

**PROGESTERONE AND THE STRIATAL
6-HYDROXYDOPAMINE MODEL OF PARKINSON'S DISEASE**

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I declare that the work described in the current thesis has been done by myself, except where indicated.

James Perry

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Abbreviations

6-OHDA	6-hydroxydopamine
ALLO	allopregnanolone
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
COMT	catechol- O-methyl transferase
DA	dopamine
DBS	deep brain stimulation
DMV	dorsal motor nucleus of the vagus nerve
DOPA decarboxylase	L-amino acid decarboxylase
DOPAC	3,4-Dihydroxyphenylacet acid
GABA	gamma-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GPe	globus pallidus, external segment
GPi	globus pallidus, internal segment
HVA	homovanillic acid
IL	interleukin
L-DOPA	L-3,4-dihydroxyphenylalanine
Maneb	manganese ethylene-bis-dithiocarbamate
MAO	monoamine oxidase
MFB	medial forebrain bundle
MMP	matrix metalloproteinase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine
NFT	neurotrophic factor
Paraquat	1,1'-dimethyl-4,4'-bipyridinium
PD	Parkinson's disease
PROG	<i>natural</i> progesterone
REM	rapid eye movement
ROS	reactive oxygen species
SN	substantia nigra

SNpc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
STAIR	Stroke Therapy Academic Industry Roundtable
TBI	traumatic brain injury
TH	tyrosine hydroxylase
TNF- α	tumor necrosis factor-alpha

Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterised by akinesia, muscular rigidity, and postural instability, due primarily to the loss of dopaminergic neurons in the substantia nigra and depletion of upstream dopamine in the striatum. Current dopaminergic treatments reduce motor symptoms, but have diminishing benefits as the disease progresses. Treatment with the neuroactive steroid *natural* progesterone (PROG) improves outcomes in many experimental models of brain injury due to its pleiotropic mechanisms of neuroprotection, many of which may also benefit PD. This thesis investigated the influence of PROG on motor impairments in the unilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesion model of PD in rats. We established a PD-like impairment with a d-amphetamine induced rotation test at day 7 after large lesions and then administered PROG (4 mg/kg or 8 mg/kg) once daily for 7 days starting at day 8. Both PROG doses markedly improved the primary outcome measure, forelimb akinesia on the adjusting steps test, with improvement sustained for six weeks after treatment had stopped. In a second study the beneficial influence of PROG (8 mg/kg) on akinesia was replicated for rats with large lesions and was extended to rats with small lesions so that the latter rats were now similar to sham operated controls. We also found that PROG modestly improved postural instability of the ipsilateral forelimb on the postural instability test, and sensorimotor integration on the whisker test, but did not improve skilled reaching accuracy on a single-pellet reaching task, forelimb use asymmetry on the cylinder test, sensory neglect on the corridor test, or rotation bias after apomorphine. Furthermore, PROG did not change striatal tyrosine hydroxylase density when assessed in rats with large lesions. This study has provided the most thorough examination to date regarding PROG's influence on motor skills in an animal model of PD. Furthermore, this study has produced novel evidence of the beneficial effects of PROG treatment on forelimb akinesia. These initial promising findings suggest that PROG is an effective therapy for akinesia and thus provides an impetus to further investigate PROG's efficacy for the treatment of PD.

Chapter 1

Introduction

This introductory chapter provides a brief orientation of the thesis. The “General Introduction” outlines each of the introductory chapters (chapters 3-5). The “Aims of the Current Study” and the experimental chapters (Chapters 5-7) are then introduced. The last section of this chapter, “Outline of the Thesis”, details the structure and direction of the thesis.

1.1. General Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder and affects around 1% of the population over the age of 55 (Lees et al., 2009). The disorder is characterised by slowness in the initiation of movement, muscular rigidity, postural instability, and tremor at rest; however, these motor impairments are often accompanied by non-motor symptoms such as cognitive impairment, sleep disturbance, and olfactory deficits (Chaudhuri et al., 2006; Dauer and Przedborski, 2003; Jankovic, 2008). The primary neuropathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), in association with the presence of intracytoplasmic inclusions called Lewy bodies which leads to substantial depletion of dopamine (DA) upstream in the striatum (Blesa et al., 2012; Shulman et al., 2011). Although the loss of DA leads to the major clinical symptoms of PD, the disorder is complex and has a widespread pathology (Dawson and Dawson, 2003). Other potentially important mechanisms in the pathophysiology of PD include inflammation, oxidative stress, mitochondria dysfunction, decreased levels of neurotrophins, and prion like cell-to-cell transfer of aggregated α -synuclein (Dunning et al., 2013; Levy et al., 2009; Macphee and Stewart, 2012; Nagatsu and Sawada, 2005).

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Despite nearly 200 hundred years of research since James Parkinson first described PD in his classic 1817 monograph “An Essay on the Shaking Palsy” (Parkinson, 2002) the cause of and especially the cure for PD are still unknown (Dauer and Przedborski, 2003; Valadas et al., 2014). However, current drug treatments such as L-3,4-dihydroxyphenylalanine (L-DOPA) that replace DA effectively reduce motor symptoms, but, with chronic use the benefits wear off and severe side effects such as dyskinesia (abnormal involuntary movements) occur in the majority of patients, especially as the condition worsens (Ahlskog and Muenter, 2001; Valadas et al., 2014). Additionally, there are no treatments that slow, halt, or reverse the degenerative process. Thus, there is clearly a need to explore new treatment options.

A novel possible treatment is *natural* progesterone (PROG), a hormone that is now clearly recognised as a neurosteroid, synthesized locally in the brain by neurons and glia (Guennoun et al., 2014). There is a substantial literature that shows that PROG treatment is beneficial in experimental models of traumatic brain injury (TBI), stroke, and several other models of brain injury and disease (see Deutsch et al., 2013 for a review). Interestingly, PROG’s neuroprotective properties are pleiotropic and include decreasing inflammation, neuronal apoptosis, and oxidative stress, increasing neurotrophic support, and improving mitochondria function (Deutsch et al., 2013; Wei and Xiao, 2013). Many of PROG’s pro-recovery effects are also plausible mechanisms of neuroprotection in PD. For example, inflammation is now considered an important mediator regarding DA degeneration in PD (Barcia, 2013). In the PD brain, activation of microglia produces pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 (Dzamko et al., 2014; Nagatsu and Sawada, 2005). It is relevant, then, that PROG is effective at reducing the expression of activated microglia and pro-inflammatory cytokines following experimental brain injury (Stein, 2008, 2012). PROG’s ability to inhibit inflammation is promising as

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current evidence suggests that other anti-inflammatory treatments may improve outcomes in PD (see Barcia, 2013 for a review). Despite numerous reports regarding PROG's beneficial influence in various experimental models of brain injury and disease, surprisingly few studies have examined PROG's influence in the context of an experimental model of PD. None of these PD studies have thoroughly examined the beneficial influence of PROG on motor skill functions or directly compared PROG treatment in models of the early and late stages of degeneration in PD.

Animal models of PD are useful to examine the beneficial influence of novel treatments. These models can be classified into those using environmental or synthetic neurotoxins, and those using *in vivo* expression of PD-related genetic mutations (Blesa et al., 2012; Duty and Jenner, 2011). The most commonly used model, and the one used in this thesis, are unilateral nigrostriatal dopamine lesions produced by intracerebral infusions of the neurotoxin 6-hydroxydopamine (6-OHDA, Blandini et al., 2008; Kirik et al., 1998). While far from perfect, the 6-OHDA model reproduces many of the key pathological features of PD, including progressive nigrostriatal degeneration, inflammation, mitochondrial dysfunction, and oxidative stress (Duty and Jenner, 2011). Additionally, the 6-OHDA model produces clinically relevant motor deficits such as akinesia, postural instability, impaired skilled reaching, and impaired sensory function (Dowd et al., 2005a; Meredith and Kang et al., 2006; Metz et al., 2003; Woodlee et al., 2008). The beneficial influence of novel treatments such as PROG can be assessed against these pathological and functional consequences of 6-OHDA lesions.

1.2. Aims of the Current Study

This brief introduction, expanded in subsequent chapters, shows that PD is a complex disorder with a number of mechanisms that contribute to the degenerative process. Current treatments for PD provide initial symptomatic relief but eventually become ineffective in

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most patients. Thus, there is an unmet need for additional treatment options. PROG is a neuroprotective agent that has improved morphological and functional outcomes in many experimental models of brain injury and disease. Despite these promising reports, PROG has received little attention in the context of PD. PROG may be beneficial in a model of PD due to its pleiotropic mechanisms such as reducing inflammation and increasing trophic support.

The main aim of the current study was to examine the beneficial influence of PROG treatment following unilateral 6-OHDA lesions that model PD degeneration and motor impairments. The unilateral 6-OHDA lesion model is useful for examining the beneficial influence of novel treatments such as PROG as it reproduces many of the pathological and functional changes observed in human PD. This study investigated whether 7 days of PROG treatment started at day 8 after 6-OHDA lesion surgery would improve motor impairments in both the short and long-term after lesion surgery. Furthermore, one experiment investigated whether PROG treatment could improve striatal tyrosine hydroxylase fibre deafferentation. In addition, the current study examined the beneficial influence of PROG following 6-OHDA lesions that model the early and late stages of PD degeneration. These aims were examined across three experiments.

The first experiment was a pilot study that established the time-course for motor impairments following unilateral 6-OHDA infusions to the dorsal striatum that produce large lesions. Establishing the time-course for impairments in our laboratory was a new task, and was essential to identifying an ideal treatment time-point for subsequent studies using PROG. Prior to surgery, rats were tested for forelimb akinesia on the adjusting steps test and skilled forepaw use on the sunflower seed handling test then given 6-OHDA lesions or sham surgery. At day 7 after surgery the dopamine depleting lesions were verified with a rotation after d-amphetamine test. To assess striatal supersensitivity contralateral rotation after apomorphine was tested at days 24 and 61. Forelimb akinesia and skilled forepaw use were

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examined at three repeated intervals between post-surgery days 15 and 58, as the emergence and progression of motor impairments following intrastriatal 6-OHDA lesions can be time-sensitive (Kirik et al., 1998). Rats were perfused at day 65 after surgery and tyrosine hydroxylase immunohistochemistry of the striatum was performed in a random sample of rats.

The second experiment examined the beneficial influence of two doses of PROG after large unilateral 6-OHDA striatal lesions. Rats were trained and tested pre-operatively on a single-pellet skilled reaching test and the adjusting steps test for forelimb akinesia then given 6-OHDA lesions or sham surgery. Rats with sufficient lesions according to the d-amphetamine rotation test at day 7 were assigned to PROG treatment groups. Rats with lesions were administered either 8 mg/kg PROG, 4 mg/kg PROG, or sesame oil vehicle once daily for 7 days starting at day 8 after surgery. Importantly, the dose was tapered by sequential halving on the sixth and seventh days of treatment. Sham surgery rats received sesame oil vehicle alone. Forelimb akinesia was investigated at four occasions between days 17 and 53 and skilled reaching was tested on days 24-26 and 49-51 post-surgery. Contralateral rotation after apomorphine was tested at days 29 and 62. Rats were perfused at day 64 and striatal tyrosine hydroxylase fibre density was examined. An additional group of lesion-only control rats were perfused at day 8 to serve as a reference for striatal tyrosine hydroxylase deafferentation at the time when PROG treatment was started.

The third experiment investigated the beneficial influence of a single dose of PROG after small and large unilateral 6-OHDA striatal lesions. This experiment included a larger battery of tests to assess the extent of PROG's beneficial influence on motor impairments. Rats were tested pre-operatively on the adjusting steps test for akinesia, postural instability test, cylinder test for forelimb use asymmetry, whisker evoked forelimb placing test for sensorimotor integration, and the corridor test for sensory neglect then given small or large 6-

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OHDA lesions or sham surgery. Rotation after d-amphetamine was tested at day 7 to confirm the lesions then PROG treatment was administered for 7 days starting at day 8 after surgery. Rats with small and large lesions and sham rats received 8 mg/kg PROG or sesame oil vehicle once daily with tapered withdrawal as previous. The above motor skills were tested at repeated intervals between days 17 and 62 and contralateral rotation after apomorphine was assessed at day 66 post-surgery. Rats were sacrificed at day 68 and the brains stored for histology at a later date due to time constraints.

1.3. Outline of the Thesis

The following chapter details the characteristics of PD and briefly describes its symptoms, pathogenesis, and current and emerging treatment approaches. Chapter three reviews the literature regarding PROG and neuroprotection with a focus on TBI and ischemia. Towards the end of this chapter PD models that have examined the beneficial influence of PROG treatment are also described. Chapter four briefly describes animal models of PD with a focus on the 6-OHDA model used in this thesis. The following three chapters (Chapters 5-7) report the findings of the three experiments conducted in this thesis. Chapter 8 provides a general discussion regarding the implications of the findings in relation to the literature reviewed in Chapters 2-4. The limitations of the current thesis and suggestions for future research are also discussed towards the end of Chapter 8.

Chapter 2

Overview of Parkinson's disease

2.1. Objectives

This chapter provides an overview of Parkinson's disease. The clinical characteristics of Parkinson's disease are described before the pathological factors that contribute to the disease are discussed. The final section examines current and emerging treatments for Parkinson's disease then discusses the need for new treatment options.

2.2. Characteristics of Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterised by a large number of motor and non-motor features. The majority of PD cases are sporadic (90%) with an unknown cause while the remaining fraction is familial where a number of causative gene mutations have been identified (Valadas et al., 2014). PD is more common in males than females which suggests an influence of sex hormones on the development of the disease (Smith and Dahodwala, 2014). PD affects an estimated 6 million patients worldwide (Dorsey et al., 2007) and as life expectancy increases the incidence of PD will rise as advancing age is the greatest risk factor (Collier et al., 2011). Current treatments provide effective relief of motor symptoms, but as the disease progresses these treatments become ineffective (Valadas et al., 2014). With an ageing population the need for improved therapies is becoming increasingly important.

2.2.1. Risk factors and evolution

Despite substantial research the causes of PD is still largely unknown. Epidemiology studies have identified advancing age as the primary risk factor for PD with a prevalence of about 1% at age 65 rising to nearly 5% at age 85 (Collier et al., 2011; de Lau and Breteler, 2006;

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Van Den Eeden et al., 2003). There is a greater risk for first degree relatives of PD patients, especially for siblings than for parents or children (Sundquist et al., 2006). Environmental studies have also associated pesticide exposure and repeated traumatic loss of consciousness as risk factors for PD (Dick et al., 2007). Interestingly, there is some evidence that smoking and caffeine use are associated with decreased risk (Qi and Li, 2014; Ritz et al., 2007). Since inflammation contributes to the pathogenesis of PD researchers have examined the relationship between non-steroidal anti-inflammatory drugs and the risk of developing the disease. Some studies have reported a decreased risk, especially for ibuprofen use (Gao et al., 2011; Wahner et al., 2007), while others have found no relationship (Driver et al., 2011). These findings have been valuable for identifying at risk groups, but they do not explain the evolution of PD once the disease process has initiated.

To explain the evolution of PD Braak and colleagues proposed a now widely accepted staging system according to the regional distribution of Lewy bodies and Lewy neurites (Braak et al., 2003, 2006). Lewy bodies and Lewy neurites are composed of aggregates of α -synuclein and are considered a pathological hallmark of PD (Braak et al., 2003). Braak et al. (2003) examined the regional distribution of Lewy pathology in the brains of people with clinically diagnosed PD and those with incidental Lewy pathology without clinical features. They found there was an association between the presence and distribution of Lewy pathology and the symptoms of PD. With these findings they were able to categorise PD into six stages. In Braak stages 1 and 2, Lewy pathology is restricted to the medulla oblongata/pontine tegmentum and the anterior olfactory structures. During these early stages patients are pre-symptomatic with respect to motor impairments. As the disease progresses to Braak stages 3 and 4 the substantia nigra (SN) and other areas of the basal mid- and forebrain become involved. During these stages the motor symptoms manifest. Finally, in Braak stages 5 and 6 the pathology presents in the neocortex which may in turn explain some of the

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cognitive symptoms associated with PD (Braak et al., 2003, 2006). The Braak staging system assumes that Lewy pathology begins in the gastrointestinal system then progresses in a predictable and characteristic sequence where it advances through the vagal nerve to the brainstem then subsequently ascends to the SN, and eventually spreads to the neocortex (Brundin et al., 2008). This characteristic spread of Lewy pathology allows for accurate prediction of disease advancement.

Although the Braak staging system is widely accepted not everyone agrees that it is a useful marker for disease progression. One challenge to the Braak system is that it lacks predictive validity. This challenge stems from observations of subjects with widespread Lewy pathology (Braak stages 5-6) whom showed no neurological impairment (Parkkinen et al., 2005, 2008). Others argue there is no relationship between Lewy pathology and disease duration and/or clinical severity which questions if the Braak system actually represents the temporal evolution of PD (Burke et al., 2008; Jellinger et al., 2008). Another challenge comes from reports where Lewy pathology has been reported throughout the brain but with preservation of the medullary nuclei which according to the Braak system should be the first affected areas (Jellinger, 2003).

Similarly, according to the Braak system the severity of the Lewy pathology in the affected areas should increase as the disease progresses. It would therefore be expected that areas affected earlier in the disease would have higher α -synuclein loads compared to areas affected later in the disease. However, one study found that in about 65% of subjects the α -synuclein loads in the SN and locus coeruleus were about equal to the loads observed in the dorsal motor nucleus of the vagus nerve (DMV), the point of departure for Lewy pathology in the brain (Attems and Jellinger, 2008). Furthermore, another study reported cases that had substantial Lewy pathology in the SN without any pathology observed in the DMV (Kalaitzakis et al., 2008).

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The Braak system assumes that Lewy pathology first appears in the peripheral nervous system such as enteric neurons in the gut then advances in a caudal-rostral direction to the DMV prior to progressing to the midbrain regions. Beach et al. (2010) have questioned this “gut to brain” spread of Lew pathology. Beach et al. propose that the pathology spreads from “brain to gut” according to their findings of decreasing density and frequency of phosphorylated α -synuclein in a rostral-caudal direction of the gastrointestinal tract. In comparison, a recent finding provided the first direct evidence of “gut to brain” spread of α -synuclein via the vagal nerve to the DMV in a rat model (Holmqvist et al., 2014). Taken together these findings suggest that Lewy pathology could spread in both an anterograde and retrograde direction although a bi-directional spread of pathology is not assumed in the Braak system.

Despite the challenges and criticisms of the Braak staging model the system seems to be valid in most cases. Kalaitzakis et al. (2008) reported that the spread of Lewy pathology was consistent with Braak staging in 53% of cases whereas others have reported a much higher compatibility of about 82% (Attems and Jellinger, 2008; Jellinger, 2003; Parkkinen et al., 2008). Therefore, the Braak staging system remains a useful system for staging the evolution of PD in the majority of cases.

2.2.2. Motor Symptoms

The cardinal motor features of PD are summarised in Table 2.1. These symptoms include tremor at rest, bradykinesia, rigidity, postural instability, and gait abnormalities (Jankovic et al., 2008; Smith et al., 2012). The presence of these characteristic motor features is used to make a clinically probable diagnosis of PD (Jankovic, 2008).

Tremor at rest is the most common and easily recognised symptom that usually manifests in the early stages of PD although it is not the most diagnostic feature of the

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disease (Fahn, 2003). Tremors typically occur asymmetrically in the hands at a frequency of 4-6Hz but can also involve the legs, lips, chin, and jaw (Jankovic, 2008). Tremors usually decrease with action although they can increase when walking. Tremor at rest occurs in most patients although approximately 11% never develop the symptom in the course of the disease (Jankovic, 2008).

Bradykinesia usually manifests in the early stages of PD and is considered the most diagnostic feature of the disorder (Jankovic, 2008). Bradykinesia is defined as the slowness of movement and is often used synonymously with akinesia and hypokinesia. Akinesia is the paucity of spontaneous movement such as arm swing during walking. Hypokinesia are movements that are both slower and smaller than normal such as micrographia (Berardelli et al., 2001). These three symptoms occur in PD although not all patients will present with each (Berardelli et al., 2001). Another cardinal feature that manifests early in the disease is rigidity. Rigidity refers to the increased resistance to passive movement that is usually accompanied by the “cogwheel” phenomenon (Jankovic, 2008). Patients may complain of muscle stiffness although this is not a major source of disability (Smith et al., 2012).

Postural instability is common in the late stages of PD and usually manifests after the onset of other motor symptoms (Jankovic, 2008). Postural instability has clinical importance as it contributes to the danger of frequent falls in PD (Jessop et al., 2006). The final cardinal feature of PD is gait impairments. A common gait impairment is “freezing”, a debilitating symptom that also contributes to the danger of falls (Giladi et al., 2001). Freezing episodes present as a sudden and transient inability to move where the patients feet appear “glued to the floor”. Freezing episodes are most common in relation to starting to walk, when turning, when walking in a narrow passage, and when approaching a destination (Jankovic, 2008).

Overview of Parkinson's disease

As stated at the start of this section, the cardinal motor features of PD are used to make a clinically probable diagnosis of PD. However, individual patients show considerable variation in the presentation of these motor manifestations and accompanying symptoms (Kang et al., 2005). The accompanying non-motor symptoms of PD are often under reported and underappreciated in clinical practice (Bonnet et al., 2012). These non-motor complications are briefly described in the following section.

Table 2-1

Cardinal Motor Features of Parkinson's disease.

Early features	Late Features
<i>Bradykinesia/akinesia</i> <ul style="list-style-type: none">• Slowness of movement, fatiguing with decreased amplitude of movement, arrests in ongoing movement• Decreased spontaneous movements such as eye blinking, swallowing, and arm swing	<i>Postural change</i> <ul style="list-style-type: none">• Flexed posture• Postural instability – retropulsion, propulsion, falls (en bloc)
<i>Tremor</i> <ul style="list-style-type: none">• Rhythmic sinusoidal movement of a body part due to regular contractions of reciprocally innervated muscles (either synchronous or alternating)• Occurs at rest	<i>Gait disorder</i> <ul style="list-style-type: none">• Shuffling, lack of arm swing• Festination: going from walking to running• Freezing: Feet 'sticking to the floor like glue', occurs with turning, gait initiation, enclosures like doorways
<i>Muscle rigidity</i> <ul style="list-style-type: none">• Increase in resistance to passive movement• 'Cogwheel'• Patients may complain of stiffness but not a major source of disability	

(Table adapted from Smith et al., 2012).

2.2.3. Non-motor Symptoms

The symptoms described in the previous section represent the cardinal motor features of PD. However, non-motor symptoms are also a common feature of PD (Table 2.2). Non-

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motor features include cognitive impairment, neuropsychiatric symptoms, sleep dysfunction, autonomic symptoms, and sensory deficits (Chaudhuri et al., 2006). Non-motor symptoms occur throughout the course of the disease and in some instances precede the motor symptoms (Bonnet et al., 2012; Slow et al., 2014; Wu et al., 2011).

Neuropsychiatric symptoms of PD include depression, anxiety, and apathy (Chaudhuri and Schapira, 2009). Depression affects up to 27.6% of patients and can occur at any stage of the disease (Ravina et al., 2007). Reports show that depression is a major contributor to reduced quality of life (Rahman et al., 2008; Visser et al., 2008). Anxiety usually exists alongside depression and can affect up to 50% of patients during the course of the disease (Bonnet et al., 2012; Chaudhuri and Schapira, 2009). Apathy is another common behavioural change in PD that affects 32% of patients (Starkstein et al., 2009). Mild cognitive impairment and frank dementia are prevalent non-motor features of PD. Mild cognitive impairment in domains such as executive function, psychomotor speed, visuospatial abilities, language, and memory have been reported in 18-55% of patients without dementia (Goldman et al., 2011). Frank dementia is common during the latter stages of PD and is the most debilitating aspect of the disorder with 83% of patients experiencing dementia after 20 years disease duration (Hely et al., 2008).

In addition to cognitive and neuropsychiatric symptoms patients often experience sleep, autonomic, and sensory dysfunction. Sleep dysfunction is present in up to 90% of PD patients and includes insomnia, hypersomnia, nightmares, sleep fragmentation, nocturnal movements, and rapid eye movement (REM) sleep behaviour disorder (Chaudhuri et al., 2010; Stacy, 2002). REM sleep behaviour disorder consists of excessive motor activity during dreaming in association with loss of skeletal muscle atonia during REM sleep which enables patients to physically enact their dreams (Chaudhuri et al., 2006). During these episodes patients can injure themselves and their bed partners. REM sleep behaviour disorder

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occurs in up to 60% of patients and can precede the motor stage of PD by several years (Chaudhuri et al., 2006; Claassen et al., 2010; Olson et al., 2000; Slow et al., 2014).

Autonomic symptoms in PD include constipation, bladder and sexual dysfunction, and increased sweating (Chaudhuri et al., 2006). Constipation is one of the most common non-motor symptoms in PD (Abbott et al., 2001). Constipation can precede the motor stage and is associated with a 4.5 fold increase in risk of developing PD (Abbott et al., 2001). Sensory symptoms such as olfaction dysfunction, pain, and paresthesia are frequent in PD (Jankovic, 2008). Hyposmia, a disorder of odour detection and discrimination could be an early sign of PD as one study found it was associated with a 10% increase risk of developing the disease 2 years later (Ponsen et al., 2004).

The symptoms described in this section show that non-motor complications often accompany the cardinal motor features of PD. Some of the non-motor symptoms such as hyposmia and REM sleep behaviour disorder can precede the motor stages of the disease and may be the earliest signs of PD.

Table 2-2

Non-motor Features of Parkinson's disease.

<p><i>Neuropsychiatric</i></p> <ul style="list-style-type: none"> • Psychosis (hallucinations, delusions, illusions) • Depression • Apathy • Anxiety • Anhedonia • Attention deficit • Impulsive and compulsive behaviours • Panic attacks • Cognitive impairments * • Dementia <p><i>Autonomic symptoms</i></p> <ul style="list-style-type: none"> • Orthostatic hypotension (related falls) • Gastrointestinal dysfunctions (constipation, fecal incontinence)* • Bladder disturbances (urgency, frequency) • Nausea • Vomiting • Drooling • Increased sweating • Sexual dysfunctions (hypersexuality, erectile dysfunction) • Dysphagia/choking 	<p><i>Sleep disorders</i></p> <ul style="list-style-type: none"> • Rapid eye movement (REM) sleep behaviour disorder (loss of atonia)* • Vivid dreaming • Restless legs syndrome • Excessive daytime somnolence • Insomnia <p><i>Sensory deficits</i></p> <ul style="list-style-type: none"> • Amosmia (olfactory deficits) * • Ageusia (taste deficits) * • Pain • Paresthesia <p><i>Other symptoms</i></p> <ul style="list-style-type: none"> • Fatigue • Diplopia • Blurred vision • Seborrhoea • Weight loss • Weight gain (possibly drug induced)
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*Nonmotor symptoms that often precede PD motor impairments (table adapted from Chaudhuri et al., 2006; Smith et al., 2012).

2.3. Pathological Factors in Parkinson's disease

PD is characterised by the depletion of striatal DA due to the degeneration of dopaminergic neurons in the SNpc (Dauer and Przedborski, 2003). This section starts with a description regarding the role of DA depletion and basal ganglia dysfunction as these are considered key to the motor impairments in PD. The factors that contribute to the death of SNpc neurons is not fully understood although inflammation, prion-like propagation of α -synuclein, oxidative stress, mitochondrial dysfunction, reduced neurotrophic factors, and vascular degeneration

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are possible candidates that contribute to the pathogenesis of PD. These mechanisms are briefly described and are summarised in Figure 2.1.

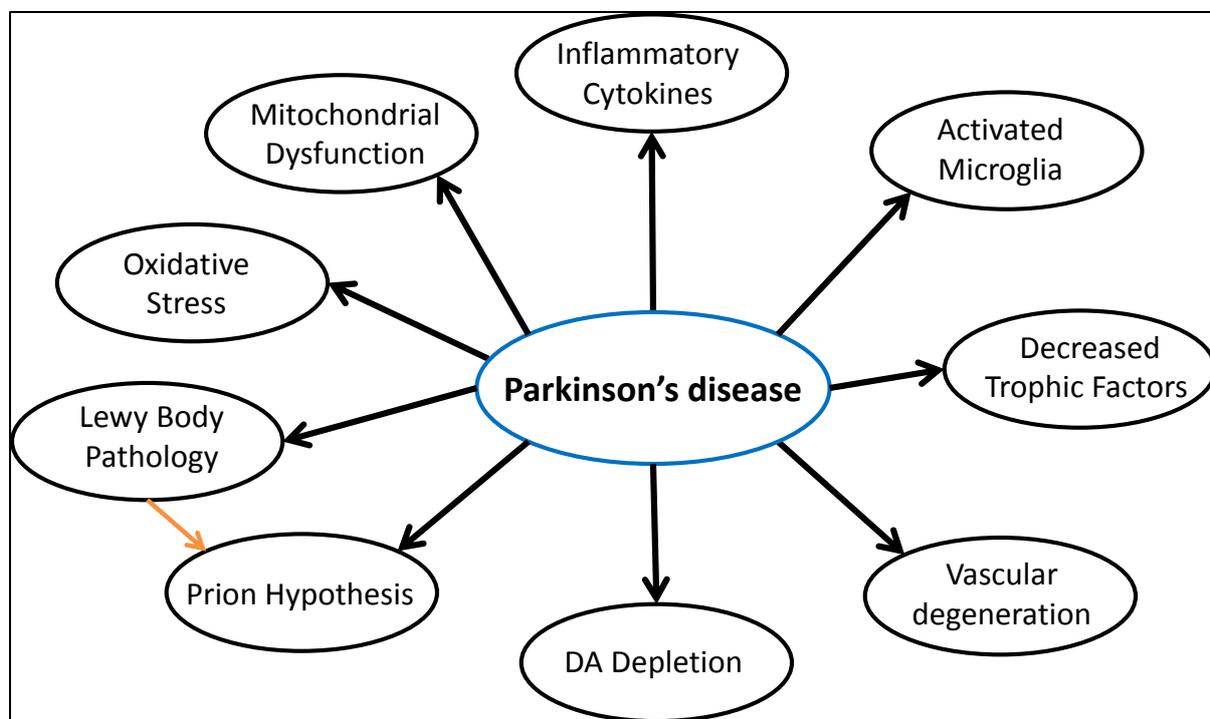


Figure 2.1. Flow chart summarising the factors that contribute to the pathogenesis of Parkinson's disease.

2.3.1. Basal ganglia and dopamine theory

There is little doubt that loss of dopaminergic neurons in the SNpc significantly contributes to the cardinal motor features of PD (Fahn and Sulzer, 2004). Degeneration of dopaminergic neurons in the SNpc leads to depletion of striatal (caudate nucleus and putamen) DA and subsequent dysfunction of the basal ganglia (Smith et al., 2012). Due to the remarkable compensatory capacity of the dopaminergic system motor symptoms do not manifest until striatal DA is depleted to ~80% and ~60% SNpc dopaminergic neurons have been lost (Dauer and Przedborski, 2003). Abnormalities of basal ganglia function due to dopamine depletion are thought primarily responsible for PD motor impairments (Galvan and Wichmann, 2008).

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Therefore, a brief description of the basal ganglia is necessary to understand the motor dysfunctions that develop in PD.

The basal ganglia are a group of interconnected structures that comprise the striatum, the globus pallidus (both the internal, GPi; and external segments, GPe), the subthalamic nucleus, and the pars reticulata (SNr) and pars compacta of the substantia nigra (Galvan and Wichmann, 2008). The basal ganglia are components of re-entrant cortico-subcortical circuits of which the motor circuit is the most researched due to its importance for movement disorders such as PD (see Figure 2.2; DeLong and Wichmann, 2007). In the classic motor circuit cortical information flows through the basal ganglia via two major striatal projection systems known as the “direct” and “indirect” pathways (Smith and Wichmann, 2014). In the normal state, activation of the direct pathway inhibits the GPi/SNr which in turn has an excitatory effect on thalamocortical interactions. Indirect pathway activation has an excitatory effect on the GPi/SNr thereby producing an inhibitory effect on thalamocortical interactions. Dopamine's net action is to reduce GPi/SNr activity, thereby facilitating activity of thalamocortical interactions leading to greater activation of the cerebral cortex which facilitates movement (Galvan and Wichmann, 2008). In the PD state, depletion of striatal DA causes insufficient activation of the direct pathway and insufficient inhibition of the indirect pathway. This imbalance leads to over-activity of the GPi/SNr with an overall inhibitory effect on thalamocortical interactions. This leads to under-activation of the cerebral cortex and subsequently clinical hypokinesia (Braak and Del Tredici, 2008).

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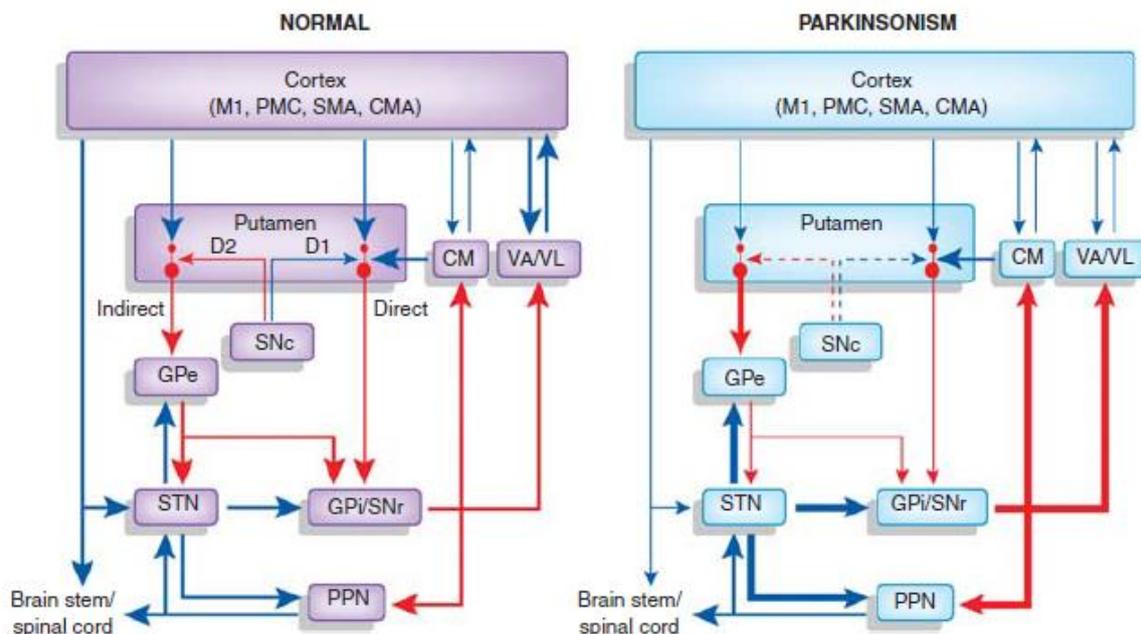


Figure 2.2. A schematic representation of the basal ganglia motor circuits in normal and parkinsonian states. The direct pathway, represented by the red lines, indicates inhibitory projections. The indirect pathway, represented by the blue lines, indicates excitatory projections. The changes in the thickness of the arrows in the parkinsonian state indicate the increase (larger arrow) or decrease (thinner arrow) in activity of specific connections. Denervation of the dopaminergic projection from the SNc to the putamen in the parkinsonian state is represented by the dashed lines. CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus, external segment; GPi, globus pallidus, internal segment; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (figure and description adapted from Galvan and Wichmann, 2008; Smith et al., 2012).

While SNpc neurons are preferentially lost in PD their degeneration are by no means exclusive. Although nigrostriatal DA degeneration and subsequent basal ganglia dysfunction significantly contributes to motor and perhaps some other impairments, numerous non-motor symptoms associated with PD cannot be explained by DA deficiency alone (Ahlskog, 2007; Bonnet et al., 2012; Shulman et al., 2011). Furthermore, the pathological changes in PD are

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not limited to DA depletion. Therefore, it is important to examine the pathogenesis of PD beyond DA deficiency alone.

2.3.2. Inflammation

Neuroinflammation is an important contributor to the pathogenesis of PD. Interest in neuroinflammation resulted from a post-mortem analysis of the SN of PD patients which revealed the presence of activated microglia, a sign of inflammation (McGeer et al., 1988). More recently, increased levels of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 have been observed in the brain, cerebrospinal fluid, and peripheral blood mononuclear cells of PD patients (see Dzamko et al., 2014 for a review). This shows that inflammation is clearly associated with the pathogenesis of PD.

Microglia play an integral role in the coordination of inflammation in PD. In the brain microglia typically exist in a resting state where they perform surveillance of the surrounding brain environment but become activated in response to cues such as brain injury or immunological stimuli (Block et al., 2007). Once activated, microglia can serve beneficial functions to neuronal survival through the release of anti-inflammatory and neurotrophic factors, and the removal of damaged neurons and foreign substances (Garden and Moller, 2006). However, microglia can become over activated which produces a self-sustaining cycle of inflammation that drives progressive neurodegeneration (Qian et al., 2010). In PD, microglia activate in response to the molecules released by damaged and dying dopaminergic neurons. Activated microglia increase the expression of pro-inflammatory cytokines such as TNF- α and IL-1 β and increase the production of harmful reactive oxygen (ROS) and nitrogen species (Dias et al., 2013). The release of large levels of proinflammatory cytokines and ROS is toxic to the neighbouring neurons, resulting in a self-sustaining cycle of neurodegeneration and inflammation (Barcia, 2013). Thus therapies that reduce neuroinflammation may be beneficial in the treatment of PD.

2.3.3. Alpha-synuclein and the prion hypothesis

A pathological hallmark of PD is α -synuclein pathology in the form of Lewy bodies and Lewy neurites (Braak et al., 2003). α -synuclein is a neuronal protein but its abnormal aggregation contributes to the pathogenesis of PD (Stefanis, 2012). Braak et al. (2004) described the regional distribution of α -synuclein and proposed that this distribution correlated with the disease stage. However, Braak et al. was unsure what mechanism was responsible for the spread of α -synuclein.

A new and exciting hypothesis is that α -synuclein is a prion-like protein and that PD is a prion-like disease (Olanow and Brundin, 2013). Prions are proteins that acquire alternative conformations that become self-propagating (Prusiner, 2014). Prions cause neurodegenerative disease by misfolding into a β -sheet rich conformation that polymerizes to form toxic oligomers and amyloid plaques (Olanow and Brundin, 2013). Like prions, α -synuclein can also adopt a β -sheet rich conformation (Prusiner, 2012). The misfolded protein acts as a template to promote conformational change in other proteins and once established the process becomes self-propagating causing a chain-reaction that leads to neurodegeneration (Olanow and Brundin, 2013; Prusiner, 2012).

The suggestion that PD might be a prion disorder stemmed from the observation of Lewy body pathology in the fetal mesencephalic tissue grafted into the putamen of PD patients. After a PD patient died 18 months after fetal mesencephalic tissue transplantation, initial histology revealed good graft survival that was well integrated to the host striatum without discernible Lewy bodies (Kordower et al., 1995). However, two independent studies reported that the actual grafted neurons of other patients who died 11-16 years after transplantation had α -synuclein positive Lewy bodies that were identical to the Lewy body pathology in PD (Kordower et al., 2008; Li et al., 2008). These patients had Lewy bodies in 2-8% of the grafted neurons which is comparable to the levels detected in non-grafted PD

patients. Furthermore, these grafts showed signs of dysfunction as there was less staining for tyrosine hydroxylase and the DA transporter. These findings were supported in a subsequent report of a patient who received grafts at 12 and 16 years before death (Li et al., 2010). In this study, they found that 2% of the 12-year old and 5% of the 16-year old grafted dopamine neurons contained Lewy bodies positive for α -synuclein. This suggests that Lewy bodies develop gradually in grafted neurons (Li et al., 2010). Since the grafts in these studies were derived from multiple genetically unrelated donors it is likely that the Lewy pathology in the transplants resulted from host-to-graft propagation (Olanow and Brundin, 2013). This host-to-graft propagation suggests that α -synuclein is prion-like and PD is a prion-like disorder.

In support of the prion hypothesis laboratory studies have shown *in vitro* and in animal models that α -synuclein is transferred from neurons to neurons (Desplats et al., 2009; Hansen et al., 2011; Lee et al., 2010; Luk et al., 2009). These studies show that α -synuclein is taken up by neurons and promotes misfolding in the host α -synuclein which leads to dopaminergic neurodegeneration (for reviews see Dunning et al., 2013; Geroge et al., 2013; Olanow and Brundin, 2013; Sato et al., 2014; Visanji et al., 2013). If PD can be confirmed as a prion like disorder it will revolutionise our understanding of the disease and will provide new targets for neuroprotective treatments.

2.3.4. Mitochondrial dysfunction, oxidative stress, and cell death

Mitochondrial dysfunction and oxidative stress are implicated in the pathogenesis of PD. Mitochondrial dysfunction was first implicated in PD after several drug users accidentally injected themselves with the heroine analogue 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP). Within days they developed parkinsonism and post-mortem analysis confirmed significant dopaminergic neuron loss in the SNpc (Langston et al., 1983). MPTP crosses the blood-brain barrier (BBB), and then is metabolized to 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ is taken up by the DA transporter into dopaminergic neurons where it inhibits complex

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I of the mitochondrial transport chain which leads to cell death (Hauser and Hastings, 2013). Similarly, decreases in mitochondrial complex I activity in the SNpc of patients with sporadic PD has been reported (Schapira et al., 1990; Hattori et al., 1991). Inhibition of complex I activity generates excessive levels of ROS which leads to oxidative stress and subsequently cell death (Subramaniam and Chesselet, 2013).

In addition to deficient complex I activity, inflammation and decreased levels of antioxidants also contributes to oxidative stress in PD. As discussed above, neuroinflammation contributes to oxidative stress as microglia when activated are a major source of ROS. The production of ROS by microglia damages neurons which in turn activate additional microglia thus creating a self-sustaining inflammatory-oxidative environment (Taylor et al., 2013). Decreased levels of the antioxidant glutathione in the SN of PD brains have been reported (Pearce et al., 1997; Perry et al., 1982; Sian et al., 1994; Sofic et al., 1992; Riederer et al., 1989). Glutathione is a molecule that is transported to the mitochondria where it functions as a major antioxidant (Hauser and Hastings, 2013). Depletion of glutathione in the SN results in selective decrease of mitochondrial I complex activity and thus contributes to oxidative stress (Dias et al., 2013). Taken together these findings suggests that deficient mitochondrial function, neuroinflammation, and decreased levels of the antioxidant glutathione all contribute to oxidative stress and subsequent cell death in PD.

The mechanisms underlying cell death in PD are thought to involve a combination of cell-autonomous (mechanisms that take place inside the dying neuron) and non-cell-autonomous mechanisms (mechanisms that originate from outside of the neuron; Hirsch et al., 2012). Hirsch et al. (2012) propose that cell-autonomous mechanisms include the build-up of damaged mitochondria and non-cell-autonomous mechanisms involve prion like misfolded proteins and inflammation. These mechanisms combine to initiate cell death and

propagate the neurodegenerative process. For a neuroprotective treatment to be effective it would need to address the multifactorial cascade that leads to cell death.

2.3.5. Neurotrophic factors

Alterations in the expression of neurotrophic factors (NTFs) have been implicated in the pathogenesis of PD. NTFs are proteins that support the survival and function of neurons (Ramaswamy et al., 2009). NTFs can enhance mitochondrial function by acting as anti-excitotoxins and anti-oxidants (Kordower and Bjorklund, 2013).

Brain-derived neurotrophic factor (BDNF) is a NTF that is required for the normal development of dopaminergic neurons and is expressed in the nigrostriatal system of adult brains (Peterson and Nutt, 2008). In post-mortem samples, BDNF expression was significantly reduced in the caudate, putamen, and SN of patients with PD (Mogi et al., 1999). In a similar study, BDNF mRNA expression was reduced by 70% in the SN (Howells et al., 2000). The reduced expression of BDNF was likely due in part to the loss of dopaminergic neurons that contain BDNF. However, the surviving DA neurons in patients expressed 20% less BDNF compared to neurons of controls (Howell et al., 2000).

Altered BDNF expression in the serum of PD patients has also been reported. In one study serum BDNF was decreased in patients that had moderately severe motor impairments compared to healthy controls (Scalzo et al., 2010). However, among patient's higher levels of serum BDNF was associated with longer disease duration and severity of motor impairments (Scalzo et al., 2010). In contrast others found that serum BDNF levels were actually increased in patients with severe motor impairments (Ventriglia et al., 2013). Taken together these findings suggest that serum BDNF levels are reduced during the early stages of the disease but increase with advancing disease duration and symptom severity. The increase of

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serum BDNF levels with disease progression suggests a compensatory role of BDNF during the latter stages of PD.

Other NTFs of interest to PD are glial cell line-derived neurotrophic factor (GDNF) and neurturin which are both essential for dopaminergic neuron survival (Peterson and Nutt, 2008). Neither GDNF nor neurturin are reduced in PD brains but both have improved outcomes in both *in vitro* and animal models of PD. Similarly, BDNF administration has improved outcomes in animal and *in vitro* models of PD (see Peterson and Nutt, 2008; Kordower and Bjorklund, 2013 for reviews). These suggest that therapies which improve NTF expression may be beneficial for the treatment of PD.

2.3.6. Vascular degeneration

Vascular degeneration also contributes to the pathology of PD. Guan et al. (2012) examined cerebrovascular markers in post-mortem PD brains and found compromised BBB, due to endothelial cell 'clusters', as well as capillaries that had a larger diameter, shorter length, and less branching in the SN, middle frontal cortex, and brain stem nuclei compared to age-matched controls. Loss of the integrity of the BBB and poorer micro-vasculature reduces oxygen supply and expose the brain to harmful agents such as pro-inflammatory cytokines, which are known to be elevated in the periphery of patients with PD (Dzamko et al., 2014; Guan et al., 2012). Thus treatments that improve the integrity of the BBB, counter inflammation and reduce exposure to free radicals would be expected to improve outcomes in PD.

2.3.7. Current and emerging therapies

Current pharmacological therapies for PD primarily focus on treatments that restore DA depletion. DA is synthesised in the brain in two stages from the essential amino acid tyrosine. First, tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by the rate-limiting

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enzyme tyrosine hydroxylase. In the second stage L-DOPA is converted to DA by the enzyme aromatic L-amino acid decarboxylase (DOPA decarboxylase; Elsworth and Roth, 1997). DA is metabolised to its inactive metabolites by the enzymes monoamine oxidase (MAO), aldehyde dehydrogenase, and catechol- O-methyl transferase (COMT; Elsworth and Roth, 1997).

Since DA does not cross the BBB its precursor L-DOPA has remained the gold-standard therapy for the motor symptoms of PD for over fifty years (Smith et al., 2012). L-DOPA readily crosses the BBB where it is converted to DA and subsequently improves motor symptoms for several hours before another dose is required (Hornykiewicz, 2002). L-DOPA is usually co-administered with a DOPA decarboxylase inhibitor (carbidopa) to decrease peripheral conversion to DA and thereby reduce side effects such as nausea and vomiting (Schapira et al., 2008). L-DOPA/carbidopa formulations can also be administered with a COMT inhibitor to extend the beneficial duration of each dose (Smith et al., 2012). Despite its beneficial influence on motor symptoms chronic L-DOPA treatment is associated with significant complications such as aggravation of hallucinations, motor fluctuations, abnormal involuntary movements (dyskinesia), and the “wearing-off” effect where, over time, the beneficial duration of each dose progressively shortens (Schapira et al., 2008; Tarazi et al., 2014). The development of these complications advances over time so that about 90% of patients with PD will experience these debilitating side-effects after 10 years of L-DOPA treatment (Ahlskog and Muentner, 2001). There are also several DA agonists available for the treatment of PD that effectively reduce motor symptoms both alone and in combination with L-DOPA. DA agonists can delay the time until L-DOPA is needed and enable a lower dose of L-DOPA to be used thereby reducing L-DOPA related motor complications (Schapira et al., 2008). However, DA agonists can induce dyskinesia in some cases and produce troubling non-motor symptoms to a greater extent than L-DOPA treatment (Smith et al., 2012).

