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## Effects of a Unilateral Injection of Botulinum Neurotoxin Subtype-A in the Subthalamic Nucleus of a Parkinsonian Rat Model

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## Abstract

Dopaminergic degeneration in Parkinson's disease (PD) leads to altered functional activity within the basal ganglia (BG) circuitry, including hyperactivity of the subthalamic nucleus (STN). Treatments restoring the BG functional circuitry often result in improvements in parkinsonian symptoms in patients and animal models. A recent study from our laboratory identified that infusing botulinum toxin (BoNT-A) into the internal globus pallidus provided a transient restoration of motor asymmetry and goal-directed locomotion in a rat model of PD. We hypothesized that infusions of BoNT-A into the STN in a parkinsonian rat model will improve motor asymmetry and locomotor abnormalities. Infusions of BoNT-A into the ipsilateral STN in unilaterally 6-hydroxydopamine lesioned rats assessed in the apomorphine rotation task, rotarod, or CatWalk apparatus revealed a dose-dependent amelioration of pathological rotations, while failing to affect spontaneous locomotion. The present results suggest that spontaneous locomotion may not dependent on the integrity of the BG functional circuitry.

## Keywords

Parkinson's disease, botulinum toxin subtype A, 6-hydroxydopamine, subthalamic nucleus, gait, rotarod, CatWalk, apomorphine, movement disorders

## Summary for Lay Audience

In Parkinson's disease dopaminergic cells die in the substantia nigra, a region part of the brains motor circuitry. This cell death leads to changes in the functional activity of this motor circuitry which leads to motor symptoms. Treatment options for Parkinson's disease try to restore or alter the activity of this circuitry to improve motor symptoms. This study investigates the effects of a potential new treatment in a rat model of Parkinson's disease. This new treatment involves injecting botulinum neurotoxin in a very small dose directly into the brain to alter the activity of the motor circuitry, which is impaired in disease, to more normal levels of activity. This study investigates the effects of various doses of botulinum toxin in the brain of a rat. This study will assess this treatments effect on drug-induced movement, forced movement, and voluntary movement. It was found that the two highest doses of botulinum toxin were successful in reducing pathological drug induced rotations, meaning that this treatment successfully changed the levels of activity in the brains motor circuitry to healthier levels. The rats did not develop impaired gait in this study, thus it is unknown whether this treatment would have improved a deficit in gait. The results are promising and the effects of this treatment on impaired gait need to be examined in a future study in animals with impaired walking.

## List of Abbreviations

- 6-OHDA: 6-hydroxydopamine
- ABC: avidin biotin peroxidase complex
- BG: basal ganglia
- BoNTs: botulinum neurotoxins
- BoNT-A: botulinum neurotoxin subtype-A
- CNS: central nervous system
- CW: clockwise
- CCW: counter-clockwise
- DAB: 3,3' -diaminobenzidine
- DBS: deep brain stimulation
- EPN: entopeduncular nucleus
- GBA:  $\beta$ -Glucocerebrosidase
- GPe: external globus pallidus
- GPi: globus pallidus interna/ internal globus pallidus
- HC: Heavy chain
- LRRK2: Leucine-rich repeat kinase 2
- LRP10: low-density lipoprotein receptor-related protein 10
- LC: locus coeruleus
- LC: light chain
- MFB: medial forebrain bundle
- MAO-B: monoamine oxidase B
- MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- OCT: optimal cutting temperature
- PD: Parkinson's disease
- PNS: peripheral nervous system
- RPM: rotations per minute
- SD: standard deviation
- SEM: standard error of mean
- SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
- SNAP-25: synaptosomal nerve-associated protein 25
- SN: Substantia nigra
- SNpc: Substantia nigra pars compacta
- SNpr: Substantia nigra pars reticulata
- TH: tyrosine hydroxylase
- LF: left front paw
- LH: left hind paw
- RF: right front paw
- RH: right hind paw

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# Chapter 1

## 1 Introduction

This chapter will provide an overview of Parkinson's disease (PD), including its symptoms, pathology, diagnosis, etiology, and treatment options. Major changes in neural circuitry involved in PD, the animal models used in PD research, and the gait impairment seen in humans with this disease and in animal models will be discussed. This chapter will then describe botulinum toxin, the molecular tool investigated as a potential new treatment for the gait impairment of PD. Finally, this chapter will conclude with the rationale of the study undertaken for this thesis.

### 1.1 Parkinson's Disease

PD is the second most common neurodegenerative disorder in the world, surpassed only by Alzheimer's disease in prevalence<sup>1</sup>. PD is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which leads to changes in functional activity in the basal ganglia (BG)<sup>2,3</sup>. This disease primarily affects older populations and its incidence rate increases with age. However individuals can develop PD earlier than in their 50's<sup>1,4</sup>. Early onset PD is due to genetic mutations, however cases of familial PD are very rare<sup>1,5</sup>. For idiopathic cases average age of disease onset is 60 years old, with 1% of the population at that age developing the disease<sup>5,6</sup>. The average life expectancy following diagnosis is 14.6 ( $\pm 7.7$ ) years with men developing PD slightly more often than women, at a ratio of 3:2<sup>5,7,8</sup>. The symptoms of PD are primarily motor related but can include non-motor symptoms as well. People suffering from PD experience a reduced quality of life and this disease places great economic burden on both patients and the healthcare system<sup>9</sup>. Additionally, average life expectancy is rising worldwide, thereby increasing the aging population of the world<sup>4</sup>. Due to this, it is estimated that by 2030, the prevalence of PD will more than double, leading to a growing economic burden unless further research is done to improve prevention, treatment, and

palliative care options<sup>1</sup>.

### 1.1.1 Symptoms

PD is primarily a movement disorder and thus the most recognizable symptoms are motor related. However, there are non-motor symptoms strongly associated with this disease as well. Motor symptoms tend to appear in the early stages of the disease and are a result of dopaminergic nigrostriatal denervation<sup>10</sup>. Intermediate and advanced stages of disease are characterized by motor fluctuations, dyskinesia, and cognitive impairment<sup>10</sup>.

There are four motor symptoms that are cardinal to PD: bradykinesia, rest tremor, rigidity, and postural instability<sup>11, 12</sup>. These symptoms are usually asymmetric in onset and manifest after approximately 80% of dopaminergic terminals in the nigrostriatal system are already lost<sup>13-15</sup>.

The foremost cardinal symptom, as it is found in every case of PD, is bradykinesia, which is defined as slowness of movement<sup>12</sup>. Bradykinesia is highly debilitating as it leads to difficulties in performing daily tasks, particularly those requiring fine motor control such as dressing, eating, and bathing<sup>11, 12</sup>. Bradykinesia impairs movement initiation, execution, and reaction times, which can lead to injury<sup>16-18</sup>. Manifestations of bradykinesia can present as difficulty with swallowing (leading to drooling), loss of facial expression, reduced blinking, monotonic and/or quiet speech, reduced arm swing while walking, and a reduction of spontaneous hand gestures<sup>17, 18</sup>.

The most recognizable symptom of PD is a tremor that is present during rest<sup>12</sup>. Rest tremor is an involuntary, slow, and coarse shaking, specifically between a frequency of 4-6 hertz, which is present in approximately 70% of patients at initial diagnosis<sup>13</sup>. Tremor ceases during voluntary movement and during deeper stages of sleep<sup>12</sup>. Tremor is typically seen in the hands, initially affecting one side, but both hands could become affected with disease progression<sup>12</sup>. One feature of tremor is a phenomenon called pill-rolling in which the index finger and thumb rub together in circular motions.

The third cardinal feature is rigidity. Rigidity is a stiffness and resistance to limb movement which is caused by excessive and continuous contraction of muscles, leading to increased muscle tone<sup>12</sup>. There are two types of rigidity seen in PD, lead-pipe rigidity

and cogwheel rigidity. Lead-pipe rigidity is a sustained resistance to movement through the whole range of motion while cogwheel rigidity is a jerky resistance in which the muscles tense and relax<sup>12</sup>. Rigidity can be associated with joint pain<sup>12</sup>. In the early stages of disease, rigidity tends to affect only one side of the body and the neck and shoulder muscles<sup>19</sup>. As the disease progresses, both sides of the body can be affected along with extremities and facial muscles leading over an overall reduced ability to move<sup>19</sup>.

The fourth cardinal feature is postural instability. Postural instability is difficulty in maintaining an upright or steady posture during both movement and standing. This feature is more common in the later stages of disease and leads to impaired balance causing patients to trip or fall<sup>12, 20</sup>. Falls in the elderly often lead to bone fractures, reduced mobility, fear/anxiety, and reduced quality of life<sup>21, 22</sup>.

PD can be associated with additional motor symptoms, called secondary motor symptoms. These most commonly include gait disturbances such as shuffling gait, fenestration, and freezing of gait<sup>12, 23</sup>.

Although primarily a movement disorder, PD is associated with many non-motor symptoms as well. Some non-motor symptoms can be present before the development of motor symptoms<sup>4, 12, 24</sup>. Non-motor symptoms of PD include autonomic dysfunction such as orthostatic hypotension, excessive sweating, constipation, and urinary disturbances<sup>25, 26</sup>. Neuropsychiatric disturbances, cognitive impairment (executive dysfunction, memory impairment, dementia, visuospatial impairment), and mood disorders (anxiety, depression, apathy) are commonly present<sup>25, 26</sup>. Sensory abnormalities (olfactory dysfunction) and sleep disturbances such as rapid eye movement sleep behavior disorder are common as well<sup>4, 10, 12, 27</sup>. The combination of motor and non-motor symptoms leads to a decreased quality of life, an increase in patients overall disability, and an economic burden placed on individuals and the health care system.

### 1.1.2 Pathology

There are two main pathological characteristics of PD. The first is dopaminergic cell death in the substantia nigra and the second of the presence of Lewy bodies/neurites in several brain areas<sup>28, 29</sup>.

At the time of a patient's death, approximately 80% of dopaminergic cells in the SNpc have degenerated compared to healthy brains<sup>28</sup>. The most affected area is the ventrolateral component of the SNpc, which contains projections to the dorsal putamen of the striatum<sup>4</sup>. The symptoms of bradykinesia and rigidity are suggested to be caused by moderate to severe dopaminergic cell death within this area<sup>14</sup>. Neuronal death also occurs in other regions, such as the locus coeruleus (LC), nucleus basalis, pedunculopontine tegmental nucleus, raphe nucleus, dorsal motor nucleus of the vagus nerve, amygdala, and hypothalamus<sup>4, 30</sup>.

Dopaminergic cell death is not the only contributor to PD pathology as Lewy body aggregates play an important role as well. When the protein  $\alpha$ -synuclein becomes abnormal and misfolds, it develops into insoluble aggregates of intracellular inclusions<sup>4, 28</sup>. They are called Lewy bodies when these inclusions are within the cell body and Lewy neurites when they are in the processes of neurons<sup>33</sup>. Lewy pathology has also been found in the spinal cord and peripheral nervous system, including the vagus nerve, sympathetic ganglia, cardiac plexus, enteric nervous system, salivary glands, adrenal medulla, cutaneous nerves, and sciatic nerve<sup>4</sup>. The Braak staging system is used to classify the degree of pathology and disease progression in PD and is based on pathological findings<sup>33, 34</sup>. This system contains six stages with each stage outlining the structures that are pathologically affected<sup>33, 34</sup>. Stages 1 and 2 are the early stages of the disease when patients have not yet developed motor symptoms but can have some non-motor symptoms present such as impaired olfaction, sleep disturbances, or constipation<sup>33-35</sup>. At these stages, Lewy neurites are more prevalent than Lewy bodies and Lewy pathology is found in lower brainstem structures (dorsal nucleus of the vagus nerve, medulla oblongata, pontine tegmentum) and the olfactory system (olfactory bulb, anterior olfactory nucleus)<sup>34</sup>. In stages 3 and 4 is when motor symptoms begin to develop<sup>35</sup>. The areas mentioned in the earlier stages show more Lewy pathology and in stage 3, the SNpc will contain Lewy inclusions as well<sup>33, 35</sup>. Pathology then spreads into the nucleus basalis of Meynert and by stage 4, has also spread into the amygdala and thalamus, along with extensive dopaminergic cell death in the SNpc<sup>33-35</sup>. In stages 5 and 6, cognitive impairment develops as the pathology has spread into the neocortex and into structures of the temporal, parietal, and frontal lobes<sup>35</sup>. There is extensive cell death in the SN, the

dorsal motor nucleus of the vagus nerve, the gigantocellular reticular nucleus, and the LC<sup>33</sup>. Stage 6 is the final and most severe stage where Lewy pathology has spread throughout the neocortex, affecting both motor and sensory areas in the brain<sup>33-35</sup>.

Other pathological mechanisms that have been examined include reduced mitochondrial activity due to impaired functioning of proteasomal and lysosomal systems<sup>36, 37</sup>.

### 1.1.3 Diagnosis

Whether a patient definitively had PD cannot be confirmed until post-mortem, when the brain is pathologically examined. A degeneration of the SNpc as well as Lewy body pathology must be found for a confirmed diagnosis of PD<sup>4, 38</sup>. However, physicians can make a fairly accurate pre-mortem diagnosis, with studies suggesting the current accuracy rate to be around 80%<sup>39</sup>. Observations that can be made for diagnosis includes symptom onset affecting the body asymmetrically, as well as a good response to levodopa are supportive of a PD diagnosis<sup>29, 38</sup>. Misdiagnosis of PD can occur, with the most common misdiagnosis being essential tremor, Alzheimer's disease, dementia with Lewy bodies, and vascular parkinsonism<sup>40</sup>. Some features that can suggest a diagnosis other than PD is the absence of rest tremor, occurrence of gait impairment early in disease, and poor response to levodopa<sup>12</sup>.

### 1.1.4 Etiology

While it is known that PD symptoms arise from dopaminergic cell death in the SNpc, it is not known what triggers this very specific cell death. The majority of cases are idiopathic and likely a result of an interaction between various environmental and genetic risk factors<sup>4</sup>.

The research on environmental factors contributing to the development of PD is correlational and the risk of development of PD due to any individual environmental factor is modest<sup>41</sup>. Some of the environmental risk factors that have been suggested to be

linked with PD are exposure to pesticides, herbicides, heavy metals, low levels of urate in blood serum, and prior head injuries<sup>5, 41, 42</sup>. Interestingly, smoking (cigarettes or smokeless tobacco) and frequent consumption of caffeinated beverages such as coffee, has been found to be protective against PD according to correlational studies<sup>41, 43</sup>.

Although PD is mostly idiopathic, approximately 5-10% of all cases are caused by a known genetic mutation<sup>5, 44</sup>. However, these cases can have a distinct presentation such as young onset, as compared with idiopathic PD, thus are considered a distinct category of PD<sup>5</sup>. Both autosomal dominant and autosomal recessive gene mutations have been linked to the development of PD with the most common genes being SNCA, Leucine-rich repeat kinase 2 (LRRK2), low-density lipoprotein receptor-related protein 10 (LRP10), and  $\beta$ -Glucocerebrosidase (GBA)<sup>4, 45, 29</sup>. The SNCA gene has been extensively studied as this gene encodes alpha-synuclein, a protein that is the main component of Lewy bodies<sup>44</sup>.

However, since very few cases of PD can be attributed to a known mutation, it is believed that environmental factors and genetic susceptibility interact together for the development of PD in the majority of cases<sup>5</sup>. The cellular mechanisms that are affected by these gene mutations that lead to the development of PD are hypothesized to be associated with lysosomal dysfunction leading to mitochondrial failure, oxidative stress, inflammation, and protein misfolding leading to protein aggregation<sup>5, 29, 46, 47</sup>.

### 1.1.5 Treatment

There is currently no cure for PD and treatment is centered on symptom management, with the most focus towards alleviating the motor symptoms<sup>4</sup>. The two general treatment options available are medications or neurosurgery. The main families of drugs used for PD treatment are levodopa, dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors<sup>48</sup>. When medication loses efficacy, as it tends to occur after about 5-6 years of use, neurosurgery can provide symptomatic relief<sup>49</sup>.

The majority of patients are treated using medication. The aim of most medications for PD treatment is to increase dopamine concentrations in the brain or directly stimulate remaining dopamine receptors<sup>4</sup>. The most effective medication for PD is L-3,4-

dihydroxyphenylalanine, otherwise known as L-DOPA or levodopa. Levodopa is a precursor of dopamine and is capable of crossing the blood-brain barrier, which dopamine cannot do<sup>50</sup>. Levodopa is usually administered with a peripheral dopamine decarboxylase inhibitor such as carbidopa or benderizine, in order to reduce the amount of levodopa that gets peripherally metabolized into dopamine. This reduces side-effects such as nausea, vomiting, and orthostatic hypotension, which occur when levodopa is converted to dopamine peripherally<sup>4, 51</sup>. It also increases the amount of levodopa that crosses the blood-brain barrier<sup>49, 50</sup>.

In the early stages of the disease, levodopa is most effective in improving rigidity, akinesia, and bradykinesia<sup>4, 11, 23, 52</sup>. Improvements in tremor with levodopa are inconsistent and variable in patients, especially at lower doses<sup>4, 23</sup>. Unfortunately, levodopa provides little improvement in certain motor symptoms such as gait and balance impairment, and does not offer relief for non-motor symptoms associated with PD<sup>23, 53, 54</sup>.

It is also important to note that the effectiveness of levodopa decreases as the disease progresses. Levodopa has a short half-life in the body, requiring patients to take the medication regularly and after long term use (typically between 4-6 years), its effects become sporadic and unpredictable<sup>55</sup>. The combination of neuronal loss from disease progression, as well as the long term use of levodopa leads to the development of motor complications such as fluctuations and dyskinesias<sup>11, 13, 23, 56</sup>. However, it is important to note that severity and disease duration have been found to be more strongly correlated with levodopa-related dyskinesia rather than duration of treatment, therefore there is no benefit in withholding or delaying initial levodopa therapy<sup>57</sup>.

In the late stages of PD, the aim is to offer as much symptom relief as possible while controlling the motor fluctuations caused by medication<sup>50</sup>. An alternative to levodopa, dopamine agonists, which bind to dopaminergic post-synaptic receptors thus having a similar effect as dopamine, are often used<sup>7, 50</sup>. While dopamine agonists are less effective at symptom management than levodopa, their effect is generally found to be sufficient in the first few years of treatment<sup>58</sup>. Another alternative to levodopa is MAO-B inhibitors, which increase the amount of dopamine in the brain by blocking the activity of MAO-B, an enzyme that breaks down dopamine<sup>50</sup>. Similar to dopamine agonist use, MAO-B inhibitors are often used to delay the use of levodopa early in disease, however MAO-B

inhibitors are associated with more adverse side-effects and are found to be less effective at motor symptom management than levodopa<sup>50</sup>.

For patients in the advanced stages of disease for whom medication is no longer sufficient in controlling their symptoms or began to experience intolerable side effects from medication, neurosurgery is a good treatment option. The most common brain regions that are targets in surgery are the STN, the internal division of the globus pallidus (GPi), the pedunculo-pontine tegmental nucleus, and ventral nucleus of the thalamus<sup>59</sup>.

One option is lesioning surgeries in which specific regions of the brain are intentionally and permanently damaged. By lesioning a specific target, over activity of that region can be suppressed. For example, lesioning the GPi, which is hyperactive in PD, has been found to improve dyskinesia and lesions of the ventralis intermedius of the thalamus have been found to improve tremor<sup>59</sup>.

However, lesion surgeries are not preferred now, and the most common neurosurgical treatment for PD is deep brain stimulation (DBS). Unlike lesioning, DBS is reversible, does not destroy tissue beyond the implantation of the device, and can be modified to the needs of the individual patient. In DBS a device called a neurostimulator is implanted into a brain region, most commonly the STN, and the electrodes in the device spread electrical impulses around the surrounding region, thus affecting neuronal activity. As long as a patient does not present with severe neuropsychiatric problems, DBS is a recommended treatment option for most motor symptoms of PD, especially tremor<sup>60</sup>. A major shortcoming of DBS is that it is not effective in improving postural instability, gait, and freezing of gait<sup>23</sup>. Therefore, there is still an unmet need in treatment options for patients who experience postural disturbances and impaired gait. These symptoms severely impair mobility, independence, and quality of life in patients.

## 1.2 Neural Circuitry Involved in Parkinson's Disease

The following section is a review of the important aspects of the neural circuitry involved in PD. Voluntary motor movement involves the communication between the motor and sensory regions of the cerebral cortex, and the BG<sup>61, 62</sup>. In particular, this thesis will focus on the neural circuitry of the BG, as this region is severely impacted by PD<sup>62</sup>.

The dysfunction of the BG leads to the motor symptoms seen in PD, which is the focus of this thesis<sup>62</sup>. The major structures and pathways of the BG will be described.

### 1.2.1 Basal Ganglia Circuitry in a Healthy Brain

The role of the BG circuitry is to process signals coming from the cerebral cortex, and produce output signals back to the cerebral cortex, allowing for the proper execution of voluntary movement through a feedback loop<sup>2, 63</sup>. The BG is a group of interconnected subcortical nuclei which span the telencephalon, diencephalon, and the midbrain<sup>62</sup>. The BG is composed of the striatum, globus pallidus, substantia nigra (SN), and subthalamic nucleus (STN)<sup>2</sup>. The striatum is made up of a dorsal portion (caudate nucleus, putamen), and ventral portion (nucleus accumbens)<sup>2</sup>. The globus pallidus can also be further differentiated into the internal division of the globus pallidus (GPi) and the external division of the globus pallidus (GPe). In rats, the equivalent of the human GPi is the entopeduncular nucleus (EPN)<sup>2</sup>. The SN can be further divided into the substantia nigra pars compacta (SNpc) and substantia nigra pars reticulata (SNpr)<sup>2</sup>.

A representation of the main connections of the BG circuitry in a healthy brain is presented in Figure 1A. The main input structure of the BG is the striatum, and the main output structures are the GPi and SNpr<sup>2, 62</sup>. The striatum receives mostly glutamatergic, thus excitatory, input from all areas of the cerebral cortex and dopaminergic input from the SNpc. The striatum sends out GABAergic (inhibitory) projections to the GPi/SNpr, and GPe<sup>64</sup>. The striatal output GABAergic neurons express D1 and D2 receptors, whose functional segregation led to the division of the striatal output into two pathways, the direct and indirect pathway<sup>2</sup>. D1 receptors are mainly located in neurons that project directly to the main output structures of the BG, the GPi/SNpr, hence the name direct pathway. Meanwhile, D2 receptors are mainly on neurons that do not directly project to the main output structures, instead projecting to the GPe, and then from GPe to the STN and finally from the STN to the GPi/SNpr, hence the name indirect pathway<sup>65</sup>.

In the direct pathway, dopamine from the SNpc exerts an excitatory effect on striatal D1 receptors. The inhibitory GABAergic striatal neurons then project to the GPi/SNpr<sup>2, 62</sup>. In the indirect pathway, dopamine, in contrast to its role in the direct pathway,

provides inhibitory effect on the striatal D2 neurons<sup>2</sup>. These D2 receptor-containing GABAergic striatal neurons project to the GPe<sup>2</sup>. In addition to receiving afferent inhibitory projections from the striatum, the GPe also receives excitatory glutamatergic projection from the STN<sup>64</sup>. The GPe also sends inhibitory projections to many other structures (GPi/SNpr, SNpc), but this thesis will focus on its main target, the STN<sup>64</sup>. The STN, the only glutamatergic nucleus of the BG, then sends excitatory output to the GPi/SNpr, the output nuclei of the BG, thus completing the indirect pathway<sup>2</sup> (Figure 1).

The main output structures of the BG, the GPi/SNpr, contain GABAergic neurons, thus send inhibitory projections primarily to the ventral anterior and ventral lateral thalamic nuclei<sup>64</sup>. The thalamic nuclei then project excitatory, glutamatergic input back to the motor cortex<sup>64</sup>.

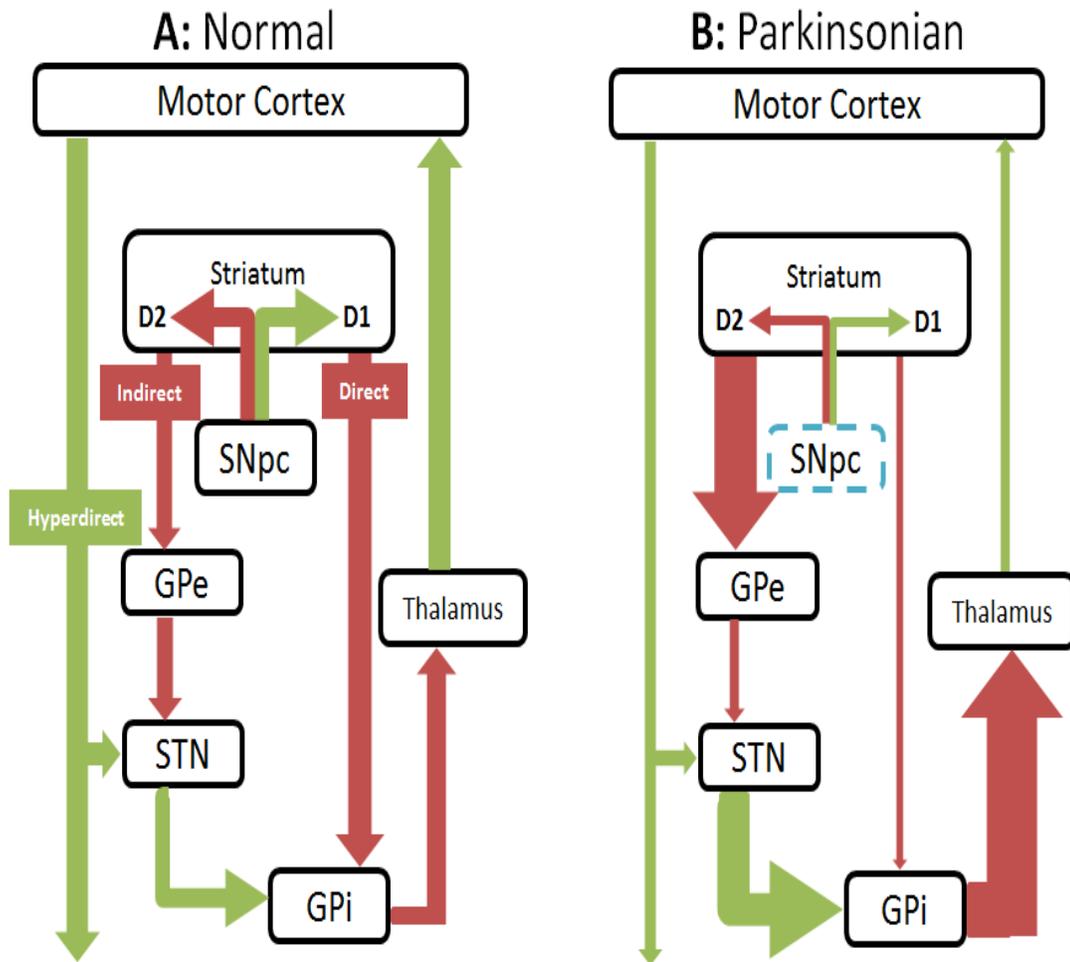
There is a recent additional pathway, called the hyper-direct pathway, which has been the subject of many recent studies examining BG circuitry. In this pathway, the STN receives excitatory glutamatergic afferents directly from the motor and premotor cortex<sup>62</sup>. This makes the STN the only other BG structure, other than the striatum, to receive significant direct cortical input<sup>2, 62</sup>. As this pathway bypasses the striatal processing of cortical input, it has been named the hyper-direct pathway<sup>66</sup>.

