Exendin-4 promotes recovery of both behavioural and neurochemical deficits in a “pre-motor” rodent model of Parkinson’s disease

Nazir Rampersaud B.A. (magna cum laude), M.Sc.
Thesis submitted for the degree of Doctor of Philosophy
The School of Pharmacy
University of London
2010
Declaration

This thesis describes research conducted at The School of Pharmacy, University of London between September 2008 and August 2010 under the supervision of Dr. Peter Whitton. I certify that the research described is original and that any parts of the work that have been conducted in collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by a suitable citation any part of this work that had already appeared in publication.

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Date: 22/02/...
Abstract

Research on Parkinson’s disease (PD) has mainly focused on the degeneration of the dopaminergic neurons of nigro-striatal (NS) pathway; however, post-mortem studies have demonstrated that other brain regions are significantly affected as well (Jellinger, 1999). These other regions include the locus coeruleus (LC) and raphe nuclei (RN), which are principal sites of noradrenergic and serotonergic synthesis, respectively. Degeneration of these crucial neuronal cell bodies is generally thought to occur prior to the deterioration of dopaminergic neurons in the NS pathway and hence predates the appearance of the motor symptoms that characterize PD. Many patients in the early “pre-motor” stage of PD suffer from comorbid depression, anxiety, and cognitive deficits. These deficits may be the result of a loss in noradrenergic and serotonergic innervation given the prominent role of these neurotransmitters in both emotional and cognitive function (Cummings et al., 1999). These psychiatric disturbances greatly affect the patient’s quality of life. We have thus set out to create a “pre-motor” rodent model of PD which mimics the early stages of the condition. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a selective noradrenergic neurotoxin, and parachloroamphetamine (pCA), a selective serotonergic neurotoxin, were utilized concomitantly with bilateral 6-hydroxydopamine (6-OHDA) injections into the striatum to produce a premotor rodent model of PD with partial deficits in the dopaminergic, noradrenergic, and serotonergic systems. Behavioral deficits were assessed using a wide array of tests including sucrose preference, open field exploration, forced swim test, and novel object recognition. Neurochemical deficiencies were assessed using in vivo microdialysis, tissue content levels, and immunohistochemistry.

We then sought to evaluate the therapeutic value of Exendin-4 (EX-4) on our premotor rodent model. It has been previously demonstrated that EX-4, a glucagon-like peptide-1
receptor (GLP-1R) agonist, is neuroprotective in rodent models of PD (Bertilsson et al., 2008; Harkavyi et al., 2008). We found that EX-4 was able to reverse all neurochemical and behavioural deficits exhibited by our model and exhibits anti-depressant like properties. EX-4 preserved the functional integrity of the dopaminergic, noradrenergic, and serotonergic systems. In conclusion, we have generated a novel animal model of PD that recapitulates certain premotor symptomology. These symptoms and causative physiology are ameliorated upon treatment with EX-4 and thus it could be used as a possible therapy for the non-motor symptoms prominent in the early stages of PD.
Acknowledgements

I would like to first thank my supervisor Dr. Peter Whitton for providing me with the opportunity to embark on such an exciting research project and continually offering his guidance and unwavering support. I would also like to express my gratitude to my second supervisor Dr. Brian Pearce who has always provided me with invaluable advice, support, and guidance throughout my studies.

I must also acknowledge Dr. Ann Kingsbury who generously offered her time and expertise in immunohistochemistry to aid my work. I would like to thank Dr. Clare Stanford for aiding me with establishing protocols and providing advice regarding behavioural testing. I am also very grateful to Dr. Rebecca Lever for allowing me to use her microscope and providing constant insightful advice. I would also like to extend my gratitude to Steve Coppard, Dave Zeraschi, Michaela Lee, and Donna Howell for keeping my animals healthy and providing a pleasant atmosphere in the BSU.

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Lastly, but most significantly, I would like to extend my gratitude to my parents for always providing their enduring love and guidance throughout my life. I would like to especially thank my mother who never stopped believing in me and always pushed me to become a better individual; without her in my life none of this would be possible.
Dedication

For my Mother
"We live in a time when the words impossible and unsolvable are no longer part of the scientific community's vocabulary. Each day we move closer to trials that will not just minimize the symptoms of disease and injury but eliminate them." - Christopher Reeve
Publications Arising from this Thesis

RAMPERSAUD, N., ABUIRMEILEH, A., HARKAVYI A., STANFORD C., KINGSBURY A.E., & WHITTON, P.S. (2010). Exendin-4 promotes recovery of both behavioral and neurochemical deficits in a "pre-motor" rodent model of Parkinson's disease with concomitant partial noradrenergic and serotonergic lesions. Article to be submitted to Journal of Neuroscience


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<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
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<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
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<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
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<td>6-OHDA</td>
<td>6- Hydroxydopamine</td>
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<tr>
<td>AADC</td>
<td>Aromatic amino acid decarboxylase</td>
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<tr>
<td>Aβ</td>
<td>Amyloid beta peptide</td>
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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
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<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>Bcl2</td>
<td>B-cell leukemia protein</td>
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>BSU</td>
<td>Biological Services Unit</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CBT</td>
<td>Cognitive behavioural therapy</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>COMT</td>
<td>Catechol-O-methyl transferase</td>
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<td>COX-2</td>
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<td>CREB</td>
<td>Cyclic adenosine 3’, 5’- monophosphate response element binding protein</td>
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<td>Corticotropin-releasing factor</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>3, 3'-Diaminobenzidine</td>
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<td>Description</td>
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<td>DAT</td>
<td>Dopamine transporter</td>
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<td>DOPA decarboxylase</td>
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<td>Desipramine</td>
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<td>3, 4- Dihydroxyphenylacetic acid</td>
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<td>Electrochemical Detection</td>
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<td>EX-4</td>
<td>Exendin-4</td>
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<td>FC</td>
<td>Frontal cortex</td>
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<td>FOXO1</td>
<td>Forkhead transcription factor</td>
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<td>FST</td>
<td>Forced Swim Test</td>
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<td>Gama-aminobutyric acid</td>
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<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
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<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
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<td>GLP-1R</td>
<td>Glucagon-like peptide-1 receptor</td>
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<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
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<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<td>HPLC-ECD</td>
<td>High pressure liquid chromatography w/ electrochemical detection</td>
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<td>i.c.</td>
<td>Intracerebral (injection)</td>
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<td>I.D.</td>
<td>Inner diameter</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>Irs2</td>
<td>Insulin receptor substrate 2</td>
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<td>LC</td>
<td>Locus coeruleus</td>
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<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
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<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MPP+</td>
<td>1-Methyl-4-phenylpyridinium</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>NA</td>
<td>Noradrenaline, norepinephrine</td>
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<td>NAT</td>
<td>Norepinephrine (noradrenaline) transporter</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOR</td>
<td>Novel object recognition</td>
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<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>NRI</td>
<td>Noradrenaline reuptake inhibitor</td>
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<td>O.D.</td>
<td>Outer diameter</td>
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<td>OFT</td>
<td>Open field test</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PC12</td>
<td>Rat Phaeochromocytoma cell line</td>
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<td>pCA</td>
<td>para-chloroamphetamine</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<td>PDX-1</td>
<td>Pancreatic-duodenal homeobox</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<td>Protein kinase A</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>RN</td>
<td>Raphé nuclei</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>s.c.</td>
<td>subcutaneous (injection)</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SERT</td>
<td>Serotonin Transporter</td>
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<td>SGZ</td>
<td>Subgranular zone</td>
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<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
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<td>SNr</td>
<td>Substantia nigra pars reticulata</td>
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<td>SNRI</td>
<td>Selective noradrenaline reuptake inhibitor</td>
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<td>Sucrose preference test</td>
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<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
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<td>SVZ</td>
<td>Subventricular zone</td>
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<td>TCA</td>
<td>Tricyclic antidepressant</td>
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<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>UCN</td>
<td>Urocortin</td>
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<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter 1: General Introduction
1.1 History of PD

The pathology now referred to as Parkinson's disease (PD) has been known since medieval times and has afflicted all global populations. The ancient Indian medical system of Ayurveda described some symptomatic features of PD under the name *Kampavata* (Manyam *et al.*, 1999). Traditional therapies in the form of herbal preparations containing anticholinergics, levodopa, and monoamine oxidase inhibitors were used in the treatment of PD in India, China, and the Amazon region of South America (Gourie-Devi *et al.*, 1991). Galen of Pergamum (AD 138-201), a prominent Roman physician and philosopher, also described several features of PD and characterized it as the "shaking palsy". However, PD was not formally recognized and its symptoms were not documented until 1817 in *An Essay on the Shaking Palsy* by the British physician James Parkinson (Parkinson, 2002). PD was then known as *paralysis agitans*, the term "Parkinson's disease" being coined later by Jean-Martin Charcot. The underlying biochemical changes in the brain were identified in the 1950s largely due to the work of Swedish scientist Arvid Carlsson, who later went on to win a Nobel Prize in Physiology or Medicine for his research on dopamine (DA) (Carlsson *et al.*, 1957). PD is a progressive disorder and the motor symptoms manifest only when approximately 70% of the DA neurons in the substantia nigra have already degenerated (Fearnley *et al.*, 1991). Although there is no current cure for the disease, there are a number of effective symptomatic therapies available (Poewe, 2006). The first specific treatment to be used for PD was L-dihydroxyphenylalanine (L-DOPA), which entered clinical practice in 1967 (Hornykiewicz, 2002). The first large study reporting the efficacy of this drug in patients with PD was published in 1968 (Cotzias, 1968).
1.2 Epidemiology

PD is the second most common neurodegenerative disorder after Alzheimer’s disease. In industrialized countries it is estimated that approximately 0.3% of the whole population and 1% of the population above the age of 60 suffer from PD (de Lau et al., 2006). Globally, PD affects around 0.1% of the population; it is an age related disorder which affects about 1-3% of people above the age of 65 and 4-5% of people above the age of 85 years, only 5-10% of PD patients are below the age of 40 (early-onset PD) (Tanner et al., 1999). Twin studies have shown that the occurrence of PD in patients under 40 years old is almost always genetically caused (Tanner, 2003). In the U.S.A, the prevalence of PD rises from 0.3% in the general population to 1% to 2% in persons whose age is 65 years or older, with some data indicating a prevalence of 4% to 5% in individuals above the age of 85 years (Weintraub et al., 2008a).