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Another current dopaminergic treatment is MAO-B inhibitors (e.g. selegiline and rasagiline) which increase synaptic DA by blocking its metabolism (Schapira, 2011). MAO-B inhibitors provide milder symptomatic relief compared to L-DOPA and DA agonists but can extend the beneficial influence of each L-DOPA dose during the more advanced stages of PD (Schapira, 2011).

An effective non-pharmacotherapy is deep brain stimulation (DBS). DBS is used as an adjunctive therapy for reducing the motor symptoms of advanced, L-DOPA -responsive PD that is not adequately controlled by medication (Okun, 2012). The procedure involves electrode implantation to the subthalamic nucleus or the internal segment of the globus pallidus (Bronstein et al., 2011). The electrode is connected to an impulse generator which delivers electrical stimulation to the target area affecting the firing rate and patterns of neurons (Okun, 2012; Tarazi et al., 2014). The DBS procedure improves motor symptoms, reduces dyskinesia and can allow for reductions in medication (Bronstein et al., 2011). However, DBS is only available to a small number of carefully selected patients who are judged as good candidates for the procedure (Bronstein et al., 2011). The procedure can have adverse side effects such as intracranial haemorrhage, infection, post-operative seizures, and sometimes cognitive impairment (Tarazi et al., 2014). Despite the improvement of motor symptoms after DBS there is little evidence that the procedure prevents the progression of PD. Eventually many patients who have had DBS develop L-DOPA resistant symptoms such as freezing of gait, postural instability, and cognitive decline (Bronstein et al., 2011).

The treatments currently available to patients can significantly reduce motor symptoms and thus improve quality of life. However, the beneficial influence of these treatments is limited and are associated with side effects that contribute to reduce quality of life. Furthermore, none of the available treatments prevent the progression of the disease. Therefore, there is an urgent need for novel therapies.

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There are several emerging therapies for PD that have shown promise in recent years. One approach is cell replacement therapy where dopaminergic neurons derived from the human embryonic brain are grafted into the striatum of patients with PD. The assumption of cell replacement therapy is that DA neurons grafted into the striatum can restore dopaminergic neurotransmission and thereby improve motor symptoms (Grealish et al., 2014). The early clinical trials of cell replacement therapy provided proof of principle that transplantation of fetal dopaminergic neurons into the striatum could reduce rigidity and bradykinesia, improve the beneficial duration of L-DOPA doses (reduced "off" period), and restore DA synthesis in the grafted area (Lindvall et al., 1988, 1990). Furthermore, one study showed that grafts provided very long-term reduction in motor impairments for two patients when examined at 15 and 18 years after transplantation (Kefalopoulou et al., 2014). Despite these very promising results many transplant recipients have not shown improvement and in some cases have developed graft-induced dyskinesia (see Barker et al., 2013 for a review). More recently, Grealish and colleagues showed that transplanted DA neurons derived from human embryonic stem cells provided widespread restoration of DA neurotransmission in a rat model of PD thus highlighting their potential use in patients with PD (Grealish et al., 2014). Although fetal and human embryonic stem cell replacement therapies are promising their efficacy will only ever be as good as the best dopaminergic drugs such as L-DOPA, but if used early they could significantly reduce the amount of medication required thereby adding years of quality life for patients (Barker et al., 2013).

An emerging neurorestorative therapy is the use of GDNF as discussed in the previous section. The efficacy of GDNF has been examined in several small clinical trials. Nutt et al. (2003) administered recombinant GDNF protein into the lateral ventricle for 8 months using mechanical pumps but found it did not improve motor symptoms and was associated with nausea and vomiting. A subsequent post-mortem analysis of one patient

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revealed that the GDNF did not diffuse out of the ventricles. Therefore, it was unlikely the GDNF reached its target tissues, the SN and striatum. In another trial Gill et al. (2003) delivered GDNF into the putamen of five patients for one year. This method of delivery produced progressive and sustained improvement in motor symptoms, increased flurodopa uptake, and did not produce side effects. Similarly, Slevin et al. (2005) found that unilateral infusions of GDNF to the putamen for six months in 10 patients produced bilateral improvement of motor symptoms with little side effects. These findings are promising although they need to be tested in larger studies. In addition, the use of GDNF is limited as it does not cross the BBB thus requiring constant intracerebral delivery. A possible solution would be to transplant cells that are genetically coded to release GDNF though the use of a viral vector. This method of *ex vivo* gene therapy has produced good results in pre-clinical models and was proved to be safe in an initial Phase I clinical trial, however, a Phase II trial did not report any beneficial influence on its primary outcome measure after bilateral putamen injections (see Kordower and Bjorklund, 2013 for a comprehensive review). Despite the negative clinical findings GDNF may prove to be an effective therapy for PD once improved routes of delivery have been identified.

2.3.8. Need for New Treatments

The pharmacotherapies currently available for PD provide alleviation of motor symptoms but with chronic use significant adverse effects such as dyskinesia and motor fluctuations occur. The current surgical treatments available also provide significant improvement of motor symptoms but are only available to a small number of ideal patients who over time develop medication resistant symptoms despite the surgical intervention. Neither of these treatments halts nor reverses the progression of the disease or the eventual worsening of symptoms. Several promising treatment approaches have emerged although further research and development of these therapies is needed before they can be safely used in patients with PD.

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Therefore, there is an urgent need to identify novel therapies that will have a beneficial influence for patients with PD.

2.4. Summary

PD is a neurodegenerative disorder that is characterised by both motor and non-motor symptoms. Current treatments, while initially efficacious for motor symptoms, eventually lose their beneficial influence resulting in significant side effects. Therefore, there is a need for additional treatment options. One such treatment could be *natural* progesterone, a neurosteroid that has improved outcomes in over 170 preclinical studies of brain injury and disease. The following chapter examines the evidence regarding the beneficial influence of exogenous progesterone's treatment after brain injury and disease. The evidence presented in this chapter will provide a compelling case for the use of progesterone in an animal model of PD.

Chapter 3

Progesterone and Neuroprotection

3.1. Objectives

This chapter provides an overview of the considerable literature over the last 25 years regarding the beneficial influence of *natural* progesterone (PROG) treatment after brain injury and disease. This first section overviews the evidence of PROG's beneficial influence in animal models of brain injury. This section briefly summarises literature reviews, key findings, and recent studies in this field with a focus on TBI and ischemia as PROG has received the most attention in these injury models. The recent Phase II and III clinical trials that have used PROG to treat TBI will also be summarised. A key section reviews the modest literature regarding the influence of PROG treatment in animal models of PD. Lastly, the neuroprotective mechanisms of PROG will be briefly described with an emphasis on those mechanisms that are most relevant for the treatment of PD. The evidence presented in this chapter will provide a compelling case for the use of PROG treatment in an animal model of PD.

3.2. Progesterone – Animal Models

Interest in PROG began in the late 1980s when Stein's group followed up evidence that female rats had less impairment than male rats after acute brain injury. They examined pseudopregnant rats (a condition in which PROG levels are high compared to estrogen) and found that high levels of PROG was associated with improved functional outcomes on a delayed-spatial alternation task and reduced cerebral edema after experimental TBI compared to normal cycling female rats (Attella et al., 1987).

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To examine more formally this hormonal basis of improved recovery after TBI the Stein group measured cerebral edema 24 hours after medial frontal cortex contusions in both male and female rats (Roof et al., 1993a). Again, cycling females in proestrus (when levels of estrogen are high but PROG is at its lowest level), were compared with pseudopregnant females. They found that female rats in proestrus had less edema than male rats. However, the pseudopregnant females, the group with highest levels of PROG, developed almost no post-injury edema. Subsequently they administered PROG or estrogen to female ovariectomized rats prior to injury and confirmed that PROG was the critical factor responsible for reduced edema. This finding showed that PROG had a protective effect following TBI. However, for PROG to be considered a realistic treatment for TBI it needed to be administered after injury.

In a series of studies the Stein group showed that exogenous PROG treatment, started 1 hour after cortical contusion injury, significantly reduced edema levels even in male rats, to equivalent levels to female rats treated with PROG (Roof et al., 1992). In addition PROG improved spatial navigation in the Morris water maze and reduced secondary neuronal loss in the mediodorsal thalamic nucleus (Roof et al., 1994), was able to reduce edema within 6 hours after injury (Roof et al., 1996), and substantially reduced lipid peroxidation suggesting an antioxidant effect of PROG (Roof et al., 1997). Furthermore, they then showed that PROG could reduce edema even when the treatment was delayed up to 24 hours after injury although the sooner PROG was given, the better the outcome (Roof et al., 1994). These studies by the Stein group showed that post-injury administration of PROG improved outcomes in rats following TBI. Their research set the stage for future investigations regarding PROG's beneficial influence following brain injury.

Many publications have now demonstrated the beneficial influence of PROG in the treatment of TBI (Cutler et al., 2007; Geddes et al., 2014; Pettus et al., 2005), ischemic stroke

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(Gibson and Murphy, 2004; Wali et al., 2014; Yousuf et al., 2014) and other injury models such as experimental brain resection (Xu et al., 2014), motor neuron disease (Deniselle et al., 2012), and Alzheimer's pathology (Carroll et al., 2010). Recent literature reviews have identified more than 170 papers using 22 different injury models from 25 independent laboratories that report beneficial effects of PROG in experimental models of brain injury and disease (Stein and Cekic, 2011; Stein, 2013). The volume of positive findings suggests that PROG may be a useful therapeutic and neuroprotective agent for improving outcomes after brain injury in general and thus possibly neurodegenerative diseases such as PD.

It is important to note that all studies considered in this thesis administered the *natural* form of PROG. PROG, a natural progestogen, should not be confused with progestins which are synthetic and often proprietary hormones such as medroxyprogesterone acetate (MPA; Provera) which is widely used as an oral contraceptive ("the pill") and in hormone therapy (Stein, 2008). Progestins such as MPA do not mimic all of the beneficial effects of PROG and in some cases can exacerbate glutamate excitotoxicity (Stein, 2013). Furthermore, current thinking is that the neuroprotective properties of PROG are due in part to its metabolism to allopregnanolone (ALLO). Most progestins including MPA do not metabolise to ALLO and therefore have a limited range of actions compared to PROG (Schumacher et al., 2014). Therefore, PROG can be expected to produce more benefits than progestins due to its far wider range of actions.

The beneficial influence of PROG after brain injury and disease has been extensively reviewed in recent publications (e.g. Deutsch et al., 2013; Guennoun et al., 2014; Schumacher et al., 2014; Sayeed and Stein, 2009). The general consensus among the reviews is that PROG treatment, when given soon after the injury, improves functional and pathological outcomes. The reviews give particular attention to experimental models of TBI and ischemic stroke as these injuries have received the most attention in the literature.

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However, extensive research has also found that PROG treatment can prevent seizures in animal models (Deutsch et al., 2013). PROG is also beneficial in models of spinal cord injury, peripheral nerve injury, motor neuron disease, demyelinating disease, Alzheimer's pathology, intracerebral hemorrhage, and meningeal edema (Deutsch et al., 2013). According to the Deutsch et al. (2013) review, there is now sufficient preclinical evidence to advance PROG to human clinical trials for TBI and stroke. Since their review two Phase III clinical trials for TBI have been completed (discussed below) although stroke trials have not yet started.

According to the reviews PROG can improve motor, cognitive, sensory, and affective behaviours after injury. For example, PROG improved rotarod performance, spontaneous locomotor activity, learning and memory in the Morris water maze and radial arm maze, neurological function, grip strength, depression and anxiety-like behaviours, and sensory deficits after brain injury (Deutsch et al., 2013; Sayeed and Stein, 2009). This suggests that PROG can improve a comprehensive range of functional outcomes. There are a wide range of neuroprotective effects that underlie PROG's ability to improve functional outcomes. These include reduced edema, inflammation, apoptosis, and oxidant activity, enhanced myelination, and increased brain derived neurotrophic factor (BDNF) to name a few (Deutsch et al., 2013; Guennoun et al., 2014; Schumacher et al., 2014; Sayeed and Stein, 2009). The neuroprotective mechanisms of PROG are discussed in more detail below.

In addition to these literature reviews, there has been a systematic review and a meta-analysis regarding PROG treatment in experimental models. A systematic review by Gibson et al. (2008) found that PROG was effective at reducing lesion volume after ischemia and TBI as long as the treatment was initiated soon after injury. However, the authors note that PROG was only effective for TBI when the studies had the highest quality score whereas PROG was effective following ischemia regardless of the quality score. A recent meta-

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analysis by Wong et al. (2013) assessed lesion volume and mortality after ischemia using individual animal data provided by authors of both published and un-published papers. They found that PROG significantly reduced lesion volume but was associated with an increase in mortality, especially for young female ovariectomized rats. In addition, Wong et al. reported a publication bias on lesion volume (i.e. studies that did not find reduced lesion volume were less likely to be published). In light of these findings the authors recommended that future experiments and meta-analyses report mortality in addition to lesion volume as death rates are particularly relevant to future clinical trials. A limitation of the systematic review and meta-analysis is that neither study assessed functional outcomes. Future reviews and meta-analyses should include functional outcomes as current recommendations from the Stroke Therapy Academic Industry Roundtable (STAIR) for stroke studies require functional assessments before proceeding to clinical trials (Fisher et al., 2009).

The reviews and meta-analysis summarised here show that PROG effectively improves functional and pathological outcomes after brain injury. In addition to these reviews, this chapter provides an overview of the key and recent studies in this field. Key examples were selected from the key literature reviews. Recent studies published after the literature reviews were also considered. Table 3.1 provides a summary of key and recent studies that have examined the beneficial influence of PROG in animal models of brain injury or disease. A brief summary regarding the key features of this table are discussed after its presentation.

Table 3-1.

Summary of Progesterone Treatment Studies in Experimental Models of Brain Injury and Disease.

Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
2010	Carroll et al.	Mice, ovx and sham female transgenic	AD	2.8mg or 25mg pellet	Subcutaneous implant for 3x10 (cyclic) or 90 (continuous) days	1. Memory	2. β -amyloid accumulation	Cyclic but not continuous treatment improved 1, 2 [#]
2008	Frye and Walf	Mice, ovx female transgenic	AD	25mg pellet	Subcutaneous implant for 6 months	1. Memory tasks	Nil	Improved hippocampus-cortex mediate memory tasks. [#]
2014	Xu et al. ⁺	Rats, adult male	Brain resection (surgical brain injury)	10, 20	-12h, +1h, +24h, +48h, +72h.	Nil	1. Edema 2. BBB permeability 3. Inflammation 4. MMP-9	Improved all outcomes. [#]
2014	Wali et al. ⁺	Rats, 12 months old males.	FCI(P)	8-32	+1h, +6h, +24h then every 24h till 7 days post injury*	1. Motor 2. Sensory 3. Memory	4. Infraction size	Improved all outcomes with 8 and 16 mg/kg doses. [#]
2014	Perez-Alvarez et al. ⁺	Rats, adult male	FCI(P)	4 combined with 0.04	+6h, +24h, +48h	1. Neurological	2. Reactive gliosis 3. Neuronal	PROG estradiol combination

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
2012	Ishrat et al.	Rats, adult males	FCI(P)	8	+1h, +6h, +24h, +48h	Nil	1. Infarct size 2. Edema 3. BDNF 4. Apoptosis 5. VEGF	Improved all outcomes. [#]
2010	Ishrat et al.	Rats, adult males	FCI(P)	8	+1h, +6h, +24h, +48h	Nil	1. Infarct size 2. BBB permeability 3. Inflammation	Improved all outcomes. [#]
2009	Ishrat et al.	Rats, adult males	FCI(P)	8	+1h, +6h, +24h, +48h	1. Motor 2. Grip strength	3. Infarct volume	Improved all outcomes. [#]
2007	Sayeed et al.	Rats, 90 day old males	FCI(P)	8	+1h, +6h, +24h, +48h	1. Motor	2. Infarct volume	Improved 1 and 2. [#]
1990	Betz and Coester	Rats, adult males	FCI(P)	2	-1h	Nil	1. Edema 2. BBB permeability	Improved 1. [#]
2005	Gibson et al.	Mice, adult male	FCI(T and P)	8	+1h, +6h, +24h	Nil	1. Edema 2. Infarct volume 3. Inflammation	Improved all outcomes. [#]
2014	Yousuf et al. ⁺	Rats, 13 month old males	FCI(T)	8-32	+1h, +6, then every 24h till 7 days post-	1. Motor 2. Sensory 3. Memory	4. Infarct size	Improved all outcomes with 8 mg/kg [#]

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
2014	Spratt et al. ⁺	Rats, adult male spontaneously hypertensive	FCI(T)	8	injury* +105mins, +6h	1. Motor 2. Sensory	3. Infarct volume	No improvement on any outcome
2006	Sayed et al.	Rats, 60 day old males	FCI(T)	8	+2h, +6h	Nil	1. Infarct volume	Improved 1. [#]
2004	Gibson and Murphy	Mice, adult male	FCI(T)	8	+1h, +6h, +24h	1. Motor 2. Memory	3. Infarct volume 4. Body weight 5. Survival	Improved 1 and 3-5. [#]
2004	Toung et al.	Rats, reproductively senescent adult females	FCI(T)	5	-30min, +2h, +6h	Nil	1. Infarct volume	No improvement
2002	Murphy et al.	Rats, adult ovx females	FCI(T)	5-20	-30min OR -30, +2h, +6	Nil	1. Infarct volume	Repeated treatment at 5 mg/kg improved 1. [#]
2000	Murphy et al.	Rats, adult ovx females	FCI(T)	30 or 60	-30mins or daily for 7-10 days prior to injury	Nil	1. Infarct volume	Exacerbated striatal infarct
1999	Chen et al.	Rats, adult male	FCI(T)	4-32	+2h	1. Motor 2. Sensory 3. Neurological	4. Infarct volume	8 mg/kg improved all outcomes [#]

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
1996	Jiang et al.	Rats, adult males	FCI(T)	4	-30min, +6h, +24h OR +2h, +6h+24h	1. Neurological	2. Body weight 3. Infarct volume	Pre and delayed treatment improved all outcomes. [#]
2014	Li et al. ⁺	Rats, 7 days old	HIE	8	-30mins	Nil	1. Neuronal structure 2. Inflammation	Improved 1 and 2. [#]
2012	Deniselle et al.	Wobbler mice, male and female	Motor neuron disease	20mg pellet	Subcutaneous implant for 18 days	Nil	1. Mitochondrial function 2. Amyloid precursor protein (indicates axon degeneration)	Improved both outcomes. [#]
2002	Gonzalez Deniselle et al.	Wobbler mice, male and female	Motor neuron disease	20mg pellet	Subcutaneous implant for 15 days	Nil	1. $\alpha 3$ subunit of Na,K-ATPase 2. Motor neuron pathology	Improved both outcomes. [#]
2014	Liu et al. ⁺	Rats, adult male	Neuropathic pain	5-20	+1min then once daily for 5 or 10 days OR +21 days then once daily for 5 or 10 days	1. Allodynia	2. Translocater protein	10 day treatment started immediately improve both outcomes. [#]
2011	Coronel et al.	Rats, adult	SCI	16	Once daily	1. Allodynia	2. Spinal	Improved both

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
		males			after injury for duration of study (28d)		noiceptive signalling	outcomes. [#]
2007	Fee et al.	Rats, adult male and female	SCI	4-16	+30mins, +6h, then once daily for 6 or 14 days	1. Locomotion	2. Spinal cord morphology	No improvement although some possible grey-matter sparing
2014	Si et al. ⁺	Rats, adult male	TBI	16	+1h, +6h, +12h	1. Neurological	2. Inflammation 3. Edema 4. BBB permeability	Improved all outcomes. [#]
2014	Robertson and Saraswati ⁺	Rats, immature males and females	TBI	10	+1h, + 6h	Nil	1. Mitochondrial respiratory control ratio 2. glutathione (oxidative stress marker)	Males: 1. Preseved, 2. Prevented loss Females: 1. Not improved 2. Loss not prevented but tissue loss reduced [#]
2014	Geddes et al. ⁺	Rats, males 28 days old at injury	TBI	4-16	+1h, +3h, +24, then every 24h till 7d post-	1. Motor 2. Memory 3. Locomotion 4. Anxiety	5. Lesion size	Improvement on all outcomes with 8-16 mg/kg. [#]

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
2013	Tang et al. ⁺	Rats, middle aged vitamin D deficient males.	TBI	16 and VDH (5µg/kg)	injury* PROG +1h, +6, then every 24h till 7 days post-injury*	1. Memory 2. Sensory strength 3. Grip strength 4. Locomotor	5. Necrotic cavity 6. Neuronal loss 7. GFAP	Improved 1. Combination with VDH also improved 6 and 7. [#]
2012	Cekic et al.	Rats, young adult males	TBI	16	VDH +1h +1h, +6h, +24h, then every 24h till up to 7d post-injury*	1. Locomotion	2. Pro-apoptotic precursors 3. NGF 4. BDNF	Improved 1-3. Decreased injury associated increase of 4. [#]
2012	Peterson et al.	Rats, adult male	TBI	10-20	+4h, then every 12h for 72h.	1. Memory	1. Cortical and hippocampal tissue loss	10 mg/kg improved both outcomes. [#]
2012	Hua et al.	Rats, adult male	TBI	16 and VDH (1, 2.5 or 5µg/kg)	PROG +1h, +6, then every 24h till 7 days post-injury*	1. Memory 2. Sensory 3. Locomotor	4. Necrotic cavity 5. Neuronal death 6. GFAP	PROG improved 1, 6. PROG + VDH more effective. [#]
2011	Barha et al.	Rats, adult male	TBI	16	VDH +1h +1h, +6, then every 24h till 7 days post-	Nil	1. Cell proliferation and cell death in the	Normalized 1. [#]

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
					injury*		dentate gyrus	
2011	Cekic et al.	Rats, aged males vitamin D normal and deficient	TBI	16	+1h, +6, then every 24h till up to 72h post-injury	1. Locomotion	2. Inflammation	Improvement on both outcomes for vitamin D normal rats only. [#]
2011	Hua et al.	Mice, adult male	TBI	16	+1h, +6h	Nil	1. Inflammation 2. Toll-like receptors	Improved 1. Did not change 2. [#]
2005	Pettus et al.	Rats, adult male	TBI	16	+1h, +6h, +24h	Nil	1. Inflammation	Improved 1. [#]
2005	Djebaili et al.	Rats, adult male	TBI	16	+1h, +6h, then every 24h till 5 days post-injury	1. Memory	2. Anti-apoptotic proteins 3. Apoptotic DNA fragmentation 4. GFAP 5. Lesion size	Improved 1-4. [#]
2005	Cutler et al.	Rats, adult male	TBI	16	+1h, +6h, +24h, then daily till 7 days post-injury (with and without*)	1. Anxiety	2. Inflammation 3. Apoptosis	Improved all outcomes although sudden withdrawal of PROG

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
2004	Djebaili et al.	Rats, adult male	TBI	16	+1h, +6h, then every 24h till 5 days post-injury	1. Memory	2. Apoptosis 3. Lesion size	increased 1 and some expressions of 2 and 3. [#] Improved 1 and 2. [#]
2003	Goss et al.	Rats, adult male	TBI	8-32	+1h, +6h, +24h, then every 24h ti 5 days post injury	1. Memory 2. Anxiety 3. Sensory	4. Lesion size	Improve 1 but not 4. Did not change or exacerbate 2 and 3. [#]
1996	Roof et al.	Rats, adult males and females	TBI	4	+1h, +6h, +24h, then every 24h till up to 7d post-injury		1. Edema	Improved 1 within 6h. Treatment delayed for 24h still effective. [#]
1994	Roof et al.	Rats, adult males	TBI	4	+1h, +6h, +24h, then every 24h till five days post-injury	1. Memory	2. Neuronal loss in mediodorsal thalamus	Improved both outcomes. [#]
2008	Gilmer et al.	Rats, young adult males	TBI(moderate)	8-16	+15mins, +6h, then	Nil	1. Edema 2. Cortical tissue	No improvement

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Year	Authors	Species	Model of injury or disease	Dose range (mg/kg)	Timing of dose	Behavioural task type	Histology	Outcomes
					every 24h till 4 days post-injury (with and without [*])		sparing	on either outcome

Abbreviations: AD=Alzheimer's disease; BBB=blood-brain barrier; BDNF=brain-derived neurotrophic factor; FCI(P)=permanent focal cerebral ischemia; FCI(T)=transient focal cerebral ischemia; GFAP=glial fibrillary acidic protein; HIE-hypoxic-ischemic encephalopathy; MMP-9=matrix metalloproteinase 9; NGF=nerve growth factor; ovx=ovariectomized; SCI=spinal cord injury; TBI=traumatic brain injury; VDH=vitamin D hormone; VEGF=vascular endothelial growth factor; ^{*}=studies that used tapered withdrawal of progesterone treatment; [#]=studies that found progesterone treatment was beneficial. ⁺=recent publications not in reviews; other examples were selected from key reviews.

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The summary presented in Table 3.1 reports 44 studies across 9 different experimental models of key and recent literature regarding PROG's influence after experimental brain injury or disease. Male animals were exclusively used in 39 of the studies while females were used in only 10 studies of which three studies used ovariectomized females (see Table 3.1). The higher proportion of studies that used either male or ovariectomized females is not surprising as normal cycling females can complicate findings in PROG treatment studies due to hormonal fluctuations during the estrus cycle.

Nine of the studies used tapered withdrawal of PROG treatment (Barha et al., 2012; Cekic et al., 2012; Cutler et al., 2005; Geddes et al., 2014; Gilmer et al., 2008; Hua et al., 2012; Tang et al., 2013; Wali et al., 2014; Yousuf et al., 2014). Each of these studies gave repeated doses of PROG for up to 7 days post-injury. Tapered withdrawal of PROG following repeated dosing is important as abruptly ceasing treatment can exacerbate the injury, produce an inflammatory rebound effect, and increase anxiety like behaviours (Cutler et al., 2005; Cutler et al., 2006). Tapered withdrawal of PROG overcomes this withdrawal syndrome (Cutler et al., 2005).

Thirty-nine of the 44 studies found that PROG treatment was beneficial. PROG improved functional and/or neuropathological outcomes following TBI in 17 studies (Barha et al., 2012; Cekic et al., 2011; Cekic et al., 2012; Cutler et al., 2005; Djebaili et al., 2004; Djebaili et al., 2005; Geddes et al., 2014; Goss et al., 2003; Hua et al., 2011; Hua et al., 2012; Peterson et al., 2012; Pettus et al., 2005; Robertson and Saraswati, 2014; Roof et al., 1994; Roof et al., 1996; Si et al., 2014; Tang et al., 2013), permanent focal cerebral ischemia in six studies (Betz and Coester, 1990; Gibson et al., 2005; Ishrat et al., 2012, 2011, 2010; Perez-Alvarez et al., 2014; Sayeed et al., 2007; Wali et al., 2014), transient focal ischemia in seven studies (Chen et al., 1999; Gibson and Murphy, 2004; Gibson et al., 2005; Jiang et al., 1996; Murphy et al., 2004; Sayeed et al., 2006; Yousuf et al., 2014), motor neuron disease in two

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studies (Deniselle et al., 2012; Gonzalez Deniselle et al., 2002), Alzheimer's pathology in two studies (Carroll et al., 2010; Frye and Walf, 2008), spinal cord injury in two studies (Coronel et al., 2011; Si et al., 2014), hypoxic-ischemic encephalopathy in one study (Li et al., 2014), surgical brain injury in one study (Xu et al., 2014), and neuropathic pain in one study (Liu et al., 2014). Taken together, these studies show that PROG has consistently improved outcomes across numerous brain injury and disease models.

Table 3.1 also shows that PROG improves functional outcomes after brain injury. For example, PROG treatment improves motor skill on the rota-rod, spatial memory and learning in the Morris water maze, and neurological severity score after TBI (e.g. Djebaili et al., 2004; Geddes et al., 2014; Si et al., 2014;). Likewise, after ischemic injury PROG treatment improves each these outcomes in addition to improving grip strength and sensory deficits in a sticky adhesive removal task (e.g. Chen et al., 1999; Ishrat et al., 2009; Wali et al., 2014; Yousuf et al., 2014). In addition, PROG improves numerous pathological consequences following brain injury. For example PROG reduces cerebral edema, inflammation, BBB permeability, and apoptosis, and increases trophic factors and neuronal survival following TBI (e.g Barha et al., 2011; Cekic et al., 2012; Culter et al., 2005; Hua et al, 2011; Roof et al., 1996; Si et al., 2014;). PROG treatment also reduces infarct volume, inflammation, BBB permeability, and apoptosis, and increases trophic factors after ischemic injury (e.g. Ishrat et al., 2009, 2010, 2012; Sayeed et al., 2007; Wali et al., 2014). Such findings show that PROG is a pleiotropic agent with complex effects that improves functional outcomes via numerous pathophysiological and biochemical mechanisms.

Despite the large number of studies reporting positive outcomes not everyone agrees that PROG is beneficial. Five of the 44 studies did not report beneficial outcomes following PROG treatment. The study by Fee et al. (2007) found no beneficial influence of PROG in a

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model of spinal cord injury although there was some possible grey-matter sparing. Another study found no benefit of PROG treatment following a moderate unilateral TBI injury, even with tapered withdrawal of PROG treatment (Gilmer et al., 2008). The authors suggested that that PROG may not be beneficial for all types of brain injuries which is a possibility that needs to be investigated further. Three studies found that PROG did not improve outcomes following transient focal cerebral ischemia (Murphy et al., 2000; Spratt et al., 2014; Toung et al., 2004). Toung et al. (2004) reported that PROG treatment did not reduce infarct volume in reproductively senescent female rats. In their study, however, they administered PROG prior to injury then abruptly stopped treatment six hours after injury. The lack of improvement in their study could be explained by the sudden withdrawal of PROG triggering a withdrawal syndrome during the post-ischemic reperfusion period. In another study, Murphy et al. (2000) found that PROG treatment actually exacerbated striatal stroke injury. In that study PROG was administered at very high doses (30 or 60 mg/kg) for up to 10 days prior to injury and then abruptly stopped. This finding is readily explained by PROG withdrawal syndrome exacerbating the injury as was confirmed by Cutler et al. (2005). Therefore, rather than weakening the case for PROG this study highlights the importance of tapered withdrawal, especially when large doses are used. More recently, Spratt et al. (2014) did not find any beneficial influence of PROG treatment administered at 105mins and again at 6 hours after ischemic injury in hypertensive rats. Therefore, it is possible that PROG is not beneficial in subjects with comorbidities such as hypertension.

In summary, the studies reported in Table 3.1 provide very strong evidence that PROG improves outcomes following brain injury or disease. There were a surprisingly small proportion of negative reports regarding the influence of PROG following brain injury. The findings of some of these negative reports are explained by PROG withdrawal syndrome that is triggered by abrupt cessation of treatment thus highlighting the importance of tapered

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withdrawal following repeated dosing of PROG. Importantly, PROG treatment improved numerous functional and neuropathological outcomes in 39 of the 44 studies across 9 different models of brain injury or disease. As discussed above, PROG has improved outcomes in over 170 pre-clinical studies across 22 injury models with few negative reports (Stein, 2013). Therefore, it is possible that PROG will be beneficial in an animal model of PD also.

3.3. Progesterone – Clinical Studies

Based on the compelling preclinical data presented earlier in this chapter two independent Phase II clinical trials using PROG to treat TBI were completed, with encouraging findings. Each of these studies were single-centre, randomized, double-blind, placebo-controlled trials (Table 3.2).

Table 3-2.

Summary of the Phase II Clinical Trials Used to Examine Progesterone’s Efficacy in TBI Patients.

Characteristics	ProTECT II (Wright et al., 2007)	Xiao et al. (2008)
Country	US	China
Glasgow coma scale (GCS) at enrolment	4 to 12	≤8
Time after injury	<11h	<8h
Deliver method (length)	Intravenous (3 days)	Intramuscular (5 days)
Outcome assessment post-injury	30 days	3 and 6 months
Patients	100	159
Primary endpoint	GOS-E; DRS; Mortality	GOS; Mortality and the modified FIM

Abbreviations: GOS=Glasgow Outcome scale; GOS-E=Glasgow Outcome Scale-extended; DRS=Disability Rating Scale; FIM=Functional Independence Measure (adapted from Wei et al., 2013).

ProTECT II (Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment) enrolled 100 patients presenting with moderate to severe brain injury (Wright et al., 2007). Patients with severe brain injury who were given 3 days of intravenous PROG had a 50% reduction in mortality at day 30 compared to placebo control patients ($p<0.06$). In comparison, patients with moderate brain injury showed significant signs of improvement on their disability rating scale outcome at day 30 after injury ($p<0.02$). These findings suggested that PROG was beneficial for both moderate and severe brain injury. These findings were supported in a second independent Phase II trial conducted in China that enrolled 159 patients

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with severe brain injury (Xiao et al., 2008). The Xiao et al. (2008) trial administered PROG intramuscularly for five days then tracked patient outcomes at 3 and 6 months after injury. PROG treated patients had significantly better survival and functional outcomes at both 3 and 6 months after injury. Therefore, in both trials PROG improved survival, but more importantly it also improved functional outcomes.

Building on the compelling preclinical evidence and the encouraging Phase II clinical trials two independent large-scale, multi-centre Phase III trials were conducted. As previously, these were randomized, double-blind, placebo-controlled trials (Table 3.3).

Table 3-3.

Summary of the Phase III Clinical Trials Used to Examine Progesterone's efficacy in TBI Patients.

Characteristics	ProTECT III (Wright et al., 2014)	SyNAPSe (Skolnick et al., 2014)
Country	US	International
Glasgow coma scale (GCS) at enrolment	4 to 12	≤8
Time after injury	<4h	<8h
Deliver method (length)	Intravenous (4 days)	Intravenous (5 days)
Primary outcome measures	GOS-E	GOS
Secondary outcome measures	Mortality, DRS	Mortality, SF-36
Patients	882	1195

Abbreviations: GOS=Glasgow Outcome Scale; GOS-E=Glasgow Outcome Scale-extended; DRS=Disability Rating Scale; SF-36=Short-Form Health Survey.

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ProTECT III enrolled 882 of the planned 1140 patients presenting with moderate-severe brain injury before the trial was stopped for futility with respect to the primary outcome measure (Wright et al., 2014). There was no significant difference between the PROG treated and placebo control groups on either the Glasgow Outcome Scale-extended primary measure or for the mortality or Disability Rating Scale secondary outcome measures. In a similar finding, the SyNAPSe (Study of a Neuroprotective Agent, Progesterone, in Severe Traumatic Brain Injury) trial enrolled 1195 patients presenting with severe brain injury (Skolnick et al., 2014). In this trial there was no significant difference between the PROG treated and placebo control groups on either the Glasgow Outcome Scale primary outcome measure or for the mortality or Short-Form Health Survey secondary outcome measures. The conclusion of these trials is that PROG showed no clinical benefit over placebo for any of the outcome measures. Despite the compelling preclinical data and encouraging Phase II clinical findings PROG did not translate to large-scale multi-centre clinical trials for human TBI.

The disappointing results of these two Phase III trials could be due to several factors such as heterogeneity of the injury, insensitive outcome measures, and characteristics of individual patients such as pre-existing conditions (Skolnick et al., 2014; Wright et al., 2014). In preclinical work using animals each of these factors can be well controlled yet they are significant contributors to outcomes in human TBI (Wright et al., 2014). Wright et al. (2014) suggests that success at translating from bench to bedside may require new clinical testing paradigms such as adaptive trial design and profiling of patients who had a response during early phase trials.

Another approach, recommended by Stein (2014), is instead of enrolling patients with many different kinds of brain injury, restrict enrolment to more limited injuries such as temporal cortex damage. These trials would better parallel the precise modelling used in high

quality laboratory research. Stein argues that performing clinical trials in this manner would save time and money as fewer patients across fewer centres would need to be enrolled. The data gathered would be more concise, easier to interpret, and thus more likely to reveal consistent treatment effects where they exist. Employing this paradigm would mean numerous smaller, more affordable, and more focused trials could be run. Conversely, Jickling and Sharp (2014) argue that preclinical models should be overhauled to better replicate the clinical picture. This would include producing heterogeneous injuries and adopting similar outcome measures in the laboratory that are used in clinical trials. Whether future clinical trials for PROG in other injury models should use the current paradigm or an alternative approach such as adaptive trial design will no doubt be debated.

Despite the negative findings in the Phase III trials for TBI, considerable evidence still exists regarding PROG's beneficial influence in models of TBI and ischemia. The convincing preclinical evidence nonetheless lends support to establish PROG's efficacy in other disease models such as PD.

3.4. Progesterone in Animal Models of Parkinson's disease

To the author's knowledge, nine studies have examined the beneficial influence of PROG treatment in animal models of PD. These studies are summarised in Table 3.4.

Table 3-4.

Summary of Progesterone Treatment Studies in Animal Models of Parkinson's disease.

Year	Authors	Species	Model of Parkinson's disease	Dose	Timing of dose	Behavioural tasks	Histology	Outcomes
2015	Yunes et al.	Rats, adults male	6-OHDA – Striatal one site	4 mg/kg	Once daily for three days starting 7 days after surgery	1. Rotation after amphetamine 2. Rotation after apomorphine	3. 3 α -hydroxysteroid oxidoreductase	Improved 1 and 2. Increased ipsilateral striatal expression of 3.
2013	Casas et al.	Rats, adult male	6-OHDA – Striatal one site	4 mg/kg	Once daily for three days starting 7 days after surgery	1. Rotation after amphetamine 2. Open field.	3. K ⁺ evoked, [³ H]-DA, [³ H]-Glutamate, and [³ H]-GABA release from striatal slices	No behavioural change, \uparrow DA, \downarrow glutamate, no change in GABA. #
2011	Casas et al.	Rats, adult male	6-OHDA – Striatal one	4 mg/kg	Once daily for	1. Rotation after apomorphine	Syringe placement only	Prevented contralateral

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Year	Authors	Species	Model of Parkinson's disease	Dose	Timing of dose	Behavioural tasks	Histology	Outcomes
			site		three days starting 7 days after surgery	2. Open field 3. Novel object recognition 4. Forced swim test		rotation, ↑object recognition, ↑forced swim test. [#]
2011	Chao et al.	Rats, adult male	6-OHDA – Striatal four sites	4 mg/kg and 8 mg/kg	Once daily for 13 days starting 24 hours after surgery	1. Spontaneous and 2. apomorphine rotation 3. Grid walking, 4. Cylinder 5. Open field	6. DA, 7. DOPAC 8. HVA, 9. 5-HIAA striatal concentrations	↑Spontaneous rotation asymmetry, ↑foot slips on grid, ↓Symmetrical forelimb use in cylinder, ↑DOPAC/DA ratio. [!]

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Year	Authors	Species	Model of Parkinson's disease	Dose	Timing of dose	Behavioural tasks	Histology	Outcomes
2002	Yu et al.	Gonadectomized male and female mice at 4 and 6 weeks of age	MA (four injections, 10 mg/kg s.c. at 2-h intervals)	0.47µg/day	Three days prior to first MA injection	Nil	1. DA 2. DOPAC 3. 5-HIAA Striatal concentrations	No change for 4 week old mice ↑DA and DOPAC for 6 week old male mice only. [#]
2001	Callier et al.	Retired breeder male mice	MPTP (four injections, 15 mg/kg i.p. at 2-h intervals)	2 µg/day	Ten days starting 5 days before MPTP injections.	Nil	1. DAT-specific binding in striatum 2. DAT mRNA in SN. 3. Striatal [³ H]-Glutamate, and [³ H]-AMPA binding	↑DAT in striatum but not SN. [#] Striatal [³ H]-Glutamate, and [³ H]-AMPA binding unchanged

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Year	Authors	Species	Model of Parkinson's disease	Dose	Timing of dose	Behavioural tasks	Histology	Outcomes
2000	Grandbois et al.	Retired breeder male mice	MPTP (four injections, 15 mg/kg i.p. at 2-h intervals)	2 µg/day	Ten days starting five days before MPTP injections.	Nil	1. DA 2. DOPCA 3. HVA striatal concentrations	↑DA, DOPAC, HVA concentrations [#]
2000	Yu and Liao	Ovariectomized female mice	MA (four injections, 10 mg/kg s.c. at 2-h intervals)	0.467 mg/day	Three days prior to first MA injection	Nil	1. DA 2. DOPAC 3. HVA 4. 5-HIAA Striatal concentrations	↑DA with PROG ↑DOPAC when combined with estradiol benzoate [#]
1992	Gomez-Mancilla and Bedard	Ovariectomized female cynomolgus monkeys	MPTP (0.3mg/kg i.v injection)	0.1 mg/day	Once daily for 7 days.	1. Canadian disability scale, 2. L-DOPA induced	Nil	PROG did not improve the beneficial influence of L-

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Year	Authors	Species	Model of Parkinson's disease	Dose	Timing of dose	Behavioural tasks	Histology	Outcomes
						dyskinesia		DOPA on 1 or 2.

Abbreviations: 5-HIAA=5-Hydroxyindoleacetic acid; 6-OHDA=6-hydroxydopamine; AMPA= α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DA=dopamine; DAT=dopamine transporter; DOPAC=3,4-Dihydroxyphenylacet acid; GABA= γ -Aminobutyric acid; HVA=homovanillic acid; MA=methamphetamine; MPTP=1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mRNA=messenger ribonucleic acid; SN=substantia nigra; #=studies that found progesterone treatment was beneficial; !=studies that found progesterone treatment exacerbated motor impairments.

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Four different models of PD have been used. Four of these studies used unilateral 6-hydroxydopamine (6-OHDA) lesions of the striatum in adult male rats (Casas et al., 2011, 2013; Chao et al., 2011; Yunes et al., 2015). Two studies used systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in adult male mice (Callier et al., 2001; Grandbois et al., 2000). One study used systemic administration of MPTP in adult female monkeys (Gomez-Mancilla & Bedard, 1992). Two studies used the less common model of systemic administration of methamphetamine and this was in gonadectomized male and female mice (Yu et al., 2002; Yu and Liao, 2000). Each of these models produces a parkinsonian syndrome by selectively killing dopaminergic neurons in the SN (see Chapter 4 for further discussion regarding animal models of PD). Seven of the nine studies found that PROG treatment was beneficial (Casas et al. 2013, 2011; Yu et al., 2002; Callier et al., 2001; Grandbois et al., 2000; Yu and Liao, 2000) while one study found that PROG was not beneficial (Gomez-Mancilla & Bedard, 1992) and one study found that PROG exacerbated impairments (Chao et al., 2011).

Casas et al. (2011) administered 4 mg/kg PROG once daily for three days starting at day 7 after unilateral striatal 6-OHDA lesion surgery in male rats. At four weeks after surgery PROG prevented depressive like behaviour in a forced swim test, improved memory deficit in a novel object recognition test, and prevented contralateral rotation after apomorphine. In a subsequent study Casas et al. (2013) examined open field behaviour and rotation after d-amphetamine at two weeks and K^+ -evoked [3H]-DA, [3H]-Glutamate, and [3H]-gamma-Aminobutyric acid (GABA) release from striatal slices at eight weeks using the same model of PD and PROG treatment. They found that PROG treatment did not influence open field or rotation after d-amphetamine behaviour but it increased DA, decreased glutamate, and did not influence GABA release in the lesioned striatum. This second study suggested a neuroprotective and neuromodulatory effect of PROG after 6-OHDA lesions. In another

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study using the same lesion and PROG treatment protocols as Casas et al. (2011, 2013) Yunes et al. (2015) found that PROG reduced rotation after d-amphetamine at two weeks and apomorphine at eight weeks after lesion surgery. PROG also increased ipsilateral striatum expression of 3α -hydroxysteroid oxidoreductase, an enzyme that catalyses PROG to its neuroactive and neuroprotective metabolite allopregnanolone (Ishrat et al., 2010). This finding suggested a sustained neuroprotective effect of PROG that could be modulated through increased expression 3α -hydroxysteroid oxidoreductase. The findings by Casas et al. (2011, 2013) and Yunes et al. (2015) are encouraging as they show that brief PROG treatment initiated seven days after lesion surgery can improve functional and pathological outcomes in the long term after lesion surgery.

The Grandbois et al. (2000) study found that low dose PROG treatment, initiated prior to MPTP, prevented loss of DA and its metabolites 3,4-Dihydroxyphenylacet acid (DOPAC) and homovanillic acid (HVA) when measured six days after MPTP administration. In a follow-up study the same group investigated the mechanism by which PROG exerted its neuroprotective activity using the same PROG and MPTP treatment methods (Callier et al., 2001). They found that PROG treatment prevented loss of dopamine transporter (DAT) in the striatum but not in the SN suggesting PROG provided its neuroprotection by blocking MPTP entry to the SN nerve terminals in the striatum which then led to increased DA in the extracellular space (Callier et al., 2001).

Improved outcomes after PROG treatment have also been observed in methamphetamine models of PD. Yu and Liao (2000) found that a low dose PROG (0.467mg/day) treatment prior to methamphetamine administration reduced loss of striatal DA in female mice ovariectomized at 6 weeks of age. In a similar study Yu et al. (2002) administered a very low dose of PROG (0.47 μ g/day) and methamphetamine as previous but to male and female mice gonadectomised at 4 and 6 weeks of age. The only beneficial

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influence they found was reduced DA and DOPAC loss for 6 week old male mice. Hence PROG's beneficial influence after methamphetamine toxicity may be age and sex dependent. The inconsistency in their studies regarding PROG's beneficial influence in female ovariectomized rats is explained by the different PROG doses they used. The beneficial influence observed in first study (Yu and Liao, 2000) used a higher dose of PROG than the second study (Yu et al. 2002). Therefore, females may require a higher dose of PROG to experience benefits in the methamphetamine model (Yu et al., 2002).

The study by Gomez-Mancilla and Bedard (1992) used monkeys with L-DOPA induced dyskinesia that had been rendered Parkinsonian by MPTP injections. PROG treatment was started approximately two-three months after the MPTP injection. PROG was administered at 0.1 mg/day for seven days then combined with L-DOPA on day 8. PROG did not reduce L-DOPA induced dyskinesia or provide any additional relief of Parkinsonism. The lack of benefit of PROG was likely due to the timing of treatment. PROG was administered after the MPTP induced neuronal death was complete thus reducing PROGs opportunity for neuroprotection. This finding suggests that PROG treatment should be started before the lesion is complete to give PROG an opportunity to provide neuroprotection. In contrast to the positive findings one study found that PROG actually exacerbated impairments after unilateral 6-OHDA lesions of the striatum which could be explained by the timing of PROG treatment after surgery (Chao et al., 2011). These authors administered PROG daily for 13 days, starting 24 hours after lesion surgery. Compared to vehicle treated rats with lesions, PROG increased hindlimb slips on an elevated grid, decreased symmetrical use of the forelimbs in a cylinder task, increased spontaneous rotation, increased DA/DOPAC ratio, and a produced non-significant trend for reduced striatal DA. However, PROG treatment did not influence rotation after apomorphine or ipsilateral forelimb use asymmetry in the cylinder. Following 6-OHDA infusions to the striatum the neurotoxin takes approximately 3 days to

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metabolise and produce SN cell death (Cicchetti et al., 2002; Walsh et al., 2011).

Administering PROG at 24 hours after surgery would have produced interactions with 6-OHDA metabolism that could have exacerbated the lesion and subsequent motor impairments. Therefore, like Casas et al. (2011, 2013), PROG treatment should be started sometime after day 3 post 6-OHDA lesion surgery.

