ROTATIONAL BEHAVIOR DURING THE POLE TEST: A NOVEL BEHAVIORAL ASSAY IN A MOUSE MODEL OF PARKINSON'S DISEASE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Rotational Behavior during the Pole Test:
A Novel Behavioral Assay in a Mouse Model of Parkinson's Disease

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Parkinson's disease (PD) is a debilitating disorder that is likely to reach pandemic proportions by the year 2040, and there is no cure. Current treatments are symptomatic in nature and often have deleterious side effects, such as L-dopa-induced dyskinesia. Neuroprotective drugs, however, target the cause of PD, which is neuronal death; these drugs may prevent motor deficits from progressing in patients with PD. The development of neuroprotective drugs, however, requires a reliable and clinically translatable animal model for PD. The classic preclinical model for testing drugs for PD is a rodent model based on intracranial injections of 6-hydroxydopamine (6-OHDA), a neurotoxin that results in the loss of dopaminergic neurons. Injection of 6-OHDA into the dorsolateral striatum of rodents results in retrograde degeneration of dopaminergic axons, resulting in the loss of dopaminergic neuronal cell bodies within the substantia nigra pars compacta (SNc), which is the brain region lost in PD. Unilateral lesioning with 6-OHDA damages only one side of the SNc, and the extent of damage can be quantified with drug-induced rotational assays. These behavioral assays, while effective, require administration of additional drugs to induce

rotations. The use of drugs, specifically apomorphine or amphetamine, to induce rotations in 6-OHDA injected rodents is problematic because of potential drug-drug interactions, especially in the setting of drug discovery. To address this issue, I developed a new behavioral assay that induces rotational behavior during a motor task without the need for additional drugs. I utilized either sham-operated or ovariectomized female mice that were injected with 6-OHDA into the dorsolateral striatum. These mice were subjected to a specialized task in which the mice were required to turn and descend down a 2-foot pole. Three trials per mouse were conducted at 5 time points, which included 2 time points prior to 6-OHDA injection and 3 time points following 6-OHDA injection; each time point was at least one week apart. I quantified several parameters utilizing this assay format, including the time to turn, time to descend, average score of descent, direction of rotations, and number of rotations. Results showed that 6-OHDA significantly increased the number of rotations in both ovariectomized and sham-operated female mice without the use of any additional drug. My data suggest that spontaneous rotations whilst performing a specialized task such as descent down a 2-foot pole are a useful measure for the discovery of preclinical neuroprotective drugs to treat PD.

DEDICATION

As an aspiring neurologist, I would like to dedicate this undergraduate thesis to all of the patients I will treat in the future, as well as their families.

ACKNOWLEDGMENTS

First, I would like to give glory to God for helping me to reach this milestone in undergraduate research; completion of this thesis is truly an answer to numerous prayers.

I would like to thank my faculty advisor, Dr. Srinivasan, for his guidance, patience, and support throughout this past year as I completed my capstone project. I also want to extend my gratitude to Sara Zarate, Gauri Pandey, Taylor Huntington, and Eric Bancroft, the graduate students who taught me in the lab and helped me with this project. Furthermore, I want to thank all the undergraduate researchers who worked in the lab alongside me, especially Doja Qaraqe, Alan Gonzalez, Sunanda Chilukuri, and Kayla Williams.

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Finally, special thanks to my family and friends for their continuous encouragement, love, and support; I am truly blessed.

NOMENCLATURE

PD	Parkinson's disease
SNc	Substantia nigra pars compacta
6-OHDA	6-hydroxydopamine
DAT	Dopamine transporter
PBS	Phosphate-buffered saline
OCT	Optimum Cutting Temperature
NGS	Normal goat serum
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area

CHAPTER I

INTRODUCTION

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by both motor and non-motor symptoms. Motor symptoms include primary symptoms, such as impaired voluntary motor control, slow movement, tremors at rest, stiffness, frail posture, as well as secondary symptoms, such as shuffling gait, cramped handwriting, weakened gripping, difficulty speaking, and eventually immobility.[1] Non-motor symptoms include depression, anxiety, irritability, difficulty with executive function, dementia, hallucinations, and more.[2] While PD used to be considered a rare disorder, it will likely reach pandemic proportions in the next 20 years; the number of global cases has doubled since 1990 to over 6 million cases, and the number is projected to climb to over 12 million cases by 2040.[3] Given that the cost of caring for a patient with PD in the United States is approximately $22,800 annually, the national economic burden of PD is expected to increase substantially in the next few decades.[4] Despite its discovery in 1817, PD is still largely untreatable.[3]

Pathophysiology

Symptoms for PD result from the gradual loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), a brain region located within the midbrain that is crucially involved in motor function.[5] The aggregation of alpha-synuclein proteins within the SNc, known as Lewy bodies, is also associated with PD; the neurotoxic buildup interrupts neuronal functioning.[5] While the exact biochemical mechanisms behind PD remain unknown, there are multiple suspected causes. Some genetic mutations have been tied to PD, including those in the PINK1

gene; mutations in PINK1 have been known to promote aggregation of alpha-synuclein.[5] Additionally, exposure to environmental toxins may also be linked to PD.[5] However, none of these suspected causes alone account for most cases of PD; instead, multiple factors likely interact to collectively result in oxidative stress, neural inflammation, and eventual neurodegeneration.[5,6]