The neurotransmitter at the center of this system is dopamine, which acts as a modulator of the BG circuitry<sup>2</sup>. The importance of the overall function of dopamine in the motor system can be understood by its role in the BG, which is disinhibition of the system. The general role of the BG is to inhibit motor systems to prevent them from being active until appropriate<sup>31</sup>. Thus, when we want to move, the inhibition of the motor system is reduced, allowing us to move. As dopamine's role in the BG is to facilitate disinhibition, normal levels of dopamine will promote motor activity, while low levels (as seen in PD) will inhibit movement thus requiring more effort for proper motor output<sup>31</sup>. When dopamine is in excess, such as when patients are taking dopamine replenishing or boosting medications, involuntary movements, known as dyskinesia, will occur<sup>31</sup>. When dopamine acts on the D1 receptors in the striatum, the role of the direct pathway is facilitated. When dopamine acts on the D2 receptors in the striatum, the indirect pathway is inhibited. When the circuitry works in tandem in a healthy brain, voluntary movement is facilitated. When dopamine is depleted, as is the case in PD due to cell death in the

SNpc, this leads to changes in the BG circuitry leading to suppression of voluntary motor function.

### 1.2.2 Basal Ganglia Circuitry in Parkinson's Disease

The dopaminergic cell death that occurs in the SNpc in this disease leads to functional changes throughout the BG circuitry<sup>2, 62</sup>. An overview of the functional changes in BG circuitry in PD is presented in Figure 1B. The loss of dopamine neurons in the SNpc leads to underactivity of the D1 and D2 receptors of the striatum<sup>63</sup>. Thus, the direct pathway contributes to the under-inhibition of the GPi/SNpr, leading to its hyperactivity in PD. The GPi/SNpr is not receiving as many inhibitory signals from the striatum, and in turn become overactive. The GPi/SNpr send inhibitory projections, thus an overactive GPi/SNpr will over-inhibit the ventrolateral thalamus. The ventrolateral thalamus, which sends excitatory projections, gets over-inhibited by the GPi/SNpr and thus sends fewer excitatory inputs to the motor cortex. The indirect pathway in PD also contributes to the over activity of the GPi/SNpr. In the indirect pathway, the loss of dopamine activity in the SNpc leads to underactivity of the D2 receptors of the striatum<sup>63</sup>. Thus, there is an increase in inhibitory input from the striatum to the GPe. The GPe gets over-inhibited and itself sends inhibitory output. Therefore, less inhibitory output is sent from the GPe to the STN. The STN, a structure that sends excitatory projections, is under-inhibited, making the STN hyperactive. A hyperactive STN then sends excitatory projections to the GPi/SNpr, thereby contributing to this area's hyperactivity through the indirect pathway<sup>2</sup>. Ultimately, there is increased inhibition of the thalamocortical motor centers, which are then unable to facilitate movement, leading to the development of PD motor symptoms such as tremor, rigidity, and bradykinesia<sup>61, 63</sup>.



**Figure 1.1 - Basal Ganglia Circuitry in a Normal and Parkinsonian Brain**

Red arrows represent inhibitory projections and green arrows represent excitatory projections. **A** represents the functional connectivity in a normal brain. The direct, indirect, and hyper-direct pathways are labelled in this figure. **B** represents the functional connectivity in a Parkinsonian brain. The blue dashed lines around the SNpc represent the dopaminergic cell death found in PD, and the thickness of the arrows represent consequential changes in the activity of the regions of the BG due to this dopaminergic cell death. Figure adapted from Bergman, H., Wichmann, T., & DeLong, M. R. (1990).

## 1.3 Animal Models of Parkinson's Disease

Animal models are an invaluable tool for studying human disorders. Experiments that would not be possible in humans can be conducted in animal models of disease, making them useful for studying pathogenesis and treatment options. There are a number of PD animal models available, each with its own advantages and disadvantages. A general disadvantage of PD animal models is that none perfectly replicate the human disease<sup>67</sup>. Most animal models of PD lack the progressive course of PD and the non-motor symptoms associated with the disease<sup>67</sup>. Certain animal models will also be associated with a specific set of symptoms but not others. Most PD animal models also lack the typical Lewy body inclusions seen in PD<sup>68</sup>. However, there is still great value in replicating certain aspects of a disease in order to closely investigate it and further our knowledge of the disease. A researcher must carefully consider and select the animal model related to the specific goals and research questions of their study. In PD, there are two main categories of animal models employed: genetic or neurotoxin models<sup>67-69</sup>.

### 1.3.1 Genetic Models of PD

Although the majority of PD cases appear idiopathic, the use of genetic animal models can be very valuable as they can provide insight into the molecular processes of PD pathology. Gene abnormalities identified in familial PD have led to the development of genetic animal models of PD, with examples of transgenic rodent models showing alpha-synuclein, LRKK2, PINK1, PARKIN, DJ-1, and ATP13A2 mutations<sup>67</sup>. It is important to note that none of the genetic rodent models fully duplicate the human PD condition. Current models often exclude Lewy body inclusions and there is an absence of consistent neuronal loss in the SNpc<sup>67</sup>. Another issue in current genetic models that requires troubleshooting is that many models show an inconsistent phenotype, even among lines with the same mutations<sup>67</sup>.

### 1.3.2 Neurotoxic Models of PD

Currently, the most widely used neurotoxic animal models of PD in the literature are 6-hydroxydopamine (6-OHDA) lesions in rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesions in mice and primates<sup>67, 70</sup>. A key difference between neurotoxic animal models of PD and the human disease is that the neurodegeneration in animal models occurs rapidly. In animal models, neurodegeneration occurs over a few days while in humans the degeneration occurs over years<sup>67</sup>. Neurotoxic models also lack Lewy body inclusions<sup>67</sup>. An advantage of neurotoxic models is their ability to robustly model the degeneration of the nigrostriatal pathway and their tendency to generate behavioural motor abnormalities<sup>67, 68</sup>.

MPTP is a highly lipophilic drug that readily crosses the blood-brain barrier and produces bilateral lesions of SN dopamine neurons<sup>70</sup>. After crossing the blood-brain barrier, MPTP is converted into MPP<sup>+</sup> by the enzyme monoamine oxidase-B in astrocytes<sup>6, 70</sup>. MPP<sup>+</sup> is then selectively taken up by dopaminergic neurons by the vesicular monoamine transporter 2 where it inhibits complex 1 of the mitochondrial electron transport chain<sup>71</sup>. This reduces ATP production and increase release of reactive oxidative species which leads to neuronal death<sup>71</sup>. The strength of this model is that the damage MPTP causes to the nigrostriatal DA pathway is identical to what's seen in PD<sup>67</sup>. MPTP affects most mammals, with the most commonly used animals being mice and monkeys. Interestingly, rats are highly resistant to MPTP, making them an unsuitable animal for use with this toxin<sup>72</sup>. MPTP mouse models are mainly used to investigate neuronal degeneration processes in PD, while the MPTP monkey models are mainly used to investigate fine motor behavioural and electrophysiological studies related to PD<sup>67, 68</sup>. This is because the MPTP mouse model often doesn't develop a level of impairment sufficient for behavioural study while the primate models do<sup>68</sup>. Behavioural testing in MPTP primate models are often considered necessary when new PD treatment options are developed<sup>73</sup>.

6-OHDA is a selective catecholaminergic neurotoxin, used to generate lesions in the nigrostriatal DA neurons<sup>67</sup>. 6-OHDA does not cross the blood-brain barrier, thus it must be injected directly into the brain<sup>74</sup>. 6-OHDA is taken up by catecholaminergic neurons via transporter-mediated reuptake, and once within the neurons, it exerts its neurotoxic

effects by generating oxidative stress and mitochondrial dysfunction, leading to cell death<sup>75</sup>. When creating a PD model, to prevent uptake into noradrenergic neurons, desipramine (a noradrenaline reuptake transporter blocker), is intraperitoneally injected half an hour before the 6-OHDA injection<sup>76</sup>. The most common sites of 6-OHDA injection are in the SN, medial forebrain bundle (MFB), or the striatum<sup>74, 77, 78</sup>. The most common animal model using 6-OHDA are rats. Unilateral lesions are most common, as bilaterally lesioned rats develop severe adipsia and aphagia<sup>78</sup>. Survival in bilaterally lesioned animals is possible but there are high mortality rates and animals require intensive post-surgical care which make this model highly inconvenient<sup>68</sup>. Although a unilaterally 6-OHDA rat model does not replicate all of the clinical features of PD, this model is successful at achieving dopamine depletion, nigral dopamine cell loss, and neurobehavioral deficits. In unilateral 6-OHDA-lesioned models, since there is a lesioned and a non-lesioned hemisphere, an individual animal is often used as its own control<sup>68</sup>. The asymmetrical lesion also allows for the use of the apomorphine-induced rotation test to gauge the extent of dopamine depletion and the efficacy of potential PD therapeutic agents and gene therapies<sup>79</sup>.

Apomorphine is a dopamine receptor agonist which stimulates D1 and D2 dopamine receptors<sup>70</sup>. Following subcutaneous injection of apomorphine, rats will rotate away from the site of lesion. This drug-induced behaviour occurs due to the development of postsynaptic hyper-sensitivity of the D1 and D2 receptors in the striatum following depletion of dopamine on the lesioned side. Rotations are said to only occur following a dopamine depletion of at least 80%<sup>79</sup>. The consensus in the literature is that an average of 7 rotations per minute over at least 10 minutes following subcutaneous injection of apomorphine represents an animal that has been sufficiently dopamine depleted<sup>76, 80, 81</sup>.

There are other behavioural, particularly motor, changes associated with the unilateral 6-OHDA lesion model. These motor deficits tend to affect the rats asymmetrically. There have been occasional findings of akinesia, rigidity, and very rarely tremor in 6-OHDA lesioned rats<sup>82, 83</sup>. Rats lesioned with 6-OHDA at the MFB have been found to be significantly worse at the rotarod task, a measure of forced motor movement<sup>84, 85</sup>. Lesioned rats fall off the rotating rod faster compared to sham lesioned rats. In the open field test, an assessment of exploratory locomotion, in which rats can freely roam showed

that 6-OHDA lesioned rats display significantly less motor activity compared to sham rats<sup>85</sup>. In general, the literature suggests that 6-OHDA lesioned rats display deficits in voluntary and forced motor tasks.

Ultimately, there is no animal model currently available that can perfectly mimic the human condition. Each model, whether genetic or neurotoxic, has its own advantages and disadvantages. For the current study, a MPTP primate model would serve better, but due to the novelty of the treatment that this thesis is exploring, preliminary studies in rodents must be done before moving onto a more expensive higher order animal species. Therefore, for the purpose of this thesis, the animal model that is selected is the unilateral 6-OHDA rat model, as this model is well suited to test the changes in motor output in an experimental therapeutic treatment.

## 1.4 Gait Impairment in Parkinson's Disease

### 1.4.1 Gait Impairment in Humans

As mentioned in the treatment section (1.1.5), there are no effective and consistent treatment options for the gait abnormalities seen in PD<sup>86</sup>. This affects the quality of life of those suffering from PD as gait impairments severely limit mobility and predisposes patients to falls which can be debilitating or even deadly in an elderly population<sup>22</sup>. Impaired gait in PD includes slowness when walking, shorter stride length, and a reduced arm swing<sup>54, 88</sup>. Gait abnormalities in PD presents as a walking pattern with short and shuffling steps, with occasional episodes of freezing of movement<sup>22, 87</sup>. Some parameters of gait have been found to be sensitive to levodopa treatment while others are not responsive to medication. For example, pace, which includes step velocity and step length, have been found to be levodopa sensitive and tend to improve following treatment<sup>54, 89</sup>. However, other parameters of gait such as duration of stride, duration of swing, and variability (step-to-step fluctuations) tends to be resistant to levodopa treatment<sup>54, 89</sup>. In terms of the effect of DBS on gait, the literature is inconsistent. Some long-term studies found that DBS of the STN resulted in consistent improvement of tremor, rigidity, and bradykinesia, but that there was a decline in the effectiveness on gait

disturbances over 3 years<sup>90,91</sup>, 5 years<sup>92,93</sup>, 8 years<sup>94</sup>, and 10 years<sup>95,96</sup>. However, a recent meta-analysis by Roper and colleagues (2016) found that both unilateral and bilateral DBS improved gait speed in PD patients<sup>97</sup>.

Overall, there is a need for new treatment options for Parkinsonian gait impairment as each patient is unique and responds to treatments differently. More treatment options are needed in order to improve the quality of life of those suffering from PD.

#### 1.4.2 Gait Assessment Tools for 6-OHDA Rat Model

A commonly used method for measuring forced motor movement is the rotarod test. The rotarod test assesses overall motor deficit in rodents by having them balance on a rotating rod<sup>84</sup>. The amount of time spent on the rod and maximum speed reached is recorded and used as a measure of the animal's performance on this forced motor task.

The most common assessment tool for gait in rodents is the CatWalk apparatus. This tool provides an automated and simultaneous quantification of both static and dynamic parameters of gait during voluntary locomotion<sup>98</sup>. The Catwalk has been established as a reliable tool in assessing gait in rodents models<sup>86,99,100</sup>. Several studies have assessed the 6-OHDA lesioned rat model using the CatWalk and have validated gait impairment in this model using this tool<sup>101,102</sup>. As the CatWalk apparatus is able to analyze changes in both static and dynamic gait parameters, and due to its established validity in measurement of 6-OHDA lesioned rat models, this test will be used in this study for gait analysis.

#### 1.4.3 Gait Impairment in the 6-OHDA Rat Model

As this study is investigating the effects of a potential new treatment for gait impairment in PD, gait impairment in the 6-OHDA animal model will be reviewed.

A study by Monville and colleagues (2006) assessed motor deficit using the rotarod test and found that the 6-OHDA lesioned rats compared to the sham-lesioned rats, performed significantly worse at 2, 4 and 6 weeks following lesion compared to pre-lesion states<sup>84</sup>. A study by Zhou and colleagues (2015) examined the differences in gait parameters before and after unilateral 6-OHDA lesion in three groups of rats. Rats were assigned to 6-OHDA lesion in the MFB, striatum, or SNpc<sup>101</sup>. Employing the Catwalk

apparatus, they found the most significant impairments were in the MFB lesion group and concluded that 6-OHDA lesions in the MFB is a suitable model for studying gait dysfunction<sup>101</sup>. These authors found that stance, step cycle, terminal dual stance, and duty cycle were increased in lesioned rats while paw pressure intensity and print area of the affected paw were decreased<sup>101</sup>. A study by Chuang and colleagues (2010) found a similar reduction in paw pressure and maximal area of paw contact in 6-OHDA lesioned rats<sup>99</sup>. In particular, these studies stressed the importance of terminal dual stance as this parameter suggest an increased duration of the postural phase, which they claimed is comparable to the freezing of gait seen in human PD<sup>99, 101</sup>. The reductions found in stride length and swing speed are believed to reflect increased muscle rigidity<sup>101</sup>. To summarize, in unilaterally MFB 6-OHDA lesioned rats, studies using the Catwalk apparatus found decreases in average speed, max contact area, mean intensity, stride length and swing speed<sup>99, 101</sup>. They also found increases in stand, swing, step cycle, duty cycle, and terminal dual stance<sup>99, 101</sup>. Table 1 defines the CatWalk gait parameters examined in this thesis. It is also important to note that asymmetries between the paws ipsilateral or contralateral to lesioned side were only detected for static gait parameters such as max contact area and mean intensity, but not for dynamic gait parameters in these studies<sup>99, 101</sup>. Overall, strong evidence indicates that unilateral injection of 6-OHDA into the MFB produces gait deficits that can be measured using the CatWalk apparatus.

**Table 1 - CatWalk Parameter Definitions**

<b>Parameter</b>	<b>Definition</b>
Average Speed (cm/s)	Distance traversed on the walkway divided by time
Cadence (steps/s)	Number of steps taken per second
Stand (s)	Duration of contact of a paw with the glass walkway
Swing (s)	Duration of no contact of a paw with the glass walkway
Swing Speed (cm/s)	Speed of a paw during swing
Step Cycle (s)	Duration of two consecutive contacts of the same paw
Duty Cycle (%)	Stand as a percentage of step cycle
Max Contact Area (cm <sup>2</sup> )	Maximum area of a paw that contacts the glass walkway
Mean Intensity	Mean pressure of a paw that contacts the glass walkway
Stride Length (cm)	Distance between two consecutive placements of the same paw
Terminal Dual Stance (s)	Duration of the second step in a step cycle of a paw

## 1.5 Botulinum Neurotoxin

Botulinum neurotoxins (BoNTs) are proteins produced by the anaerobic bacterium *Clostridium botulinum*<sup>103</sup>. BoNTs are the most acutely lethal toxin currently known in existence and infections with the toxin producing bacteria causes botulism, a paralytic illness<sup>104</sup>. There are eight distinct subtypes of BoNTs, named type A through H. The subtypes vary in the animal species they affect (e.g., humans, cattle, horses, fish, birds), which proteins they cleave, the location of cleavage along the protein, and the severity of paralysis the subtype causes<sup>104</sup>. Only subtypes A and B are used commercially in humans, with type A (BoNT-A) being the most common and potent of the two<sup>105</sup>. Subtypes A and B are used medically to treat diseases characterized by overactive muscles (muscle spasms) or cosmetically, with the most common brand names being Botox, Dysport, and Xeomin. Although BoNTs are the deadliest toxins in the world, at proper dosages their mechanisms of action make them very useful biological tools that are commonly used medically, cosmetically, and in research.

### 1.5.1 Mechanism of Action

BoNTs are produced as a 150 kDA polypeptide made up of a 100 kDA heavy chain (HC) and 50 kDA light chain (LC) linked together by a disulfide bond<sup>105</sup>. The main effect of BoNTs is cleaving proteins involved in releasing vesicles filled with neurotransmitters from the presynaptic membrane<sup>105</sup>. It does this through a series of steps. The first step is binding to specific nerve or neuron terminals<sup>106</sup>. This is mediated by the HC portion of the molecule. The HC contains a carboxyl terminus and an amino terminus. The carboxyl terminus is responsible for recognizing and binding to specific presynaptic nerve terminals<sup>107</sup>. The second step is internalization of the toxin into the cell, mediated by the amino terminus of the HC<sup>107</sup>. Once the toxin is inside the cell, the increase in acidity causes the disulphide bond to break, which separates the LC from the HC<sup>107</sup>. The LC is a zinc metalloprotease and the active portion of the toxin. The LC, depending on its subtype, will cleave a specific component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Each BoNT subtype acts at

different SNARE proteins and cleavage sites<sup>108</sup>. BoNT-A specifically cleaves synaptosomal nerve-associated protein 25 (SNAP-25)<sup>107</sup>. By cleaving these proteins which mediate vesicle fusion and release from the presynaptic membrane, a vesicle filled with neurotransmitter cannot be released, thus the effect of the neurotransmitter is blocked<sup>107</sup>. However, this blockage wears off as the LC loses its activity and the SNARE proteins regenerate<sup>107</sup>. To illustrate this point with a real-life example, this is why people who get cosmetic Botox injections need to repeat the procedure every few months, as BoNTs do not have a permanent effect.

### 1.5.2 BoNT use in the Peripheral Nervous System

In the peripheral nervous system (PNS), BoNT-A has a potent effect on cholinergic terminals<sup>107</sup>. By preventing the release of acetylcholine from axon endings at neuromuscular junctions, nerve signaling stops. This stop leads to flaccid paralysis<sup>107</sup>. The most common use of BoNT-A is as a cosmetic treatment for the reduction of the appearance of wrinkles. There are many other uses for BoNT-A injections in the PNS. BoNT-A is used as a treatment for disorders of muscle spasticity (in the neck, eyelids, genitals, limbs, vocal cords), disorders of hyperactive nerves (excessive sweating, neuropathic pain), to relax clenching muscles (oesophagus, jaw, urinary tract, bladder), and migraine relief<sup>109-117</sup>.

BoNT-A use in the PNS has been found to be an overall very safe procedure. The most common negative side effect of BoNT-A is the paralysis of unintended muscles caused by injection into the wrong areas. The safety of BoNT-A use in the PNS can be attested by the frequency and high number of cosmetic injections carried out each year. Yearly injections of BoNT-A treatment over 12 years has been found to be consistently safe with only a few instances of bad reactions<sup>118</sup>. The safety of BoNT-A in the PNS is well established, but investigations of the use and safety of BoNT-A in the central nervous system (CNS) are ongoing.

### 1.5.3 BoNT use in the Central Nervous System

In the PNS, BoNT-A blocks the presynaptic release of the neurotransmitter acetylcholine. In the CNS, it has been found that BoNT-A can exert its effects on multiple neurotransmitters, including glutamate,  $\gamma$ -aminobutyric acid (GABA), glycine, noradrenaline and dopamine<sup>106, 119-121</sup>. BoNT-A preferentially inhibits the release of excitatory neurotransmitters to a much stronger degree over inhibitory neurotransmitters<sup>120-122</sup>. Ashton and Dolly (1988) found that BoNT-A's ability in blocking presynaptic release of GABA compared to acetylcholine is significantly weaker<sup>120</sup>. Another study, by Bigalke and colleagues (1981) found approximately 80% blockage at 10 ug/mL of BoNT-A for the excitatory neurotransmitter's acetylcholine, noradrenalin, and glycine in preparations of rat brain and spinal cord tissue<sup>119</sup>. For the inhibitory neurotransmitter GABA however, in order to achieve a comparable level of inhibition as seen with the excitatory neurotransmitters, a 5x higher dose of BoNT-A was required<sup>119</sup>. Due to BoNT-A's specificity as an excitatory neurotransmitter blocker and its transient effects, this molecule has great potential for application in the CNS by preventing the pre-synaptic release of excitatory neurotransmitters. Studies investigating the application of BoNTs in the CNS in rodent models of disease have found that BoNTs can serve as a modulator for pain<sup>123</sup>, epilepsy<sup>124</sup>, and PD<sup>69</sup>.

### 1.5.4 BoNT-A Use as a Treatment for PD

BoNT-A is already used as an effective and safe treatment option for tremor in PD<sup>125</sup>. Injecting BoNT-A peripherally reduces tremor by blocking cholinergic activity in specific muscle groups. However, due to BoNT-A's ability to block the release of excitatory neurotransmitters, it has great potential to be used in the CNS as a molecular tool to reduce over-excitation in specific brain regions. As discussed in Chapter 1.2.2, the activity of the BG circuitry in PD is abnormal. Several studies have examined the effects of BoNT-A administered directly in the brain in parkinsonian animal models. A study by Wree and colleagues (2011) examined the results of the apomorphine rotation test in unilaterally 6-OHDA lesioned rats who received an ipsilateral injection of BoNT-A (1ng or 2ng dose) in the striatum. They found a significant reduction in pathological drug-

induced rotational behaviour for up to 6 months in rats that received a BoNT-A injection<sup>69</sup>.

Another study by Antipova and colleagues (2013) examined the effects of an ipsilateral injection of BoNT-A (1 or 2ng) in the striatum of unilaterally 6-OHDA lesioned rats. A reduction in apomorphine rotations was found but no motor improvement was found in the rotarod or open-field tests<sup>126</sup>. Although Antipova (2013) did not see improvements in forced or voluntary movement, they found neither neuronal death in the striatum nor evidence of an inflammatory response following BoNT-A injections<sup>126</sup>. The results of prior studies are promising as they demonstrate that a BoNT-A injection directly in the brain, at the doses they tested, does not cause tissue damage while also improving some aspects of motor performance. A recent study by Tsang, Rajakumar, and Jog (2019) examined the effects of a BoNT-A injection in the GPi of 6-OHDA lesioned rats compared with sham lesioned rats<sup>127</sup>. An injection of 0.5ng of BoNT-A in the GPi significantly reduced pathological rotations in the apomorphine rotation test and improved various parameters of gait as measured by the CatWalk XT apparatus. They found that rat's speed, body speed variation, cadence and walking pattern was returned to pre-lesioned performance, and the improvements persisted for up to 1-month post BoNT-A injection<sup>127</sup>. These results suggest that BoNT-A injections at the GPi can improve impaired gait in hemi-parkinsonian rats<sup>127</sup>. Administration of BoNT-A directly in the brain has potential as a new treatment option for the impaired gait seen in PD. It is of great importance to continue research on this topic as there are no treatment options currently available for the gait impairment seen in PD. Further research must be done to examine the effects of BoNT-A injections at various brain sites to determine the optimal site(s) of injection which yield the most motor improvement. The effects at different doses need to be examined as well.

## 1.6 Rationale

PD affects many people as it is the second most common neurodegenerative disorder in the population. PD is increasing in prevalence as the global aging population continues to increase as well. PD is a debilitating disorder which greatly affects patient's quality of

life and although there are reasonably effective treatment options available for most symptoms, not all patients benefit from the current options available. There is still a need for novel treatment options, particularly to improve the gait impairment seen in PD as there are no strong treatment options available for this symptom.