The studies reviewed here show that PROG treatment can protect mid brain dopamine and improve some functional outcomes in animal models of PD. However, the literature regarding PROG's beneficial influence in animal models of PD is scarce leaving a number of critical questions unanswered. First, none of the studies thoroughly examined PROG's influence on motor impairments. The Chao et al. (2011) study is good in that it included several motor skill tests, but it only tested rats in the short-term and gave PROG too soon after lesion surgery. Casas et al. (2011, 2013) and Yunes et al. (2015) investigated affective and cognitive behaviour but only examined motor skills using drug induced rotation and open field. The Gomez-Mancilla and Bedard (1992) study assessed relief of Parkinsonian symptoms in monkeys but gave PROG too long after MPTP lesions. A broad examination of motor skills is required to demonstrate which symptoms might be improved by PROG treatment. Second, none of the studies examined functional impairments in both the short and long-term after lesion surgery. Testing motor skills in both the short and long-term is essential to demonstrate both initial and sustained benefit of PROG treatment. Finally, none of the reviewed studies directly compared PROG's influence following small and large dopamine depleting lesions. Investigating PROG's influence after small and large lesions will determine if PROG is beneficial at different stages of striatal-SN degeneration. Each of these questions is addressed in the experimental chapters of this thesis (Chapters 5-7).

3.5. Neuroprotective Mechanisms of Progesterone

This section provides a brief overview regarding the mechanisms that underlie PROG neuroprotection (Fig. 3.1). This section places an emphasis on those mechanisms that are particularly relevant to the treatment of PD.

As discussed above there is substantial preclinical evidence that shows PROG is beneficial in the treatment of TBI (Culter et al., 2005; Cekic et al., 2012; Geddes et al., 2014), ischemia (Ishrat et al., 2012; Wali et al., 2014; Yousuf et al., 2014), and models of neurodegenerative disease such as Alzheimer's pathology (Frye and Walf, 2008) and motor neuron disease (Deniselle et al., 2012). It is likely that the observed benefits in these models are due to the interacting pleiotropic actions that underlie PROG's neuroprotection. These pleiotropic actions are briefly described below.

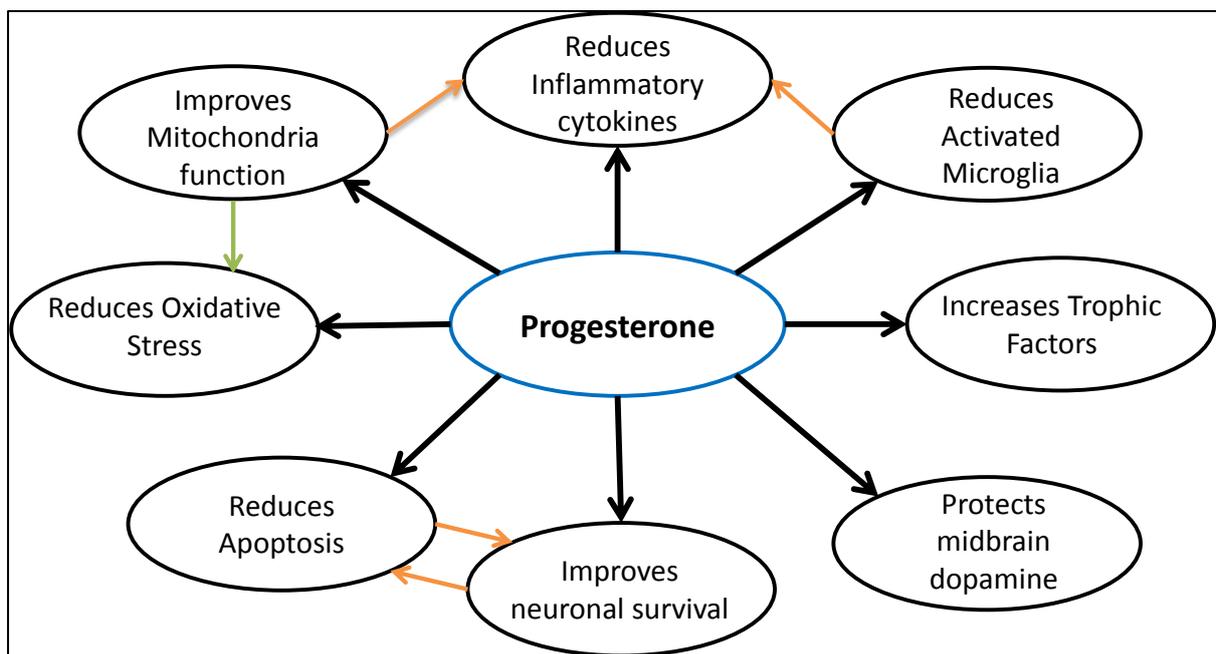


Figure 3.1. Flow chart summarising progesterone's neuroprotective mechanisms that are relevant to the pathology of Parkinson's disease (figure adapted from Sayeed and Stein, 2009).

Inflammation. Inflammation is a key contributor to secondary loss after brain injury. PROG's actions on the inflammatory pathway contribute to neuroprotection following brain injury (Deutsch et al., 2013). Activated microglia are cells that have pro-inflammatory effects after injury or disease. PROG is known to reduce microglia activation in models of spinal cord injury (Labombarda et al., 2011) and demyelinating disease (Garay et al., 2012). Interestingly, in one study PROG actually increased the expression of activated microglia after TBI although edema was still reduced (Grossman et al., 2004). The production of pro-inflammatory cytokines such as TNF- α and IL-1 is another inflammatory response to brain injury and disease (Qian and Flood, 2008). PROG reduces the production of pro-inflammatory cytokines after TBI (Chen et al., 2008; Cutler et al., 2007; Pettus et al., 2005), ischemia (Gibson et al., 2005; Ishrat et al., 2010; Jiang et al., 2009), and demyelinating disease (Garay et al., 2012). Reduction of inflammation is critical for the treatment of brain injury and disease as prolonged inflammation can promote additional injury and neurodegeneration (Skaper, 2007). Furthermore, activated microglia and increased expression of pro-inflammatory cytokines are implicated in the neurodegenerative process in PD (Peterson and Flood, 2012). Therefore, PROG treatment may be beneficial in PD by reducing inflammation.

Lipid peroxidation, mitochondrial function, and oxidative stress. Another mechanism underlying PROG's neuroprotection is the reduction of oxidative stress. After TBI PROG reduces lipid peroxidation (Roof et al., 1997), prevents reduction of the antioxidant glutathione (Robertson and Sarawati, 2014), and normalises mitochondrial respiration (Roberts et al., 2006). Following ischemic injury PROG administration attenuated the stroke-induced expression of inducible nitric oxide synthase and the production of inducible nitric oxide synthase-derived nitric oxide (Coughlan et al., 2005). In addition, PROG attenuated the ischemia-induced reduction of glutathione in one study

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(Ozacmak and Sayan, 2009). In another study, it decreased lipid peroxidation and increased the expression of the antioxidants glutathione, superoxide dismutase, and catalase after ischemic injury (Aggarwal et al., 2008). PROG treatment prevented the increase of mitochondrial nitric oxide synthase and prevented reduction of mitochondrial respiratory complex I in the Wobbler mouse model of amyotrophic lateral sclerosis (Deniselle et al., 2012). Reducing oxidative stress is relevant to the treatment of PD as reactive oxygen species and nitric oxide are generated by activated microglia and deficient mitochondrial complex I respiration (including reduced levels of glutathione), which in turn mediate neurodegeneration (Dias et al., 2013; Peterson and Flood, 2012; Subramaniam and Chesselet, 2013).

Neuronal apoptosis and survival. Another beneficial influence of PROG after brain injury is to enhance neuronal survival and decrease apoptosis. After TBI PROG reduced neuronal loss in the CA1 and CA3 subfields of the hippocampus (Robertson et al., 2006), reduced secondary neuronal loss in the mediodorsal thalamic nucleus (Roof et al., 1994) and normalized cell proliferation and cell death in the dentate gyrus (Barha et al., 2011). Similarly, PROG improved survival of pyramidal neurons in the CA1 and CA2 sub-regions of hippocampus after ischemia (Morali et al., 2005). Related to neuronal survival is the well-established finding that PROG reduces infarct volume after ischemia (e.g. Ishrat et al., 2009, 2010, 2012; Murphy et al., 2002; Wali et al., 2014; Yousuf et al., 2014). The increased survival of neurons may be due in part to PROG's influence on apoptosis. PROG reduces pro-apoptotic factors such as caspase-3, bax, and bad (Cutler et al., 2007; Djebaili et al., 2005; Yao et al., 2005) and increases the anti-apoptotic factors Bcl-2 and Bcl-X_L in the injured cortex after TBI (Yao et al., 2005). Likewise, following ischemia PROG reduces caspase-3 expression (Ishrat et al., 2012). The mechanisms regarding neuronal cell death in PD are not fully understood although it is thought to involve a combination of Lewy body

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pathology, mitochondrial dysfunction, oxidative stress, and inflammation (Hirsch et al., 2012; Levy et al., 2009). PROG administration can improve several of these triggers which would in turn improve neuronal survival in PD.

Neurotrophic support. Neurotrophic factors promote development, survival, restoration, and growth in the central nervous system (Althaus et al., 2008; Smith et al., 2012). PROG treatment upregulates brain-derived neurotrophic factor (BDNF) following TBI, while simultaneously downregulating the expression of the proBDNF and proNGF which are pro-apoptotic precursors to BDNF and nerve growth factor (NGF; Cekic et al., 2012). PROG also upregulates the expression of BDNF after ischemia (Coughlan et al., 2009), spinal cord injury (De Nicola et al., 2006), and motor neuron disease (Meyer et al., 2013). PROG's ability to increase the expression of BDNF is interesting as this neurotrophic factor is a target of interest for the treatment of PD (Smith et al., 2012).

Midbrain dopamine. Loss of nigrostriatal DA is one of the pathological hallmarks of PD (Dauer and Przedborski, 2003). PROG can protect midbrain DA in models of PD. For example, exogenous PROG administered prior to MPTP or methamphetamine prevented loss of striatal DA (Grandbois et al., 2000; Yu et al., 2002; Yu and Liao, 2000). Additionally, PROG when administered for three days starting at day 7 after 6-OHDA lesions improved K^+ -evoked release of DA from striatal slices. Protection of midbrain DA by PROG would improve outcomes in PD.

Edema and blood-brain barrier. Cerebral edema is a serious side effect of brain injury. Edema can produce intracranial swelling, free radicals, and subsequently neuronal death. Controlling edema can improve functional outcomes (Roof et al., 1996). PROG when administered after injury reduces cerebral edema following TBI (Grossman et al., 2004; Roof et al., 1992, 1996), ischemia (Betz and Coester, 1990; Gibson et al., 2005), and experimental

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surgical brain injury (Xu et al., 2014). PROG reduces cerebral edema through several mechanisms. PROG preserves BBB integrity which prevents the indiscriminate flow of water, ions, and inflammatory factors across the membrane (Stein et al., 2008). PROG reduces expression of aquaporin-4, a molecule that controls water drainage, in the area around the lesion and lateral ventricles (Guo et al., 2006). PROG also reduces the second phase of edema which involves the accumulation of fluid inside neurons and reactive astrocytes (Guo et al., 2006; Stein et al., 2008). Although not usually associated with PD, PROG's ability to control edema contributes to the growing evidence that PROG is a pleiotropic agent. Interestingly, there is speculation that altered aquaporins may contribute to the pathology of PD (Foglio and Fabrizio, 2010; Fukuda and Badaut, 2012). Aquaporins may represent a novel therapeutic target for PD; however, their role in PD pathology is poorly understood.

Breakdown of the BBB contributes to neuronal injury after TBI and ischemia (Ishrat et al., 2010). PROG preserves BBB integrity by reducing the expression of matrix metalloproteinases (MMPs) after ischemia and experimental surgical brain injury (Ishrat et al., 2010; Xu et al., 2014). MMPs are agents that contribute to the breakdown of BBB integrity by degrading endothelial tight junction proteins (Ishrat et al., 2010). PROG could improve BBB integrity in PD as altered expression of MMPs (Rosenberg, 2009) and damage to endothelial cells (Guan et al., 2012) have been implicated in the disease.

3.6. Summary

The evidence presented in this chapter shows that PROG is a pleiotropic agent that produces complex effects on multiple systems to produce neuroprotection. PROG has improved functional and pathological outcomes in over 170 preclinical studies with few reports of negative findings (Stein et al., 2013). The substantial preclinical evidence shows that PROG

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improves outcomes by attenuating the complex pathology that accompanies brain injury and disease. Similarly, PD is a disorder with a complex pathology that includes midbrain DA loss, activation of microglia, production of pro-inflammatory cytokines, oxidative stress, and mitochondrial dysfunction. PROG as a pleiotropic agent has improved each of these PD related pathologies in other models of brain injury and disease. The pleiotropic actions of this neurosteroid combined with the compelling preclinical data make PROG an attractive candidate for investigation in an animal model of PD.

To date few studies have examined the beneficial influence of PROG in an animal model of PD. None of these studies have thoroughly examined PROG's influence on motor impairments nor have any directly compared PROG treatment following small and large dopamine depleting lesions. To address these shortcomings a suitable animal model of PD is required. The following chapter (Chapter 4) discusses animal models of PD and provides a justification for the model used in this thesis.

Chapter 4

Animal Models of Parkinson's disease

4.1. Objectives

An appropriate animal model of PD is required to examine the beneficial influence of PROG. Animal models of PD have traditionally involved the systemic or intracerebral administration of neurotoxins or pesticides that reproduce most of the essential features of the disease. However, with recognition that about 10% of all PD cases are familial several genetic rodent models have also been developed. This chapter describes the most popular neurotoxin and pesticide models and briefly describes some of the more common genetic models, highlighting the strengths and disadvantages of each. Also described is the newer prion-like model. A justification for the model used in this thesis is then provided. Table 4.1 summarises the key features of each model.

Table 4-1.

Animal Models of Parkinson's disease.

	Model	Motor impairments	Nigrostriatal damage	Lewy pathology	Other pathology	Advantages	Disadvantages
Toxin-based	6-OHDA rats	Akinesia, rotation, skilled forelimb use	Loss of DA/TH and SNpc neurons	No	Inflammation, Mitochondrial dysfunction, oxidative stress	Progressive SN degeneration after striatal injections, highly reproducible	Requires stereotaxic injection, little Lewy pathology
	MPTP mice	Bradykinesia, reduced locomotion	Loss of DA/TH and SNpc neurons	No	Inflammation, Mitochondrial dysfunction, oxidative stress	Easy to perform, highly reproducible, bilateral lesion	Non-progressive model of cell death, little Lewy pathology
	MPTP primate	Bradykinesia, akinesia, rigidity, postural tremor	Loss of DA/TH and SNpc neurons	No	Inflammation, Mitochondrial dysfunction, oxidative stress	Closely replicates PD motor impairments, bilateral lesion	Non-progressive model of cell death, no Lewy pathology
Pesticide-based	Rotenone	Reduced locomotion, rigidity	Loss of DA/TH and SNpc neurons	Yes	Mitochondrial dysfunction, oxidative stress, inflammation	Ecological validity, Lewy pathology, bilateral lesion	Substantial morbidity and mortality. Low reproducibility.
	Paraquat/maneb	Reduced locomotion	Loss of DA/TH and SNpc	No	Mitochondrial dysfunction,	Ecological validity,	Inconsistent lesions, high

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	Model	Motor impairments	Nigrostriatal damage	Lewy pathology	Other pathology	Advantages	Disadvantages
Genetic-based	α -synuclein-viral-vector overexpression	Akinesia, rotation	neurons Loss of DA/TH and SNpc neurons	Yes	oxidative stress Inflammation	bilateral lesion Lewy pathology, progressive model of cell death	mortality Variable motor impairments, requires further predictive value validation.
	Mutations - α -synuclein, LRKK2, PINK1, PARKIN, DJ-1	Reduced locomotor activity	Little, inconsistent	No	Mitochondrial dysfunction, altered reactive oxygen species production.	Studying contribution of mutations to PD pathogenesis	Little or no nigrostriatal degeneration. Limited motor impairments
Prion-like model	α -synuclein-inoculation	Motor coordination, grip strength	Loss of DA/TH and SNpc neurons	Yes		Prion-like propagation of Lewy pathology, progressive model of cell death	Requires further development, prion-hypothesis not confirmed.

Abbreviations: 6-OHDA=6-hydroxydopamine; DA=dopamine; MPTP=the 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine; PD=Parkinson's disease; SNpc=substantia nigra pars compacta; TH=tyrosine hydroxylase (Table adapted from Blesa and Przedborski, 2012, 2014).

4.2. Neurotoxin Models

Neurotoxic models are the most common animal models of PD. The most popular neurotoxins are the 6-hydroxydopamine (6-OHDA) model in rats and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in mice.

6-OHDA

Ungerstedt (1968) first used the 6-OHDA model almost fifty years ago to lesion the dopaminergic nigrostriatal pathway in the rat. The 6-OHDA model remains widely used (Duty and Jenner, 2011) and is the model used in this thesis. Dopaminergic lesions produced by 6-OHDA are highly reproducible and can be varied to yield different degrees of nigrostriatal denervation depending on the dosage and site chosen for infusion (Blandini et al., 2008; Kirik et al., 1998). 6-OHDA does not cross the blood-brain barrier (BBB) and so requires direct infusion to the brain. The neurotoxin can be injected into the cell bodies in the SN or into the medial forebrain bundle (MFB) to produce a 'complete' lesion or it can be injected to the terminal region of the striatum to produce a 'partial' lesion (Blandini et al., 2008). Unilateral infusions of 6-OHDA are almost always used as bilateral lesions produce severe aphagia and adipsia, requiring the rat to be tube feed to keep it alive (Sakai and Gash, 1994). Furthermore, unilateral lesions allow each rat to serve as its own control as the impaired side can be compared to the intact unimpaired side.

After its injection 6-OHDA is taken up by the DA and to a lesser extent the noradrenalin transporters where it selectively destroys catecholaminergic neurons by a combined effect of reactive oxygen species (ROS) and quinones (Blesa and Przedborski, 2012; Duty and Jenner, 2011). The model cannot replicate PD but it does mimic the essential features of the disease including progressive striatal deafferentation and nigral cell loss, motor impairments, and pathogenesis such as inflammation, mitochondrial dysfunction, and

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oxidative stress (Baldini et al., 2008; Cicchetti et al., 2002; Jin et al., 2008; Walsh et al., 2011). The model, however, does not address pathology elsewhere in the brain or induce Lewy bodies which is considered a pathological hallmark of PD (Blesa and Przedborski, 2012, 2014; Braak et al., 2006). The formation of Lewy body pathology is desirable but not essential. Also, a small number of PD cases occur without classic Lewy bodies and the pathology is not observed in the parkin-related familial form of PD (Dauer and Przedborski, 2003). The 6-OHDA model shows good predictive validity as all drugs in clinical use excluding antimuscarinic drugs have demonstrated efficacy in this model (Duty and Jenner, 2011).

Unilaterally injecting 6-OHDA to the MFB or SN produces a 'complete' lesion that models the late stages of PD degeneration (Duty and Jenner, 2011). Injection of 6-OHDA to the MFB or SN causes near total degeneration (>90%) of dopaminergic neurons that begin to die within 12 hours of injection in conjunction with near complete striatal DA depletion that is established within 2-3 days after surgery (Baldini et al., 2008; Deumens et al., 2002; Walsh et al., 2011). This rapid onset of cell death is a disadvantage of this model as neurodegeneration is progressive in PD. However, rats with complete lesions display profound and stable motor impairments that can be used to test the efficacy of symptomatic treatments (Duty and Jenner, 2011). Rats with complete lesions display marked rotation in response to peripheral administration of DA agonists and robust impairments on tests of forelimb akinesia, skilled reaching, and forelimb use asymmetry (Metz et al., 2001; Olsson et al., 1995; Woodlee et al., 2008; see experimental chapters 5-7 for a detailed description of the motor skill tests used in this thesis).

In order to achieve a more progressive and less severe lesion the 6-OHDA neurotoxin can be unilaterally injected to the striatum (Kirik et al., 1998). The intrastriatal injection causes rapid striatal tyrosine hydroxylase (TH, the rate limiting enzyme in DA synthesis)

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denervation followed by a progressive cell loss due to retrograde transport of the 6-OHDA neurotoxin from the striatum to the SN (Berger et al., 1991). A smaller lesion that models the earlier stages of PD degeneration can be produced by two injections whereas a substantial yet not complete lesion that models the later stages can be achieved by four injections made along rostro-caudal extent of the dorsal striatum (Decressac et al., 2012; Kirik et al., 1998; Shin et al., 2014). Previous studies have shown that striatal denervation occurs within 6-24 hours but reaches its maximum at about 1 week after striatal 6-OHDA lesion surgery (Blandini et al., 2007; Grealish et al., 2008; Rosenblad et al., 2000). In comparison, dopaminergic cell bodies in the SN slowly degenerate over several weeks after striatal lesions (Cicchetti et al., 2002; Walsh et al., 2011). Similar to the MFB model, large lesions also produce robust drug induced rotation bias and impairments on tests of forelimb akinesia, skilled reaching, and forelimb use asymmetry (Kirik et al., 1998; Plowman et al., 2014; Shin et al., 2014) while small lesions produce less substantial deficits (Decressac et al., 2012; Shin et al., 2014). Importantly, the development of motor impairments after intrastriatal lesions is more gradual than the MFB model (Decressac et al., 2012; Kirik et al., 1998). The slower evolving lesion and progressive motor impairments achieved by intrastriatal 6-OHDA injections is excellent for testing novel therapies that are designed to interfere with the degenerative process (Blandini et al., 2008).

MPTP

MPTP is another commonly used neurotoxin to model PD. As discussed in Chapter 2, the selective dopaminergic neurotoxicity of MPTP was discovered when a group of drug users accidentally injected themselves with MPTP, an analogue by-product of heroin production (Langston et al., 1983). MPTP readily crosses the BBB then is converted to its toxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺) which subsequently kills dopaminergic neurons (Hauser and Hastings, 2013). MPTP can be systemically injected to produce bilateral

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degeneration in monkeys and selected strains of mice although rats are relatively insensitive to its toxic effects (Duty and Jenner, 2011; Sonsalla and Heikkila, 1988). MPTP also causes oxidative stress, mitochondria dysfunction, and inflammation, key pathogenic factors of PD (Jackson-Lewis et al., 2012). However, MPTP administration does not produce Lewy bodies (Blesa and Przedborski, 2014). The most common procedures for inducing MPTP neurotoxicity in mice involve either a single high dose injection, giving 80-90% striatal DA depletion, or one lower dose injection given every 2 h for a total of four doses, leading to 60-90% DA depletion (Duty and Jenner, 2011; Jackson-Lewis and Przedborski, 2007). Cell death is rapid after MPTP, emerging within 12-72 hours and reaching maximum within one week post injections (Jackson-Lewis et al., 1995; Jackson-Lewis and Przedborski, 2007; Novikova et al., 2006).

MPTP treated mice show signs of akinesia, catalepsy, and reduced locomotion, although these impairments can be transient thereby reducing the usefulness of this model to study therapeutic interventions (Duty and Jenner, 2011; Taylor et al., 2010). On the other hand the MPTP monkey displays a parkinsonian syndrome consisting of akinesia, bradykinesia, rigidity, and postural tremor thus closely replicating the cardinal motor features of PD, but like MPTP in the mouse the SN cell death and the onset of symptoms is rapid (Blesa and Przedborski, 2012). The MPTP mouse and primate are highly useful models of PD although the rapid onset of cell death and motor impairments limits the therapeutic window for administration of novel treatments aimed at neuroprotection.

4.3. Pesticide Models

Another approach to modelling PD in animals is with the administration of pesticides. This approach has validity as lifetime exposure to pesticides has been associated with an increased risk of PD (Dick et al., 2007) although others have argued that the evidence for this link is

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inconclusive (Berry et al., 2010). Nonetheless, several studies have shown in rodents that systemic administration of the herbicide paraquat alone or in combination with the fungicide maneb can cause a PD like syndrome. Similarly, systemic or intracerebral administration of the herbicide/insecticide rotenone can also model PD.

Paraquat/maneb

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) exerts its toxicity through induction of redox cycling which in turn generates harmful ROS (Day et al., 1999). Paraquat also induces the activation of microglia, a marker of inflammation in PD (Peng et al., 2009). In comparison, maneb (manganese ethylene-bis-dithiocarbamate) mediates toxicity by selective inhibition of complex III of the mitochondrial respiratory chain (Zhang et al., 2003). Following systemic administration of paraquat, mice show reduced ambulatory activity and modest loss of striatal TH and SN neurons but no Lewy pathology (Brooks et al., 1999; McCormack et al., 2002; Rappold et al., 2011). In comparison, systemic administration of maneb alone reduced locomotor activity in older rats for up to 3 months after the last injection but had little influence on striatal TH or the number of SNpc cells (Thiruchelvam et al., 2003). However, maneb when co-administered with paraquat produced enhanced toxicity in mice although the nigrostriatal degeneration was only modest (Thiruchelvam et al., 2000, 2003). The strength of the paraquat/maneb model is its ecological validity with respect to pesticide exposure increasing the risk of PD in humans. However, the use of this model is limited due to high mortality and inconsistent findings such as SN cell loss without depletion of striatal DA, a hallmark of PD (Duty and Jenner, 2011; Miller, 2007).

Rotenone

Rotenone readily crosses the BBB where it inhibits complex I of the mitochondria electron transport chain leading to reduced adenosine triphosphate production and electron leakage that can form harmful ROS and subsequently oxidative stress (Johnson and Bobrovskaya, 2015). The first demonstration of the rotenone model showed that chronic systemic administration in rats caused selective nigrostriatal dopaminergic degeneration, Lewy pathology, hypokinesia, and rigidity (Betarbet et al., 2000). Systemic administration also produced selective activation of microglia in the SN and striatum thus mimicking PD inflammation (Sherer et al., 2003). However, systemic administration is also associated with marked peripheral organ toxicity which confounds findings and contributes to the high rates of morbidity and mortality observed in this model (Ravenstijn et al., 2008).

To overcome these limitations some studies have unilaterally injected rotenone into the nigrostriatal pathway. Injection to the striatum, MFB or the SN and ventral tegmental area caused progressive dopaminergic lesions with no associated peripheral organ toxicity or mortality (Mulcahy et al., 2011; Ravenstijn et al., 2008; Xiong et al., 2009). However, the intracerebral rotenone model can be difficult to reproduce, and can cause liquefactive necrosis and glia scarring at the injection site (Blesa and Przedborski, 2014; Johnson and Bobrovskaya, 2015). We have previously trialled the MFB and SN/VTA rotenone models in our laboratory but were unable to produce any detectable motor impairments (Ravenstijn et al., 2008; Xiong et al., 2009). However, we have not trialled the newer striatal infusion model which could be a better alternative to the MFB and SN/VTA models (Mulcahy et al., 2011). Thus the variable nature of the rotenone model in the literature and in our laboratory prevented its use in the current study.

4.4. Genetic Models

Monogenetic mutation models of PD have been developed to better simulate the mechanisms underlying the rare familial forms of the disease. These models usually employ mice bearing mutations of PD associated genes such as the autosomal dominant α -synuclein and LRRK2 genes or the autosomal recessive genes PINK1/Parkin and DJ-1 (Valadas et al., 2014). Other approaches have used viral-vector driven overexpression of α -synuclein to reproduce the pathological features of PD.

Genetic mutation models

Genetic models reproduce several pathogenic factors of PD including mitochondrial dysfunction, altered ROS production, and protein misfolding but often fail to produce nigrostriatal degeneration (Blesa and Przedborski, 2014). For example, α -synuclein mutations cause a rare form of autosomal dominant PD and was the first gene linked to familial PD (Blandini and Armentero, 2012). To date, several α -synuclein transgenic mouse models have been developed. In these mice, some motor impairment occurs along with decreased levels of striatal TH or DA but with little or no nigrostriatal degeneration (Chesselet, 2008). Similarly, mutations of the other main PD associated genes LRRK2, PINK1/Parkin, and DJ-1 produce some motor impairment but often fail to produce dopaminergic neuron degeneration (see Blesa and Przedborski, 2014 for a review). Therefore, genetic models are valuable for studying how genetic mutations contribute to the pathogenesis of PD but are less suitable for screening therapies aimed at improving motor symptoms related to nigrostriatal dopaminergic degeneration.

Viral-vector α -synuclein overexpression model

Another approach to modelling PD is via viral-vector driven overexpression of α -synuclein. In these models a viral-vector expressing either wild type or mutated human α -synuclein is

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unilaterally injected in rats to the dopaminergic regions of the SN. In contrast to the transgenic α -synuclein models discussed above, viral-vector mediated overexpression of α -synuclein produces reliable dopaminergic degeneration and α -synuclein pathology (Decressac et al., 2011, 2012; Kirik et al., 2002). In this model the degeneration of SN cell bodies and denervation of striatal TH is progressive, reaching a maximum of about 50-70% at 8 weeks after surgery (Decressac et al., 2011, 2012; Kirik et al., 2002; Yamada et al., 2004). However, some recovery of SN cell bodies and striatal TH occurs over longer periods of about 6 months after surgery (Kirik et al., 2002). This model also produces activated microglia and pro-inflammatory cytokines, key features of PD (Chung et al., 2009). Impaired motor skills are also observed in the α -synuclein overexpression model but its occurrence can be variable (Decressac et al., 2012; Kirik et al., 2002).

Taken together these studies show that the α -synuclein model closely recapitulates the key features of PD i.e. progressive dopaminergic neurodegeneration, α -synuclein pathology, inflammation, and motor impairments. However, others argue that the model may require further assessment regarding its predicative validity before it can be used to evaluate treatments (Lindgren et al., 2012). A recent study found that impaired forelimb use asymmetry was reduced by L-DOPA treatment after α -synuclein overexpression (Van der Perren et al., 2015). This finding improves the predictive validity of this model encouraging further study to examine the influence of novel treatments.

4.5. Prion-like α -synuclein Models

As discussed in Chapter 2, it has been proposed that aggregated α -synuclein is a prion-like protein and that PD is a prion-like disease (Olanow and Brundin, 2013). As a result prion-like models have been investigated and are the latest approach to model PD.

Several studies have shown that α -synuclein can spread via cell to cell transmission and produces a PD like impairment in mice models. In one study young asymptomatic α -synuclein transgenic mice that received injections into the neostriatum of brain homogenates of α -synuclein derived from older transgenic mice already expressing α -synuclein pathology had accelerated formation of Lewy pathology, earlier onset of motor impairments, and reduced TH expression in the SN (Luk et al., 2012b). In a similar study wild type non-transgenic mice given a single inoculation of synthetic α -synuclein fibrils into the striatum showed cell to cell transmission of α -synuclein, PD like Lewy pathology, progressive loss of dopaminergic neurons in the SN and DA in the striatum, and reduced rotarod performance and motor strength (Luk et al., 2012a). This finding was supported by a recent study that also observed prion-like spread of α -synuclein and impaired rotarod performance and motor strength after SN or striatal injections (Masuda-Suzukake et al., 2014). Another recent study found that injections into the SN or striatum of pathological α -synuclein, purified from post-mortem PD brains, caused progressive nigrostriatal degeneration and triggered the pathological conversion of host α -synuclein in both mice and monkeys (Recasens et al., 2014). Taken together these findings suggest that exposure to small quantities of pathological α -synuclein can cause a prion-like spread of α -synuclein and a PD like syndrome in mice and monkeys. The prion model is potentially a close replication of PD although its use for examining novel treatments is currently limited as the model requires further development and, importantly, the prion hypothesis of PD has not yet been confirmed.

4.6. Animal Model Used in this Thesis

An ideal model of PD should recapitulate the cardinal features of the disease. These features should include some of the key aspects of pathogenesis, motor impairments, neurochemistry, and pathology to PD (Duty & Jenner, 2011). The model should preferably produce at least some progressive impairment, as is the case with PD. In addition, the choice of the model

also depends on the aim of the study. The primary aim of the current thesis was to examine PROG's influence on motor impairments in a clinically relevant model of PD. The model used in this thesis, therefore, should be suitable for testing novel therapeutics. The four-site unilateral intrastriatal 6-OHDA lesion model is widely used and has been found suitable for testing novel treatments aimed at symptomatic reduction (Blandini and Armentero, 2012; Blesa and Przedborski, 2012, 2014; Dauer and Przedborski, 2003; Kirik et al., 1998). The intrastriatal model has some progressive injury characteristics and thus provides a therapeutic time-window with which to administer PROG. In addition, the two-site intrastriatal 6-OHDA lesion model can be used to produce smaller lesions and consequently less severe motor impairments if required (Descressac et al., 2012; Kirik et al., 1998; Shin et al., 2014). Thus, the ability of the intrastriatal model to mimic many of the essential features of PD supports its use as a clinically relevant model with which to examine PROG's influence on motor impairments.

4.7. Summary

There are numerous animal models of PD available to the researcher, each with strengths and disadvantages. A given model should not be used exclusively but rather selected on the basis of being relevant for the question being asked. If a substantial and reproducible dopaminergic lesion is required for the purpose of testing a novel therapy then a neurotoxin model is suitable. Conversely, pesticide models may be better suited to test the influence of environmental toxins on the pathogenesis of PD, while genetic models are appropriate for investigating the role of specific mutations. The newer viral-vector mediated expression of α -synuclein and prion-like models replicate most of the key features of PD although both models perhaps require further development and validation before they are suitable for testing novel therapeutics.

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Of the models reviewed in this chapter the intrastriatal 6-OHDA model was selected as most suitable for examining PROG's beneficial influence at this point in time. This model recapitulates the essential features of PD and has been found suitable for testing novel therapies. The following chapter reports the first of three experiments presented in this thesis. This experiment established the time-course for motor-impairments in the intrastriatal 6-OHDA model and in doing so identified a suitable treatment time-point for subsequent experiments using PROG.

Chapter 5

Study One: Motor Impairments in the Intrastratial 6-OHDA Model

5.1. Objectives

This chapter reports the first of three experiments (see also: Chapters 6 and 7) and involves a pilot investigation to establish the unilateral striatal 6-OHDA lesion model of PD. The main objective of this chapter was to determine the time-course for motor impairments in this model. The second objective was to identify a suitable treatment time-point for subsequent experiments using PROG. Motor function tests of akinesia, skilled forepaw use, and drug induced rotation are investigated. Rats were tested pre-surgery on the adjusting steps test for akinesia, and the sunflower seed handling test for skilled forepaw use, then given 6-OHDA lesions or sham surgery. Ipsilateral rotation after d-amphetamine was used to verify the functional effect of the lesion at day 7 after surgery. Post-operative impairments in the adjusting steps test were measured on days 15-17, 28-30, and 49-51 and in the sunflower seed test on days 20-22, 34-36, 56-58. Contralateral rotation after apomorphine was measured at days 24 and 61. Rats were sacrificed at day 65 for tyrosine hydroxylase immunohistochemistry of the striatum. This chapter begins with a brief justification of the 6-OHDA lesion model and the motor skills tests used in this experiment.

5.2. Introduction

As discussed in Chapter 4, an ideal model of PD should recapitulate the key features of the disease including similar neurochemistry, pathology, and motor impairments (Duty and Jenner, 2011). The model used in this thesis should also be suitable for investigating novel treatments as the primary aim of this thesis is to examine PROG's influence on motor

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impairments in a clinically relevant model of PD. As stated in Chapter 4, the four-site unilateral intrastratial 6-OHDA partial lesion model is ideal as it mimics many of the essential features of PD and has been found suitable for testing novel treatments aimed at symptomatic reduction (Blandini and Armentero, 2012; Blesa and Przedborski, 2012; 2014; Dauer and Przedborski, 2003; Kirik et al., 1998). Before examining PROG in the context of this model the time-course of motor impairments needed to be established in a pilot experiment.

Rats were tested for forelimb akinesia using the adjusting steps test described by Olsson et al. (1995). The adjusting steps test is useful because it is one of the few tests able to detect motor deficits after moderate dopamine depletion following unilateral 6-OHDA lesions (Decressac et al., 2012; Kirik et al., 1998; Krishnamurthi et al., 2009; Torres et al., 2008). Additionally, previous studies have shown that forelimb stepping in the adjusting steps test decreases as the dopamine content is reduced (Kirik et al., 2001b; Winkler et al., 1996). The adjusting steps test was selected as the primary behavioural outcome measure as akinesia is a cardinal motor feature of clinical PD (Dauer and Przedborski, 2003; Olsson et al., 1995). Furthermore, the presence of symptoms associated with akinesia significantly contributes to reduced quality of life in PD patients (Rahman et al., 2008).

Loss of manual dexterity of the hands is another debilitating motor impairment observed in PD patients (Klein et al., 2012). Rats demonstrate the equivalent of manual dexterity when they skilfully handle food items such as pasta and sunflower seeds (Whishaw and Coles, 1996). To assess skilled forepaw use rats were assessed using a modified version of the pasta handling and sunflower seed handling tests. Intact rats handle lengths of uncooked vermicelli by repeatedly adjusting their forepaw hold on the pasta piece whereas 6-OHDA lesioned rats make fewer adjustments with their impaired paw (Allred et al., 2008). Prior to this pilot study we trialled the vermicelli handling test without success as our rats

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adopted a handling style that did not allow the experimenter to observe forepaw adjustments. Instead, we used a modification of the sunflower seed handling test as forepaw adjustments could be observed when shelling and eating the seeds (Kane et al., 2011). Rats typically consume a sunflower seed by holding the seed at each end, biting the corner, splitting the seed longitudinally into two pieces, and then consuming the seed. Rats with 6-OHDA lesion take longer to eat the seed, produce more shell pieces, and should make fewer adjustments with the impaired forepaw as compared to the vermicelli handling test (Allred et al., 2008; Kane et al., 2011).

The most common motor behaviour assessment of unilateral 6-OHDA lesions is the rotational behaviour induced by systemic administration of the indirect dopamine agonist d-amphetamine, which induces a circling behaviour towards the lesion side (Torres and Dunnett, 2007; Ungerstedt and Arbuthnott, 1970). This ipsilateral to lesion rotation occurs because the dopamine release induced by d-amphetamine favours the intact side thus producing an imbalance in dopamine transmission and subsequent movement imbalance (Duty and Jenner, 2011). Following partial intrastratial 6-OHDA lesions rotations after d-amphetamine are consistently observed after about 50% of dopaminergic neurons in the SN are lost (Kirik et al., 1998). However, similar rates of rotation are sometimes observed over a wide range of SN cell loss following partial lesions (e.g. Fang et al., 2006; Sun et al., 2010) but rats with larger lesions tend to rotate more (Kirik et al., 1998, 2001b). The d-amphetamine rotation test is therefore useful as an ante-mortem validation of the lesion but may not be reliable at detecting functional improvements in partial lesion models (Duty and Jenner, 2011). Hence rats were tested for rotations after d-amphetamine at day 7 after surgery to validate the presence of the 6-OHDA striatal lesion.

Rats were also tested for rotation after apomorphine at days 24 and 61 after surgery. The test is similar to d-amphetamine rotations other than rats will rotate in the contralateral to

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lesion direction as a result of denervation-induced dopamine receptor supersensitivity in the lesioned hemisphere (Ungerstedt, 1971). The dopamine receptor supersensitivity required to produce contralateral rotations after apomorphine typically occurs only after there is >90% nigral cell loss such as is observed in the MFB complete lesion model. Partial lesion models usually produce <90% SN cell loss although contralateral rotations after apomorphine will occur if there is localized dopamine loss of >90% in the circumscribed area of the striatal lesion (Kirik et al., 1998). Rotation after apomorphine is therefore a useful ante-mortem assay for supersensitivity induced by large 6-OHDA striatal lesions and was therefore included.

The main objective for this pilot experiment was to determine the time-course for motor impairments following partial unilateral 6-OHDA lesions of the dorsal striatum. Previous research has shown that stable impairments in the adjusting steps test typically appear within 2-3 weeks following partial lesions (Decressac et al., 2012; Fang et al., 2006; Kirik et al., 1998) although others have reported a substantial impairment within 24 hours of lesion surgery (Grealish et al., 2008). Impaired use of the forepaws requires more extensive lesions than is required to detect akinesia impairment (Kirik et al., 1998). Given the progressive nature of the partial lesion model it is possible that deficits in skilled forepaw use in the sunflower seed handling test could take additional time to emerge compared to the adjusting steps test. Rotation after d-amphetamine emerges within 7 to 14 days after lesion surgery whereas rotation after apomorphine requires about 3 weeks to emerge (Fang et al., 2006; Kirik et al., 1998). These findings indicate that the appearance of motor impairments is time-sensitive following partial lesions. It is therefore necessary to establish the time-course for motor impairments in our rats using our surgical and behavioural testing techniques prior to examining the influence of PROG.

The second objective of this experiment was to identify a suitable time-point to initiate PROG treatment in subsequent experiments. The optimal treatment time-point would

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be when impairments are first present but are not yet fully developed. The onset of motor symptoms in PD patients occurs when about 60% SNpc neurons have been lost and there is a concomitant reduction of striatal DA by about 80% (Dauer and Przedborski, 2003). Note that delaying PROG treatment until impairments are first present is consistent with current treatment approaches where treatment is not started until a patient presents with symptoms (Reichmann, 2008). The delay in treatment until after significant dopaminergic loss has occurred is because a diagnosis can only be made in the presence of the cardinal features of PD due to the lack of early disease identifying biomarkers (Sharma et al., 2013). Although treatments can be delayed when symptoms are relatively mild the current recommendations are to initiate treatment early as this leads to better long-term outcomes (Schapira et al., 2008). Therefore, the best time-point to initiate PROG treatment in our model was taken to be when motor impairments were first present but not yet fully developed.

5.3. Materials and Methods

5.3.1. Animals

Twenty male Wistar rats were used, weighing between 518g and 628g at the day of surgery. The rats were 7-10 months old at surgery and 9-12 months old at perfusion. Testing occurred during the dark phase of the rats reversed light cycle (lights on at 2000hrs). Following surgery rats were housed individually for the remainder of the experiment with food and water available *ab libitum*. All protocols in this study followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee, University of Canterbury.

5.3.2. Experimental design

The experiment used a between groups design. Rats received unilateral 6-OHDA lesions of the striatum. Lesions were verified at day 7 with a d-amphetamine rotation test and post-

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surgery motor skills were evaluated between days 15 and 61. Rats were euthanized at day 65 for histology (Fig. 5.1).

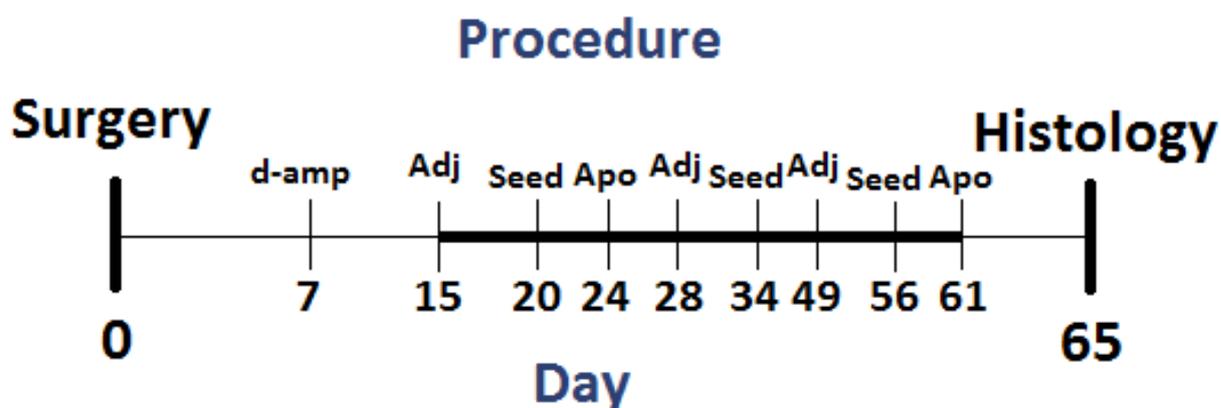


Figure 5.1. Post-surgery experimental timeline. Rats received unilateral lesions with four 6-OHDA infusions in the striatum. Lesion rats rotating in the ipsilateral direction at > 6 turns/min on day 7 after d-amphetamine were tested for impairments in akinesia, skilled forepaw use, and rotations after apomorphine between days 15 and 61. Rats were sacrificed for TH immunohistochemistry on day 65. Adj, adjusting steps test; Apo, apomorphine rotation test; d-amp, d-amphetamine rotation test; Seed, sunflower seed handling test.

5.3.3. Surgery

All surgical procedures were performed under general anaesthesia using isoflurane inhalation (4-5% for induction at 1.5 L/min oxygen; 2-3% for maintenance at 1 L/min oxygen). Rats received 5mg/kg carprofen i.p 1 hour prior to surgery for long term pain relief. Rats were also given 2ml Hartmann's solution i.p at the start of surgery and an additional 1ml at the end of surgery to mitigate fluid loss. Core body temperature was monitored and maintained at 37°C (+/- 1°C) via an automatic temperature regulating system (FHC, Bowdoin, Maine, USA).

Once anesthetised, the rat's head was shaved and placed in a stereotaxic frame (atraumatic ear bars, Kopf) with the incisor bar adjusted to achieve flat skull. The incised scalp was retracted to reveal bregma and lambda (anterior and posterior landmarks on the skull), and burr holes drilled above the striatum. Unilateral striatal lesions were achieved by

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four 2µl injections of freshly prepared 6-OHDA solution (3 µg/ul freebase dissolved in a vehicle solution of 0.2 mg/ml L-ascorbic acid in 0.9% w/v NaCl). The following four coordinates were used, as described by Kirik et al. (1998): AP 1.3mm, ML ±2.7mm; AP 0.4mm, ML ±3.1mm; AP -0.4mm, ML ±4.3mm; AP -1.3mm, ML ±4.7mm (from bregma) and DV -5.0mm from dura at each site (Fig. 5.2). Sham surgery rats received infusions of the vehicle solution alone. Infusions were made using an automated Stoelting microinfusion pump and a 10-µl Hamilton syringe (Reno, NV, USA) syringe fitted with PE20 tubing and a 28 gauge cannula (Plastics One). Infusions were made at a rate of 1µl/min with the cannula left in place for a further minute at each site to allow diffusion of the 6-OHDA solution. The wound was closed with surgical silk and mepivacaine (4mg/ml) and Emla cream applied topically for pain relief.

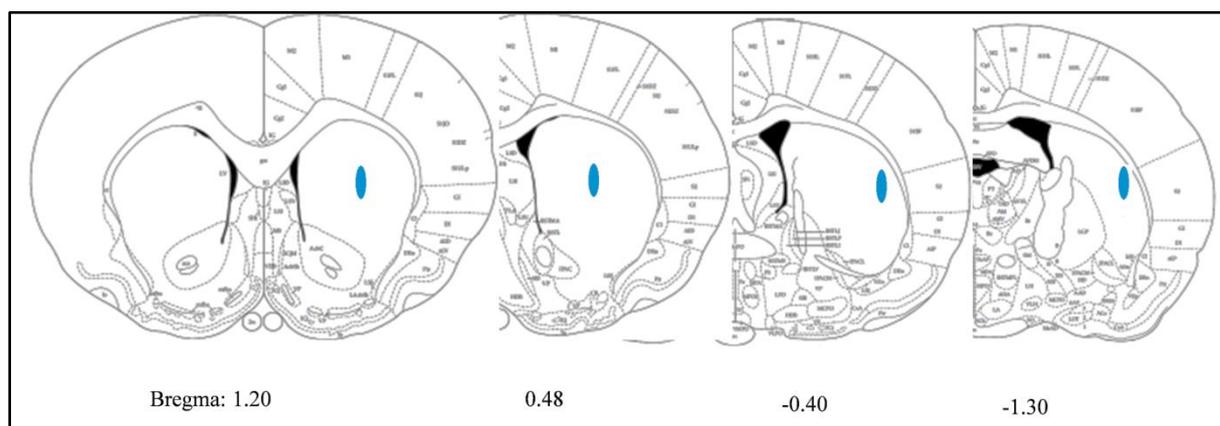


Figure 5.2. Target striatal infusion sites (blue ovals) for 6-OHDA lesion surgery. Numbers indicate distance from bregma in millimetres (plates adapted from Paxinos and Watson, 1998).