Sex Differences

While being above the age of 65 is one of the most well-known risk factors for developing PD, there is a significant disparity between sexes as well.[7,8] PD affects both men and women, but it is twice as prevalent in men.[9] Male susceptibility to PD is likely enhanced due to biological differences in brain physiology and endocrinological function. The female nigrostriatal pathway has an increased endogenous concentration of dopamine, which translates to better performance in dopamine-related motor and cognitive functioning relative to men.[10] PD is associated with reduced dopamine transmission within the nigrostriatal pathway; thus, naturally higher levels of dopamine in the female brain may be partially responsible for their reduced susceptibility to PD.[8,10] Furthermore, sex hormones may also impact the progression of neurodegenerative diseases. Some research has suggested that estrogen is actually neuroprotective, and can reduce dopaminergic neuron loss, whereas testosterone has a detrimental effect.[8] Understanding the mechanism by which estrogen exerts neuroprotection could allow for the development of neuroprotective drugs to treat PD in humans.

Neuroprotective Drugs

Current treatments do not focus on the underlying causes of PD, but rather target symptoms. Because many symptoms result from reduced dopamine levels within the striatum, dopamine agonists such as L-3,4-dihydroxyphenylalanine (L-dopa) are often administered to

patients.[11] While drugs like L-dopa can be helpful initially, they are associated with harmful side effects; they also tend to lose effectiveness over time.[11]

Alternatively, neuroprotective drugs have become a favored area of research regarding neurodegenerative diseases; these drugs are designed to slow down degeneration of neurons and provide protection against future damage.[12] In patients with PD, neuroprotective drugs may specifically help to reduce cell death in striatal dopaminergic neurons. Currently, there are no neuroprotective drugs available as treatment for PD. Interestingly, nicotine and tobacco usage are inversely correlated with the development of this disease because of associated neuroprotective side effects.[13] Due to the dangerous side effects and the likelihood of addiction, nicotine would cause more harm than benefit as a treatment for PD. Cytisine, which is a smoking-cessation drug with similar structural and functional properties as nicotine, lacks addictive properties and may be a viable neuroprotective treatment option for PD.[14]

Models of Parkinson's Disease

In order to develop better neuroprotective treatments for PD, a biological model that replicates the disease in humans must be developed. The gold standard model for PD is the 6-hydroxydopamine (6-OHDA) rat model, with 6-OHDA administered either unilaterally or bilaterally.[6] 6-OHDA is a neurotoxin that specifically causes cell death in dopaminergic neurons of the substantia nigra pars compacta (SNc), the same area of the brain affected in humans with PD.[6] Using stereotaxic injection, the toxin can be administered to the rodent's striatum, a section of the forebrain integral to the nigrostriatal pathway.[15] In this pathway, dopaminergic neurons in the SNc project to the striatum, releasing dopamine upon firing. When the neurotransmitter is no longer needed in the synapse, it is cleared away using a dopamine transporter (DAT), which returns dopamine to the presynaptic neurons originating in the substantia nigra.[15] Because 6-

OHDA is similar in structure to dopamine, the DAT returns the toxin to the neurons, leading to neuron death in the substantia nigra, as seen in patients with PD.[6] While the nigrostriatal pathway does not initiate movement, it is vital for regulating motor function; thus, when dopaminergic neurons die, affected individuals begin to experience dyskinesia.[5]

While the 6-OHDA model of PD reproduces many aspects of the disease, it tends to cause neuronal death quickly rather than the realistic slow onset of symptoms, and it fails to cause the aggregation of proteins.[16] Alpha-synuclein overexpression has recently become another model for PD. Alpha-synuclein is the primary constituent of Lewy bodies; injecting viral vectors carrying the alpha-synuclein gene into the substantia nigra can lead to the artificial agglutination of the protein in rodents.[16] Overexpression of alpha-synuclein leads to gradual neurodegeneration, at a rate more comparable to that of PD in humans. However, neurodegeneration in the alpha-synuclein model is only moderate compared to that seen in the 6-OHDA models, and motor dysfunction is less apparent as well.[16]

Estrous Cycling and the Role of Estrogen

Ovariectomies can be performed on female mouse models of PD in order to better understand the effect of the absence of estrogen on the development of PD, as seen clinically in men or postmenopausal women. Ovariectomy, or excision of the ovaries, removes the primary source of estrogen in the female mouse; the success of ovariectomies can be determined through vaginal smearing and cytological analysis.[17] The mouse estrous cycle, comparable to the human menstrual cycle, lasts approximately 4 days, and has 4 definitive stages: proestrus, estrus, metestrus, and diestrus.[18] The stage is determined by the predominant cell types present in the female reproductive tract, which is in turn determined by varying levels of sex hormones.[18] During the proestrus stage, estrogen, luteinizing hormone, and follicle-stimulating hormone are

all at their peak concentration; nucleated epithelial cells predominate in vaginal smears at this stage.[19] The subsequent stage is estrus. During estrus, prolactin reaches its peak concentration while all the other hormones drop to low levels; vaginal smears are characterized by showing predominantly cornified epithelial cells.[19] The third stage, metestrus, is characterized by a slight increase in estrogen and progesterone; vaginal smears exhibit nucleated epithelial cells, cornified epithelial cells, and neutrophils.[19] Finally, during diestrus, estrogen levels are at their lowest concentration whereas progesterone levels are at their highest concentration; neutrophils predominate in vaginal smears at this stage.[19]

