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Sequence Learning in a 6-OHDA Mouse Model of Parkinson's Disease

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Abstract

Parkinson's disease (PD), a neurodegenerative disorder originating in the dopaminergic cells of the basal ganglia, is characterized by severe motor impairments such as tremors, bradykinesia, and postural instability. However, non-motor symptoms impacting sensory systems and cognition are consistently pointed to as more greatly affecting quality of life for PD patients. Cognitive impairments in PD can include changes in reward processing, depression, apathy, and increases in risk-taking behavior. Additionally, learning and memory deficits are seen in PD, specifically in motor sequence learning. Often, the neural correlates of sequence learning impairments are traced to the substantia nigra. However, there is some evidence in the literature that the ventral tegmental area (VTA) could also play a role in sequence learning deficits. In the present study we investigate the potential role of the VTA in sequence learning using a unilateral 6-OHDA lesion model of PD in mice. In comparison to control animals (n=3), some lesion animals (2 out of n=3) experienced an impairment in performance and rate of learning on a sequence task post-lesion surgery. This impairment also occurred without significant changes in motor skill measured by gait analysis and a cylinder test of forelimb laterality. This indicates that a lesion to the VTA may impact sequence learning without causing motor impairment- pointing to a more direct role of the VTA in sequence learning. This result contributes to our understanding of the neural correlates of cognitive impairments in PD, and could provide a basis for more targeted treatment of PD.

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Literature Review

Parkinson's Disease

Parkinson's disease (PD) is the second-most commonly occurring neurodegenerative disease, with more than ten million people affected worldwide. In the United States, one million people currently living with the disease and an additional 90,000 are diagnosed per year. In the US alone, the healthcare costs associated with PD are estimated around $52 billion per year, with therapeutic treatments costing up to $100,000 per patient (PD Foundation, 2019). In terms of prevalence, PD is more common in men than women, and the way that PD affects men and women differentiates across age ranges. The rate of PD diagnosis increases with age, a primary risk factor, and is most common in adults 60+ years of age (Marras et al, 2018) (Dexter & Jenner, 2013). With such a high burden on patients and caregivers, as well as socioeconomic cost, there is great motivation within the biological and medical fields to research PD and its potential treatments.

PD is a progressive neurodegenerative disorder, meaning that it is associated with the gradual death of neural cells. While many of the cellular mechanisms of PD remain to be fully explained, there are key features of its pathophysiology. PD is characterized by the loss of dopamine-producing cells in the nigra-striatal region, causing dysfunction in the basal ganglia and limbic system. These areas are located centrally in the brain, deep within the cerebral hemispheres. The basal ganglia are composed of the caudate nucleus, putamen, substantia nigra (SN), nucleus accumbens (NAc), globus pallidus (GP), and subthalamic nucleus (STN) (see **Figure 1**). Several limbic structures are implicated

progressively in later stages of Parkinson's disease, including the ventral tegmental area (VTA), hypothalamus, cingulate gyrus, hippocampus, and amygdala (Banwinkler et al, 2022). Dysfunction in these areas of the brain is deeply connected to the mechanisms and roles the dopamine system plays in the brain, leading to the typical Parkinsonian phenotype.

The Dopamine System and PD

Dopamine is a neurotransmitter, a chemical that plays a key role in the passage of electrochemical information from neuron to neuron. Dopamine-producing neurons are centered in the midbrain, in the substantia nigra (SN) and ventral tegmental area (VTA). Parkinson's Disease is associated with the death of dopaminergic cells in the SN pars compacta (SNc) and VTA. As dopaminergic cell death begins in these areas and progresses to other downstream neural regions of the brain, we see specific deficits in PD patients related to the role of dopamine in affected areas.

Dopamine has several roles within the nervous system, ranging from motor control to reward processing. There are three main pathways of the dopamine system that contribute to these functions, including the nigrostriatal, mesolimbic, and mesocortical pathways. The nigrostriatal pathway originates from the SNc, and projects downstream in the basal ganglia to the caudate and putamen nuclei, part of the dorsal striatum. This pathway largely contributes to movement through the 'direct' and 'indirect' dopamine pathways, which help initiate and suppress movement, respectively. In PD, the nigrostriatal pathway is depressed through the loss of dopamine in the SNc. This leads to

hypokinetic dysfunction such as bradykinesia- the characteristic slowness of movements in PD - as well as a resting tremor (Young & Sonne, 2018) (Speranza et al, 2021).

The mesolimbic dopamine system is a more complex pathway, originating in the VTA and sending out several connections to regions such as the prefrontal cortex (PFC), amygdala, cingulate gyrus, hippocampus, nucleus accumbens (NAc), insular cortex, and olfactory bulb (Li & Jasanoff, 2020). The mesolimbic pathway plays a crucial role in reward behavior, specifically controlling motivational behaviors through incentive salience (Berridge, 2007). Projections from the VTA to the NAc are particularly important in this reward function. In terms of other downstream projections, the dopaminergic projections from the VTA to the amygdala and cingulate gyrus play a significant role in affect and emotional processing. The hippocampal DA cells have been shown to play a key role in memory formation and learning through the long-term facilitation of glutamatergic projections and basal ganglia circuitry (Wise, 2004). Notably, the mesolimbic dopamine system is a key mediator of addiction due to its roles in motivational behavior and reward valuation (Evans et al, 2006). In the context of Parkinson's disease, the loss of dopaminergic cells in this pathway can lead to changes in reward processing, including a lack of motivation and altered impulse control. Altered impulse control often leads to impulse control disorder and increased risky decision making in PD patients (Buelow et al, 2014) (Drew et al, 2020) (Haagensen et al, 2020) (Kobayashi et al, 2019). PD results in impaired cognitive flexibility and memory deficits that are linked to the dysfunction of learning and reward circuitry in the mesolimbic dopamine system (Bonito-Oliva et al, 2014) (Costa et al, 2012) (Grospe et al, 2018).

The mesocortical pathway, the third main division of the dopamine system, also originates in the VTA. However, it projects to the PFC, which contributes to complex decision making and executive functioning (Swanson, 1982) (Liu et al, 2019). Dopamine neurons from this pathway interact with several different neural and psychiatric disorders. For example, dopamine deficiency in the PFC is highly associated with schizophrenia, causing key symptoms such as psychosis (Winton-Brown et al, 2014). Additionally, dopamine dysfunction and deficiency are associated with the cognitive effects on attention and processing in ADHD (Leo et al, 2003). In PD, the mesocortical pathway results in several cognitive changes, including increased apathy, depression, and impaired cognitive flexibility (Lawrence et al, 2011) (Müller et al, 2013) (Grospe et al, 2018). Further discussion of cognitive and reward-related impairments in PD can be found in the 'Non-Motor Symptoms of PD' section of the paper.

Parkinson's disease impacts all three of these pathways, as their origins all include either the SNc or the VTA. The diverse functions of dopaminergic neurons in the central nervous system contribute to various manifestations of symptoms in PD, including both motor and non-motor changes in behavior and mental processing.

Motor Symptoms of PD

Much of the literature on Parkinson's disease investigates the motor symptoms of the disease. There are key motoric changes in Parkinson's Disease that are related to the neurodegeneration of the dopamine system. More than 50% of PD patients will develop motor impairments within five years of diagnosis (Bjornestad et al, 2016). Key motor impairments include resting tremors that may lessen when making intentional

movements, as well as bradykinesia and rigidity (Dexter & Jenner, 2013). Progressively, dyskinesia and difficulty with motor control occur. This can cause dysphagia, falls, and instability that is dangerous to those afflicted with PD (Kalia & Lang, 2015). Kalia and Lang (2015) describe the emergence of two motor subtypes in PD- tremor dominant and non-tremor dominant PD. Non-tremor dominant PD manifests as an akinetic-rigid phenotype with postural instability. It is important to note that in PD, postural instability is not a result of vestibular, visual, cerebellar, or proprioception-based deficits (Gibb & Lees, 1988). Non-tremor dominant PD has a higher functional disability and a faster rate of progression than tremor dominant PD (Jankovic et al, 1990).

Motor symptoms can also have differential manifestations based on sex. For example, women are three times as likely to experience motor fluctuations and dyskinesia (Bjornestad et al, 2016) (Bloem et al, 2021). However, it is unclear if this increased risk of dyskinesia is due to average lower body-weight of females or other factors such as genetic predisposition (Bjornestad et al, 2016). Additionally, women are less likely to experience rigidity compared to men but are more likely to develop non-tremor dominant PD (Georgiev et al, 2017). There is also evidence that treatment of PD with dopamine agonists such as levodopa medication can result in sex-dependent motor complications, with men developing less medication-related dyskinesia and less instances of early 'wearing off' of levodopa treatment (Colombo et al, 2015).

Non-Motor Symptoms of PD

While motor symptoms of PD may be more noticeable to the average observer of a PD patient, non-motor symptoms can be equally debilitating. In both humans and

animal models of PD, these symptoms often have an earlier onset than motor symptoms, making them factors in PD diagnosis (Li et al, 2013). Non-motor symptoms such as fatigue, depression, and sensory issues were the most related to reduced scoring on a quality-of-life survey assessment in PD patients. When comparing motor and non-motor deficits, both mental and physical quality of life scores were better explained by non-motor deficits at diagnosis and after 3 years (Müller et al, 2013). Sex-dependent differences in PD also occur in non-motor symptoms. For example, while women are more prone to depression in PD, men experience more overall impairment in cognition (Bloem et al, 2021). The remainder of this section will discuss more specific non-motor symptoms in PD, focusing in on cognitive and reward processing-related changes in PD patients.