5.3.4. Statement regarding use of desipramine

The neurotoxin 6-OHDA is relatively selective for the catecholaminergic neurons DA and NA (Dauer and Przedborski, 2003). To protect the NAergic system from the toxic effects of 6-OHDA studies sometimes administer the NA transport blocker desipramine prior to surgery (e.g. Breit et al., 2007; Casas et al., 2011; Shin et al., 2014). However, desipramine pre-

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treatment is not always used in the 6-OHDA model (e.g. Decressac et al., 2012; Grealish et al., 2014; Krishnamurthi et al., 2009; Walsh et al., 2010). In the current thesis desipramine was not used. First, we recognise that PD is a multi-system disease affecting numerous neurotransmitter systems including NA although the striatal target is poorly innervated by NAergic fibres (Delaville et al., 2011). There is also evidence implicating the degeneration of the NAergic system for PD symptoms so their protection may be inadvisable (see Delaville et al., 2011 for a review).

5.3.5. Rotation after d-amphetamine

Rotation after d-amphetamine sulphate (3 mg/kg i.p.; Sigma) was tested at day 7 as an ante mortem validation of the 6-OHDA lesion. The apparatus consisted of a clear acrylic glass box (48cm long by 29cm wide by 25cm high) with bedding material on the floor and a colour CCD camera (Imaging Source) mounted above (Fig. 5.3). Rats were allowed 5mins to habituate to the box before d-amphetamine was administered. Following the injection rats were returned to the testing box for 5mins before recording began. This time allowed the rat to overcome the increased activity associated with the manipulation. Rotation was recorded via video tracking software (OpenControl, Aguiar et al., 2007) for 90mins. Scores are expressed as net full body turns/min. Ipsilateral (to lesion) turns were assigned positive values whereas contralateral turns were given negative values. Lesion rats will preferentially turn in the ipsilateral (to lesion) direction. Sham surgery rats do not show a preference. The criterion for a successful lesion was ipsilateral rotations at > 6 turns/min (Schwarz et al., 2006; Torres et al., 2007). Only lesion rats meeting this criterion were included in the experiment.



Figure 5.3. The apparatus used for the d-amphetamine and apomorphine rotation tests.

5.3.6. Rotation after apomorphine

Rotation following apomorphine hydrochloride (0.25 mg/kg s.c., Sigma) was assessed at days 24 and 61. The same procedure for rotation after d-amphetamine was followed except rotation was recorded for 60mins. Lesion rats will preferentially turn in the contralateral (to lesion) direction. Sham rats do not show a preference.

5.3.7. Adjusting steps test: Akinesia

Forelimb akinesia was tested using the adjusting steps test. Rats were tested pre-surgery and at days 15-17, 28-30, and 49-51. The apparatus consisted of a 102cm long by 50cm wide smooth table surface. Rats were handled on the three days prior to pre-surgery testing to familiarise them with the experimenters grip and the testing procedure. For each trial the experimenter used one hand to raise the hind quarter of the rat vertically to a “wheelbarrow”

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like position and the other hand to gently restrain the forelimb not to be tested while the unrestrained forepaw touched the table surface and was weight bearing (Fig. 5.4). The rat was slowly moved sideways (90cm in 5s) along the table surface in the medial direction with respect to the unrestrained forepaw (i.e. the rat is pulled to the left when the right paw is restrained). The number of adjusting steps made with the unrestrained forepaw was counted. The test was repeated twice for each forelimb on each of three consecutive days. The average number of steps for each forelimb over these six trials represented the rats score. Data were expressed as *contralateral forelimb steps/total steps x 100%*. An unimpaired rat would score 50% on this task. Lower scores indicate larger akinesia impairment.

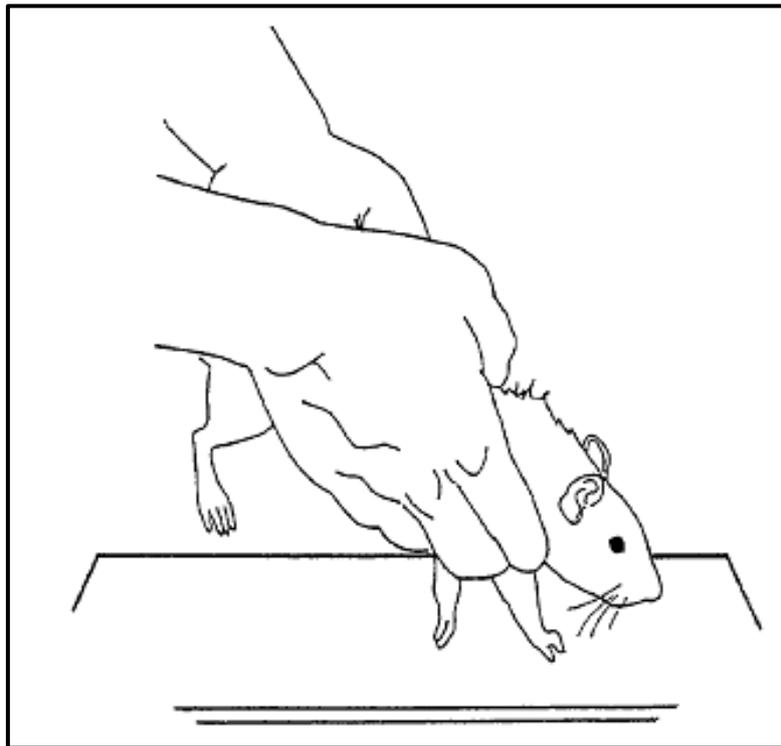


Figure 5.4. Example of the grip and positioning of the rat during the adjusting steps test (adapted from Tillerson et al., 2001).

5.3.8. Sunflower seed handling test: Skilled forepaw use

Skilled use of the forepaws was tested using the sunflower seed handling test. Rats were tested pre-surgery and at post-surgery days 20-22, 34-36, 56-58. The apparatus consisted of a

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clear acrylic glass box (48cm long by 29cm wide by 25cm high) with a wooden block inserted to restrict the rat to one half of the testing arena (Fig. 5.5). The box overlapped the edge of the table to allow the experimenter to video record (CCD colour camera, Imaging Source) the rat from below. To encourage eating the rat's food was removed for 1 hour prior to the test. Prior to testing rats were introduced to the sunflower seeds by providing each rat with 5 seeds per day for two days. Rats were habituated to the testing apparatus for 5mins per day for three days by placing the rat in the centre of the testing arena and placing five seeds in the corner.

Following habituation rats were tested once per day for three days at each testing occasion. Rats were placed in the centre of the box with five seeds placed in one corner. The number of shell pieces produced, the time taken to consume the seeds, and the number of adjustments made with each forepaw per seed was counted. An adjustment was defined as any visible released and re-grasp of the seed or any reformation of the grip on the seed using extension-flexion and/or abduction-adduction of the digits. For each session the scores for the five seeds were added together. Scores were taken as the average of the three sessions. Lesion rats were expected to make fewer adjustments with the impaired paw, take longer to consume seeds, and produce more shell pieces compared to shams. Due to technical difficulties with the recording equipment the forepaw adjustments and time spent eating the seeds were not able to be coded. Therefore, the forepaw adjustments and time spent eating the seeds measures are not reported.



Figure 5.5. The sunflower seed handling test. The image shows performance during pre-surgery testing.

5.3.9. Perfusion and immunohistochemistry

Rats were deeply anaesthetised with sodium pentobarbital (300mg/kg i.p.) at day 65 and transcardially perfused with 120ml cold saline followed by 180ml 4% paraformaldehyde (pH 7.4). Brains were removed and post-fixed overnight in the same fixative then transferred to a cryo-protection solution (20% glycerol, 80% PB with 0.05% sodium azide, pH 7.4) until sectioning. Brains were cut into serial sections of 30 μm using a freezing sledge microtome and stored at -20°C in a freezing solution (30% glycerol, 30% ethylene glycol, 40% PB ,pH 7.4) until processing.

Tyrosine hydroxylase (TH) immunohistochemistry was performed on free-floating sections. Sections were rinsed several times in 0.1M phosphate buffered saline containing 0.2% Triton X-100 (PBS-tx) between each incubation period. All incubation solutions contained PBS-tx. Endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 for 10min. Non-specific binding of the secondary antibody was blocked using 5%

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normal goat serum (NGS, Gibco, New Zealand) solution for 30mins. The sections were incubated overnight at 4°C under gentle agitation with the rabbit polyclonal anti-TH primary antibody (Millipore, AB152) diluted at 1:1000 with 1% NGS. The following day, sections were incubated for 1 hour with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, BA-1000) diluted at 1:400 with 1% NGS followed by 1 hour with avidin-biotin-peroxidase complex with 1% NGS (ABC Elite, Vector Laboratories) and visualised with 0.03% 3,3-diaminobenzidinetetrahydrochloride containing 0.01% H₂O₂ in 0.1M phosphate buffer. Sections were mounted on gelatine coated slides, dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped using DPX mounting medium.

5.3.10. Quantification of tyrosine hydroxylase immunohistochemistry

Photomicrographs of the striatum and ventral midbrain were taken using a Nikon Eclipse E800 microscope fitted with a Nikon DS-Fi1 camera and were analysed using ImageJ software (National Institute of Health, USA). The density of TH immunostaining was measured in three coronal sections in the striatum (AP: 0.7mm, -0.3mm, -0.7mm from bregma). To measure specific TH staining each section was corrected for non-specific background staining by subtracting the mean grey value of the medial corpus callosum (spanning both hemispheres) from the mean grey value of the striatum on each section.

5.3.11. Statistical analysis

Statistical analyses using Statistica (version 12.0) were conducted to determine if striatal 6-OHDA lesions produced significant impairment in akinesia (adjusting steps test), skilled forepaw use (sunflower seed handling test), and rotation after d-amphetamine and apomorphine compared with sham surgery rats. Differences in pre-surgery adjusting steps, and sunflower seed tests and in striatal TH density, and rotation after d-amphetamine were

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analysed using an independent samples t-test. A separate Lesion (6-OHDA vs. Sham) x Time (two or three time-points) ANOVA assessed the adjusting steps, sunflower seed handling, and apomorphine rotation test followed by *post-hoc* Newman-Keuls multiple comparisons tests where appropriate. Statistical significance was set at $p < 0.05$. All data are expressed as means \pm SEM.

5.4. Results

5.4.1. Rotation after d-amphetamine

At day 7, lesions produced substantially increased ipsilateral rotations ($t(16) = 15.34$, $p < 0.001$) after d-amphetamine confirming the success of the lesion (Fig. 5.6). Due to heteroscedasticity the analysis was followed up with a Mann-Whitney U test which found the same conclusions ($Z = 3.51$, $p < 0.001$). Two of the lesion surgery rats did not rotate at the required >6 turns/min and were excluded from this analysis and the remainder of the study.

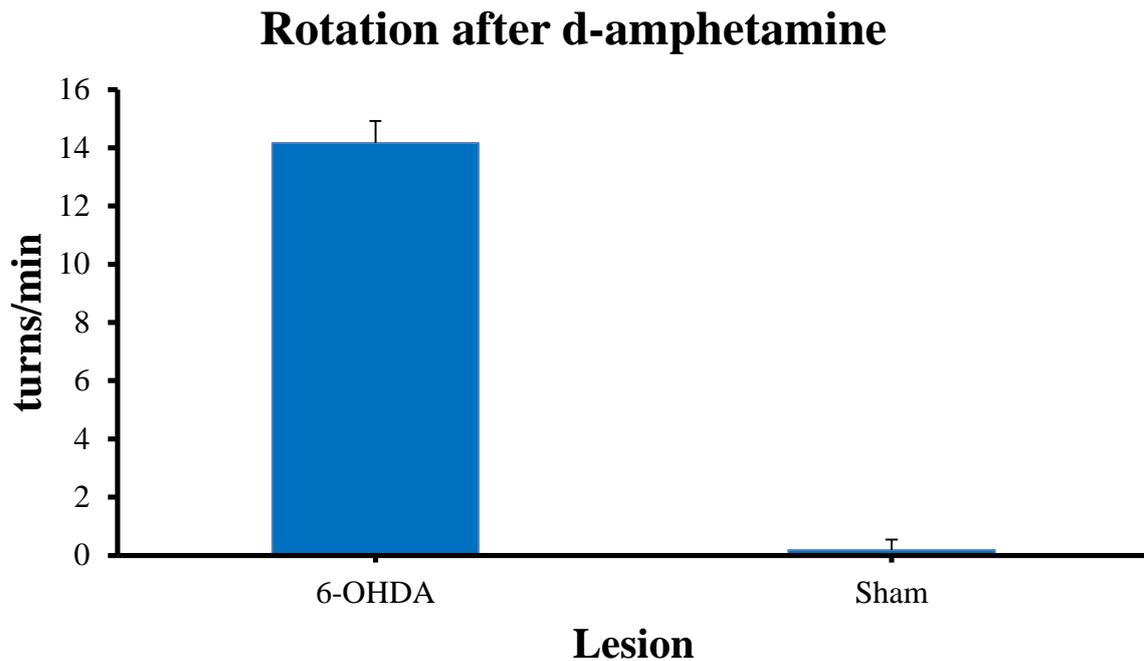


Figure 5.6. Mean turns/min after d-amphetamine at post-surgery day 7. Error bars denote SEM.

5.4.2. Adjusting steps test: Akinesia

Prior to surgery forelimb stepping did not differ between lesion and sham surgery groups ($p > 0.20$) in the adjusting steps test. Post-surgery, lesions produced substantially impaired forelimb stepping compared to shams (Lesion, $F(1, 16) = 139.57, p < 0.001$; Fig. 5.7). There was also a main effect of Time ($F(1, 16) = 9.82, p < 0.001$) and a significant Time x Lesion interaction ($F(2, 32) = 8.60, p = 0.001$) due to the increasingly poor performance of the lesion rats. Post-hoc Newman-Keuls confirmed that forelimb stepping for lesion rats was significantly reduced on each test occasion.

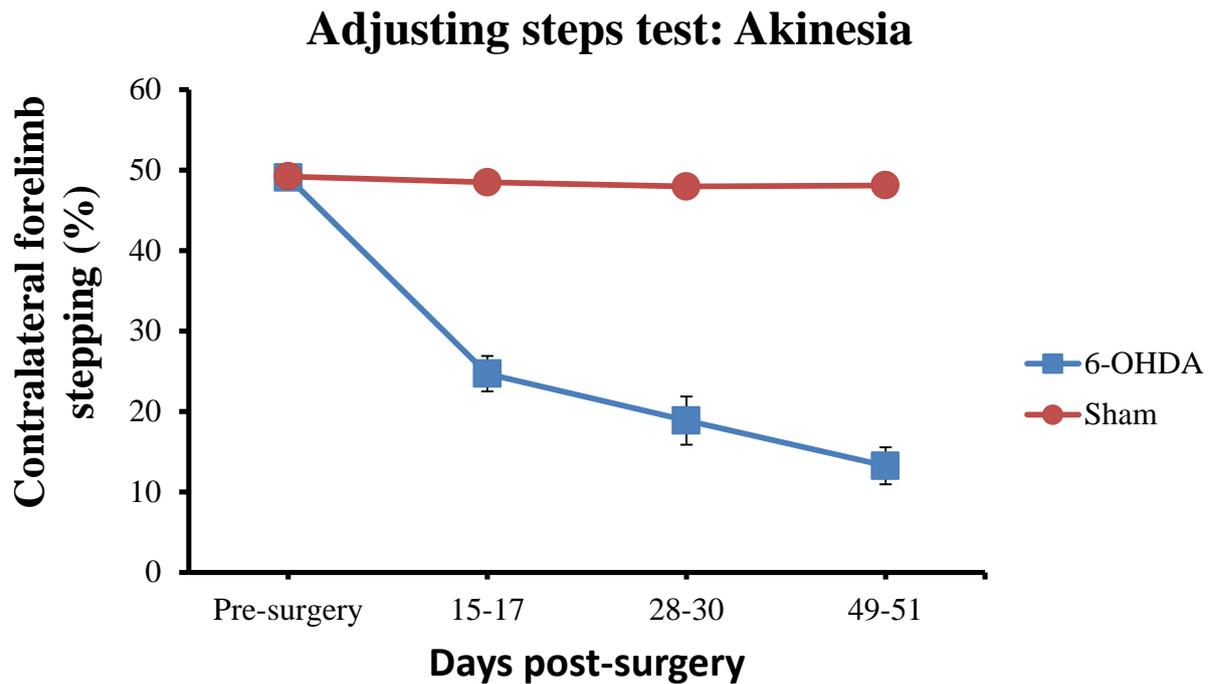


Figure 5.7. Contralateral forelimb stepping in the adjusting steps test. Error bars denote SEM.

5.4.3. Sunflower seed handling test: Skilled forepaw use

Prior to surgery the number of shell pieces produced did not differ between lesion and sham groups ($p > 0.20$) in the sunflower seed handling test. Following surgery rats with lesions produced more shell pieces than sham (Lesion, $F(1, 16) = 14.38$, $p = 0.002$; Fig. 5.8). There was a main effect of Time ($F(2, 32) = 4.05$, $p = 0.03$) due to decreasing shell pieces produced over testing occasions. The Time x Lesion interaction was not significant ($p > 0.20$).

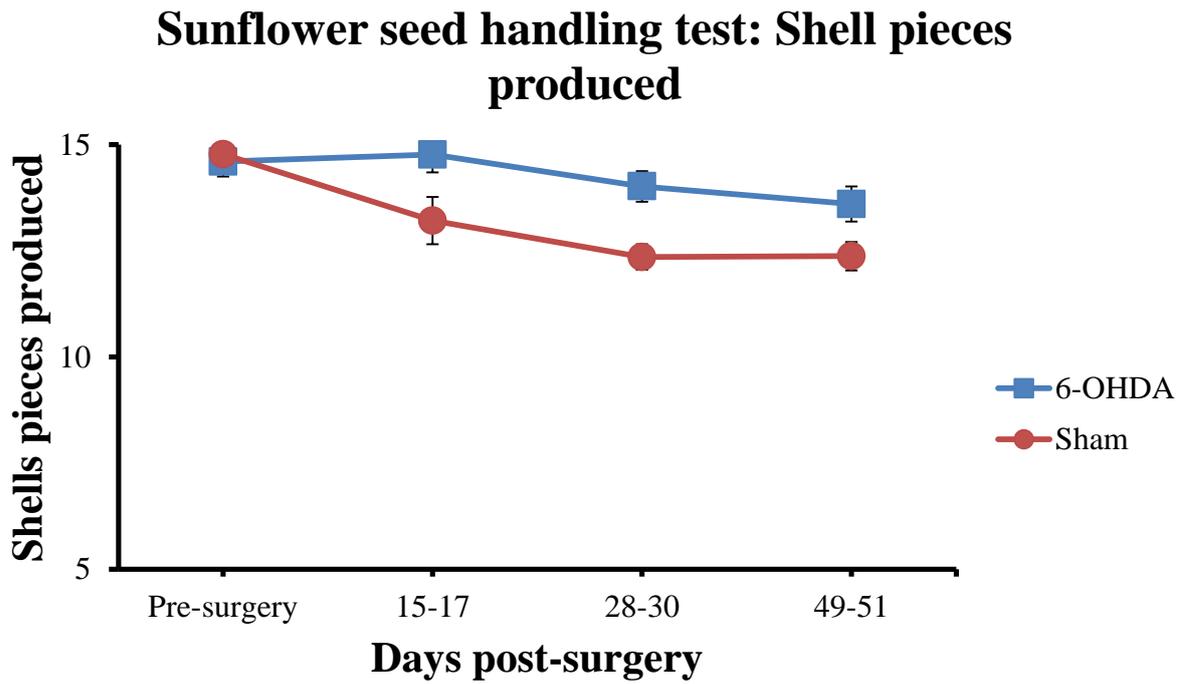


Figure 5.8. The mean number of shells leftover in the sunflower seed handling test. Error bars denote SEM.

5.4.4. Rotation after apomorphine

Lesions induced significant contralateral rotations after apomorphine (Lesion, $F(1, 16) = 15.47, p = 0.001$; Fig. 5.9). Neither the effect of Time ($F(1, 16) = 2.42, p = 0.12$) nor the Time x Lesion interaction ($F(1, 16) = 1.61, p = .19$) were significant.

Rotation after apomorphine

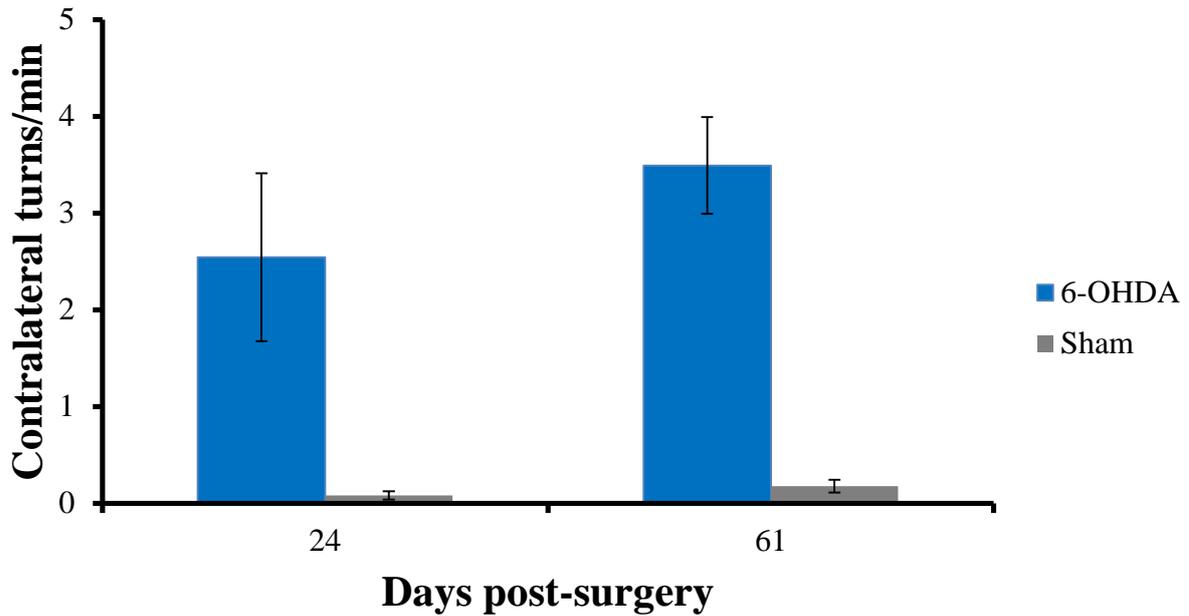


Figure 5.9. Mean turns/min after apomorphine. Error bars denote SEM. Note: Contralateral turns are expressed as positive values.

5.4.5. Tyrosine hydroxylase

To pilot the tyrosine hydroxylase immunohistochemistry procedure a random selection of seven lesion and three sham rats was used. At day 65, the 6-OHDA lesions significantly reduced TH density in the striatum compared to sham ($t(8) = 17.98, p < 0.001$; Fig. 5.10).

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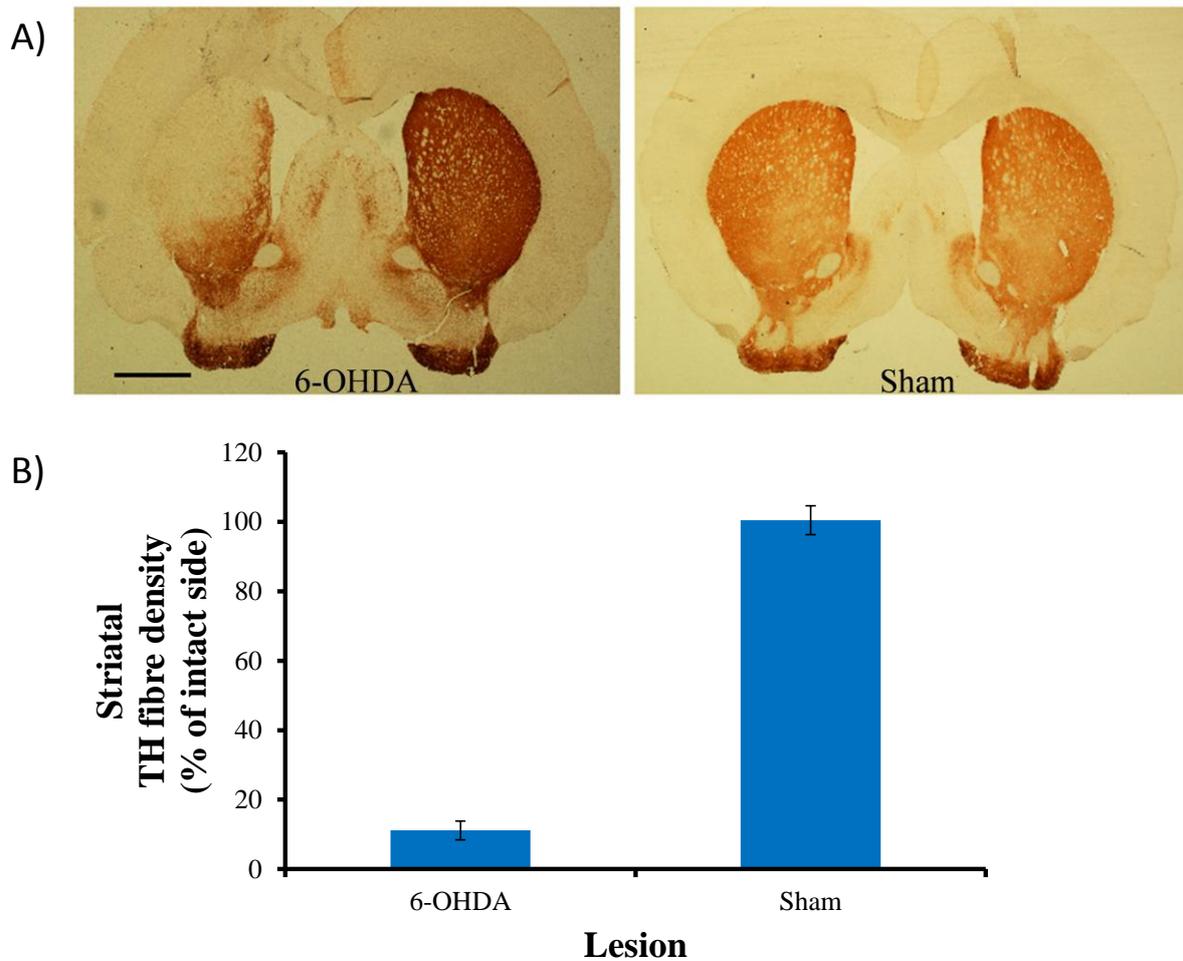


Figure 5.10. Striatal TH density at day 65. (A) Representative photomicrographs for a 6-OHDA lesion and sham rat (B) Figure showing % TH fibre density. Scale bar represents 2mm.

5.5. Summary and discussion

Unilateral 6-OHDA lesions of the striatum produced substantial akinesia impairments in the adjusting steps test, and increased rotation after d-amphetamine and apomorphine administration. Lesions also increased the number of shell pieces produced in the sunflower seed handling test. The lesions substantially decreased TH density in the striatum.

Tyrosine hydroxylase

6-OHDA infusions produced a substantial lesion resulting in ~89% reduction in TH fibre density in the striatum at day 65 post-surgery. Previous studies have found ~80-90% reduction in TH striatal fibre density at two, eight, 12, and 15 weeks after lesion surgery (Dowd et al., 2005a; Grealish et al., 2008; 2010; Kirik et al., 1998; Walsh et al., 2011). Other studies reported more moderate reductions between ~50 – 80% at 12 weeks and seven months after surgery (Kirik et al., 2001a, 2001b). The current finding of a substantial depletion of striatal TH at day 65 after the 6-OHDA lesion is consistent with previous reports of the four-site 6-OHDA lesion model.

Rotation after d-amphetamine

At 7 days post-lesion 6-OHDA produced substantial ipsilateral rotation after d-amphetamine administration. Sham rats did not have a rotation bias so this effect is attributable to the unilateral 6-OHDA neurotoxin, not non-specific damage caused by the vehicle. The presence of this rotation bias confirmed dopamine lesions and a deficit soon after surgery. This finding is consistent with the original report of the unilateral four-site 6-OHDA lesion of the striatum by Kirik et al. (1998) where they found substantially increased rotation after d-amphetamine at day 7 after lesion surgery. The rate of rotation they reported (12.1 turns/min) was comparable to our findings (14.2 turns/min). Recent studies employing the four-site intrastratial 6-OHDA lesion model have tested rotation after d-amphetamine at different times after surgery. Similar rates of rotation to the current experiment have been found when rats were first tested at two, three, four, and five-six weeks after surgery (Breysse et al., 2007; Carlsson et al., 2009; Grealish et al., 2008, 2010; Kirik et al., 2001a; Shin et al., 2014). In these studies the rate of rotation varied between ~11–18 turns/min with the exception of Grealish et al. (2008) who found a moderate rotation rate of ~5.5 turns/min. In their study the

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rate of rotation was more moderate as they included all rats that displayed an ipsilateral rotation bias regardless of the rate of rotation. The day at which rotation after d-amphetamine is first tested depends on the aim of the study. The current thesis tested rotation at day 7 as a dopamine lesion effect needed to be detected soon after surgery prior to PROG treatments and subsequent tests of motor impairments (see Chapter 6).

Adjusting steps test: Akinesia

Lesions produced substantial forelimb akinesia impairments in the adjusting steps test whereas sham rats were unimpaired. This impairment in forelimb akinesia was evident at the start of testing on this task at day 15 but it also worsened steadily across subsequent testing. This finding shows that forelimb akinesia impairment can get progressively worse in this unilateral 6-OHDA striatal lesion model.

Contralateral to lesion forelimb stepping is usually moderate to substantially reduced after four-site intrastratial 6-OHDA lesions. The current experiment found that the percentage of contralateral forelimb steps out of the total steps made for both forelimbs was reduced to about 25% at week two, 19% at week 4, and 13% at week 7. Unimpaired rats would score 50% whereas substantially impaired rats would score $\leq 15\%$ in this task (Winkler et al., 2006). Therefore, in the current experiment rats with lesions had moderately impaired forelimb stepping at week two which progressed to a substantial impairment at week 7 after surgery.

Kirik et al. (1998) found that contralateral forelimb stepping was moderately reduced at week three after surgery which had decreased further at week eight. Kirik et al. (2001a) found that contralateral forelimb stepping was moderately reduced at week one and remained stable over 12 weeks of testing. When tested at three to four weeks after surgery moderate to substantially reduced contralateral forelimb stepping has been reported (Dupre et al., 2008;

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Fang et al., 2006; Shin et al., 2014; Sun et al., 2013; Winkler et al., 2002). A stable but substantial impairment by day two after surgery has also been reported (Grealish et al., 2008). Interestingly, Kirik et al. (2001b) found that rats with both moderate and substantial forelimb stepping impairment at one month showed partial recovery when tested again at three and seven months post-surgery. This was possibly due to spontaneous sprouting of dopaminergic fibres and TH recovery in the striatum that can occur several months after partial 6-OHDA lesions (Stanic et al., 2003).

These studies show that impaired forelimb stepping in the adjusting steps is typically evident within three weeks after surgery. The impairment is often stable for up to three months although longer term testing can show partial recovery (e.g. Kirik et al., 2001b). However, testing over several weeks can show some progressive impairment (e.g. Kirik et al., 1998). Forelimb akinesia in the current experiment is consistent with previous studies as a forelimb stepping impairment was found at two weeks that worsened over subsequent testing.

Sunflower seed handling test: Skilled forepaw use

As expected rats made many forepaw adjustments on the sunflower seeds during handling and eating. These adjustments were too rapid to be reliably scored. Time spent handling and eating the seeds also could not be scored as rats regularly moved out of view of the camera. However, the number of shell pieces produced was easily scored. Lesion rats produced more shell pieces during sunflower seed handling compared to sham rats. The number of shell pieces produced decreased at subsequent testing occasions for both lesion and sham rats.

Impaired grip on pasta pieces has been observed in rats with unilateral 6-OHDA lesions (Allred et al., 2008). If present in the current experiment impaired grip on the sunflower seed could account for the increase shell pieces leftover. According to Whishaw and Coles (1996) intact rats grip sunflower seeds with a symmetrical paw hold and curl digit

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five (the “little finger”) around the bottom of the seed. Differences in forepaw grip on the seed do not likely account for the increased shells leftover in the current experiment as casual observations revealed that both lesion and sham rats held the sunflower seed with a typical grip. Typical grip styles do not exclude 6-OHDA lesion rats from having impaired contralateral forepaw adjustments as observed in the vermicelli pasta handling test (Allred et al., 2008). The increase in shells leftover among rats with lesions is not likely a result of forepaw use alone as shelling and eating requires an interaction between the forepaws and oral motor function (Kane et al., 2011). This finding suggests there may be some impairment in skilled forepaw use in our model although differentiating this deficit from oral motor function requires testing in a different task such as single-pellet skilled reaching (see Chapter 6).

Rotation after apomorphine

Lesions produced significant contralateral rotation after apomorphine that was evident at day 24 and had not diminished at day 61 post-surgery. This finding suggests there was substantial striatal deafferentation that had produced a persisting supersensitivity by day 24. The occurrence of contralateral rotation was, however, variable at day 24 with three of the ten lesion rats not showing a contralateral rotation bias. At day 61 all lesion rats preferentially rotated in the contralateral direction. So the time-course of this supersensitivity is variable.

Previous studies have shown increased contralateral rotation after apomorphine at two through to 12 weeks after four-site intrastratial 6-OHDA lesion surgery (Kirik et al., 1998, 2001a). In addition, differential rates of rotation have been found in rats with moderate and severe impairment on the adjusting steps test. Rats that were severely impaired on the adjusting steps test rotated substantially after apomorphine whereas moderately impaired rats showed intermediate rotation at six weeks after surgery (Kirik et al., 2001b). In another study,

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rats that had severely impaired forelimb stepping rotated substantially at six weeks, but, rotation had recovered at three and six months post-lesion (Winkler et al., 2006). Consistent with these reports the current experiment found increased contralateral rotation at day 24 that was maintained at day 61.

Comparison of behavioural tests

In the pilot study, impaired forelimb stepping on the adjusting steps test worsened over time, which is due to the striatal 6-OHDA infusions causing progressive degeneration of dopaminergic neurons in the SNpc (Walsh et al., 2011). This phenomenon is due to the sensitivity of the adjusting steps test to relatively small changes in dopamine depletion (Chang et al., 1999; Kirik et al., 1998; Olsson et al., 1995). The adjusting steps test provides a reliable assessment of dopamine depletion although impaired forelimb stepping is not exclusively dopamine associated (Chang et al., 1999; Pinna et al., 2007, 2014). Conversely, impaired skilled use of the forelimbs in the seed handling test is associated with both dopaminergic and non-dopaminergic changes after 6-OHDA lesions, such as ipsilateral motor cortex dysfunction in the areas responsible for skilled forelimb use (Plowman et al., 2014; Viaro et al., 2011). In treatment studies it would therefore be useful to include skilled forelimb task to assess neuronal plasticity beyond dopamine neurotransmission. By contrast, drug-induced rotation is most closely associated with dopamine depletion. The rotational bias produced by a pharmacological challenge with a dopamine agonist is dependent on asymmetrical dopamine neurotransmission (Ungerstedt & Arbuthnott, 1970). Drug induced rotation tests provide a gross assessment of dopamine related behavioural impairment but have limited translational value as the rotational bias is a pharmacological phenomenon. In treatment studies rotation after d-amphetamine will be useful as an ante-mortem validation of the 6-OHDA lesion prior to treatment with PROG whereas rotation after apomorphine is

useful as an ante-mortem indication of extensive dopamine lesions. **Identification of**

PROG treatment time-point and assessment of motor impairments

The findings of this experiment indicated that a dopamine depletion was present at day 7 after surgery as shown by increased rotation after d-amphetamine which is an indication of asymmetrical dopamine depletion. Impairments in forelimb akinesia in the adjusting steps test, the primary outcome measure, were observed at day 15 and progressively worsened thereafter. These findings support the starting of PROG treatment as soon as possible after lesion verification in the d-amphetamine rotation test. PROG treatment should be started at day 8 to avoid interactions with d-amphetamine and to ensure any beneficial effects are due to neuroprotection and not interactions with 6-OHDA neurotoxin metabolism (see Chapter 6 for further discussion). Starting PROG treatment at day 8 is ideal as a PD like impairment is present as shown by increased rotation after d-amphetamine but not yet fully developed as evidenced by progressive forelimb akinesia impairment. PROG's influence on motor impairments should be assessed in both drug induced rotation and clinically relevant non-drugged measures of motor function.

Rotational behaviour after d-amphetamine and apomorphine has long been the 'gold standard' with which to screen potential therapeutic candidates (Pienaar et al., 2012). It is suggested that the reduction of rotation intensity after therapeutic intervention is evidence of treatment efficacy (Pinna and Morelli, 2014). For example, transplants of dopaminergic cells in 6-OHDA lesion rats reduces rotation after d-amphetamine to levels observed in intact rats and improves forelimb akinesia, sensory neglect, and forelimb use asymmetry (Grealish et al., 2010; Mukhida et al., 2001; Torres et al., 2008). However, skilled reaching is not improved despite substantial reduction in rotation after d-amphetamine and apomorphine (Torres et al., 2008). Furthermore, the intensity of rotation after d-amphetamine and apomorphine was not correlated with motor assessments of skilled reaching and skilled

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ladder rung walking (Metz and Whishaw, 2002). These findings show that drug induced rotation and impaired skilled motor functions are independent consequences of 6-OHDA lesions. A common criticism of drug induced rotation tests is that the model is a pharmacological phenomenon whereas the impairments observed in clinical PD are present without drug induction (Meredith and Kang, 2006). In light of these findings current recommendations are to combine drug induced rotation tests with more clinically relevant measures to fully assess loss and recovery of function (Meredith and Kang, 2006; Metz and Whishaw, 2002; Pinenaar et al., 2012).

The adjusting steps test is widely used and is considered a clinically relevant measure of akinesia, a cardinal feature of PD (e.g. Bordia et al., 2015; Pinenaar et al., 2012; Schallert et al., 1992; Shin et al., 2014). Furthermore, the adjusting steps test produces the most reliable behavioural readout following partial 6-OHDA lesions (Duty and Jenner, 2011). The reliability and clinical relevance of the adjusting steps test support its use as the primary outcome measure in PROG treatment studies. The adjusting steps test should be used in conjunction with drug induced rotation and skilled reaching tests to fully assess loss and recovery of function.

Subsequent experiments involving PROG treatment should assess motor skills in both the short and longer term after lesion surgery. The rationale for assessing the relatively immediate and longer term consequences of PROG is to demonstrate sustained benefit of the treatment as this is critical to the development of new neuroprotective agents (Fisher et al., 1999, 2009). In the current and subsequent experiments using PROG (see Chapters 6 and 7) motor skills were assessed between two and eight weeks after lesion surgery as this time period captures the relatively early to late stages after striatal 6-OHDA lesions, respectively (Kirik et al., 1998).

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In summary, this pilot experiment has established the time-course for motor impairments in our unilateral 6-OHDA partial lesion model of PD. Additionally, we have identified day 8 as a suitable time to start PROG treatment in later experiments. This experiment established the efficacy of the adjusting steps test as a reliable measure of akinesia. A possible deficit in skilled forepaw use was also found which suggests later experiments examining PROG's influence should include a behavioural task involving skilled forelimb use. The utility of the d-amphetamine rotation test as an ante-mortem verification of the lesion was established and was used in subsequent experiments to determine the presence of a PD like impairment before PROG is administered. Increased rotation after apomorphine was also found in our model and will be a useful assay of PROG's influence on 6-OHDA lesion induced supersensitivity.

Chapter 6

Study Two: Progesterone after Large Intrastratial 6-OHDA Lesions

6.1. Objectives

This chapter reports the second of three experiments (see also: Chapters 5 and 7) and involves PROG treatment in the 6-OHDA model of PD. The objective of this experiment was to examine PROG's influence on akinesia, skilled reaching, and rotation after apomorphine following 6-OHDA lesions. Rats were tested preoperatively on the adjusting steps test for akinesia (as previously) and a single pellet skilled reaching task (a new version compared to the previous sunflower seed test) and then given 6-OHDA lesions or sham surgery. As before, the success of the lesion was confirmed at day 7 with a d-amphetamine rotation test. Eight days after surgery injections of either 8 mg/kg PROG, 4mg/kg PROG or sesame oil vehicle were given once daily for 7 days with tapered withdrawal by sequential halving on the sixth and seventh days of treatment. The first injection was given intraperitoneal (i.p) and the remainder were given subcutaneous (s.c) Rats were tested on the adjusting steps test on post-surgery days 17-19, 27-29, 39-41, and 53-55, on the skilled reaching test on days 24-26 and 49-51, and the apomorphine rotation test at days 29 and 62. Rats were sacrificed at day 64 for tyrosine hydroxylase immunohistochemistry of the striatum. An additional group of rats designated 'lesion-only control' were euthanized at day 8 to serve as a reference for the analysis of tyrosine hydroxylase at the time when PROG treatment was started. This chapter begins with a brief recap of the justification for examining the influence of PROG in an animal model of PD.

6.2. Introduction

As discussed in Chapter 3, there is considerable evidence that the neurosteroid PROG improves functional and neurological outcomes in animal models of TBI and ischemia (see Deutsh et al., 2013; Stein et al., 2008 for reviews). PROG offers neuroprotection by reducing cerebral edema, decreasing proBDNF and proNGF, increasing BDNF, enhancing neuronal survival, decreasing neuronal apoptosis, reducing oxidative stress, and attenuating neuroinflammation in brain injury models (Deutsh et al., 2013). Despite the increasing evidence for the beneficial effects of PROG following brain injury PROG's influence has been examined in only a few pre-clinical studies of PD (see Chapter 3). Many of the pro-recovery effects of PROG listed are plausible mechanisms of neuroprotection in the context of PD also (see Figure 3.1 in Chapter 3). For example, activated microglia, a sign of neuroinflammation, has been reported in clinical PD brains and animal models including those using 6-OHDA lesions (see Hirsch and Hunot, 2009 for a review). Neuroinflammation is thought to contribute to the cascade that leads to neurodegeneration in PD (Barcia, 2013). PROG has reduced the expression of activated microglia and other markers of inflammation in models of TBI, ischemia, spinal crush injury, demyelinating disease, and Alzheimer's disease (Deutsch et al., 2013; Wei and Xiao, 2013). PROG, therefore, could offer neuroprotection after 6-OHDA lesions by reducing the expression of activated microglia. To examine PROG's influence on motor impairments following unilateral 6-OHDA lesions we used the four-site intrastratial model as established in the pilot study (see Chapter 5).

To examine PROG's efficacy on motor impairments we tested forelimb akinesia, skilled reaching, and rotation after apomorphine. Forelimb akinesia in the adjusting steps test was used as the primary behavioural outcome measure as akinesia is a cardinal feature of PD and contributes to poorer quality of life in PD patients (Olsson et al., 1995; Rahman et al.,

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2008). Contralateral rotation after apomorphine was used as an ante-mortem indication of PROG's influence on 6-OHDA striatal lesion induced supersensitivity.

We found a possible impairment in skilled forepaw use following striatal 6-OHDA lesions (see study one, Chapter 5). We examined skilled forepaw use further in the current PROG experiment as impairments in fine motor control are a debilitating symptom in PD patients (Klein et al., 2012). Instead of the previous sunflower seed handling test we used a single-pellet reach-to-eat task as a measure of skilled forelimb use. This task was selected as a rat's skilled reaching for a food target is phylogenetically and structurally similar to skilled reaching in humans (Klein et al., 2012). Previous studies have shown that unilateral 6-OHDA lesions impair skilled reaching performance of the contralateral forelimb in the single-pellet reaching task (Klein et al., 2007; Metz et al., 2003; Plowman et al., 2013, 2014).

When testing the efficacy of neuroprotective agents it is crucial to administer the drug so that it is affecting the underlying disease pathology rather than interacting with the neurotoxin's metabolism. It is not known if PROG will interact with 6-OHDA metabolism but to ensure that any beneficial effects of PROG are due to neuroprotection and not interactions with 6-OHDA metabolism we used a treatment protocol whereby PROG treatment was not started until the 6-OHDA neurotoxin had metabolized and the cell death cascade had commenced. This approach to examining neuroprotective agents in PD models has been used elsewhere (Hung et al., 2012). Previous studies have shown that SN cell death emerges from 3 days after four-site intrastratial 6-OHDA infusions and then progressively worsens thereafter (Cicchetti et al., 2002; Walsh et al., 2011). Therefore, PROG treatment should not be started before day 3 post-lesion. In addition, PROG treatment should not be started until a PD like impairment is present, as discussed in Chapter 5. In the pilot experiment (see Chapter 5) we found a PD like impairment was present at post-surgery day 7 as evidenced by increased rotation after d-amphetamine. To avoid interactions with d-

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amphetamine we started PROG treatment at day 8 after surgery. At this point 6-OHDA metabolism had terminated, the cell death cascade had commenced, and a PD like impairment was present.

The PROG doses used in the current experiment were 8 mg/kg and 4 mg/kg. These doses were selected as they represent common doses used in the literature that have improved functional and neurological outcomes in rat models of TBI (Grossman et al., 2004; Roof et al., 1996; Shear et al., 2002), and stroke (Jiang et al., 1996; Sayeed et al., 2007; Yousuf et al., 2014). PROG was administered once daily for seven days. This length of treatment represents a standard treatment regime in TBI and stroke models (e.g. Geddes et al., 2014; Yousuf et al., 2014). Suddenly withdrawing PROG after repeated dosing can exacerbate the lesion effects, produce an inflammatory rebound effect, and increase anxiety like behaviours (Cutler et al., 2005; Cutler et al., 2006). We therefore used tapered withdrawal by sequential halving of the dose on the sixth and seventh days of treatment as this has been shown to overcome the PROG withdrawal syndrome (Cutler et al., 2005).

The novel aim of this study was to examine the beneficial influence of PROG treatment in the 6-OHDA striatal lesion model of PD. As discussed the pleiotropic actions of PROG may be beneficial in the 6-OHDA lesion model by reducing neuroinflammation, oxidative stress, and neuronal apoptosis, and increasing trophic support. This experiment represents the first study in this field to examine whether PROG reduces striatal 6-OHDA lesion induced impairments on akinesia, skilled reaching, and rotation after apomorphine. Furthermore, this experiment investigated PROG's influence on motor impairments in terms of both short and longer term outcomes.

6.3. Materials and Methods

6.3.1. Animals

Fifty-nine male Wistar rats were used, weighing between 531g and 756g at the day of surgery. The lesion control, sacrificed at day 8 after surgery, were ~7 months old. The rats used in the main experiment were 8-13 months old at surgery and 10-15 months old at perfusion. Testing occurred during the dark phase of the rats reversed light cycle (lights on at 2000hrs). Rats were housed individually for five days of post-surgery recovery after which they were housed in groups of 3-4 for the remainder of the study. All protocols in this study followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee University of Canterbury. Two rats were lost after surgery and four rats were euthanized several weeks after surgery due to respiratory distress due to unknown reasons.