Behavioral Assays

Behavioral assays are a quantitative method to ensure the validity of a given model. While no model has consistently demonstrated all of the motor and non-motor symptoms of PD in humans, various tests can be used to measure the range of symptoms that are present in models.[20]

Tests such as the open field test and apomorphine-induced rotations test allow for observation of general activity, locomotion, as well as anxiety or stress levels. The apomorphine-induced rotations test in particular highlights the damage done to the nigrostriatal pathway in rodents with unilateral lesioning.[21] Intraperitoneal injections of apomorphine or amphetamine, a dopamine agonist and synergist, respectively, will cause heightened activity in the dopaminergic neurons of the nigrostriatal pathway. If one side of the brain has been lesioned with 6-OHDA, unbalanced dopamine release will occur, resulting in one-sided movement, or rotational behavior; a greater degree of damage on one side will result in an increased amount of rotation.[22] The open field test involves placing the mouse in an arena with a designated inner zone; the mouse is then monitored for its total distance traveled, average speed, and the amount of times it

enters into and remains in the inner zone.[23] This test allows for measurement of both motor and non-motor characteristics, including locomotor ability and anxiety levels.[23]

Tests such as the pole test, cylinder test, challenging beam test, and grip strength test allow for verification of motor symptoms in PD models. In the pole test, the mouse must turn down and descend a 2-ft pole; this test is used to measure motor coordination and bradykinesia in the mice.[24] The cylinder test is one in which the mouse is placed in a glass cylinder and removed after rearing up to touch the sides of the cylinder 20 times. This test verifies whether the mouse exhibits asymmetry between the use of their limbs when touching the glass.[24] During the challenging beam test, the mouse must traverse an increasingly narrow beam, which is covered with a raised mesh grid; motor coordination is measured in this test based on the mouse's speed, head orientation, and the number of times a foot slips through the grid.[24] Lastly, the grip strength test is used to determine the extent of muscle weakness in one limb compared to the other, which again reflects the extent of damage to the nigrostriatal pathway on one side of the brain.[25] Given that the 6-OHDA model exhibits a clinically translatable amount of neurodegeneration, verified through measured motor and non-motor deficits, it can be utilized as a model for PD in humans. This provides the necessary foundation for therapeutic testing.

Objectives

My primary objective was to develop a mouse model of Parkinson's disease using unilateral stereotaxic striatal injections of 6-OHDA, then perform ovariectomies on half of the mice to determine the effect of estrogen, or lack thereof. With these animal models, I then conducted various behavioral assays on the mice to determine a new method of quantifying PD-related pathophysiology in mouse models. In my study, I developed a novel assay based on spontaneous rotational behavior that occurs as the mouse descends down a pole. This new assay

provides several advantages, as it provides simultaneous measurements of motor coordination and dopamine release without the need for injection of additional drugs.

CHAPTER II

METHODS

Experimental Timeline and Overview

This project took place over the course of 6 weeks, using a cohort of 9 female mice. Out of these, 5 females were ovariectomized and 4 females received sham surgeries. Sham surgeries and ovariectomies took place on the third day of week 1. Additionally, all of the mice received intrastriatal injections of 6-OHDA on the fourth day of week 3. Behavioral training took place one day before behavioral testing throughout the project timeline. Behavioral testing occurred on the third day of week 1 prior to ovariectomies and sham surgeries, the third day of week 2, the third day each of week 4, 5, and 6; the last three days of behavior testing occurred after intrastriatal injections of 6-OHDA. Vaginal smears were collected every day throughout the experiment timeline, beginning on the first day of week 1. The mice were sacrificed after behavioral testing ended on the third day of week 6. Figure 1 depicts the timeline details for behavioral testing, ovariectomies, sham surgeries, and 6-OHDA injections. Behavior tests that were performed included the novel object recognition test, apomorphine rotations, cylinder test, ladder test, and pole test. For the purposes of this capstone project, the pole test will be the only behavioral protocol explained.

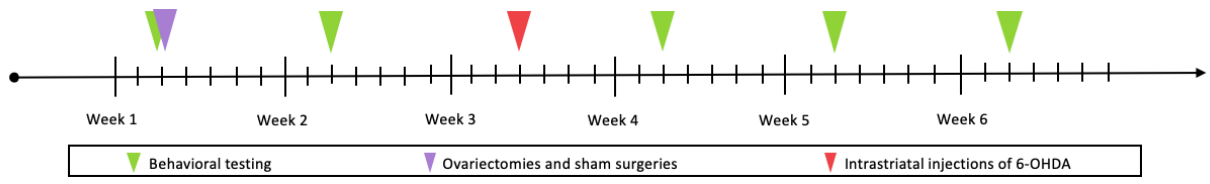

Figure 1. Experiment Timeline

Chemicals and Materials

Nine female CL57BL/6 mice were utilized in this experiment; they were maintained in the Medical Research and Education Building at the Texas A&M Health Science center in the vivarium. Mouse cages included a wire rack for holding food and a water dispenser bottle. Both ovariectomies and sham surgeries required isoflurane and 100% oxygen for anesthetization. Mice were weighed on a scale, and clippers were used to shave fur around the incision site. During surgery, iodine, 70% ethanol, a scalpel, cotton swabs, and eye lubricant were utilized. After surgery, mice were placed in their home cage on a heating pad, and given peanut butter and water.