PD occurs in all parts of the world; however there are prominent cross-cultural variations most likely due to dissimilarity in environmental exposures or the difference in susceptibility gene distribution (Van Den Eeden et al., 2003). PD exists both as an idiopathic and a familial disorder, and the contribution of environmental and genetic factors in the genesis of the disease is still not completely understood. PD appears to be more common in people of European ancestry than in those of African ancestry. Those of East Asian ancestry have an intermediate risk (Zhang et al., 1993). It is more common in rural than urban areas, and men are affected slightly more often than women. There have been several major epidemiological studies carried out in such countries as Finland, Holland, Spain, United Kingdom and United States (de Lau et al., 2004; Marion, 2001). All of these show similar patterns concluding that occurrence increases with age and that it is more common in males than in females with an estimated ratio of
2:1. In addition, PD is one of the few diseases that appear to occur less amongst the population who smoke tobacco (Veldman et al., 1998).

1.3 Motor and Non-Motor Symptoms of PD

The cardinal motor symptoms of PD are resting tremor, rigidity, bradykinesia, and postural instability. These symptoms emerge when almost all (around 80%) nigrostriatal dopaminergic innervation is lost (Tissingh et al., 1998). Resting tremor is the most apparent and well known symptom of PD although 30% of patients have little perceptible tremor and are referred to as akinetic-rigids. Rigidity is characterized by stiffness and increased muscle tone (Obeso et al., 2000; Quinn, 1997). Bradykinesia/akinesia is slowness and absence, respectively, of coordinated voluntary movements. Postural instability, which is characterized by a loss of postural reflexes, leads to balance impairment and falls. Other motor symptoms include dystonias which are abnormal, painful muscle contractions usually in the feet but may include other skeletal muscles. Such dystonic episodes are acutely painful and disabling for a patient. Since muscles in the face are also affected, the swallowing reflex is impaired; therefore, PD patients often drool. Impaired ability to swallow can lead to aspiration, pneumonia, and ultimately death. Speech disturbances and cognitive decline are common in patients with PD: these include decreased verbal fluency and cognitive disturbance especially related to comprehension of emotional content of speech and of facial expression (Pell, 1996). Even in the early stages of PD, subtle cognitive deficits can be identified, with patients exhibiting difficulty with frontal executive functioning in the form of bradyphrenia, impaired visuospatial processing, and deficits in attention (Lees et al., 1983). There are also various neuropsychiatric disturbances associated with PD such as mood and anxiety disorders, fatigue and apathy, anhedonia, psychosis, dementia, and
sleep disorders (Ferreri et al., 2006). One or more of the psychiatric symptoms are reported by more than 60% of patients with PD at some point in the course of their disease (Aarsland et al., 1999). Depression and anxiety affect more than 50% of patients with PD and in most cases it is thought that these affective disorders precede the motor symptoms of the condition (Cummings, 1992; Cummings et al., 1999; Shiba et al., 2000; Tolosa et al., 2007). There is also a correlation between depressive manifestation and greater motor deficits as PD pathology progresses (Ishihara et al., 2006; Schuurman et al., 2002). It is important to note that these neuropsychiatric deficits are most likely not only due to degeneration of dopaminergic neurons in the nigrostriatal pathway but could also be the result of a loss of serotonergic, noradrenergic, and cholinergic innervation in other regions of the brain (Braak et al., 2003; Chaudhuri et al., 2006; Schrag, 2004; Ziemssen et al., 2007). Mild cognitive impairment is present in 15-20% of early PD patients (Schrag et al., 2000). Dementia and psychosis are common in the late stages of the disease and the latter presents most commonly in the form of visual and auditory hallucinations (Williams et al., 2005). Sleep dysfunction is present in 60–98% of PD patients and is characterized by excessive daytime sleepiness, sleep attacks, advanced sleep phase syndrome, early morning awakenings, and rapid eye movement sleep behavior disorder (RBD) (Diederich et al., 2005). These sleep disorders can precede PD by many years (Postuma et al., 2009). Autonomic dysfunction is also common in PD and include nausea, constipation, urogenital problems, orthostatic hypotension, and excessive sweating (Park et al., 2009). Sensory deficits in PD include visual changes, pain, impaired olfaction, and restless legs syndrome (RLS). Hyposmia is present in up to 90% of PD patients and is one of the earliest symptoms of the pathology (Katzenschlager et al., 2004).
1.4 Diagnosis

Currently, the main PD diagnostic method is neurological examination based on patient presentation of clinical symptoms and previous medical history (Rao et al., 2006). There is no single laboratory or blood test which can be used to correctly diagnose PD. In addition, only 75% of PD cases are confirmed at autopsy (Gelb et al., 1999). Early signs and symptoms of PD may sometimes be interpreted as the effects of normal aging. The physician may need to observe the person for some time until it is certain that the symptoms are consistent. Neurologists usually look for unequivocal bradykinesia, a shuffling or dragging of the feet, and absence of arm swing, with a mild flexion of the arm at the elbow. The existing criteria for diagnosing PD require the presence of at least two of the following cardinal symptoms of the disease: resting tremor, bradykinesia, rigidity, or postural instability, with the exclusion of other potential causes of secondary parkinsonism (de Lau et al., 2006). Thus the diagnosis of PD is established when at least two motor symptoms are found, at least one being tremor or bradykinesia, and when patients respond positively to L-DOPA therapy (Gelb et al., 1999). The unified PD rating scale exists and helps to diagnose and measure the degree of severity of the disease. Doctors may sometimes request brain scans or laboratory tests in order to rule out other diseases. Demonstration of normal striatal dopamine-transporter uptake with dopamine transporter (DAT) single photon emission computed tomography (SPECT) can aid in determining whether or not anti-parkinsonian treatment is required (Scherfler et al., 2007). It has recently been reported that ultra-sound and high-resolution diffusion tensor imaging (DTI) of the caudal substantia nigra (SN) has 100% sensitivity and specificity for distinguishing patients with early PD from healthy controls (Vaillancourt et al., 2009; Woitalla et al., 2010). Despite the advances in imaging technology to aid diagnosis, these scans often fail to detect abnormalities in patients with clinical PD (Ribeiro et al., 2002).
The use of odor-identification tests, such as the University of Pennsylvania smell inventory test, can aid in diagnosis as hyposmia is one of the earliest symptoms of the pathology (Katzenschlager et al., 2004).

1.5 Pathophysiology

1.5.1 Dopamine

DA is the predominant catecholamine neurotransmitter in the mammalian brain where it has a variety of roles including cognition, locomotion, emotion, positive reinforcement of learning, food intake, and endocrine function. Roles of DA are not restricted to the central nervous system (CNS). In the periphery it serves as a modulator of cardiac function and vascular tone, as well as gastrointestinal motility and renal function. It is also involved in catecholamine release and hormone secretion. DA was first discovered in 1952 by the Swedish scientist Arvid Carlsson. The name “dopamine” came from its precursor in the biosynthetic pathway which is L-DOPA and also because it is a monoamine. Arvid Carlsson was awarded the Nobel Prize for demonstrating that DA was a neurotransmitter in its own right and not just a precursor of noradrenaline and adrenaline. Classical neurotransmitters like catecholamines are manufactured in one or several biochemical steps. These synthetic pathways are important firstly because they offer a regulatory mechanism for the amount of neurotransmitter available for release and secondly because the pathway can be altered by adding various drugs or precursors. The rate limiting step in production of DA is catalyzed by tyrosine hydroxylase (TH) where tyrosine is converted to dihydroxy-phenyl-alanine (DOPA). DOPA is then decarboxylated by aromatic amino acid decarboxylase (AADC) to form DA. Neurons that use DA as their neurotransmitter only possess TH and AADC unlike, for example noradrenergic nerves that also possess DA β-hydroxylase which in turn adds a hydroxyl
group to DA to form noradrenaline. There are several mechanisms of transmitter inactivation after release. The major mechanism is reuptake back into the nerve terminal via DA transporters present in the membrane. Released DA also undergoes enzymatic breakdown by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

There are five subtypes of DA receptors. D₁ and D₅ are stimulatory and are known as D₁-like. They are similar to each other in that both stimulate the production of cyclic adenosine monophosphate (cAMP), whereas D₂, D₃ and D₄ represent a separate group called D₂-like and inhibit cAMP production (Kebabian et al., 1979). All DA receptors are metabotropic, meaning they are G-protein-linked. D₁ and D₂ were the first to be discovered and are the most common types found in brain areas such as striatum (STR) and the nucleus accumbens which are the major termination sites of the nigrostriatal and mesolimbic DA pathways. Although DA receptors are widely distributed in the brain, different areas have different receptor type densities, reflecting different functional roles. D₁ are excitatory postsynaptic receptors whereas D₂ are inhibitory autoreceptors and are coupled negatively to adenylate cyclase (AC). These D₂ autoreceptors are normally found presynaptically except in case of pituitary gland, one of the functions of which is to secrete the hormone prolactin. Here, D₂-receptors are found postsynaptically and their function is to inhibit prolactin release by the pituitary. DA receptors are not only expressed in the CNS; for example, DA receptor subtypes D₁, D₂, D₄ and D₅ have been characterized in the human pulmonary artery where they are thought to mediate vascular tone (Cantello et al., 1989). In rats, DA receptors were found in blood vessels of most major organs and more specifically dopamine D₄ receptors were shown to be present in the atria of both rat and human hearts. DA has positive inotropic but not chronotropic effects on the heart and increases cardiac output (Cantello et al., 1989; Hussain et al., 2001). All types of DA receptors are also found in the kidneys. DA acting via the
receptors found in renal vasculature and the renal parenchyma is able to modulate kidney function (Aperia, 2000).

1.5.2 Noradrenaline

Noradrenaline (NA) is a catecholamine that functions both as a hormone and neurotransmitter. As a stress hormone, it is released along with adrenaline into the bloodstream from the adrenal medulla under sympathetic activation (Rang, 2003a). This activation usually takes the form of the “fight or flight response”, hence NA is able to increase heart rate, blood pressure, glycogenolysis in the liver, lipolysis in adipose tissue, and stimulate relaxation of bronchial smooth muscle. As a neurotransmitter, NA synthesis originates from the locus coeruleus (LC) and the lateral tegmental area in the brainstem. These regions project afferents to various areas of the brain including the hypothalamus, cerebellum, spinal cord, frontal cortex, striatum, amygdala, and hippocampus. NA is usually excitatory in nature and has been implicated in governing arousal, alertness, motivation, and emotional state (Tanaka et al., 2000).