As discussed in section 1.2, the dopaminergic cell death in the SNpc leads to functional changes throughout the BG circuitry. DBS is one treatment option for PD that modulates the activity of certain areas of the BG circuitry to provide symptomatic relief. Another way to change the activity of specific targets in the BG circuitry is through the use of a molecular tool. As discussed in section 1.5, BoNT-A has the ability to prevent presynaptic neurotransmitter release. The mechanisms of BoNT-A have been extensively studied and described. This toxin is already safely used in medicine, cosmetics, and research. However, as BoNT-A preferentially blocks glutamatergic input, the injection sites for this treatment in the BG are limited. The two areas of the BG that receive glutamatergic input are the STN and GPi. Both of these areas are good candidate sites for BoNT-A injection as both of these sites are hyperactive in PD. Therefore, by injecting BoNT-A at these sites and blocking the excitatory input they receive, their levels of activity can be reduced to more normal levels, thereby improving motor symptoms. The site of injection that this thesis will examine is the STN, as our laboratory has previously examined the effects of a BoNT-A injection into the EPN (rodent equivalent of the human GPi) on gait performance in a PD rat model. In that study by Tsang, Rajakumar, and Jog (2019), an injection of 0.5ng of BoNT-A at the EPN in unilaterally MFB 6-OHDA lesioned rats significantly reduced pathological rotations in the apomorphine rotation test and improved some parameters of gait as measured by the CatWalk XT apparatus. The beneficial effects of the toxin lasted for up to 3 months. Specifically, in terms of gait, the lesioned rat's performance was comparable to that of sham lesioned rats in their walking speed, cadence, and walking pattern<sup>127</sup>. This study provided promising results for the use of BoNT-A injections in the BG as a treatment for gait impairment.

The current study explores the effects of a BoNT-A injection at the STN. The STN has great potential as a target site as the majority of DBS electrode implantations are done at this site. In addition, several studies have found that lesions or functional inactivation of the STN led to improvement in PD symptoms in MPTP lesioned monkeys<sup>128, 129</sup>.

Ultimately, the literature suggests that reducing the hyperactivity of the STN can lead to improvements in motor output. The hyper-direct pathway provides glutamatergic input into the STN. However, the GABAergic input from the GPe is reduced substantially. Hence, it is possible that the reduction of the glutamate input into the STN may equalize the two inputs.

Since this is a novel treatment, as with the majority of preclinical studies, the efficacy should be assessed in an animal model of PD. In section 1.3, the possible animal models of PD are discussed, with the unilateral MFB 6-OHDA lesioned rat model selected as the animal model of choice for this study. Previous studies have confirmed both extensive dopamine depletion and motor impairment in this model, making it suitable for examining motor output following a novel treatment option.

### 1.6.1 Hypothesis

The hypothesis of the current study is that the injection of BoNT-A into the ipsilateral STN of a unilaterally 6-OHDA lesioned PD rat model will improve its apomorphine-induced pathological rotations and affect gait abnormalities.

Rationale for the predicted effect is as follows: injection of BoNT-A into the STN will lead to a reduction of STN hyperactivity through the blockage of excitatory input coming from the cortex. This will lead to a reduction of the over-excitation of the EPN, leading to a reduction in the over-inhibition of the ventrolateral thalamus, thereby alleviating motor abnormalities.

### 1.6.2 Objectives

The objectives of this thesis were to assess the behavioural outcomes of a single injection of BoNT-A at the STN in a unilateral MFB 6-OHDA lesioned rat model of PD. The specific objectives of this study were as follows:

- 1) To confirm dopamine depletion in the striatum and SN in the PD animal model used.
- 2) To evaluate the dose response of BoNT-A injection at the STN in the behavioural tasks used. Four doses (0.5ng, 1ng, 2ng, and 4ng) were examined.

- 3) To evaluate changes in drug induced rotations using the apomorphine rotation test before and following injection of BoNT-A into the STN at various time points (1-week post-injection, 1-month post-injection, 2-months post-injection, 3-months post-injection).
- 4) To evaluate changes in forced motor movement using the rotarod test at baseline, after 6-OHDA lesion, and following injection of BoNT-A into the STN at various time points (1-week post-injection, 1-month post-injection, 2-months post-injection, 3-months post-injection).
- 5) To evaluate changes in voluntary motor movement using the CatWalk apparatus at baseline, after 6-OHDA lesion, and following injection of BoNT-A into the STN at various time points (1-week post-injection, 1-month post-injection, 2-months post-injection, 3-months post-injection).

### 1.6.3 Predictions

The predictions were as follows:

- 1) 6-OHDA injection at the MFB would cause dopaminergic depletion in the striatum and SN.
- 2) Injection of BoNT-A at the STN would reduce pathological drug induced rotations in 6-OHDA lesioned rats.
- 3) Injection of BoNT-A at the STN would lead to improvements in the amount of time spent on the rotarod, the forced motor movement task, in 6-OHDA lesioned rats.
- 4) Injection of BoNT-A at the STN would lead to improvements in gait in the voluntary motor movement task as measured by the CatWalk in 6-OHDA lesioned rats.
- 5) The effects of the BoNT-A injection would be transient, with a peak effect at around the 1-month post BoNT-A injection time-point and motor performance returning to post-lesion around the 3-months post BoNT-A injection time-point.

As BoNT-A has never been injected at the STN before, there are no predictions as to which dose will be most effective.

## Chapter 2

### 2 Materials and Methods

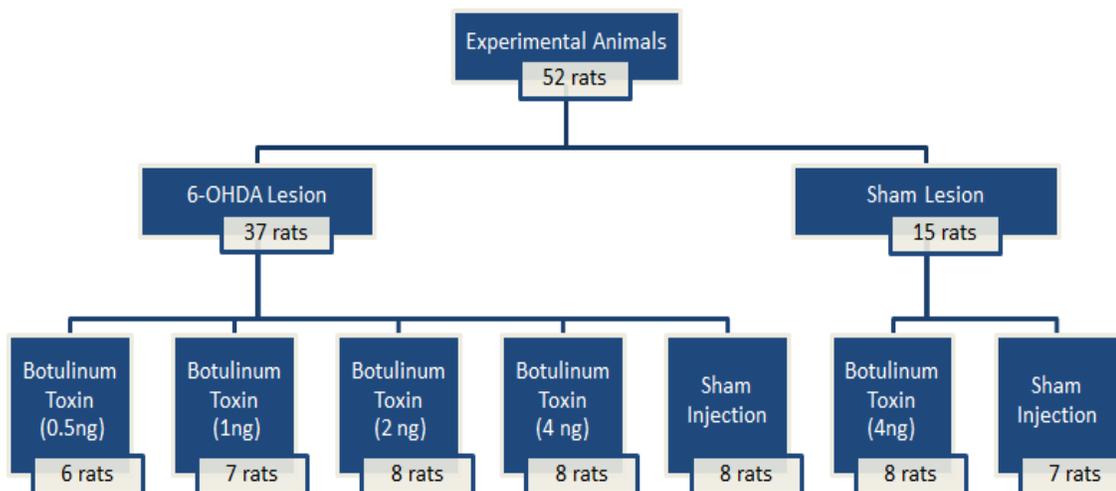
This chapter describes the methods and materials utilized in this study. All experimental work was approved under the protocol, AUP 2015-087, and conducted in accordance with the guidelines of the Animal Care Committee at Western University (Appendix 1). All regulatory materials permitting the use of BoNT-A and sodium pentobarbital in rats were approved (Appendix 2 & 3).



**Figure 2.1 - Timeline of the Experimental Protocol**

## 2.1 Experimental Animals

Adult male Sprague-Dawley rats (Charles River Canada), weighing between 250-300 grams at the time of the first surgery, were used in this study. Rats were housed 2-3 per cage, however, under the circumstance that one of the animals in the pair was sacrificed early, the surviving animal was housed alone. Animals were housed in a temperature and humidity-controlled room ( $22\pm 1^{\circ}\text{C}$ ) under 12 hour light/12 hour dark conditions with free access to food and water.



**Figure 2.2 - Breakdown of Experimental Conditions that Animals were Assigned to**

## 2.2 Surgery

This section will describe the procedures for each surgery the animals went through. First, the procedures common to both surgeries will be described and the following sections will detail specificities associated with the particular surgery.

### 2.2.1 General Surgery Procedures

All animals were subcutaneously injected with Metacam (1 mg/kg), an anti-inflammatory medication for pain management, 15 minutes before the start of each surgery as well as a dose the day following surgery. Animals were anaesthetized through

an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). The animal's body heat was monitored through the use of a rectal thermometer and thermoregulation was maintained between 36-38°C using a heat lamp, both during surgery and recovery. Level of sedation was monitored using the toe pinch withdrawal reflex. Once the withdrawal reflex was absent, indicating that the rat was deeply sedated, lubricating eye-drops were applied, and the area of the scalp where the incision would be made was shaved with an electric razor. The animal was then placed and secured onto a stereotaxic frame (Kopf Instruments). The surgical site was cleaned with surgical soap, isopropyl alcohol and a chlorhexidine solution before incision. A midline skin incision of approximately 2 cm was made and all soft tissue from the surface of the skull was removed to expose bregma. A burr hole of approximately 1 mm was drilled through the skull at the designated coordinates for the surgery to allow for the needle to go through into the desired brain region. Stereotaxic coordinates were calculated from bregma according to the rat brain atlas of Paxinos and Watson (2009).

### 2.2.2 First Surgery - MFB Lesion using 6-OHDA

To render the animals hemi-Parkinsonian, they received a unilateral infusion of 6-OHDA into the right MFB. Approximately 30 minutes before the infusion of 6-OHDA into the brain, all animals were intraperitoneally injected with desipramine hydrochloride (25 mg/kg, Sigma), a selective norepinephrine reuptake inhibitor. 6-OHDA lesions both noradrenergic and dopaminergic terminals thus the injection of desipramine protects the noradrenergic terminals and thus causing a lesion selectively for dopaminergic terminals.

After a burr hole was drilled in the skull at the MFB coordinates (AP -1.8, ML -2, DV - 8.3) (Appendix 4), a 10µl Hamilton syringe (Cole-Parmer) was lowered and a 4 µL solution containing 8 µg 6-OHDA /rat (100 mg of 6-OHDA dissolved 50 mL solvent consisting of 0.9% saline and 0.1% ascorbic acid; Sigma) was delivered over 5 minutes. The needle was then left in place for 5 minutes and then slowly withdrawn over another 5 minutes.

Control animals receiving a sham lesion went through the same procedure as the 6-OHDA lesioned animals but received an equivalent volume of 0.9% saline containing

0.1% ascorbic acid.

### 2.2.3 Second Surgery - BoNT-A Injection at the STN

Three weeks after animals received their first lesioning/control surgery, they underwent the experimental treatment surgery in which either BoNT-A or vehicle was injected at the STN. BoNT-A (List, Campbell, USA) was injected into the STN (AP -3.6, ML -2.5, DV -8.0) (Appendix 5) using a 31G injection cannula attached to a 10  $\mu$ L Hamilton syringe by polyethylene tubing. All doses were injected over 5 minutes with a volume of 0.5  $\mu$ L. The cannula was left in place an additional 5 minutes after injection and then retracted over an additional 5 minutes.

All doses were dissolved in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Animals receiving a BoNT-A injection were assigned to one of four doses.

1) 0.5 ng (0.5  $\mu$ L of 1 ng/ $\mu$ L BoNT-A)

2) 1 ng (0.5  $\mu$ L of 2 ng/ $\mu$ L BoNT-A)

3) 2 ng (0.5  $\mu$ L of 4 ng/ $\mu$ L BoNT-A)

4) 4 ng (0.5  $\mu$ L of 8 ng/ $\mu$ L BoNT-A)

Control animal received an injection of 0.5  $\mu$ L of PBS with 0.1% BSA.

## 2.3 Behavioural Measures

The purpose of the behavioural measures conducted was to evaluate the changes in animals motor activity between baseline performance (before any surgeries), after inducing a PD state (lesioning/sham surgery), and after the treatment intervention (BoNT-A/sham injection).

### 2.3.1 Apomorphine Rotation Test

The purpose of the apomorphine rotation task is to assess the quality of the 6-OHDA lesion. It is also a measure used to assess drug-induced movement. The animals were assessed after two weeks have passed from their 6-OHDA or sham surgery. After the rats

are subcutaneously injected with apomorphine (0.25 mg/kg; Sigma), and since the MFB was unilaterally lesioned, they would rotate away from the lesioned side (see Chapter 1.3.2.2 for details). The right MFB was lesioned, therefore the rats would rotate counter-clockwise (CCW).

After receiving the apomorphine injection, a rat would be placed into a polypropylene cage (14 inches x 20 inches, 8 inches tall). A video camera setup recorded the behaviour for 30 minutes. The videos were transferred to a desktop computer, and the rat's rotational behaviour was quantified over 10 minutes of video footage. Clockwise (CW) and CCW rotations would be counted and each animal would receive a score based on their net rotations. This net score is divided by 10 to determine the average number of rotations per minute (rpm) for each rat. A sufficiently lesioned, and thus a successful parkinsonian model, should have a score of at least 7 rpm. If an animal that had been assigned to the lesioned group did not meet that score, it was removed from the study and sacrificed.

The rotation test was repeated at 1-week, 1-month, 2-months, and 3-months after animals received their BoNT-A injection surgery to assess changes in drug-induced movement.

### 2.3.2 Rotarod

To assess rat's performance on a task of forced motor movement, the rotarod apparatus (Panlab, Harvard Apparatus) was used. The rotarod is a standard test of motor coordination, balance and fatigue in rodents in which the animals are placed on rotating rods and the time until the animal falls off the rod is recorded.

In this study, the animals were placed on the rod under acceleration mode, in which the rotational speed of the rod will increase from 4 to 40 rpm over 5 minutes. Therefore, after every 8 seconds, the rpm of the rod increases by 1 rotation. Three trials per rat were collected with an interval of at least 30 minutes between trials. The maximum amount of time (in seconds) and the maximum speed of the rod (rpm) reached before a rat fell off were recorded. Those three trials per animal would be averaged into one score for statistical analysis. Both time and speed were examined because the difficulty of the task

for the rat increases over time. As time passes, the speed of the rod increases. For example, at 0 seconds, the rod starts at 4rpm. After 30 seconds, the rpm is 7. After another 30 seconds (1-minute total) have passed, the speed of the rod is 11, and after another 60 seconds have passed (2 minutes in), the rpm is increased to 18.

### 2.3.3 Catwalk

To assess the gait of voluntarily moving rats, the CatWalk XT (Noldus, Wageningen, Netherlands) apparatus was used. The Catwalk method is an automated and computerized gait-analysis technique that allows objective quantification of multiple static and dynamic gait parameters. The apparatus consists of a glass walkway, surrounded by Plexiglas walls except for the two ends to serve as openings (entrance and exit). In a dark room, a fluorescent light shines onto the walkway from one side, and as the light is reflected downward, the footprints as the rat freely walks across the walkway are recorded by a camera mounted underneath the glass. The principle of this method is based on an optical technique in which the light of a fluorescent tube is completely internally reflected in the glass walkway. The light leaves the glass and illuminates the areas of contact as the animal crosses the walkway. The paw contacts are visualized, signals are digitized, and then stored on a computer for analysis.

For proper gait analysis, there are two criteria that must be met when collecting data<sup>130</sup>. 1) The animals must cross the walkway within 8 seconds with no interruptions in their gait or turning around, and 2) a minimum of three crossings per animal are required.

Two whole body parameters were examined, average speed and cadence. The rest of the gait parameters measure each paw, left front limb (LF), left hind limb (LH), right front limb (RF), and right hind limb (RH) separately.

## 2.4 Perfusion and Brain Extraction

After all behavioural data was collected, animals were sacrificed with an intraperitoneal injection of 1 mL of sodium pentobarbital (250 mg/kg). After injection, animal's reflexes were assessed using the toe pinch reflex. Once all reflexes were absent,

the animal underwent a trans-cardiac perfusion. An incision in the abdomen was made to enter the body cavity and extended into the thoracic cavity. Once the heart was exposed, a needle attached to tubing and a pump, was inserted through a small incision made in the left ventricle into the aorta. An incision was made in the right atrium to allow for blood and other fluids to drain from the animal. 200 mL of 0.9% saline was pumped to clear the blood, followed by 400 mL of 4% paraformaldehyde to cause tissue fixation. The rat's brain was removed and put into a specimen bottle containing 4% paraformaldehyde for at least 24 hours. The brain was then transferred into a specimen bottle containing 30% sucrose for cryoprotection.

## 2.5 Immunohistochemistry

Brains were blocked for the region of interest and cut using a freezing microtome after being mounted with optimal cutting temperature (OCT) compound. Coronal brain sections of 40  $\mu\text{m}$  thickness were cut. All incubations on the rotator were done at room temperature. Whenever washing sections is mentioned, the washes were done three times in phosphate buffer (PB) for 5 minutes each on a shaker at low speed. Images were acquired on a Nikon DS-Qi2 microscope.

### 2.5.1 Thionin Nissl Staining

The STN is a small region and it is possible to miss this site during the BoNT-A injection surgery. In addition to the site being small, the brain atlas is for Sprague Dawley rats between 250-350g. Due to the nature of this study's timeline, most animals are over 400g at the time of their second surgery, thus the coordinates may be slightly off. In order to ensure that the needle and injection was at the STN, a 0.25% thionin nissl stain was performed in order to visualize the needle track leading to the STN.

First, STN brain sections were selected and mounted onto 1 mm thick Superfrost plus slides (VWR International). The slides were left to air dry overnight. The next day, the slides containing sections were de-lipidated through transfer in 70%, 95% and then in 100% ethanol twice for 2 minutes each, followed by submersion in 100% xylene twice

for 15 minutes each. The slides were then transferred in decreasing concentration of ethanol (100% to 95% to 70%) for 2 minutes each, followed by a 2-minute submersion in distilled water. The slides were then placed into a 0.25% thionin solution (Sigma-Aldrich) for 10 seconds. The slides were then dipped between 5-10 times in distilled water in order to dilute the blue stain to its desired intensity. The slides then went through dehydration through transfer in increasing concentration of ethanol (75% to 95% to 100%-1 to 100%-2) for 2 minutes each, followed by two consecutive submersions in 100% xylene for 5 minutes. No. 1 cover glasses (VWR International) coated with xylene-based mounting medium (Triangle Biomedical Sciences Inc) were applied to the surface of the slides.

### 2.5.2 TH Staining

Staining for tyrosine hydroxylase (TH) was done for verification of 6-OHDA lesioning. TH staining was done at the striatum, substantia nigra, and locus coeruleus. Sections with regions of interest were selected, washed, and then put on a rotator for a maximum of 2 hours with blocking solution (0.001% Triton X-100, 15% BSA and 10% non-immune serum from the animal which corresponded to species of the secondary antibody). Sections were then directly put into the 1<sup>0</sup> antibody solution (antibody diluted in distilled H<sub>2</sub>O containing 15% BSA and animal serum) for at least 14 hours on a rotator and the next day, were washed. Sections were then put into the 2<sup>0</sup> antibody (diluted in PB containing 15% BSA and animal serum) for maximum 1 hour on a rotator. The 2<sup>0</sup> antibody was either an Alexa Fluor conjugated secondary antibody (Invitrogen by Thermo Fisher Scientific) or a biotinylated secondary antibody (Vector Laboratories). After, sections were washed and put into avidin biotin peroxidase complex (ABC) (Vectastain Elite ABC-HRP Kit, Vector Laboratories) solution for 1 hour on the rotator for amplification of signal. Sections were washed and then put into TSA Biotin System (PerkinElmer), with 3% H<sub>2</sub>O<sub>2</sub> added in a time-dependent manner. A timer used to ensure that sections were kept in the solution for the same amount of time in the solution at 10 minutes each. Following washes, TH was detected by the 3,3'-diaminobenzidine (DAB) method for 5 minutes (Sigma-Aldrich). Sections were then mounted onto 1 mm thick

Superfrost plus slides and left for at least 24 hours to dry. Once dry, sections were then dehydrated by transferring slides in increasing concentration of ethanol (75% to 95% to 100%-1 to 100%-2) for 2 minutes each, followed by two consecutive submersions in 100% xylene for 5 minutes. Cover glasses coated with xylene-based mounting medium were applied to the surface of the slides.

## 2.6 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20. All data is expressed as mean  $\pm$  standard error of mean. A critical value for significance of  $p < 0.05$  was used throughout the study. If the assumption of normality was violated, the intended tests would be run regardless as the regular/repeated-measures one-way ANOVA is fairly robust to deviations from normality. Normality also implies that the data follow a normal distribution. In the case of the apomorphine rotation test, for example, a normal distribution may not be expected. At the post-lesion time point for example, it is expected that all lesioned animals will rotate at least 7 times per minute or more while the sham-lesioned animals will not rotate (thus their scores will be around 0 rpm). The scores will therefore be on either extreme (0 rpm or 7rpm), with no Gaussian distribution. In this case, it is expected that the data should not be normal, and violations of normality are expected in this case. Any violations of normality will be reported in the results section for transparency.

### 2.6.1 Analysis of Apomorphine Data

The first section of the apomorphine rotation test results were analyzed using a one-way ANOVA. In the first section, the scores of every condition were compared at the same time-point. In the second section of the apomorphine rotation test results, a repeated measures one-way ANOVA was run. In the second section, the scores of each condition (separately) were compared across every time point. For both sections, the ANOVA was followed by a Bonferroni post-hoc test. Normality was assessed using the Shapiro-Wilk's test. Sphericity was assessed using Mauchly's test of sphericity, and homogeneity of

variances was assessed by Levene's test for equality of variances. In cases where the assumption of homogeneity was violated ( $p < .05$ ), the results were interpreted using the Welch ANOVA and Games-Howell post-hoc test.

### 2.6.2 Analysis of Rotarod Data

A comparison between all animal groups at the pre-lesion and post-lesion time points was done to determine if the 6-OHDA rat model induced any significant impairment on this forced motor task compared to sham lesioned animals. At pre-lesion, before animals have undergone any surgery, it would be expected that there is no significant difference between animal groups.

A one-way ANOVA was run. The scores for each condition at both time points were assessed for normality using the Shapiro-Wilk's test. Homogeneity of variances was assessed by Levene's test for equality of variances. In cases where the assumption of homogeneity was violated ( $p < .05$ ), the results were interpreted using the Welch ANOVA and Games-Howell post-hoc test.

### 2.6.3 Analysis of Catwalk Data

A comparison between all animal groups at the baseline and post-lesion time point was done to determine if the 6-OHDA rat model induced significant motor impairment compared to sham lesioned animals. At pre-lesion, before animals have undergone any surgery, it would be expected that there is no significant difference between animal groups. At the post-lesion time point however, based on our laboratories previous study, all lesioned groups are expected to perform worse than the sham lesioned groups<sup>127</sup>.

Two of the CatWalk parameters that were presented are whole body measures (average speed and cadence), meaning that an overall score was presented for the whole body. Those two parameters will be presented first in the CatWalk results section. A one-way ANOVA for both time points (pre- and post-lesion) was run, with a Bonferroni post-hoc test for multiple comparisons. For the rest of the CatWalk parameters, there is a score for each individual paw. Therefore, a one-way ANOVA with a Bonferroni post-hoc was run for every paw at both time points. For all analyses, normality was assessed using the

Shapiro-Wilk's test and homogeneity of variances was assessed by Levene's test for equality of variances. In cases where the assumption of homogeneity was violated ( $p < .05$ ), the results were interpreted using the Welch ANOVA and Games-Howell post-hoc test.

## Chapter 3

### 3 Results

#### 3.1 Verification of 6-OHDA Lesion

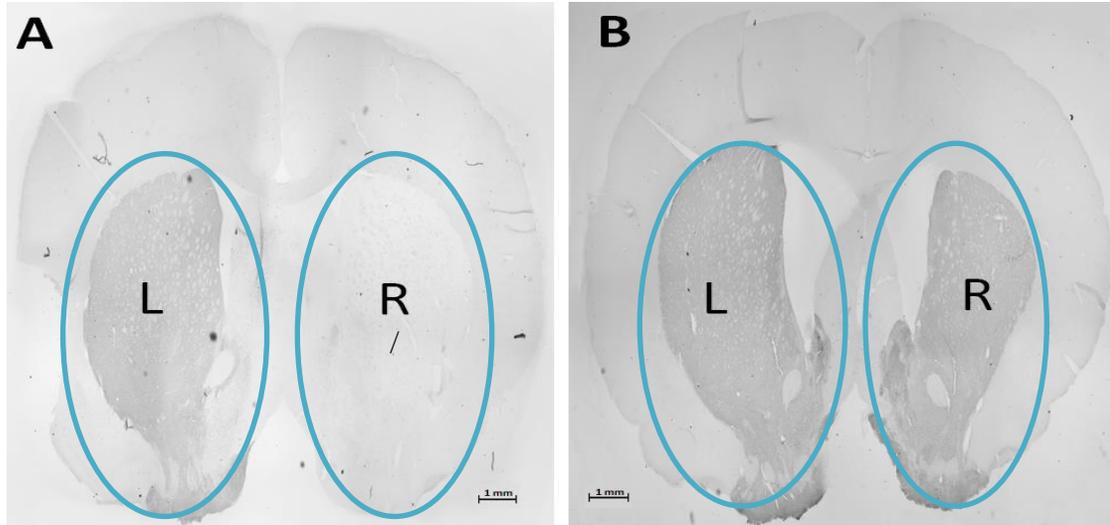
Unilateral 6-OHDA lesioning of the MFB was visually verified in a random selection of lesioned animals through staining of tyrosine hydroxylase (TH)-positive terminals in the striatum (Figure 3.1 A) and cell bodies in the SN (Figure 3.2 B). Sections from sham-operated animals were also labeled for TH-positive terminals in the striatum (Figure 3.1 B) and SN (Figure 3.2 B). 7 randomly selected 6-OHDA lesioned animals (20% of the total lesioned animals) were stained for TH. 3 randomly selected sham lesioned animals (20% of the total sham animals) were stained for TH. 6-OHDA lesioning of the right MFB resulted in a reduction in TH immunoreactivity in the ipsilateral striatum and SN while a sham saline injection did not result in a reduction in TH immunoreactivity on both sides in the striatum and SN.

Additionally, to confirm that the injection of desipramine successfully prevented damaged to noradrenergic terminals, labeling of TH-positive neurons in the LC was examined. The LC of a representative 6-OHDA lesioned animal is presented in Figure 3.3 A, and the LC of a representative sham-operated animal is shown in Figure 3.3 B.

Visual inspection comparing the lesioned and non-lesioned side in 6-OHDA lesioned animals as well as comparison with sham lesioned animals suggests that there was a substantial depletion of dopaminergic cells in the striatum and SN.