Vaginal smearing required 0.9% saline, 70% ethanol, and double-distilled water; the saline was purified through a filter prior to beginning. Three Falcon 50 mL tubes were utilized to hold the saline, ethanol, and waste during the smearing process. A one-mL eyedropper was used to inject saline and collect cells. Smear samples were collected using a 48-well cell plate. Samples were viewed on a 25x75 mm glass slide and stained with crystal violet stain. Glycerol was used to cover the smear, and a 24 x 60 mm coverslip was placed on top. An Olympus CK2 light microscope was used to visualize vaginal smear cell morphology.

For stereotaxic injection of 6-OHDA, mice were anesthetized with isoflurane administered through a Kent Scientific SomnoSuite Low Flow Anesthesia System. For surgery, clippers, a scalpel, rat-toothed forceps, scissors, and eye lubricant were also used. Lesions were made using a Kopf Instruments stereotaxic apparatus. 10 μg of 6-OHDA in 0.9% saline and 0.2% ascorbic acid was administered via glass-pulled pipettes.

The pole test utilized a pole comprised of a rod that was 2 feet long and 0.5 inches in diameter. A 3-inch x 3-inch piece of cardboard was screwed to the top of the rod. The base plate

was approximately 8 inches long, 4.5 inches wide, and ¾ of an inch tall. Wood shavings were used for bedding, and all were placed in the bottom half of a rat cage. Rescue animal cleaner was used to clean the pole and rat cage. A 4K Ultra-HD digital video camera was used to record behavior on test days, and video recordings were stored on a 32-gigabyte or 64-gigabyte SanDisk memory card.

For transcardial perfusion, isoflurane inhalant was used to deeply anesthetize the mice. The perfusion took place in a fume hood; a scalpel, scissors, and 24-gauge injection needle were used. Gibco phosphate-buffered saline (PBS) and 10% formalin were injected. After perfusion, a spatula, VWR 15 mL tube, and 10% formalin, and 30% sucrose were used for brain tissue storage.

Brain slices were obtained using a Microm HM 550 cryostat, a single-edge razor blade, and Fisher Healthcare optimum cutting temperature (OCT) embedding medium. Slices were stored in 1% sodium azide in a 24-well cell plate.

The immunofluorescence staining process required a 24-well cell plate, Gibco PBS, a Thermo Scientific MaxQ 2506 Lab Shaker, small paintbrushes, normal goat serum (NGS), Sigma Triton X-100, and both primary and secondary antibodies. The primary antibody used was chicken anti-tyrosine hydroxylase, diluted 1:1500. The secondary antibody used was goat anti-chicken 594, diluted 1:2000. Slices were mounted on a 25x75 mm glass slide using fluoromount and a 24 x 60 mm coverslip; the edges were sealed with nail polish. Slides were imaged using an Olympus confocal scanning laser microscope.

Ovariectomy and Sham Surgery Procedures

Ovariectomy Procedure

Five female mice underwent ovariectomies. The mice were weighed immediately prior to surgery. Mice were then anesthetized with isoflurane in an induction chamber; they were removed when breathing slowed down to one deep breath per second. Fur on the back was shaved with a clipper. During surgery, mice remained anesthetized using vaporized isoflurane mixed with 100% oxygen. Mice were placed in the prone position, and two 1-cm incisions were made parallel to each side of the lumbar vertebrae. A 1-cm incision was made just behind the peritoneum on the erector spinae muscle to reach the ovaries. On each side, uterine blood vessels distal to the ovaries were ligated. Then part of the uterine horns, the ovaries, the oviducts and the surrounding fat were all excised. The incision was then closed and the mouse returned to its home cage. Mice were given peanut butter and water daily in small Petri dishes following surgery.

Sham Surgery Procedure

Four female mice underwent sham surgeries. The same protocol in the Ovariectomy Procedure section above was followed, but uterine vessels, ovaries, oviducts, uterine horns, and surrounding fat remained intact for each mouse. Then the incision was reclosed, and the mouse was returned to its home cage. The mice were also supplemented with peanut butter and water.

Vaginal Smear Protocol

To determine the estrous cycle phases occurring in both the ovariectomized and sham-operated mice, vaginal smears were collected daily during the experiment. Prior to collecting smears, a 0.9% filtered saline solution was prepared. Three 50 mL beakers were used, one filled with the filtered saline solution, one filled with ethanol, and one left empty for waste disposal. A

48-well cell plate was labeled with identification numbers for each mouse. To begin, the cage was opened with the wire rack left in place. The first mouse was removed from the cage and placed on the wire rack, then grasped by the scruff of the neck by the handler's non-dominant hand to immobilize the arms and head of the mouse. If the mouse urinated, it was allowed to finish and then was cleaned with double-distilled water. An eyedropper with a rubber bulb was filled ¾ full with saline solution, then inserted into the vagina of the mouse. The contents of the eyedropper were released inside the vagina, then sucked back into the eyedropper. This was repeated 4-5 times until enough cells were contained in the saline solution. The eyedropper was removed and the mouse was placed back in the cage. The solution was then dispensed into the appropriate well for that mouse, and the eyedropper was cleaned using filtered saline, 70% ethanol, then filtered saline again. The procedure was repeated for all the female mice.