6.3.2. Experimental design

The experiment used a between groups design with the experimenter blinded to the PROG treatment group of the lesion rats. Matched rats were assigned to groups (4 mg/kg PROG, 8 mg/kg PROG, vehicle, or sham) according to their pre-surgery hit rate performance on the skilled reaching test. The lesion-only control group were handled prior to surgery but were not trained or tested on the skilled reaching or adjusting steps test. The sequence of 6-OHDA lesion surgery, PROG treatment, and motor skills testing is shown in Fig. 6.1.

Study Two: Progesterone after Large Intrastratial 6-OHDA Lesions

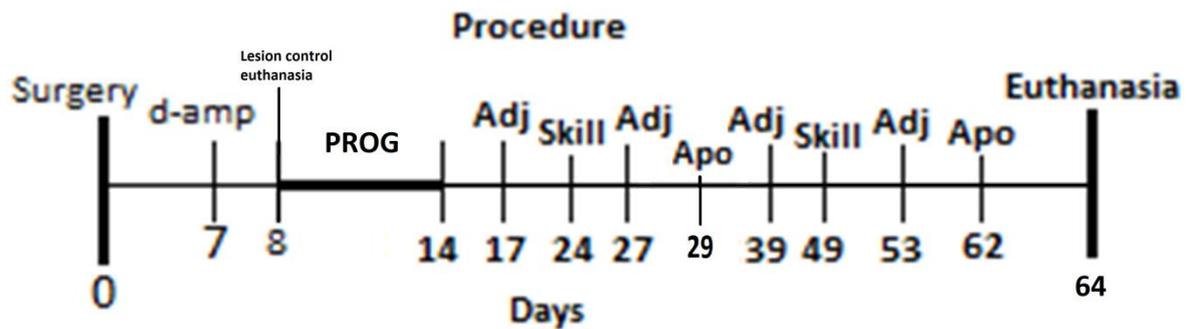


Figure 6.1. Experimental timeline. Rats were unilaterally lesioned with a 4-site 6-OHDA lesion of the striatum. Lesion rats rotating in the ipsilateral (to lesion) direction at the criterion of >6 turns/min at day 7 after d-amphetamine were matched to treatment groups according to baseline skilled reaching hit rate. Progesterone was given once daily on days 8-14. Akinesia in an adjusting steps test and skilled forelimb use in a single pellet reaching task were tested between days 17 and 53. Contralateral (to lesion) rotation after apomorphine were measured at days 29 and 62. Lesion control rats were euthanized at day 8 and all other rats were euthanized at day 64 for histology. Adj, adjusting steps test; Apo, apomorphine rotation test; d-amp, d-amphetamine rotation test; PROG, progesterone; skill, single pellet skilled reaching test.

6.3.3. Surgery

The same surgery protocol as described in Chapter 5 was used except rats were operated on the side contralateral to their preferred paw as identified in the pre-surgery skilled reaching task. All lesion control rats received infusions on the right side.

6.3.4. Rotation after d-amphetamine and apomorphine

Rats were tested for rotation after d-amphetamine at post-surgery day 7 and apomorphine at days 29 and 62 using the same procedure as described in chapter 5.

6.3.5. Progesterone treatment

Eight days after surgery rats with 6-OHDA lesions were given either 4 mg/kg PROG (Sigma, Castle Hill, Australia, $n = 11$), 8 mg/kg PROG ($n = 9$) or vehicle ($n = 10$) once daily for seven days. Tapered withdrawal of the PROG treatment in both groups was used by sequentially

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halving the dose on the sixth and seventh days of treatment. Sham rats ($n = 8$) received injections of the vehicle alone. PROG was dissolved in sesame oil (Sigma) and injected at a volume of 0.4 ml/kg. The first injection was given i.p. to allow for rapid absorption and the remainder were given s.c, with injections being administered between 10:00am and noon each day. Lesion control rats ($n = 7$) did not receive any injections. These treatment procedures were suggested by Prof. Don Stein and Assistant Prof. Iqbal Sayeed of Emory University, Atlanta, Georgia who are experts regarding PROG neuroprotection (personal communications). The bottles containing the PROG and vehicle solutions were labelled by an independent investigator who kept the code in a locked safe that could not be accessed by the experimenter responsible for the assessment of outcomes. The experimenter remained blinded to the treatment condition for all lesion rats until all data had been collected and analysed.

6.3.6. Adjusting steps test: Akinesia

Forelimb akinesia was measured pre-surgery and at post-surgery days 17-19, 27-29, 39-41, and 53-55 using the same procedure as described in Chapter 5.

6.3.7. Single pellet-reaching task: Skilled reaching

Skilled reaching was tested using a “reach to eat” task. Rats were tested pre-surgery and at post-surgery days 24-26 and 49-51.

Food restriction. Prior to pre-surgery training rats were gradually food deprived to 90-95% of their *ad libitum* body weight by food restriction. Five days prior to training rats were habituated to the pellet reaching targets. Each rat received twenty 45mg dustless precision chocolate-flavoured pellets (Bioserve, Frenchtown, NJ, USA) in their home cage about 1 hour prior to daily feeding. These standardised pellets (size, shape, weight) ensured consistency of the reaching target. Rats were returned to free-feeding following pre-surgery

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testing. In post-surgery sessions rats were again food deprived to ~ 95% of their *ad libitum* body weight over the four days prior to testing.

Reaching box. The reaching box was made of clear acrylic glass measuring 21cm long x 14cm wide x 16cm high with a hinged lid (Fig. 6.2). The floor was made of 0.9cm² grids so that dropped pellets fell and could not be retrieved. The front of the apparatus consisted of a removable clear window. The centre of the habituation window used to establish forelimb preferences contained a vertical slot measuring 1.8cm wide that extended from the floor to a height of 12cm (Fig. 6.2 A, B). A 4cm deep platform was mounted 3.25cm above the floor in front of the slot on the outside of the window. The four wells used to hold pellets were located 2cm from the inside edge of the window. The centre of the two medial wells was located 0.25cm either side of the centre of the slot. The centre of the lateral wells was placed in line with the edges of the vertical slot. The location of the lateral wells encouraged use of the contralateral paw as the paw pronates medially to grasp and it prevents a rat from retrieving the pellet with its tongue. A different window was used for testing and contained two vertical slots each located 1.5cm from the side walls of the box (Fig. 6.2 C, D). The location of the slots prevented the rat from using the paw contralateral to the side wall thus permitting independent assessment of each forelimb. In front of each of these testing slots there was a 1.5cm deep notch that extended 0.5cm past the medial edge of the slot. This notch meant that the rat could not slide the pellet to its mouth from the platform, instead requiring the pellet to be grasped and lifted.

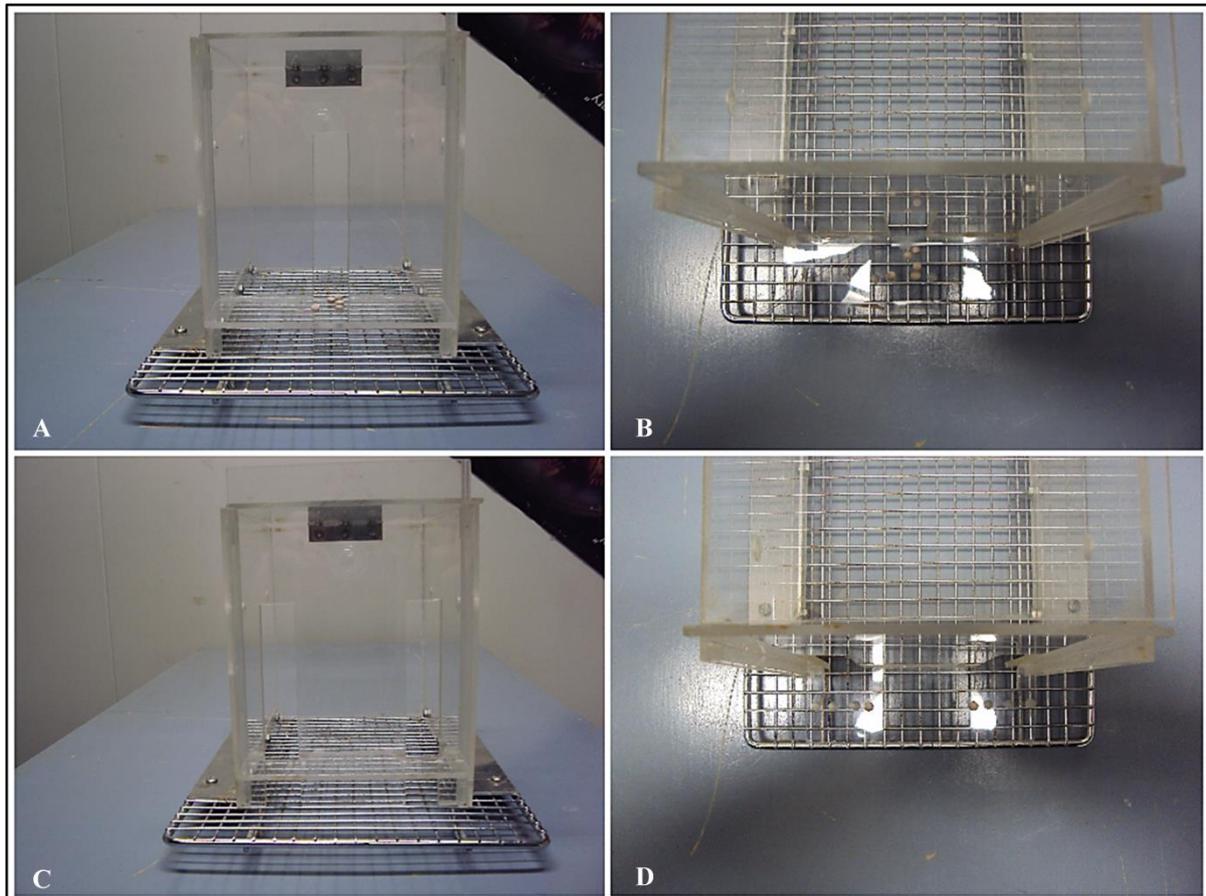


Figure 6.2. The reaching box apparatus used for single pellet skilled reaching. Views of the habituation (A, B) and testing windows (C, D). The habituation window consisted of a single central vertical slot whereas the testing window had two vertical slots located near the side walls of the reaching box.

Training. Rats were trained in the skilled reaching box for one 10min session per day for four weeks. The first phase familiarised the rat with the reaching box and the location of the pellets using the habituation window. Rats were placed in the box with several pellets placed on the platform within reach of the rat's tongue. Rats were required to eat pellets from the platform before progressing to reach training, which took two sessions. The second phase shaped reaching behaviour. A reach was defined as any advance of the forepaw through the slot towards the target. The pellets were moved away from the slot to encourage use of the paws. Rats required up to two sessions to develop a preference for reaching. The third phase of training established the rats paw dominance. For each trial rats were presented with one

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pellet in each of the four wells. Rats were free to reach with either paw. Paw dominance was defined as the paw used to take the pellet in at least 70% of trials for three consecutive days.

The final phase trained skilled reaching of the dominant forelimb using the window with two side slots (i.e. the “testing” window). Each session consisted of 20 trials with one pellet per trial placed in the most medial well of the slot ipsilateral to the dominant paw. On each trial the rat was required to reset its body posture by moving away from the slot before presentation of the next pellet. This ensured that each trial consisted of a discrete reach. If the rat reached when a pellet was not present it was required to move away before the next pellet was presented. Reaching when a pellet was not present was minimised by withholding pellets on random trials. This encouraged the rat to probe the slot by sniffing and reach only when a pellet was present (Fig. 6.3). After the rat was making discrete reaches it received daily sessions consisting of 10 warm-up trials followed by 20 scored trials. The rat was trained until its average “hit” rate for the preceding three sessions was 70%. Finally, the rat received three videotaped sessions (Panasonic digital video camera recorder; Model SDR-H85) that served as the rat’s average baseline performance. Rats were matched to groups according to their baseline hit rate on this task.

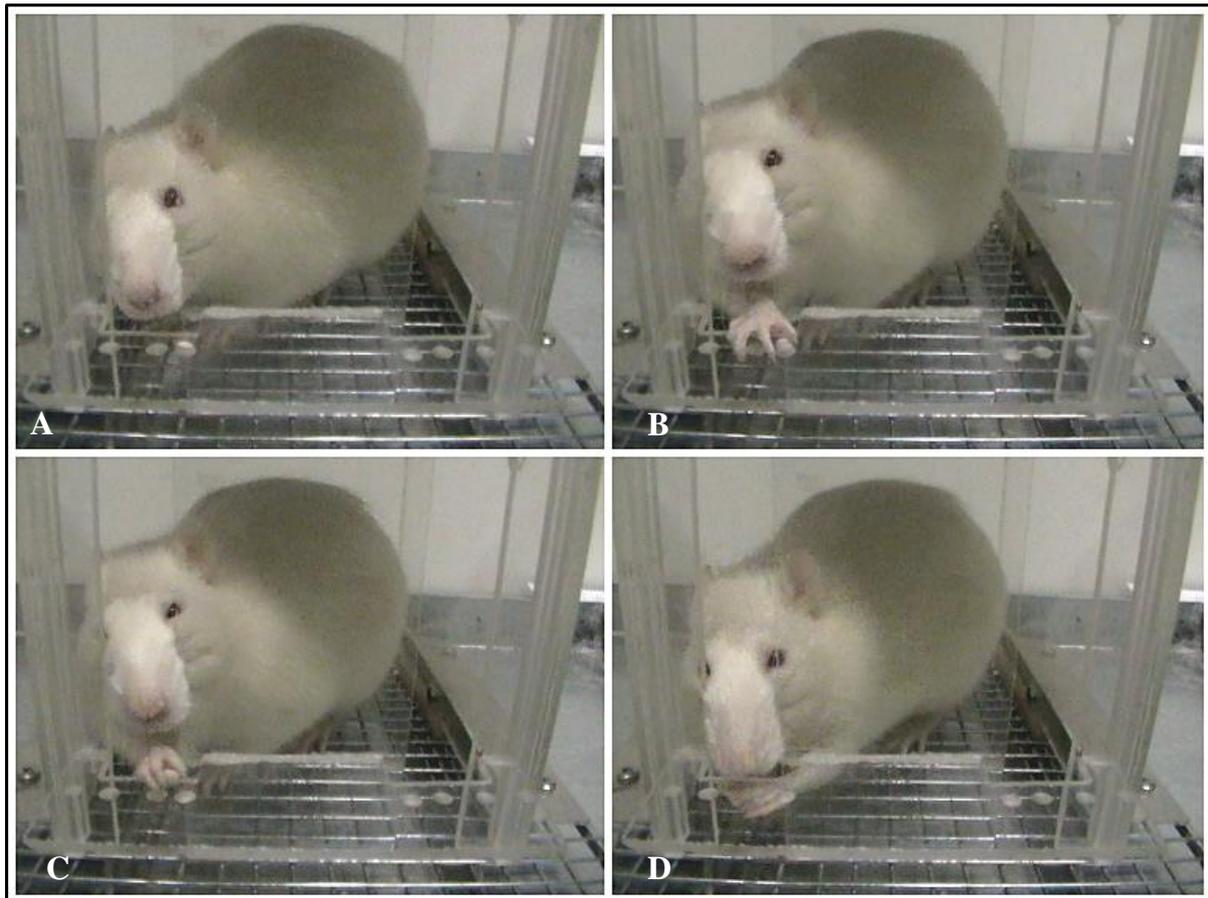


Figure 6.3. Example of a successful skilled reaching sequence during pre-surgery testing. The rat orients to the pellet by sniffing (A) then advances its paw through the slot (B) to grasp the pellet (C) then retracts its paw to eat the pellet (D).

Scoring reaching success. Two end-point measures were used to quantify skilled reaching accuracy. A “success” occurred when in one reach attempt the rat grasped the pellet, brought it back inside the box, and ate it. If the rat required more than one reach to grasp and eat the pellet then these trials were added to the “success” trials to derive an overall “hit” rate. If the pellet was dropped or dislodged from the well it was considered an “unsuccessful” reach. Rats were allowed up to five reaches per trial after which the pellet was removed and the trial counted as an unsuccessful reach. Three sessions were averaged at each testing time-point. The “success” rate was calculated as $number\ of\ success/20 \times 100\%$. The “hit” rate was calculated as $number\ of\ hits/20 \times 100\%$.

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Post-surgery testing. Rats were tested between days 24-26 and days 49-51. To ensure equivalency in the recency of skilled reaching experience, all rats received “refresher” sessions on the three days prior to testing. Rats received one refresher session per day consisting of 20 non-scored trials.

6.3.8. Perfusion and immunohistochemistry

Lesion-only control rats were euthanized at day 8 at the time of day when PROG treatment would have been administered i.e. between 10:00am and noon. The remaining rats were euthanized at day 64. The same perfusion, TH immunohistochemistry, and quantification procedures as described in Chapter 5 were used.

6.3.9. Statistical analysis

Statistical analyses (Statistica software, version 12.0) were conducted to determine if the impairments in akinesia (adjusting steps test), skilled reaching (single-pellet skilled reaching task), and rotation after apomorphine produced by striatal 6-OHDA lesions were reduced by PROG treatment. Differences in pre-surgery performance on the adjusting steps and skilled reaching tasks, rotation after d-amphetamine, and striatal TH density were analysed by a one-way ANOVA (five groups; 4 mg/kg PROG, 8 mg/kg PROG, vehicle, sham, and lesion-only control). Differences in post-surgery skilled reaching performance were also analysed with a one-way ANOVA as only days 49-51 could be analysed due to a feeding error at the earlier testing time-point (see below). The Group x Time interaction in post-surgery adjusting steps test and rotation after apomorphine was analysed by a two-way ANOVA (pre-surgery data were not included due to differences in variances). One 8 mg/kg PROG rat was excluded from the analysis for the rotation after apomorphine test due to a video tracking error. *Post-hoc* Newman-Keuls multiple comparisons were used for significant main effects and interactions. Statistical significance was set at $p < 0.05$. All data are expressed as means \pm SEM.

6.4. Results

6.4.1. Rotation after d-amphetamine

At post-surgery day 7 nine of the lesion rats did not show a rotation bias at the required >6 turns/min and were excluded from the study. The remainder produced substantially increased ipsilateral rotations ($F(4, 40) = 59.01, p < 0.001$) confirming the success of the lesion (Fig. 6.4). Post-hoc Newman-Keuls confirmed that all lesion groups rotated significantly more than sham (all $p < 0.001$) but did not rotate differently from each other (all $p > 0.20$).

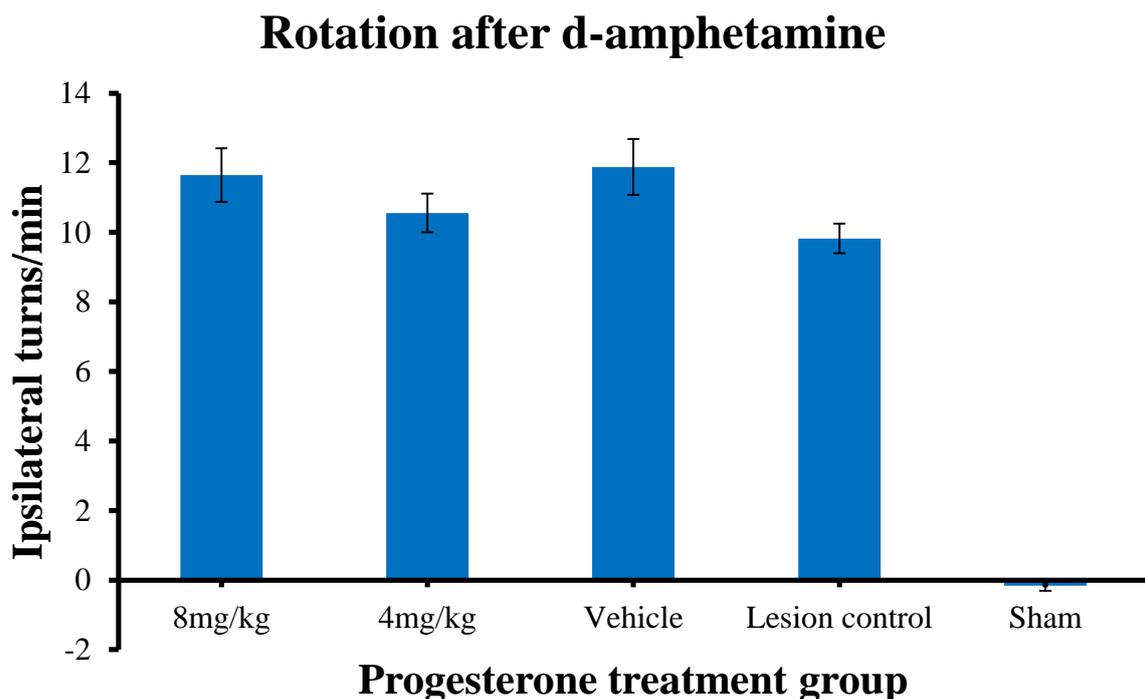


Figure 6.4. Mean turns/min after d-amphetamine at day 7. Error bars denote SEM.

6.4.2. Adjusting steps test: Akinesia

Prior to surgery forelimb stepping did not differ between lesion and sham surgery groups ($p > 0.20$). Post-surgery, lesions caused substantially impaired forelimb stepping (Lesion, $F(3,$

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34) = 19.24, $p < 0.001$; Fig. 6.5). Post-hoc Newman-Keuls showed that all lesion groups made significantly fewer steps than the sham group (all $p < 0.001$) but both 4 mg/kg PROG and 8 mg/kg PROG treatment groups were less impaired with an intermediate level of impairment compared to the vehicle-treated lesion group (both $p < 0.01$). Forelimb stepping did not differ between the two PROG treatment groups ($p > 0.20$). Neither the main effect of Time ($p > 0.20$) nor the Time x Group interaction ($p > 0.20$) were significant, although the mean values for the vehicle-treated group worsened over time.

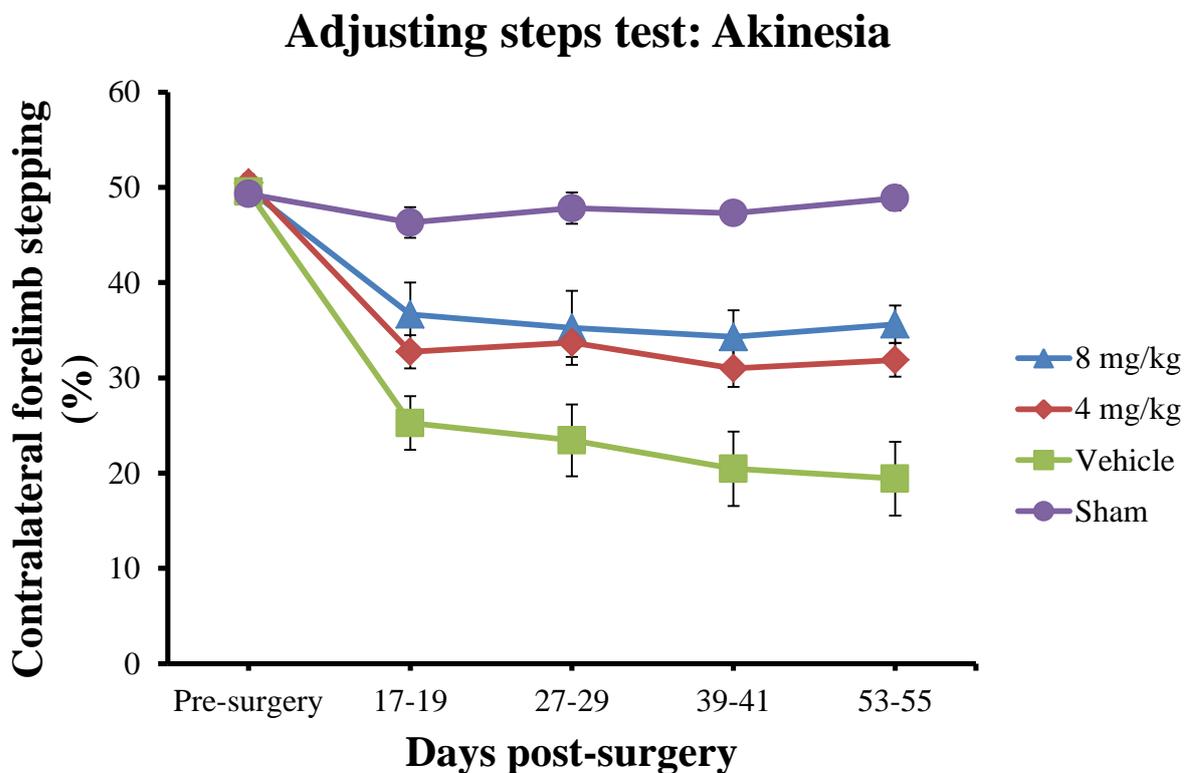


Figure 6.5. Contralateral forelimb stepping in the adjusting steps test. Error bars denote SEM.

6.4.3. Single-pellet reaching task: Skilled reaching

At the day 24-26 testing session many of the rats would not participate in the task. It was discovered that these rats had inadvertently received additional food from laboratory staff and hence were not motivated. The day 24-26 data are therefore not reported. At the day 49-51 time-point all rats were adequately food-deprived and readily engaged in the task.

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Preoperatively the “success” rate during skilled reaching did not differ between lesion and sham surgery rats ($p>0.20$). At days 49-51 after surgery, lesions reduced the “success” rate compared to sham ($F(3, 34) = 3.75$ $p = 0.02$; Fig. 6.6). Post-hoc Newman-Keuls revealed that 4 mg/kg PROG, 8 mg/kg PROG and vehicle treated lesion groups had a lower “success” rate than the sham group (all $p<0.05$). Neither 4 mg/kg PROG nor 8 mg/kg PROG treatment improved the “success” rate compared to vehicle treated lesion rats (both $p>0.20$).

Prior to surgery the “hit” rate during skilled reaching did not differ between lesion and sham surgery rats ($p>0.20$). At post-surgery testing, the lesion effect was significant ($F(3, 34) = 2.94$, $p = 0.047$; Fig. 6.7) and post-hoc Newman-Keuls showed that 4 mg/kg PROG, 8 mg/kg PROG and vehicle treated lesion groups produced less “hits” than the sham group (all $p<0.05$). As with the more stringent “success” measure, treatment with 4 mg/kg PROG or 8 mg/kg PROG did not improve the “hit” rate compared to vehicle treated lesion rats (both $p>0.20$).

Skilled reaching: Success rate

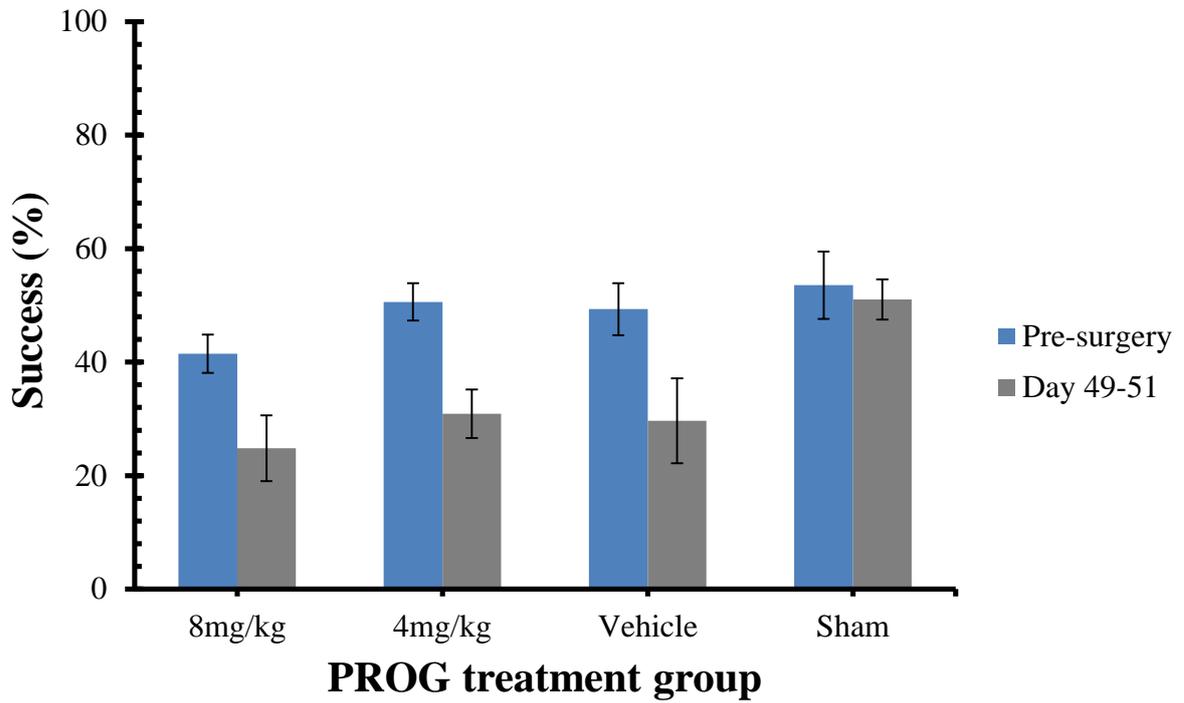


Figure 6.6. Success rate at post-surgery days 49-51 during single-pellet skilled reaching. Error bars denote SEM.

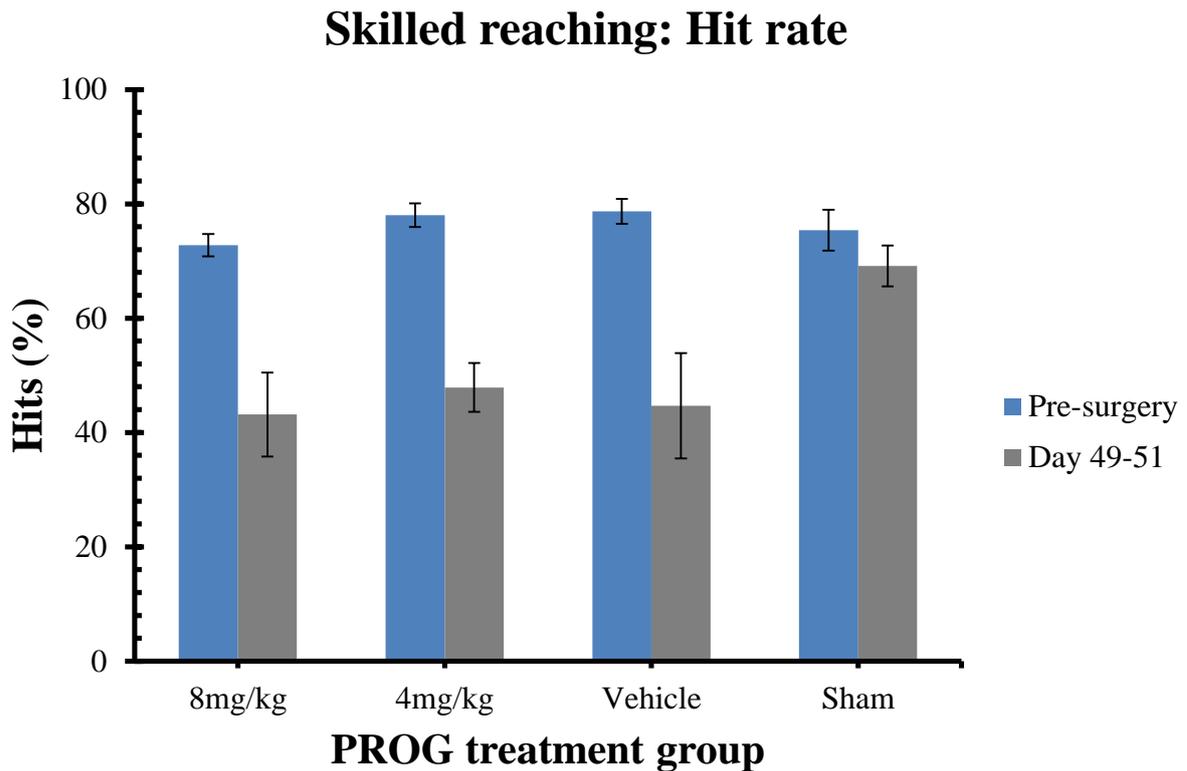


Figure 6.7. Hit rate at post-surgery days 49-51 during single-pellet skilled reaching. Error bars denote SEM.

6.4.4. Rotation after apomorphine

Lesions increased contralateral rotation after apomorphine compared to sham surgery rats ($F(3, 33) = 4.39, p = 0.01$; Fig. 6.8). Post-hoc Newman-Keuls showed that all lesion groups rotated more than sham (all $p < 0.05$) but 4 mg/kg PROG or 8 mg/kg PROG treatment did not significantly influence rotation (both $p > 0.20$). Neither the main effect of Time ($p > 0.20$) nor the Group x Time interaction ($p > 0.20$) were significant.

Rotation after apomorphine

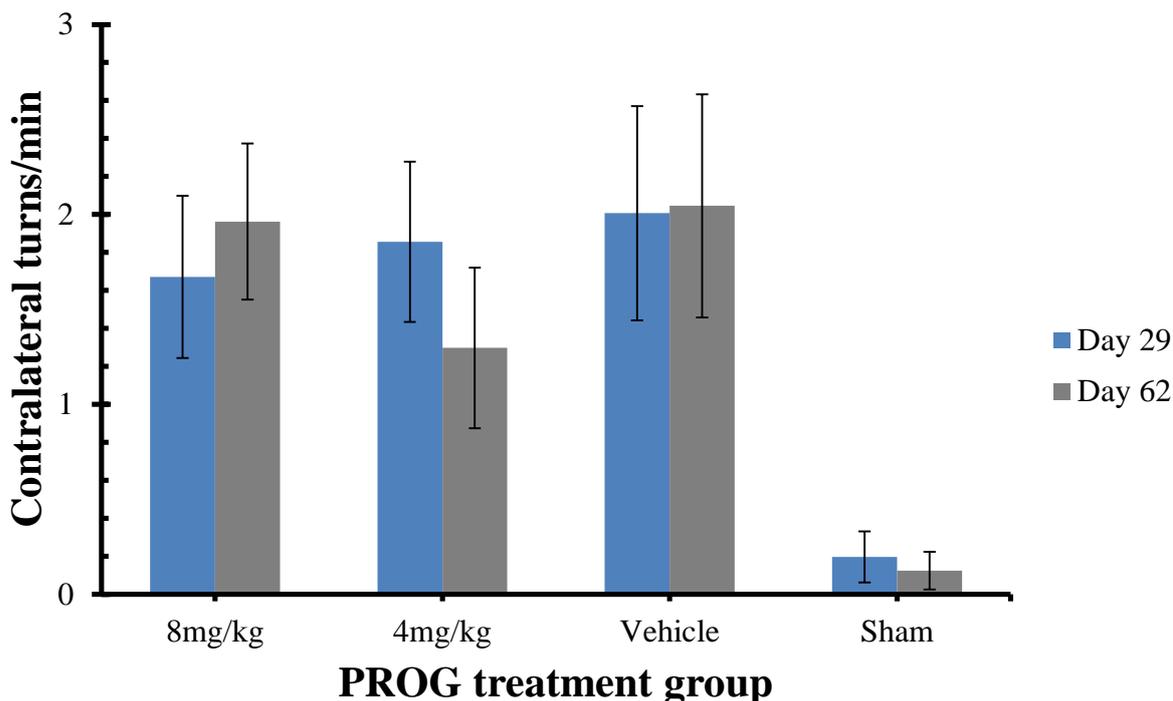


Figure 6.8. Mean turns/min after apomorphine. Error bars denote SEM. Note: Contralateral turns are expressed as positive values.

6.4.5. Tyrosine hydroxylase

6-OHDA lesions significantly reduced TH density in the striatum ($F(4, 39) = 48.27, p < 0.001$; Fig. 6.9). Post-hoc Newman-Keuls showed that all lesion groups had significantly lower striatal TH density compared to shams (all $p < 0.001$) and this effect was not changed by 4 mg/kg PROG or 8 mg/kg PROG treatment (all $p > 0.20$). The TH density of lesion control rats that were euthanized at day 8 (and thus had not received any treatment) did not differ from the 8 mg/kg PROG ($p = 0.14$), 4 mg/kg PROG ($p > 0.20$) or vehicle ($p > 0.20$) treated groups that were euthanized at day 64. One vehicle treated lesion rat was excluded from the analysis due to damage to the tissue sections that did not allow for reliable quantification.

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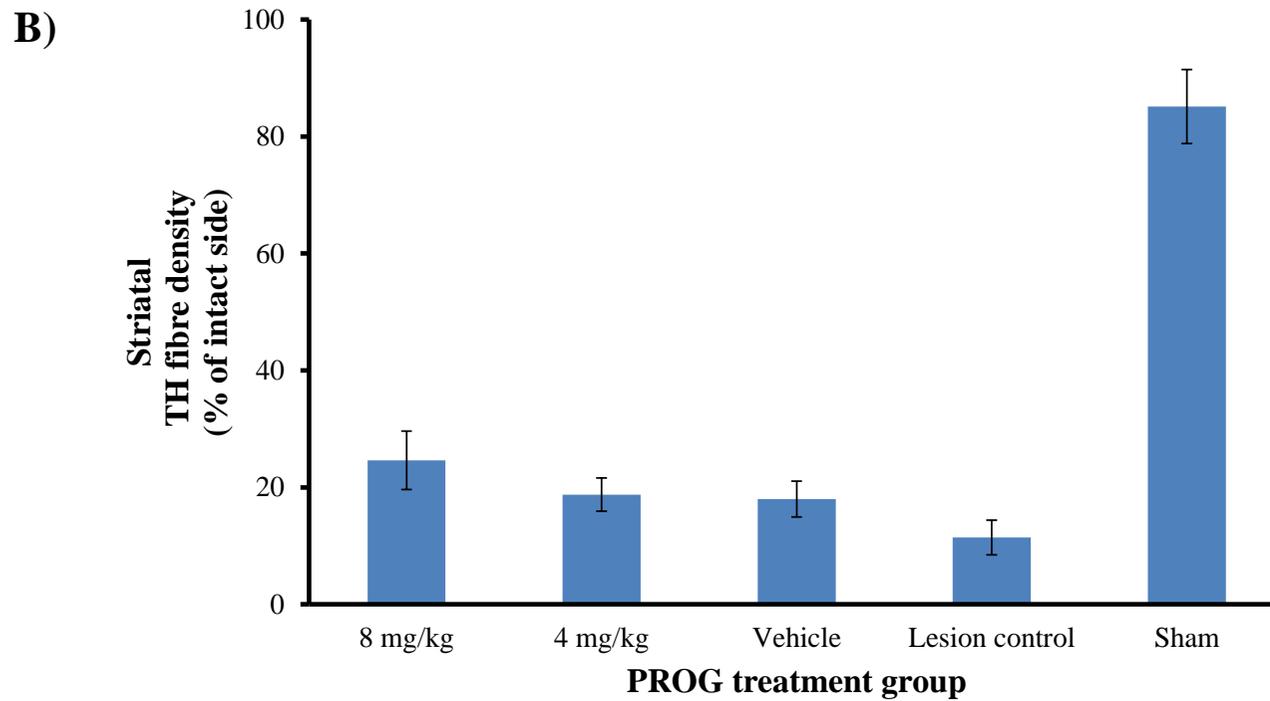


Figure 6.9. Striatal TH density at day 64 in all groups except lesion-only control (day 8).(A) Representative photomicrographs for a 8 mg/kg PROG, 4 mg/kg PROG, vehicle, lesion control, and sham rat (B) Figure showing % TH density. Scale bar represents 2mm.

6.5. Summary and discussion

Lesions produced substantial rotation after d-amphetamine and apomorphine, marked forelimb akinesia and skilled reaching impairments, and decreased TH density in the striatum. Both 4 mg/kg and 8 mg/kg PROG doses successfully reduced forelimb akinesia at all post-surgery testing occasions, but did not change skilled reaching, rotation after apomorphine, or TH density.

Tyrosine hydroxylase

The TH density of sham rats was 85% of the non-operated side. This was a little lower than expected and is likely due to one sham rat having a score of just 46% although this rat did not show impairment on any behavioural task. At day 8 the TH density in the striatum of lesion-only control rats was reduced to ~11%. At day 64 the TH density was similar for 8 mg/kg PROG (25%), 4 mg/kg PROG (18%), and vehicle (19%) groups. The 8 mg/kg PROG group tended to have less TH fibre reduction than the other lesion groups, but, this difference was not significant. There was a trend for the day 64 rats to have less reduction in TH density compared to lesion control rats although this difference was not significant for any group. Hence striatal TH fibre degeneration was probably maximal by day 8, and that PROG treatment was not able to induce sprouting and/or regeneration of TH positive fibres that could be detected seven weeks after PROG treatment had stopped. Here it should be noted that the rats in the lesion control group were ~7-8 months old on the day of perfusion while the remaining rats in the day 64 survival groups were 10-15 months old at the day of sacrifice. This could be a limitation of the current experiment as nigrostriatal levels of dopamine and its metabolites can change with ageing although significant changes are not usually observed until the rat is aged 18 months or older (Friedemann & Gerhardt, 1992; Girogi et al., 1987).

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These findings are consistent with previous reports that TH fibre degeneration is complete approximately one week after intrastratial 6-OHDA lesion surgery (Cicchetti et al., 2002; Rosenblad et al., 2000) and support the success of the lesion method used. Previous studies have shown that significant reduction in TH density has emerged at 6 hours (Walsh et al., 2011) and 24 hours (Blandini et al., 2007; Grealish et al., 2008) followed by a progressive decline in TH density that became maximal at one to two weeks after surgery. Similar to the current experiment, Kirik et al. (2001a) found substantial reduction in TH density at two weeks that had not declined further at 12 weeks after surgery.

Rotation after d-amphetamine

The success of the lesion was also shown by the substantial rotation at day 7 after d-amphetamine injections. All lesion groups rotated at similar levels that were not significantly different from each other. This confirmed that a functional impairment was similar across the 6-OHDA lesion groups as an ante-mortem validation of the 6-OHDA lesion prior to PROG for treatment groups and thus probable also for the lesion-only control rats which showed substantial reduction of striatal TH density at day 8 after surgery. The increased ipsilateral rotation observed at day 7 was comparable to previous reports as discussed in Chapter 5. For example, substantially increased rotation after d-amphetamine at a similar level to the current experiment has been observed when rats were tested at one or two weeks after surgery (Kirik et al., 1998, 2001a). That is, the reduction in striatal TH at day 8 and increased ipsilateral rotation after d-amphetamine at day 7 confirm that a PD like impairment was present at the time of the first PROG injection.

Adjusting steps test primary outcome measure: Akinesia

Lesions produced substantial contralateral forelimb akinesia whereas sham surgery had no effect. The forelimb akinesia impairment was evident at the start of testing at day 17 in all

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lesion groups and was maintained during subsequent testing. Impaired forelimb stepping in the vehicle treated lesion rats tended to worsen across subsequent tests although this change was not significant. The 4 mg/kg PROG and 8 mg/kg PROG treatment groups showed comparable performance to each other and were consistently impaired compared to shams at all post-surgery time-points. However, the PROG groups were substantially less impaired than vehicle treated rats with lesions at all post-surgery tests. The moderate forelimb akinesia impairment in the vehicle-treated lesion group was consistent with the previous studies discussed in study one (Chapter 5). For example, moderately impaired forelimb stepping at 2 to 8 weeks after surgery has been reported elsewhere (Dupre et al., 2008; Kirik et al., 1998)

In the present study PROG was administered on days 8 – 14 then stopped. Assessment of forelimb akinesia began at day 17. Both 4 mg/kg PROG and 8 mg/kg PROG groups experienced reduced forelimb akinesia compared to vehicle rats that was evident at day 17 and maintained at subsequent testing at days 27, 39, and 53. This suggests that PROG treatment gave long-term reduction in akinesia impairments in the adjusting steps test. This improvement is not simply symptomatic relief as the akinesia improvement was still evident 6 weeks after PROG treatment had stopped. The mechanism of action underlying this sparing of akinesia impairment is not clear but it does not involve striatal TH density. While the benefit of PROG was relatively immediate and long-lasting it was only partial. It would be interesting to know whether continued or repeated treatment regimes of PROG would have a greater benefit on this measure.

Other studies have also shown that forelimb akinesia can be improved independent of striatal dopaminergic innervation. A previous report used a novel diketopiperazine administered daily for 14 days starting 2 weeks after a two-site 6-OHDA striatal lesion (Krishnamurthi et al., 2009). Krishnamurthi et al. (2009) found long-lasting reduction in forelimb akinesia at 3 – 8 weeks after the treatment had been completed that was independent

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of striatal and substantia nigra TH. This novel diketopiperazine was also beneficial on forelimb stepping after central administration to the lateral ventricles starting 2 hours after lesion surgery. In another study, both voluntary and forced running wheel exercise started two weeks prior to partial 6-OHDA striatal lesions and maintained for four weeks after surgery improved forelimb akinesia recovery without sparing striatal dopamine transporters or substantia nigra TH positive cell bodies (O'Dell et al., 2007).

Single-pellet skilled reaching

The reduction in akinesia impairment found by PROG treated lesion rats did not extend to skilled reaching accuracy irrespective of the dose used. 6-OHDA lesions significantly reduced the “hit” and “success” rates at days 49-51 in the single-pellet skilled reaching task. Sham surgery did not impair either measure.

In the current experiment impaired skilled reaching accuracy was confirmed at week 7 after 6-OHDA lesion surgery. This finding is consistent with previous reports employing the single-pellet skilled reaching task in the four-site intrastratial model where impaired skilled reaching accuracy was observed at week 6 after surgery (Plowman et al., 2013, 2014).

Unfortunately, a procedural error meant that the test at 2-3 weeks post-lesion could not be assessed. In comparison, the Montoya staircase test is another test of skilled paw reaching that studies have used following unilateral 6-OHDA lesions (Montoya et al., 1991). Impaired skilled reaching accuracy has been observed on the staircase test at 2, 6, and 12 weeks after four-site 6-OHDA lesions (Kirik et al., 1998, 2001a, 2001b). However, Kirik et al. (2001b) found that only rats with severe TH degeneration were impaired.

The inability of PROG treatment to improve skilled reaching accuracy is similar to previous studies where therapeutic interventions have not been effective and therefore this symptom represents a difficult impairment to reverse. Skilled reaching accuracy was not

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improved by L-DOPA on a pasta matrix reaching test (Metz et al., 2001) or ethanol on a single-pellet reaching task (Metz et al., 2003) following MFB lesions. Skilled reaching was also not improved on the staircase test after post-lesion infusions of glial cell-line derived neurotrophic factor (GDNF; Kirik et al., 2001a) or grafts of fetal dopaminergic neurons both with or without continuous exposure to GDNF (Winkler et al., 2006) in the four-site model. Similarly, pre-lesion treatment with the neuroprotective agent parkin in a single-site striatal model did not improve skilled reaching (Vercammen et al., 2006). Therefore, protecting or improving dopamine transmission alone may not be sufficient to improve skilled reaching after dopamine depleting lesions. However, improved skilled reaching on the staircase test has been reported in the MFB model following post-surgery grafts of rat embryonic ventral mesencephalic tissue when the reward contingency of the reaching task was high (Corderio et al., 2010) and after pre-lesion treatment with a lentiviral vector expressing GDNF (Dowd et al., 2005b). Thus, varying the nature of the task and improving the brains resilience prior to injury may be important factors for skilled reaching.

Rats require training to develop the necessary skill to execute successful reaches in skilled reaching tasks (Klein et al., 2012). Following unilateral 6-OHDA lesions rats make compensatory adjustments to achieve successful reaches. These compensatory adjustments are largely learned and are different from the reaching technique learned prior to surgery (Metz et al., 2002). The motor skill learning required to make successful reaches is experienced based and thus several studies have found improved skilled reaching following experiential therapy. Six weeks of daily skilled reaching rehabilitation on a single-pellet task started 6 weeks after four-site striatal lesion surgery substantially improved reaching accuracy (Plowman et al., 2014). Similarly, post-surgery rehabilitation for several weeks in an easy tray reaching task also improved single-pellet skilled reaching performance after MFB lesions (Vergara-Aragon et al., 2003). Rats pre-trained on a single-pellet task then

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housed prior to MFB lesions in an enriched environment for six weeks with toys and various food items that required differing types of forepaw manipulation had improved skilled reaching accuracy after several weeks of post-lesion testing (Jadavji et al., 2006).