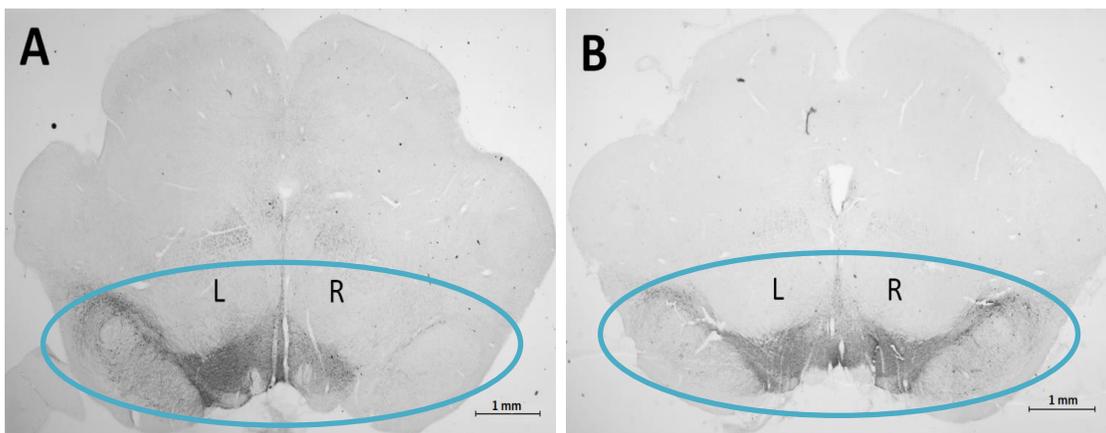
#### 3.2 Verification of STN Injection Site

In order to verify that BoNT-A injection was at the STN, a Nissl stain was performed in coronal sections to visualize the needle track leading to the STN. The needle track of a representative section is shown in Figure 3.4. This method provides a visual confirmation of whether the STN was accurately targeted.



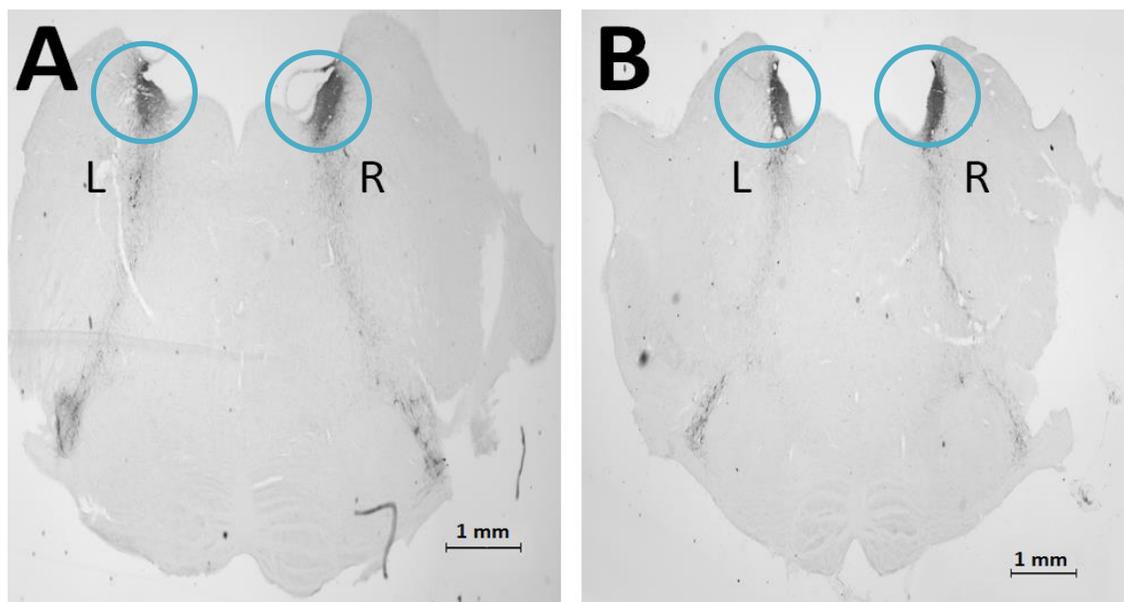
**Figure 3.1 - TH Staining of the Striatum**

TH staining in the striatum of a representative 6-OHDA lesioned animal (A) and a representative sham lesioned animal (B). The contralateral/left striatum (L) and ipsilateral/right striatum (R) are labelled. The areas of interest (striatum) are outlined in a blue oval. 6-OHDA lesioning of the right MFB resulted in a reduction in TH immunoreactivity in the ipsilateral striatum while a sham saline injection did not result in a reduction in TH immunoreactivity on both sides. Scale bars: 1 mm.



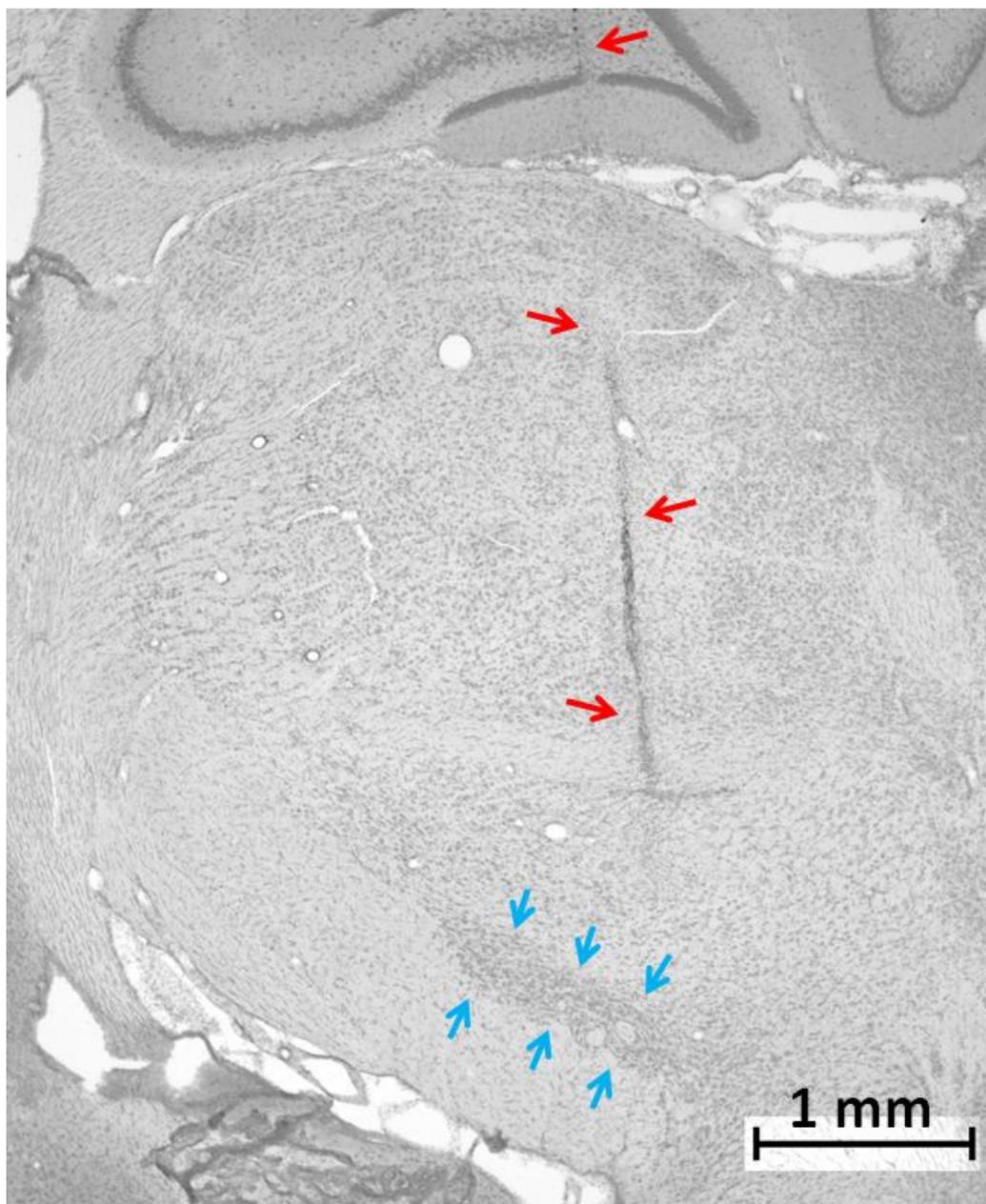
**Figure 3.2 - TH Staining of the STN**

TH staining in the SN of a representative 6-OHDA lesioned animal (A) and a representative sham lesioned animal (B). The contralateral/left SN (L) and ipsilateral/right SN (R) are labelled. The areas of interest (SN) are outlined in blue ovals. 6-OHDA lesioning of the right MFB resulted in a reduction in TH immunoreactivity in the ipsilateral SN while a sham saline injection did not result in a reduction in TH immunoreactivity on both sides. Scale bars: 1 mm.



**Figure 3.3 - TH Staining of the LC**

TH staining in the LC (the cluster of darkly stained cells at the top) of a representative 6-OHDA lesioned animal (A) and a representative sham lesioned animal (B). The contralateral/left LC (L) and ipsilateral/right LC (R) are labelled. The areas of interest are outlined in blue ovals. 6-OHDA lesioning of the right MFB did not result in a reduction in TH immunoreactivity in the LC in both lesioned and sham lesioned animals with the injection of desipramine 30 mins before 6-OHDA injection, thus sparing noradrenergic terminals in the LC. Scale bars: 1 mm.



**Figure 3.4 - Nissl Stain for Needle Track to STN Confirmation**

Thionin Nissl staining to visual the needle track in a representative animal that received injection into the STN (AP -3.6, ML -2.5, DV -8.0). Red arrows are pointing to the needle track and the blue arrows point to the neuron cluster that composes the STN. The needle track leads to the medial portion of the STN. Due to the volume injected ( $0.5 \mu\text{l}$  would occupy  $0.5 \text{ mm}^3$ ), it is likely the STN (volume in a rat is  $0.8 \text{ mm} \pm 0.1$ ), was sufficiently flooded with BoNT-A<sup>152</sup>. Scale bar: 1 mm.

### 3.3 Apomorphine Rotation Test Results

#### 3.3.1 Effect of BoNT-A at Each Time Point

This section will report on the results of the all animal groups compared at the same time point. Comparing the scores (rpm) of each animal group at the same time-point will provide insight into the effect of BoNT-A on pathological rotations at each time point. This allows for a comparison of the efficacy of each dose of BoNT-A in reducing pathological rotations at each time point.

At every time point, the lesion/sham BoNT-A injection group and lesion/0.5ng BoNT-A injection group did not significantly reduce in the number of pathological drug-induced rotations. At every time point, those two groups were not significantly different from each other. Additionally, at every time point, the sham lesion/4ng BoNT-A group and sham lesion/sham BoNT-A group were not significantly different from each other in their rotational score.

At the post-lesion time point, lesioned groups rotated at 7 rpm or higher, while sham lesioned animals remain at 0 rpm (Figure 3.5 A). According to multiple comparisons with Bonferroni post-hoc, none of the lesioned groups were significantly different from each other. Likewise, neither of the sham lesioned groups were significantly different from each other. As expected, all of the lesioned animals rotated at significantly higher rates than the sham lesioned animals [ $F(6, 45) = 88.654, p < .001$ ]. The scores on the apomorphine rotation test were normally distributed in every group, except for the lesion + 2ng BoNT-A group ( $p = .008$ ). There was homogeneity of variances.

At the 1-week post treatment injection time point, there were significant differences between various groups [ $F(6, 45) = 19.481, p < .001$ ] (Figure 3.5 B). According to Bonferroni post-hoc, the lesion/sham BoNT-A group, lesion/0.5ng BoNT-A group, and lesion/1ng BoNT-A group, did not differ significantly in their rpm at this time point. However, compared to the lesion/sham BoNT-A group, the lesion/2ng BoNT-A ( $p < .001$ ) group, and lesion/4ng BoNT-A group ( $P < .001$ ) had significantly fewer pathological rotations. At this time point, there were no significant differences between the lesion/2ng BoNT-A, lesion/4ng BoNT-A and the two sham lesioned groups (4ng

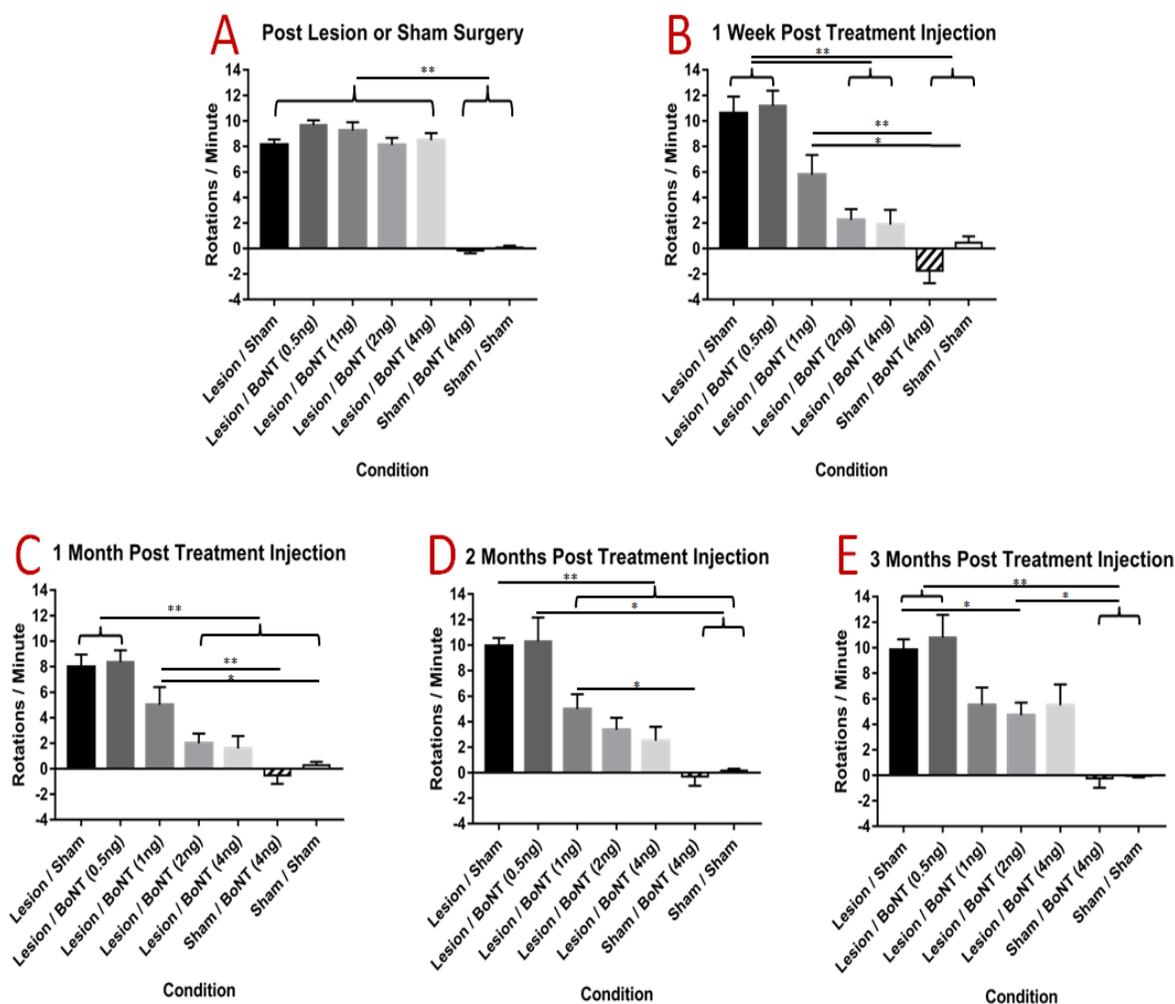
BoNT-A and sham BoNT-A). There were also no significant differences between the sham lesioned animals who received a 4ng BoNT-A or sham BoNT-A injection ( $p = .47$ ). Therefore, at this time point, the lesioned animals who received the two highest doses of BoNT-A (2 or 4ng) rotated at comparable rates to that of the sham lesioned animals. The scores on the apomorphine rotation test were normally distributed for every group, except for the lesion/4ng BoNT-A group ( $p = .02$ ), sham/4ng BoNT-A group ( $p = .041$ ), and sham/sham BoNT-A group ( $p = .03$ ). There was homogeneity of variances.

At the 1-month post-treatment injection time point, there were significant differences between the various animal groups [ $F(6, 45) = 15.51, p < .001$ ] (Figure 3.5 C). The lesioned animals who received a 1ng ( $p < .001$ ), 2ng ( $p < .001$ ), or 4ng ( $p < .001$ ) injection of BoNT-A continue to show a significantly lower number of pathological rotations compared to the lesion/sham group at this time point. However, only the lesioned animals who received either 2ng or 4ng of BoNT-A were found to not be significantly different from either of the sham lesioned groups. The scores on the apomorphine rotation test were normally distributed for every group, except for the lesion/4ng BoNT-A group ( $p = .02$ ), sham/4ng BoNT-A group ( $p < .001$ ), and sham/sham BoNT-A group ( $p = .002$ ). There was homogeneity of variances.

At the 2 months post-treatment injection time point, there were significant differences between various animals, Welch's  $F(6, 17.913) = 38.807, p < .001$ . (Figure 3.5 D). The lesioned animals who received a 1ng, 2ng, or 4ng injection of BoNT-A continued to rotate below 7 rpm and the scores of these groups were significantly lower compared to the lesion/sham group at this time point. However, only the lesioned animals who received either 2ng or 4ng of BoNT-A were found to not be significantly different from either of the sham lesioned groups. The scores on the apomorphine rotation test were normally distributed for every group, except for the sham/4ng BoNT-A group ( $p = .02$ ). The assumption of homogeneity of variances was violated ( $p = .013$ ), therefore significance was interpreted using the Welch ANOVA and the Games-Howell post-hoc was used to interpret the multiple comparisons.

At the 3 months post-treatment injection time point, there were significant differences between various groups [Welch's  $F(6, 17.43) = 30.677, p < .001$ ] (Figure 3.5 E). While all of the lesioned groups that received an injection of BoNT-A at a dose of 1ng or higher

continued to rotate below the pathological threshold of 7 rpm, the number of rotations were returning to pathological baseline. Only the lesion/2ng BoNT-A group was found to still be significantly different from the lesion/sham BoNT-A ( $p = .019$ ). The scores on the apomorphine rotation test were normally distributed for every group, except for the lesion/sham BoNT-A group ( $p = .038$ ), and the sham/sham group ( $p = .01$ ). The assumption of homogeneity of variances was violated ( $p = .007$ ), therefore significance was interpreted using the Welch ANOVA and the Games-Howell post-hoc was used to interpret the multiple comparisons.



**Figure 3.5 - Apomorphine Rotations of Every Group Compared at the Same Time Point**

A comparison of apomorphine (0.25 mg/kg, SC) induced rotations between all groups at the same time point. Positive values on y-axis represent counter-clockwise rotations and negative values represent clockwise rotations. Lesion/Sham (n=8), lesion/0.5ng BoNT-A (n=6), lesion/1ng BoNT-A (n=7), lesion/2ng BoNT-A (n=7), lesion/4ng BoNT-A (n=8), sham/4ng BoNT (n=8), sham/sham (n=7). Apomorphine rotations 2 weeks post-lesion or sham surgery (A), 1-week post BoNT-A injection (B), 1-month post BoNT-A injection (C), 2 months post BoNT-A injection (D), 3 months and post BoNT-A injection (E). All results are presented as mean  $\pm$  SEM. One-way ANOVA and post hoc Bonferroni test were performed, except for the 2-month (D) and 3-month (E) post injection time points where a Games-Howell post-hoc test was performed, \* $p < 0.05$ , \*\* $p < 0.005$ .

### 3.3.2 Effect of BoNT-A Over Time

This section will report on the results of the apomorphine rotation test scores of each animal group separately across time points. Comparing the performance of one group across time will provide insight to the effect of BoNT-A on pathological rotations over time for each group. The results of the control groups will be presented first, followed by the results of the experimental groups.

#### 3.3.2.1 Control Groups Results

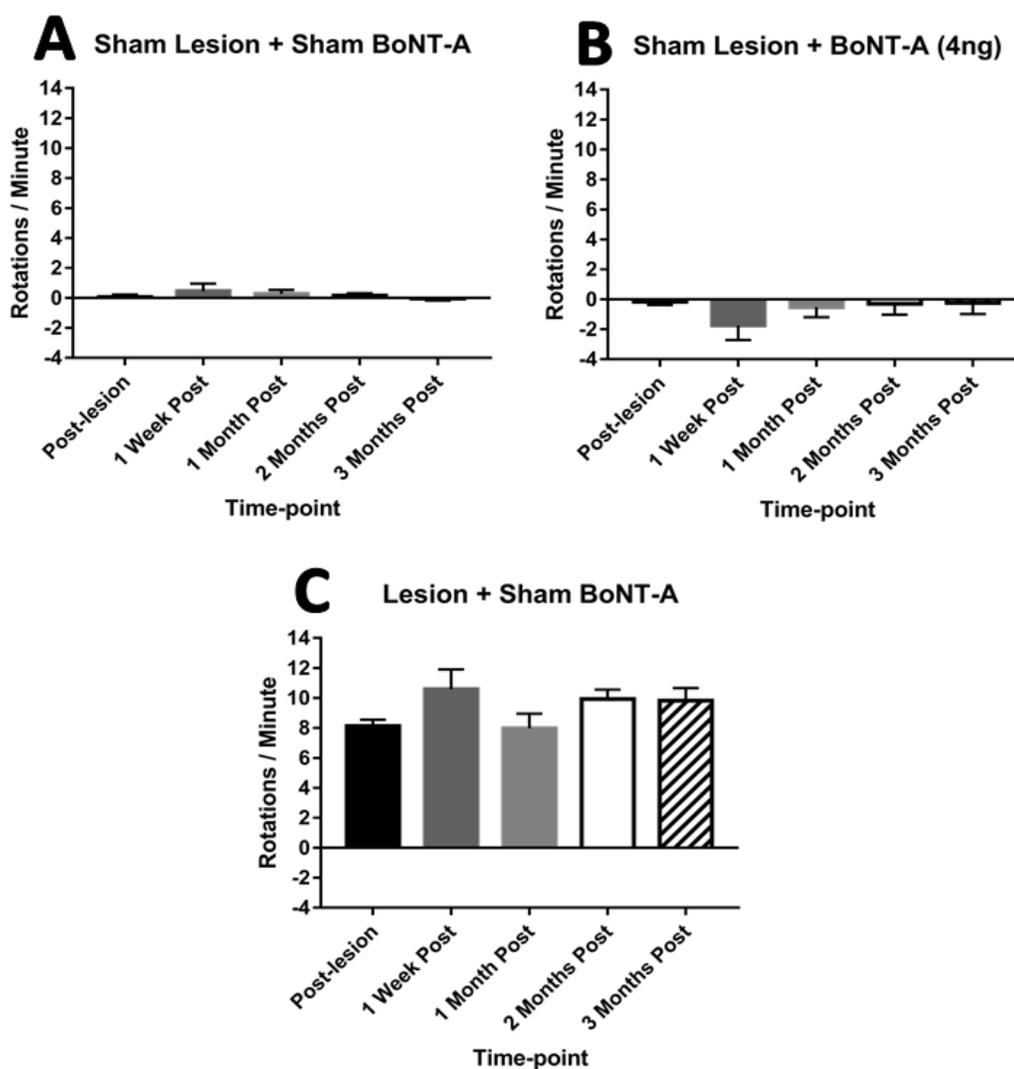
Sham lesioned animals displayed no significant rotational behaviour at any time-point, regardless of whether animals received a 4ng dose of BoNT-A or sham injection during the treatment surgery (Figure 3.6 A-B).

A sham BoNT-A injection in sham lesioned animals did not cause significant changes in drug-induced rotational behaviour at any time point [ $F(1.406, 8.433) = .934, p = .396$ ] (Figure 3.6 A). Rotations were normally distributed, except at the 1-week ( $p = .03$ ), 1 month ( $p < .002$ ), and 3 month post ( $p = .01$ ) time points. The assumption of sphericity had been violated,  $\chi^2(9) = 19.558, p = .029$ , thus the results were interpreted and reported using the Greenhouse-Geisser adjustment. Epsilon ( $\epsilon$ ) was 0.351 and used to correct the one-way repeated measures ANOVA.

An injection of the highest dose of BoNT-A examined in the study (4ng) in sham lesioned rats did not cause significant changes in drug-induced rotational behaviour at any time point [ $F(1.618, 11.323) = 2.856, p = .106$ ] (Figure 3.6 B). Rotations were normally distributed except at the 1-week ( $p = .041$ ), 1-month ( $p < .001$ ), and 2-month post ( $p = .019$ ) time points. The assumption of sphericity had been violated,  $\chi^2(9) = 24.632, p = .005$ , thus the results were interpreted and reported using the Greenhouse-Geisser adjustment. Epsilon ( $\epsilon$ ) was 0.404 and used to correct the one-way repeated measures ANOVA.

A sham BoNT-A injection in lesioned rats led to no reduction in pathological rotational behaviour at any post-treatment time point, remaining at above 7 rpm (Figure 3.6 C). Although rpm was found to be statistically significantly different across time [ $F(4, 28) = 3.708, p = .015$ ], upon examining the Bonferroni post-hoc, no significant

differences were found between time points. However, the difference between the 1-week post and 1-month post-treatment injection time points were close to approaching significance ( $p = .053$ ). This may account for why significance was found at the tests of within-subjects effects level but not at the pairwise comparison level. Rotations were normally distributed at each time point, except at 3 months post ( $p = .038$ ) and the assumption of sphericity had not been violated.



**Figure 3.6 - Apomorphine Rotations across Time Points in Control Groups**

Changes in apomorphine (0.25 mg/kg, s.c) induced rotations before and after BoNT-A or sham injection at the STN. Positive values on the y-axis represent counter-clockwise rotations and negative values represent clockwise rotations. (A)  $n=7$ , (B)  $n=8$ , (C)  $n=8$ . A sham injection in place of BoNT-A in sham lesioned rats did not cause any significant changes in rotations at any time point (A). An injection of 4ng of BoNT-A in sham lesioned rats did not cause any significant changes in rotational behaviour (B), and a sham injection in place of BoNT-A in 6-OHDA lesioned rats also did not cause any changes in rotations at any time point (C). All sham lesioned rats remained at around 0 rotations/minute, while 6-OHDA lesioned rat always remained at above 7 rotations/minute. All results are presented as mean  $\pm$  SEM. A repeated measures one-way ANOVA and post-hoc Bonferroni test was performed.

### 3.3.2.1 Experimental Groups Results

Injection of apomorphine (0.25 mg/kg) caused at least 7 CCW rpm in the 6-OHDA lesioned groups at the post-lesion time point (Figure 3.7 A-D). Lesioned animals displayed an average number of 8.7 rpm CCW at post-lesion.