To visualize cell morphology, a drop of saline containing cells obtained from each mouse was placed on a glass slide and dried at room temperature. The slide was completely covered with crystal violet stain for six minutes, then rinsed with double-distilled water. Excess water was removed, then approximately 15 μL of glycerol was placed on top of the smear and the smear was covered with a glass coverslip. The cells were viewed immediately under a light microscope, and pictures were taken at 10x and 20x magnification.

Stereotaxic Injection of 6-OHDA

Two weeks after ovariectomies and sham surgeries, all 9 mice were stereotaxically unilaterally injected with 6-OHDA. Mice were anesthetized with isoflurane in an induction chamber until they were breathing one deep breath per second. The fur on their head between their ears and nose was shaved with clippers, and they were placed on the stereotaxic apparatus. Mice were continuously anesthetized during surgery using isoflurane. Once immobilized, the

skin of the head was incised and reflected laterally from the midline. A craniotomy was performed 0.8 mm anteroposteriorly, 2.0 mm mediolaterally, and 2.4 mm dorsoventrally. The neurotoxin, which was 10 μg of 6-OHDA in 0.9% saline and 0.2% ascorbic acid, was injected unilaterally into the mouse striatum via glass-pulled pipette at a rate of 750 nL/min. After injection, the needle was carefully removed, and the head was sutured shut. After surgery, mice were placed back in their home cage on a warming pad and supplemented with peanut butter and water.

Pole Test and Analysis

For pole test, the mice received two days of training prior to the first day of behavioral testing, then one day of training prior to each subsequent test day. For both training and testing, the pole used was 2 feet long and 0.5 inches in diameter. There was a cardboard topper and a heavy base plate at the bottom. The pole was placed in a clean rat cage surrounded with bedding.

Training Days

The pole was tipped until almost horizontal, and the mouse was placed on the pole nose-up toward the cardboard topper. When each mouse was placed on the pole, the tail was manually stabilized while the pole was returned to the vertical position. Following release, the mouse had to turn nose-down on the pole and then descend until all four limbs were on the base plate. In cases where the mouse failed to turn within 15 seconds, the mouse was manually turned by gently pulling the tail up toward the cardboard topper. This trial was repeated three times for each mouse. Following each training session, the mouse was returned to its home cage. In between littermates of the same cage, urine and fecal matter was removed from the pole and base plate with a clean cloth, while for mice from different cages, the pole was thoroughly wiped down with rescue animal cleaner.

Testing Days

Prior to testing, the digital video camera was set up on the tripod so that the entire pole test apparatus was visible. Once recording had begun, a sign with the day number, date, cohort number, test, and mouse identification number was passed in front of the camera. With the pole held almost horizontally, the mouse was placed on the pole nose-up toward the cardboard topper. Their tail was manually stabilized until the pole was vertical again. When the pole reached the vertical position and the hand was removed from the pole, then a timer was started. If the mouse turned on its own and descended, then it was placed on the pole again for its second trial. If the mouse failed to turn after 60 seconds, only then would the mouse be guided to turn down by having its tail pulled up toward the top of the pole. Each mouse underwent three trials. Prior to recording the next mouse, a sign with updated information for that mouse was passed in front of the camera. Protocol for cleaning the pole between littermates of the same cage and mice of different cages was the same as training days. After all mice finished testing, the camera recording was stopped.

Analysis

The pole test videos were manually analyzed. For each trial, each mouse was analyzed according to its time to turn down while on the pole, the time it took to descend the pole, and the average score of descent. The start time for each trial began when the mouse was placed vertically in the starting position (nose-up), after the hand was removed from the pole. The time to turn was defined as the amount of time it took from start time for the mouse to turn 180° so it was facing nose-down, after bringing its hind limb around to reposition itself on the pole. See Figure 2.

Figure 2. Example of mouse turning 180° and bringing hind paw around.

The time to descend was recorded as the time after turning until the time the mouse touched the base plate at the bottom of the pole with all four limbs. The score of descent was given based on a scale from 0 to 5, with 5 being the best. The scoring system was as follows: 0 = mouse stayed at top for 60+ seconds; 1 = mouse fell; 2 = mouse descended backwards; 3 = mouse descended sideways; 4 = mouse turned after descending halfway; 5 = mouse turned at top and descended. For each mouse, the data from all three trials were averaged.

Due to observation of spontaneous rotations, particularly after unilateral lesioning with 6-OHDA, the number of rotations for each trial was also retrospectively quantified. One rotation was defined as one 360° turn around the pole, measured only during the time it took for the mouse to descend. The number of rotations recorded was also averaged from the three trials for each mouse.

Sampling and Statistics

OriginLab software was utilized to perform two-way repeated measures ANOVA tests (surgical treatment*time) for significance testing in time to turn, time to descend, average score, and number of rotations for each group of females, ovariectomized or sham-operated. Significance testing was done for the weeks prior to 6-OHDA (week 1 and 2) and the weeks after 6-OHDA (weeks 4, 5, and 6) for each of the four data sets.