NA was first isolated from the adrenal medulla by Polish physiologist Napoleon Cybulski in 1895. NA is synthesized by a series of enzymatic steps from the amino acid tyrosine (Rang, 2003a). Tyrosine is first converted to dihydroxy-phenyl-alanine (DOPA) by TH; thus TH is the rate limiting step for both the production of DA and NA. DOPA is then decarboxylated by aromatic amino acid decarboxylase (AADC) to form DA. DA is then converted into NA by dopamine β-hydroxylase which adds a hydroxyl group to DA to form noradrenaline. There are several mechanisms of transmitter inactivation after release. The major mechanism is reuptake back into the nerve terminal via NA transporters (NAT) present in the membrane. Released NA also undergoes enzymatic
breakdown by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). COMT breakdown produces Normetanephrine, whereas MAO generates 3,4-Dihydroxymandelic acid, Vanillylmandelic acid (3-Methoxy-4-hydroxymandelic acid), and 3-Methoxy-4-hydroxyphenylethylene glycol.

NA exerts its effects through activation of adrenergic receptors. Adrenergic receptors are G-protein coupled receptors which are divided into two main groups, α and β, with several subtypes (Rang, 2003a). α receptors have the subtypes α1 (a Gq coupled receptor) and α2 (a Gi coupled receptor) whereas β receptors have the subtypes β1, β2 and β3. All three β subtypes are linked to Gs proteins (although β2 also couples to Gi), which in turn are linked to AC. Agonist binding thus causes a rise in the intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include cAMP-dependent protein kinase (PKA), which mediates some of the intracellular events following hormone binding. Activation of both α and β receptors will generally cause a sympathetic response ("fight or flight response") in the PNS whereas CNS activation produces alterations in attention, arousal, cognition, and emotional disposition.

1.5.3 Serotonin

Serotonin (5-HT) is a prominent monoamine neurotransmitter biochemically derived from the amino acid tryptophan. It is mostly present in the CNS, gastrointestinal tract, and platlets. 5-HT has various functions in the CNS including the regulation of mood, appetite, sleep, muscle contraction, and several cognitive functions including memory and learning (Rang, 2003b). In the PNS, 5-HT functions to regulate GI motility, blood clotting, vasoconstriction, and cell growth. Vittorio Erspamer was the first person to isolate 5-HT from enterochromaffin cells in 1935. In the CNS, the neuronal cell bodies
of the raphe nuclei (RN) are the principal sources of 5-HT release in the brain. 5-HT afferents innervate numerous regions of the brain including the frontal cortex, hippocampus, and striatum. 5-HT is synthesized from tryptophan by two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC). The TPH-mediated reaction is the rate-limiting step in the pathway (Rang, 2003b). There are several mechanisms of transmitter inactivation after release. The major mechanism is reuptake back into the nerve terminal via 5-HT transporters (SERT) present in the membrane. Released 5-HT also undergoes enzymatic breakdown and is converted into 5-Hydroxyindoleacetic acid (5-HIAA), by MAO.

There are seven families of 5-HT receptor (5-HT₁-5HT₇) that mediate both excitatory and inhibitory neurotransmission (Hoyer et al., 1994). With the exception of the 5-HT₃ receptor, a ligand gated ion channel, all 5-HT receptors are G protein-coupled receptors that activate an intracellular second messenger cascade. 5-HT receptors modulate the release of many neurotransmitters, including glutamate, GABA, DA, adrenaline / NA, and acetylcholine, as well as many hormones, including oxytocin, prolactin, vasopressin, cortisol, corticotropin, and substance P, among others. 5-HT receptors influence various biological and neurological processes such as aggression, anxiety, appetite, cognition, learning, memory, mood, nausea, sleep, and thermoregulation (Berger et al., 2009). The 5-HT receptors are the target of a variety of pharmaceutical and illicit drugs, including many antidepressants, antipsychotics, anorectics, antiemetics, gastroprokinetic agents, antimigraine agents, hallucinogens, and entactogens.

1.5.4 Functional Neuroanatomy and the Parkinsonian State

The motor symptoms of PD result from a massive loss of dopaminergic neurons in the substantia nigra in the brain (Herrero et al., 2002). Pigmentation is lost in the pars
compacta region of the substantia nigra (SNpc) due to the loss of neuromelanin containing dopamine cells. Neuromelanin is thought to be a by-product of monoamine degradation (Zigmond, 2002). The neurons from substantia nigra (SN) project to the striatum and their loss leads to alterations in the neural circuits in the basal ganglia which are responsible for movement. According to the classic model of PD, the internal segment of the globus pallidus has an increased inhibitory drive to the thalamus that reduces the excitatory thalamic drive to the cerebral cortex (Figure 1.1) (Albin et al., 1989). This has been confirmed by various brain imaging techniques. More specifically DA depletion leads to inhibition of the direct pathway and disinhibition of the indirect pathway which ultimately leads to hypokinetic movement. The striatum modulates voluntary movement by these two pathways. In the direct pathway, striatal neurons directly inhibit the neurons in the globus pallidus internus which in turn inhibit thalamic neurons responsible for activation of the pre-motor cortex. Therefore, the direct pathway facilitates movement and anything inhibiting it causes akinesia. In the indirect pathway, striatal neurons inhibit neurons in the globus pallidus externus which in turn inhibit the subthalamic nucleus, both pathways then meet at the level of globus pallidus internus with the excitation of neurons in this region by the subthalamic neurons. Ultimately, these series of excitations and inhibitions result in an inhibition of movement by the indirect pathway, therefore damage to this as in PD results in inability to switch to new voluntary motor programs. The muscle rigidity and the resting tremor in PD are caused by abnormal synchronous oscillating neuronal activity within the basal ganglia (Jankovic, 2006).
Figure 1.1a: Basal Ganglia in normal and Parkinsonian state. This simplified diagram of the neural circuitry in the basal ganglia illustrates that loss of DA leads to alterations in the neural circuits, ultimately leading to excessive inhibition of the thalamus and therefore reduced excitation of the motor cortex.

Although the motor deficits arising in PD can largely be explained by deficiency of the dopaminergic system in the nigrostriatal pathway, other non-motor features of PD may be explained by neuronal loss in other brain regions (Braak et al., 2003; Chan-Palay et al., 1989; Jellinger, 1999; Kish, 2003). Extensive cell loss has been documented in the locus coeruleus, dorsal nuclei of the vagus, raphe nuclei, nucleus basalis of Meynert, and some other catecholaminergic brain stem structures including the ventrotegmental area (VTA) (Chan-Palay et al., 1989; Damier et al., 1999; Kish, 2003). Nerve-cell loss in these regions and also the substantia nigra is characterized by the presence of three subtypes of intraneuronal inclusions: the Lewy body, the pale body, and the Lewy neurite. Lewy bodies are divided into two categories based on their morphology - classical (brain-stem) and cortical. Classical Lewy bodies are spherical in structure and
measure 8–30 μm with a hyaline core surrounded by a peripheral pale-staining halo. It is composed ultrastructurally of 7–20-nm wide filaments with dense granular material and vesicular structures. Pale bodies are large rounded eosinophilic structures that often displace neuromelanin and are the predecessor of the Lewy body (Lees et al., 2009). An abnormal and insoluble aggregated form of the presynaptic protein α-synuclein (AS) is the main component of Lewy bodies. AS antibodies stain Lewy bodies and Lewy neurites, and have become the standard immunohistochemical method for diagnostic purposes (Wakabayashi et al., 2007). Cortical Lewy bodies do not possess the inner core and halo, and are common in small-to-medium-sized pyramidal neurons of layers V and VI of the temporal, frontal, parietal, insular cortices, cingulum, and entorhinal cortex. These cortical Lewy bodies are present in small numbers in almost all cases of PD (Halliday et al., 2008).

Braak and his research group have proposed a six stage scheme describing the rostral progression of AS pathology from brainstem regions to more cortical areas via vulnerable axonal pathways (Braak et al., 2006; Braak et al., 2003). The investigators suggested that the disease process begins in the gastric autonomic plexus of Meissner and the olfactory nerve endings, and then spreads to specific regions of the medulla oblongata and the anterior olfactory nucleus (Stage 1) (Braak et al., 2006; Braak et al., 2003). From the lower brainstem, the disease process gradually ascends into more rostral brainstem structures such as the LC and RN (Stage 2). Eventually, the pathology affects the pars compacta of the substantia nigra (Stage 3). Cortical pathology is restricted to the temporal mesocortex in the following stage of the disease, then extends into the neocortex and finally into the first-order sensory association and premotor neocortical areas (Stages 4-6). Lamina 1 spinal-cord neurons may also be involved in the early phase of the disease contributing to autonomic dysfunction (Braak et al., 2007). Several
research groups have validated this staging system, although at least 15% of patients with Parkinson's disease do not conform to this pattern (Kalaitzakis et al., 2008; Parkkinen et al., 2008). This observation does not negate the theory as it is not a requirement for lower stage structures to be completely degenerated before higher order structures are affected (Burke et al., 2008). In addition, different neuronal populations may vary in their genetic susceptibility to the pathologic process of AS deposition. Lewy body pathology in olfactory bulb and the dorsal motor nucleus of the vagus nerve is believed to be responsible for some features of sensory and autonomic dysfunction present in PD. LC and RN alterations are hypothesized to underlie the depression and sleep disturbances present in PD patients (Braak et al., 2005).
Figure 1.1b: Patterns of abnormal immunostaining for AS identified by Braak and colleagues. Six patterns of immunostaining were observed. In the pattern with the least extent of abnormal staining, involvement was observed only in region 1, which contains the dorsal motor nucleus of the vagus. In the pattern with the next most limited distribution, staining was observed, in addition to region 1, in region 2, which included the LC and other “gain setting nuclei.” In the pattern with the next most involvement, abnormal staining was observed not only in regions 1 and 2, but also region 3, which included the SN and the amygdala. Because each succeeding pattern of increased rostral involvement included pathology in the adjacent more caudal regions, Braak and colleagues proposed that PD begins in region 1 (stage 1), then proceeds rostrally to region 2 (stage 2), and so on. Clinical signs of parkinsonism and Lewy pathology in the mesencephalon, both of which are now required for a definitive diagnosis of PD, are hypothesized to occur late in the disease, at stage 3. 4 = region 4 including mesocortex and thalamus; 5 = region 5 including neocortex high order association; 6 = region 6 including neocortex, primary and secondary. (Figure and caption taken from Burke et al. 2008 and Braak et al. 2004)
1.6 Causative Factors

1.6.1 Environmental

The development of clinical motor symptoms in PD is preceded by an asymptomatic phase during which there is a progressive loss of dopaminergic neurons. Hallmark motor symptoms appear when this loss exceeds a certain threshold. It has been suggested by some research groups that this pre-symptomatic phase may start very early in life and may be the result of developmental insults such as pesticide exposure at an early age or intrauterine infections (Cory-Slechta et al., 2005; Logroscino, 2005). Other research groups hypothesize that it may start a few years before disease onset. Recent positron emission tomography (PET) studies assessing striatal fluorodopa $^{18}$F uptake suggest that the duration of the latent period is approximately 6 years (Hilker et al., 2005).