There are several affect-related changes observed in PD, including increased apathy and depression. These are closely linked (but not limited to) the dopaminergic system and its various reward correlates. Muhammed et al. (2016) investigated the role that dopamine medication plays with apathy in relation to pupil dilation and saccadic velocity. The authors found that PD patients' apathy score could be predicted by reduced pupillary changes to incentives, indicating that PD patients with apathy have lower sensitivity to monetary reward. Furthermore, when PD patients were off their dopaminergic medication, there was again a reduced pupillary response and saccadic velocity, indicating that medication increased their sensitivity to reward (Muhammed et al, 2016). In support of this analysis of monetary reward, Lawrence et al. (2011) addresses the neural correlates of these changes. PD patients with apathy had reduced PET scan activity reward-related regions of the brain (the VTA, ventromedial PFC,

striatum, amygdala, and midbrain) in response to monetary reward (Lawrence et al, 2011).

Depression is also a common non-motor symptom of PD, with 50% of PD patients also having major depressive disorder (MDD) (Herzallah et al, 2017). Since it has been noted in the past that PD patients with MDD have increased rate of cognitive decline and impairment, researchers have attempted to tease apart if this is due to MDD or PD individually. They find that patients with MDD (both with and without PD) have reduced learning accuracy specifically with positive feedback trials. Non-MDD subjects had normal learning accuracy. Parkinson's patients (with and without MDD) had a slower response time, which initially could be attributed to motor impairments, but mathematical modeling showed that this slowing is more due to PD patients needing more time to accumulate evidence to make a response. This is indicative of impaired decision-making processes as opposed to purely motor effects (Herzallah et al, 2017). Another study by Timmer et al. found that PD patients with a history of depression had impaired reward learning and a lower neural response to unexpected reward, a pattern that did not occur in PD patients without a history of depression (Timmer et al, 2017). Timmer et al. (2017) links depression to impairments in reward learning and correlated brain activity in PD. This study has very similar results to Herzallah et al. (2017), where there was reduced learning accuracy specifically in positive feedback trials for subjects with MDD (with or without PD). Together, these constitute a strong argument for depression as a causal role of reward impairments in PD.

In addition to affective changes, the treatment of PD with dopamine agonists or levodopa can also impact the cognition of patients, increasing risky behavior and

impairing impulse control. Interestingly, several studies have found an interaction between apathy (discussed above) and impulse control disorder (ICD) in PD. Individuals with PD and apathy are more likely to make riskier decisions. Buelow et al. (2014) argues that this is likely due to sensitivity to lower frequency losses and higher short-term gains (but ultimately less beneficial long-term results) in the BART card deck task. Additionally, even when given more learning trials to accumulate information about various rewarding (or not reward) outcomes, PD patients with apathy still chose more disadvantageous outcomes long-term, linking apathy to risky decision making (Buelow et al, 2014). When investigating neural correlates of ICD in PD, an fMRI study shows that dopamine replacement therapy impairs impulse control in patients with PD and ICD by decreasing ventral striatal activity. It also shows that patients with ICD are likely to have baseline reduced functional connectivity between frontal areas and the STN. This provides a neural mechanism to explain how impulse control impairments may arise in patients on dopamine replacement therapy based on changes in basal ganglia reward circuitry (Haagensen et al, 2020).

There are notable impairments in learning, memory, and cognitive flexibility in PD, originating in the mesocortical and mesolimbic dopamine systems. These cognitive impairments can be produced in rodent studies using MPTP and 6-OHDA lesions. Both 6-OHDA and MPTP lesions seem to be effective at modeling PD deficits, but the MPTP lesion does seem to be a more robust and accurate model of early cognitive impacts in PD such as habit learning and memory deficits. 6-OHDA lesions of the substantia nigra are more effective at modeling motor deficits in a 2005 study (Ferro et al, 2005). Nonetheless, 6-OHDA lesions have been used to model cognition-related deficits. In a

2014 paper, Bonito-Oliva et al. investigate how bilateral 6-OHDA lesions impact long-term recognition memory and plasticity in the mouse hippocampus. They find that the lesion does induce long term- but not short term- deficits in recognition memory in a novel object recognition test. Additionally, they find that LTP is decreased following partial dopamine depletion (Bonito-Oliva et al, 2014). It is important to note that these memory deficits are indeed due to dopamine depletion, since levodopa treatment rescues these deficits (Bonito-Oliva et al, 2014) (Costa et al, 2012). Cognitive flexibility and learning deficits in PD have also been modeled using 6-OHDA lesions. Rats with lesions in the dorsomedial striatum were not impaired in the acquisition of tasks but were impaired in reversal learning. The dopamine depletion led to perseveration of old choice patterns and difficulties in maintaining new patterns, including regressive errors (Grospe et al, 2018). Modeling of cognitive impairments through lesion studies not only speaks to the transferability of rodent studies to human experience, but also provides an opportunity to investigate more specific cognition-related changes in PD.

Sequence Learning in PD

For the present study, we focus on another cognitive aspect of PD: sequence learning. In several studies, cognitive and motor sequence learning (MSL) have been shown to be impaired in patients with PD. This study narrows our investigation to motor sequence learning. Motor sequence learning arises from the mesolimbic and mesocortical dopamine systems. Imaging studies of MSL show activation in the visual and motor loops of the brain, including their origins in the basal ganglia and downstream projections to the dorsolateral PFC and supplementary motor area (Alexander et al, 1986) (Nakahara et al, 2001) (Garr, 2019). PD patients show overarching changes in neural activity during

MSL tasks, such as an increase in activity pre-supplementary motor area, hippocampus, and anterior cingulate cortex. Conversely, decreases in neural activity occur in occipital lobe, parietal lobe, and dorsolateral PFC for PD patients (Carbon et al, 2010).

Impairments in behavioral MSL tasks accompany these changes in neural activations. In a 2001 study, Krebs et al. use a visuomotor MSL task primarily focusing on arm and forearm movements. PD patients in this study had difficulty guiding movements, but also had an overall decrease in rate of learning, with even greater impairment in novelty and early acquisitional phases of learning (Krebs et al, 2001). Another behavioral task used to test MSL in Parkinson's patients is the button-press task (Mochizuki-Kawai et al, 2010). Here, subjects had to press eight pairs of buttons with as much accuracy and speed as possible. Like the previous study described, PD patients had difficulty performing these sequences, especially when learning a new sequence. In addition to showing impairments in sequence learning, this study also highlights difficulties in cognitive flexibility that arise in PD (Mochizuki-Kawai et al, 2010). For many decades, the exact neural substrates of these cognitive processes remained unclear. More recent studies have attributed this change in behavior to regions in the basal ganglia, specifically the substantia nigra. Compared to healthy controls, people with PD have increased substantia nigra activity during sequence tasks, as measured by an fMRI (Tzvi et al, 2021). The way MSL deficits emerge in PD are also dependent on the stage of disease progression. PD patients' performance and rate of learning on MSL tasks decrease as they progress in the disease (Stephan et al, 2011). Thus, patients with a higher amount of neurodegeneration and dysfunction will have much greater impairment than patients who are in the early stages of PD.

PD-related impairments in sequence learning have also been modeled in experiments with rodents. He et al. (2022) utilizes an alpha-synuclein model of PD in mice. In addition to dopaminergic cell death, PD is also characterized by mutations of intracellular alpha-synuclein inclusions (Galvin et al, 2001). In the alpha-synuclein model, He et al find that, in a two-step lever press sequence (right then left press), performance and rate of learning of the correct sequence significantly decreased (He et al, 2022). Our experiment uses a similar two-step sequence to test MSL in mice with lesions to the VTA.

VTA in Sequence Learning

While there are many studies focusing on MSL in PD, the body of research looking specifically at the VTA's role in sequence learning is much less extensive. The VTA's role in MSL largely connects to its dopaminergic projections to the cortex, specifically the primary motor cortex (Hosp et al, 2019). One study found that the VTA reacts differentially to motor sequence tasks and reward. Here, VTA to motor cortex projections were activated during successful sequence learning acquisition, but not in failure to learn the task or once mice reached plateau performance. In this same study, researchers noted that dopaminergic VTA cells that did not project to the motor cortex activated in response to food rewards and skilled reaching towards these food rewards (Leemburg et al, 2018). Following the PD model, a 6-OHDA lesion study to the VTA also produced impairment in MSL in the acquisition phase- not in the maintenance of learned sequences (Hosp et al, 2011) (Cousineau et al, 2022). Therefore, impairment of sequence learning in PD may be linked to both suppression of skill acquisition as well as reward

sensitivity. The current study sought to model sequence learning in a 6-OHDA lesion model of PD, measured behaviorally by a nose-poke task.

6-OHDA Lesion Model of PD

To investigate potential roles of the VTA in sequence learning, we utilized a 6-hydroxydopamine (6-OHDA) neurotoxicity lesion model in mice. The unilateral 6-OHDA lesion model was first established by Ungerstedt and Arbuthnott in 1970 to produce impaired motor activity in PD at a lower mortality rate than a bilateral lesion (Ungerstedt & Arbuthnott, 1970). This lesion is most used in the nigrostriatal and mesolimbic pathways to model PD in rats and mice. 6-OHDA targets catecholaminergic neurons, or neurons that produce either noradrenaline or dopamine. It can be used in varying concentrations and quantities to produce large or more acutely targeted lesions. Early use of this lesion showed that lesions can be neurotransmitter-specific depending on the location of the injection. For example, dopamine neurons can be targeted through the SNc or VTA, and noradrenergic neurons can be targeted through the locus coeruleus (Kostrzewa & Jacobowitz, 1974). In this case, 6-OHDA targeted dopamine neurons of the VTA.