Transcardial Perfusion and Brain Slicing

Transcardial Perfusion

After all testing was complete, mice were deeply anesthetized using isoflurane. Then they were placed in the supine position on a platform within a fume hood for transcardial perfusion. An incision was made perpendicular to the midline just below the ribcage. Another incision was made through the skin and ribs from the most lateral aspect of the ventral ribcage toward the clavicle. A second incision was made on the contralateral side through the ribcage so that the front-facing ribcage could be reflected cranially, exposing the entire thoracic cavity. With the heart visible, a needle containing PBS was inserted into the left ventricle, and approximately 1.5 mL of PBS were injected to flush out the circulatory system. The vena cava was then transected to exsanguinate the mouse. Once PBS had been injected, a needle containing 1.5 mL of 10% formalin was inserted into the left ventricle, and its contents were released into the circulatory system. After 10 minutes of formalin profusion, the needle was removed and the mouse was decapitated. A sagittal incision was made through the skin on along the length of the skull and the skin was reflected laterally from the midline. The skull was transected between the eyes and down each side toward the dorsal aspect of the head. The top of the skull was then removed, exposing the brain. The brain was removed from the skull with a spatula and placed in a 15 mL tube filled with 10% formalin; the tube remained in a 4°C cooler overnight. The following day, the brain was transferred to a 30% sucrose solution and stored in a 4°C cooler until needed.

Brain Slicing

The brain was retrieved from its storage site in 30% sucrose and sliced using a cryostat machine set to -24°C. The olfactory bulbs and cerebellum were removed, and the brain was mounted rostral-side up on a chuck with OCT embedding medium. OCT medium was used to

cover the brain. When the brain and OCT medium were fully frozen, the chuck was mounted to the cryostat slicing machine. The slicer was set to cut at a thickness of 100 μm, and the first few slices were used to adjust the brain so it was properly positioned. Then, 100 μm brain slices were collected and placed in a 24-well cell plate. As the slicer approached the midbrain, 40 μm slices were collected and placed in the 24-well cell plate. After the brain slices were collected, the slices were put in 1% sodium azide and stored in a 4°C cooler until needed.

Immunofluorescence Staining and Imaging

Immunofluorescence Staining

For each mouse, 2-4 midbrain slices were selected and transferred via a small paintbrush into a 24-well cell plate. The slices were washed twice with PBS for 5 minutes each on an agitator. Then slices were placed in a blocking and permeabilizing solution consisting of 10% NGS and 0.5% Triton X-100 in PBS. Slices remained in the blocking and permeabilizing solution for one hour, and then they were washed twice more in PBS for 5 minutes with agitation. The slices were incubated in the primary antibody solution, which had 1% NGS, 0.05% Triton X-100, and a chicken anti-tyrosine hydroxylase antibody diluted 1:1500. The brain slices remained in the primary antibody solution overnight with agitation in a 4°C cooler.

The following day, the slices were removed from the primary antibody solution and washed twice with PBS for 5 minutes each with agitation. The slices were then incubated in a secondary antibody solution for one hour; the solution consisted of 1% NGS, 0.05% Triton X-100, and a goat anti-chicken 594 antibody diluted 1:2000. After secondary antibody incubation, the slices were washed twice with PBS for 5 minutes with agitation. The slices were transferred onto glass slides labeled with the date, mouse number, and antibody information. Once dry, the

slices were covered in fluoromount, and a coverslip was placed on top. The slides were left to fully dry, and then sealed with nail polish around the edges.

Imaging

Slides were imaged using confocal scanning laser microscopy. Images were taken at 10x magnification, and tyrosine hydroxylase (TH), a marker for dopaminergic neurons, was tagged and fluorescently highlighted in the images. Images of the substantia nigra pars compacta (SNc) and the adjacent ventral tegmental area (VTA), a part of the brain involved in reward, were taken on both the unlesioned side of the brain and the side lesioned with 6-OHDA. Four images were taken for each brain slice, and the images were spliced together to show a representation of dopaminergic neuron levels within the midbrain.

CHAPTER III

RESULTS

Vaginal Smears

Vaginal smears from both ovariectomized and sham-operated groups of females were observed under a microscope and compared based on the present cell morphologies. Analysis of vaginal smears from sham-operated females revealed that the cell morphology varied on a daily basis, corresponding with sequential phases of estrous cycling in the mice. Figure 3 shows vaginal smears from a female mouse who received a sham surgery and was still undergoing estrous cycling. Image A shows an abundance of nucleated epithelial cells, indicating proestrus. Image B shows an abundance of cornified epithelial cells, indicating estrus. Image C shows a combination of nucleated epithelial cells, cornified epithelial cells, and neutrophils, indicating metestrus. Image D shows an abundance of neutrophils, indicating diestrus.

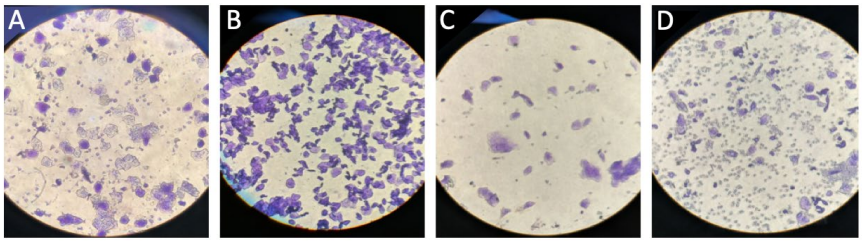

Figure 3. Images of vaginal smears from a sham-operated female over 4 consecutive days.

Vaginal smears from ovariectomized females showed regularity in cell morphology from day to day, as seen in Figure 4. Images E, F, G, and H in Figure 4 all show an abundance of neutrophils, indicating that the mouse was in a prolonged stage of diestrus. Figures 3 and 4 show

a stark contrast in the cell morphologies presented in the ovariectomized females versus the sham-operated females over the length of one estrous cycle.