Injection of 0.5ng of BoNT-A in lesioned rats did not cause significant differences in pathological rotations at any time point [ $F(4,20) = 29.29$ ,  $p = .244$ ] (Figure 3.7 A). Rotations were normally distributed at each time point, and the assumption of sphericity had not been violated.

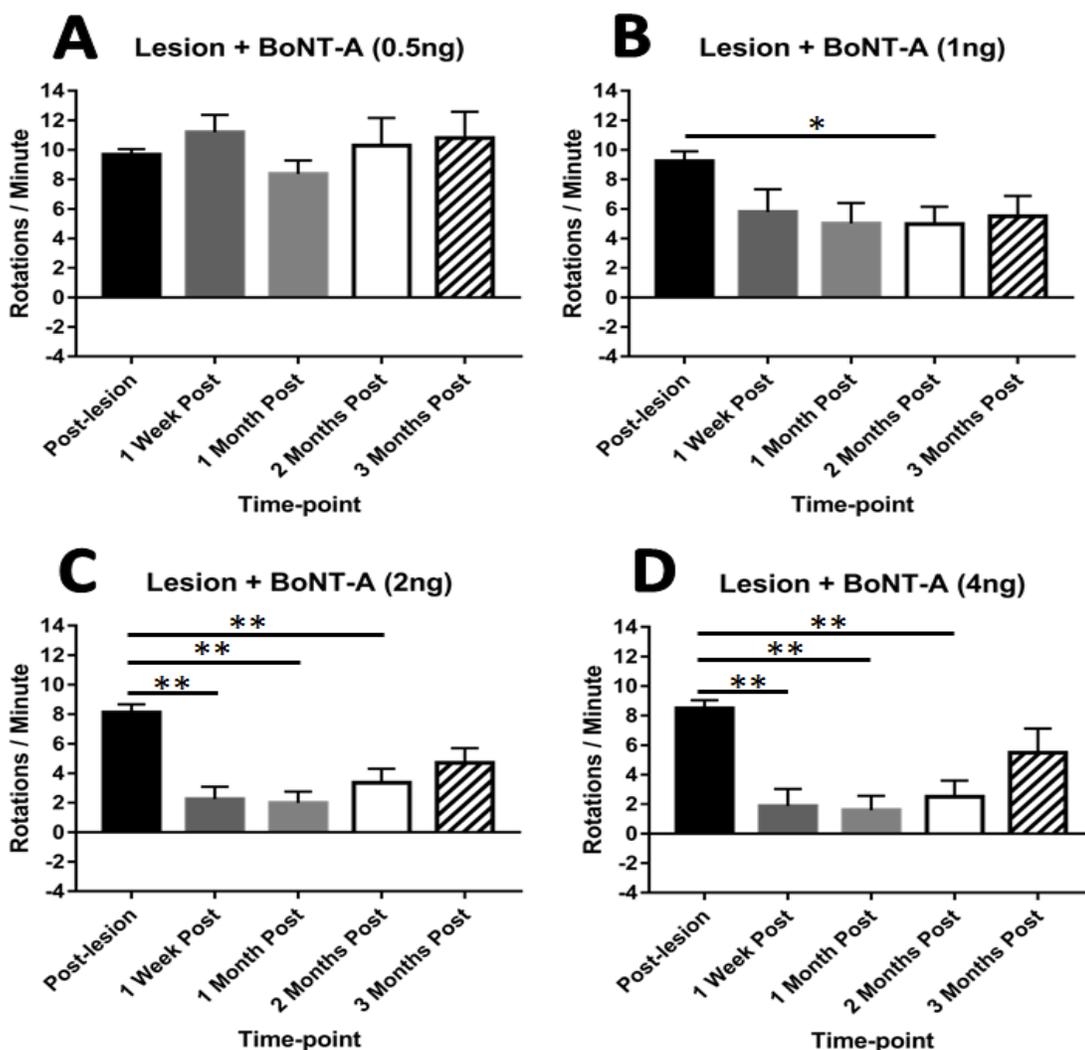
Injection of 1ng of BoNT-A in lesioned rats significantly reduced the number of pathological drug-induced rpm [ $F(4, 24) = 5.076$ ,  $p = .004$ ]. However, the only time point where there was a significant reduction in rotations from post-lesion according to Bonferroni post-hoc, was at 2-months post-treatment ( $p = .046$ ) (Figure 3.7 B). Rotations were normally distributed at each time point, and the assumption of sphericity had not been violated.

Injection of 2ng of BoNT-A in lesioned rats significantly reduced the number of pathological drug-induced rpm [ $F(4, 28) = 12.526$ ,  $p < .001$ ]. Bonferroni post-hoc revealed that rpm significantly decreased from post-lesion to 1-week post BoNT-A injection ( $p = .005$ ), post-lesion to 1-month post BoNT-A injection ( $p = .011$ ), and from post-lesion to 2-months post BoNT-A injection ( $p = .013$ ) (Figure 3.7 C). Rotations were normally distributed at each time point, except at the post-lesion time point where the data was not normally distributed ( $p = .008$ ). The assumption of sphericity had not been violated.

Injection of 4ng of BoNT-A in lesioned rats significantly reduced the number of pathological drug-induced rpm [ $F(1.823, 12.759) = 16.68$ ,  $p < .001$ ]. Bonferroni post-hoc revealed that rpm was significantly decreased from post-lesion to 1-week post BoNT-A injection ( $p = .004$ ), post-lesion to 1-month post BoNT-A injection ( $p = .001$ ), and from post-lesion to 2-months post BoNT-A injection ( $p = .009$ ) (Figure 3.7 D). Rotations were normally distributed, except at the post-lesion ( $p = .045$ ), 1-week post ( $p = .002$ ) and 1-month post ( $p = .02$ ) time points. The assumption of sphericity had been violated,  $\chi^2(9) = 22.629$ ,  $p = .01$ , thus the results were interpreted and reported using the Greenhouse-

Geisser adjustment. Epsilon ( $\epsilon$ ) was 0.456 and used to correct the one-way repeated measures ANOVA.

Changes in apomorphine induced rotations in 6-OHDA lesioned rats before and after



BoNT-A injections into the ipsilateral STN. Positive values on the y-axis represent

### Figure 3.7 - Apomorphine Rotations Across Time Points in Experimental Groups

counter-clockwise rotations and negative values represent clockwise rotations. (A)  $n=6$ , (B)  $n=7$ , (C) and (D)  $n=8$ . An injection of 0.5ng of BoNT-A did not cause a reduction in rotations at any time-point (A). An injection of 1ng of BoNT-A only caused a significant reduction in rotations at the 2-month time point (B). Both the 2ng and 4ng doses were successful in significantly reducing pathological rotations at the 1-week, 1-month, and 2-month post-injection time points (C and D). All results are presented as mean  $\pm$  SEM. Asterisks indicate a significant difference between the time points according to repeated measures one-way ANOVA and post-hoc Bonferroni test, \* $p<0.05$ , \*\* $p<0.005$ .

## 3.4 Rotarod Results

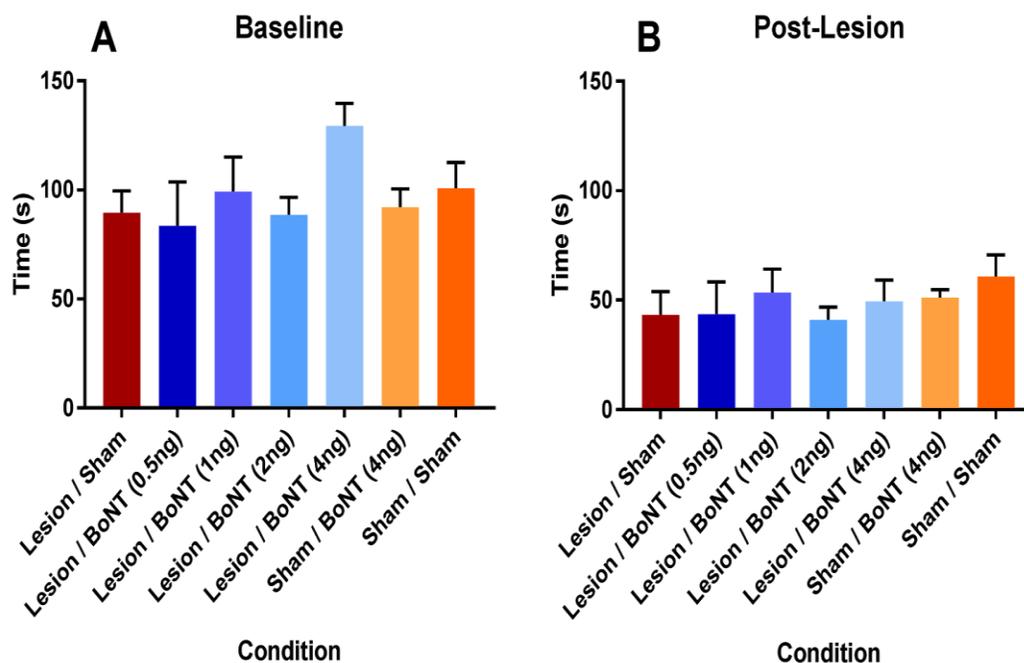
The amount of time (seconds) spent on the rotarod, as well as speed (rpm) reached before a rat fell off was recorded and analyzed. At the pre-lesion (baseline) time point, all animals are naive as none have undergone any surgeries. At the post-lesion time point, all animals have either undergone a unilateral 6-OHDA lesion or sham lesion.

### 3.4.1 Maximum Time Spent on Rotarod

The average time spent on the rod at the pre-lesion time point for lesioned animals was 98.8 (SD  $\pm$  36.7) seconds and for sham lesioned animals was 96.2 (SD  $\pm$  26.7) seconds. The amount of time animals spent on the accelerating rod before falling off was not significantly different between the animal groups at the pre-lesion time point [F (6, 45) = 1.86,  $p$  = .146] (Figure 3.8 A) or the post-lesion time point [F (6, 45) = .527,  $p$  = .785] (Figure 3.8 B).

As 6-OHDA lesioned animals did not perform significantly worse than sham lesioned animals, the results suggest that the 6-OHDA PD animal model did not produce an impairment in the amount of time spent on an accelerating rotating rod before falling off.

The scores for the rotarod test of all 7 animal groups at both the pre-lesion and post-lesion time points were normally distributed and there was homogeneity of variances for all groups at both time points.



**Figure 3.8 - Time Spent on Rotarod at Baseline and Post-Lesion Time Points**

At the pre-lesion time point, before animals undergone any surgery, there were no significant differences between any groups in the amount of time spent on an accelerating rod before falling off (A). At the post-lesion time point, there was no significant difference between groups in the amount of time spent on an accelerating rod before falling off (B). 6-OHDA lesioned animals did not perform significantly better or worse than sham lesioned animals, thus the 6-OHDA PD animal model did not produce an impairment in this forced motor task. All results are presented as mean  $\pm$  SEM. A one-way ANOVA was performed, followed by Bonferroni post-hoc.

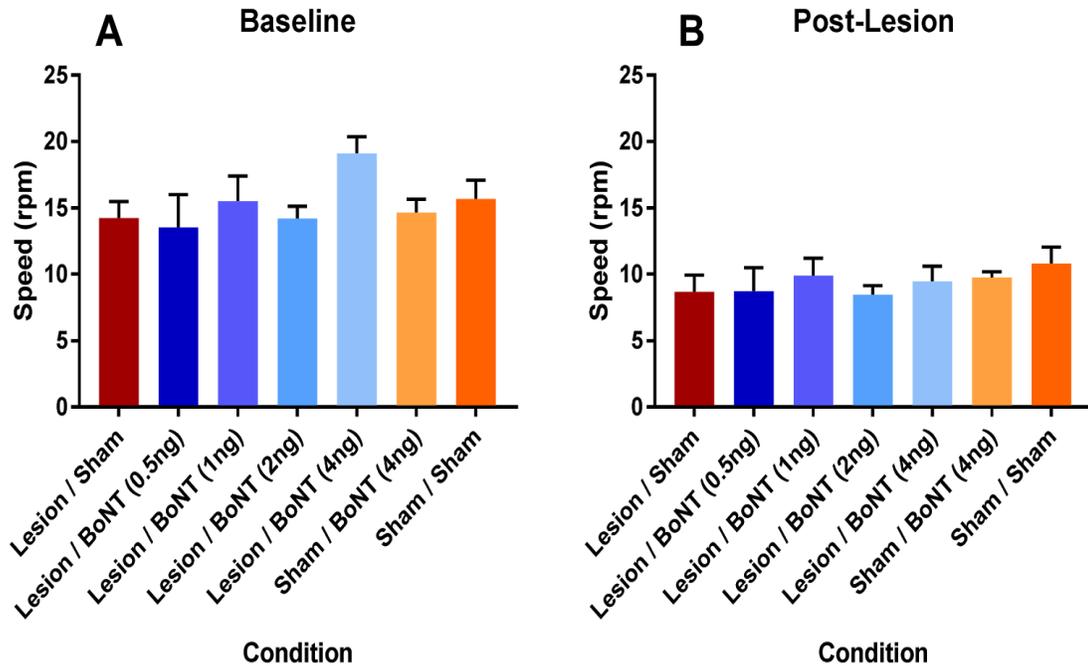
### 3.4.2 Maximum Speed Reached on Rotarod

The average speed reached at baseline (pre-lesion) time point for animals assigned to the lesion-group was 15.4 (SD  $\pm$  4.4) rpm while for the sham lesioned group was 15.1 (SD  $\pm$  3.2) rpm. The speed reached before falling off was not significantly different between groups at the pre-lesion time point [F (6, 45) = 1.701,  $p = .143$ ] (Figure 3.9 A) nor at the 2-week post-lesion time point [F (6, 45) = .537,  $p = .777$ ] (Figure 3.9 B).

As the 6-OHDA lesioned animals did not perform significantly worse than sham lesioned animals, the results suggest that the 6-OHDA PD animal model did not produce an impairment in the speed reached nor the time spent on an accelerating rotating rod before falling off.

The scores for the rotarod test of all 7 animal groups at both the pre-lesion and post-lesion time points were normally distributed and there was homogeneity of variances for all groups at both time points.

As the 6-OHDA lesioned animals scores on both time spent on the rod and max speed researched were not significantly different from sham lesioned animals, the data for post BoNT-A injection time points (1-week, 1-month, 2-months, 3-months post BoNT-A injection) was not shown in the results section. If the PD animal model did not produce a deficit in this task, then the effects of a BoNT-A injection cannot be interpreted properly. However, the full data of the rotarod task for both time and speed is presented in Appendix 6 for transparency. For the data presented in the appendix, a one-way ANOVA was run by comparing the scores of all conditions at the same time point and no significant differences were found throughout. Thus, at all time points, all of the animal groups were not significantly different from each other.



**Figure 3.9 - Speed Reached on Rotarod at Baseline and Post Lesion Time Points**

At the pre-lesion time point, before animals undergone any surgery, there were no significant differences between any groups in the speed reached on an accelerating rod before falling off (A). At 2-weeks post-lesion, there was no significant difference between groups in the speed reached on an accelerating rod before falling off (B). 6-OHDA lesioned animals did not perform significantly better or worse than sham lesioned animals, thus the 6-OHDA PD animal model did not produce an impairment in this forced motor task. All results are presented as mean  $\pm$  SEM. A one-way ANOVA was performed, followed by Bonferroni post-hoc.

## 3.5 CatWalk Results

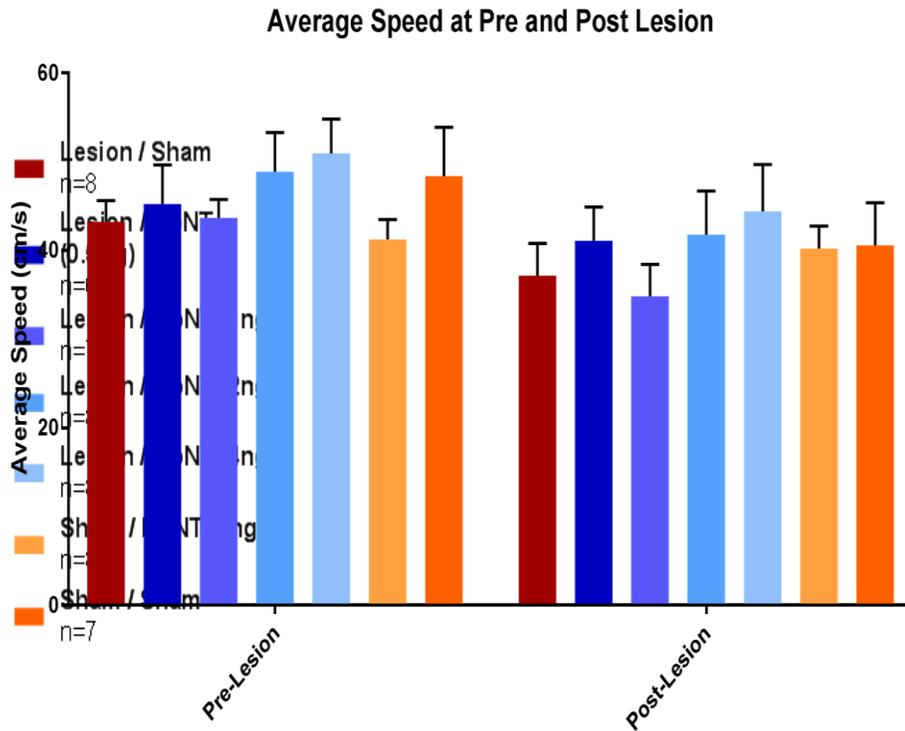
Throughout the CatWalk results, animal groups are reflected according to the figure legend with warm colours representing control groups (red: 6-OHDA lesion animals + Sham BoNT-A injection, light orange: sham lesion + 4ng BoNT-A injection, dark orange: sham lesion + sham BoNT-A injection). Cool colours (shades of blue) represent the experimental groups. All animals in the experimental groups are 6-OHDA lesioned and receive one of four doses of BoNT-A (0.5ng-4ng). The shade of blue gets lighter the higher the dose.

### 3.5.1 Average Speed

Average speed is the distance traversed on the walkway divided by time. No significant differences were found in average speed between any animal groups at any time point (Figure 3.10). None of the five 6-OHDA lesioned group's average speed was better or worse than of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit of average compared to sham lesioned animals at any post-lesion time point. At both the pre-lesion and post-lesion time points, the data was normally distributed for all animal groups ( $p > .05$ ) and there was homogeneity of variances ( $p > .05$ ).

### 3.5.2 Cadence

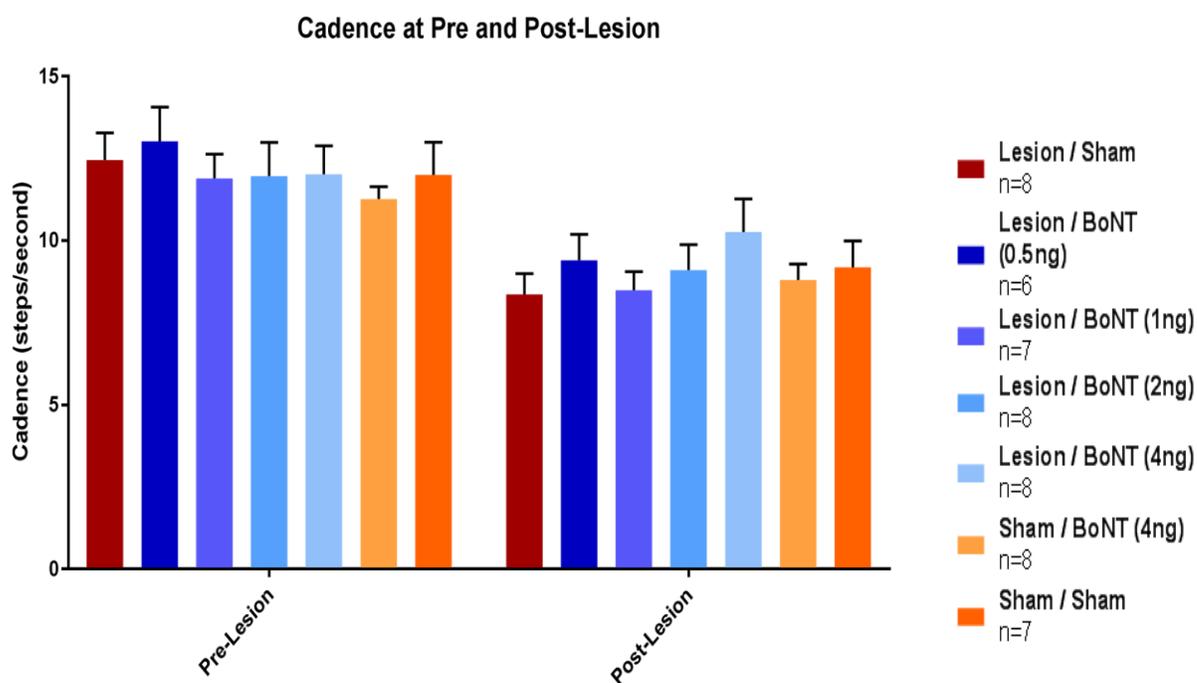
Cadence is the number of steps taken per second. No significant differences in cadence were found between any animal groups at any time point (Figure 3.11). None of the five 6-OHDA lesioned groups, cadence was better or worse than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit of cadence compared to sham lesioned animals at the post-lesion time point. At both the pre-lesion and post-lesion time points, the data was normally distributed for all animal groups ( $p > .05$ ) and there was homogeneity of variances ( $p > .05$ ).



**Figure 3.10 - Average Speed at Pre and Post Lesion Time Points**

Changes in average speed before any

surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups. No differences pre-lesion would be expected as animals have not undergone any surgery, however at the post-lesion time point a difference should be expected between the lesioned groups and the sham groups. No difference in average speed at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned (control) animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA was performed on both time points.

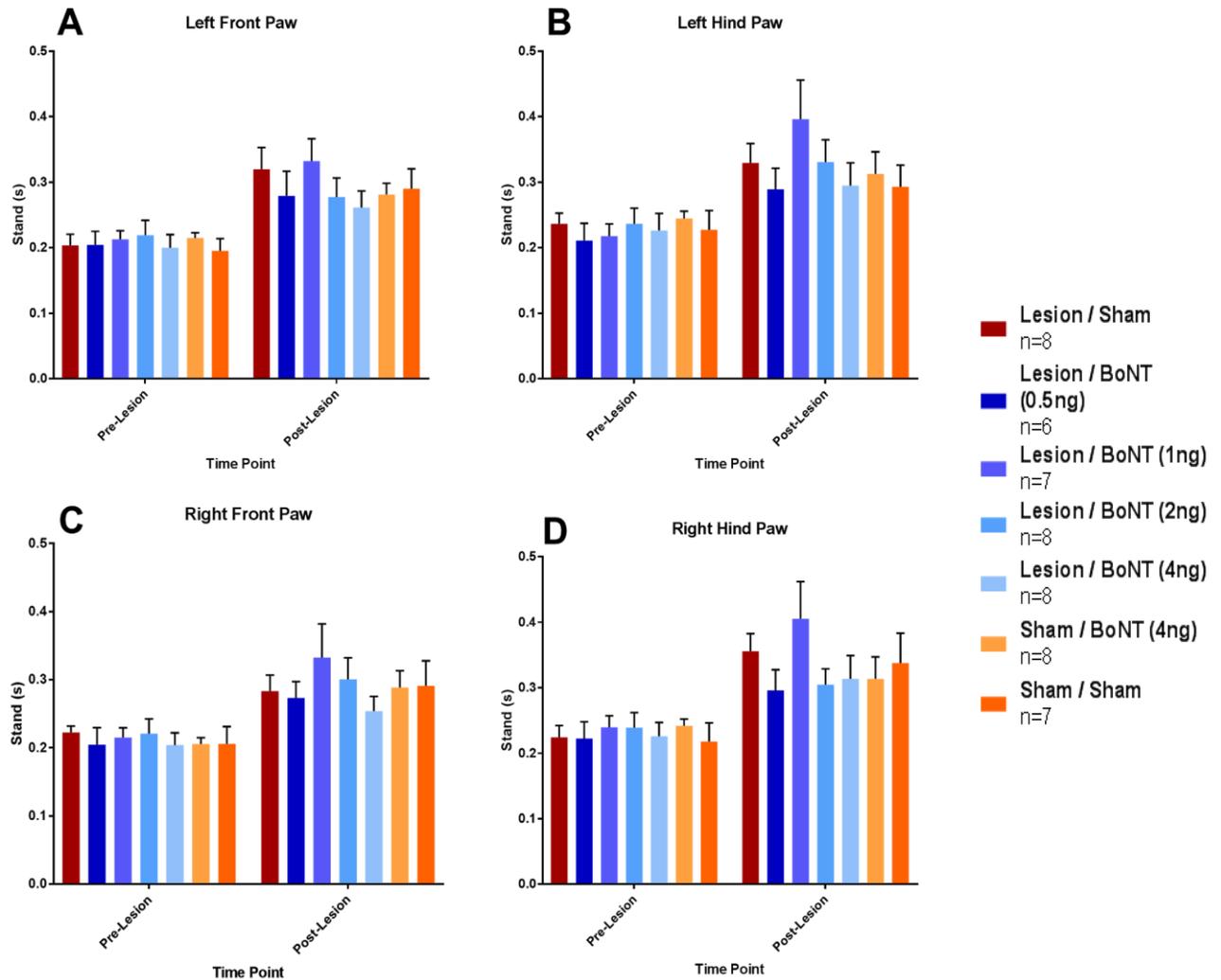


**Figure 3.11 - Cadence at Pre and Post Lesion Time Points**

Changes in cadence before any surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups. No differences pre-lesion would be expected as animals have not undergone any surgery, however at the post-lesion time point a difference should be expected between the lesioned groups and the sham groups. No difference in average speed at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned (control) animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA was performed on both time points.

### 3.5.3 Stand

Stand is the duration of contact of a paw with the walkway in seconds. No significant differences in stand were found between any animal groups at any time point for any of the paws (Figure 3.12 A-D). None of the five 6-OHDA lesioned groups stand scores were significantly different than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit in the stand parameter compared to sham lesioned animals at the post-lesion time point. At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/sham group ( $p = .041$ ) and the RF paw of the lesion/0.5ng BoNT group ( $p = .036$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/sham group ( $p = .013$ ) and the sham lesion/4ng BoNT group for every paw except the LF, LH ( $p = .015$ ), RF ( $p = .015$ ), RH ( $p = .005$ ). There was homogeneity of variances at pre-lesion for every paw except the RF paw ( $p = .046$ ), and there was homogeneity of variances at post-lesion for every paw.