As previously stated, the 6-OHDA model of PD results in the death of catecholaminergic cells. The mechanism by which this death occurs is accomplished in two phases- first, the build-up of the neurotoxin in the cell. This is followed by a perturbation to the neuron's homeostasis that results in cell damage (Simola et al, 2007). However, 6-OHDA is limited by its inability to cross the blood-brain barrier. Therefore, it must be directly injected into the desired site of the lesion. Once injected in the

extracellular space of the desired nucleus, dopamine (or noradrenaline) transporters (DAT) uptake the neurotoxin into the cell due to its structural similarities to dopamine (Simola et al, 2007). Inside the cell, 6-OHDA causes oxidative stress that results in cell death, or apoptosis. Oxidation of the 6-OHDA neurotoxin leads to the production of reactive oxygen species that attack nucleophilic groups within the cell (Padiglia et al, 1997) (Palumbo et al, 1999). The increase in reactive species endogenously results in depletion of antioxidant enzymes, which impairs cell structure and metabolism (Blum et al, 2001). This change in cellular homeostasis leads to autophagy and apoptosis of dopaminergic cells.

The neurotoxic effect of 6-OHDA injection to dopaminergic nuclei in the midbrain- such as the VTA or SNc- results in behavioral phenotypes that mirror the effects of PD. 6-OHDA injections in rodents produce motor impairments characteristic of PD, such as impaired motor control, rotations, tremors, akinesia, and dyskinesia (Simola et al, 2007). Injections to different areas of the subcortex result in slightly different behavioral phenotypes, which can be used to model the functions and related deficits in these areas. While the 6-OHDA model has been used to study motor impairments in PD, it can also be used to study non-motor cognitive effects. The rat model of 6-OHDA neurotoxicity has been shown to decrease self-care and motivational behaviors, which are often associated with depressive symptoms in PD (Mendes-Pinheiro et al, 2021). In our experiment, we use the unilateral 6-OHDA model to investigate the potential role of the VTA in sequence-learning behaviors, and how this role may be disrupted in PD.

Materials and Methodology

Subjects

Subjects included C57/BL mice (n=11, 6 male, 5 female), split into 3 separate cohorts (n=3-4 per cohort). All mice were born on August 12th, 2023, and data was collected throughout the fall and spring semesters of the 2023-2024 academic year.

Lesion Surgery

Mice were split into sham (n=4) and lesion groups (n=7). All mice were anesthetized with isoflurane and treated with saline and carprofen pre-surgery. The lesion group received a 2-microliter injection of 6-OHDA HCl dissolved in water to either the right or left ventral tegmental area (VTA) via stereotaxic surgery (AP = -3.16, ML = +/- 0.50, DV = -4.60). The incision site and skull were cleaned with an antiseptic povidone-iodine solution. The sham group received an incision to the skull and drill hole, but no 6-OHDA injection.

Following surgery, mice were treated with ketoprofen (1mL/gram of mass) for 3 days. They were monitored for any abnormalities in healing or motor behavior. Four lesioned mice did not survive post-surgery, leaving three mice (n=3) in the final lesion group. Due to complications with the FED3 device, one sham mouse did not have a full pre- and post-surgery dataset completed. This mouse was left out of analysis, leaving three (n=3) mice in the final sham group. **Table 1** summarizes the final cohort of mice utilized for data analysis of the sequence task.

FED-3 Testing

FED3 Device

The FED3 device (Nguyen et al, 2016) is an open-source device (see **Figure 2**) that can be used to measure various behaviors in mice. The device has two nose poke holes and a delivery well where sucrose pellets are delivered. A screen on the side of the device shows the program that is running, along with counters for the number of left and right pokes. Data was saved onto a microSD card, then transferred to a password-protected computer for data analysis.

Acclimation

Mice were individually housed throughout the duration of the experiment. Following isolation, a FED3 device on a free feeding program was run for ~3 days. Subsequently, mice were trained on an FR1 feeding schedule with either the left or right nose poke active. Active sides were counterbalanced across cohorts. The FR1 feeding schedule was run for approximately 5 to 7 days.

Sequence Task

Following training, mice were tested on a sequence task. The task requires mice to nose poke in a right-left or left-right sequence on a FR1 schedule for a food pellet reward. The active side is defined as the first of two sides after which mice poke and receive a pellet. For example, a mouse with right side active would poke a right-left sequence and receive a food pellet reward. Active sides were counterbalanced across the original

cohorts, and the task ran for 5-7 days. A visual diagram of the task can be found in **Figure 11.**

Cylinder Test

The cylinder test is used to test spontaneous rotations and laterality of weight bearing motions in a novel environment (Brooks & Dunnett, 2009) (Heuer et al, 2012). The mice were placed into a clear, cylindrical plastic container, and their movements were recorded with a video camera for five minutes. Cylinder testing took place before and after lesion surgery. Following testing, videos were coded for rotations and forelimb touches to the walls of the cylinder.

Previous literature indicates that mice with unilateral lesions will often rotate in the direction of the lesion. In addition, mice tend to utilize the forelimb ipsilateral to the lesion (Schallert et al, 2000) (Alvarez-Fischer et al, 2008). By measuring these behaviors before and after surgery, we can test the accuracy of the lesion as well as its potential impact on motor behavior. The timescale of behavior measurement is in accordance with previous literature showing that these effects occur predominantly within the first five minutes of habituation to a novel environment.

Gait Analysis

Gait analysis is a common measure of motor ability in Parkinson's disease models (Brooks & Dunnett, 2009) (Heuer et al, 2012). The fore and hind paws were painted with different colors of non-toxic water-based paint, and the mice then ran across white absorbent paper in a straight line through a small corridor. The footprints were measured

for stride length and base width in control and lesion groups to assess for any gait changes.

Staining, Immunohistochemistry, and Imaging

Following the completion of FED3 data collection post-surgery, mice were sacrificed and perfused. Following at least 24 hours of fixation in a 4% paraformaldehyde solution, brains were sliced at 50um thickness with a Cryostat.

Free-floating brain slices were first blocked with an antibody diluent buffer (Sigma-Aldrich U3635) for 2hrs, followed by exposure to a primary antibody for tyrosine hydroxylase (1:500, Sigma-Aldrich AB152) for 72hrs. Slices were then washed (3x) using an antibody diluent buffer and exposed to secondary antibody (1:1000, Sigma-Aldrich SAB4600084) for 2hrs. Slices were lastly washed (3x) and mounted with DAPI (Sigma-Aldrich F6057) for cellular visualization. Slices were imaged using a confocal microscope, with the blue channel showing DAPI (all cell bodies) and the red channel showing TH staining (dopaminergic cell bodies). See **Figures 3 through 5** for further information.

Data Analysis

Cylinder and Gait Analysis

The web cam footage of the cylinder test was scored for left and right rotations, as well as touches to the wall of the cylinder with the left or right forelimb. A rotation was scored as a full 180 degree turn from the initiation of a change in direction. For both

rotations and forelimb laterality, we measured the proportion of left and right events and compared these values pre- and post-surgery.

In gait analysis, paw prints were measured for base length and width of three consecutive strides. The length and width of these three strides were averaged for each animal before and after surgery. Any major changes in length or width before and after surgery were noted.

Sequence Task

Data from the FED3 devices was analyzed using FED3Viz and Microsoft Excel. Any concatenation of data files, as well as graphs for acclimation and FR1 training were generated via FED3Viz. The sequence task data was analyzed using Microsoft Excel. We calculated the number of occurrences of each type of task event (i.e., left-right, left-left, right-left, and right-right sequences). Utilizing this data, we plotted the various events by time, fitting a linear regression to the correct task event. Comparisons of the slope of these regressions were used as an indication of changes in the rate of learning. Additionally, we quantified performance as the final proportion of each task event and compared these values before and after surgery.

Results

Images

Sham

Figure 3 shows a stained image of the midbrain in a control animal (#19, male). Blue (DAPI) shows stained nuclei, while red (TH) shows dopaminergic cells. In this image, dopaminergic cell bodies show the VTA medially and SNc laterally.

Figure 4 highlights a closer image of cell bodies in the VTA in a control animal (#19, male). The overlap between dopaminergic cell bodies in red and blue stained cell bodies indicates a confirmation of staining accuracy.

Lesion

Figure 5 is a stained image of the midbrain in a lesioned animal (#20, male). The red staining of dopaminergic cells in this image confirms lesioning of the right VTA, as there are no red cells in this portion. 6-OHDA appears to have also spread into the right SNc and part of the dorsal left VTA.

Figure 6 is another example of a lesioned animal's brain slice (#29, female). Again, the image shows an absence of dopaminergic cells in the right VTA where the lesion injection was delivered. In contrast with Figure 5, the lesion is contained to the right VTA, with no additional spreading to the left side. There also seems to be minor sparing of dopamine neurons in the SNc.

Sequence Task

Sham Surgery Animals

Figure 7 depicts the performance of control mice as a function of time before (7a) and after (7b) the sham surgery. Individual event types are graphed, with the correct poke sequence fitted with a linear regression. Further numeric summaries of this data can be found in **Tables 2 and 3**. Statistical analyses such as t-tests were not performed on this data due to the low statistical power of our dataset.