The earliest hypothesis on the pathogenesis of PD was based upon the fact that mitochondrial complex I (NADH dehydrogenase) inhibitors such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) and rotenone were able to induce parkinsonian symptoms leading to a selective loss of dopaminergic neurons in mouse (Seniuk et al., 1990) and primate models (Forno et al., 1986) of the disease. The toxic effect of the former compound was first inadvertently demonstrated in humans when a group of heroin addicts self-administered incorrectly synthesized heroin, which in fact was MPTP. This caused them to develop acute symptoms of PD overnight (Langston et al., 1983). The early animal models of PD did not fully mimic the condition because there were no Lewy bodies present; however, more recently, groups have utilized continuous infusion of rotenone and MPTP to produce a better representation of the pathology (Betarbet et al., 2000). These animal models suggest that sporadic PD might be caused by environmental toxins acting on mitochondrial complex I and thereby inhibiting its
function. Inhibiting complex I has two major consequences: firstly, ATP depletion and therefore impairment of all energy dependent processes and secondly, generation of free radicals. This has been shown by analyzing post mortem PD brains which show increased levels of lipid peroxidation markers and protein nitration in the SN suggesting increased levels of oxidative stress (Andersen, 2004). In mice and rat neuron–glial cell cultures, a non-toxic or minimally toxic concentration of rotenone and the inflammatory agent lipopolysaccharide (LPS) synergistically induced dopaminergic degeneration (Gao et al., 2003). This finding has led researchers to suggest that pro-inflammatory agents such as lipopolysaccharide might be an environmental factor in the development of PD (Niehaus et al., 2003). The activation of brain microglia has been implicated in rotenone neurotoxicity, and these cells also release reactive oxygen species as well as inflammatory factors (Gao et al., 2002; Liu et al., 2003). Studies in vitro have also suggested that a number of pesticides (alone or in combination with certain metals) may induce a conformational change in α-synuclein and accelerate the formation of α-synuclein fibrils. Pesticides known to induce this effect are hydrophobic and include rotenone, dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxy-acetic acid, dieldrin, diethylthiocarbamate, paraquat, maneb, trifluralin, parathion, and imidazoldinethione (Brown et al., 2006; Uversky et al., 2001). Several epidemiological studies have demonstrated a strong link between pesticide/environmental toxin exposure and incidence of PD development (Ascherio et al., 2006; Priyadarshi et al., 2001).

1.6.2 Genetic

PD had been thought of as a prototypic non-genetic disorder for many years. A major advance in understanding the underlying mechanisms of the pathology involved in PD came from identification of inherited forms of the condition that are clinically similar to the idiopathic forms. Gain-of-function mutations in the SNCA (AS) and leucine-rich
repeat kinase 2 (LRRK-2) (dardarin) genes results in autosomal dominant parkinsonism. The phenotype of patients with AS (SNCA -PARK1/PARK4) point mutations is that of L-DOPA-responsive parkinsonism in patients with a relatively young age at onset, rapid progression, and high prevalence of dementia, psychiatric, and autonomic disturbances. Patients with duplications in the gene resemble those with idiopathic PD, and those with triplications have earlier onset, faster disease progression, severe dementia, and frequent dysautonomia. There is consensus that any change in the levels of AS expression or the presence of mutations in AS has a toxic effect on DA neurons. AS monomers interact under certain circumstances to form protofibrils or fibrillar β-pleated sheets (Giasson et al., 2001). Toxicity caused by protofibrils may involve the leakage of dopamine from synaptic vesicles because of perforation of the vesicular membranes by these protofibrils (Vekrellis et al., 2004). AS is a major component of Lewy bodies not only in rare familial forms but also in the brains of sporadic PD cases without any family history of the disease (Duda et al., 2002). Although point mutations and gene triplications of AS produce a pathology that mimics sporadic PD, the incidence of these aberrations are very rare (Polymeropoulos et al., 1997; Singleton et al., 2003).

Over 75 mutations in LRRK-2—a kinase encoding the protein dardarin—have been reported; however, only eight of these have been deemed pathogenic (Dachsel et al., 2010). The most common of these—the Gly2019Ser mutation—has a worldwide frequency of 1% in sporadic cases and 4% in patients with hereditary parkinsonism (Healy et al., 2008; Paisan-Ruiz et al., 2004). In north African Arabs, almost a third of all patients diagnosed with parkinsonism have an LRRK-2 mutation, which is also common in Ashkenazi Jews (28% of hereditary cases) and in the Portuguese population (Healy et al., 2008). The clinical presentation closely resembles sporadic PD, but the progression is mild with less likelihood to develop dementia. Loss-of-function mutations
in four genes (*Parkin, DJ-1, PINK1, and ATP13A2*) cause recessive early onset parkinsonism (age of onset <40 years). *Parkin (PRKN, PARK2)* mutations are the second most common genetic cause of L-DOPA-responsive parkinsonism, whereas mutations in the other three genes are rare. Parkin protein localizes, although not predominantly, to the synapse and associates with membranes. Its main function is as an ubiquitin ligase in the cellular ubiquitination protein degradation pathway. The parkin protein mediates the engulfment of dysfunctional mitochondria by autophagosomes (Narendra *et al.*, 2008). Failure to remove dysfunctional mitochondria may therefore be an important pathogenetic factor (Mata *et al.*, 2004). Severe and selective degeneration in the SNpc but without Lewy bodies has been described in patients with parkin mutations, suggesting that the disease may differ in some important ways from typical idiopathic PD. Mutations in the *PINK1* gene (*PARK6*) have also been identified as a cause of autosomal recessive early-onset parkinsonism. This gene links PD to mitochondrial dysfunction and oxidative stress as it encodes a primarily mitochondrial protein kinase. PINK1 shares the same mitochondrial pathway as parkin (Clark *et al.*, 2006; Park *et al.*, 2006). A dysfunction of mitochondria could be the key reason for at least some of the autosomal recessive forms of parkinsonism (Schapira, 1994).

Mutations have been identified in a DJ-1 (Bonifati *et al.*, 2003). It encodes a ubiquitous, highly conserved protein which is thought to be involved in chaperone function and cell redox potentials. It appears that overexpression of this gene protects cells against the effect of mitochondrial complex I inhibitors and oxidative stress caused by hydrogen peroxide and thus it is likely that mutations in it may lead to a higher risk of PD.

The *ATP13A2* gene is mapped at the PARK9 locus and is responsible for Kufor-Rakeb disease, a recessive, juvenile-onset, atypical Parkinsonism with pyramidal degeneration and cognitive dysfunction (Ramirez *et al.*, 2006). The *ATP13A2* gene encodes a large
lysosomal P-type adenosine triphosphatase, which is involved in the lysosomal degradation pathway that clears SNCA aggregates. Lysosomal dysfunction caused by mutations in this gene might contribute to the pathogenesis of parkinsonism.

1.6.3 Inflammatory

In addition to these environmental and genetic factors the idea that PD might be caused by neuroinflammation has recently received a lot of attention. Initially, the evidence was first presented by McGeer and his group in 1988 where they showed that major histocompatibility complex (MHC) molecules were upregulated in postmortem brains of PD patients (McGeer et al., 1988). It was later reported that all types of proinflammatory cytokines were released by activated microglial cells, for example tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Mogi et al., 1994; Muller et al., 1998). Upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) were also reported. The inflammatory response is further amplified and sustained by factors released from dying dopaminergic cells leading to a lethal cascade and irreversible destruction of the nigrostriatal pathway (Orr et al., 2002; Whitton, 2007; Whitton, 2010). Although commonly inflammatory reactions in the CNS are considered detrimental, there is evidence to suggest that they can be beneficial and even neuroprotective (Hohlfeld et al., 2006). For example, it was demonstrated that T cells and other cells of the immune system are capable of producing neurotrophic factors such as brain derived neurotrophic factor (BDNF) which promotes neuronal growth and survival (Kerschensteiner et al., 2003). It is now becoming increasingly evident that immune cells can release both neurodestructive and neuroprotective molecules during CNS inflammation and it is the balance between the two that ultimately determines the net outcome of a neuroimmune interaction (Kerschensteiner et al., 2009)
1.7 Current Treatments

1.7.1 Pharmacological

L-DOPA:

As mentioned previously, the symptoms of PD only appear when around 80% of dopaminergic neurons are lost; therefore, by the time the pharmacological treatment is started, it is purely symptomatic and directed at minimizing the motor impairments associated with the condition. L-DOPA is the amine precursor of DA which is administered orally and is absorbed from the small intestine. It remains the most common pharmacological treatment. L-DOPA in the periphery can be metabolized to DA and not reach its desired destination, therefore it must be administered with peripherally active DOPA decarboxylase (DDC) inhibitors such as carbidopa or benserazide. L-DOPA is also partly metabolized by COMT and an enzyme antagonist such as entacapone may also be given to maximize L-DOPA delivery to the target site. The peripheral DDC inhibitors are designed not to cross the blood brain barrier (BBB) therefore when L-DOPA reaches the brain it is only then converted to DA. Although L-DOPA treatment seems like the most sensible option there are numerous complications. Potential adverse effects associated with L-DOPA treatment include nausea and vomiting, hypotension, arrhythmias, extreme emotional states and psychotic episodes. Long term problems include dyskinesias and motor fluctuations which appear after 2-5 years of treatment in about 30-50% of patients and in alarming 80-100% of patients after 10 years of treatment (Uitti et al., 1996). Other risks associated with L-DOPA treatment that have been reported include an increased incidence of malignant melanoma (Fiala et al., 2003) and higher incidence of stroke and coronary heart disease due to elevated levels of homocysteine (Postuma et al., 2009).
**Dopamine Agonists:**

Another strategy in PD treatment is to stimulate DA receptors in the STR directly. Several DA agonists are available clinically and include rotigotine, piribedil, bromocriptine, pergolide, pramipexole, and ropinirole. The advantage of these drugs is that they are not affected by pharmacokinetic shortcomings seen with L-DOPA. There is no competition with dietary neutral amino acids for absorption in the gut and no competition for penetration of the BBB. DA agonists have substantially longer elimination half-lives than L-DOPA, which allows for more prolonged stimulation of receptors. Since these drugs are specific at targeting DA receptors it was hoped they would provide more selective therapeutic benefits and reduce some of the unwanted effects seen in long term L-DOPA therapy. In fact, they were shown to produce more behavioural complications including psychosis and autonomic side effects like orthostatic hypotension as well as more frequent gait disorders such as freezing episodes. But they still do provide a better therapeutic window and a reduced profile of diskynesias compared to L-DOPA therapy (Richard *et al.*, 1997; Whone *et al.*, 2003).