**Figure 3.12 - Stand Score of Every Paw at Pre and Post Lesion Time Points**

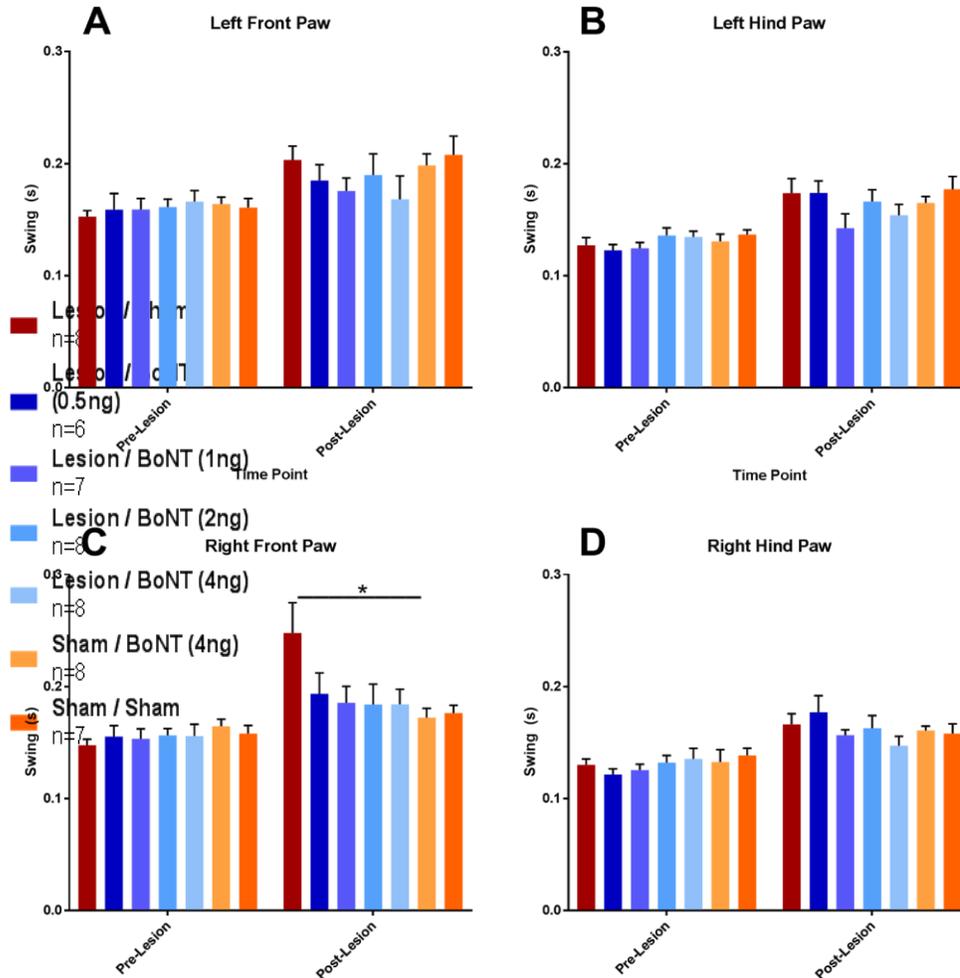
Changes in stand (seconds) before surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups in the left front paw (A), left hind paw (B), right front paw (C), and right hind paw (D). No difference in stand at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points.

### 3.5.4 Swing

Swing is the duration of no contact of a paw with the walkway in seconds. No significant differences in stand were found between any animal groups at any time point for the LF, LH, and RH paws (Figure 3.12. A, B, D). However, a significant difference was found at the RF paw at the post-lesion time point  $F(6, 45) = 2.437$ ,  $p = .04$  (Figure 3.13 C). According to Bonferroni post-hoc, the lesion/sham group and the sham lesion/4ng BoNT-A group were significantly different ( $p = .042$ ).

However, this finding of a significant difference between a lesioned group and a sham lesioned group does not mean that the 6-OHDA model was successful in inducing a motor deficit. As none of the other four lesioned groups showed a significant increase in swing, it is likely that this significant finding was due to high variability in the lesion/sham group. In order to confidently claim that the 6-OHDA lesion PD model was successful in producing a deficit in the swing parameter, the other four 6-OHDA lesioned groups should also show a deficit compared to the two sham lesioned groups.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/0.5ng BoNT-A group ( $p = .022$ ), the LH paw of the sham/4ng BoNT-A group ( $p = .009$ ), and the RH paw of the lesion/4ng BoNT-A ( $p = .034$ ) and sham/4ng BoNT-A group ( $p = .009$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/2ng BoNT-A group ( $p = .024$ ) and the LH paw of the lesion/sham group ( $p = .048$ ). There was homogeneity of variances at pre- and post-lesion for every paw.



**Figure 3.13 - Swing Score of Every Paw at Pre and Post Lesion Time Points**

Changes in swing (seconds) before

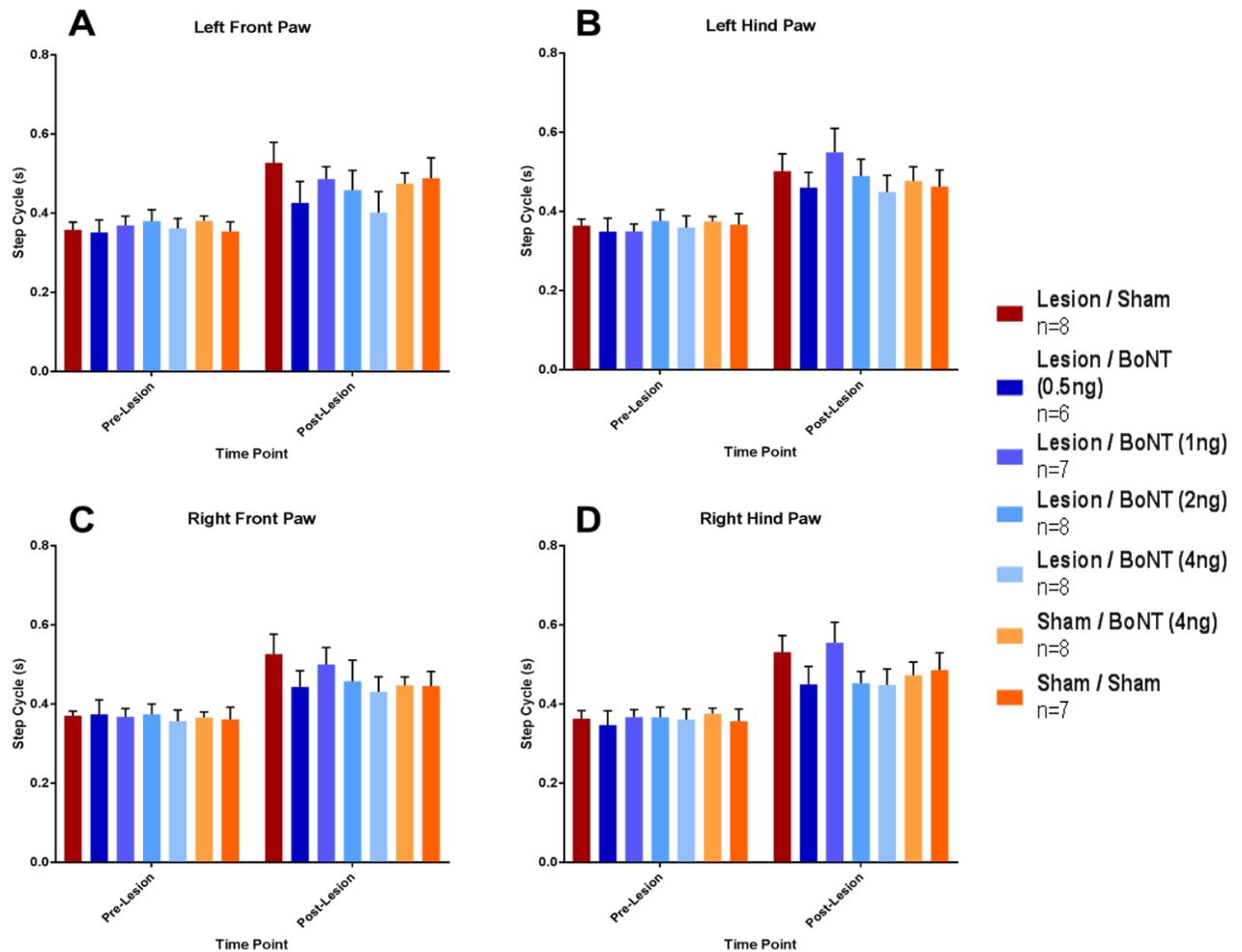
surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre- or post-lesion between all groups in the left front paw (A), left hind paw (B), or right hind paw (D). A significant difference was found at the post-lesion time point in the right front paw between the lesion/sham group and the sham/4ng BoNT-A group (C). Due to the fact that only one lesioned group showed a significant difference with a sham lesioned group, the likely reason for this finding is due to high variability of the data in the lesion/sham group. To make the claim that the 6-OHDA lesion was successful in causing a deficit in the swing parameter, all lesioned group should have a higher swing score than both sham lesioned groups. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points,  $p < 0.05^*$ .

### 3.5.5 Step Cycle

Step cycle is the time in seconds between two initial contacts of the same paw. No

significant differences in stand were found between any animal groups at any time point for any of the paws (Figure 3.14 A-D). None of the five 6-OHDA lesioned groups step cycle scores were significantly different than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit in the step cycle parameter compared to sham lesioned animals at the post-lesion time point.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/0.5ng BoNT-A group ( $p = .025$ ), the RF paw of the lesion/0.5ng BoNT group ( $p = .009$ ), and the RH paw of the lesion/0.5ng BoNT group ( $p = .013$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/sham group ( $p = .046$ ). There was homogeneity of variances at pre- and post-lesion for every paw.



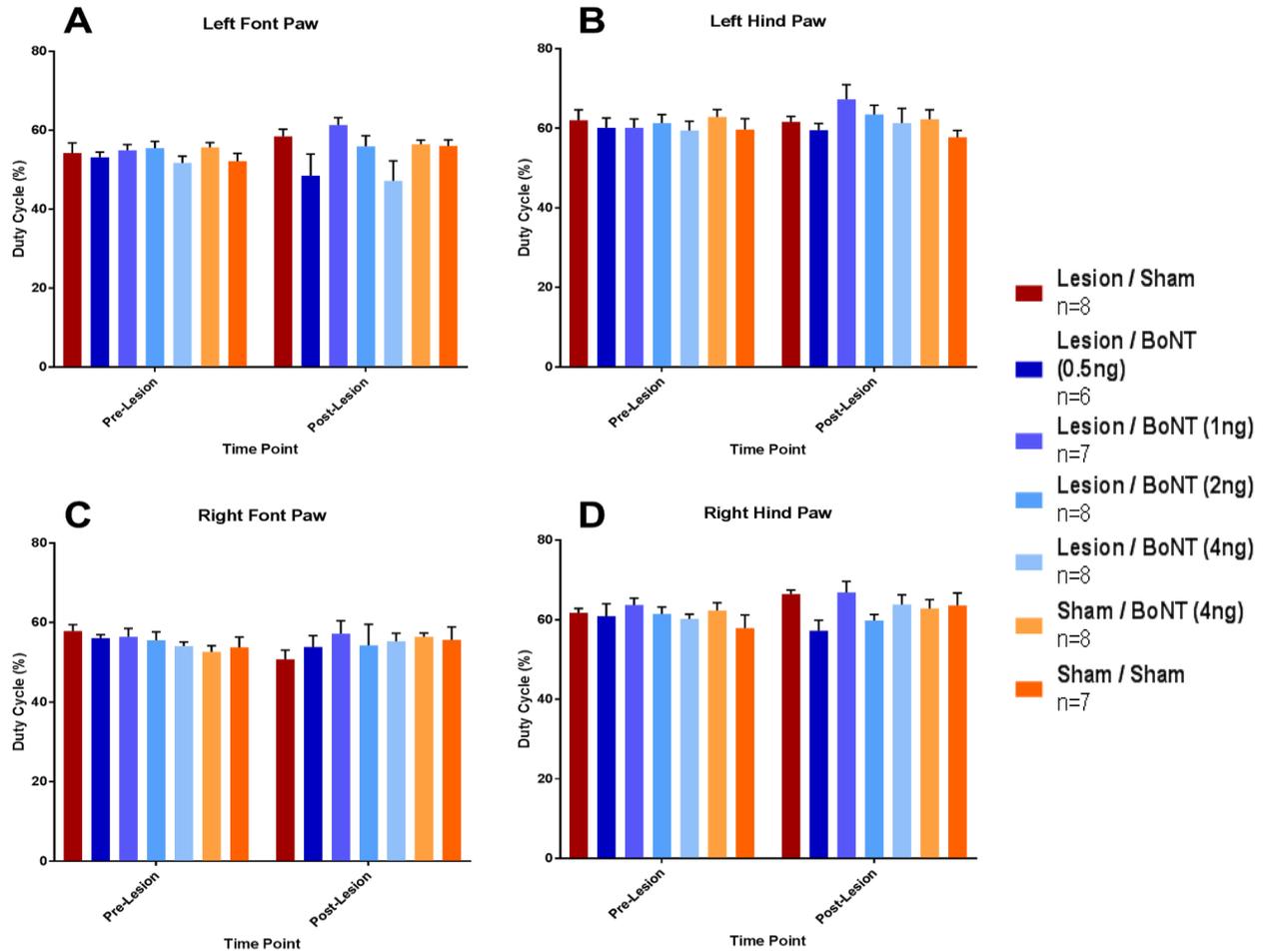
**Figure 3.14 - Step Cycle of Every Paw at Pre and Post Lesion Time Points**

Changes in step cycle (seconds) before surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups in the left front paw (A), left hind paw (B), right front paw (C), and right hind paw (D). No difference in step cycle at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points.

### 3.5.6 Duty Cycle

Duty cycle expresses stand as a percentage of step cycle. No significant differences in duty cycle were found between any animal groups at any time point for any of the paws (Figure 3.15 A-D). None of the five 6-OHDA lesioned groups duty cycle scores were significantly different than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit in the duty cycle parameter compared to sham lesioned animals at the post-lesion time point.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/sham group ( $p = .049$ ), the LF paw of the sham/4ng BoNT group ( $p = .011$ ), and the RF paw of the lesion/1ng BoNT group ( $p = .044$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/4ng BoNT group ( $p = .013$ ), and the RF paw of the lesion/2ng BoNT-A group ( $p = .009$ ). There was homogeneity of variances at pre- and post-lesion for every paw, except for the LF paw at pre-lesion ( $p = .013$ ) and post-lesion ( $p = .009$ ).



**Figure 3.15 - Duty Cycle of Every Paw at Pre and Post Lesion Time Points**

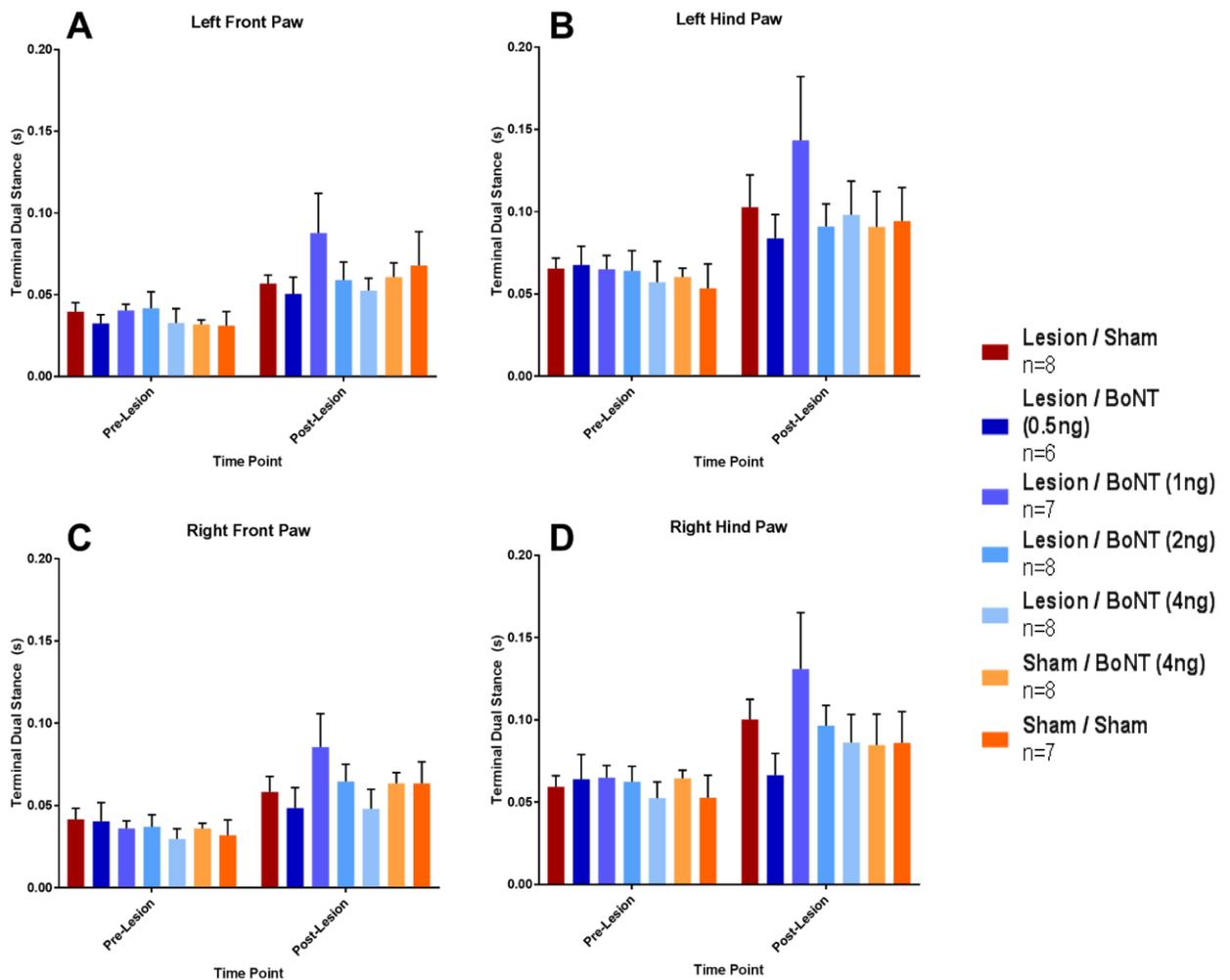
Changes in duty cycle (%) before surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups in the left front paw (A), left hind paw (B), right front paw (C), and right hind paw (D). No difference in duty cycle at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points.

### 3.5.7 Terminal Dual Stance

Terminal dual stance is the duration in seconds of the second step in a step cycle of a paw that the contralateral paw is also in contact with the walkway. No significant differences in terminal dual stance were found between any animal groups at any time point for any of the paws (Figure 3.16 A-D). None of the five 6-OHDA lesioned groups terminal dual stance scores were significantly different than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit in the terminal dual stance parameter compared to sham lesioned animals at the post-lesion time point.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LH paw of the lesion/4ng BoNT-A group ( $p = .007$ ), the RF paw of the lesion/0.5ng BoNT group ( $p = .011$ ), and the RF paw of the lesion/4ng BoNT group ( $p = .039$ ).

At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the sham/4ng BoNT ( $p = .023$ ) and sham/sham group ( $p = .012$ ), and the RH paw of the lesion/sham ( $p = .007$ ), lesion/2ng BoNT-A ( $p = .022$ ), and sham/4ng BoNT-A ( $p = .001$ ) group. There was homogeneity of variances at pre- and post-lesion for every paw, except for the LF paw at pre-lesion ( $p = .018$ ) and post-lesion ( $p < .001$ ).



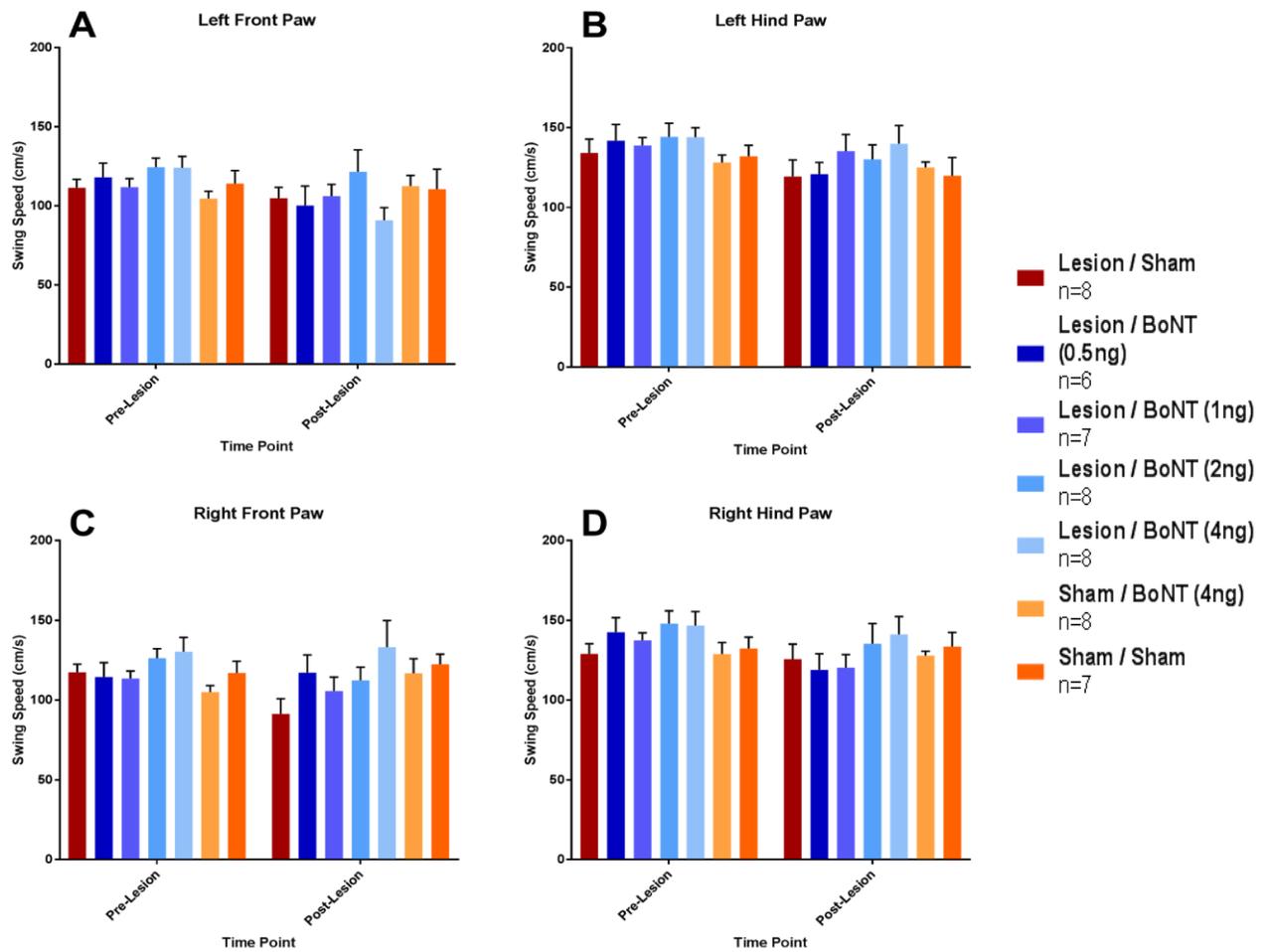
**Figure 3.16 - Terminal Dual Stance of Every Paw at Pre and Post Lesion Time Points**

Changes in terminal dual stance (seconds) before surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups in the left front paw (A), left hind paw (B), right front paw (C), and right hind paw (D). No difference in terminal dual stance at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points.

### 3.5.8 Swing Speed

Swing speed is the speed in centimeters per second of a paw during swing. No significant differences in terminal dual stance were found between any animal groups at any time point for any of the paws (Figure 3.17 A-D). None of the five 6-OHDA lesioned groups swing speed scores were significantly different than that of the two sham lesioned groups. This suggests that the PD animal model used did not produce a quantifiable deficit in the swing speed parameter compared to sham lesioned animals at the post-lesion time point.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the lesion/0.5ng BoNT-A group ( $p = .045$ ), LH paw of the lesion/1ng BoNT-A group ( $p = .034$ ), and RF paw of the lesion/0.5ng BoNT-A group ( $p = .049$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the RF paw of the lesion/1ng BoNT ( $p = .007$ ). There was homogeneity of variances at pre- and post-lesion for every paw.



**Figure 3.17 - Swing Speed of Every Paw at Pre and Post Lesion Time Points**

Changes in swing speed (cm/second) before surgery and after 6-OHDA or sham lesion surgery. No significant differences were found at pre-lesion or at post-lesion between all animal groups in the left front paw (A), left hind paw (B), right front paw (C), and right hind paw (D). No difference in swing speed at the post-lesion time point indicates that the 6-OHDA lesioned animals have not developed a motor impairment, as measured by the CatWalk, compared to sham lesioned animals. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points.