Table 2 summarizes the performance of sham animals before and after surgery based on poke event type. Performance is represented by the final proportion of each event type compared to the total number of pokes. The correct poke sequence is highlighted in green, while the most common error event type is highlighted in red. For mice #18 and #27, the proportion of correct poke sequences increases after surgery, while mouse #19 experiences a slight decrease. The most common error type corresponds to the second poke side in the correct sequence. This is expected, since mice are initially trained on FR1 and predict the correct poke to be a single poke preceding delivery of a food pellet. Therefore, they associate the second step in the sequence. In the control animals there is variability in the proportions of error events, with some increasing in proportion and other decreasing post-surgery. Graphical representation of this data can be found in **Figure 9.**

To quantify learning in the sham mice, we fit a linear regression line to the correct poke sequence data. This regression represents the rate of learning in number of correct pokes per day. The slope of the regression is reported in **Table 3** alongside R^2 values that

evaluate how accurately the regression fits the dataset. Because the VTA is still intact in control animals, we would expect to see an increase in learning rate due to acclimation in learning to the sequence task that continues from the pre-surgery to the post-surgery data. For mice #19 and #27, the rate of correct pokes per day increased after sham surgery. Mouse #18 experienced a decrease in this rate. However, even though the rate of the correct poke sequence regression decreased, **Table 2** shows that the final proportion of the right-left sequence events increased. This may indicate that an improvement in performance is still occurring, even if the rate of the correct sequence was lower post-surgery. Graphical representation of this data can be found in **Figure 10**.

Lesion Surgery Animals

Figure 8 depicts the performance of lesioned mice as a function of time before (8a) and after (8b) the lesion surgery. Individual event types are graphed, with the correct poke sequence fitted with a linear regression. Further numeric summaries of this data can be found in **Tables 4 and 5**. Again, statistical analyses such as t-tests were not performed on this data due to the low statistical power of our dataset.

In **Table 4**, we quantify the performance of lesioned mice as a function of the final proportion of poke event types. After surgery, the proportion of correct poke sequences decreased for mice #20 and #29, but not number #28. Complications with the FED3 device in the pre-surgery data collection for mouse #28 led to a low number of pokes registered by the device. If the device had functioned normally (as it had with the other two lesioned mice), we hypothesize that #28 would likely follow the pattern of

mice #20 and #29, with an overall decrease in performance on the correct poke sequence. There is also an increase in the most common error type for all lesioned mice.

Table 5 summarizes learning in lesioned mice with a linear regression of the correct poke sequence in each animal. R^2 values for each slope measure how accurately the regression fits the dataset. Mirroring our results in Table 4, mice #20 and #29 experienced a decrease in the slope of the correct poke sequence regression, possibly indicative of a learning impairment post lesion surgery. Similarly to the previous table, mouse #28 had an overall increase in the learning rate post-surgery, but this is likely due to complications with the FED3 device pre-surgery that did not occur in the post-surgery data collection session.

Cylinder Test

For the cylinder test, we collected the number of left and right rotations, as well as left and right touches of the forelimb to the wall of the cylinder. From this, we measured the proportion of each of these metrics. A paired, unequal variance t-test was performed to measure the statistical significance of any potential differences pre- and -post surgery for both sham and lesion animals. It should be noted, however, that this t-test lacks statistical power, as the sample sizes are not large.

There was no significant change ($p > 0.05$) in the proportion of left rotations or right rotations before and after surgery for both sham and lesion groups. Additionally, there was no significant change in the proportion of left touches or right touches before and after surgery for both sham and lesion groups. A further summary of this data can be

found in **Tables 6 and 7**. Data was collected from all mice that completed some or all of cylinder testing (may include mice not used for the sequence task).

Gait Analysis

From our gait analysis data, we collected the average stride length and width of three consecutive strides for both the hind and forelimbs. Again, a paired, unequal-variance t-test was performed on these metrics to compare gait before and after surgery. Again, while this statistical test was performed, it lacks statistical power due to low sample sizes.

For forelimbs, there was no significant difference ($p > 0.05$) in the average stride length before and after surgery. The same pattern held for average stride width of forelimbs. In terms of hind limbs, there was also no significant change in the average stride length or width comparing pre- and post-surgery. A further summary of this data can be found in **Tables 8 and 9**. Data was collected from all mice that completed some or all of gait testing (may include mice not used for the sequence task).

Discussion

Our experiment sought to investigate potential roles of the VTA in motor sequence learning through a unilateral 6-OHDA model of Parkinson's Disease in mice. Two out of three lesioned mice experienced an impairment in performance and rate of learning of a sequence task on the FED3 device (see **Figures 7 through 10** and **Tables 2 through 5**). This impairment occurred without significant changes in gait or forelimb laterality as measured by gait analysis and a cylinder test (see Tables **6 through 9**). While the lack of differences in motor performance is promising to conclude that impairments in sequence task are because of the VTA's role in learning, the lack of statistical power in these measurements warrants future confirmation of this result. Furthermore, when comparing the total number of poke events pre- and post- surgery in lesioned animals, there is not a large difference in the number of pokes post-surgery. For the one lesion mouse (#29) that experienced a decrease in the number of pokes post-surgery, the number of pokes went from 2054 to 1879, only a 9 percent change in pokes from baseline. Therefore, it does not appear that deficits in the sequence task are simply due to a lower frequency of nose poke responses. This also holds with the idea that VTA lesions would be less likely to influence nigrostriatal pathways that are in greater control of motor behavior.

As previously mentioned, the VTA activates in response to both reward and motor skill acquisition (Leemburg et al, 2018). If the impairments in rate of learning and performance on the sequence task are not due to potential motor deficits induced by the lesion, then it possible that sequence learning impairments are due to the VTA's role in

reward valuation and motivation. However, confirmation of this hypothesis should be tested through further FED3 tasks such as a progressive ratio to analyze the break points where lesioned mice stop performing for sucrose pellets before and after surgery. This would allow for more targeted analysis of reward behavior and motivation. If reward valuation or motivation is not the cause of the deficits in performance on the task, then it would be reasonable to conclude that the impairments on the sequence task are due to the lesion's damaging of sequence learning acquisition substrates.

Even though there were not any apparent changes in motor ability in our lesioned mice, there is still a chance that impairment in sequence learning was more due to SNc depletion that occurred as a side-effect of using a high volume of 6-OHDA. For example, in **Figure 5** there appears to be depletion of dopaminergic cells in the right SNc for mouse #20. In **Figure 6**, mouse #29 also has depletion of some dopaminergic cells of the SNc, but not as complete as mouse #20. 6-OHDA may also have trans-synaptic effects that could explain dopaminergic depletion in areas that were not intended to be lesioned. The death of dopaminergic neurons in the VTA may influence the activity of post-synaptic targets of these neurons outside of the VTA.

Limitations

As discussed before, in lesioned mice there was a greater spread of the 6-OHDA lesion that caused death of dopaminergic neurons in the SNc as well as the VTA. Therefore, we cannot conclude with certainty that impairments in sequence learning were due to eliminating the role of VTA dopamine neurons in sequence learning- even though there were not any measured differences in motor function typically characteristic of the

SNc. There is also limitation inherent to the mice already having learned the sequence task prior to surgeries. However, with this experimental model we were able to minimize the number of animals used for the study while observing intra-subject effects.

Additionally, the size of sham and lesion cohorts was small, leading to low statistical power of our analyses. Due to the death of lesioned mice throughout our experiment, the active sides for the correct sequence task are not balanced in the final lesion cohort. This could also complicate our analysis by not having proper counterbalancing of active sides in the sequence task. Another extension of a small cohort sizes is the inability to make conclusions or analyses based on the sex of mice. Because of the differential manifestation of cognitive symptoms in PD (Bloem et al, 2021), it would be valuable to investigate if there are differences in sequence learning across sexes in a PD model.

Future Directions

To improve on the current study, future research should include larger cohorts of mice, potentially including both male and female mice to investigate differences in sequence learning across sexes. A smaller volume of 6-OHDA should be injected into the brain for a more targeted lesion to the VTA. This way, a stronger connection of the VTA to sequence learning could be drawn if the lesion does not spill over into unintended regions such as the SNc. To further test the role of the VTA in reward sensitivity and motivation as a cause of impairments in sequence learning performance, different feeding schedules such as a progressive ratio could also be utilized. A progressive ratio task

focuses on reward motivation and sensitivity by comparing break points in performance across lesion and control groups.

There is a gap in the literature regarding the VTA's role in motor sequence learning. While this study attempts to bridge that gap, further studies of the VTA in MSL would expand the body of knowledge regarding functions of the VTA. Understanding the role of the VTA in sequence learning also has implications for human treatment of PD. If there is greater knowledge of the neural correlates behind cognitive impairments in Parkinson's disease, there is potential to develop more targeted treatments of PD. Since PD treatments can result in cognitive changes such as increased impulsivity and risky decision making (Buelow et al, 2014) (Haagensen et al, 2020), future research should test the role of common pharmacological PD treatments such as dopamine agonists on sequence learning.

Other ways to expand the research completed in the present study could inject 6-OHDA at downstream targets of the VTA, to obtain a more detailed understanding of circuit-level functions in motor sequence learning. Additionally, other lesion methods such as chemogenetics could be used to create 'reversible' lesions to the VTA. Chemogenetics uses cell type-specific labeling of cells that are pharmacologically reversible because they are activated by a specific type of matching cognate drug on a genetically engineered receptor (Sternson & Bleakman, 2020). Therefore, the use of chemogenetics has broader implications for both testing of PD models experimentally and treatment of PD itself clinically. A reversible lesion would allow for non-permanent testing of sequence learning in the VTA that does not have other long-term effects on reward learning or motivation.

Conclusion

In the present study, we sought to investigate a potential role of the VTA in sequence learning using a 6-OHDA model of Parkinson's disease. While the statistical power of this study is limited to due small group sizes, it may provide a basis for further study of the VTA in sequence learning. In two out of three lesioned animals, performance and rate of learning on a sequence task were impaired. The 6-OHDA model produced a change in cognitive processing while seemingly leaving basic motor tasks such as rotational behavior, gait, or number of total pokes unaffected. Future studies should increase the sample size of control and lesion groups, as well as utilizing a more targeted and lower volume lesion to further address trends discussed in this paper. Additionally, future research should include different feeding schedules such as progressive ratio to investigate possible reward and motivation-related explanations for the results we see in this study.