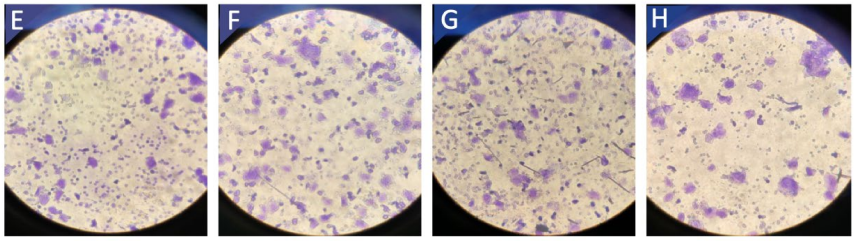

Figure 4. Images of vaginal smears from an ovariectomized female over 4 consecutive days.

One of the mice continued cycling despite removal of the ovaries; all other ovariectomized females showed estrous cycle disruption. The behavioral data for this anomalous mouse were discounted.

Midbrain Staining

Midbrain slices were stained so as to highlight tyrosine hydroxylase (TH) within the substantia nigra pars compacta (SNc) and nearby ventral tegmental area (VTA). TH is an enzyme required for the production of dopamine, thus is more highly concentrated in dopaminergic neurons. The images in Figure 5 were taken from midbrain slices from a sham-operated female and an ovariectomized female. TH is stained bright yellow in the pictures, indicating where dopaminergic neurons and their associated processes lie. The right side of the brains received an intrastriatal injection of 6-OHDA while the left sides of the brain were left unlesioned. As can be observed in Figure 5, the concentration of TH, and thereby dopaminergic neurons, is greatly reduced in the lesioned SNc of both the sham-operated and ovariectomized females. Some neurodegeneration also appears to have occurred in the VTA simply due to its proximity. Comparative quantification of TH concentration in the SNc is pending.

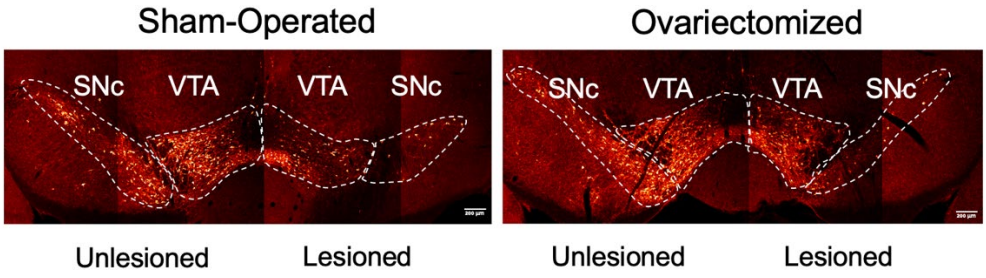

Figure 5. Midbrain slices highlighting unilateral lesioning in both groups of female mice.

Pole Test

Due to anomalous estrous cycling in one ovariectomized female, pole test behavioral data were analyzed for only 8 of the 9 females. Each female completed three trials for each testing day, and testing was conducted once a week for 5 of the 6 total experiment weeks. The mice were analyzed based on their time to turn, time to descend, and quality of descent, and the average of the three trials was recorded for each mouse; results were then separated according to the type of surgery each mouse received. Figure 6 below shows graphical results from the pole test. Blue indicates data from sham-operated females, while red indicates data from ovariectomized females; the green arrow indicates the time point at which all the mice received unilateral intrastriatal injections of 6-OHDA. Figure 6 graph A illustrates the average time to turn for each of the groups of mice over course of the experiment. Figure 6 graph B illustrates the average time to descend for each of the groups of mice over the course of the experiment. Figure 6 graph C indicates the score of descent for each of the groups of mice over the course of the experiment, with 5 indicating the highest possible score.

While no apparent pattern can be seen for either average time to turn or average score, it appears that the average time to descend increased for both groups of females after both types of surgery as well as after receiving injections of 6-OHDA. However, statistical analysis showed

that no significant difference between sham-operated females or ovariectomized females existed either before 6-OHDA or after 6-OHDA for average time to turn, average time to descend, or average score. P-values are indicated on each graph in Figure 6.

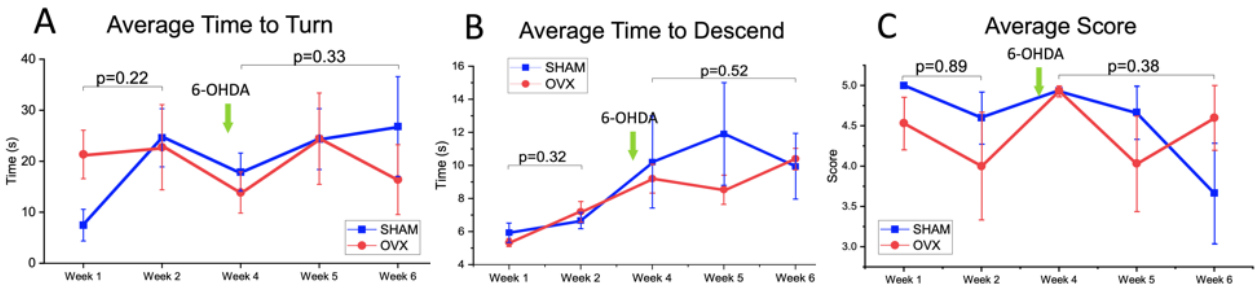

Figure 6. Graphical representation of average time to turn (A), average time to descend (B), and average score of descent (C) for both sham-operated and ovariectomized mice.

