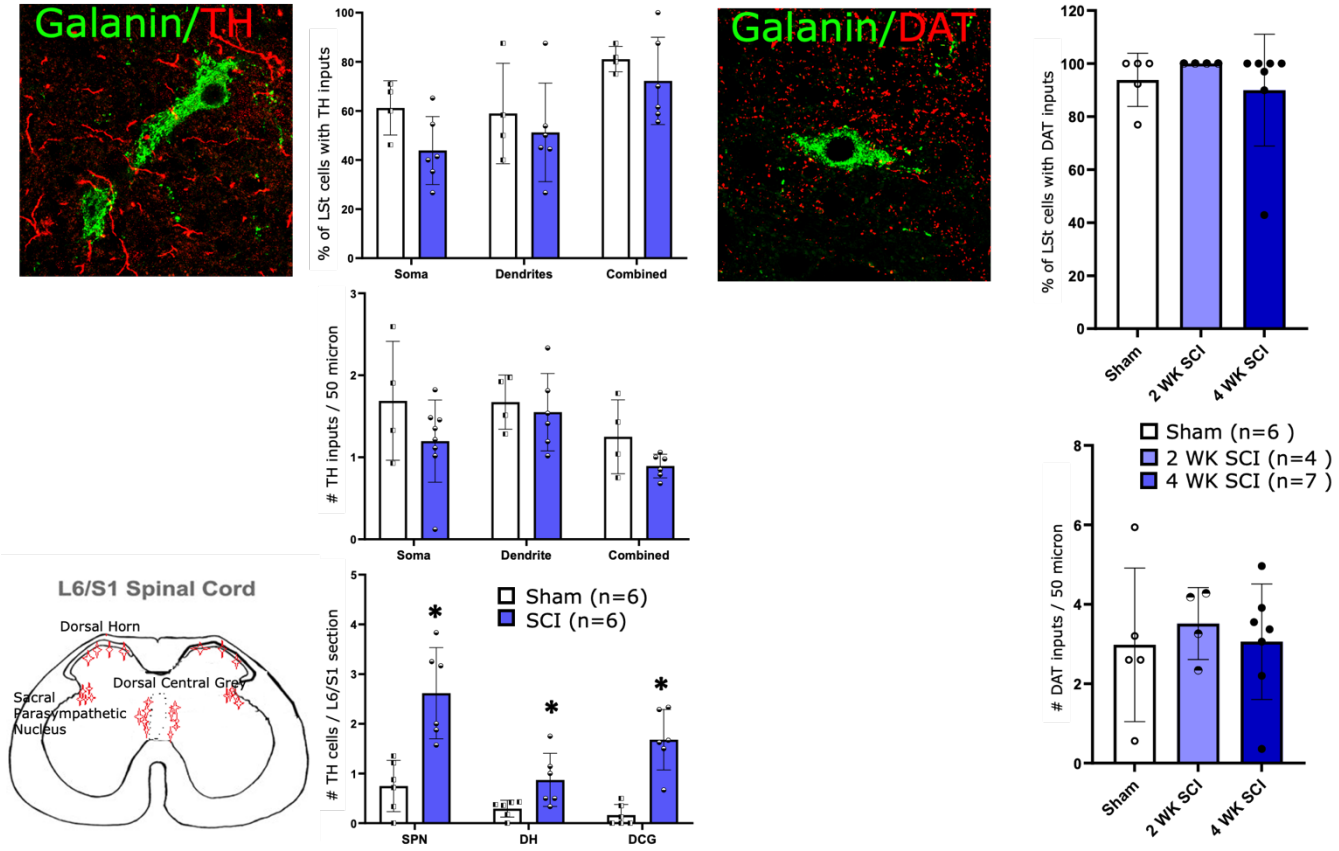
Major Task 3 (Specific Aim 1b): To test the hypothesis that chronic treatment with D3 agonist pramipexole restores ejaculatory function following SCI.
Progress: These studies are planned for third budget year and are currently in progress.

Major Task 4 (Specific Aim 2): *To examine localization of D3 receptors in and dopaminergic innervation of neurons controlling ejaculatory reflexes, and effects of SCI on dopaminergic transmission.*
Subtask 4.1: Complete sham and SCI surgery at T5-6 levels and postoperative care. Determine locomotor activity weekly. At 2, 6, or 12 weeks after sham or SCI surgeries, perfuse animals with 4% paraformaldehyde and collect spinal cords, brains, and sperm. 6 groups of rats will be included, each containing N=8 rats; hence N=48 animals are included in Aim 2.
Progress: Subtask 4.1 surgeries are planned for the third reporting year.