Figures

Figure 1

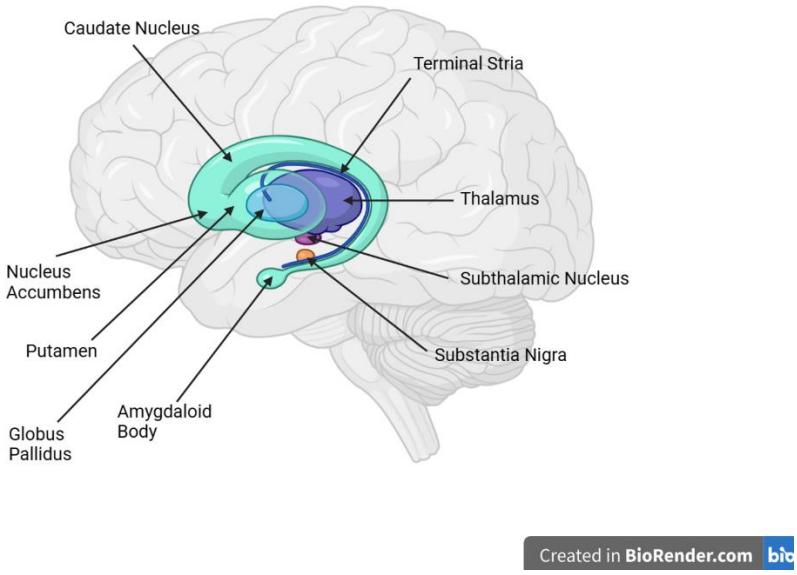


Figure 1. Diagram of the Basal Ganglia: *Image depicting the main structures of the basal ganglia from a lateral view of the brain. Created with BioRender.*

Figure 2

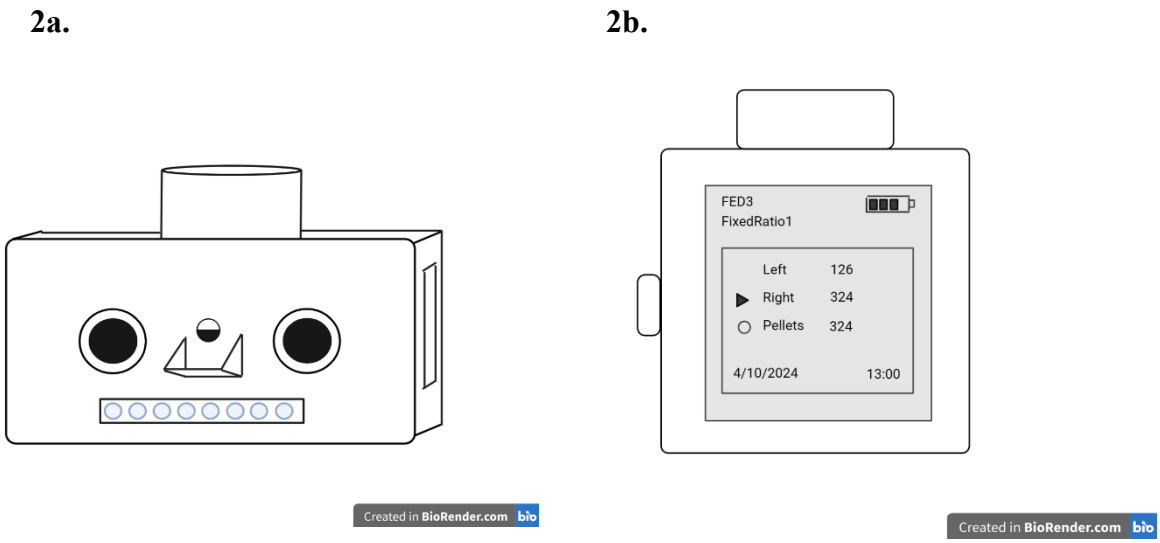
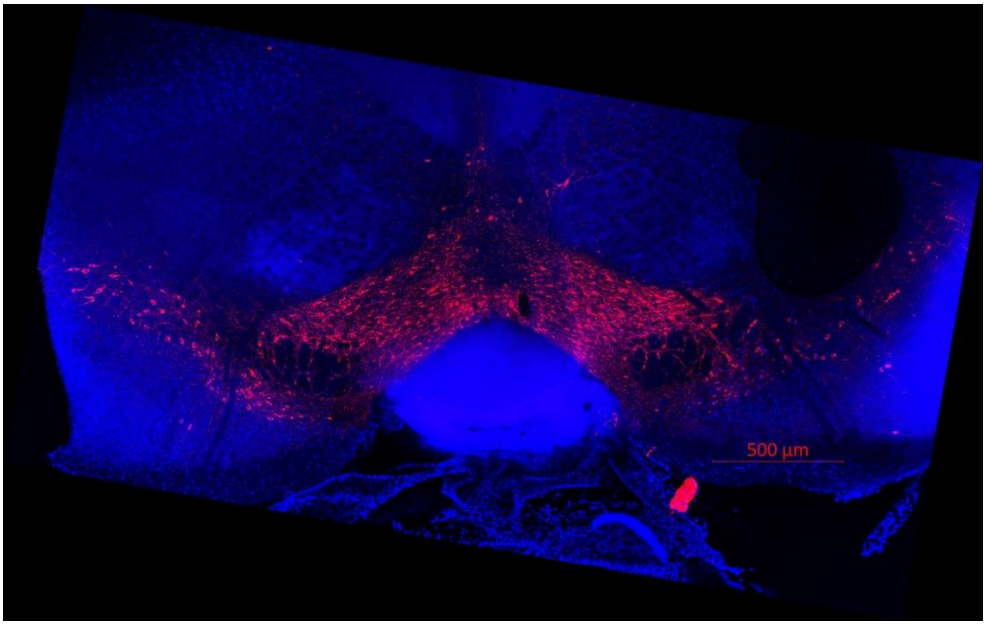


Figure 2. FED3 Device*: Graphical depiction of the FED3 Device. Created with BioRender.*

Figure 3

3a.



3b.

[IMAGE REMOVED FOR COPYRIGHT PURPOSES]

Figure 3: Stained image of a control animal VTA*: (3a) Stained image of the VTA in a control animal (#19, male). Blue (DAPI) shows stained cell bodies, while red (TH) shows dopaminergic cell bodies. (3b) Coronal image of the mouse brain from a brain atlas (Paxinos & Franklin, 2001). The VTA is outlined in blue.*

Figure 4

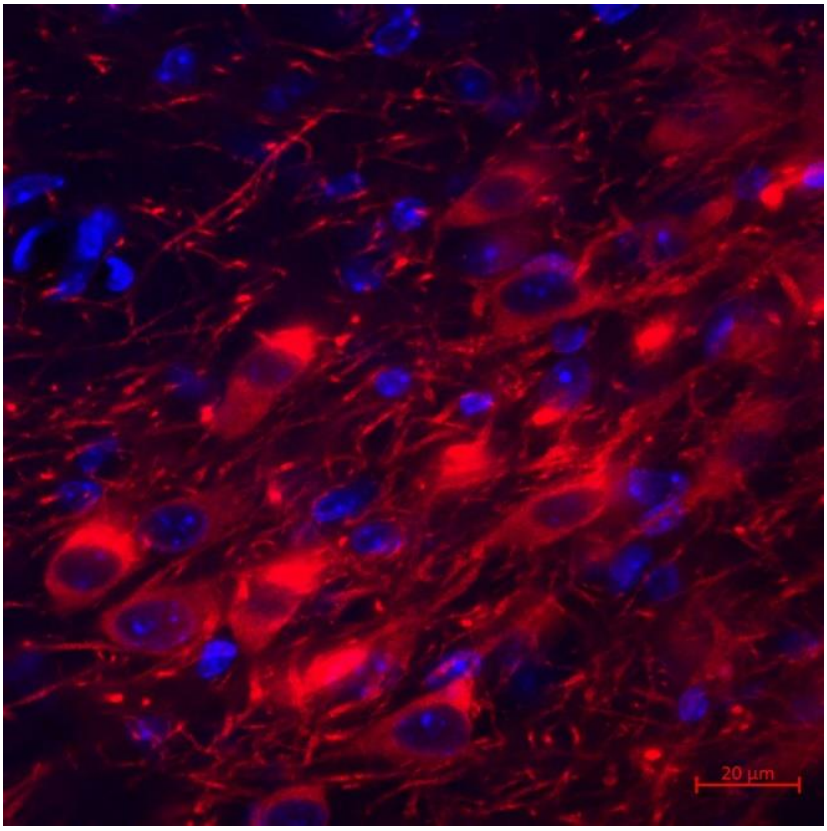


Figure 4. Dopaminergic cells of the VTA: *This image depicts a zoomed-in portion of the VTA, highlighting specific neuronal cell bodies. Blue (DAPI) shows stained cell bodies, while red (TH) shows dopaminergic cell bodies. Note the overlap between dopaminergic cell bodies in red and blue stained cell bodies- indicating confirmation of stain accuracy.*