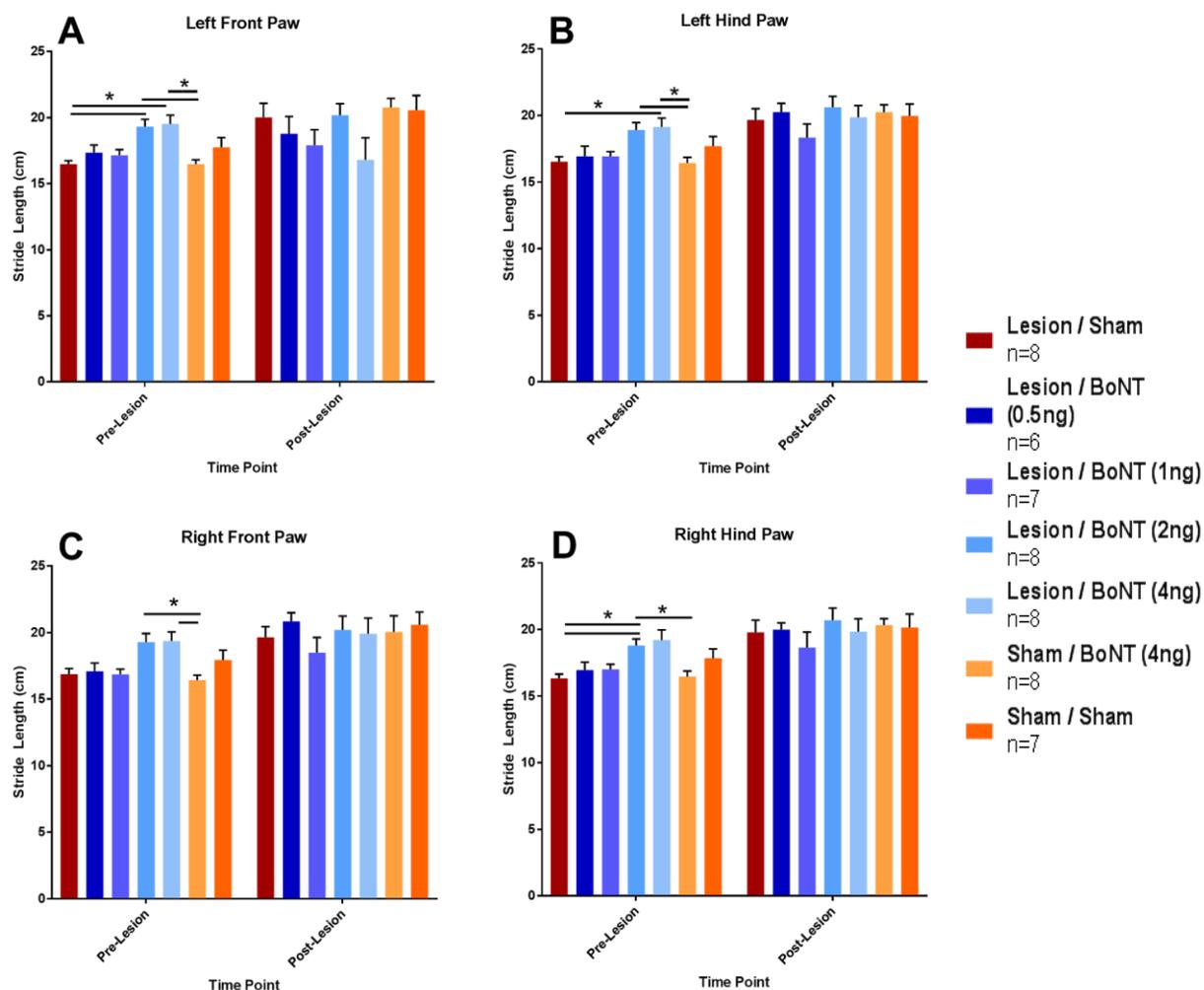
### 3.5.9 Stride Length

Stride length is the distance between successive placements of the same paw. Interestingly, significant differences between animal groups in every paw was present at pre-lesion. In the LF paws [Welch's  $F(6, 19.125) = 5.455, p = .002$ ], a significant difference was found between the lesion/sham group and lesion/2ng BoNT-A group ( $p = .014$ ), the lesion/sham group and lesion/4ng BoNT-A group ( $p = .025$ ), the lesion/2ng BoNT-A and sham/4ng group ( $p = .026$ ), and finally, the lesion/4ng BoNT-A and sham/4ng group ( $p = .015$ ) (Figure 3.18 A). In the LH paws [ $F(6, 45) = 4.175, p = .002$ ], a significant difference was found between the lesion/sham group and lesion/4ng BoNT-A group ( $p = .028$ ), the lesion/2ng BoNT-A and sham/4ng group ( $p = .047$ ), and the lesion/4ng BoNT-A and sham/4ng group ( $p = .019$ ) (Figure 3.18 B). In the RF paws [ $F(6, 45) = 4.691, p = .001$ ], a significant difference was found between the lesion/2ng BoNT-A group and sham/4ng BoNT-A group ( $p = .013$ ), and the lesion/4ng BoNT-A ( $p = .01$ ) (Figure 3.18 C). In the RH paws [Welch's  $F(6, 19.383) = 4.086, p = .009$ ] a significant difference was found between the lesion/sham group and lesion/2ng BoNT-A group ( $p = 0.39$ ), the lesion/sham group and lesion/4ng BoNT-A group ( $p = .007$ ) and the lesion/4ng BoNT-A group and sham/4ng BoNT-A group ( $p = .012$ ) (Figure 3.18 D). However, no significant differences in stride length were found between any animal groups at the post-lesion time point (Figure 3.18 A-D).

The results suggest that at baseline before animals have undergone any surgery, that their stride length was fundamentally different between groups. However, at the post-lesion time point, there are no significant differences. One explanation is that there was particularly high variability in the data for this feature at that time point. Ultimately however, as there are no significant differences at the post-lesion time point in any of the paws between animal groups, the 6-OHDA model not successful in inducing a motor deficit in the stride length parameter.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the sham/sham group ( $p = .044$ ), the RF paw of the sham/sham group ( $p = .043$ ), and the RH paw of the sham/sham group ( $p = .007$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the sham/4ng BoNT-A group ( $p = .03$ ), the LH paw

of the lesion/4ng BoNT-A group ( $p = .045$ ), the RF paw of the sham/4ng BoNT-A group ( $p = .002$ ), and the RH paw of the sham/4ng BoNT-A group ( $p = .039$ ). There was homogeneity of variances at the pre-lesion time point for the LH and RF paw but not for the LF ( $p = .017$ ) and RH ( $p = .019$ ) paws. There was homogeneity of variances at the post-lesion time point for every paw.



**Figure 3.18 - Stride Length of Every Paw at Pre and Post Lesion Time Points**

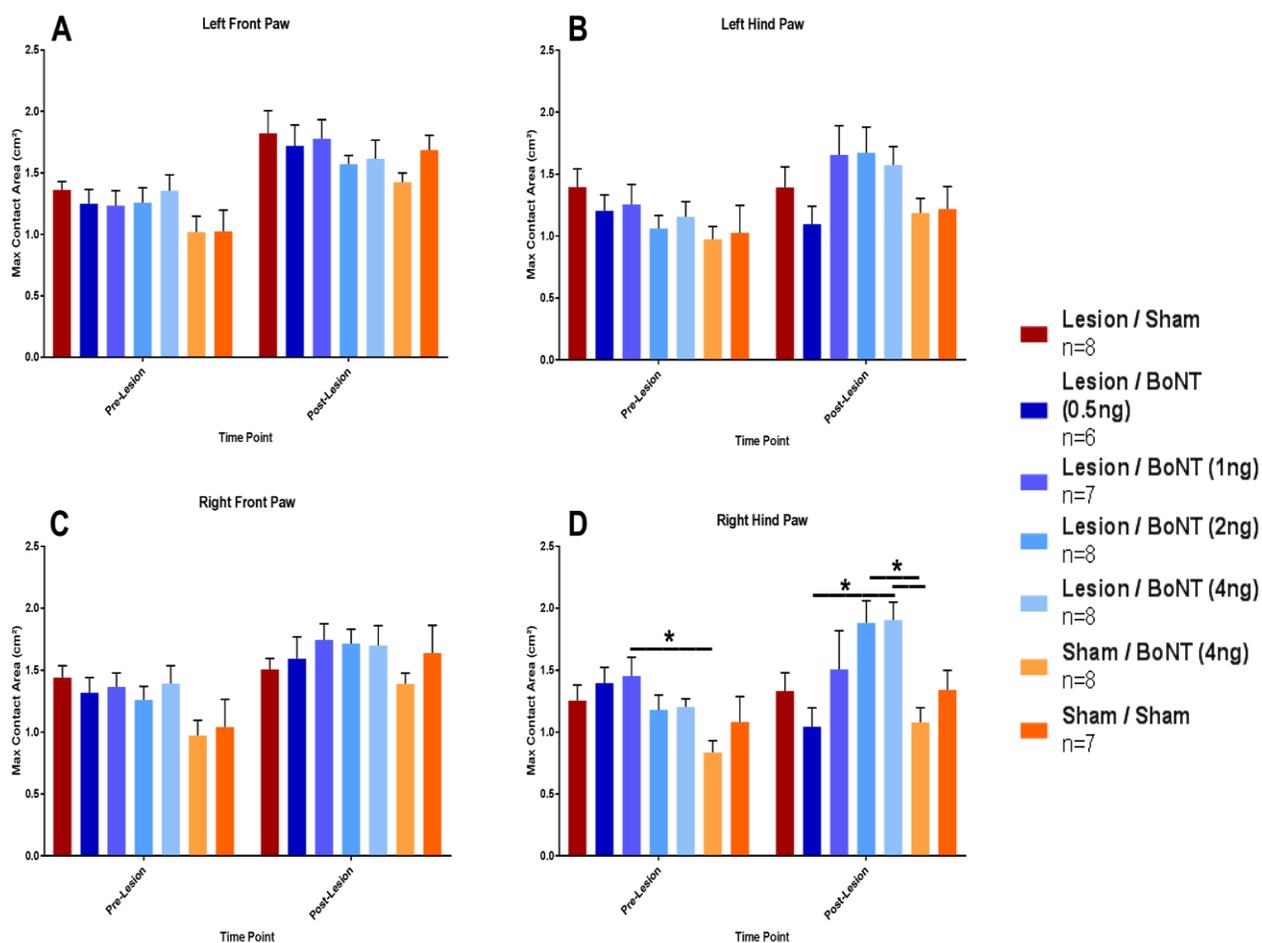
Changes in stride length (cm) before surgery and after 6-OHDA or sham lesion surgery. Significant differences were found at the pre-lesion time point for the left front paw (A), left hind paw (B), and right hind paw (D). No significant differences were found at the post-lesion time point. As no differences were found between the lesioned or sham lesioned animal groups at post-lesion, the 6-OHDA animal model was not successful in causing a deficit in the stride length parameter. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points,  $p < 0.05^*$ .

### 3.5.10 Max Contact Area

Max contact area ( $\text{cm}^2$ ) is the maximum surface area of a paw that contacts the glass walkway. There are no significant differences in max contact area between groups at both the pre-lesion and post-lesion time points for the LF, LH, and RF paws (Figure 3.19 A-C). A significant difference between groups was found for the RH paw at both pre-lesion [ $F(6, 45) = 2.46, p = .038$ ] and post-lesion [ $F(6, 45) = 3.747, p = .004$ ] (Figure 3.19 D). According to Bonferroni post-hoc, at pre-lesion in the RF paw, the lesion/1ng BoNT-A group and the sham lesion/4ng BoNT-A group are significantly different ( $p = .034$ ). At post-lesion time, differences in the RF paw was found between the following groups, the lesion/0.5ng BoNT-A and lesion/4ng BoNT-A group ( $p = .048$ ), the lesion/2ng BoNT-A and sham/4ng BoNT-A group ( $p = .044$ ), and finally the lesion/4ng BoNT-A group and sham/4ng BoNT-A group ( $p = .034$ ).

Upon examination of Figure 3.19 D, it is clear that there is a high amount of variability in the data, which could explain the finding of a significant difference between a lesioned group and a sham lesioned group. Although differences were found between lesioned groups and sham groups at the post-lesion time point, it is clear that the 6-OHDA lesion PD model was not successful in producing a deficit in the max contact area parameter. All of the lesioned groups should produce a similar level of deficit, however the literature states that a decrease in max contact area should be seen in 6-OHDA lesioned animals as PD animals are exerting less pressure with their affected paws<sup>99, 101</sup>. Another reason why the differences found in this parameter is due to variability in the data is because a significant difference was found between two groups that were both lesioned, the lesion/0.5ng BoNT-A and the lesion/4ng BoNT-A groups.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the RF paw of the sham/sham group ( $p = .036$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the RF paw of the sham/4ng BoNT-A group ( $p = .022$ ) and the sham/sham group ( $p = .006$ ). There was homogeneity of variances at the pre- and post-lesion time points for every paw.



**Figure 3.19 - Max Contact Area of Every Paw at Pre and Post Lesion Time Points**

Changes in max contact area (cm<sup>2</sup>) before surgery and after 6-OHDA or sham lesion surgery. No significant difference at pre- or post-lesion were found between animal groups for the LF (A), LH (B), or RF (C) paws. Significant differences were found at the pre- and post-lesion time point for the RH paw (D).

The 6-OHDA animal model was not successful in causing a deficit in the max contact area parameter as there is not a consistent deficit among all lesioned groups. The significant differences found in this parameter are likely due to high variability in the data. The inconsistency in scores among the 6-OHDA groups supports this as well as the finding that there is a significant difference between the two groups that were both lesioned (lesion/0.5ng BoNT-A and lesion/4ng BoNT-A). All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni post-hoc test was performed for every paw at both time points,  $p < 0.05^*$ .

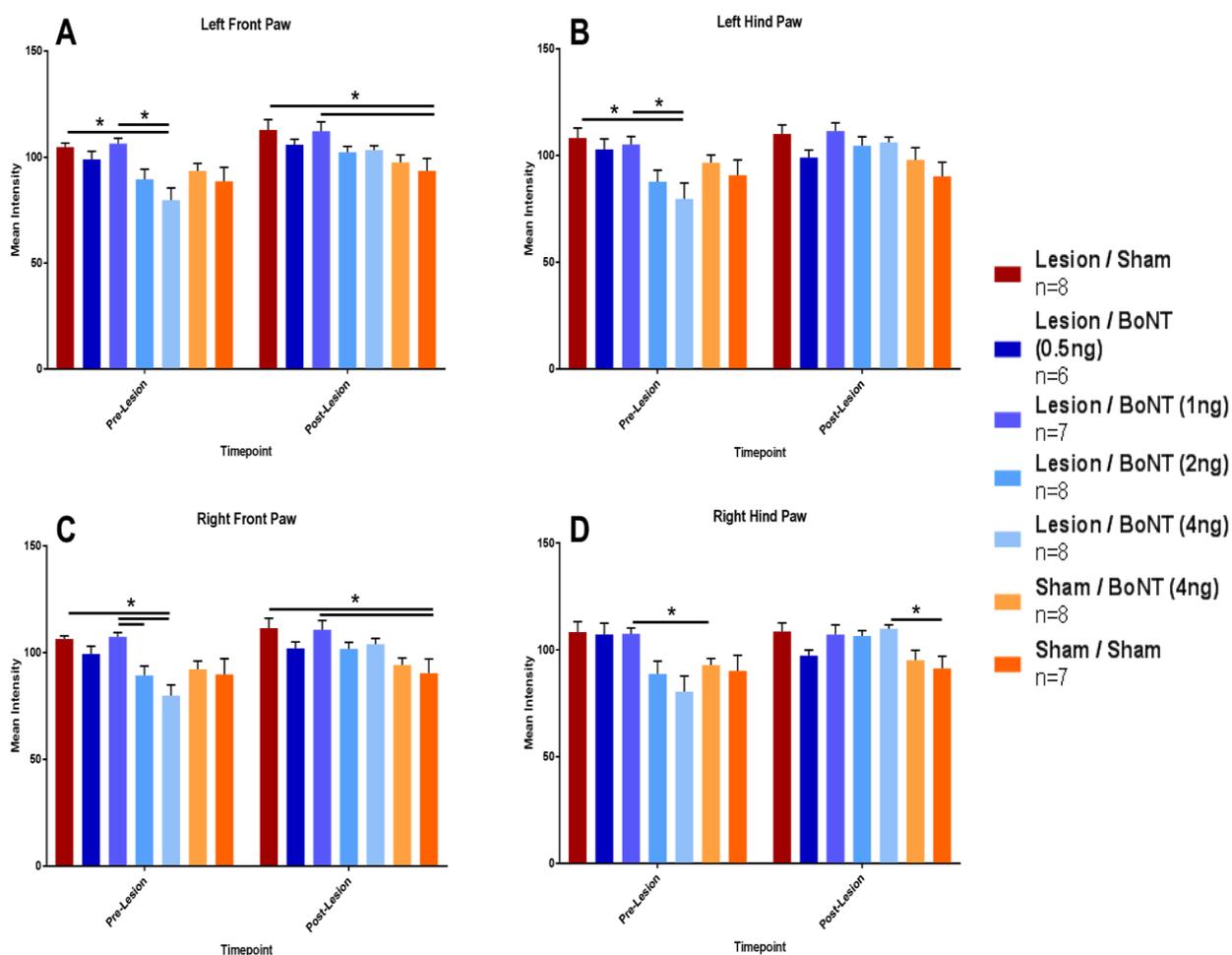
### 3.5.11 Mean Intensity

Mean intensity is the mean pressure of a paw that contacts the walkway. At the pre-lesion time point significant differences in mean intensity were found for the LF [Welch's  $F(6, 19.147) = 5.129, p = .003$ ](Figure 3.20 A), LH [ $F(6, 45) = 3.633, p = .005$ ](Figure 3.20 B), RF [Welch's  $F(6, 18.881) = 7.556, p < .001$ ](Figure 3.20 C), and RH paws [Welch's  $F(6, 19.396) = 4.244, p = .007$ ](Figure 3.20 D). According to Bonferroni (or Games-Howell post-hoc when homogeneity of variances was violated), at pre-lesion there were significant differences in max contact area of the LF paw between the lesion/sham and lesion/4ng BoNT-A groups ( $p = .031$ ), and lesion/1ng BoNT-A and lesion/4ng BoNT-A groups ( $p = .022$ ). In the LH paw, there were significant differences between the lesion/sham and lesion/4ng BoNT-A groups ( $p = .009$ ) and the lesion/1ng BoNT-A and lesion/4ng BoNT-A groups ( $p = .043$ ). In the RF paw, there were significant differences between the lesion/sham and lesion/4ng BoNT-A groups ( $p = .01$ ), the lesion/1ng BoNT-A and lesion/2ng BoNT-A groups ( $p = .043$ ), and the lesion/1ng BoNT-A and lesion/4ng BoNT-A groups ( $p = .008$ ). In the RH paw, there was a significant difference between the lesion/1ng BoNT-A and sham/4ng BoNT-A groups ( $p = .047$ ).

At the post-lesion time point significant differences in mean intensity were found for the LF [ $F(6, 45) = 3.285, p = .009$ ](Figure 3.20 A), LH [ $F(6, 45) = 2.615, p = .029$ ](Figure 3.20 B), RF [ $F(6, 45) = 3.527, p = .006$ ](Figure 3.20 C), and RH paws [ $F(6, 45) = 3.564, p = .006$ ](Figure 3.20 D). According to Bonferroni post-hoc, at post-lesion there were significant differences in max contact area of the LF paw between the lesion/sham and sham/sham groups ( $p = .023$ ), and lesion/1ng BoNT-A and sham/sham groups ( $p = .043$ ). In the LH paw, although a significance was found using the ANOVA, Bonferroni post-hoc did not find significant differences between any groups. Some group comparisons were close to reaching the  $p=.05$  threshold but did not make it. In the RF paw, there were significant differences between the lesion/sham and sham/sham groups ( $p = .017$ ), and the lesion/1ng BoNT-A and sham/sham groups ( $p = .032$ ). In the RH paw, there was a significant difference between the lesion/4ng BoNT-A and sham/sham groups ( $p = .037$ ). Finding differences between groups at pre-lesion indicates that there was a high degree of variability between animals before any surgery. Although differences were

found between lesioned groups and sham groups at the post-lesion time point, it is clear that the 6-OHDA lesion PD model was not successful in producing a deficit in the mean intensity parameter. Based on prior studies, a decrease in intensity should be expected in 6-OHDA lesioned animals (Chuang et al. (2010) and Zhou et al. (2015), not a general increase in the lesioned groups as seen in the results of this study.

At the pre-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the LF paw of the sham/sham group ( $p = .043$ ) and the RF paw of the lesion/1ng BoNT-A group ( $p = .032$ ). At the post-lesion time point the data was normally distributed for all 7 animal groups for every paw, except for the RF paw of the lesion/2ng BoNT-A group ( $p = .035$ ). There was homogeneity of variances at the pre-lesion time point for the LH paw but not for the LF ( $p = .009$ ), RF ( $p = .001$ ) and RH paws ( $p = .014$ ). At the post-lesion time point, there was homogeneity of variances for all paws.



**Figure 3.20 - Mean intensity of Every Paw at Pre and Post Lesion Time Points**

Changes in mean intensity before surgery and after 6-OHDA or sham lesion surgery. Significant differences at pre- and post-lesion were found between animal groups for the LF (A), RF (C), and RH(D) paws. Significant differences were found only at the pre-lesion time point for the LH paw (B). The 6-OHDA animal model was not successful in producing a deficit in the mean intensity parameter as there is not a consistent deficit among all lesioned groups when compared to sham lesioned animals. Also, prior studies have found a decrease in mean intensity in 6-OHDA lesioned animals, not an increase as the results from this study show. The significant differences found in this parameter are likely due to high variability in the data. All results are presented as mean  $\pm$  SEM. A one-way ANOVA and Bonferroni or Games-Howell post-hoc test was performed for every paw at both time points,  $p < 0.05^*$ .

## Chapter 4

### 4 Discussion

Unilateral 6-OHDA lesions resulted in pathological rotations following apomorphine injection. A dose of 2-4ng of BoNT-A is sufficient in reducing pathological drug-induced rotations, for up to 3 months, as by that time point, in both the 2ng and 4ng groups, the number of pathological rotations began to return to baseline performance and were not significantly different from the pre-lesion time point.

Despite the emergence of apomorphine-induced rotations, forced movement as measured by the rotarod test and spontaneous locomotion as measured using the CatWalk did not produce motor deficits or gait abnormalities in our MFB 6-OHDA lesioned model. Testing spontaneous locomotion, the majority of CatWalk parameters did not find any significant differences between animal groups at the pre-lesion and post-lesion time points in any paws. The instances where significant differences were found between animal groups was likely due to the high degree of variability in the data. This interpretation is supported by the fact that the significant findings between parameters were not consistent. To support the claim that the 6-OHDA model causes a deficit in a particular gait parameter, at the post-lesion time point, all animals assigned to a lesioned group should show the deficit when compared to the sham lesioned animals. Additionally, no differences should be found between two different groups that were both assigned to the lesioned condition. Yet there were instances, such as with the stride length, max contact area, and mean intensity parameters when significant differences were found between the two groups in which animals were 6-OHDA lesioned.

#### 4.1 Discussion of Apomorphine Test Results

The timeline of BoNT-A effect seen in the results of this study follow a similar timeline of peak effects and wearing off to what is seen involving injections in peripheral targets in a clinical setting<sup>131-133</sup>. In agreement with this study, the peak effect of BoNT-A when used as a clinical therapeutic for tremor through peripheral injection is at 1-month post-injection and wears off at around 3-4 months post-injection<sup>131-133</sup>.

Overall, 2-4ng of BoNT-A injected into the STN was successful in reducing pathological drug-induced rotations. However, in combination with the results of this study, the published literature suggests that the EPN is the superior site of injection for the use of BoNT-A in the brain. A prior study in the lab by Tsang, Rajakumar, and Jog (2019) examined the effects of a BoNT-A injection in the brain of 6-OHDA lesioned rats. In that study, they found a greater reduction in pathological drug-induced rotations in the apomorphine rotation test when a dose of BoNT-A was injected into the EPN<sup>127</sup>. Additionally, this stronger reduction in pathological rotations was achieved with a dose 4x lower than what was needed in the current study to achieve a lasting reduction in pathological rotations (0.5ng versus 2ng). The difference in dose needed to reduce pathological rotations is likely due to the difference in firing activity of the glutamatergic projections coming to the area that received the BoNT-A injection<sup>145</sup>. The activity of the glutamatergic cortical projections coming to the STN is reduced in PD, while the glutamatergic projections from the STN to the GPi/SNpr are increased in activity in PD<sup>145</sup>. Therefore, if the activity of the cortical projections are already reduced in a diseased state, to cause further reduction in its level of activity would require a high dose. The EPN, on the other hand receives a higher firing rate of glutamatergic input coming from a hyper-active STN in the disease brain, therefore an effect can be seen with a lower dose of BoNT-A<sup>145</sup>. The current literature thus suggests that an injection in the EPN (rodent equivalent of the human GPi/SNpr) is much more effective than an injection in the STN.

## 4.2 Lack of Impairment in 6-OHDA Rat Model

This section will discuss possible explanations as to why the 6-OHDA rat model of PD did not produce a motor deficit in the rotarod and CatWalk tasks in the current study.

### 4.2.1 Lack of Impairment in Rotarod Task

A difference in the maximum speed or the amount of time spent on an accelerating rod was not seen between 6-OHDA lesioned animals and sham lesioned animals at the pre and post-lesion time points. Therefore, lesioned animals did not show a motor deficit

in this specific task. One possible explanation for why a deficit in lesioned animals was not found could be due to the rotarod protocol that was used. Rotarod has a fixed speed function where the rod rotates at a steady speed and an acceleration function where the rod increases in speed from 4 rpm to 40 rpm over a 5-minute period. This study used the acceleration function as this was the protocol that was most commonly used in the literature with the 6-OHDA lesion rat model<sup>85, 134, 141</sup>. Carvalho et al., (2013) as well as Spooren et al., (2000) used the accelerating rotarod in their studies examining unilaterally 6-OHDA lesioned animals. Campos et al., (2013) also used the accelerating rotarod in bilaterally 6-OHDA lesioned animals. Spooren et al., (2000) had no control group (sham or no lesion) to compare performance with for rotarod, and Campos et al., (2013) used a bilateral lesion which produces a more severe deficit. Therefore, the results of this thesis did not agree with the deficit found in the Carvalho et al., (2013) study, whose methods are comparable with the study presented in this thesis.

A study by Monville and colleagues (2006) compared the relative power, reliability, and sensitivity of the fixed speeds and accelerating protocols of the rotarod task in 6-OHDA and sham lesioned animals. Their results suggest that the fixed speed protocol is more sensitive in detecting the presence of a lesion while the accelerating protocol is a more discriminative test to correlate motor deficits against lesion size<sup>84</sup>. Although the rotarod assessment was not conducted, based on the extent of motor deficits found in 6-OHDA lesioned animals in of our previous study (Tsang, Rajakumar & Jog, 2019), it was predicted that the 6-OHDA animal model would have produced a strong and obvious motor deficit. Thus, based on a priori expectations, the accelerating protocol was selected as the more appropriate protocol. It may be the case that the fixed speed protocol would have in fact been the more appropriate protocol to use in this study, as Monville et al (2006) recommend the fixed speed protocol in experiments where maximum sensitivity is required to detect small changes in performance. A study by Haddadi et al., (2014) supports this as they used the fixed speed rotarod protocol in unilaterally 6-OHDA lesioned rats and found a significant reduction in the amount of time spent on the rod compared to sham lesioned and non-operated animals. The accelerating protocol is also relatively more difficult for animals to perform. To illustrate the difference between the protocols, in a fixed speed function the rpm is selected and stays consistent, but with the

accelerating function, after every 8 seconds the rpm increases by 1. Therefore, at 1 second, the speed is 4rpm, at 33 seconds the rpm is 8, at 1 minute the rpm is 11, and at 2 mins the rpm is 19. Rats that might be able to stay on the rod for a long amount of time at a lower fixed speed, say 8 rpm, might have incredible difficulty at higher speeds.