Other safety issues concerning the use of DA agonists include excessive daytime somnolence and sleep attacks (Richard *et al.*, 1997), pathological gambling, and related impulse control disorders where there is an inability to resist an impulse desire despite negative consequences. These include compulsive shopping, compulsive eating, and hypersexuality (Galpern *et al.*, 2007).

**Anticholinergic Drugs:**

Muscarinic receptor antagonists were the first drugs introduced for the treatment of PD (Hobson *et al.*, 2002) and are still in the clinic especially in the early stages of the condition. The pharmacological rationale for this approach was the demonstration of functional antagonism between the DA and acetylcholine (ACh) systems in the STR.
(Polymeropoulos et al., 1997). Indeed, D\textsubscript{2} receptor activation reduces the release of ACh. In PD, DA depletion is accompanied by an increased activity of the striatal cholinergic system, subsequent rearrangement of the striatal circuitry, and appearance of the motor symptoms. Recent evidence suggests that loss of dopaminergic afferents does not exert its effect on cholinergic signalling directly by diminished D\textsubscript{2} receptor activity. Rather, it has been proposed that DA depletion triggers a reduction in the efficacy of the M\textsubscript{4} autoreceptors and these are responsible for increases in ACh signaling (Ding et al., 2006). Hence anticholinergic agents were intended to correct this imbalance, and although were shown to be beneficial, were also associated with various neuropsychiatric side effects which often led to patient withdrawal which in turn led to worsening of the symptoms (Katzenschlager et al., 2003).

**Catechol-O-methyltransferase (COMT) inhibitors:**

COMT is one of two enzymes that are responsible for DA metabolism. It is an intracellular enzyme located in postsynaptic neurons. Any compound having a catechol structure is a substrate, including L-DOPA. COMT inhibitors act peripherally to inhibit metabolism of L-DOPA to 3-O-methyl-DOPA (Jorga et al., 1997). The strategy here is to increase the half-life and bioavailability of L-DOPA increasing the duration of its effect. There are two drugs in this class which have been approved for PD treatment. Tolcapone was introduced in 1997, followed by entacapone in 1999. Both drugs increase the bioavailability of L-DOPA but also have a number of adverse effects. The major side effects of tolcapone include diarrhoea, which was the most frequent reason given for patient withdrawal from long-term trials. The other event that occurs between 6 to 12 weeks after the start of tolcapone is the elevation of alanine aminotransferase and aspartate aminotransferase, with three times the normal levels. Although enzyme levels return to normal in two to four weeks, three deaths from acute hepatic failure have been
reported in association with the use of tolcapone. Consequently, the use of tolcapone became limited (Obeso et al., 2000). Prescribing requirements include liver-function testing before starting the drug and monitoring every two weeks for one year and every four weeks for six months and every two months for the lifetime of the patient (Lew et al., 2007). Entacapone has been documented to cause hypotension, benign urine discoloration, constipation and diarrhoea but no liver toxicity has been reported (Rinne et al., 1998).

**Monoamine oxidase (MAO) inhibitors:**

MAO is an intracellular enzyme located in the outer mitochondrial membrane which plays a major role in oxidative deamination of monoamines such as DA. There are two isoforms of the enzyme, MAO-A and MAO-B. The B-isoform is primarily present in the brain and is found in astrocytes but not neuronal cells (Westlund et al., 1985). Inhibition of the enzyme would be expected to increase amounts of both intracellular and extracellular DA, and therefore serve as another pharmacotherapeutic strategy in the treatment of PD. The most well known MAO inhibitor used in PD is selegiline which was introduced in 1989, followed by rasagiline, a more B-type selective compound and also a new formulation of the oral form of selegiline branded Zydis (Deane et al., 2004).

One of the main problems with non-specific MAO inhibitors is the so called "cheese reaction". Foods rich in tyramine such as chocolate, red wine, and most notably cheese may cause episodes of hypertensive crises. This occurs because inhibitors block the function of hepatic MAO-A, which leads to the accumulation of tyramine in the bloodstream. It is thought that this increased circulating tyramine causes displacement of NA from synaptic vesicles leading to a hypertensive crisis. This problem was partly solved with the introduction of selective type-B agents. The other potential dangerous
interaction may arise if a non-selective MAO drug is used in conjunction with a tricyclic antidepressant or a selective serotonin re-uptake inhibitor. This will lead to elevated 5-HT levels and may lead to the so called "serotonin syndrome". Clinical features include motor symptoms, including movement disorders, myoclonus and tremor, muscle rigidity, hyperreflexia; mental status changes, including agitation, confusion, disorientation and restlessness; and autonomic instability, including low-grade fever, nausea, diarrhoea, headache, shivering, flushing, diaphoresis, tachycardia, tachypnea, blood pressure changes, and pupil dilation (Bodner et al., 1995).

### 1.7.2 Surgical

When pharmacological treatment is no longer enough to control the symptoms, surgical intervention may help certain patients. Nowadays the most common surgical procedure performed is deep brain stimulation (DBS) involving implantation of electrodes and selective stimulation of certain brain areas. DBS has progressively replaced brain lesioning such as thallamotomies and pallidotomies over the past twenty years. The first target in the modern era of DBS was the ventral intermediate thalamus (VIT) (Blum et al., 2001). VIT stimulation provided a positive effect on tremor but no improvement in rigidity, gait or balance. Limited effectiveness of VIT simulation led to application of this procedure to other brain areas in a search for a better target. Following an observation that MPTP induced lesion of the STN appeared to improve the symptoms of PD (Bergman et al., 1990), the STN has progressively become the most popular target for DBS. Although stimulation of the STN appears to have a positive effect on all symptoms of PD, it does come at a price. As expected for a procedure of this level of complexity the disadvantages are numerous. The first and most obvious one is the cost. Secondly, there are many potential complications during surgery. The main risks
associated with the procedure itself are intracranial bleeding and infection (Wider et al., 2008). In rare cases seizures are also observed. Hardware problems such as lead fracture and battery failure have also been reported (Blomstedt et al., 2005). In addition there are also target specific side effects which for the STN may include motor speech disturbances, neuropsychiatric problems such as mood changes, confusion, and apathy, eyelid opening apraxia and weight gain (Limousin et al., 2008).

### 1.7.3 Experimental Strategies

Many different classes of drugs were tested in the clinic after promising animal model studies. These include serotonin receptor antagonists after observations that serotonergic system was overactive in PD (Fox et al., 2000); N-methyl-D-aspartate (NMDA) receptor antagonists and GABA_A agonists based on the fact that there is an imbalance of the glutamate/GABA system in the basal ganglia; adenosine A_2_A receptor antagonists because they were shown to influence both the striatal cholinergic and GABA systems; α_2-adrenergic antagonists based on beneficial effects in animals models; opioid antagonists, after observations that opioid peptide transmission was upregulated in PD sufferers (Piccini et al., 2005) and finally cannabinoid receptor antagonists because these are expressed on GABAergic neurones and inhibit the release of GABA. However, none were proven to be truly beneficial. Some were associated with severe and unpredictable side effect profiles, while others gave very limited efficacy (Linazasoro, 2004).

### 1.7.4 Regenerative Strategies

**Glial cell derived neurotrophic factor (GDNF):**

Neurotrophic factors are essential for neuronal survival and differentiation during development and for maintenance of normal function during adulthood. GDNF is one of
the most potent neurotrophic factors for dopaminergic neurons as demonstrated in animal models (Goke et al., 1993). GDNF delivery is however a major problem due to its inability to cross the BBB. Intracerebroventricular (ICV) delivery has been explored as the method for delivering GDNF into the brain, but poor diffusion from the site of injection into the brain parenchyma and the occurrence of serious side-effects such as nausea, loss of appetite, cutaneous sensory disturbances, and psychiatric symptoms limit this delivery approach in PD patients (Kordower et al., 2008; Nutt et al., 2003). Both animal and human studies demonstrate that direct intrastriatal administration reduces the occurrence of unwanted effects seen with the ICV method, although the degree of symptomatic relief in clinical trials varies from major improvements to no significant differences. This is possibly because, for a noticeable clinical benefit, GDNF probably has to be delivered continuously for several years (Yasuhara et al., 2007). Direct localised delivery can be performed using several approaches such as a microinfusion pump, cell-based or direct gene delivery. Gill et al. demonstrated, in a small open-label phase I safety trial, that direct infusion of GDNF via catheter into the putamen of PD patients produced significant improvement of motor function and dopamine storage (Gill et al., 2003; Patel et al., 2007). The results of this small trial; however, were not replicated in a larger randomized control trial (Lang et al., 2006). This discrepancy could be due to technical differences between the open-label and controlled study (ex. catheter sizing) (Patel et al., 2007). A major drawback to this surgical approach is that efficient CNS delivery using a pump will require implantation of several catheters on each side of the brain in addition to an infusion pump implanted in the abdominal cavity, making this procedure cumbersome and prone to complications (Patel et al., 2007; Zurn et al., 2001). Direct GDNF gene delivery to the target site leads to efficient local synthesis, but does not offer the capacity to completely turn off protein synthesis in case of adverse effects. The same problem limits the use of neural progenitor cells to deliver
GDNF to the target site. In addition, the ability of these cells to migrate poses a risk of further complications (Yasuhara et al., 2007).