Figure 5

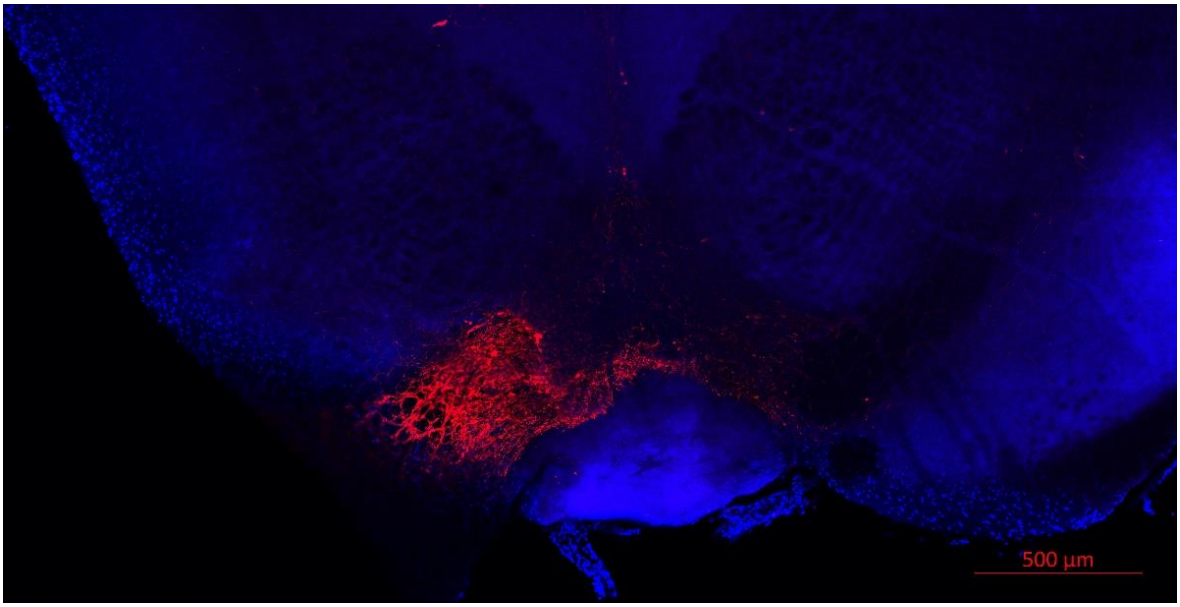


Figure 5. Lesion Image, Mouse #20*: Stained image of mouse #20 (male). Mouse received a 6-OHDA lesion to the right side of the VTA. Blue (DAPI) shows stained cell bodies, while red (TH) shows dopaminergic cell bodies.*

Figure 6

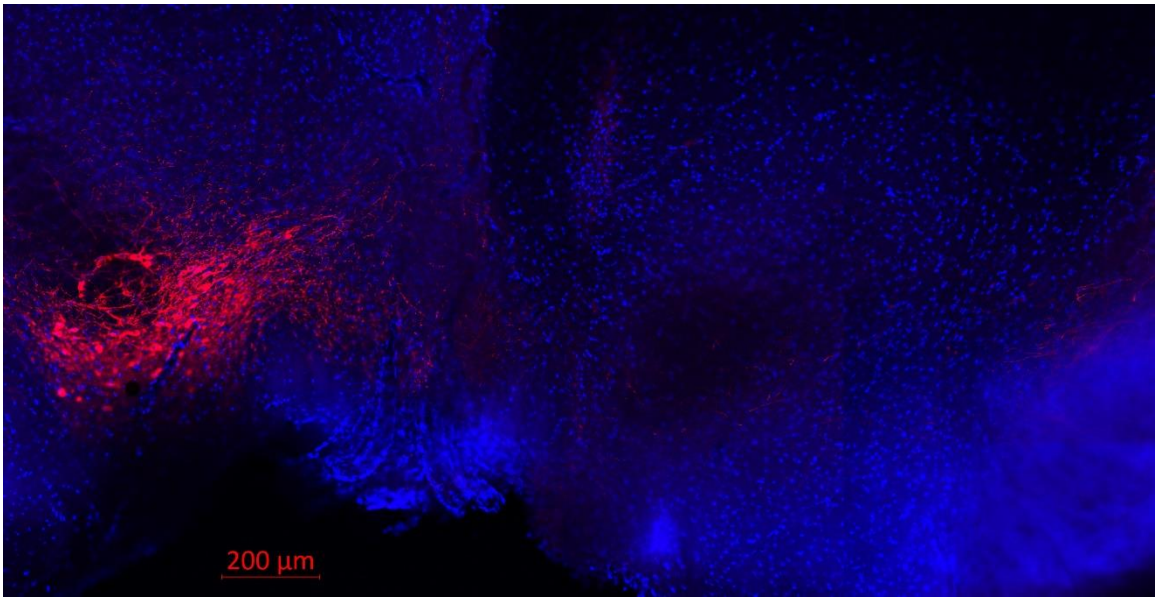
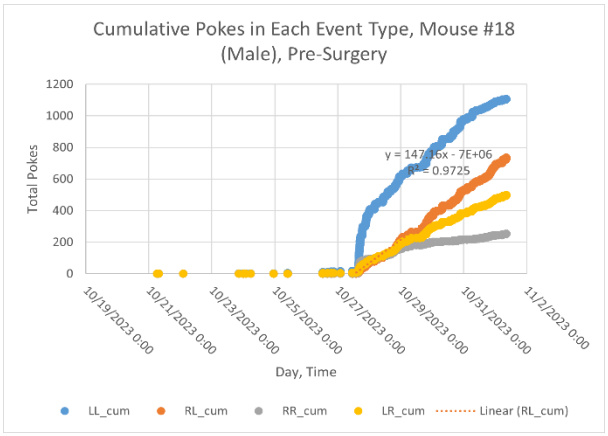


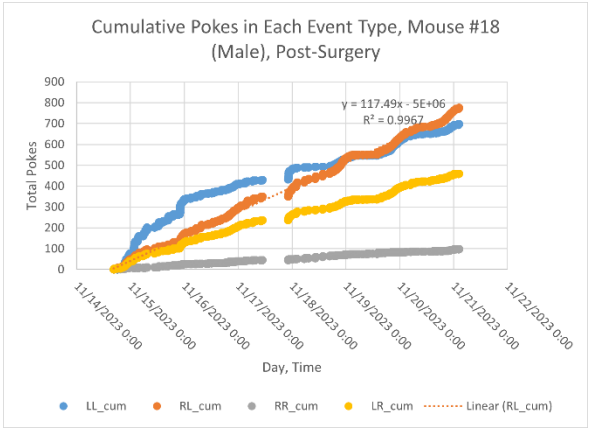
Figure 5. Lesion Image, Mouse #29: *Stained image of mouse #29 (female). Mouse received a 6-OHDA lesion to the right side of the VTA. Blue (DAPI) shows stained cell bodies, while red (TH) shows dopaminergic cell bodies.*

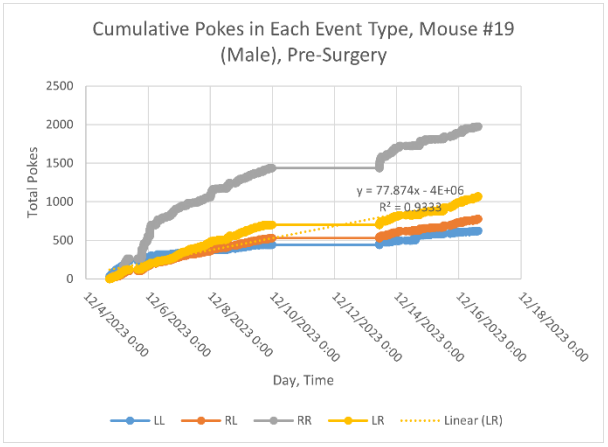
Figure 7

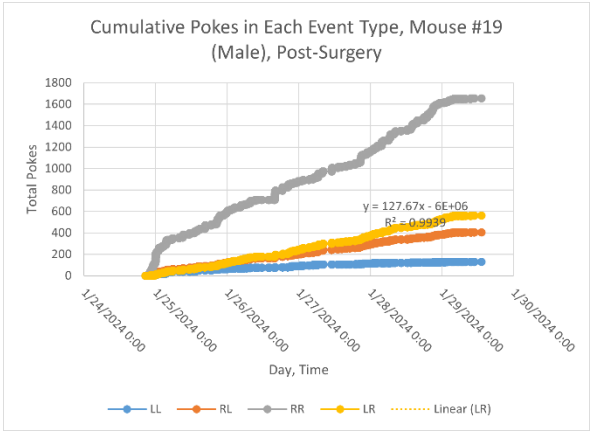
7a. Pre-Surgery

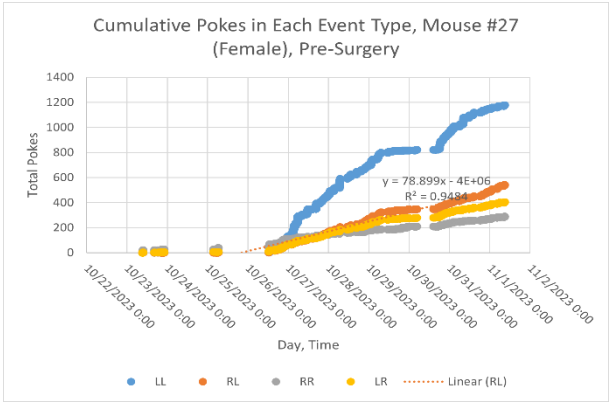
7b. Post-Surgery











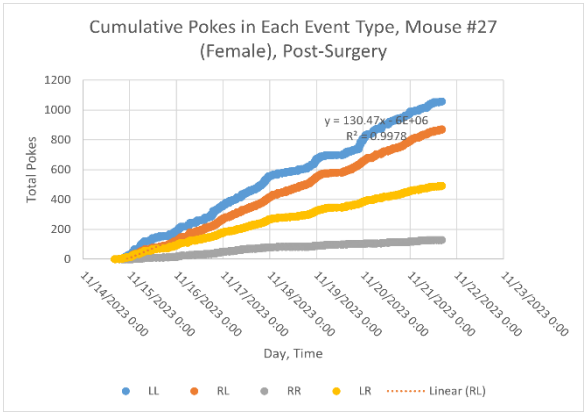
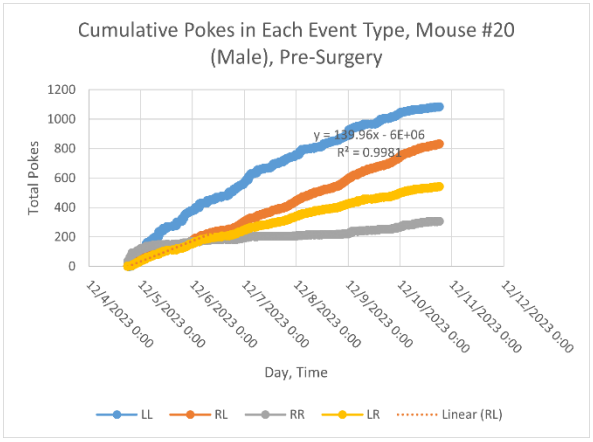