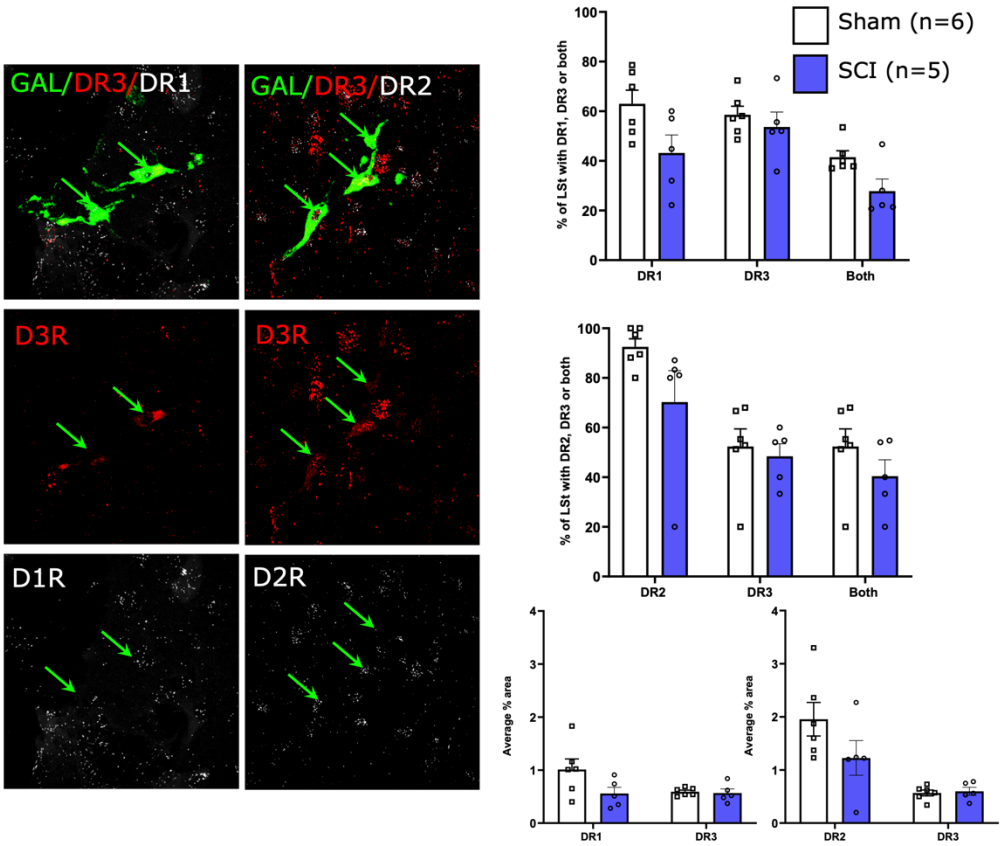
Subtask 4.2: Section Spinal cords coronally at 14 µm using cryostat and collected in parralel series on microscope slides. Brains and sperm are stored for future experiments.
Subtask 4.3: Process spinal cord sections for immunofluorescence for TH (Mouse anti-TH; Millipore MAB5280) or DAT (Mouse anti-DAT; Millipore MAB369); in combination with galanin (marker for LSt cells; Rabbit anti Galanin; Peninsula Labs T4334) or ChAT (Sheep anti-ChAT; Millipore AB1582); and together with synaptophysin (Rabbit anti-synaptophysin; Invitrogen MA5-14532) using Dylight 488, 550 and 630-conjugated secondary antibodies and tissue sample amplification reagents.
Subtask 4.4. Capture confocal and fluorescent microscope images and contact image analyses.
Subtask 4.5: Process spinal cord sections will be processed for FISH using RNAscope probes for rat *D3*, *galanin* and *ChAT*.
Subtask 4.6. Capture confocal and fluorescent microscope images and contact image analyses.

Progress: Even though the surgeries in Subtask 4.1 are scheduled for the third reporting year, progress towards subtasks 4.3-4.4 and 4.5-4.6 was made using tissues that had already been collected in a similar manner as described and for prior studies not related to this award.

Progress Subtask 4.3: Process spinal cord sections for immunofluorescence.
Progress: Spinal cord sections that were stored from a prior experiment including Sham and Spinal cord injured (SCI) male rats, with identical treatment to what is proposed in this award (weeks after mid-thoracic contusion injury), and collected at 1, 2, and 4 weeks after injury. These spinal cords hadn't been collected RNAse-free and thus couldn't be used for RNAscope (Subtask 4.5). Therefore, tissues were sectioned for immunofluorescence using freezing microtome (35 µm coronal sections) and immunoprocessed for TH (tyrosine hydroxylase) or DAT (dopamine transporter) and galanin (LSt cell marker). Images were captured using Confocal microscope and putative dopaminergic contacts onto LSt soma and dendrites were analyzed. Analysis is currently still in progress, but findings thus far show that a majority of LSt cells receive doaminergic inputs on soma and/or dendrites containing TH (4 weeks after injury) or DAT (2 or 4 weeks after injury). Moreover, SCI didn't affect the dopaminergic inputs to LSt cells, at 2 or 4 weeks after injury. However, SCI increased expression of TH specifcially in neurons in L6/S1 spinal levels as more TH-immunoreactive cells were observed in the sacral parasympathetic nucleus, dorsal central grey and dorsal horn at the L6/S1 spinal level. TH-immunoreacitve cells were not noted in any other spinal level and DAT-immunoreactive neurons were not observed in any spinal level including L6/S1. See Figure below.