**Cell Transplantation:**

The idea of developing transplant cell therapies for PD is based firstly on assumptions that it is the nigrostriatal dopaminergic neuronal pathway that degenerates, and that if new neurones are somehow introduced into the SNc they would re-grow and re-innervate the dennervated STR. Extensive animal studies have demonstrated that indeed transplanted neurons are able to survive and re-establish striatal connections and restore baseline DA synthesis and release (Dunnett et al., 1999). Very promising results were demonstrated in 6-hydroxydopamine (6-OHDA) treated rats where there were improvements with both the motor problems and L-DOPA induced dyskinesias (Hurtig et al., 2000). As for human studies, initially a series of small open-label ones were conducted to assess the therapeutic potential of this strategy. Fetal dopaminergic neurons were used and showed remarkable long-lasting benefits in many PD patients. However, following these results, two double-blind sham surgery controlled clinical studies failed to reproduce the previously obtained data and there was only limited efficacy. In addition a significant number of patients developed severe off-state dyskinesias after the procedure (Freed et al., 2001; Olanow et al., 2003). Stem cells, especially human embryonic stem cells, provide an unlimited supply of dopaminergic neurons and are capable of differentiating into DA neurons in the laboratory, although cell survival and behavioural improvement is limited and the potential risk of tumour formation remains (Laguna Goya et al., 2008).
1.8 Animal Models of PD

Animal models are important tools in experimental medical science to better understand the pathogenesis of human diseases. Once developed, these models can be exploited to test therapeutic approaches for treating functional disturbances observed in the disease of interest. On the basis of experimental and clinical findings, PD was the first neurological disease to be modelled and subsequently treated with neurotransmitter replacement therapies. Agents that cause selective damage to catecholaminergic neurons have been used to mimic the condition in experimental animals. There are several models of PD that are used to screen potential new therapeutics. A few prominent examples are listed below:

Reserpine model: It was observed that systemic administration of this compound to rabbits caused akinesia by depleting the levels of catecholamines in the brain; furthermore it was shown that levodopa alleviated these symptoms suggesting that behavioral recovery depended on dopamine (Carlsson et al., 1957; Hornykiewicz, 1963). The main problem with this model is that changes in catecholamine levels are temporary and reserpine does not cause any morphological changes in the nigrostriatal tract.

6-hydroxydopamine model (6-OHDA): 6-OHDA was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways (Sachs et al., 1975). 6-OHDA uses the same dopamine transport system as DA and NA and therefore produces specific neurodegeneration. Systemically administered 6-OHDA cannot cross the blood brain barrier (BBB); therefore, it has to be delivered directly into the substantia nigra, medial forebrain bundle or the STR stereotaxically (Perese et al., 1989). Neurons start degenerating within 24 hours and striatal dopamine becomes depleted in 2-3 days. Normally 6-OHDA is injected into one hemisphere to produce a unilateral lesion, firstly because bilaterally lesioned animals are difficult to maintain and
secondly because the intact side serves as an internal control. Unilateral lesions also lead to asymmetric circling behaviour when tested with a DA agonist such as apomorphine. The number of turns that the animal performs indicates the severity of the lesion and can be easily quantified (Ungerstedt, 1971). Although 6-OHDA produces lesions in many different species of animals, the most commonly used are rats due to well established stereotaxic techniques and also low maintenance costs. The mechanism of 6-OHDA toxicity is thought to be both an increase in oxidative stress during its metabolism yielding an increase in reactive oxygen species (ROS) production and also its ability to inhibit mitochondrial complex I (Perumal et al., 1992; Sachs et al., 1975). The acute nature of this experimental model differs from the progressive degeneration of neurons in the actual PD and it does not accurately mimic all the clinical and pathological feature characteristic of the condition. One such example would be the absence of cytoplasmic inclusions like those observed in human patients, but despite these limitations it is widely used to test the effects of experimental compounds, evaluate the efficacy of cell transplantation and various neurotrophic factors that promote degenerating nigral neuron survival (Dunnett et al., 1999)

1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) model: The effect of this compound was first inadvertently demonstrated in humans when a group of heroin addicts consumed incorrectly synthesized heroin which was in fact MPTP and developed clinical symptoms similar to sporadic PD overnight (Langston et al., 1999). Researchers went on to develop an animal model. After administration, MPTP crosses the BBB and is converted by astrocytes to its active metabolite MPP⁺ which is taken up selectively by the dopamine neurons via its affinity for the dopamine transporter. MPP⁺ toxicity is believed to be due to inhibition of mitochondrial complex I, thus resulting in oxidative stress (Nicklas et al., 1985). The downside to treatment with MPTP is that it does not
produce consistent lesions in all animal species and is more commonly used in mice and primate but not rats.

**Methamphetamine model:** The amphetamines are psychostimulatory drugs with high addictive potential. Such activity is associated with a powerful DA releasing mechanism. At very high doses the drug causes neurotoxicity in rodents and primates (Wagner *et al.*, 1980). As in the case of the reserpine model, the methamphetamine does not induce any morphological changes in the SN and does not lead to loss of nigral cell bodies (Fibiger *et al.*, 1971).

**Rotenone model:** Rotenone is a pesticide that is extracted from certain plant roots and when it is systemically and chronically administered to rats, they seem to develop many features of PD such as degeneration of the DA neurons in the SNC and the appearance of cytoplasmic inclusions reminiscent of Lewy bodies, which stain with antibodies for ubiquitin and AS (Betarbet *et al.*, 2000). It has been established that rotenone is a high-affinity specific inhibitor of mitochondrial complex I (Betarbet *et al.*, 2000). Rats treated with rotenone through chronic systemic exposure from a subcutaneously implanted osmotic pump show symptoms similar to those of human PD, including bradykinesia, postural instability, unsteady gait, and some evidence of tremor. These symptoms improved when the DA agonist apomorphine was administered to the rotenone treated rats. This parkinsonian pathology is; however, limited to the Lewis rat strain (Sherer *et al.*, 2003).

**Lipopolysacharide (LPS) model:** LPS is a gram-negative bacterial endotoxin and potent microglial cell activator. It is now well established as an effective initiator of dopaminergic neuronal loss and PD symptoms in experimental animal models. The toxin works by binding to a cell membrane component known as a toll-like receptor on microglial cells triggering the activation of NFκB which is a pro-inflammatory
transcription factor which subsequently upregulates pro-inflammatory gene pathways (Liu et al., 2000). Interestingly, this microglia-mediated neuronal cell loss is only observed when LPS is injected into the SNpc and not when administered in the hippocampus or cortex. Microglial cells in the SNpc seem to be activated up to 8 times more than they are in other regions of the brain (Kim et al., 2000).

**Proteasome Inhibition Model:** Dysfunction of the ubiquitin-proteasome system has been implicated in the etiology of PD (Dawson et al., 2003). Proteasomal enzyme activity and subunit number is significantly lower in SNc of PD patients (Cook et al., 2009). McNaught et al. showed that rats treated systemically with proteasome inhibitors, namely epoxomicin and synthetic (Z-Ile-Glu(OtBu)-Ala-Leu-al (PSI), exhibited PD-like systems and pathology (McNaught et al., 2004). Rats displayed cardinal locomotor dysfunction including bradykinesia, tremor, rigidity, and postural instability. Cell loss was observed in the SNc, LC, nucleus basalis of Meynert, and the dorsal motor nucleus of the vagus. This neurodegeneration was accompanied by ubiquitin/AS positive inclusions that resemble Lewy bodies. However, these findings have not been consistently replicated (Bove et al., 2006; Kordower et al., 2008; Nair et al., 2006; Schapira et al., 2006). The reason for the lack of consistent results is largely unknown; however, it has been suggested that there might be variation in either the composition of the inhibitor itself or the brain bioavailability of the toxin (McNaught et al., 2006).
1.9 Glucagon Family and Exenatide

1.9.1 Introduction to the glucagon family of peptides

Processing of the proglucagon gene gives rise to 29 amino acid glucagon itself and a number of biologically active peptides including glicentin, oxyntomodulin (OXM), glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2). Both OXM and glicentin contain the whole 29 amino acid sequence of glucagon and a C-terminal 8-amino acid extension called intervening peptide-1 (IP-1). Compared to OXM (37 amino acids), glicentin (69 amino acids) also contains the N-terminal extension called glicentin-related pancreatic polypeptide (GRPP). OXM has recently been found to suppress appetite and a recent clinical study found that it could be used as a treatment for obesity (Wynne et al., 2006b). The mechanism of action of OXM is poorly understood. It has been shown to bind to both the glucagon-like peptide 1 receptor (GLP-1R) and the glucagon receptor, but it is likely that its effects are mediated by a novel receptor (Wynne et al., 2006a). Effects of glicentin are not well understood, but it is thought to be implicated in the growth of intestinal mucosa by mechanisms involving the GLP-1R (Ayachi et al., 2005). On the other hand, a lot more is known about functions of GLP-1 and its mechanisms of action. GLP-1 functions are discussed further in this section and summarized in figure 1.2.
**Figure 1.2**: Structure of proglucagon gene fragment contains sequences coding for several biologically active peptides: Glicentin related pancreatic polypeptide (GRPP), glucagon, intervening peptide 1 (IP1), glucagon-like peptide 1 (GLP-1), intervening peptide 2 (IP2) and glucagon-like peptide 2 (GLP-2). Figure also summarizes the known functions of GLP-1, adapted from (Drucker, 2001).