In clinical PD skilled reaching is not improved by L-DOPA medications (Melvin et al., 2005; Sacrey et al., 2011), accompanying preferred musical pieces that improve other aspects of motor function (Sacrey et al., 2009, 2011), or pallidal deep brain stimulation (Melvin et al., 2005). These clinical findings together with skilled reaching impairments observed in the 6-OHDA lesion model show that few therapies are able to improve skilled reaching accuracy. The most effective therapies for skilled reaching in the 6-OHDA lesion model involve either motor skill training or treatment prior to lesion surgery.

6-OHDA lesions of the nigrostriatal pathway may induce skilled reaching deficit due to dysfunction of the motor cortex area responsible for forelimb motor function. For example, reduction in the size of intracortical microstimulation forelimb representations in the motor cortex ipsilateral to lesion can be a consequence of unilateral 6-OHDA lesions (Plowman et al., 2014; Viaro et al., 2011). However, others have reported preserved forelimb representations following unilateral 6-OHDA lesions (Brown et al., 2009; Metz et al., 2004). This inconsistency could be explained by the use of desipramine to protect noradrenergic fibres in the Metz et al. (2004) study and the mild dopaminergic denervation observed after two striatal infusions in the Brown et al. (2009) study. Due to the substantial striatal TH lesions in the current experiment it is possible that motor cortex forelimb representations were reduced although this was not measured.

Unilateral MFB 6-OHDA lesions cause greater dysfunction of the rostral than the caudal region of the motor cortex (Viaro et al., 2011). This explains, in part, why lesions impair skilled reaching accuracy as the complex movements involved in reach-to-eat tasks

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are represented in the rostral forelimb area (Viaro et al., 2011). The onset of motor cortex dysfunction occurs within a few days after MFB lesions (Viaro et al., 2011) and has been observed several months after four-site striatal lesions (Plowman et al., 2014). This suggests that cortex dysfunction after striatal dopamine depleting lesions is rapid and enduring.

Synaptic dysfunction in the frontal cortex is thought to contribute to skilled reaching impairment as motormap topography of forelimb movement representations is highly sensitive to the number and strength of synapses in this region (Plowman et al., 2014). Indeed, reductions in synaptophysin and synapse numbers have been reported within the frontal cortex ipsilateral to 6-OHDA infusions to the SNpc (Hou et al., 2010). Skilled reaching training produces synaptogenesis and enhances synaptic strength (see Adkins et al., 2006 for a review) which may account for the beneficial influence of experiential therapy on skilled reaching accuracy. PROG treatment on the other hand can promote synaptogenesis in the CA1 region of the hippocampus after global ischemia in rats (Zhao et al., 2011). However, to the best of our knowledge it is not known if PROG can induce synaptogenesis in the frontal cortex after experimental injury.

Delaying PROG treatment until day 8 after surgery possibly accounts for why PROG was unable to improve skilled reaching accuracy in the current experiment. As reported by Viaro et al. (2011) the onset of motor cortex dysfunction is rapid and progressive reaching maximum at about two weeks after MFB lesions. Therefore, PROG was unable to prevent skilled reaching impairment as it is likely that motor cortex dysfunction was reaching its peak at the time of the first PROG injection.

Although PROG treatment did not improve the endpoint measures of skilled reaching it is not known if the qualitative aspects of skilled reaching were changed. The behaviour during single-pellet skilled reaching can be subdivided into a number of qualitative

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movement elements. In rats with 6-OHDA lesions the qualitative aspects of a reach are impaired even when a successful reach is made (Klein et al., 2012). Qualitative analyses can distinguish compensatory reaching behaviour from the original reaching behaviour (Alaverdashvili and Whishaw, 2013). The examination of reach quality requires high speed video capture which was not available in the current experiment. Future studies examining PROG's influence on skilled reaching should include a qualitative analysis of the movement elements. A qualitative analysis would elucidate PROG's ability to preserve normal reach function from compensatory reach behaviour for successful reaches. Preserved normal reach function is clinically significant as compensatory behaviour can limit range of motion and produce joint stiffness and pain in the forelimbs (Alaverdashvili and Whishaw, 2013).

Rotation after apomorphine

Lesions produced increased contralateral rotation after apomorphine at day 29 that was maintained when tested again at day 62 indicating that 6-OHDA had produced supersensitivity in the lesioned striatum. Sham surgery did not produce a rotation bias at either testing occasion. PROG treatment did not reverse apomorphine induced rotation at either testing occasion. At day 62 there was a trend for the 4 mg/kg PROG group to rotate less than their rotation at day 29 and less than other lesion groups although these differences were not significant. However, this decreasing rotation among 4 mg/kg PROG rats resulted from two rats that had a clear contralateral rotation bias at day 29 but no bias at day 62. As discussed in study one (Chapter 5) the increased contralateral rotation observed at days 29 and 62 is consistent with previous findings that have tested rotation after apomorphine at similar days after surgery (e.g. Kirik et al., 1998, 2001a).

The inability of PROG to reduce contralateral rotation after apomorphine is readily explained by the TH density finding. As discussed above, TH density in the striatum was

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substantial at day 8 for lesion control rats and did not change for PROG and vehicle treated lesion rats at day 64. Supersensitivity occurs after substantial loss of TH fibres and thus apomorphine administration favours stimulation of the lesion side to produce contralateral rotation (Duty and Jenner, 2011). PROG treatment could not reduce rotation after apomorphine as the striatal TH fibre degeneration was already complete at the time of the first PROG injection.

Summary

The present experiment reported the first study in this field regarding PROG's beneficial influence on akinesia, skilled reaching, and rotation after apomorphine in the unilateral 6-OHDA striatal lesion model of PD. We found that 7 days of 4 mg/kg PROG and 8 mg/kg PROG treatment, with tapered withdrawal, started at day 8 after surgery was able to reduce by roughly half the lesion induced akinesia in the primary outcome measure adjusting steps task in both short and long-term after striatal 6-OHDA lesion surgery. This functional improvement was not merely symptomatic relief as the beneficial influence of PROG was observed up to 6 weeks after treatment had stopped. PROG, however, did not improve skilled reaching accuracy in the single-pellet reaching task or rotation after apomorphine. PROG, therefore, was beneficial for relatively simple but not complex or drug induced motor impairments. By day 8 after surgery we observed substantial striatal TH fibre deafferentation in the lesion control rats. At day 64 striatal TH density was not significantly different between the 4 mg/kg PROG, 8 mg/kg PROG or vehicle groups nor were these groups different from the lesion-only control rats sacrificed at day 8 post-surgery. This suggests that striatal TH fibre deafferentation, which was maximal by day 8, was not changed by PROG treatment. Therefore, the beneficial influence of PROG regarding forelimb akinesia was independent of striatal TH fibre innervation.

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The current experiment examined PROG's influence following 6-OHDA lesions that modelled the late stages of PD degeneration. However, it is not known if PROG would also be beneficial following 6-OHDA lesions that model the earlier stages of PD degeneration. Furthermore, the assessment of skilled reaching in the current experiment was time consuming and precluded testing of additional motor impairments. Therefore, it is not known if the beneficial influence of PROG would extend to additional measures of motor impairments. To answer these questions the following experiment (Chapter 7) examines PROG's influence on a battery of motor skills tests following 6-OHDA lesions that modelled the early and late stages of PD degeneration.

Chapter 7

Study Three: Progesterone after Small and Large Intrastriatal 6-OHDA lesions

7.1. Objectives

This chapter reports the third of three experiments (see also: Chapters 5 and 6) and compared the effects of PROG treatment after small and large unilateral 6-OHDA lesions of the striatum. The small and large lesions modelled the early and late stages of SN-striatal PD degeneration. As before, the success of the lesion was confirmed at day 7 with a d-amphetamine rotation test, and PROG injections (8 mg/kg or sesame oil vehicle) made on day 8 once daily for 7 days with tapered withdrawal by sequential halving on the sixth and seventh days of treatment. Outcomes were assessed using a battery of motor skill tests on which the rats had been familiarized pre-operatively: the adjusting steps test for akinesia, postural instability test, whisker evoked forelimb placing test for sensorimotor integration, cylinder test for forelimb use asymmetry, and the corridor test for sensory neglect. Rats were tested for: akinesia on days 17-19, 27-29, 39-41, and 56-58; postural instability at days 20, 42, and 59; sensorimotor integration at days 17, 27, 39, and 56; forelimb use asymmetry at days 20, 42, 59; sensory neglect on days 22-23, 44-45, and 61-62; in addition, rotation after apomorphine was tested at day 66, just prior to sacrifice at day 68. Cylinder testing at day 42 was conducted by Meisha Nicolson (Honours project student), under the supervision of the author and primary supervisor. Brains have been stored for histology at a later date due to time constraints. This chapter begins with a brief rationale for examining PROG's influence following small and large lesions and a justification for the motor skill tests used.

7.2. Introduction

In Chapter 6 we found that both 8 mg/kg and 4 mg/kg PROG treatment regimes gave long lasting reductions in akinesia impairments (adjusting steps) following large unilateral striatal 6-OHDA lesions that modelled the later stages of PD degeneration. In the current experiment we examined whether PROG treatment would also be beneficial after small lesions that model the early stages of PD degeneration. To examine PROG's influence during the early stages of PD degeneration we used the two site unilateral striatal 6-OHDA model (small lesions) as described by Decressac et al. (2012). In practice PROG's influence at the early stages of PD degeneration could be examined by administering PROG within 24 hours after large lesion surgery. As discussed in Chapter 6, this method is not preferred when assessing neuroprotective agents after 6-OHDA lesions. .

The PROG dose used in the current experiment was 8 mg/kg. We found no significant difference between 8 mg/kg and 4 mg/kg in study two (see Chapter 6) although 8 mg/kg tended to be more beneficial in the adjusting steps test. According to the PROG studies reviewed in Chapter 3 (see table 3.1) 8 mg/kg PROG was the most commonly used dose (14 studies) while fewer studies had used 4 mg/kg (7 studies) thus supporting the use of 8 mg/kg in the current experiment. As previously, PROG was administered once daily for seven days with tapered withdrawal by sequential halving on the sixth and seventh days of treatment. Sham rats also received PROG to determine its effects in the absence of a 6-OHDA lesion.

As study two (see Chapter 6) showed that PROG treatment was beneficial for forelimb akinesia in the adjusting steps test, but not skilled reaching or rotation after apomorphine, in the current experiment we examined whether the beneficial effects of PROG on forelimb akinesia would extend to benefits on measures of postural instability, forelimb use asymmetry, sensorimotor integration, and sensory neglect.

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Postural instability is a common impairment in PD patients that contributes to the danger of frequent falls, which become increasingly problematic as the disease progresses (Jessop et al., 2006). The postural instability test following 6-OHDA lesions in rats followed that described by Woodlee et al. (2008). This test used in rats is similar to the “pull” or “push” test used in clinical exams of postural instability where the neurologist gently pushes or pulls a standing patient by the shoulders. The change in the centre of gravity requires the patient to make a catch-up step to maintain their balance. Patients with PD often present as having their feet “glued to the ground” and cannot make the needed catch-up step requiring the neurologist to catch them to prevent a fall (Woodlee et al., 2008). In the rat’s test, one forelimb is gently moved forward over a rough surface until a catch-up step is made. Woodlee et al. (2008) found that the contralateral forelimb of rats with unilateral 6-OHDA lesions of the MFB required further displacement to trigger a catch-up step compared to sham rats. They also found that the ipsilateral forelimb of lesion rats had enhanced compensatory function requiring less displacement to trigger a catch-up step. To date no study has examined postural instability in this test following small or large unilateral 6-OHDA lesions of the striatum.

Following unilateral lesions of the nigrostriatal pathway rats develop a behavioural asymmetry where they preferentially use the ipsilateral (“good”) forelimb to make wall contacts during vertical exploration of a cylinder (Schallert et al., 2000). To examine this forelimb use asymmetry we used the cylinder test, also described by Woodlee et al. (2008). The cylinder apparatus is preferred over walls in an open field or the rat’s home cage as it is more likely to encourage rearing and exploration with the forepaws. Increased use of the ipsilateral forelimb in the cylinder test has been found following unilateral 6-OHDA lesions of the MFB, (Schallert et al., 2000; Shin et al., 2012; Woodlee et al., 2008) and both large and small 6-OHDA lesions of the striatum (Carlsson et al., 2009; Shin et al., 2014). The

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cylinder test is a clinically relevant test because L-DOPA and bromocriptine given at therapeutic doses reduce forelimb use asymmetry after MFB lesions (Lundblad et al., 2002). Furthermore, decreased forelimb use asymmetry has been found following midbrain dopaminergic grafts in rats with large striatal (Grealish et al., 2010) and MFB 6-OHDA lesions (Torres et al., 2008). The sensitivity of the cylinder test to therapeutic interventions supports its use in the current PROG treatment experiment.

Deficits in sensorimotor integration also occur in PD patients (Abbruzzese and Berardelli, 2003). This problem can be mimicked in rats by holding them aloft whereupon they instinctively attempt to place their forelimbs upon nearby surfaces they sense (Woodlee et al., 2005). Placing can be triggered by stimulation of the vibrissae on the same or contralateral side of the forelimb being tested (Meredith and Kang, 2006). Rats with unilateral 6-OHDA lesions place the impaired paw less often than intact rats in response to vibrissae stimulation suggesting that motor responses following sensory stimulation require intact dopaminergic innervation of the dorsal striatum (Meredith and Kang, 2006). This reduction in whisker evoked forelimb placement indicates sensorimotor integration impairment. To examine sensorimotor integration impairment following striatal 6-OHDA lesions and the influence of PROG treatment we used the whisker evoked forelimb placement test described by Woodlee et al. (2005). Impaired forelimb placements following vibrissae stimulation have been observed after MFB (Woodlee et al., 2005, 2008; Schallert et al., 2000) and large striatal 6-OHDA lesions (Grealish et al., 2008).

Rats with unilateral 6-OHDA lesions also demonstrate sensory neglect where they fail to orient to food items presented on the contralateral side of their body (Dowd et al., 2005a). We used the corridor test described by Dowd et al., (2005a) to examine sensory neglect after small and large lesions and the potential influence of PROG. In their study, Dowd et al. (2005a) placed rats with either MFB or large striatal 6-OHDA lesions in a long narrow

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corridor lined with pairs of pots containing sugar pellets and then allowed the rats to freely explore and retrieve pellets. Rats with both lesion types neglected pellets from the contralateral side of their body, instead selectively retrieving pellets from the ipsilateral side. This sensory neglect test is sensitive to neuroprotective, neuroreparative, and symptomatic treatments. For example, ventral mesencephalic cell suspension transplants reduced this side bias for rats with large striatal but not MFB 6-OHDA lesions (Dowd et al., 2005a). Further research by Dowd et al. (2005b) showed that rats with MFB lesions did not develop a side retrieval bias when treated with a lentiviral vector expressing glial cell-line derived neurotrophic factor prior to lesion surgery. Fitzsimmons et al. (2006) found that systemic apomorphine administration significantly reduced the side bias among rats with large 6-OHDA lesions of the striatum.

In summary, this study examined the beneficial influence of PROG treatment after small and large 6-OHDA lesions of the striatum. As shown in Chapter 6, PROG gave long lasting reductions in forelimb akinesia after large lesions that modelled the later stages of PD degeneration. However, it is not known if PROG will reduce motor impairments after small lesions that model the early stages of degeneration in PD. Furthermore, it is not known if the beneficial effects found in Chapter 6 would extend to measures of postural instability, forelimb use asymmetry, sensorimotor integration, and sensory neglect. This experiment is original on two counts. First, it represents the first experiment to directly compare PROG treatment after small and large 6-OHDA lesions of the striatum. Secondly, this experiment provides the most comprehensive examination to date of PROG's influence on motor impairments after 6-OHDA lesions. We anticipated that PROG treatment would reduce motor impairments following both small and large unilateral 6-OHDA lesions of the striatum.

7.3. Materials and Methods

7.3.1. Animals

Subjects were 62 male Wistar rats weighing between 528g and 760g at the day of surgery. The rats were 6-12 months old at surgery and 9-14 months old at perfusion. Rats were housed individually for five days of recovery then housed in groups of 3-4 for the remainder of the experiment. Testing took place during the dark phase of the rats reversed light cycle (lights on at 2000hrs). All protocols in this study followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee University of Canterbury. One rat did not recover from the surgery and was euthanized. Two rats were euthanized several weeks after surgery after developing growths under the skin.

7.3.2. Design

The experiment used a 3 (lesion size) x 2 (PROG treatment) factorial design with the experimenter blind to the PROG treatment group of the lesion and sham rats until all analyses had been conducted. The sequence of surgery and testing is shown in Figure 7.1.

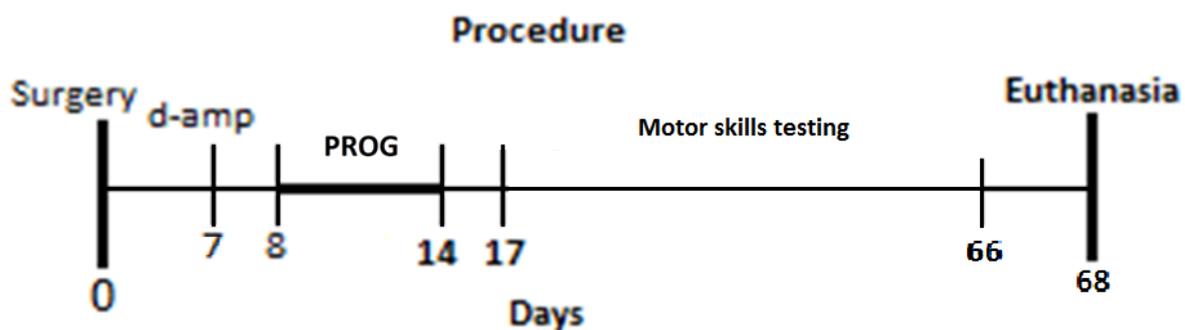


Figure 7.1. Experimental design. Rats received either small or large unilateral lesions with 6-OHDA targeting the striatum or sham surgery. Rotation after d-amphetamine was measured at day 7. Progesterone was given once daily on days 8-14. Motor skills were tested between days 17 and 66. The motor skills testing period included the adjusting steps, postural instability, whisker, cylinder, corridor, and rotation after apomorphine tests. All rats were euthanized at day 68. Note: d-amp, d-amphetamine rotation test; PROG, progesterone.

7.3.3. Surgery

The same general surgical procedure as described in Chapter 5 was used except rats were operated on the side contralateral to their pre-surgery retrieval bias in the corridor test. Rats received either two (small lesions) or four (large lesions) unilateral infusions of 6-OHDA to the dorsal striatum. The infusion co-ordinates for the large lesions were as described in Chapter 5. The following co-ordinates for the small lesions were used, as described by Decressac et al. (2012): AP 1.2mm, ML \pm 2.5mm; AP 0.2mm, ML \pm 3.8mm (from bregma) and DV -5.0mm from dura (Fig. 7.2). Sham rats received four infusions of the vehicle solution alone to exclude the surgical effects in the presumably more severe model.

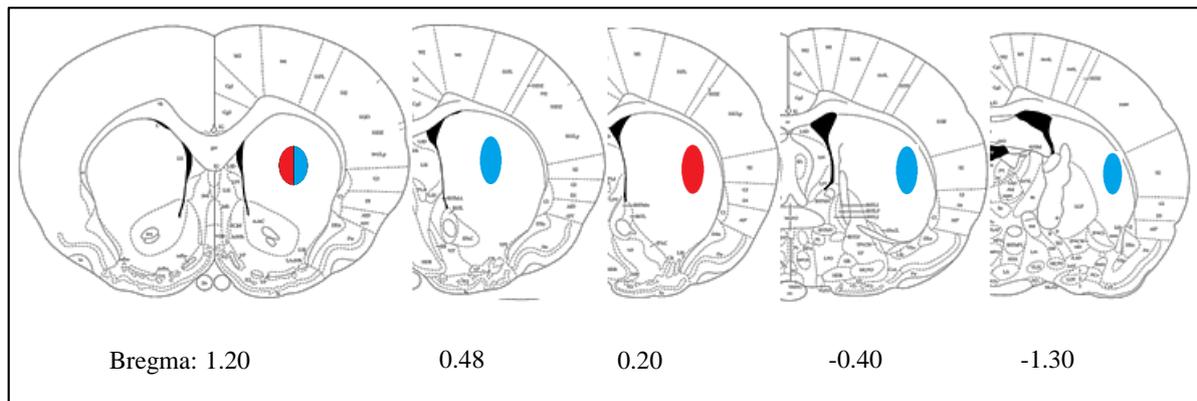


Figure 7.2. Target infusion sites for 6-OHDA lesion surgery. Rats received either two (small lesions, red ovals) or four (large lesions, blue ovals) unilateral infusions to the dorsal striatum. Sham rats received four infusions of the vehicle alone (plates adapted from Paxinos and Watson, 1998).

7.3.4. Rotation after d-amphetamine and apomorphine

Rotation after d-amphetamine was assessed at day 7 and apomorphine at day 66 using the same method as described in Chapter 5. In the d-amphetamine test, rats with large lesions were required to rotate at >6 turns/min to be included in the study. The small striatal lesions were the same as described by Decressac et al. (2012) who did not find significant rotation

Study Three: Progesterone after Small and Large Intrastriatal 6-OHDA lesions after d-amphetamine. Therefore, all rats with small lesions were included in the study irrespective of their degree of rotation bias when tested with d-amphetamine at day 7.

7.3.5. Progesterone treatment

Eight days after surgery the small lesion, large lesion, and sham rats were randomly assigned to receive either 8 mg/kg PROG or sesame oil vehicle once daily for seven days. The same treatment protocol as described in Chapter 6 was used with tapered withdrawal by sequential halving on the sixth and seventh day of treatment. There were $n = 11$ rats with small, and $n = 11 - 12$ rats with large lesions, and $n = 7$ sham rats assigned to PROG and oil vehicle treatment groups.

7.3.6. Adjusting steps: Akinesia

Forelimb akinesia was assessed pre-surgery and at post-surgery days 17-19, 27-29, 39-41, and 56-58 using the same method as described in Chapter 5.

7.3.7. Postural instability test

The postural instability test was conducted pre-surgery and at post-surgery days 20, 42, and 59. The apparatus consisted of rough textured grip tape (Safety-Walk, 3M brand) alongside a ruler (Fig. 7.3). Rats were held with the same grip as the adjusting steps test except the free forepaw was placed weight bearing on the grip tape. The purpose of the rough surface was to induce stepping rather than dragging or bracing in response to an imposed shift of the rat's weight. The tip of the rat's nose was aligned with the zero mark on the ruler. The rat's centre of gravity was gently moved forward over the unrestrained weight bearing forelimb until a "catch-up" step to regain its centre of gravity was made. The new position of the nose tip indicated the displacement required to trigger a catch-up step. The average displacement (cm) of three trials for each forelimb was measured at each testing day. Lesions are expected to increase displacement of the contralateral forelimb and decrease displacement of the

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ipsilateral forelimb (Woodlee et al., 2008). Larger rats may require a greater shift in their centre of gravity to trigger a catch-up step. Rats were weighed on each day of testing to ensure body weights did not differ between groups.



Figure 7.3. The postural instability test. (A) The apparatus used in this experiment. The grey area is the bench top and the orange area is the grip tape used for the test. (B) An example of a rat at the start of a trial before being moved forward and (C) the rat after making a catch-up step (images B and C adapted from Woodlee et al., 2008).

7.3.8. Whisker-evoked forelimb placement: Sensorimotor integration

Sensorimotor integration was evaluated in the forelimb placement test pre-surgery and at days 17, 27, 39, and 56. In the “same-side” test rats were held with the same grip as the adjusting steps test except the forelimb to be tested hung free. The rat’s vibrissae ipsilateral to the tested paw were brushed against the side of the table to elicit a forelimb placing response (Fig. 7.4-A). In the “cross-midline” test the rat was held with same grip as above except it was turned sideways so the vibrissae to be brushed were perpendicular to the table surface. The downwardly turned forelimb was restrained and the downwardly turned vibrissae were brushed against the table surface to evoke a forelimb placement contralateral to the stimulated vibrissae (Fig. 7.4-B). Both the same-side, and cross-midline, tests were repeated 10 times for each forelimb at each testing occasion. Rats received the “same-side” test first then were given a 15min break before receiving the “cross-midline” test. The number of placements for each forelimb on each test was counted. Only trials in which the rat was compliant and did not struggle were counted. Struggling during testing was rare as each rat

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was habituated to the procedure over three sessions prior to pre-surgery testing. Lesions will reduce contralateral to lesion forelimb placements but not impair ipsilateral forelimb placements. Sham rats will place both forelimbs on ~100% of trials.

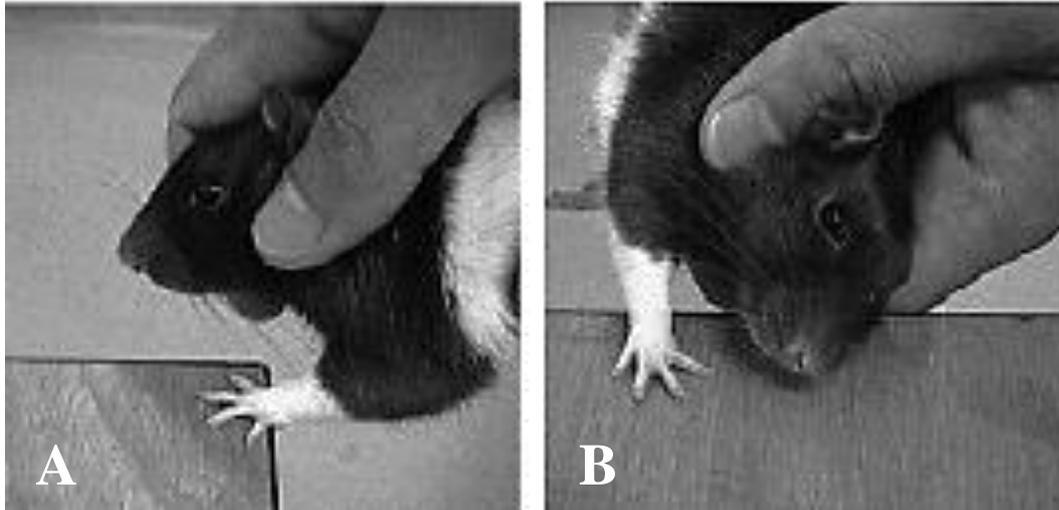


Figure 7.4. Examples of a rat performing (A) the same-side and (B) cross-midline whisker evoked forelimb placing test (image adapted from Woodlee et al., 2005. Note: Hooded rats were not used in the current study).

7.3.9. Cylinder test: Forelimb use asymmetry

Forelimb use asymmetry during vertical exploration was analysed in the cylinder test pre-surgery and at day 42. The apparatus consisted of a flexible plastic sheet shaped to a cylinder (29cm diameter x 30cm high) that was flanked by mirrors to assist viewing (Fig. 7.5). Rats were placed in the cylinder and the number of weight bearing wall contacts made during rearing using the ipsilateral, contralateral, and both forepaws simultaneously was video recorded (Panasonic digital video camera recorder; Model SDR-H85). Forelimb use asymmetry was calculated as the number of ipsilateral forepaw touches plus $\frac{1}{2}$ the number of both paw touches, divided by the total number of touches (total = ipsilateral + contralateral + both). This provided an asymmetry percentage score where 50% indicated a rat that explored symmetrically with both forelimbs. Higher scores ($>50\%$) indicate greater reliance on the

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ipsilateral forelimb while lower scores (<50%) represent greater reliance on the contralateral forelimb. Rats were recorded for 20 touches of any paw or a maximum trial time of 5mins.

Also measured was the number of “serial-stepping” behaviours with each forelimb. Serial-stepping was defined as an independent forepaw touch followed by several rapid lateral weight-shifting touches with the same limb without using the other forelimb. A serial step was counted as long as the sequence of steps was continuous and not broken by pauses lasting longer than 1 second or the animal pushing back to an unsupported rearing position. When calculating the asymmetry score above, any serial steps were counted as individual paw touches. The serial-stepping events were analysed independently as the percentage of serial-stepping behaviour out of the total number of independent forelimb uses for each forelimb. The final measure was the number of rears made during the trial as an assessment of vertical exploratory activity.



Figure 7.5. The cylinder apparatus used to assess forelimb use asymmetry. The images depict a rat during per-surgery testing. (Left) An example of a right forepaw touch while the rat faces the camera and (Right) a right forepaw touch while the rat faces away from the camera visualised with the aid of the mirror.

7.3.10. Corridor test: Sensory neglect

Sensory neglect was measured in the corridor test. Rats were tested pre-surgery and on days 22-23, 44-45, and 61-62. The apparatus consisted of a long rectangular box (150cm long x 12cm wide x 28cm deep) made from white painted medium density fibreboard (Fig. 7.6). Ten adjacent pairs of small white plastic lids (2cm diameter x 0.9cm deep) were positioned along the floor of the corridor and were held in place with Blu-tack. The lid pairs were spaced at 12cm intervals with the first pair placed 30cm from the starting end of the corridor. Each lid was filled with approximately 10 chocolate pellets (45mg Dustless precision, Bioserve).

Prior to the habituation and testing day rats were food restricted for 48hrs by once a day ration of 18g food per rat. Rats were returned to free-feeding in between testing occasions. On the two days prior to habituation rats were introduced to the chocolate pellets. Each rat received 20 pellets in their home cage 1hr before daily feeding. Rats were habituated to the corridor for 10mins on the two days prior to pre-surgery testing where chocolate pellets were scattered along the floor and the rat was free to explore. For testing the rat was first placed in an identical but empty corridor for 5mins habituation before being transferred to the start end of a corridor with food wells. The rat was free to explore and eat pellets at will and the number of 'retrievals' were counted. A retrieval was defined as the rat poking its nose into a lid regardless of the number of pellets eaten. A new retrieval could only be made when the rat visited a new lid. The number of retrievals made on the left and right side of the rat's body axis was counted. Trials were ended when 20 retrievals had been made or a maximum 5mins had elapsed. Rats were tested on two consecutive days and the scores of the two trials were added together. Scores were expressed as a percentage of contralateral to lesion retrievals of the total retrievals made from both sides of the rat's body axis. Intact rats generally display a side bias for retrievals. All rats were operated on the contralateral to

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“preferred side” to separate the effects of the lesions from the rat’s innate bias. Post-surgery, lesions will reduce retrievals from the contralateral side.

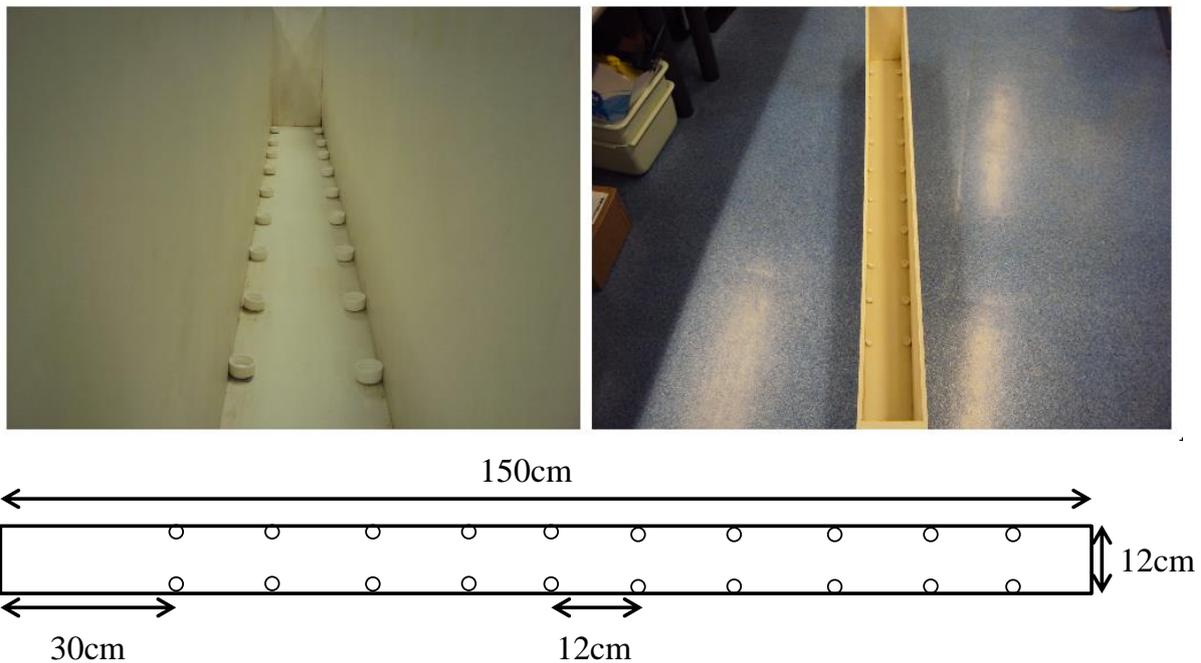


Figure 7.6. The corridor test apparatus. (Top left) View from inside and (Top right) above the corridor. (Bottom) Schematic diagram showing the dimensions of the corridor.

7.3.11. Perfusion

Rats were perfused on day 68 using the same procedure as described in Chapter 5. Due to time constraints no tyrosine hydroxylase histology was completed on these rats. The brains have been saved for histology at a later date.

7.3.12. Statistical analysis

Statistical analyses using Statistica software (version 12.0) were conducted to determine if the impairments in akinesia (adjusting steps test), postural instability (postural instability test), sensorimotor integration (whisker test), forelimb use asymmetry (cylinder test), sensory neglect (corridor test), and rotation after apomorphine produced by small and large striatal 6-OHDA lesions were reduced by PROG treatment. Individual outcome variables were analysed by an ANOVA (Lesion by Treatment) and the addition of the repeated measure of

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Time was included as appropriate. Pre-surgery performance on the adjusting steps, postural instability, whisker, and corridor tests were analysed separately from post-surgery testing. Pre and post-surgery performance in the cylinder test was analysed together as the sham rats forelimb use asymmetry appeared to change from pre to post-surgery testing. Rotation after d-amphetamine at post-surgery day 7 was analysed by an ANOVA (Lesion by Treatment). *Post-hoc* Newman-Keuls multiple comparisons tests were used for significant main effects and interactions. Statistical significance was set at $p < 0.05$. All data are expressed as means \pm SEM.

7.4. Results

7.4.1. Rotation after d-amphetamine

At post-surgery day 7 both lesion sizes produced significant ipsilateral rotations ($F(2, 53) = 65.47, p < 0.001$; Fig. 7.7) after d-amphetamine administration. Post-hoc Newman-Keuls showed that rats with a large lesion showed a rotation bias almost twice that of rats with small lesions, but rats with small lesions rotated more than sham rats which showed no rotation bias ($p < 0.001$ for all groups). No main effect of PROG or any interactions were significant (all $p > 0.20$).

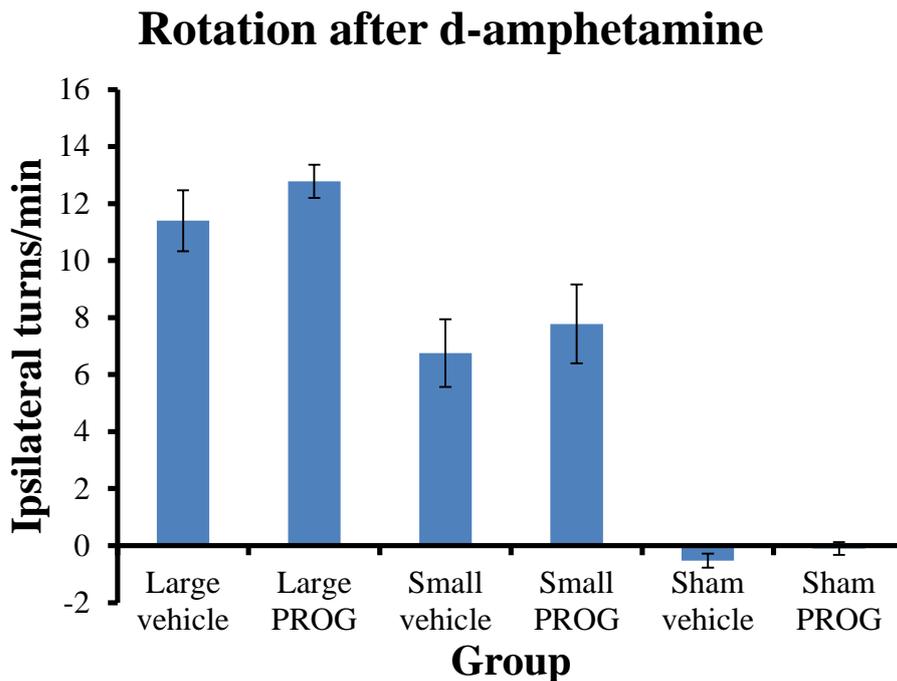


Figure 7.7. Ipsilateral rotations after d-amphetamine at day 7 in the six groups.

7.4.2. Adjusting steps test: Akinesia

Prior to surgery contralateral forelimb stepping did not differ significantly across the lesion groups (Lesion, $F(2, 53) = 2.45, p = 0.10$) in the adjusting steps test. However, there was a small but significant Treatment effect ($F(1, 53) = 6.43, p = 0.014$) where rats belonging to the future PROG group made marginally fewer forelimb steps ($M = 49.33\%$) compared to future vehicle rats ($M = 50.55\%$). The Lesion x Treatment interaction was not significant ($F(2, 53) = 1.92, p = 0.16$).

Following surgery large lesions produced substantially impaired contralateral forelimb stepping while small lesions produced an intermediate deficit (Lesion, $F(2, 53) = 51.94, p < 0.001$; Fig. 7.8). Notably, PROG significantly reduced this impairment for both lesion groups without affecting the sham lesion group (Lesion x Treatment, $F(2, 53) = 6.12, p = 0.004$). As a result, a mild and non-significant impairment remained evident in the rats with small lesions while the improvement after PROG in rats with large lesions meant that they

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now performed equivalently to vehicle treated rats with small lesions. There was also a Time x Lesion x Treatment interaction ($F(6,159) = 2.37, p=0.032$), due primarily to increasingly poor performance over time in the non-treated vehicle rats with large lesions.

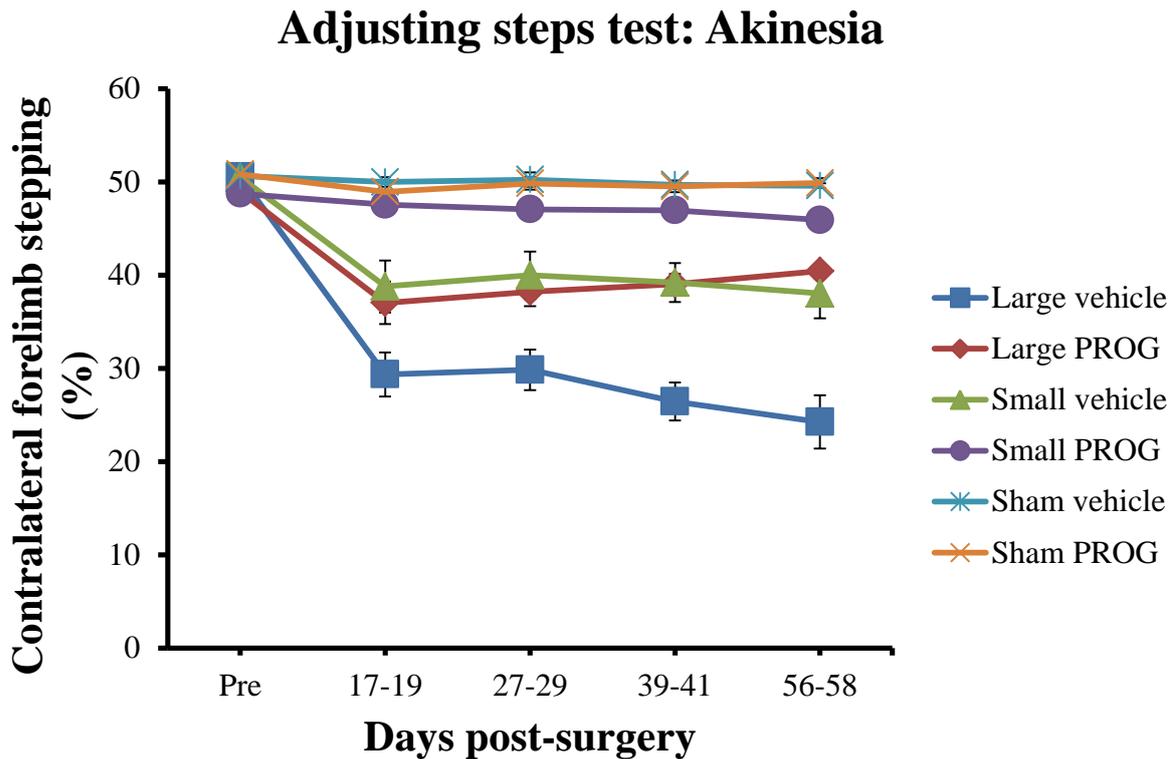


Figure 7.8. Contralateral forelimb stepping in the adjusting steps test. Error bars denote SEM.

7.4.3. Postural instability test

First, body weights are assessed as these may influence performance on this test. Prior to surgery the body weight of the rats did not differ between groups and neither the Lesion nor Treatment effects, nor the interaction, were significant (all $p>0.20$; Fig. 7.9). Body weights also did not differ between lesion or treatment groups following surgery (both $p>0.20$). There was a significant Time x Lesion interaction ($F(4, 106) = 3.02, p = 0.02$) due to relatively increasing body weight in all groups. Post-hoc Newman-Keuls showed that body weights did

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not differ between groups at any testing occasion (all $p>0.20$). No other interactions were significant (all $p>0.20$).

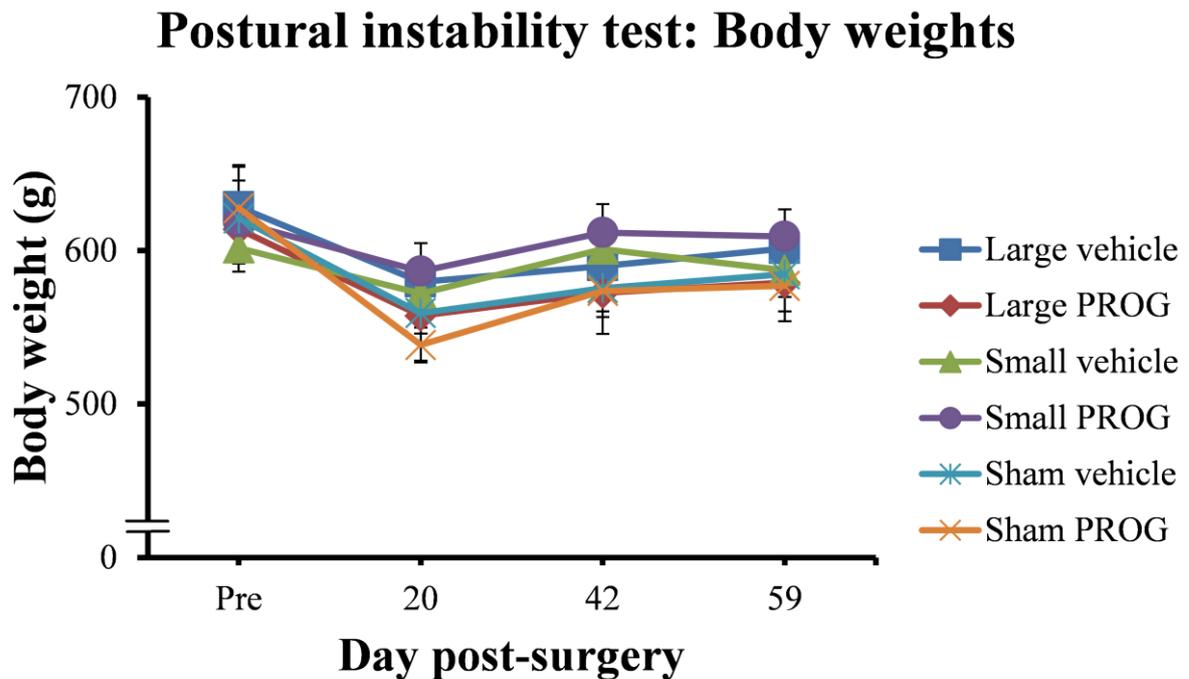


Figure 7.9. Body weights on each day of testing in the postural instability test. Error bars denote SEM.

Pre-operatively the displacement required to trigger a catch-up step in the contralateral forelimb did not differ significantly between lesion groups ($F(2, 53) = 2.69, p = 0.08$; Fig. 7.10). There was however a significant main effect of Treatment ($F(1, 53) = 4.57, p = 0.04$) where the future PROG rats ($M = 5.06\text{cm}$) required marginally more displacement than the future vehicle rats ($M = 4.71\text{cm}$) to trigger a catch-up step. The Lesion x Treatment interaction was not significant ($p>0.20$).

Following surgery, large lesions substantially increased the displacement required to trigger a catch up step in the contralateral forelimb while small lesions produced an

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intermediate impairment (Lesion $F(2, 53) = 25.52, p < 0.001$; Fig. 7.10). Relative to sham, the large lesion effect increased over time while the small lesion effect dissipated (Time x Lesion $F(4, 106) = 7.96, p < 0.001$). There were, however, no PROG treatment effects or Time x Lesion x Treatment interaction (both $p > 0.20$).

Prior to surgery the displacement required to trigger a catch-up step in the ipsilateral forelimb did not differ between groups. Neither the Lesion ($F(2, 53) = 1.77, p = 0.18$; Fig. 7.10), the Treatment main effect ($p > 0.20$) nor the interaction were significant ($p > 0.20$). Post-operatively, large lesions decreased the displacement required to trigger a catch up step in the ipsilateral forelimb while small lesions produced an intermediate impairment (Lesion, $F(2, 53) = 13.45, p < 0.001$; Fig 7.10). In this instance, PROG reduced this impairment for the large lesion group only (Lesion x Treatment $F(2, 53) = 5.32, p = 0.01$). The Time x Lesion x Treatment interaction was not significant ($p > 0.20$). There was a significant Time x Lesion interaction ($F(4, 106) = 3.52, p = 0.01$) due to increasing displacement for the sham group.