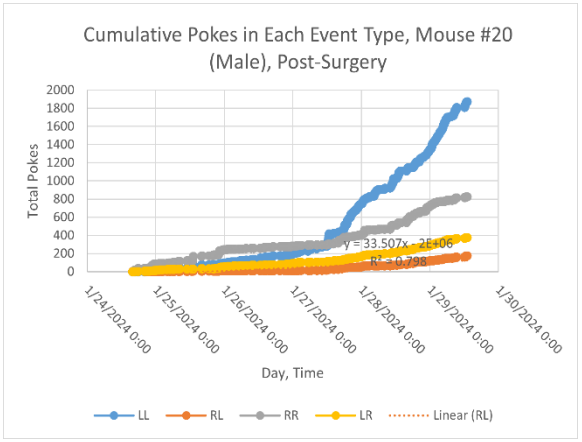
Figure 4. Sequence Task- Sham Surgery: *Graphs of total number of pokes versus time for each sham surgery animal, before (a) and after (b) surgery. Linear regression lines are shown for the correct poke sequence event type. Performance and rate of learning metrics are shown in more detail tables 2 and 3 below.*

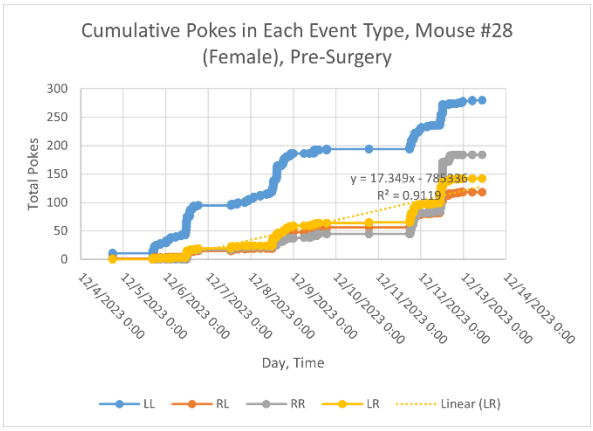
Figure 8

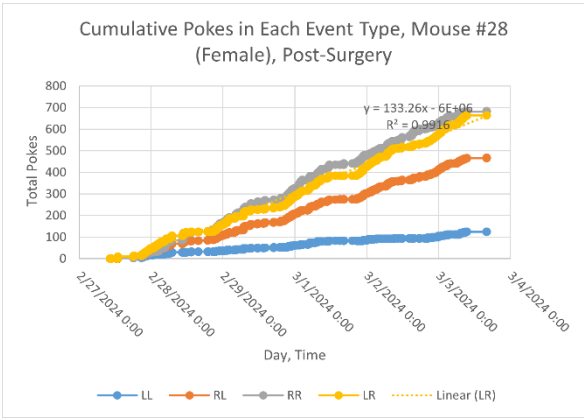
8a. Pre-Lesion

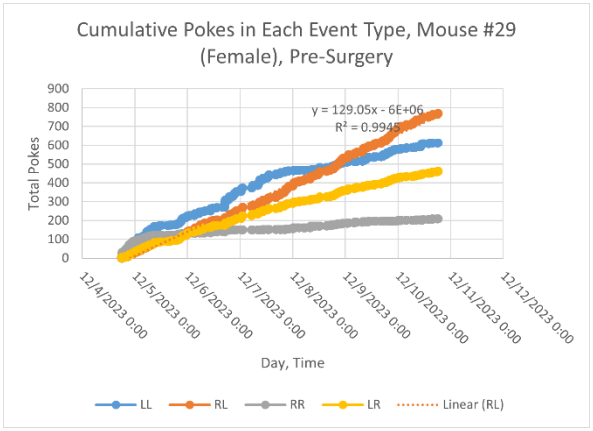
8b. Post-Lesion











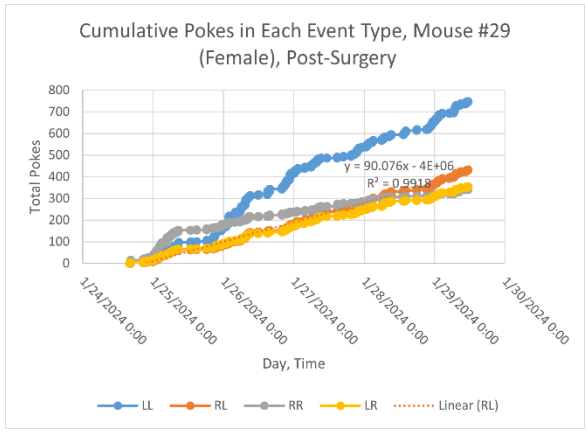


Figure 8. Sequence Task- Lesion Surgery: *Graphs of total number of pokes versus time for each lesion surgery animal, before (a) and after (b) surgery. Linear regression lines are shown for the correct poke sequence event type. Performance and rate of learning metrics are shown in more detail in tables 4 and 5 below.*

Figure 9

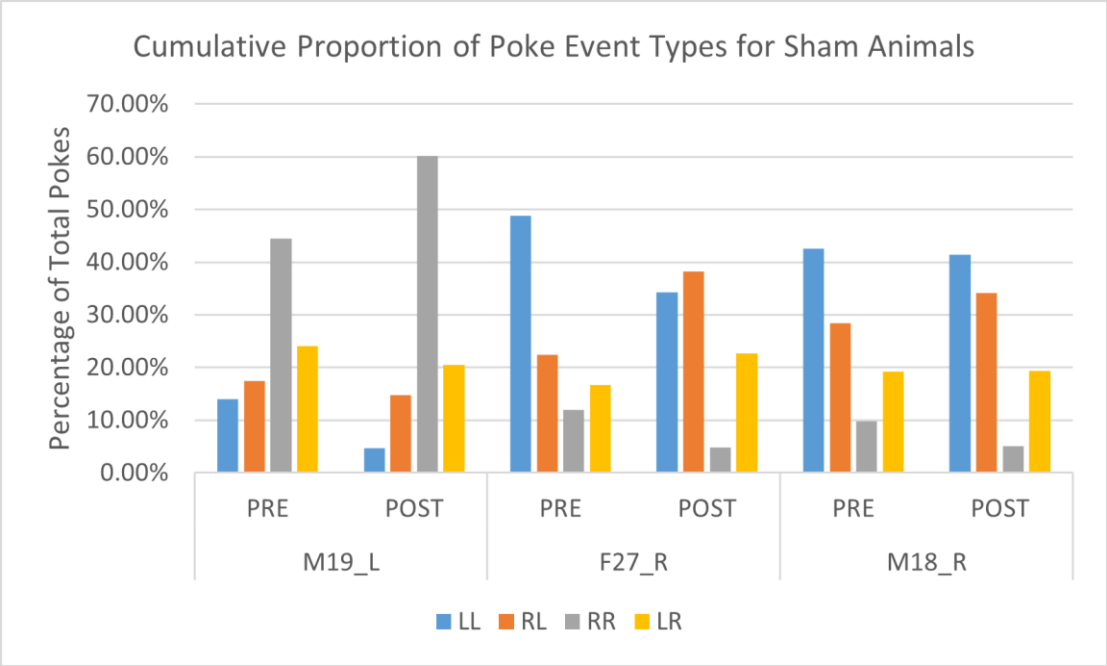


Figure 9. Bar Graph of Poke Event Types for Sham Animals*: Graph summarizing the poke event types pre- and post-surgery for sham animals. Calculations are made based on the total number of pokes in a certain event type divided by the total number of pokes in a data collection period (i.e. number of left-left pokes pre-surgery / total pokes pre-surgery = percentage graphed for LL PRE).*