Interestingly, while analyzing video recordings of the pole test, spontaneous rotation was observed as the mice descended the pole, particularly after the mice received intrastriatal injections of 6-OHDA. This phenomenon has not been previously described in similar studies utilizing the pole test and 6-OHDA rodent models, so I went back through all the videos and quantified the number of 360° rotations around the pole for each mouse during the time of descent. During the weeks prior to 6-OHDA injections, the average number of rotations was relatively low, collectively less than one turn as the mice descended. However, the number of rotations immediately increased in both groups after receiving 6-OHDA injections; some of the mice rotated around the pole as many as 5 times before reaching the base. Figure 7 below illustrates the increase in rotations observed in the mice. Blue indicates data from sham-operated

females, while red indicates data from ovariectomized females; the green arrow indicates the time point at which all the mice received unilateral intrastriatal injections of 6-OHDA.

In addition to the observable increase in rotations for each group, statistical analysis revealed a significant difference in the average number of rotations of sham-operated and ovariectomized females, with more rotation occurring in ovariectomized females (p=0.006). P-values are also displayed in Figure 7.

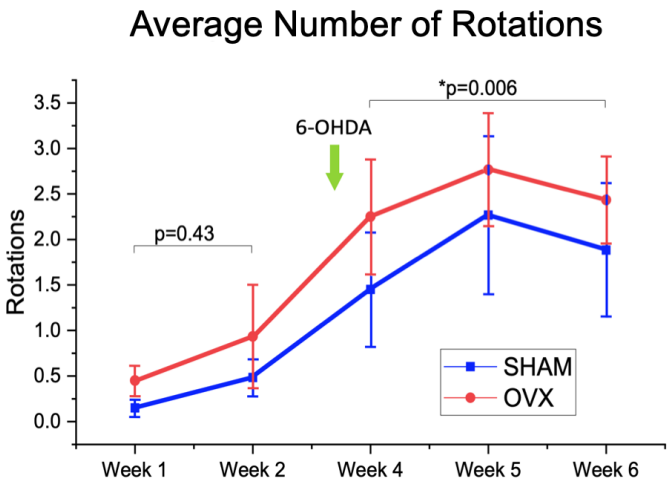

Figure 7. Graphical representation of average number of rotations for both sham-operated and ovariectomized mice.

CHAPTER IV

CONCLUSION

Data Implications

Results from vaginal smearing indicate that ovariectomy interrupts estrous cycling in female mice. Estrous cycling is dependent upon sex hormone fluctuation, similar to menstrual cycling in humans. By removing the primary source of estrogen in our female mice, we impeded their ability to continue cycling. Given this visible change, we were then able to compare sham-operated mice and ovariectomized mice as having estrogen and lacking estrogen, respectively.

Intrastriatal injection of 6-OHDA causes retrograde degeneration of dopaminergic neurons in the SNc. Midbrain slice staining shows that the lesioned side of the brain has qualitatively lower amounts of TH, indicating fewer dopaminergic neurons and less dopamine unilaterally. Such a sizeable dopamine imbalance could be responsible for the spontaneous rotational behavior observed during the pole test. The task requires the mouse to turn its entire body upside down, descend, and dismount the pole; this requires extensive motor regulation, which likely induces dopamine release in the SNc. With a neurotoxin-induced dopamine imbalance in this brain region, motor function will consequently be imbalanced. Accordingly, a substantial dopamine imbalance presents as rotational behavior.

The significantly increased number of rotations seen in ovariectomized female mice supports the belief that estrogen is neuroprotective. The ovariectomized females, who had little to no estrogen, showed a greater degree of rotational behavior; this indicates a greater imbalance in dopaminergic neurons. Assuming these mice had a typical amount of dopaminergic neurons to begin with, the heightened imbalance suggests that they experienced more extensive

neurodegeneration when subjected to intrastriatal injections of 6-OHDA than did their estrogen-intact counterparts.

Because rotational behavior usually requires extensive dopamine release, as achieved using drugs such as apomorphine or amphetamines, the spontaneous rotations observed during the pole test are extraordinary. Results support the viability of the test, indicating that the pole test is successful in causing dopamine release in the SNc. Furthermore, the spontaneous rotational behavior provides an exciting new alternative to drug-induced behavior testing. This is especially advantageous in the realm of drug testing. The pole test not only provides measurements for motor capability, but also an assessment of dopamine release without the need for additional drugs, sparing the concern about drug-drug interactions that may arise in other models.

Future Directions

Having observed a significant increase in rotational behavior in ovariectomized females, the next step is to quantify and compare TH concentrations in both ovariectomized and sham-operated females. If the behavioral imbalance is supported by corresponding TH concentration imbalance, then this would further promote the impression that estrogen has a neuroprotective role.

If this is true, then estrogen may become a valuable component in a neuroprotective drug for PD. Future research will likely focus on the benefit of adding estrogen or estrogen derivatives to PD drugs undergoing testing, such as cytisine. One drawback to using estrogen as a drug is that it naturally causes feminizing effects, or the formation of female secondary sex characteristics, which can be undesirable. However, a non-feminizing derivative of estrogen may provide a useful addition to a therapeutic drug for PD.

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