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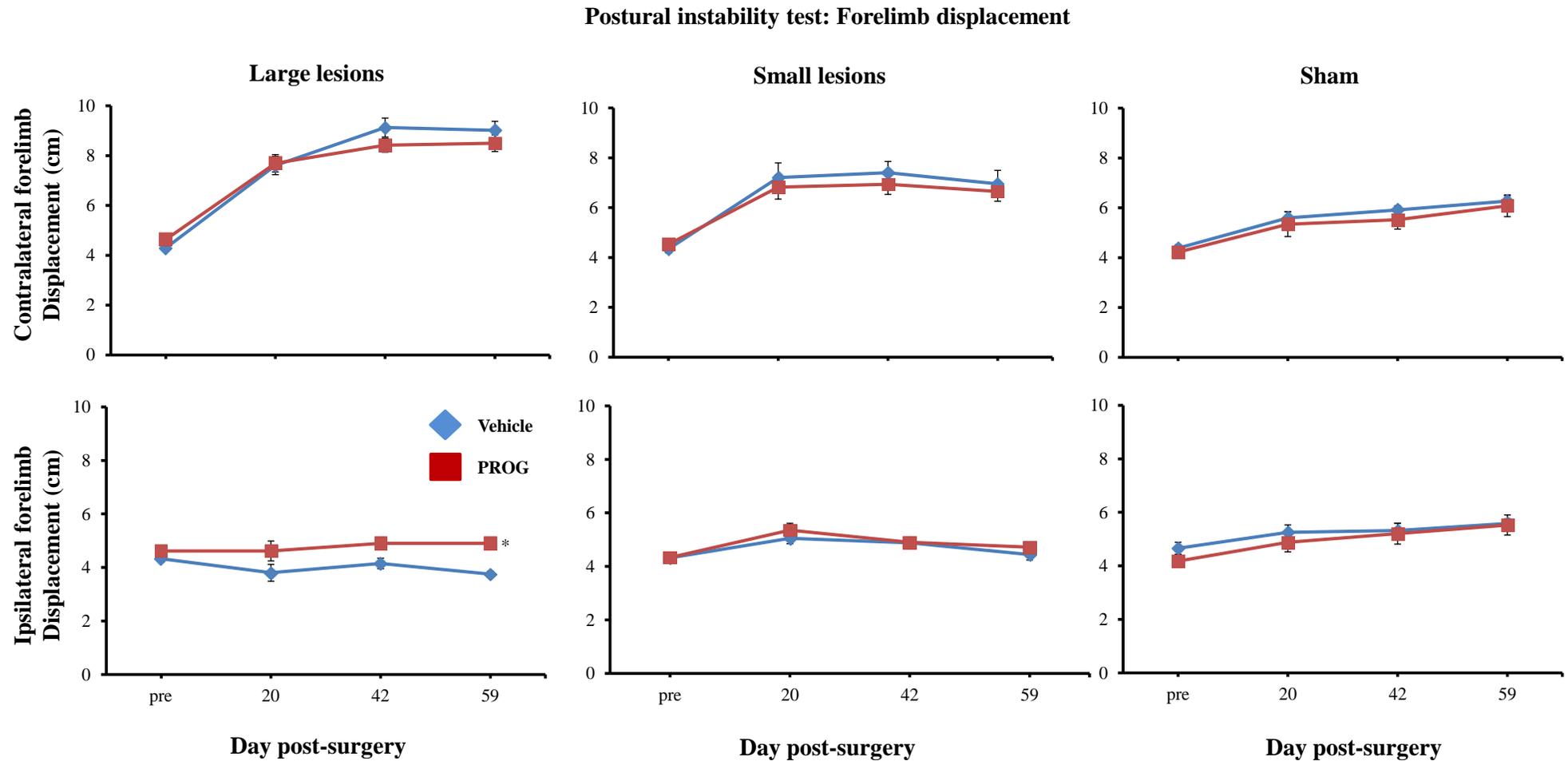


Figure 7.10. Forelimb displacement in the postural instability test. (Top row) Contralateral forelimb and (Bottom row) ipsilateral forelimb displacements. Error bars denote SEM. * $p < 0.05$ compared to ipsilateral forelimb of vehicle treated rats with large lesions.

7.4.4. Whisker-evoked forelimb placement: Sensorimotor integration

Prior to surgery in all rats, and post-surgery in sham rats, successful forelimb placements occurred at rates greater than 95% for both forelimbs regardless of the type of vibrissae stimulation (same-side or cross-midline). Following surgery, ipsilateral forelimb placements for rats with both large and small lesions occurred at rates close to 100%. The results for ipsilateral forelimb placements are not shown. The analysis of post-surgery data excluded shams as these groups showed no variation at several testing occasions due to having the maximum score of 100% for successful forelimb placements.

In the lesion groups same-side vibrissae stimulation produced substantially impaired contralateral forelimb placements after large lesions while small lesions produced an intermediate deficit (Lesion, $F(1,41) = 29.12, p < 0.001$; Fig. 7.11). PROG did not significantly reduce this impairment for rats with small or large lesions (Treatment, $F(1,41) = 1.93, p = .17$; Lesion x Treatment, $F(1, 41) = 0.36, p = 0.56$). There was a main effect of Time (Time, $F(3, 123) = 3.19, p = 0.03$) due to improving performance from day 27 to day 56 (Post-hoc Newman-Keuls, $p = 0.02$). There were no significant interactions for Treatment x Time, ($p > 0.20$); Lesion by Time ($p > 0.20$); or Treatment x Lesion x Time ($p < 0.20$). However, the rats with small lesions and treated with PROG produced performance that was similar to the Sham groups by day 56 ($t(23) = 1.55, p = 0.14$) whereas the group with small lesions given vehicle remained impaired compared to the Sham groups at day 56 ($t(23) = 3.12, p = 0.005$).

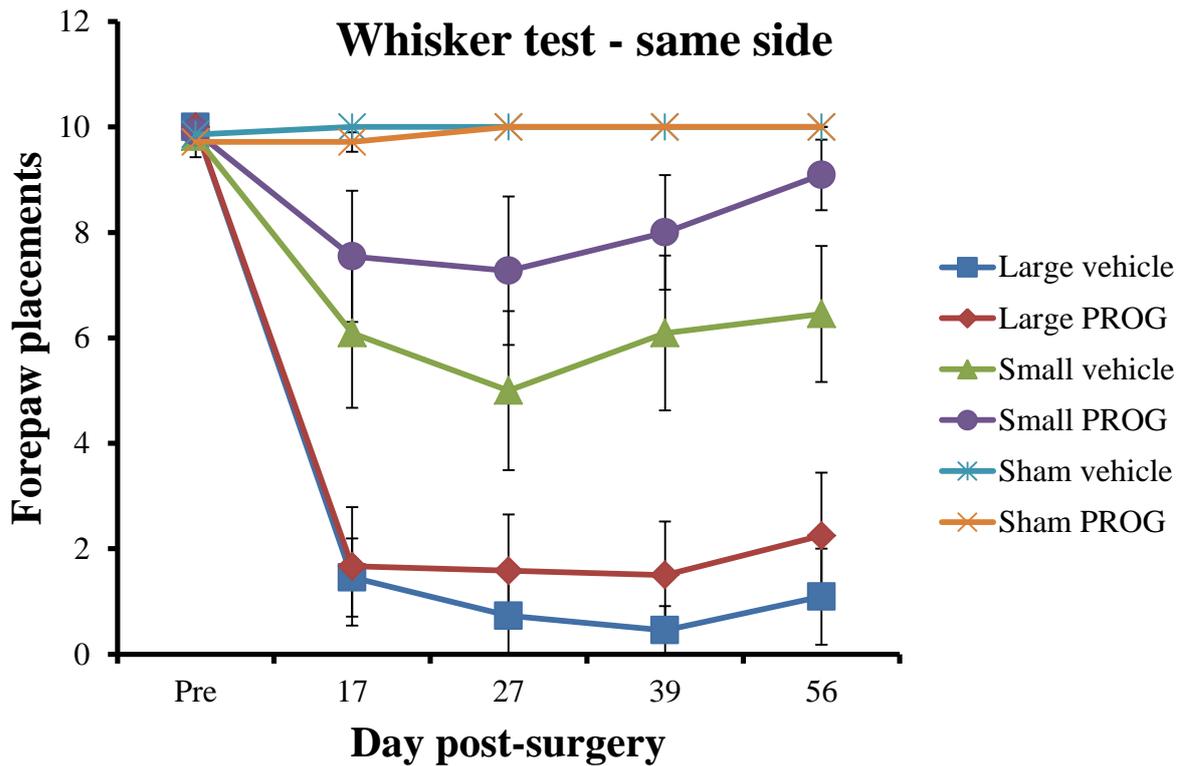


Figure 7.11. Contralateral forelimb placements on the “same side” whisker test. Error bars denote SEM.

Following cross-midline vibrissae stimulation large lesions produced substantially impaired forelimb placements while small lesions produced an intermediate deficit (Lesion, $F(1, 41) = 32.76, p < 0.001$; Fig. 7.12). In this instance, PROG significantly reduced poor performance over time shown by vehicle-treated rats with both small and large lesions (Time x Treatment interaction, $F(3, 123) = 3.32, p = .02$). There was no Treatment ($F(1,41) = 3.10, p = .09$) or Time ($p > 0.20$) main effect. Despite the appearance that PROG had a greater effect after small than large lesions, neither the Time x Lesion ($F(3, 123) = 1.72, p = 0.17$), Lesion x Treatment ($p > 0.20$) nor Time x Lesion x Treatment ($p > 0.20$) interactions were significant.

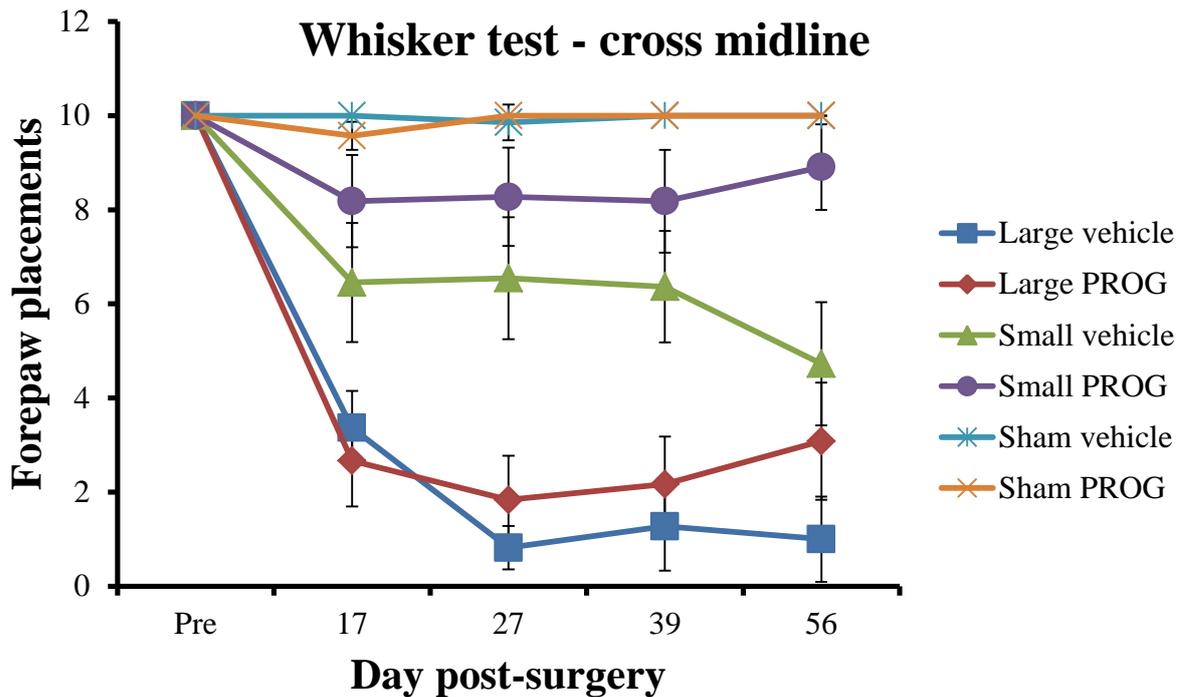


Figure 7.12. Contralateral forelimb placements in the “cross midline” whisker test. Error bars denote SEM.

7.4.5. Cylinder test: Forelimb use asymmetry

One vehicle treated rat from the large lesion group was excluded from the analysis as it would not participate in the task during post-surgery testing. Due to time constraints only the pre-surgery and day 42 videos were coded. There was a significant main effect for lesion (Lesion, $F(2, 52) = 3.89, p = 0.03$; Fig. 7.13). Post-hoc Newman-Keuls showed that, collapsed across the two testing sessions, rats with large ($p = 0.03$) but not small ($p > 0.20$) lesions had a greater reliance on the ipsilateral forelimb during the cylinder test compared to sham. There was a significant Lesion x Time interaction ($F(2, 52) = 6.35, p = 0.003$) due to increasing forelimb use asymmetry from pre to post-surgery in all lesion groups including shams, but a more marked change in the group with large lesions. Post-hoc Newman-Keuls showed that pre-surgery forelimb use asymmetry was not significantly different between all lesion and sham groups ($p > 0.20$). However, at day 42 the rats with large lesions had an

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increased asymmetry compared to small lesion and sham groups ($p < 0.05$), but forelimb use asymmetry for rats with small lesions did not differ from sham ($p > 0.20$). There was no effect of PROG (Treatment, $F(1, 52) = 1.25, p > 0.20$; Treatment x Time, $F(1, 52) = 0.67, p > 0.20$; Time x Lesion x Treatment, $F(2, 52) = 0.39, p > 0.20$).

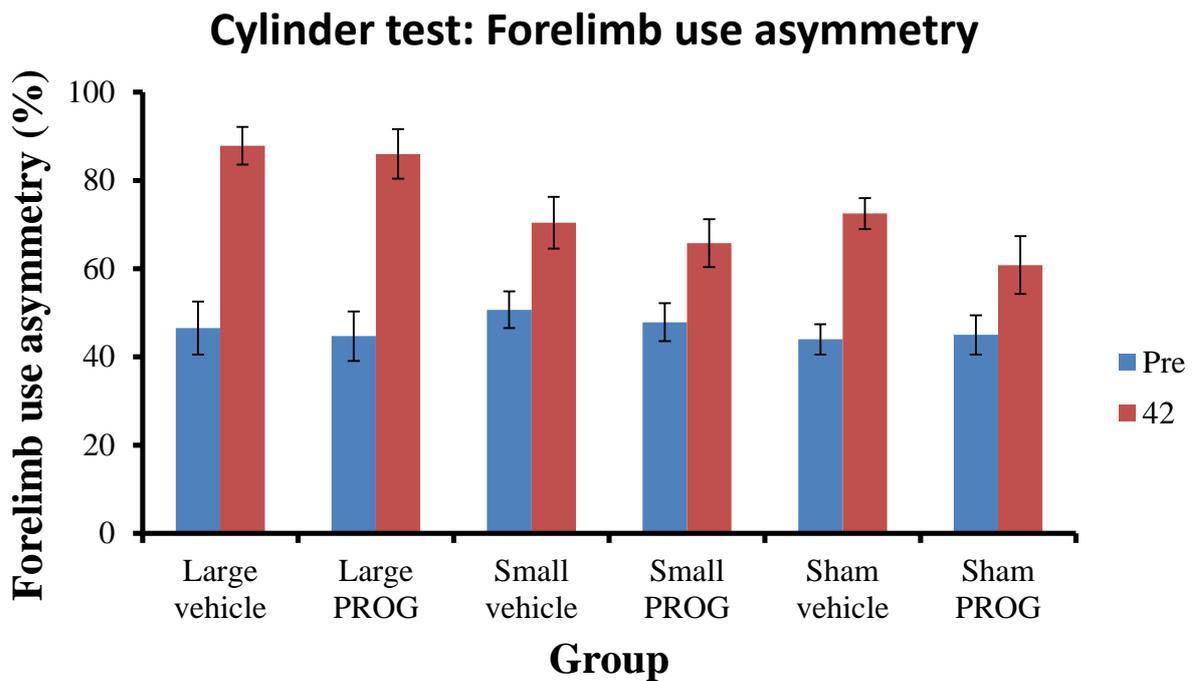


Figure 7.13. Forelimb use asymmetry in the cylinder test. Error bars denote SEM.

The occurrence of serial stepping in both forelimbs prior to surgery, and in the contralateral forelimb post-surgery was rare and did not occur in most groups. Due to the rare occurrence of this behaviour these data are not shown. After surgery, large lesions produced significantly increased ipsilateral forelimb serial stepping behaviour (Lesion, $F(2, 52) = 4.04, p = 0.02$; Fig. 7.14). Post-hoc Newman-Keuls revealed that large lesions made more serial steps than both small lesion and sham (both $p < 0.05$). Serial stepping in rats with small lesion was low at levels comparable to sham ($p > 0.20$). Neither the Treatment main effect nor the Lesion x Treatment interaction were significant ($p > 0.20$).

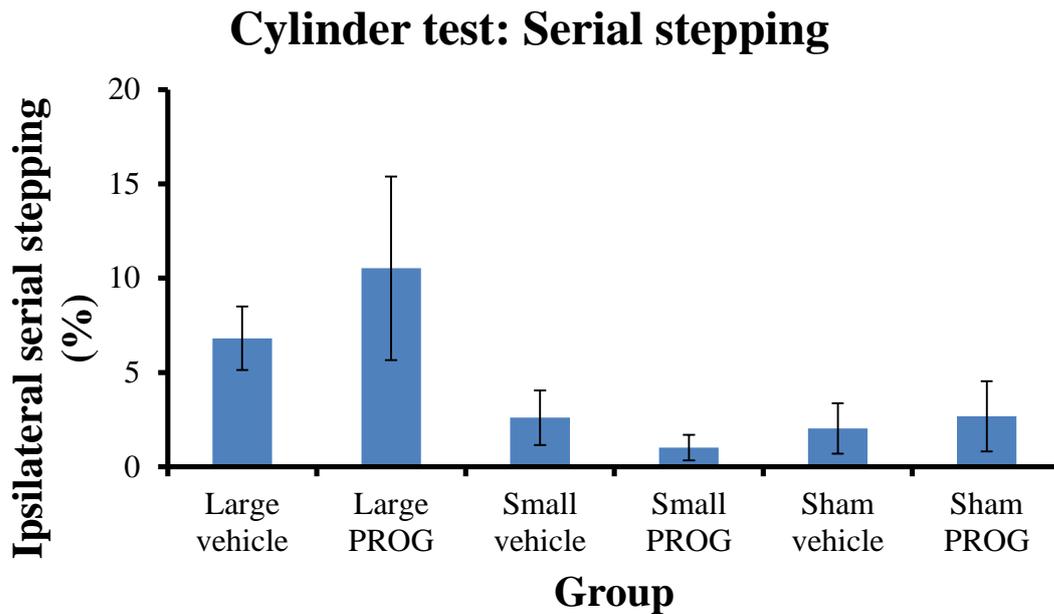


Figure 7.14. Ipsilateral forelimb serial stepping behaviour at day 42. Error bars denote SEM.

Rats with large and small lesions reared less frequently in the cylinder compared to shams (Lesion, $F(2, 52) = 4.26, p = 0.02$; Fig. 7.15). There was a significant main effect of Time due to decreasing rears from pre to post-surgery ($F(1, 52) = 18.85, p < 0.001$) although the Lesion x Time interaction was not significant ($p > 0.20$). PROG treatment did not change rearing in the cylinder (Treatment, $p < 0.20$). No significant interactions were found for Lesion x Treatment ($F(2, 52) = 2.03, p = 0.14$), Time x Treatment ($F(1, 52) = 1.82, p = 0.18$), or Time x Lesion x Treatment ($F(2, 52) = 2.25, p = 0.12$).

Cylinder test: Rearing

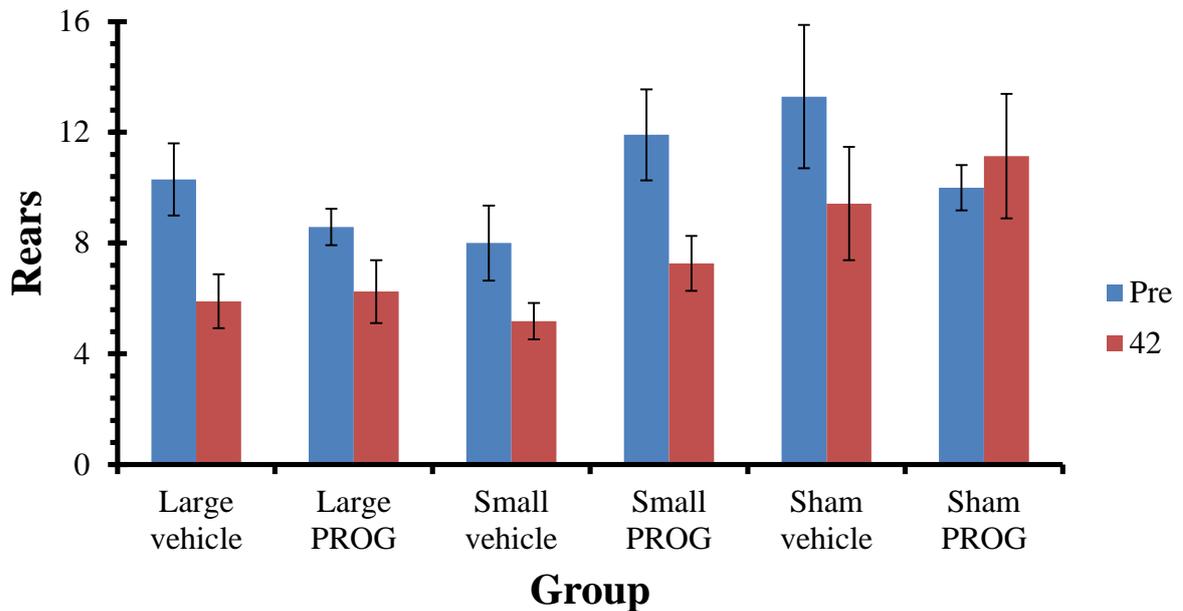


Figure 7.15. Number of rears in the cylinder test. Error bars denote SEM.

7.4.6. Corridor test: Sensory neglect

Prior to surgery contralateral retrievals did not differ between groups in the corridor test.

Neither the main effects of Lesion, Treatment nor the Lesion x Treatment interaction were significant (all $p > 0.20$).

Following surgery, large lesions substantially reduced contralateral retrievals in the corridor while small lesions produced an intermediate impairment (Lesion, $F(2, 53) = 24.23$, $p < 0.001$; Fig. 7.16). PROG treatment did not reduce this bias ($p > 0.20$). There was improved performance over time ($F(2, 106) = 5.08$, $p < 0.001$) but no interaction with PROG or lesion (all $p > 0.20$).

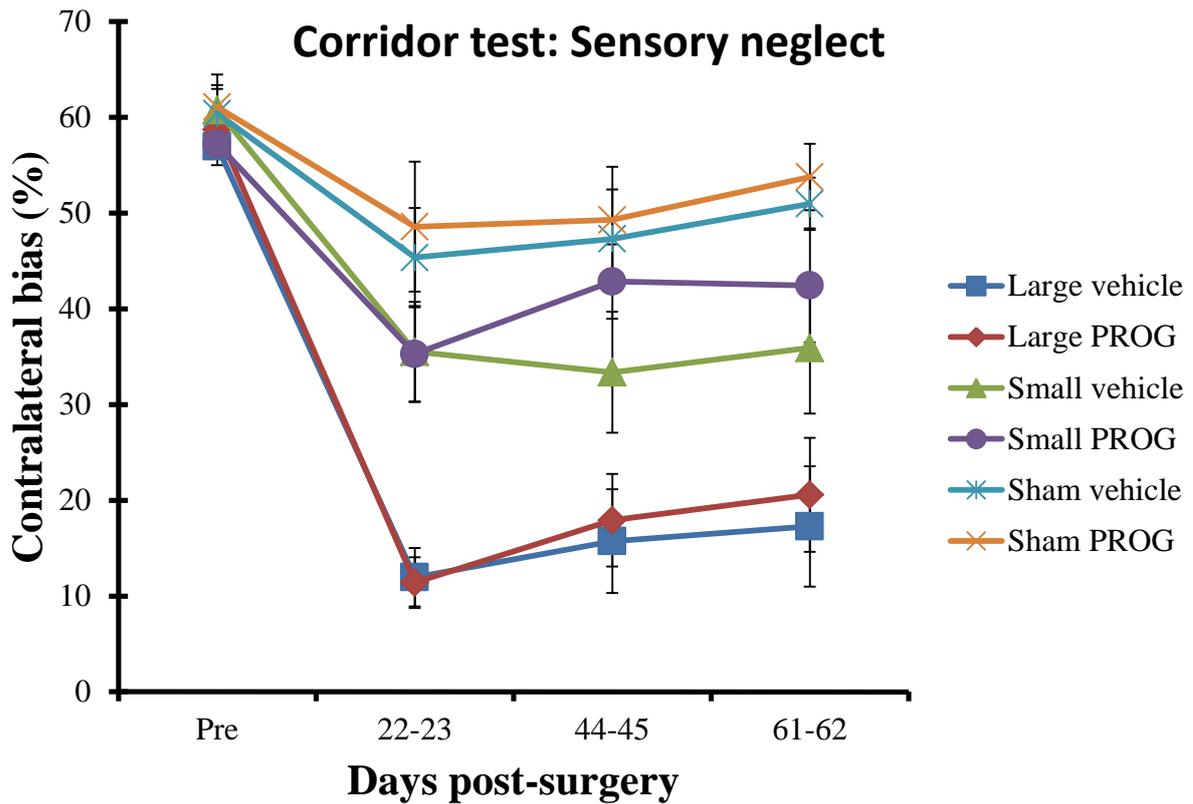


Figure 7.16. Contralateral retrieval bias in the corridor test. Error bars denote SEM.

7.4.7. Rotation after apomorphine

At day 66 only large lesions produced significantly increased contralateral rotation (Lesion, $F(2, 52) = 27.04, p < 0.001$; Fig. 7.17) after apomorphine administration. Post-hoc Newman-Keuls revealed that rats with large lesions rotated significantly more than the small lesion and sham groups ($p < 0.001$). Rats with small lesions did not rotate more than sham ($p > 0.20$). Neither the main effect for Treatment nor the Lesion x Treatment interaction were significant (both $p > 0.20$). One large lesion vehicle rat was excluded from the analysis due to a video tracking error.

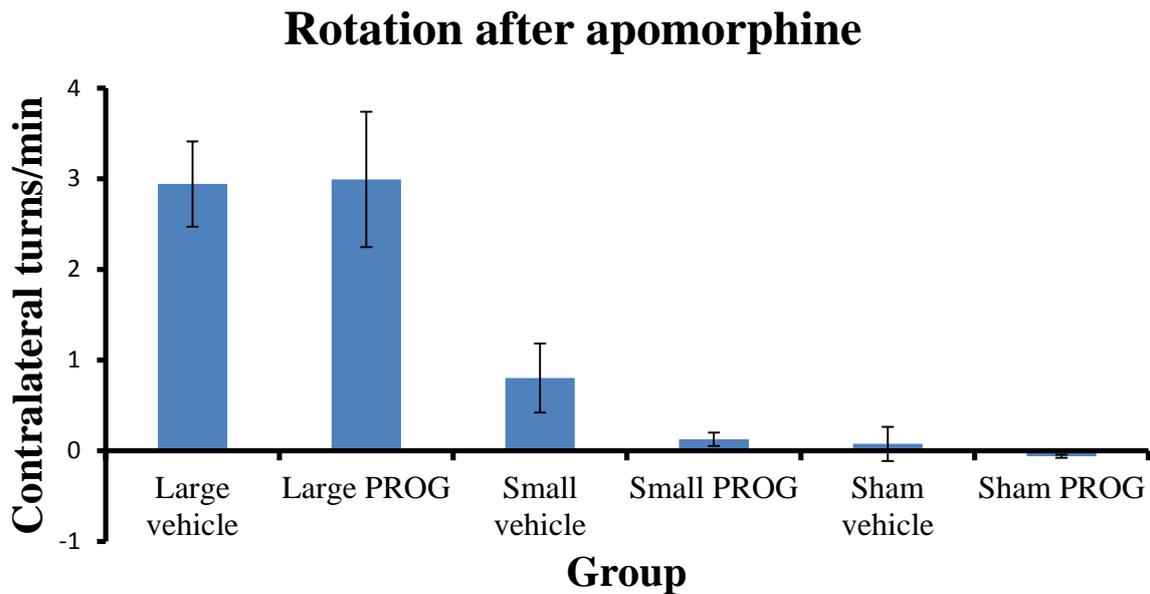


Figure 7.17. Rotation after apomorphine at day 66. Error bars denote SEM. Note: Contralateral turns are expressed as positive values.

7.5. Summary and Discussion

Large lesions produced substantial impairments on rotation after d-amphetamine and apomorphine, forelimb akinesia, postural instability, forelimb use asymmetry, sensorimotor integration, and sensory neglect. Small lesions produced intermediate impairments on forelimb akinesia, postural instability, sensorimotor integration, and sensory neglect but no impairment for forelimb use asymmetry and rotation after apomorphine. PROG treatment significantly reduced the forelimb akinesia impairment for rats with small and large lesions. PROG treatment also reduced ipsilateral but not contralateral forelimb impairment in the postural instability test for rats with large lesions, and prevented the increasingly poor performance for rats with small and large lesions in the cross midline whisker test but not significantly for the same side whisker test. PROG treatment in sham rats did not have any effects relative to the sesame oil vehicle on any test.

Lesion validation: Rotation after d-amphetamine

Due to time constraints the 6-OHDA lesions were not examined with tyrosine hydroxylase immunohistochemistry. Instead, we assessed rotation after d-amphetamine as an ante-mortem validation of the lesion (Grealish et al., 2008). At day 7 after surgery rats with small and large lesions produced significant ipsilateral rotation after d-amphetamine whereas sham rats did not rotate as expected. Rats with large lesions rotated significantly more than rats with small lesions.

The substantially increased ipsilateral rotation observed in rats with large lesions is comparable to previous reports as discussed in Chapter 5 (e.g. Kirik et al., 1998; Shin et al., 2014). Furthermore, the magnitude of rotation in the current experiment (~12 turns/min) is similar to the magnitude of rotation found in studies one (~14 turns/min; Chapter 5) and two (~11 turns/min; Chapter 6) suggesting the extent of large striatal lesions were comparable across experiments at day 7 after surgery.

In accordance with previous reports using small intrastratial 6-OHDA lesions we found that our rats had moderately increased ipsilateral rotation of ~7 turns/min. For example, Kirik et al. (1998) reported moderate rotation of ~5 turns/min at day 7 after surgery. More recently, Sadan et al. (2009) reported ~3 turns/min at week 2 and Shin et al. (2014) observed ~6 – 7 turns/min at week 4 post-surgery. One discrepancy to these studies was reported by Decressac et al. (2012) who found that rats with small striatal 6-OHDA lesions had low but not significantly increased rotation at 3 and 8 weeks after surgery.

The current experiment has reported moderate and substantial rotation after d-amphetamine at day 7 for rats with small and large lesions, respectively. The significant difference in the rate of rotation between rats with small and large lesions confirmed a graded PD like impairment prior to PROG treatment at day 8.

Adjusting steps test primary outcome measure: Akinesia

The adjusting steps task was selected as the primary outcome measure as akinesia is a cardinal motor feature of PD (Jankovic, 2008). Prior to surgery forelimb stepping did not differ between lesion groups. There was a small but significant difference between PROG treatment groups where rats belonging to the PROG group made fewer steps compared to vehicle rats. While this difference was unexpected it is not problematic as the fewer steps made by PROG rats prior to surgery goes against the hypothesis.

Following surgery rats with large lesions had substantially impaired forelimb stepping while rats with small lesions were moderately impaired. Forelimb stepping for rats with large lesions worsened across subsequent testing while rats with small lesions maintained a stable impairment. PROG treatment markedly improved the forelimb stepping impairment for rats with large lesions to levels similar to vehicle treated rats with small lesions. Furthermore, PROG treatment significantly improved forelimb stepping for rats with small lesions to a level comparable to sham rats. The beneficial influence of PROG on akinesia was evident at the first day of testing in the adjusting steps test and was maintained across subsequent testing, preventing the decline seen over time in rats with large lesions.

Impaired forelimb stepping in the vehicle treated rats with large lesions is consistent with the previous studies discussed in Chapter 5 (e.g. Dowd and Dunnett, 2005; Kirik et al., 1998; Shin et al., 2014). Moderately Impaired forelimb stepping in the vehicle treated rats with small lesions is also consistent with previous research. Small striatal 6-OHDA lesions moderately impaired forelimb stepping at 3-14 weeks post-surgery in several studies (e.g. Decressac et al., 2012; Gong et al., 2012; Kirik et al., 1998; Shin et al., 2014) although some have reported more substantial impairment (Krishnamurthi et al., 2009; Moloney et al., 2010). Additionally, single 6-OHDA infusions to the striatum that produce moderate TH

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depletion also cause moderately impaired forelimb stepping (e.g. Depino et al., 2003; Khan et al., 2010; Kirik et al., 1998, 2000). In each of these studies and the current thesis the 6-OHDA infusions were made to the dorsolateral striatum. Hence, DA in this region of the caudate putamen appears to mediate stepping in the adjusting steps test (Chang et al., 1999)

Consistent with study two (Chapter 6), the beneficial influence of PROG for rats with large lesions was replicated here and shown to extend to rats with small lesions, reducing impairment in the latter rats to levels comparable to sham rats. For both small and large lesion groups PROG's influence was evident at the first assessment at day 17 and did not diminish over subsequent testing at days 27, 39, and 56. Thus, as with study two, the improved forelimb akinesia observed was not merely symptomatic relief as the beneficial influence of PROG was maintained for six weeks after treatment had stopped. The mechanism underlying this improvement is not clear as histology has not been performed due to time restrictions. However, it is not likely to involve striatal TH fibre innervation as striatal deafferentation would have been complete at the time of the first PROG injection given the findings in study two.

Postural instability test

The postural instability test is a newer assay of motor impairment following nigrostriatal dopamine depletion (Woodlee et al., 2008). As described earlier in this chapter Woodlee et al. (2008) reported that unilateral MFB lesions produce severe postural instability of the contralateral forelimb but enhanced the function of the ipsilateral forelimb. In other PD model studies increased postural instability of both forelimbs in the postural instability test has been found following systemic administration of rotenone that causes bilateral dopamine depletion (Cannon et al., 1999; Tapias et al., 2014) although a single unilateral intrastratial

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infusion did not change the displacement required to trigger a catch-up step for either forelimb (Carriere et al., 2014).

Performance in the postural instability test is dependent on the body weight and size of the rat as would be expected according to the physics of maintaining centre of gravity. Woodlee et al. (2008) found that body weight was the best predictor with heavier rats requiring further displacement to trigger a catch-up step. At each day of testing rat's weights were unlikely to have an influence because body weights did not differ between groups at any testing day.

To the best of our knowledge the current experiment is the first report that employs the postural instability test in the context of unilateral intrastratial 6-OHDA lesions. In line with the original report of Woodlee et al. (2008) who used MFB lesions, we found that both large and small striatal lesions induced postural instability of the contralateral forelimb with large striatal lesions producing a greater impairment. Consistent with Woodlee et al. (2008) we also found that sham operated rats tended to require further displacement to trigger a catch-up step compared to their pre-surgery scores. Unexpectedly, prior to surgery rats belonging to the PROG groups required more displacement to trigger a catch-up step in the contralateral forelimb compared to vehicle treated rats. This difference is not problematic, however, as it works against the hypothesis that PROG would reduce contralateral forelimb displacements. Nonetheless, PROG treatment did not reduce postural instability impairment of the contralateral forelimb for either small or large lesion rats.

Consistent with Woodlee et al. (2008) both small and large lesions reduced the displacement required to trigger a catch-up step for the ipsilateral forelimb. This "enhanced" compensatory function is not surprising as previous reports show that the effects of unilateral dopamine depleting lesions are not restricted to the lesion hemisphere (Miklyeva et al.,

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2007; Vergara-Aragon et al., 2003). Similar to the “enhanced” function observed in the current experiment, the speed with which rats with unilateral 6-OHDA MFB lesions remove adhesive labels from their ipsilateral forelimbs is enhanced compared to sham operated animals (Schallert et al., 1982, 1983). According to Woodlee et al. (2008) the enhanced function of the ipsilateral forelimb is not due to dopaminergic reorganisation of the contralateral to lesion hemisphere as they did not find an up regulation of d-amphetamine induced c-fos expression in the striatum. However, this does not rule out other possible changes to the dopaminergic system contralateral to the 6-OHDA lesion (discussed below).

The necessity of the rat to compensate with their “good” ipsilateral forelimb for initiating and maintaining most of their daily movements could account for the enhanced function of the ipsilateral forelimb in the postural instability test. For example, in one study, rats with large striatal 6-OHDA lesions relied mostly on their good limbs to defend their posture and to initiate or terminate movements (Miklyaeva et al., 1995). In another study, the experiential influence of compensating with the “good” forelimb increased dendritic arborisation in the motor cortex of the contralateral to lesion hemisphere after 6-OHDA MFB lesions (Miklyaeva et al., 2007). Together these studies show that rats with extensive 6-OHDA lesions compensate with their “good” limbs which can lead to enhanced function of the ipsilateral forelimb and related morphological changes in the contralateral to lesion hemisphere. PROG treatment normalised the enhanced function of the ipsilateral forelimb in the postural instability test for rats with large but not small lesions. This suggests that rats with large lesions treated with PROG did not need to compensate as much with the “good” forelimb compared to vehicle treated rats and hence maintained normal function in the postural instability test. In support of this, the substantial reduction in forelimb akinesia for PROG treated rats with large lesions would mean that these rats could make greater use of their impaired forelimb thus relying less on the good forelimb.

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Another possibility is that PROG normalised some dopaminergic changes in the contralateral to lesion hemisphere as there are reports that unilateral 6-OHDA lesions can increase dopaminergic signalling in the intact hemisphere. For example, increased extracellular dopamine in striatal dialysates in the contralateral to lesion hemisphere has been observed following 6-OHDA infusions to the SN (Robinson and Whishaw, 1988). Additionally, there is evidence that large 6-OHDA lesions increase striatal dopamine content in the non-lesion hemisphere (Warencya and McKenzie, 1987). More recently, increased TH expression in the contralateral to lesion hemisphere has been reported following MFB 6-OHDA lesions (Schlachetzki et al., 2014). Importantly, a recent study that examined PROG's influence following small striatal 6-OHDA lesions found an increase in potassium-evoked [³H]-dopamine release in the contralateral to lesion striatum that was normalised by PROG treatment (Casas et al., 2013). Taken together these reports show that the contralateral to lesion hemisphere can undergo increased dopaminergic signalling following unilateral 6-OHDA lesions that can be normalised by PROG treatment. Therefore, it is possible that the normalised function of the ipsilateral forelimb for rats with large lesions was due to normalised dopaminergic function in the contralateral to lesion hemisphere.

Here we present the first report regarding postural instability impairments on the postural instability test after small and large unilateral 6-OHDA lesions of the striatum. We found that large lesions produced substantial postural instability of the contralateral forelimb and substantially enhanced compensatory function of the ipsilateral forelimb while small lesions produced moderate changes. PROG treatment did not reduce postural instability of the contralateral forelimb for rats with either small or large lesions but normalised the function of the ipsilateral forelimb for rats with large lesions.

Cylinder test: Forelimb use asymmetry

The cylinder test is a common assay of forelimb use asymmetry following unilateral 6-OHDA lesions. The standard measure is the number of weight bearing forepaw touches against the cylinder wall (Schallert et al., 2000) where unilateral 6-OHDA lesions increase the proportion of ipsilateral forepaw touches (e.g. Woodlee et al., 2008).

In the current experiment, at day 42 after surgery all groups including sham showed greater preference for use of the ipsilateral forelimb for weight shifting movements during spontaneous vertical exploration compared to pre-surgery testing. At day 42 rats with large but not small lesions showed greater ipsilateral forelimb use preference compared to sham rats suggesting that only large lesions increased ipsilateral forelimb use asymmetry. PROG treatment did not change forelimb use asymmetry for small or large lesion or sham groups.

The increased ipsilateral forelimb use asymmetry observed in rats with large lesions is consistent with previous research where moderate – severe ipsilateral forelimb use asymmetry has been reported following large 6-OHDA striatal lesions (e.g. Carlsson et al., 2009; Grealish et al., 2010; Henning et al., 2007; Kirik et al., 2000; Shin et al., 2014). We observed an increase in ipsilateral forelimb use asymmetry for rats with small lesions although this was not significantly different from sham. In support of this result Decressac et al. (2012) likewise did not find increased ipsilateral forelimb use asymmetry after small 6-OHDA lesions. However, other studies have found moderately increased ipsilateral forelimb use asymmetry after small (Shin et al., 2014; Spieles-Engemann et al., 2010; Yang et al., 2009) and single-site striatal 6-OHDA lesions (Grant et al., 2009; Vercammen et al., 2006). The increased ipsilateral forelimb use asymmetry observed in sham rats has not been observed in most studies where sham operated rats were tested (e.g. Grealish et al., 2010;

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Shin et al., 2014; Spieles-Engemann et al., 2010) although one study has reported an increased bias for single-site striatal sham operated rats (Grant et al., 2009).

A newer secondary measure during vertical exploration in the cylinder is the occurrence of serial stepping where rats make several rapid lateral weight shifting steps with the ipsilateral forelimb. This measure to the best of our knowledge has only been reported by Woodlee et al. (2008) who found that 65% of all ipsilateral forelimb use occurred during serial stepping after MFB 6-OHDA lesions. In the current experiment we found that serial stepping was slightly (~6-10%) yet significantly increased for rats with large lesions compared to sham. Rats with small lesions and sham rats had very low levels (~2%) of serial stepping. PROG treatment did not influence serial stepping for any group.

The discrepancy between the magnitudes of serial stepping observed between the current experiment and Woodlee et al. (2008) is likely due to the different 6-OHDA lesions used. The MFB lesions used by Woodlee et al. produce complete denervation of nigrostriatal dopamine whereas the small and large striatal lesions used in the present experiment cause partial and subtotal depletion, respectively. Perhaps more extensive 6-OHDA lesions are required to produce substantial ipsilateral forelimb serial-stepping in the cylinder test.

The cylinder test also allowed for the determination of spontaneous rearing activity. Although rearing frequency in the cylinder test is not often reported several studies have found decreased rearing after 6-OHDA MFB lesions (Rauch et al., 2010, Santiago et al., 2010; Shi et al., 2004). Consistent with these studies we found that rats with small and large lesions reared less often than sham rats. However, PROG treatment did not influence rearing in the cylinder for any group.

One possible explanation for decreased rearing could be habituation to the testing environment. In support of this we observed a marked decrease in rearing at day 42 compared

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to pre-surgery performance. However, it is likely that the decreased rearing can also be attributed to dopamine depletion as rats with small and large lesions reared significantly less than sham. Therefore, decreased rearing in the cylinder among rats with lesions is more likely a movement related impairment as a consequence of the 6-OHDA neurotoxin.

Impaired forelimb use in the cylinder test is sensitive to both extensive and partial dopamine depleting lesions (Shin et al., 2014; Shi et al., 2004). As reported in the introduction, therapy's that improve dopamine transmission decrease forelimb use asymmetry. In the current experiment PROG was unable to improve forelimb use asymmetry in the cylinder test. This may be consistent with the inability of PROG treatment to improve striatal TH deafferentation, given the findings in study two (Chapter 6).

Whisker-evoked forelimb placement: Sensorimotor integration

In the current experiment we tested whisker evoked forelimb placement for both forelimbs upon stimulation of the vibrissae on each side of the rats head (Woodlee et al., 2005). Testing forelimb placement by both same-side and cross-midline vibrissae stimulation allowed us to distinguish if the impairment was primarily sensory or motor in nature. If the deficit was due primarily to sensory impairment then stimulation of the "bad" vibrissae would not evoke forelimb placement of either the impaired or the intact forelimb while stimulation of the "good" vibrissae would evoke placement of both forelimbs. In contrast, if the deficit was due primarily to motor impairment then the impaired forelimb would not place in response to stimulation of the "good" or "bad" vibrissae while placement of the intact forelimb would occur regardless of which vibrissae were stimulated.

In the current experiment we found that placement of the forelimb contralateral to the lesion was substantially impaired in rats with large lesions while rats with small lesions had an intermediate deficit. Impaired contralateral forelimb placement occurred in response to

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same-side and cross-midline vibrissae stimulation. In comparison, the ipsilateral forelimb for rats with lesions, and both forelimbs for shams, was unimpaired in both versions of the test. Together these findings show that impaired contralateral forelimb placing in rats with small and large lesions was due primarily to motor rather than sensory deficit. This finding is consistent with reports that found impaired forelimb placing on the same-side and cross-midline test after MFB 6-OHDA lesions (Anstrom et al., 2007; Woodlee et al., 2005).

The moderate impairment observed in rats with small lesions is consistent with previous research that used the same-side whisker test after small 6-OHDA striatal lesions (Grant et al., 2009). Following 6-OHDA lesions the same-side version is the most commonly used forelimb placing test. Similar to rats with large lesions in this experiment, substantial impairment has been reported after MFB (Monville et al., 2005; Schallert et al., 2000; Woodlee et al., 2008) and large striatal 6-OHDA lesions (Grealish et al., 2008). Furthermore, the moderate impairment after small striatal 6-OHDA lesions found in the current experiment has also been reported elsewhere (McCoy et al., 2008; Pienaar et al., 2008).

PROG treatment did not improve forelimb placement following same-side vibrissae stimulation for rats with small or large lesions. However, in the cross-midline test PROG prevented the increasing impairment evident in rats with small and large lesions. The mechanism underlying this prevention of increasing impairment is not clear although it suggests that PROG preserved some motor programs responsible for forelimb placing in the lesion hemisphere that could be accessed via vibrissae sensory input to the intact hemisphere. Therefore, despite forelimb placement impairment being primarily a motor deficit, PROG may have provided some sensory compensation.

Corridor test: Sensory neglect

The corridor test is a newer assay of sensory neglect following nigrostriatal dopamine depletion (Dowd et al., 2005a). Rats with unilateral 6-OHDA lesions will neglect flavoured food pellets placed on the contralateral to lesion side of the corridor (Dowd et al., 2005a; Fitzsimmons et al., 2006). An examination of the literature revealed that 12 studies have used the corridor test following unilateral 6-OHDA lesions in rats. Substantial impairments have been observed after SNpc (Jouve et al., 2010), MFB (Cederfjall et al., 2012, 2013; Corderio et al., 2010; Decressac et al., 2012; Dowd et al., 2005a; 2005b; Grealish et al., 2008; Torres et al., 2008), and large striatal lesions (Dowd et al., 2005a; Fitzsimmons et al., 2006; Grealish et al., 2008; Shin et al., 2014). Moderate impairments have been observed after small (Decressac et al., 2012) and single site striatal lesions (Moloney et al., 2010) although one study found no impairment after small striatal lesions (Shin et al., 2014). This discrepancy can readily be explained by the extent of TH reduction observed in these studies as impairment in the corridor is probably dependent on nigrostriatal dopamine depletion. Decressac et al. (2012) found that impairment in the corridor test was significantly correlated with the extent of SN cell and striatal fibre TH reduction. In their study they found that the threshold for impairment was SN and striatal TH reduction to approximately 20%. In the former studies (Decressac et al., 2012; Moloney et al., 2010) TH positive cells in the SN and fibres in the striatum were reduced on average to 30 – 35% with a number of rats expressing TH below the required threshold for impairment. In the latter study (Shin et al., 2014) TH reduction was less severe varying between 60 – 90% with almost all rats expressing TH above the required impairment threshold. Interventions that improve dopamine transmission should therefore reduce sensory neglect impairment in the corridor test. Indeed, previous research shows that systemic administration of the dopamine agonist apomorphine (Fitzsimmons et al., 2006) and post-lesion grafts of embryonic dopaminergic cells (Corderio

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et al., 2010; Dowd et al., 2005a; Torres et al., 2008) ameliorate sensory neglect impairment in the corridor test after unilateral 6-OHDA lesions. Similarly, an adeno-associated viral vector that co-expressed the DOPA pre-cursors TH and GTP cyclohydrolase-1 administered to the striatum after 6-OHDA MFB lesions also reduced sensory neglect in the corridor test (Cederfjall et al., 2012, 2013).

Consistent with previous studies we found that large lesions produced substantial sensory neglect in the corridor test (e.g. Dowd et al., 2005a; Shin et al., 2014) while small lesions produced an intermediate impairment (e.g. Decressac et al., 2012; Moloney et al., 2010). All rats in the current experiment received lesions on the side opposite to their pre-operative preferred retrieval bias side. Strikingly, this innate bias was reversed by 6-OHDA lesions. Thus, consistent with the original report of Dowd et al. (2005a), rats with unilateral intrastratial 6-OHDA lesions neglected their previously preferred side. However, PROG treatment did not reduce sensory neglect impairment for rats with small or large lesions.