### 1.9.2 Exenatide

Exendin-4 (EX-4) which is a naturally occurring form of exenatide was originally isolated from salivary secretions of the lizard *Heloderma suspectum* (Eng *et al*., 1992) also known as the Gila monster. In the lizard, EX-4 circulates during ingestion of its prey. Since the lizard only feeds about four times a year, it uses EX-4 to “switch on” its pancreas to start producing insulin after a meal. EX-4 has 53% homology to mammalian GLP-1 and exerts its effect by activating GLP-1Rs. As previously mentioned, in mammals GLP-1 is processed from a proglucagon gene found in L-cells in the small intestine, whereas EX-4 is transcribed from a distinct gene only expressed in the salivary glands of the lizard (Chen *et al*., 1997). In mammals, GLP-1 is degraded by dipeptidyl-peptidase-IV and its plasma half life is around 1.5 minutes, but EX-4 appears to show
resistance to degradation by the same enzyme and has a much longer plasma half life. Some of the physiological effects of EX-4 are not only glucose-dependent enhancement of insulin secretion but also inhibition of glucagon secretion and reduction of food intake by slowing gastric emptying (Kolterman et al., 2003) which is normally accelerated in people with type 2 diabetes. EX-4 was approved by the FDA in 2005 as adjunctive therapy for patients with type 2 diabetes who have not achieved optimal glycaemic control on metformin or sulfonylurea. EX-4 has been shown to increase β-islet mass by promoting its proliferation and neogenesis from precursor cells in both in vitro and in vivo models (Tourrel et al., 2002). EX-4 appears to also exert effects in the CNS. There is evidence suggesting that GLP-1 present in the bloodstream can cross the BBB and enter the brain (Orskov et al., 1996). Although it is a large molecule, EX-4 is highly lipophilic (Kastin et al., 2003).

1.9.3 Glucagon-like peptide 1 receptor

The GLP-1R is a classic seven transmembrane domain G-protein coupled receptor. It was first cloned and characterized from the rat pancreatic islets and later from human pancreatic insulinoma and gut tumor cell lines (Freed et al., 2001; Thorens, 1992). The rat and the human GLP-1Rs show 95% amino acid homology and are 90% identical, differing at 42 amino acid positions (Tibaduiza et al., 2001). Human GLP-1R is a 64-kDa protein and its gene is located on the long arm of chromosome 6p21 (Stoffel et al., 1993). All members of the glucagon family of receptors are positively coupled to AC through Gsα subunit with subsequent production of cAMP, although they are also capable of signalling through other transduction pathways (see next section). The receptor is not only found in the pancreas but also in several regions of the CNS in both the human and the rat brain (Satoh et al., 2000). Initial findings demonstrated their presence in the hypothalamus suggesting a role in regulation of food intake, although
receptor expression was later demonstrated in several other brain regions such as thalamus, brain stem, lateral septum, the subfornical organ, and the area postrema. In addition, specific neuronal binding sites have been identified in the striatum, hippocampus, and cerebral cortex although at lower densities (Calvo et al., 1995; Goke et al., 1995). GLP-1 is also produced by neuronal cells in discrete regions of the CNS (Jin et al., 2004) and circulating GLP-1 was found to readily enter the brain (Orskov et al., 1996).

1.9.4 Signal Transduction

As mentioned previously, GLP-1Rs are metabotropic G-protein coupled receptors. Agonist engagement at the extracellular site activates AC through an interaction of the \( G_{\alpha} \) subunit with the membrane bound enzyme. Activation of AC gives rise to increased production of cAMP (Drucker et al., 1987) which then activates a signalling cascade. The two most characterized pathways are the cAMP-dependent PKA pathway and a PKA independent – exchange proteins directly activated by cAMP (Epac) pathway. PKA is a ubiquitous serine/threonine phosphorylating enzyme (Kolterman et al., 2003). In its inactive form it is composed of one regulatory subunit bound to two catalytic subunits. PKA mediated activation of cAMP response element binding protein (CREB) was shown to be involved in the protective effects associated with GLP-1R activation in β-cells (Sarkar et al., 2007). The PKA independent portion of the signalling cascade forms a part of a large family of related nonkinase effectors which have been shown to activate the Ras superfamily of effector proteins. There are two variants of this pathway known as Epac 1 and Epac 2 and both exhibit high selectivity for activation by cAMP over other cyclic nucleotides. Both have been found in rat islets and also in β-cell lines. Although these can be distinguished as two separate pathways, their precise roles and importance are still elusive (Leech et al., 2000; Mori et al., 2002). The neuroprotective mechanism
involving GLP-1R is thought to be cAMP dependent, but pancreatic receptors are also known to utilize other signalling cascades (summarized in figure 1.3). The activation of important transcription factor known as pancreatic-duodenal homeobox (PDX-1) that was shown to be involved in β-cell survival, is stimulated via PKA which induces its translocation to the nucleus (Caretti et al., 2008). A G_q subunit was shown to activate phospholipase C (PLC) pathway, leading to protein kinase C (PKC) activity and an increase in intracellular Ca^{2+} (Wan et al., 2004). PKC is thought to activate nuclear factor kappa B (NF-κB) which appears to be anti-apoptotic (Fowler et al., 1996). The phosphoinositide-3-kinase (PI3K) pathway activated by the G_pγ subunit activates another important pathway implicated in β-cell growth and survival (Buteau et al., 2003).
Figure 1.3: Diagram to demonstrate possible events downstream of GLP-1R activation in β cells. GLP-1Rs are capable of signaling through several signal transduction pathways including PLC/NF-κB, MAPK/ERK, PI3 kinase, cAMP/CREB pathways. *PDX-1 transcription factor is specific to β-cells as well as Ca2+ induced insulin vesicular fusion.

1.10 Rationale for using EX-4 for the treatment of PD

1.10.1 Control of β-cell proliferation

Initially, positive effects on β-cell proliferation following GLP-1R activation were demonstrated in vitro (Buteau et al., 2001) and in vivo in partial pancreatectomy model of type 2 diabetes (Caretti et al., 2008). It was later shown that growth promoting effect of GLP-1R in β-cells was dependent on transactivation of epidermal-growth factor-receptor leading to PI3K expression and ultimately activating its downstream effectors (figure 1.3). The more long term effect of GLP-1 on β-cell proliferation is thought to be
associated with upregulated expression of insulin receptor substrate 2 (Irs2) gene. Irs2 prevents diabetes in mice via promoting β-cell replication, survival and function especially during metabolic stress. In these experiments, EX-4 was shown to promote β-cell function, but not in Irs2 knockout mice, although it was able to stimulate the remaining β-cells to secrete insulin and thereby delay the onset of diabetes (Kim et al., 2006). GLP-1R stimulation has also been shown to inhibit the expression of a forkhead transcription factor (FoxO1) through phosphorylation-mediated nuclear exclusion and exendin-4 had no effect on β-cell proliferation in transgenic mice constitutively expressing nuclear localization of FoxO1 (Buteau et al., 2006). In fact it was shown that nuclear localization of PDX-1 and FoxO1 are mutually exclusive (Kitamura et al., 2002) thus demonstrating another mechanism by which GLP-1R stimulation may lead to PDX-1 upregulation and ultimately β-cell proliferation. Mice with specific ablation of PDX-1 expression fail to respond to EX-4 treatment, and there is no positive trophic effect on β-cells (Chesselet et al., 2005). The actions of GLP-1 on cell proliferation are complemented by also promoting cell survival. Treatment with EX-4 has been shown to protect β-cells against apoptosis and consequently preserve cell mass (Wang et al., 2002). β-cell survival was accompanied by a reduction in caspase-3 expression (Farilla et al., 2002) and upregulated expression of an anti-apoptotic B-cell leukemia protein (Bcl2) through NF-κB activation (Li et al., 2003).

1.10.2 Cardio-protective effects

Beneficial effects of GLP-1 on the myocardial function are becoming increasingly evident. Both in vitro and in vivo models of myocardial ischaemia demonstrate that infusion of GLP-1 reduces infarct size (Bose et al., 2005). GLP-1 improves myocardial contractility and the uptake of glucose in normal and postischaemic rat hearts (Zhao et al., 2006). In dogs with pacing-induced dilated cardiomyopathy, GLP-1 infusion was
able to improve left ventricular performance, stroke volume, and cardiac output (Nikolaidis et al., 2004a). It was demonstrated that a 72 hour infusion of GLP-1 improved left ventricular ejection volume in patients after myocardial infarction (Nikolaidis et al., 2004b) therefore in humans with type 2 diabetes and congestive heart failure, GLP-1 may be of dual benefit. More recently, protective effects of EX-4 were demonstrated against ischaemia reperfusion injury in the rat heart where EX-4 reduced infarct size by more than 50% (Sonne et al., 2008).

1.10.3 Neuro-protective effects

Intestinally derived peptides such as GLP-1 are classified as both hormones and growth factors since they can modulate various cellular processes such as cell division, growth and differentiation. The first evidence that EX-4 might have neuroprotective properties appeared in 2002. Both EX-4 and its endogenous peptide analogue GLP-1 were shown to be neuroprotective. It was demonstrated that activating GLP-1Rs in PC12 cell lines caused neurite outgrowth and the mechanism appeared to be cAMP dependent where signals which stimulated its production were shown to protect neurons against glutamate induced excitotoxicity (Perry et al., 2002a). It was also later demonstrated that EX-4 completely protected cultured hippocampal neurons against glutamate induced excitotoxicity and preserved basal forebrain choline acetyl transferase immunoreactivity in an animal model of Alzheimer’s disease (Perry et al., 2002a). The effects were GLP-1R mediated as co-incubating cells with a specific antagonist blocked the protective effects. The same group later showed that EX-4 decreased the amount of endogenous amyloid beta peptide (Aβ) and protected cultured neurons against apoptosis induced by Aβ and iron (Li et al., 2010; Perry et al., 2003). In the same year it was also demonstrated that EX-4 enhanced spatial learning in mice and protected hippocampal
neurons against kainate induced apoptosis (During et al., 2003). Later that year another group showed that GLP-1R activation caused rapid TH transcription in brainstem catecholaminergic neurons and was involved in regulating sympathetic outflow leading to downstream activation of cardiovascular responses in vivo (Yamamoto et al., 1998). Recently, while investigating the possibility that GLP-1R stimulation may influence axonal structure and function, beneficial effects of EX-4 were demonstrated in an animal model of peripheral sensory neuropathy induced by pyridoxine (Perry et al., 2007). Experiments by the same group also show the effectiveness of EX-4 in both a stroke model and an MPTP mouse model of PD (Li et al., 2009). The results from this study offer further support to the idea that EX-4 works by activating GLP-1R by testing the drug in GLP-1R knock outs (Li et al., 2009; Vaillancourt et al., 2009). Data generated in our lab demonstrate the potent dose-dependent neuroprotective and neurorestorative action of EX-4 given peripherally a week after toxin administration in two distinct animal models of PD (Harkavyi et al., 2008). Recently, it was shown that EX-4 prevents MPTP-induced microglial activation and expression of matrix metalloproteinase-3 in the SNc and striatum (Kim et al., 2009).