#### 4.2.2 Inconsistency and Lack of Confirmation of Noradrenergic Cell Sparing

A possible explanation for why this study did not find a gait deficit in unilaterally 6-OHDA lesioned animals could be because this study injected desipramine before the infusion of 6-OHDA. Desipramine is a noradrenaline reuptake transporter blocker and thus prevents uptake of 6-OHDA into noradrenergic neurons and degeneration of these cells. Consequently, our 6-OHDA PD model possesses specific depletion of dopamine without altering noradrenergic neuronal function. Gait abnormalities of PD is one of the least responsive symptoms to pharmacological dopamine replacing therapy (levodopa) which is remarkably effective in most other motor deficits of PD<sup>23, 53, 54</sup>. Noradrenergic projections from the LC modulates the activity of the brainstem and spinal cord motor pattern generators, thus affecting gait<sup>145, 146</sup>. This is supported by the finding that noradrenergic dysfunction in the LC is most closely linked to the symptom of freezing of gait in PD<sup>146, 147</sup>. Unfortunately, many preclinical studies do not state whether or not desipramine was injected before the infusion of 6-OHDA when creating animal models. One of the studies cited in the introduction as support for the validity of the use of the CatWalk apparatus in gait assessment in a 6-OHDA model explicitly stated in their discussion that they did not inject desipramine before 6-OHDA lesion<sup>101</sup>. Therefore, the noradrenergic cells in the LC were not protected in their animals. Zhou et al., (2015) investigated gait changes using the CatWalk apparatus in three different unilateral 6-OHDA rat models and found deficits in various gait parameters. However, Zhou et al., (2015) concluded that the gait deficits they found could be due to the loss of noradrenergic neurons of the LC. Additionally, a study by Westin et al., (2012), in bilaterally 6-OHDA lesioned rats also did not state whether desipramine was injected before 6-OHDA lesion. Vandeputte et al., (2010) confirmed deficits in gait using CatWalk in unilaterally 6-OHDA lesioned

rats, but it is unknown if desipramine was injected. Chang et al., (2003) who found that unilateral 6-OHDA lesioned rats produced impaired treadmill walking, a different task for measuring gait impairment, also did not state whether desipramine pre-treatment was given. Chuang et al., (2010) found gait deficits in a unilateral 6-OHDA model but did not state whether desipramine was administered. It is also important to note that they injected a very high dose of 6-OHDA (30  $\mu$ g) in the MFB, a dose 3.75x higher than what was used in this study.

There are studies that have injected desipramine, as stated in their methods and found motor deficits. However, the motor deficits found are not specifically gait related. Metz et al., (2005) stated desipramine was injected in their unilaterally 6-OHDA lesioned rats and found a motor deficit. However, the motor assessments in that study involved solely the forelimbs such as the forelimb reaching task and ladder walking task<sup>137</sup>. The ladder walking task measures deficits in limb coordination and limb placing by assessing a rat's ability to navigate across a runway with irregularly spaced rungs<sup>137</sup>. This study also did not confirm protection of noradrenergic cells in the LC through immunolabeling. Similarly, Shi, Woodward, & Chang (2006) stated desipramine injection in their methods and found a motor deficit unilaterally 6-OHDA lesioned rats, but the motor task they used was the cylinder test, which only measures limb-use asymmetry and not gait.

There are studies that have examined gait deficit in 6-OHDA lesioned animals and stated that desipramine was injected in their methods. A study by Vlamings et al., (2007) found deficits in gait using the CatWalk apparatus, however, this study used a bilaterally lesioned 6-OHDA model, not a unilateral lesion and the authors lesioned the striatum directly, not the MFB. The distinction is important as it is well-established that rats with bilateral 6-OHDA lesions caused impairments in more behavioral motor paradigms than unilateral 6-OHDA lesions<sup>139</sup>. A bilateral lesion is more severe than a unilateral lesion and prevents possible compensatory effects from the intact side<sup>139</sup>. Furthermore, Vlamings et al., (2007) failed to show TH staining at the LC to confirm that noradrenergic neurons were protected. Tsang et al., (2019) stated in their methods that desipramine was injected and demonstrated a deficit in gait in unilaterally 6-OHDA lesioned rats. However, histological confirmation of LC neurons being protected was not documented. Considering the poor solubility of desipramine, it is not certain that the

noradrenergic cells in the LC were, in fact, protected.

Thus far, no studies have confirmed a gait deficit in a unilaterally 6-OHDA lesioned rats who had desipramine injected and confirmed sparing of noradrenergic cells in the LC. Therefore, future studies should verify the role of concomitant noradrenergic neuronal loss in gait performance in preclinical PD models.

### 4.2.3 MFB Coordinate Selection

Another explanation for why a deficit in gait was not found in this study while other studies with 6-OHDA models found gait deficits could be due to the MFB coordinates selected for 6-OHDA lesion (Table 2). There is variability in the effect of 6-OHDA lesion based on the coordinates used to inject into the MFB. It has been found that lesions of the ventrolateral area of the striatum lead to pronounced effects on movement initiation, sensorimotor orientation, and skilled motor behaviour, while lesions of the dorsomedial area of the striatum had more general effects on locomotion and drug-induced turning behaviour<sup>143, 145-149</sup>. It may be the case that due to the MFB coordinates used in this study, there was a heterogeneous lesion of dopamine fibers across different areas of the striatum leading to a behavioural phenotype in which animals show impairment in drug-induced turning but not gait.

**Table 2: Comparison of MFB Coordinates used for Unilateral 6-OHDA Lesion**

	Anterior-Posterior (AP)	Medial-Lateral (ML)	Dorsal-Ventral (DV)
This study's coordinates	-1.8	-2	-8.3
Carvalho et al., (2013)	- 4.4	-1	-7.8
Chuang et al., (2010)	- 4.4	-1.2	-7.8
Haddadi et al., (2014)	-5	-2.1	-7.7
Metz & Whishaw (2002)	-4	-1.5	-8.5
Monville et al., (2006)	-4.4	-1	-7.8
Tsang et al., (2019)	-1.8	- 2	-8.3
Vandeputte et al., (2010)	-3.4	-1.6	-8.2
Wree et al., (2011)	- 2.3	-1.5	-9
Yuan et al., (2005)	-3.2	-1.5	-8.7
Zhou et al., (2015)	-4.4	-1.1	-7.8

The most commonly used MFB coordinates used in studies that this thesis cited are: AP -4.4, ML -1 or -1.5, DV -7.8. Following comparison with previous studies, the MFB coordinates used in this study are relatively more anterior. A study by Metz & Whishaw (2002) which investigated the relationship between drug-induced rotation rates and scores on two tests of motor behavior in rats unilaterally lesioned with 6-OHDA in the nigrostriatal bundle supports this explanation. These authors found that the intensity of apomorphine drug-induced rotations did not correlate with their measures of motor

performance. The motor assessments in their study included end point and qualitative measures of forelimbs and hindlimbs assessed in a skilled reaching task and a skilled ladder rung walking task<sup>142</sup>. It was concluded that while scores on apomorphine rotations tests are an indicator of the extent of dopamine depletion following 6-OHDA lesion, they are a poor predictor of motor impairment<sup>142</sup>. The authors therefore suggested that drug-induced rotational behaviour and skilled movement should be considered independent effects of 6-OHDA lesions<sup>142</sup>. The results from three other independent studies also suggest that rotation intensity and motor impairments are at most, only weakly related<sup>162-164</sup>. The explanation provided for the poor correlation between rotational behaviour and motor skill relates to variability in lesion site, as variations in needle insertion site may cause damage to fibers that are differentially involved in rotational behaviour versus skilled movements<sup>142, 147-151</sup>. These results support the earlier claim made in this section that there is variability in the outcomes of 6-OHDA lesion based on the MFB coordinates due to the somatotopy of the MFB. Whole body movements used in rotational behaviour, forelimb movements used for reaching, and movements using all four limbs for walking are partially independent. The observation made in Metz and Wishaw's (2002) study that the rats that showed fewer rotations had a smaller number of residual intact dopaminergic cells in the lateral part of the substantia nigra, whereas none of the high rotators showed remaining cell bodies in that area, supports that claim as well<sup>142</sup>.

Based on these findings, it is likely that the lack of a motor deficit seen in the rotarod and CatWalk tasks, while there was the presence of apomorphine induced rotations in the 6-OHDA lesioned animals, may be partly due to the MFB coordinates used in the current study.

#### 4.2.4 Difference in Type of Locomotion Measured

Although this study and the previous study done in this laboratory (Tsang et al., 2019) used the same MFB coordinates for the 6-OHDA lesion, there is a discrepancy in the measurement of a motor deficit. This study did not find a gait impairment using the CatWalk apparatus while the previous study did. As previously discussed, this discrepancy may be due to a lack of noradrenergic cell protection in the LC as this was

not confirmed in the previous study. Another possible explanation for this discrepancy in the expression and measurement of gait may be due to the effects of food restriction in the animals. After all, food deprivation changes animal's motivation during behavioural tasks. Animals that are food deprived and trained to expect a food reward at the end of a task are engaging in goal-oriented locomotion, not purely spontaneous locomotion. While the CatWalk measures voluntary movement, the previous study from our laboratory measured goal-oriented movement, while the current study measured spontaneous movement. In the current study true spontaneous movement was assessed because animals were not food deprived or given a reward for crossing the Catwalk. Animals walked from one end of the Catwalk to the other end in this study with no motivational prompts. Goal directed activities and appetitive motivation are associated with striatal dopamine<sup>148</sup>. It is well known that the ventromedial striatum is highly innervated by dopaminergic fibers and implicated in reward and motivation<sup>149-153</sup>. Manipulating dopamine levels in the ventromedial striatum can affect performance on multiple tasks believed to measure motivated behavior, including conditioned reinforcement, Pavlovian-instrumental transfer paradigms, effort-based decision-making tasks, and progressive ratio schedules<sup>154-158</sup>. Thus, it is strongly suggested that causing a dopamine depletion in the striatum will have an impairment in goal directed locomotion, but not spontaneous movement. This may be the reason why, although the same MFB coordinates were used in this laboratory's previous study, due to food restricting the animals, this led to voluntary, yet goal directed locomotion as hungry animals were motivated by received a food reward when crossing the CatWalk. In the current study, motivation did not play a role, thus the locomotion was both voluntary and spontaneous. It may be the case that striatal dopamine depletion does not cause a deficit in spontaneous locomotion sensitive enough to be measured by the CatWalk.

This finding is supported in human PD as it is well known that striatal dopamine depletion is known to affect patient's cognitive motivation, and disorders of motivation (such as apathy) are common in PD<sup>159</sup>. This phenomenon is reflected in humans as it has been found that the basal ganglia and supplementary motor area, which play a role in the affective organization of goal-directed locomotion, are impaired in PD<sup>160-161</sup>.

## 4.3 Conclusion

### 4.3.1 Limitations

The greatest limitation of this study is that no deficit in gait was found in the lesioned animals compared to sham lesioned animals. Therefore, the treatment outcomes of this intervention in the forced motor task (rotarod) and voluntary motor task (CatWalk) carried out in this study cannot be evaluated. The methods require refinement by adjusting the MFB lesion coordinates used and adding a skilled reaching task like the cylinder test. By adding a skilled reaching task, it can be determined if a motor impairment that develops is exclusive to rotational behaviour, forelimb usage, or gait.

### 4.3.2 Significance

This was the first study that attempted to examine the effects of a BoNT-A injection at the STN. The goal of this study was to assess drug-induced movement through the apomorphine rotation test, forced movement through the rotarod, and spontaneous locomotion using the CatWalk apparatus. This study found that an injection of at least 2ng of BoNT-A was successful in reducing pathological drug induced rotation for up to 3 months. The results of the apomorphine induced rotation test found in this study agree with a previous study published from our laboratory. Tsang et al., (2019) injected BoNT-A into the EPN and found that the EPN is a superior site to achieve transient symptomatic relief following intracerebral injections of BoNT-A. The current study found reductions in pathological rotations starting at a dose of 2ng of BoNT-A, while Tsang et al., (2019) found a reduction in pathological rotations at a dose of 0.5ng. Therefore, a dose 4x lower than what was needed to achieve reductions in pathological rotations in this study, was sufficient in reducing pathological rotations and improving gait. However, the study by Tsang et al. (2019) used a CatWalk testing paradigm that utilized food restricted rats trained using food reward, hence studying goal-directed locomotion, while the current study employed animals without food restriction where locomotion is unlikely influenced by motivation for food reward. While our results point to a notion that spontaneous locomotion might not be influenced by functional integrity of BG circuitry, results favors

that BoNT-A injections into the EPN would have better potential for treating PD, particularly validation in primate models of PD.

### 4.3.3 Future Directions

Unfortunately, as a behavioral motor deficit was not found in the PD animal model, whether BoNT-A injections into the STN would have improved motor symptoms is currently unknown. However, a future study may be conducted with adjusted MFB coordinates in which both drug-induced rotations, which indicate dopaminergic depletion in the striatum and SN, occur as well as motor deficits. Then it can be assessed whether BoNT-A at the STN improves motor output and can potentially be used as a treatment option in PD. Once motor outcomes are assessed in a PD animal model demonstrating a motor deficit, further comparison can be made with other studies investigating the outcomes of BoNT-A at other injection sites. From there, it can be determined which site is the best target for neuromodulation using BoNT-A in treatment outcomes. Overall, the results suggest that BoNT-A injection in the central nervous system is a viable treatment option with promising potential.

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# Appendices

## Appendix 1 - Animal Use Protocol Approval



2015-087:6:

AUP Number: 2015-087

AUP Title: *Understanding the effects of botulinum toxin on the basal ganglia in a rodent model of Parkinson disease by behavioural studies and multi-neuronal recordings.*  
 Yearly Renewal Date: 03/01/2020

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2015-087 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

- 1) Animals used in this research project will be cared for in alignment with:
  - a) Western's Senate MAPPs 7.12, 7.10, and 7.15  
[http://www.uwo.ca/univsec/policies\\_procedures/research.html](http://www.uwo.ca/univsec/policies_procedures/research.html)
  - b) University Council on Animal Care Policies and related Animal Care Committee procedures  
[http://uwo.ca/research/services/animalethics/animal\\_care\\_and\\_use\\_policies.html](http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html)
- 2) As per UCAC's Animal Use Protocols Policy,
  - a) this AUP accurately represents intended animal use;
  - b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
  - c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
  - d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC. [http://uwo.ca/research/services/animalethics/animal\\_use\\_protocols.html](http://uwo.ca/research/services/animalethics/animal_use_protocols.html)
- 3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
  - a) be made familiar with and have direct access to this AUP;
  - b) complete all required CCAC mandatory training ([ training@uwo.ca] training@uwo.ca); and
  - c) be overseen by me to ensure appropriate care and use of animals.
- 4) As per MAPP 7.15,
  - a) Practice will align with approved AUP elements;
  - b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
  - c) UCAC policies and related ACC procedures will be followed, including but not limited to:
    - i) Research Animal Procurement
    - ii) Animal Care and Use Records
    - iii) Sick Animal Response
    - iv) Continuing Care Visits
- 5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, <http://www.uwo.ca/hr/learning/required/index.html>

Submitted by: Copeman, Laura  
 on behalf of the Animal Care Committee  
 University Council on Animal Care

## Appendix 2 - Health Canada Ketamine Use Approval



[Redacted]

2019-03-11

Your file    Votre référence

Our file    Notre référence

HC6-70-498-8  
HC6-53-10-126

Animal Care and Veterinary Services  
University of Western Ontario  
Medical Sciences Bldg

[Redacted]

Attention: Dr. Emily Truscott

Dear Madam:

Authorization No. 47641.01.19  
Mandar S. Jog, MD  
Department of  
Clinical Neurological Sciences  
Faculty of Neuroscience  
London Health Sciences Centre

[Redacted]

This letter authorizes you to honour the requisition that will be prepared over the signature of Dr. Mandar S. Jog of the above address for the following controlled substance, which will be used for a scientific purpose:

160 mL (16 g)

Ketamine (100 mg/mL)

This authorization may be used to supply up to the total quantity of the above-mentioned substance for the period ending March 11, 2020.

Yours sincerely,

Kim Barber  
Associate Manager  
Authorizations Division  
Office of Controlled Substances

cc: Dr. Mandar S. Jog  
Contact for Authorization:

[Redacted]



## Appendix 3 - Health Canada Sodium Pentobarbital Use Approval



[Redacted]

2019-03-11

Your file    Votre référence

Our file    Notre référence

HC6-70-498-8  
HC6-53-10-126

Animal Care and Veterinary Services  
University of Western Ontario

[Redacted]

Attention: Dr. Emily Truscott

Dear Madam:

Authorization No. 47654.01.19  
Mandar S. Jog, MD  
Department of  
Clinical Neurological Sciences  
Faculty of Neuroscience  
London Health Sciences Centre

[Redacted]

This letter authorizes you to honour the requisition that will be prepared over the signature of Dr. Mandar S. Jog of the above address for the following controlled substance, which will be used for a scientific purpose:

250 mL (135 g)

Sodium pentobarbital (540 mg/mL)

This authorization may be used to supply up to the total quantity of the above-mentioned substance for the period ending March 11, 2020.

Yours sincerely,

Kim Barber  
Associate Manager  
Authorizations Division  
Office of Controlled Substances

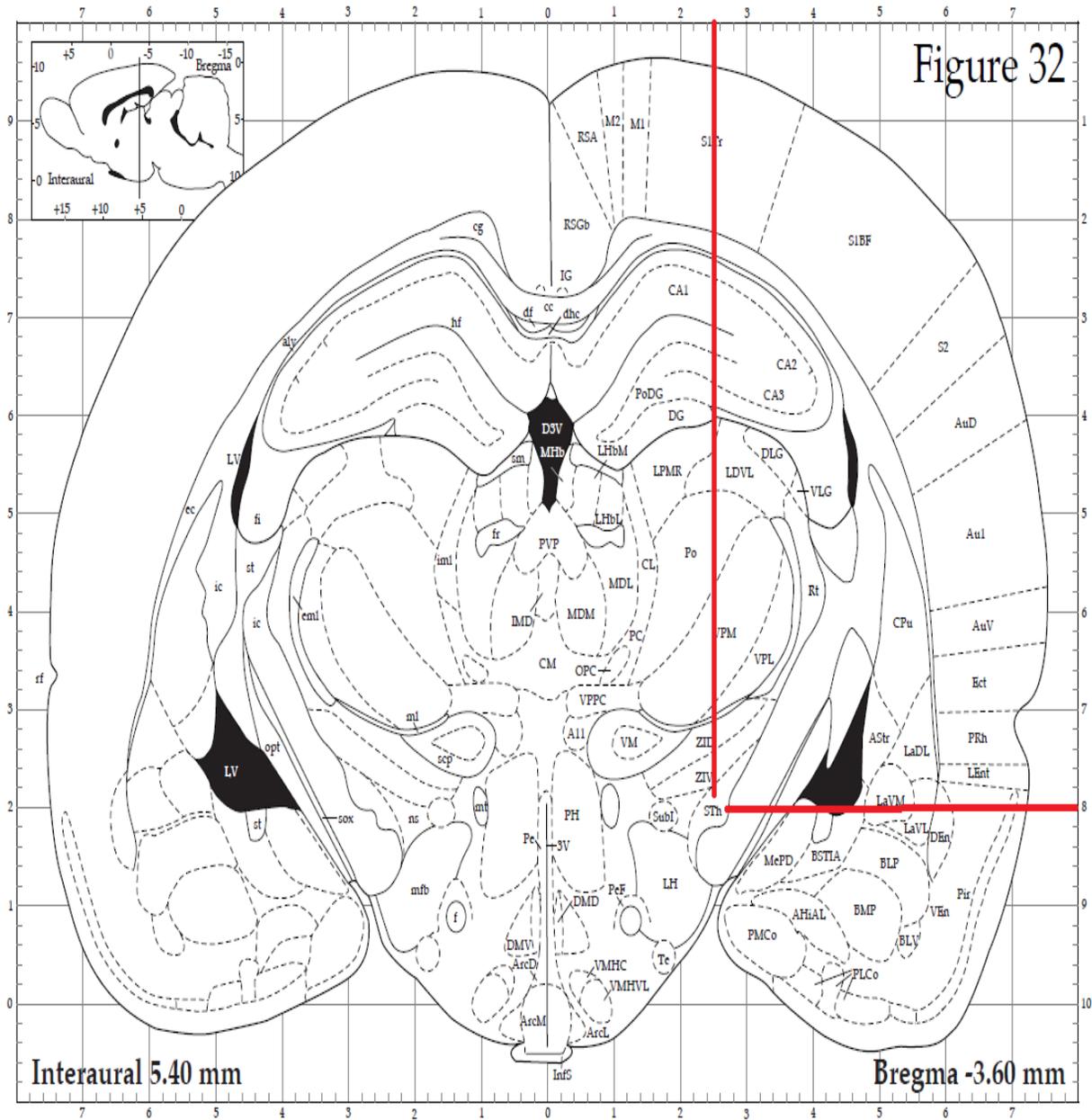
cc: Dr. Mandar S. Jog  
Contact for Authorization:

[Redacted]

Canada



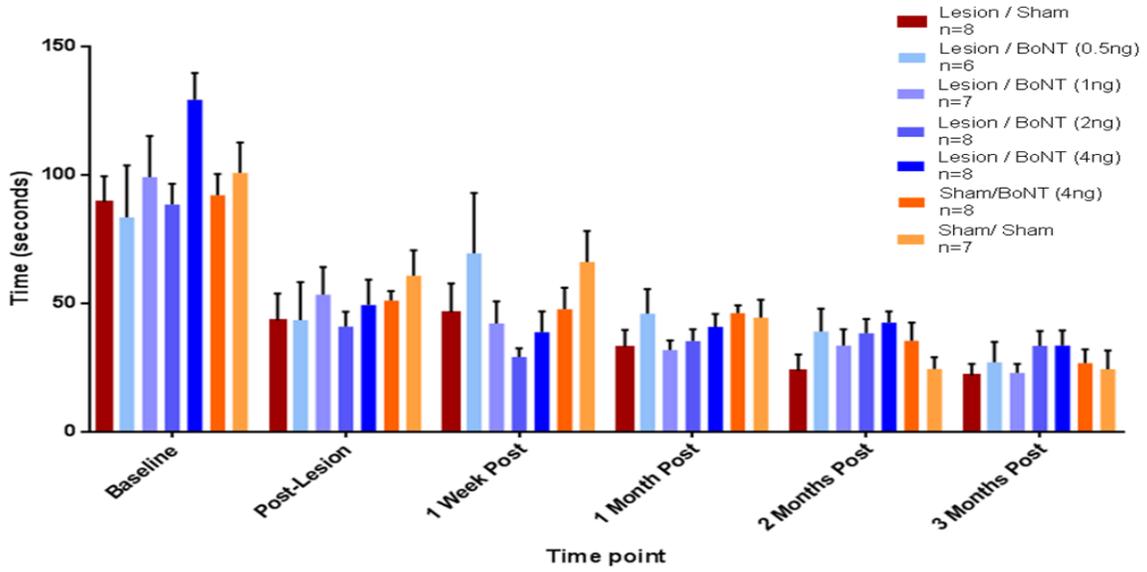
Appendix 5 - Stereotaxic Surgery Coordinates for the Subthalamic Nucleus



Appendix 6 - Full Rotarod Data

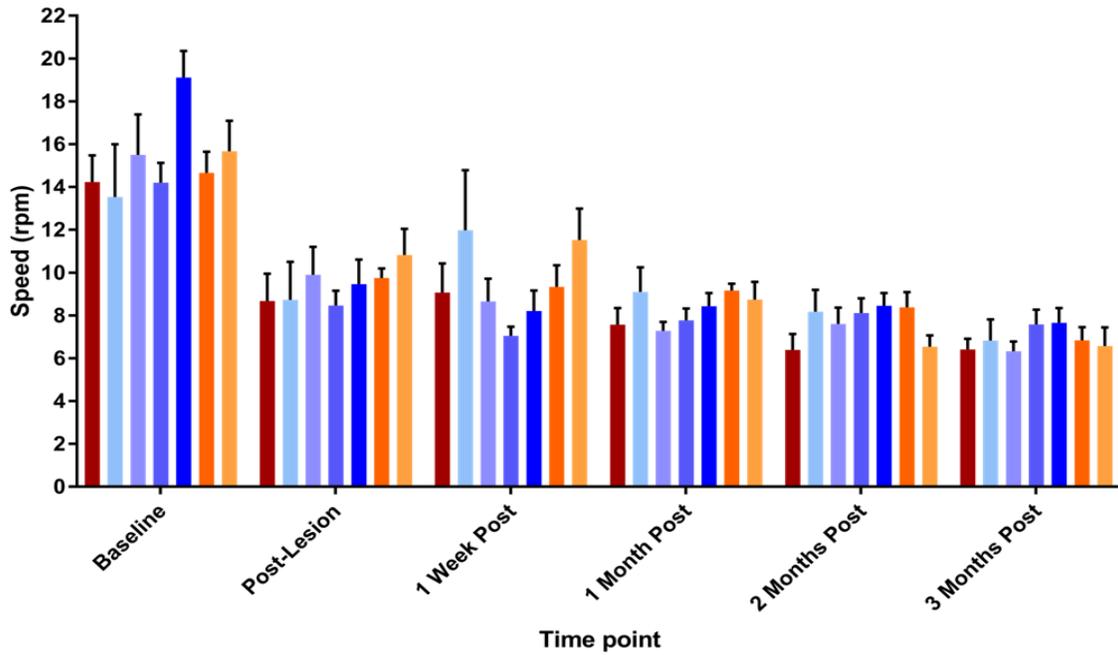
**A**

Rotarod – Time on Rod of Every Condition and Each Time Point



**B**

Rotarod – Speed Reached of Every Condition and Each Time Point



## Curriculum Vitae

**Name:** Olga Khazov

**Post-secondary Education and Degrees:** Ryerson University  
Toronto, Ontario, Canada  
2011-2016 B.A, Major in Psychology, Minor in Biology

Western University  
London, Ontario, Canada  
2017-Present, M.Sc Neuroscience.

**Honours and Awards:** Mitacs Accelerate Industry Grant  
2018-2019

Mitacs Accelerate, Graduate Research Internship Program  
2017-2018

Western Graduate Research Scholarship  
2017-2019

Dean's List, Faculty of Arts  
2012 - 2014

**Related Work Experience:** Summer Research Associate  
Movement Disorders Center – London, ON  
May 2017 – September 2017

Work Study Research Assistant  
Stress and Healthy Aging Research Lab – Toronto, ON  
2013 – 2014