In study two (Chapter 6) we found that PROG treatment did not prevent additional striatal TH fibre degeneration as the lesion was already maximal at the time of the first PROG injection (day 8 after surgery). In the current experiment it is likely that striatal TH fibre deafferentation was complete at the time of the first PROG injection for at least rats with large lesions and potentially for rats with small lesions as the first PROG injection was also applied at day 8 after surgery. Therefore, the inability of PROG to reduce sensory neglect impairment in the current experiment may reflect, at least in part, lack of improvement in DA transmission.

Rotation after apomorphine

Rats with large lesions had increased contralateral rotation after apomorphine at day 66 post-surgery. Neither sham nor rats with small lesions had a rotation bias. PROG treatment did not change rotation for any group.

The increased contralateral rotation among rats with large lesions is similar to previous reports that have noted low to moderate rotation following large striatal 6-OHDA lesions (Dowd and Dunnett, 2005; Kirik et al., 1998, 2000b; Rosenblad et al., 2000). Reports regarding two-site striatal 6-OHDA lesions are sparse but the current finding is consistent with one other study with small lesions where insufficient DA depletion resulted in no effect on rotation after apomorphine (Kirik et al., 1998).

The inability of PROG to reduce contralateral rotation after apomorphine after large lesions is in accordance with study two (Chapter 6). As discussed in Chapter 6 and above, this is not surprising as PROG was unable to repair or protect striatal TH fibre denervation that was already maximal at the time of the first PROG injection.

Spontaneous recovery of function after 6-OHDA lesions

In the current experiment we observed some spontaneous recovery of motor function in several tests. Sensory neglect in the corridor test improved from first testing at day 22 to the last test as day 61 post-surgery. This improvement was more apparent for rats with large than small lesions. Similarly, forelimb placement following same-side vibrissae stimulation improved across post-surgery testing occasions for rats with small and large lesions. In the postural instability test the increased displacement of the contralateral forelimb that was evident at day 20 for rats with small lesions recovered over subsequent testing at days 42 and 59 post-surgery to levels similar to their pre-surgery performance. Importantly, the adjusting

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steps test which was our primary outcome measure produced a stable impairment for rats with small lesions and a progressive impairment for rats with large lesions. The adjusting steps test is considered the most reliable behavioural readout following small and large 6-OHDA lesions of the striatum although recovery can occur over extended testing periods (Duty and Jenner, 2011).

The spontaneous recovery of motor function reported in this experiment is not surprising as motor function can recover over several weeks after striatal 6-OHDA lesions, especially when the lesion is moderate (Stanic et al., 2003). The recovery of function found in the current experiment is similar to previous reports that have used small and large striatal 6-OHDA lesions. For example, recovery of forelimb akinesia (Kirik et al., 2001b), forelimb use asymmetry (Kirik et al., 2000a) and rotation after d-amphetamine (Kirik et al., 2001a) have been reported after large striatal 6-OHDA lesions. Furthermore, after small striatal 6-OHDA lesions recovery of rotation after d-amphetamine and forelimb use asymmetry has also been found (Yang et al., 2009). In these studies and the current experiment motor function was assessed in both the short and long-term after surgery which improves the chances of detecting spontaneous recovery following 6-OHDA lesions and determines the relative long-term consequences of any putative treatment.

Summary

The current experiment reports the first study in this field to examine PROG's beneficial influence on an extensive series of motor tests, including forelimb akinesia, postural instability, sensorimotor integration, forelimb use asymmetry, sensory neglect, and rotation after apomorphine following small and large 6-OHDA lesions of the striatum. We found that 7 days of 8 mg/kg PROG treatment with tapered withdrawal reduced forelimb akinesia in both the short and long-term for rats with small and large lesions. This replicated the findings

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of study two (Chapter 6) where PROG provided long term reduction in forelimb akinesia for rats with large lesions. Additionally, this finding was extended to rats with small lesions suggesting that PROG is also beneficial in lesions that model the earlier stages of PD degeneration. In accordance with study two the improved forelimb akinesia was not merely symptomatic relief as the beneficial influence of PROG was observed up to six weeks after treatment had stopped.

Importantly, PROG did not exacerbate impairments for rats with lesions nor did it change motor skills for sham rats which suggest that PROG is not detrimental on any of the motor tests used in this experiment. In addition to improved forelimb akinesia, PROG prevented increasing impairment across subsequent testing sessions in the cross-midline whisker test for rats with small and large lesions and normalised ipsilateral forelimb displacement in the postural instability test for rats with large lesions. However, PROG did not influence forelimb use asymmetry, same-side whisker evoked forelimb placing, sensory neglect, or rotation after apomorphine.

The neural mechanisms underlying PROG's beneficial influence after 6-OHDA lesions is unclear. Due to time restrictions histology has not yet been completed on these rats. However, the improved functional outcomes observed in this experiment are likely to be independent of striatal TH fibre innervation given the finding in study two that PROG treatment did not change striatal TH density.

This chapter has reported the first experiment in this field that directly compares PROG treatment after small and large 6-OHDA lesions of the striatum. Furthermore, this experiment has provided the most comprehensive examination to date of PROG's influence on motor impairments after 6-OHDA lesions. Here we have found that PROG treatment is beneficial following both small and large 6-OHDA lesions of the striatum. The most benefit

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was observed on the adjusting steps test primary outcome measure of forelimb akineisa where the observed improvement was long-lasting.

Chapter 8

General Discussion

8.1. Summary

This thesis examined the beneficial influence of post-lesion treatment with the neurosteroid *natural* progesterone (PROG) in the unilateral intrastriatal 6-hydroxydopamine (6-OHDA) model of Parkinson's disease (PD). The current study has shown for the first time that PROG treatment markedly reduced motor impairments following small and large 6-OHDA lesions that modelled the earlier and later stages of PD degeneration, respectively. Table 8.1 summarises the key findings of this thesis. As shown in Table 8.1, the lesions produced substantial rotation bias after d-amphetamine which confirmed a functional dopamine (DA) impairment prior to PROG treatment. The substantial forelimb akinesia produced by lesions on the adjusting steps test was markedly improved by PROG treatment for rats with small and large lesions; this benefit after large lesions was replicated across two experiments. The beneficial influence of PROG for forelimb akinesia was evident at the first testing occasion and maintained for several weeks after treatment had stopped. Skilled reaching was moderately impaired for rats with large lesions, but PROG did not improve this deficit. Similarly, lesions induced a substantial rotation bias after apomorphine that was not reduced by PROG. PROG treatment did not improve postural instability of the contralateral forelimb but did improve the compensatory function of the ipsilateral forelimb for rats with large lesions on the postural instability test. Sensorimotor integration on the whisker test was markedly impaired for rats with small and large lesions. PROG improved forelimb placements for rats with small lesions on both versions of the whisker test and prevented the increasing impairment over time on the "cross-midline" test. Only large lesions increased forelimb use asymmetry on the cylinder test which was not improved by PROG treatment.

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Rats with large lesions displayed substantial sensory neglect on the corridor test whereas rats with small lesions had a moderate impairment, but PROG did not attenuate this sensory neglect deficit for either lesion size. Lastly, large lesions severely reduced tyrosine hydroxylase (TH) fibre density in the striatum. The considerable reduction in striatal TH was evident at the time when PROG treatment was initiated and was maintained at the end of testing several weeks later. However, treatment with PROG did not change striatal TH density. Taken together these findings are the first in this field to demonstrate some beneficial influence of PROG treatment on motor impairments in the short-term and long-term after small and large 6-OHDA lesions of the striatum.

Table 8-1

*Summary of Key Findings**

Study 2: <i>PROG</i> dose response after large lesions Task	Main effects	Effect size	Study 3: <i>PROG</i> after small and large lesions Task	Main effects	Effect size
Rotation bias after d-amphetamine	Large lesion	✓✓✓✓	Rotation bias after d-amphetamine	Large lesion	✓✓✓✓
	Lesion-only control	✓✓✓✓		Small lesion	✓✓✓
	8 mg/kg PROG	No tested		Large PROG	No tested
	4 mg/kg PROG	No tested		Small PROG	No tested
Akinesia: Adjusting steps test	Large lesion	✓✓✓✓	Akinesia: Adjusting steps test	Large lesion	✓✓✓✓
	8 mg/kg PROG	✓✓		Small lesion	✓✓✓
	4 mg/kg PROG	✓✓		Large PROG	✓✓✓
				Small PROG	✓✓
Skilled reaching: Success rate	Large lesion	✓✓	Skilled reaching: Success rate	Not measured	
	8 mg/kg PROG	✗			
	4 mg/kg PROG	✗			
Rotation bias after apomorphine	Large lesion	✓✓✓	Rotation bias after apomorphine	Large lesion	✓✓✓✓
	8 mg/kg PROG	✗		Small lesion	✓✓
	4 mg/kg PROG	✗		Large PROG	✗
				Small PROG	✓
Postural instability: Contralateral forelimb	Not measured		Postural instability: Contralateral forelimb	Large lesion	✓✓✓✓
				Small lesion	✓✓
				Large PROG	✗
				Small PROG	✗

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Study 2: <i>PROG</i> <i>dose response after</i> <i>large lesions</i> Task	Main effects	Effect size	Study 3: <i>PROG</i> <i>after small and</i> <i>large lesions</i> Task	Main effects	Effect size
Postural instability: Ipsilateral forelimb	Not measured		Postural instability: Ipsilateral forelimb	Large lesion Small lesion Large PROG Small PROG	✓✓✓ ✗ ✓✓ ✗
Sensorimotor integration: Whisker test (same side)	Not measured		Sensorimotor integration: Whisker test (same side)	Large lesion Small lesion Large PROG Small PROG	✓✓✓✓ ✓✓ ✗ ✓
Sensorimotor integration: Whisker test (cross midline)	Not measured		Sensorimotor integration: Whisker test (cross midline)	Large lesion Small lesion Large PROG Small PROG	✓✓✓✓ ✓✓✓ ✗ ✓
Forelimb use asymmetry: Cylinder test	Not measured		Forelimb use asymmetry: Cylinder test	Large lesion Small lesion Large PROG Small PROG	✓✓ ✗ ✗ ✗
Sensory neglect: Corridor test	Not measured		Sensory neglect: Corridor test	Large lesion Small lesion Large PROG Small PROG	✓✓✓ ✓✓ ✗ ✗
Striatal tyrosine- hydroxylase fibre density	Large lesion Lesion-only control 8 mg/kg PROG 4 mg/kg PROG	✓✓✓✓ ✓✓✓✓ ✗ ✗	Striatal tyrosine- hydroxylase fibre density	Not measured	

*This table summarises the key findings from the PROG studies (Chapters 6 and 7). d = Cohen's pairwise effect size. ✓, $d \geq 0.5$; ✓✓, $d \geq 0.8$; ✓✓✓, $d \geq 1.5$; ✓✓✓✓, $d \geq 3.0$; ✗, $d < 0.5$. See Appendix A for numerical values. Note: In study 2, the large lesion effect is compared to sham and the potential PROG treatment benefits are compared to large lesions. In study 3, the large and small lesion effects are compared to sham and the potential PROG treatment benefit is compared to the corresponding lesion group.

8.2. 6-OHDA Lesions Impair Motor Skills

This section summarises the changes that were produced by 6-OHDA lesions in this study. For a detailed summary and discussion of these findings see Chapters 5-7.

The intrastriatal 6-OHDA lesions used in this study impaired motor skills, as expected. Prior to treatment with PROG we validated the success of the lesions with a test of rotation after d-amphetamine. Consistent with previous research we found that at day 7 after surgery large lesions produced substantial ipsilateral rotation after d-amphetamine administration whereas small lesions caused moderately increased rotation (Kirik et al., 1998; Shin et al., 2014). Importantly, the difference in the rate of rotation between rats with small and large lesions confirmed that we were successful in producing a graded PD like impairment. In a similar test, when striatal supersensitivity to a DA agonist was examined, we found that only large lesions increased contralateral rotation after apomorphine (Kirik et al., 1998). The success of the lesion method was further validated in studies one and two (Chapters 5 and 6) with striatal TH density immunohistochemistry. As expected, we found that striatal TH density was markedly reduced at day 8 and that this reduction was maintained at 9 weeks after large lesion surgery (Cicchetti et al., 2002; Grealish et al., 2010; Kirik et al., 1998; Rosenblad et al., 2000). Taken together the d-amphetamine rotation test and striatal TH fibre measures show that our lesion method was successful at producing a functional DA impairment.

Akinesia is a cardinal motor feature of PD so the current study selected a priori, and tested first after PROG, the adjusting steps test for forelimb akinesia as the primary outcome measure (Dauer and Przedborski, 2003; Jankovic, 2008; Olsson et al., 1995). The large 6-OHDA lesions produced moderately severe contralateral forelimb akinesia that, like other studies, was maintained at repeated testing intervals between weeks 2 and 8 after surgery

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(Dowd and Dunnett, 2005; Kirik et al., 1998, 2000a, 2000b; Shin et al., 2014). We also observed that small lesions produced moderate contralateral forelimb akinesia that was maintained at repeated testing intervals (Decressac et al., 2012; Kirik et al., 1998; Shin et al., 2014). These findings further support the validity of our lesion methods as impaired forelimb stepping in the adjusting steps test is a standard finding after intrastriatal 6-OHDA lesions.

In study two we included a single-pellet skilled reaching task as a measure of complex motor skill. The single-pellet reaching task is most often used in conjunction with MFB lesions, which substantially impair reaching accuracy on this task (Klein et al., 2007; Metz et al., 2003; Vergara-Aragon et al., 2003). Similarly, others have reported that reaching accuracy in this task is substantially impaired after large striatal lesions (Plowman et al., 2013, 2014). In the current study we found that large lesions reduced skilled reaching accuracy to about 60% of pre-surgery performance. This reduction is less severe than after the large striatal lesions made by Plowman et al. (2013, 2014), who found that reaching accuracy was reduced to about 20% of baseline levels. This difference is, however, readily explained by the different pre-surgery training criterion used between our studies. In the Plowman et al. studies rats were trained to a minimum of 35% accuracy with an average group accuracy of about 45%. In the current study we trained all rats to >60% accuracy with the group average being >70%, which is line with previous reports (Alaverdashvili and Whishaw, 2008; Vergara-Aragon et al., 2003). It is likely that the more extensive pre-surgery training used in our study preserved post-lesion skilled reaching accuracy to greater degree than the less extensive training used by Plowman et al. The extensive pre-surgery training used in the current study shows that our skilled reaching task was robust and therefore suitable for assessing skilled reaching accuracy.

In study three we used the cylinder test to examine spontaneous forelimb use asymmetry (Schallert et al., 2000; Woodlee et al., 2008). As discussed in Chapter 7, we found

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that large lesions increased preferential use of the ipsilateral forelimb compared to sham rats (Carlsson et al., 2009; Henning et al., 2007; Shin et al., 2014) whereas small lesions did not (Decressac et al., 2012). We also extended the cylinder test to include the newer measure of serial stepping (Woodlee et al., 2008). We found that serial stepping was slightly but significantly increased for rats with large lesions only. In comparison, Woodlee et al. (2008) found substantial serial stepping occurred after the more extensive MFB lesions. The method used for the cylinder test in this study was consistent with previous reports (e.g. Decressac et al., 2012; Grealish et al., 2010; Woodlee et al., 2008) although due to time-restrictions we only coded the pre-surgery and day 42 post-surgery videos. Examination of the other post-surgery time-points may reveal additional differences once these videos are scored.

In study three we assessed sensory neglect in the corridor test (Dowd et al., 2005a). Consistent with previous research we found that large lesions markedly reduced sugar pellet retrievals from the contralateral to lesion side of the corridor (Dowd et al., 2005a; Fitzsimmons et al., 2006) while small lesions produced an intermediate impairment (Decressac et al., 2012) although others have not found an impairment after small lesions (Shin et al., 2014). The corridor test procedure used in this study was the same as previous reports where two test sessions were added together to determine a rats score (Cederfjall et al., 2012, 2013; Dowd et al., 2005a; Fitzsimmons et al., 2006). However, others have used a single test session (Decressac et al., 2012; Grealish et al., 2008; Torres et al., 2008) or have used the two most similar of three sessions (Shin et al., 2014). In our opinion any of these methods is suitable as our casual observations revealed that rats tended to show a consistent bias across test sessions.

In study three we used the same-side and cross-midline whisker evoked forelimb placing tests to examine sensorimotor integration (Woodlee et al., 2005). On the same-side whisker test we found that large lesions substantially impaired forelimb placement whereas

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small lesions produced a moderate deficit (Grant et al., 2009; Grealish et al., 2008; McCoy et al., 2008; Pienaar et al., 2008). Similarly, on the cross-midline whisker test rats with large lesions were substantially impaired while rats with small lesions had an intermediate deficit (Anstrom et al., 2007; McCoy et al., 2008; Pienaar et al., 2008; Woodlee et al., 2005). The inclusion of the cross-midline test in this study in the context of a PD model is novel and to the best of our knowledge no study has employed this test after large striatal lesions. Testing forelimb placement by both same-side and cross-midline vibrissae stimulation is important as it allows the experimenter to distinguish if the impairment is primarily sensory or motor in nature. As discussed in Chapter 7, impairment on both versions of the test confirmed that the deficit was primarily due to a motor than sensory deficit.

Lastly, in study three we examined postural instability in the newer postural instability test (Woodlee et al., 2008). Similar to Woodlee et al. (2008) who used MFB lesions we found that both small and large lesions produced postural instability of the contralateral forelimb and enhanced compensatory function of the ipsilateral forelimb. We found that large lesions produced a greater change in both forelimbs compared to rats with small lesions. The inclusion of this test is a new addition to this field as to the best of our knowledge the postural instability test has not been used after small and large striatal lesions.

Taken together this summary shows that we were successful at producing motor impairments in the unilateral intrastriatal 6-OHDA lesion model of PD. We found that large lesions produced substantial impairments on rotation after d-amphetamine and apomorphine, forelimb akinesia, skilled reaching, postural instability, forelimb use asymmetry, sensorimotor integration, and sensory neglect. In comparison, small lesions produced lesser impairments on forelimb akinesia, postural instability, sensorimotor integration, and sensory neglect, but no impairment for forelimb use asymmetry and rotation after apomorphine. The

difference in severity of deficits between small and large lesions confirms that we successfully produced a graded PD-like impairment in our rats.

8.3. Progesterone Improves Some Motor Skills

The current thesis has produced novel evidence regarding the beneficial influence of PROG on motor impairments after intrastriatal 6-OHDA lesions. The most striking finding was on the adjusting steps test for forelimb akinesia. In study two we found that 7 days of treatment with 4 mg/kg PROG and 8 mg/kg PROG, started at day 8 after surgery with tapered withdrawal on the sixth and seventh days of treatment, reduced forelimb akinesia in both the short-term and long-term after large striatal lesions that modelled the late stages of PD degeneration. We replicated this finding in study three using large lesions and 8 mg/kg PROG. In addition, we extended this finding to rats with small striatal lesions that modelled the earlier stages of PD degeneration. Taken together these findings show that PROG reduces forelimb akinesia in the adjusting steps test after small and large 6-OHDA lesions of the striatum. Furthermore, the beneficial influence of PROG was not simply symptomatic relief as the improvement on forelimb akinesia was sustained but did not improve further during the six weeks of testing after the treatment had stopped. The lack of further improvement supports the role for progesterone in neuroprotection, rather than promoting brain recovery. This finding is very promising, although it needs to be confirmed by independent replication in another laboratory.

We also found that PROG provided some benefits on the postural instability and whisker evoked forelimb placing tests. Specifically, the enhanced compensatory function of the ‘good’ ipsilateral forelimb in rats with large lesions was normalised by PROG treatment on the postural instability test. In addition, PROG prevented the increasing impairment evident in rats with small and large lesions in the cross-midline version of the whisker test.

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It is important to recognise that while PROG improved forelimb akinesia, whisker evoked forelimb placing, and compensatory function of the ‘good’ forelimb, PROG did not improve performance in other tasks. PROG treatment did not improve skilled reaching accuracy on the single-pellet reaching task, forelimb use asymmetry on the cylinder test, sensory neglect on the corridor test, or rotation after apomorphine. The variation in the treatment effects across tasks was possibly caused by the stage of neuronal degeneration when the treatment was started. That is, at day 8 when PROG treatment was started the lesion induced impairments on these tasks may have progressed beyond the point for PROG to be beneficial. A positive note is that PROG did not exacerbate impairments for rats with lesions nor did it change motor skills for sham operated rats which suggests that PROG was not harmful on any of the motor skills tests used in this study.

As discussed in Chapters 6 and 7, the neural mechanisms underlying the beneficial influence of PROG are not clear. The benefits we observed were independent of striatal TH as we found that PROG did not improve striatal TH density when measured at 7 weeks after the treatment had stopped. However, this negative result does not rule out other potential dopaminergic factors such as the number of TH positive cells in the SN and VTA. Thus, the treatment effects of PROG are possibly mediated through neuroprotection of dopaminergic neurons, because improved forelimb stepping in the adjusting steps test is related to dopamine function but it may not be dopamine specific. Furthermore, the beneficial influence of PROG could have been mediated through non-dopaminergic mechanisms. For example, acute administration of non-dopaminergic agents such as adenosine A_{2A} receptor or metabotropic glutamate R5 receptor antagonists improves forelimb stepping in the adjusting steps test in rats with 6-OHDA lesions (Ambrosi et al., 2010; Pinna et al., 2007, 2010). In another study 14 days administration of a novel diketopiperazine provided sustained long-term improvement in the adjusting steps test independent of TH (Krishnamurthi et al., 2009).

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Therefore, it is possible that the mechanisms responsible for the beneficial influence of PROG in this study were non-dopaminergic although further work elucidating these mechanisms is required.

Previous studies have shown that administration of low doses of PROG prevented MPTP-induced DA depletion when PROG was administered prior to and continued during MPTP injections in mice (Callier et al., 2001; Grandbois et al., 2000). Similarly, others have found that low doses of PROG also prevented DA depletion when PROG was administered prior to toxic injections of methamphetamine in mice (Yu et al., 2002; Yu and Liao, 2000). However, studies that have administered PROG after 6-OHDA lesions have shown inconsistent results regarding DA protection. Chao et al. (2011) found that neither 4 mg/kg PROG nor 8 mg/kg PROG protected DA depletion when administered once daily for 13 days starting 24 hours after large 6-OHDA striatal lesions. In comparison, Casas et al. (2013) reported that 4 mg/kg PROG administered once daily for three days from day 7 after small 6-OHDA striatal lesions improved K^+ evoked [3H]-DA from striatal slices. In study two we found that PROG was unable to protect striatal TH as the fibre denervation was already complete at the time of the first PROG injection at day 8 after large striatal lesions. Taken together it is possible that the timing of treatment initiation and the size of the DA lesion are important factors. Therefore, it appears that PROG can increase the brains resilience to the DA depleting effects of neurotoxins when administered either before MPTP or methamphetamine lesions (Callier et al., 2001; Grandbois et al., 2000; Yu et al., 2002; Yu and Liao, 2000) or when administered after small striatal 6-OHDA lesions (Casas et al., 2013) that produce mild striatal TH depletion. However, PROG does not prevent DA or TH loss when it is administered from 24 hours (Chao et al., 2011) or 8 days (study two) after large striatal 6-OHDA lesions that are known to substantially deplete striatal TH within 6-24 hours of surgery which then reach maximum depletion within 7-10 days (Cicchetti et al.,

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2002; Rosenblad et al., 2000; Walsh et al., 2011). Therefore, it would be worthwhile examining TH of the stored brain sections for study three of the current thesis as this experiment included both small and large striatal lesions.

Previous studies regarding the beneficial influence of PROG after DA lesions have provided limited assessments of motor function outcomes. In one study the contralateral rotation bias after apomorphine was reversed to an ipsilateral bias following 4 mg/kg PROG administered once daily for three days from day 7 after small striatal lesions (Casas et al., 2011). However, Chao et al. (2011) found no beneficial influence of PROG on rotational asymmetry after apomorphine. Similarly, Casas et al. (2013) found that PROG was not beneficial for rotational asymmetry after d-amphetamine. However, Casas et al. (2011) found that PROG improved a mild memory deficit in a novel object recognition test, and reversed depressive-like behaviour in a forced swim test after small 6-OHDA lesions. In contrast, Chao et al. (2011) found that PROG actually exacerbated spontaneous rotational asymmetry, hind limb foot slips on an elevated grid test, and symmetrical use of the forelimbs in the cylinder test when examined between days 4 and 13 after large 6-OHDA lesions. The findings of Chao et al. are inconsistent with our study as we found that PROG improved several motor skills (forelimb akinesia, enhanced compensatory function of the ipsilateral forelimb, and forelimb placements in the whisker test) but did not exacerbate other motor impairments. This inconsistency may be explained by the timing of PROG treatment after lesion surgery. As stated in Chapter 3, it takes approximately 3 days for 6-OHDA to metabolise and produce SN cell death following infusions to the striatum (Cicchetti et al., 2002; Walsh et al., 2011). Administering PROG at 24 hours after surgery in the Chao et al. (2011) study likely produced interactions with 6-OHDA metabolism that exacerbated the lesion and subsequent motor impairments. In comparison, the current study, and that of Casas et al. (2011, 2013), found that PROG improved some outcomes without exacerbating

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impairments when treatment was started 7 or 8 days after 6-OHDA lesion surgery. Therefore, it appears that the timing of PROG treatment after 6-OHDA infusions to the striatum is a critical factor. Here we have identified that 7 days of PROG treatment with tapered withdrawal started at day 8 after lesion surgery is an effective treatment regime.

The current study is an improvement on previous research in this field. Casas et al. (2011, 2013) examined affective and cognitive behaviour but did not thoroughly examine motor skills after small striatal 6-OHDA lesions. In comparison, Chao et al. (2011) examined several motor skills but only in the short term and after large 6-OHDA striatal lesion surgery. The current thesis has improved on these studies on three counts. First, we examined motor skills on a larger battery of tests. Second, we investigated both the short and longer term outcomes of PROG treatment. Lastly, we provided the first direct comparison regarding the beneficial influence of PROG treatment after small and large 6-OHDA lesions of the striatum. Taken together the current study has provided the most comprehensive examination to date regarding PROG's influence on motor impairments after 6-OHDA lesions.

Here we have shown that PROG treatment improves forelimb akinesia after 6-OHDA lesions. However, it is essential to consider this finding in the wider context of the PD treatment problem. As discussed in Chapter 2, L-DOPA is currently the gold-standard treatment for the motor complications of PD (Smith et al., 2012). Therefore, it needs to be considered if PROG will offer any benefit over L-DOPA. In the current study PROG improved forelimb akinesia after large striatal lesions although these rats were still impaired compared to sham rats. In comparison, PROG improved forelimb akinesia for the small lesion group so that these rats were similar to sham rats. In all groups PROG had a beneficial influence of a large effect size (Table 8.1). Several studies have examined the beneficial influence of acute L-DOPA administration on the adjusting steps test after 6-OHDA lesions of the MFB. In two studies severely impaired forelimb stepping was improved to about 50%

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of unimpaired performance after L-DOPA with approximate effect sizes ($d = \sim 1.79-2.09$) similar to the effect sizes we obtained in our study with PROG (Chang et al., 1999; Olsson et al., 1995). In comparison, three other studies found that L-DOPA administration returned severely impaired forelimb stepping to about unimpaired levels with effect sizes larger ($d = \sim 3.10$) than we found in our study (Kasture et al., 2009; Pinna et al., 2007, 2010). Therefore, it appears that acute administration of L-DOPA in rats with MFB lesions produces an effect equal to or greater than the effect we found with short term PROG treatment in rats with small and large striatal lesions.

However, the beneficial influence of L-DOPA treatment only lasts for up to several hours and as PD progresses the beneficial duration of each dose decreases and intolerable side effects such as dyskinesia occur (Hornykiewicz, 2002; Tarazi et al., 2014). In comparison, we found that a short course of PROG treatment provided long-term improvement in forelimb akinesia. Thus treatment with PROG clinically may prevent the further worsening of akinesia, although PROG's efficacy may vary with the stage of neurodegeneration at the time of treatment. Importantly, PROG has not been associated with any serious side effects in over 1000 patients administered PROG in Phase II and III clinical trials for TBI (Skolnick et al., 2014; Wright et al., 2006, 2014; Xiao et al., 2008). Therefore, this raises the possibility that PROG could be used as a safe adjunct therapy alongside L-DOPA for the treatment of akinesia. If effective, PROG has the potential to reduce the dose of L-DOPA required and/or to increase the beneficial duration of each dose which would reduce the negative side effects associated with chronic L-DOPA use.

PROG's potential as an adjunct therapy for akinesia should first be investigated in an animal model that examines the dose response and beneficial duration of L-DOPA in rats treated with PROG. Following positive findings we think that a small clinical trial would be warranted as PROG is a safe agent to give to people as discussed above. Although PROG did

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not successfully translate to the treatment of TBI in recent Phase III trials (Skolnic et al., 2014; Wright et al., 2014) it is still worthwhile trialling PROG for other brain injuries and diseases due to its safety and the extensive pre-clinical evidence regarding its beneficial influence in over 20 models of brain injury and disease as discussed in Chapter 3. However, like others (Stein, 2014; Wright et al., 2014), I believe that future clinical trials should consider adaptive trial design to improve translation from the laboratory to the clinic (see Chow and Chang, 2008 for a review of adaptive trial design methods). The benefit of PROG treatment is that it may be a suitable adjunct therapy that would be simple and cheap to administer, and is an invasively minimal approach that may improve PD motor symptoms. By comparison other promising treatments such as cell replacement therapy and glial cell-line derived neurotrophic factor therapy are highly invasive and expensive, and can be associated with negative side effects such as graft induced dyskinesia (Kordower and Bjorklund, 2013; Lindvall and Bjorklund, 2014).

8.4. Limitations and Future Directions

The current study examined the beneficial influence of PROG in a rodent model of PD. As discussed in Chapter 4, an ideal rodent model of PD should recapitulate as many of the key features of PD as possible. The 6-OHDA model used in this thesis closely mimics nigrostriatal DA degeneration and the key motor features of PD and thus is useful for examining novel treatments aimed at reduction of motor symptoms (Kirik et al., 1998). However, there is not a single model that exactly replicates PD, thus multiple models that reproduce different aspects of the disease should be used for drug development. The 6-OHDA model, like many others, does not reproduce the degeneration of other neural systems relevant to PD pathology. Indeed, degeneration of the noradrenergic, serotonergic, cholinergic, and many other neural systems also occurs (Braak et al., 2003; Delaville et al., 2011; Halliday et al., 2011). Furthermore, the 6-OHDA model does not produce Lewy

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pathology which is a key pathological hallmark of PD (Braak et al., 2006). These non-dopaminergic changes have not been well modelled in animals. In recognition of this limitation an interesting recent study found that combined 6-OHDA and noradrenalin lesions produced significantly worse motor impairments than DA lesion alone (Shin et al., 2014). Similarly, combined intranigral administration of AAV- α -synuclein and rotenone is a promising model that produces reliable motor impairments, nigrostriatal degeneration, and α -synucleinopathy (Mulcahy et al., 2012). Another new approach closely replicates Lewy pathology through prion-like cell-to-cell transfer of aggregated α -synuclein (Luk and Lee, 2014). These newer approaches to modelling PD could better replicate different aspects of the human disease. Despite the limitations of the 6-OHDA model its use to study the beneficial influence of PROG was appropriate as virtually all drugs currently in clinical use have shown efficacy in this model (Duty and Jenner, 2011). It would nonetheless be advisable to examine PROG in these newer models that mimic different aspects of PD pathology.

Time constraints in the current study prevented additional histology from being conducted. Analysis of TH positive cell bodies in the SN and VTA were intended to be part of study two (Chapter 6) as the cells in the SN and to a lesser extent in the VTA are selectively killed by intrastriatal 6-OHDA lesions (Kirik et al., 1998; Grealish et al., 2008). Furthermore, due to time restrictions no histology was conducted for study three (Chapter 7). The lesions in study three were validated with a rotation after d-amphetamine test, as per previous research (Grealish et al., 2008, 2014; Torres et al., 2007). However, histology work is needed to evaluate the extent of the small and large lesions and to assess the beneficial influence of PROG on striatal and nigral TH. The stained brain sections from study two and stored brain sections from study three will be processed for TH histology in the future.

Another limitation was the lack of histology regarding the mechanisms that contributed to PROG's beneficial influence on motor impairments. Therefore, it is not known

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if PROG treatment is influencing the disease process produced by 6-OHDA lesions. Future work should investigate markers of inflammation as this is a key pathogenic factor in PD (Barcia, 2013; Peterson and Flood, 2012). Previous studies have shown that expression of activated microglia and pro-inflammatory cytokines are increased after 6-OHDA lesions (Ambrosi et al., 2010; Cicchetti et al., 2002; Jin et al., 2008; Walsh et al., 2011). Importantly, PROG has reduced expression of activated microglia and pro-inflammatory cytokines in other models of experimental brain injury (Cutler et al., 2007; Garay et al., 2012; Ishrat et al., 2010; Labombarda et al., 2011). To investigate PROG's beneficial influence on inflammation future studies should stain brain sections against CD11b/OX-42, a marker of activated microglia. We have piloted the CD11b protocol with moderate success but further work is needed to optimize this protocol in our laboratory. The stored brain sections provide an opportunity to perform the CD11b procedure in future. This work may tell us if PROG treatment changed activated microglia expression and thus influenced the underlying degenerative process.

In addition to inflammation, other factors that contribute to the pathology of PD should be investigated as the mechanisms underlying PROG's neuroprotection are pleiotropic (Stein, 2008). Future studies should examine PROG's influence on brain-derived neurotrophic factor, mitochondria function, oxidative stress, apoptosis, and, blood-brain barrier integrity as PROG has improved each of these PD related pathophysiological factors in other experimental models of brain injury and disease (Deutsch et al., 2013; Sayeed and Stein, 2009).

Another limitation was the lack of pharmacokinetics, pharmacodynamics, and blood plasma levels of PROG. Examining these factors of PROG would provide important data regarding the absorption, half-life, central uptake, and reduction of PROG to its active metabolite allopregnanolone. This information would be useful for optimizing dosage in

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future studies and help interpret the dose independent efficacy of PROG on the adjusting steps test in Experiment two (see Chapter 6).

The current study provided a thorough examination regarding the beneficial influence of PROG on motor impairments. However, it is not known if PROG would have benefited non-motor impairments which also contribute to reduced quality of life in PD (Chaudhuri et al., 2010). Studies that investigate non-motor impairments often employ a small bilateral 6-OHDA lesion that does not produce obvious motor impairments (MacDowell and Chesselet, 2012). For example, anhedonia, anxiety-like, and depressive-like behaviour have been reported after these lesions while motor behaviour was not affected (Santiago et al., 2010; Tadaiesky et al., 2008). This small bilateral lesion paradigm was not appropriate for the current study as the aim of this thesis was to examine overt motor impairments. It would be interesting to examine the beneficial influence of PROG on non-motor symptoms using these small bilateral lesions in a future study.

The current study showed that PROG treatment considerably improved forelimb akinesia, and to a lesser extent sensorimotor integration and compensatory function of the ipsilateral forelimb. However, PROG did not improve skilled reaching, sensory neglect, forelimb use asymmetry, or rotation after apomorphine. It is possible that the PROG treated rats could have shown some improvement in these and other tests if they had ‘learned to use progesterone’. This suggestion echoes the phenomenon known as ‘learning to use the transplant’, where an animal must undergo specific training in order to produce a functional benefit of a neural graft, especially for complex tasks (Döbrössy and Dunnett, 2001). For example, Mayer et al. (1992) pre-trained rats on a complex lateralised-nose poking task prior to unilateral ibotenic acid lesions of the striatum. Half of the rats received striatal grafts then all rats were re-tested on the task six months later. Both grafted and non-grafted rats with lesions were severely impaired whereas sham rats rapidly reacquired the task. However, the

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graft group markedly improved only after repeated testing whereas the non-grafted lesion group did not. This suggests that the grafted rats needed to learn to use their transplants through experience in the task. In other words, the grafts provided a necessary substrate for new sensorimotor learning to occur (Reddington et al., 2014). In the current study it is possible that PROG treatment also provided a substrate for new motor skill learning to occur. However, most of the motor tasks used in this study were relatively simple and administered briefly at each testing occasion. PROG treatment may have shown efficacy in a task if the rats were given the opportunity to ‘learn to use progesterone’ through repeated and massed testing across days. This is especially relevant for the skilled reaching task which was the most complex motor skill test used in this study. It would therefore be of interest to investigate the beneficial influence of PROG in complex motor skill learning tasks that provide the rat with an opportunity to ‘learn to use progesterone’.

The current study has provided initial promising evidence regarding the beneficial influence of PROG in an animal model of PD. The Stroke Academic Industry Roundtable (STAIR) have developed a set of recommendations (Table 8.2) to guide the preclinical development of novel drug therapies prior to clinical trials (Fisher et al., 1999, 2009). Although these recommendations were developed for the study of stroke, these general principles can be applied to other models of brain injury and disease (Deutsch et al., 2013). These criteria can be used to inform the direction of future research based on the promising findings of this study.

Table 8-2.

Summary of STAIR Criteria Relevant to Parkinson's disease Studies

1. Adequate dose-response curve✓
2. Therapeutic time window defined✓
3. Histological✓ and functional✓✓ outcome measures in both short and long-term
4. Study of young then aged animals with comorbidities if that is the intended population for clinical trials
5. Studied in both male and female animals
6. Fundamentals of good scientific inquiry satisfied (e.g. random assignment, inclusion/exclusion criteria, blinded assessment of outcome etc).✓✓

Note: Check marks indicate criteria satisfied in current study. ✓=partially satisfied; ✓✓=fully satisfied (Table adapted from Fisher et al., 2009, see reference for full criteria relevant to stroke).

The first criterion, adequate dose-response curve, was partially addressed in study two where we compared two PROG doses. We found that both doses were more or less equally beneficial on forelimb akinesia although we did not identify the minimum effective or maximum tolerable dose. It would be worthwhile investigating the influence of PROG across several doses to determine the dose-response curve. The doses examined should range between 2 mg/kg and 32 mg/kg as dose-response studies for TBI and ischemic injury have found that 8 mg/kg and 16 mg/kg were effective whereas 32 mg/kg provided little benefits in comparison (Goss et al., 2003; Wali et al., 2014; Yousuf et al., 2014).

The second criterion, define the therapeutic time-window, was also partially addressed in study three where we compared small and large lesions that modelled the earlier and later stages of PD degeneration, respectively. PROG treatment was beneficial for both

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small and large lesions which suggest that treatment can be initiated at the earlier or later stages of PD degeneration. For both lesion types we initiated treatment at day 8 after surgery. Delaying treatment till day 8 was suitable for this study as the aim was to initiate treatment once a PD like impairment was present. However, it is not known if initiating treatment at different time-points after surgery would also be beneficial although one study found that PROG treatment started 24 hours after large intrastriatal 6-OHDA lesions exacerbated motor symptoms (Chao et al., 2011). A future experiment that varies the time-point for treatment initiation would provide additional data regarding the therapeutic time-window for PROG treatment in the 6-OHDA model of PD.

Related to the therapeutic time-window is the duration of PROG treatment. In the current study we administered PROG for 7 days with tapered withdrawal on the sixth and seventh days of treatment. This treatment method was appropriate as it is the best treatment regime in acute brain injury models and because there is little data regarding chronic PROG administration (Cutler et al., 2005, 2006). Unlike acute brain injury, PD is a chronic and progressive disorder. Therefore, building on the promising results of the 7 day treatment regime, future studies should examine chronic dosing and/or repeated treatment intervals as these would be relevant to the treatment of PD.

The third criterion, acute and long-term assessment of histological and function outcomes, was moderately and well addressed in this study, respectively. We assessed motor impairments in both the short and longer-term after lesion surgery. We showed that PROG treatment improved forelimb akinesia when tested several days after the last PROG injection. Furthermore, this benefit was sustained at repeated testing intervals for six-seven weeks after PROG treatment had stopped. The demonstration of sustained functional benefit of PROG treatment is especially encouraging as STAIR recommendations emphasise improvements in functional outcomes (Fisher et al., 2009). We provided initial long-term histology although as

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discussed above additional histology work is needed to fully assess the beneficial influence of PROG.

STAIR recommends that studies first use young healthy animals then move to testing older animals with comorbidities if that is the target population. The approximate lifespan of the rats used in the current study is 2-3.5 years (Sengupta, 2011). Therefore, the current study used healthy adult rats (about 8 months old at surgery) which are more clinically relevant compared to previous research which used young rats (~3 months old at surgery). It should be noted that the rats used in this study weighed ~600g (max 700g) at the end of the experiment with no difference between experimental groups (see Chapter 7, Fig. 7.9). However, the rats were not showing obvious signs of obesity. In future studies it would be worthwhile comparing the beneficial influence of PROG in lean and large rats as obesity can exacerbate dopamine depleting lesions (Sriram et al, 2002). The current study did not include aged rats due to time restrictions. Examining the influence of PROG in aged rats is particularly relevant as PD is primarily a disorder of the elderly (Van Den Eeden et al., 2003). Similarly, STAIR also recommends that male and female animals are studied. The current study used male rats as the hormonal cycling of female rats would have complicated findings regarding PROG treatment. However, future investigations should include both normal cycling and ovariectomized female rats to show that the beneficial influence of PROG also extends to female animals.

Lastly, the current study satisfied STAIR recommendations for good scientific inquiry. That is, this study provided clear inclusion and exclusion criteria according to the rotation after d-amphetamine test and all rats that were excluded from the study were reported with reasons provided. Matched groups of rats according to pre-surgery behavioural performance were randomly assigned to treatments by an independent investigator with assessment of outcomes performed blinded to the treatment condition to which a rat

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belonged. STAIR also recommends that studies report how the size of the experiment was planned. In the current study the number of rats used in the different conditions was conservative and derived on the basis that little had been established with respect to PROG and parkinsonian symptoms. The findings of this study can be applied to future research to assist sample size calculations. Taken together the execution of this study is regarded as high in quality as it adhered relatively well to the STAIR recommendations for good scientific inquiry.

As discussed above, the key direction for future research is to investigate PROG's potential as an adjunct therapy for the treatment of akinesia. Following positive findings in an animal model it would be appropriate to examine the beneficial influence of PROG in a pilot open-label clinical trial as no serious adverse events have been found in 1000+ patients tested thus far in clinical trials for TBI. If beneficial, PROG would be a safe adjunct treatment for akinesia.

8.5. Conclusion

The current thesis has provided the most thorough examination to date regarding PROG's influence on motor skills in an animal model of PD. Furthermore, this study has produced novel evidence of the beneficial effects of PROG treatment on forelimb akinesia.

Importantly, the improved forelimb akinesia was not merely symptomatic relief as the benefit was sustained for up to six weeks after PROG treatment had stopped. The mechanisms responsible for the improved akinesia are not clear but it appears to be independent of striatal TH density. These initial findings are encouraging as PROG is a safe agent and is unlikely to have adverse side effects when used as a clinical tool in older patients. It is proposed that PROG be considered as a safe, and low-cost, adjunct therapy to treat akinesia in a pilot clinical trial once an additional animal study is complete. Taken together, the encouraging

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findings of the current study represent a critical step in understanding the beneficial influence of PROG on motor impairments in an animal model of PD.

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Appendix A

Cohen's d pairwise effect sizes for studies two and three

Table 1.

*Cohen's d Pairwise Effect Sizes for Study 2: Progesterone after large intrastriatal lesions.**

Task	Effect	Cohen's d	95% CI	Power (%)
Rotational bias after d-amphetamine	Lesion	6.22	-7.21, -5.24	100
	8 mg/kg	0.09	-1.04, 0.86	5
	4 mg/kg	0.6	-1.50, 0.30	26
	Lesion only control	11.98	-13.07, -10.98	100
Akinesia: Adjusting steps test	Lesion	3.11	2.12, 4.09	100
	8 mg/kg	1.4	0.45, 2.35	82
	4 mg/kg	1.33	0.43, 2.22	82
Skilled reaching: Success rate	Lesion	1.13	0.14, 2.11	61
	8 mg/kg	0.23	-1.18, 0.72	8
	4 mg/kg	0.06	-0.83, 0.96	5
Skilled reaching: Hit rate	Lesion	1.07	0.09, 2.06	57
	8 mg/kg	0.06	-1.01, 0.89	5
	4 mg/kg	0.14	-0.76, 1.04	6
Rotational bias after apomorphine	Lesion	1.52	0.53, 2.50	85
	8 mg/kg	0.15	-0.83, 1.14	6
	4 mg/kg	0.32	-0.58, 1.22,	11
Striatal tyrosine-hydroxylase fibre	Lesion	4.8	3.79, 5.81	100
	8 mg/kg	0.53	-0.45, 1.51	19

Task	Effect	Cohen's d	95% CI	Power (%)
density	4 mg/kg	0.08	-0.85, 1.01	5
	Lesion only control	5.19	4.10, 6.28	100

* Note: The large lesion effect is compared to sham and the potential PROG treatment benefits are compared to large lesions.

Table 2.

*Cohen's d Pairwise Effect Sizes for Study 3: Progesterone after small and large intrastriatal lesions.**

Task	Effect	Cohen's d	95% CI	Power (%)
Rotational bias after d-amphetamine	Large lesion	4.9	4.08, 5.72	100
	Small lesion	2.68	1.86, 3.51	100
	Large PROG	0.48	-0.45, 1.35	19
	Small PROG	0.24	-0.64, 1.11	8
Akinesia: Adjusting steps test	Large lesion	4.84	4.01, 5.66	100
	Small lesion	2.02	1.20, 2.85	100
	Large PROG	2.09	1.23, 2.94	100
	Small PROG	1.36	0.48, 2.23	86
Postural instability: Contralateral forelimb	Large lesion	3.28	2.46, 4.11	100
	Small lesion	1.19	0.37, 2.01	81
	Large PROG	0.43	-0.42, 1.28	17
	Small PROG	0.22	-0.66, 1.09	8
Postural instability: Ipsilateral forelimb	Large lesion	1.91	1.09, 2.73	99
	Small lesion	0.41	-0.41, 1.23	16
	Large PROG	1.17	0.32, 2.03	76

Task	Effect	Cohen's d	95% CI	Power (%)
	Small PROG	0.38	-0.49, 1.26	14
Sensorimotor integration: Whisker test (same side)	Large lesion	6.45	5.63, 7.27	100
	Small lesion	1.41	0.58, 2.23	92
	Large PROG	0.28	-0.58, 1.13	10
	Small PROG	0.53	-0.35, 1.40	22
Sensorimotor integration: Whisker test (cross midline)	Large lesion	5.88	5.06, 6.70	100
	Small lesion	1.62	0.80, 2.44	97
	Large PROG	0.3	-0.55, 1.16	11
	Small PROG	0.71	-0.16, 1.59	36
Forelimb use asymmetry: Cylinder test	Large lesion	1.49	0.65, 2.34	93
	Small lesion	0.22	-0.60, 1.04	8
	Large PROG	0.11	-0.77, 0.99	6
	Small PROG	0.25	-0.63, 1.12	9
Sensory neglect: Corridor test	Large lesion	2.65	1.83, 3.47	100
	Small lesion	0.95	0.13, 1.77	62
	Large PROG	0.11	-0.74, 0.97	6
	Small PROG	0.31	-0.57, 1.18	11
Rotational bias after apomorphine	Large lesion	-3.77	-4.62, -2.93	100
	Small lesion	-0.91	-1.73, -0.09	58
	Large PROG	-0.12	-1.00, 0.76	6
	Small PROG	-0.74	-1.61, 0.14	38

*Note: The large and small lesion effects are compared to sham and the potential PROG treatment benefit is compared to the corresponding lesion group.