Progress Subtask 4.5: Process spinal cord sections for FISH.
Spinal cord sections (14 µm coronal sections) that were stored from a prior experiment including Sham and Spinal cord injured (SCI) male rats, with identical treatment to what is proposed in this award (4-5 weeks after mid-thoracic contusion injury), were processed using RNAscope for dopamine 3, dopamine 2, and dopamine 1 receptors together with galanin (marker for spinal ejaculation generator cells, which are referred to as lumbar spinothalamic or LSt cells). Confocal analysis and Fiji image analyses were conducted (Sham n=6; SCI n=5). Approximately 60% of LSt express D3 receptors, 70-90% of LSt express D2 and 40-60% of LSt express D1 receptors, while approximately 50% of LSt cells express more than one of the dopamine receptor subtypes. Hence, dopamine can influence LSt cells via different receptors and modes of release to either activate or inhibit LSt cell activity. No effects of SCI were detected on percentages of LSt cells expressing receptors, nor on dopamine receptor expression within LSt cells. See Figure next page.



Currently in Progress:

1. RNAscope analysis for rat *dopamine receptors* and *ChAT*: This part of the study will be completed in the third budget year and will include D3, D2, and D1 receptor analysis.
2. Immunostaining for D3 receptors to determine the cellular localization within LSt cells (i.e. post- or presynaptic labeling). This is a new experiment to be completed in the third budget year. However, finding reliable antibodies to visualize D3 receptors in spinal cord has been very challenging.
3. Analysis for immunolabeling of TH inputs to LSt cells at 1 and 4 weeks after SCI is currently ongoing.

Note: If these in progress experiments confirm the data reported above and again show that SCI had no effect on any of the outcome measures (receptor expression or dopamine inputs) at the included time points after injury, we will not conduct further SCI surgeries as outlined in Subtask 4.1 to include a 12-week timepoint and instead report on the data shown above and thus complete Major Task 4 using these previously collected tissues.

Opportunities for training and professional development

Training and development were offered to PhD student and Research Technician, including attendance of National Neurotrauma Symposium and Society for Neuroscience conference, local annual symposium of the Brain Health Research Institute at Kent State University, and numerous webinars and training modules offered virtually.

Dissemination of results:

Dr. Coolen has delivered several oral presentations including at the Neurotrauma Symposium of the National Neurotrauma Society in June 2023 and has presented preliminary findings of the studies included in this project.

Plans during next reporting period:

Subtask 2.2: Complete sham and SCI surgeries, measure locomotor activity, and administer the D3 receptor agonist pramipexole at one of three dosages (0.1, 0.3 or 1 mg/kg) or saline vehicle at 6 weeks post-surgery. Sexual reflexes will be examined. This will include 8 groups (12 rats/group) of male Sprague Dawley rats for a total N = 96.

Upon completion of Subtask 2.2, we will then conduct Subtask 2.1 and 2.3.

Subtask 4.1: Complete sham and SCI surgery at T5-6 levels and postoperative care. Determine locomotor activity weekly. At 2, 6, or 12 weeks after sham or SCI surgeries, perfuse animals with 4% paraformaldehyde and collect spinal cords, brains, and sperm. 6 groups of rats will be included, each containing N=8 rats; hence N=48 animals are included in Aim 2.
Subtask 4.2: Section Spinal cords coronally at 14 µm using cryostat and collected in parralel series on microscope slides.
Subtask 4.3: Process spinal cord sections for immunofluorescence for TH, galanin and synaptophysin.
Subtask 4.4. Capture confocal and fluorescent microscope images and contact image analyses.
Please see Note above regarding reporting on our current data instead.

4. Impact

Nothing to report.

5. Changes/Problems

Nothing to report.

6. Products

Abstracts that were submitted during this reporting year, but will be presented at Conferences that take place during the third reporting year:

Thywill Ettey, Rachel Rice, and Lique M. Coolen. Dopamine D3 receptor activation of the spinal ejaculation generator facilitates sexual reflexes. BHRI 11th Annual Neuroscience Symposium at Kent State University. October 26-27, 2023. Abstract and Poster # 18

Thywill Ettey, Rachel Rice, and Lique M. Coolen. Dopamine D3 receptor activation of the spinal ejaculation generator facilitates sexual reflexes. Society for neuroscience annual meeting, Washington DC. November 11-15, 2023. Abstract # 11482. Poster # PSTR137.13

7. Participants & Other Collaborating Organizations

Name:	*Lique M Coolen*
Project Role:	*Project Director*
Researcher Identifier (e.g. ORCID ID):	*ORCID ID 0000-0003-2920-1116*
Nearest person month worked:	*1.2*
Contribution to Project:	*Dr. Coolen oversees all aspects of the projects and has supervised and trained the Research Technician and graduate student.*
Funding Support:	*N/A*

Name:	*Rachel Rice*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*12*
Contribution to Project:	*Ms. Rice conducts RNAscope, immunostaining, confocal microscopy and image analysis*
Funding Support:	*N/A*

Name:	*Thywill Ettey*
Project Role:	*PhD Student- Graduate Assistant-Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Ettey conduct pharmacological studies and is involved in all aspects of these studies*
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No changes; Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. Special Reporting Requirements

Nothing to Report.

9. Appendices

None included.