Figure 10

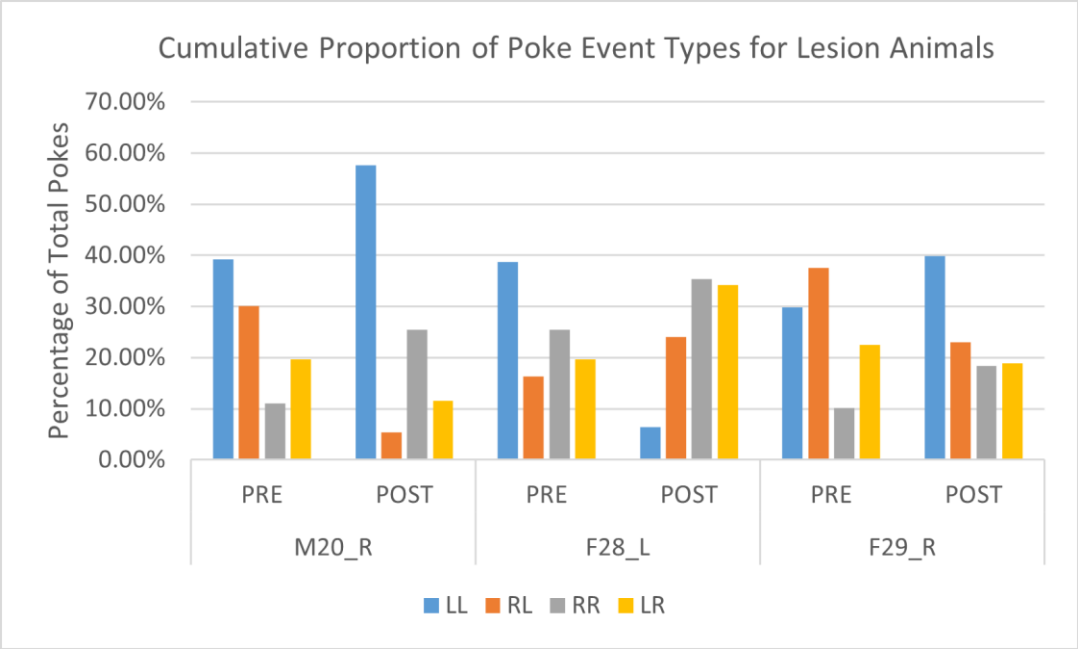


Figure 10. Bar Graph of Poke Event Types for Lesion Animals*: Graph summarizing the poke event types pre- and post-surgery for lesion animals. Calculations are made based on the total number of pokes in a certain event type divided by the total number of pokes in a data collection period (i.e. number of left-left pokes pre-surgery / total pokes pre-surgery = percentage graphed for LL PRE).*

Figure 11

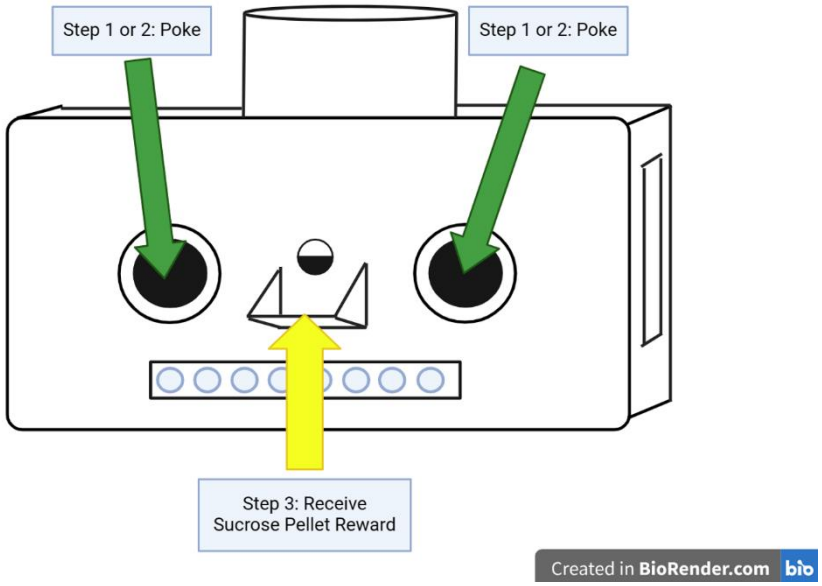


Figure 11. Sequence Task Diagram: *Depiction of sequence task on a FED3 device. Mice are assigned either a left-right sequence or right-left poke sequence. Once they complete their assigned correct poke sequence, a sucrose pellet is delivered as a reward. Image created with BioRender.*

Tables

Mouse ID #	Sex	Condition	Active Side	Poke Sequence
18	M	Sham	Right	Right-Left
19	M	Sham	Right	Right-Left
20	M	Lesion	Right	Right-Left
27	F	Sham	Right	Right-Left
28	F	Lesion	Left	Left-Right
29	F	Lesion	Right	Right-Left

Table 1. Final cohort of mice utilized for sequence task data analysis.

Sex, ID		LL	RL	RR	LR	Total # Pokes
M, 18	Pre	42.62%	28.36%	9.79%	19.23%	2595
	Post	41.48%	34.17%	5.03%	19.32%	2546
M, 19	Pre	14.04%	17.50%	44.42%	24.04%	4446
	Post	4.72%	14.71%	60.12%	20.45%	2753
F, 27	Pre	48.82%	22.46%	11.98%	16.74%	2413
	Post	34.33%	38.17%	4.87%	22.63%	2033

Table 2. Final Percentage of Event Types for Sham Animals*: Table summarizing the percentage of each poke event type (Left-Left, Right-Left, Right-Right, and Left-Right) at the end of each data collection period, pre- and post- surgery. The correct poke sequence is highlighted in green, while the most common error type is highlighted in red.*

Sex, ID		Learning Rate (correct pokes/day)	R^2
M, 18	Pre	147.16	0.9725
	Post	117.49	0.9967
M, 19	Pre	77.874	0.9333
	Post	127.67	0.9939
F, 27	Pre	78.899	0.9484
	Post	130.74	0.9978

Table 3. Linear Regressions of the Correct Poke Sequence for Sham Animals*: This table reports the slope of the linear regression line fitted to the correct sequence data in Figure 7 above, indicating the rate of learning. R^2 values are included to show the accuracy of these regressions.*

Sex, ID		LL	RL	RR	LR	Total # Pokes
M, 20	Pre	39.18%	30.12%	11.09%	19.61%	2769
	Post	57.60%	5.38%	25.42%	11.60%	3250
F, 28	Pre	38.67%	16.30%	25.41%	19.61%	724
	Post	6.44%	24.06%	35.29%	34.21%	1941
F, 29	Pre	29.84%	37.49%	10.18%	22.49%	2054
	Post	39.81%	23.04%	18.31%	18.84%	1879

***Table 4**. **Final Percentage of Event Types for Lesioned Animals**: Table summarizing the percentage of each poke event type (Left-Left, Right-Left, Right-Right, and Left-Right) at the end of each data collection period, pre- and post- surgery. The correct poke sequence is highlighted in green, while the most common error type is highlighted in red.*

Sex, ID		Learning Rate (correct pokes/day)	R^2
M, 20	Pre	139.96	0.9981
	Post	33.507	0.798
F, 28	Pre	17.349	0.9119
	Post	133.26	0.9916
F, 29	Pre	129.05	0.9945
	Post	90.076	0.9918

***Table 5**. **Linear Regressions of the Correct Poke Sequence for Lesioned Animals**: This table reports the slope of the linear regression line fitted to the correct sequence data in Figure 8 above, indicating the rate of learning. R^2 values are included to show the accuracy of these regressions.*

GAIT ANALYSIS – SHAM ANIMALS					
FORELIMBS					
Stride Length (cm)			**Stride Width (cm)**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
6.73	6.00	0.837	1.53	1.17	0.333
5.40	5.7		1.53	1.67	
4.67	5.53		1.37	2.03	
	6.97			1.97	
HINDLIMBS					
Stride Length (cm)			**Stride Width (cm)**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
6.40	5.27	0.705	1.57	2.23	0.305
4.47	6.63		2.07	2.33	
5.83	5.66		2.53	2.56	
	7.43			2.63	

Table 6. Gait Analysis Data for Sham Animals: *Table shows the measurements of stride length and stride width for the fore- and hindlimbs of sham animals. P-values are reported based on an unequal variance, two-tailed t-test.*

GAIT ANALYSIS – LESION ANIMALS					
FORELIMBS					
Stride Length (cm)			**Stride Width (cm)**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
3.43	7.16	0.097	1.77	2.76	0.090
6.60	6.56		1.47	2.35	
5.57			2.20		
5.50			1.80		
HINDLIMBS					
Stride Length (cm)			**Stride Width (cm)**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
3.93	6.80	0.289	2.67	2.75	0.629
6.57	5.90		2.20	2.56	
6.17			2.62		
5.06			2.80		

Table 7. Gait Analysis Data for Lesion Animals: *Table shows the measurements of stride length and stride width for the fore- and hindlimbs of lesioned animals. P-values are reported based on an unequal variance, two-tailed t-test.*

CYLINDER TEST - SHAM ANIMALS					
Proportion of Left Touches			**Proportion of Right Touches**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
0.48	0.57	0.267	0.52	0.43	0.267
0.52	0.56		0.48	0.44	
0.58	0.60		0.42	0.40	
	0.56			0.44	
Proportion of Left Rotations			**Proportion of Right Rotations**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
0.47	0.00	0.775	0.53	1.00	0.775
0.59	0.88		0.41	0.13	
0.38	0.50		0.62	0.50	
	0.31			0.69	

Table 8. Cylinder Test Data for Sham Animals: *Table shows the proportion of left and right touches, as well as left and right rotations for sham animals in the cylinder test. The proportion of each metric is compared to the total number of the metric (i.e. proportion of left touches = left touches / (left + right touches)). P-values are reported based on an unequal variance, two-tailed t-test.*

CYLINDER TEST- LESION ANIMALS					
Proportion of Left Touches			**Proportion of Right Touches**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
0.45	0.00	0.383	0.55	1.00	0.383
0.63	0.55		0.37	0.45	
0.58	0.49		0.42	0.51	
0.49			0.51		
Proportion of Left Rotations			**Proportion of Right Rotations**		
Pre	**Post**	**p-value**	**Pre**	**Post**	**p-value**
0.33	0.00	0.216	0.67	1.00	0.216
0.50	0.43		0.50	0.57	
0.38	0.13		0.63	0.87	
0.43			0.57		

Table 9: Cylinder Test Data for Lesion Animals: *Table shows the proportion of left and right touches, as well as left and right rotations for lesioned animals in the cylinder test. The proportion of each metric is compared to the total number of the metric (i.e. proportion of left touches = left touches / (left + right touches)). P-values are reported based on an unequal variance, two-tailed t-test.*

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