1.10.4 Modulation of Neurogenesis

The majority of neurons are created before birth. The first indication of adult neurogenesis was presented more than four decades ago but now there is accumulating evidence of ongoing processes that generate new functional neurons from neural stem cells in certain regions of both rodent and human brain. Neurogenesis in the mammalian brain occurs throughout life and has been clearly demonstrated at two locations under normal conditions: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Neurons born in the SVZ migrate great distances to the olfactory bulb where they become granule neurons and periglomerular neurons, whereas
neurons born in the SGZ migrate into the granule cell layer to become dentate granule cells (Zhao et al., 2008). Recently, several papers presented evidence of a slow rate of neuronal turnover in SN and the STR and it was demonstrated that $D_3$ receptors were involved in modulation of neurogenesis and restoration of the nigrostriatal pathway in the rat 6-OHDA model of PD (Borta et al., 2007; Van Kampen et al., 2006; Van Kampen et al., 2004). It was also demonstrated that the rate of this turnover appears to be upregulated during partial injury and that dopaminergic fibres originating in the SN project to the SVZ and when damaged, the rate of neurogenesis was reduced (Freundlieb et al., 2006; Zhao et al., 2003). Also, recently it was proposed that EX-4 may promote neurogenesis in the SVZ as measured by cellular bromodeoxyuridine (BrdU) uptake in rats. Moreover, the same group suggested that GLP-1Rs found in neurogenic regions of the brain such as the SVZ may be responsible for neurorestorative effects of EX-4 in vivo (Bertilsson et al., 2008). EX-4 has also been shown to initiate neurogenesis in primary culture from adult mouse hypothalamus (Belsham et al., 2009). Although modulation of neurogenesis in the adult brain appears an attractive option, there are debates and controversies over the modulation of adult neurogenesis in neurological disorders especially with studies using BrdU labelling for studying it (Taupin, 2007). For example, in brains of human Alzheimer’s disease (AD) patients, it was shown that DNA replication without cell replication precedes neuronal death (Yang et al., 2001) therefore some of the data observed using immunohistochemistry for cell cycle proteins and BrdU labelling in patients and animal models of AD may not demonstrate neurogenesis but rather cells which duplicated their DNA without completing the cell cycle (Yang et al., 2001). Evidence for neurogenesis in the SN is also controversial with some studies reporting increased levels in MPTP models (Zhao et al., 2003) while others when using 6-OHDA treated rats report no evidence of neurogenesis in SN (Frielingsdorf et al., 2004). Results obtained from human PD patients demonstrate reduced cell proliferation in SVZ and
SGZ and changes in adult neurogenesis resulting from neurodegenerative disorders likely depend on selected neuronal populations affected and the role of neurogenesis needs further clarification (Zhao et al., 2008).

1.11 Aims of the Project

PD is widely attributed to neuropathy of dopaminergic neurons in the nigrostriatal (NS) pathway (Hirsch et al., 1988; Olanow et al., 1999). Degeneration of noradrenergic and serotonergic neurons in the brain may actually precede the DA lesion but has received comparatively little attention (Braak et al., 2003; Chan-Palay et al., 1989; Jellinger, 1999; Kish, 2003). It is hypothesized that damage to all these neuronal systems would contribute to comorbid anxiety, depression, and cognitive deficits experienced by PD patients (Chaudhuri et al., 2006). These affective disorders and subtle cognitive deficits are observed early on in the pathology and greatly deteriorate the patient’s quality of life (Burn, 2002; Nilsson et al., 2001; Schuurman et al., 2002; Spiegel et al., 2006; Weintraub et al., 2008b; Ziemssen et al., 2007). The aim of this project was to develop and validate a premotor neurochemical/behavioural rodent model of PD that will allow a greater understanding of non-dopaminergic deficits with the future goal of improved treatment.

Generation of models which mimic the non-motor symptoms of PD constitute a relatively unstudied but important field (Branchi et al., 2008; Tadaiesky et al., 2008; Taylor et al., 2009). Robust animal models form a key substrate through which novel compounds may be tested. Although animal models are frequently criticized for failing to mimic PD in the clinic, there are as yet no obvious alternatives to this strategy. Therefore, the development of novel models to study emotional and cognitive aspects of PD will make a valuable addition through which the lives of patients may be improved.
We have utilized the selective toxins N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) and parachloroamphetetamine (pCA) to create partial lesions of the noradrenergic and serotonine systems, respectively. DSP-4 has long been used to create lesions of NA afferents stemming from the LC (Archer et al., 2003; Archer et al., 2001; Archer et al., 2006; Ross et al., 1974; Ross, 1976; Srinivasan et al., 2003). DSP-4 lesion has also been shown to decrease dopamine β hydroxylase activity in the LC (Ross et al., 1974; Ross, 1976). pCA has been used extensively to target serotonergic afferents and reduce overall 5-HT and 5-HIAA content (Kornum et al., 2006; Leonard, 1976). These lesions were then followed by a bilateral i.c. injection of 6-OHDA to create a partial dopaminergic deficiency and mimic the premotor parkinsonian condition (Branchi et al., 2008; Lee et al., 1996; Lindner et al., 1999; Roedter et al., 2001; Sauer et al., 1994; Tadaiesky et al., 2008). This sequential administration of the toxins was performed to duplicate the supposed progression of the clinical pathology. We then assessed the neurochemical and behavioral deficits produced by our model 2 weeks following 6-OHDA administration. Behavioral deficits were assessed using a wide array of tests including sucrose preference, open field exploration, forced swim test, and novel object recognition. Neurochemical deficiencies were assessed using in vivo microdialysis, tissue content levels, and immunohistochemistry.

Recent experiments strongly suggest that stimulation of GLP-1 receptors by the peptide, exendin-4 (EX-4), is neuroprotective in several systems, even as a late-stage intervention in rodent models of PD (Bertilsson et al., 2008; Harkavyi et al., 2008; Kim et al., 2009). We sought to evaluate the therapeutic value of Exendin-4 (EX-4) on our premotor rodent model. EX-4 was administered one week following 6-OHDA treatment for a period of seven days. This treatment protocol is highly relevant to the clinical condition since therapeutic intervention is only begun after the neurotoxin lesions have been allowed to
progress. After the EX-4 treatment period, all behavioural and neurochemical tests were performed to assess the treatment efficacy of EX-4.
Chapter 2: Materials and Methods
2.1 Chemicals and Reagents

All chemicals and reagents used in subsequent experiments were purchased from Sigma-Aldrich, Inc. (Sigma-Aldrich, UK) unless otherwise indicated.

2.2 Animals and Husbandry

Male albino Wistar rats (180-250 g) were purchased from Harlan Laboratories, Inc., UK and group-housed (n = 4 per cage) in the Biological Services Unit (BSU) of the university. The BSU maintained conditions of constant humidity (40-60%), temperature (18-22°C), and a 12 hr light-dark cycle (light presented from 0700-1900 hrs daily) in accordance with Home Office regulations. Access to food (standard rodent diet) and water was ad libitum. Animals used were subjected to a 7-day habituation and handling period prior to experimental usage. All experimental procedures were conducted in strict adherence to the terms of the 1986 Animals (Scientific Procedures) Act.

2.3 Stereotaxic surgery

Stereotaxic surgery was performed to administer a 6-OHDA injection bilaterally, thereby inducing a parkinsonian model of PD. Animals were anaesthetised using Isoflurane (5% v/v in O₂ for induction and 2% v/v in O₂ for maintenance delivered through a fitted anesthetic nose mask; Abbot Laboratories Ltd. Kent) and secured using blunt ear bars to a stereotaxic frame (David Kopf, U.S.A.). A sterile blade was used to expose the surface of skull. This was accomplished by laterally reflecting the two skin flaps and subsequently clearing away excess membranous material and blood. The bregma was then located and referenced with a black fine tip marker. Stereotaxic coordinates from the atlas of Paxinos and Watson (1982) were used to locate the ventrolateral area of the dorsal striatum (from bregma in mm; AP 1.1 mm, ML 3.2 mm and DV -7.2 mm). This area is analogous to the putamen in primates, which incurs the greatest dopaminergic
deficit in PD patients. Once located, a dental drill fitted with a tungsten carbide burr tip (2mm) was used to drill an insertion point through the skull, exposing the dura. A Hamilton 10 µl syringe (Hamilton Company, U.S.) was used to administer the intracerebral (i.c.) injections.

2.3.1 6-OHDA Injection

Prior to receiving an i.c. injection of 6-OHDA, animals were given intraperitoneal (i.p.) injections of both pargyline (a monoamine oxidase-B inhibitor (MAO-B)) (50 mg/kg) and desipramine (DMI, a noradrenergic reuptake inhibitor (NRI) (25 mg/kg) to ensure maximal selectivity of 6-OHDA towards dopaminergic neurons. Both pargyline and DMI were dissolved 12 mg/ml in saline (0.9% NaCl). Animals were secured to the stereotaxic frame (in the manner described in section 2.3) 15 minutes following the i.p. injections. 15 µg of 6-OHDA or vehicle (saline) was injected into the left and right striatum of each rat at a flow rate of 1µl/min to induce partial destruction of the nigrostriatal dopaminergic system. 6-OHDA was dissolved 5mg/ml in saline containing 0.2% ascorbic acid. After the injection was complete, the needle was kept in place for an additional 5 minutes to ensure adequate local diffusion of the neurotoxin. The needle was then slowly retracted to prevent any unwanted back flow. The site of incision was closed using surgical sutures or Michel clips (Harvard Apparatus, U.S.). Immediately following surgery the animal was placed on a heating pad and observed closely until it completely recovered from anaesthesia.

2.3.2 DSP-4 Intraperitoneal Injection

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), is an adrenergic neurotoxin that induces acute and relatively selective degeneration of both central and peripheral noradrenergic nerve terminals (Ross et al., 1974). It was administered at a dose of 25 mg/kg via i.p. injection four days prior to 6-OHDA i.c. neurotoxic insult. The dose of 25
mg/kg was selected to induce partial degeneration of the noradrenergic nerve terminals and cell bodies in the locus coeruleus (LC). DSP-4 was dissolved 10mg/ml in H₂O.

2.3.3 pCA Intraperitoneal Injection

Para-chloroamphetamine (pCA), acts as a serotonergic neurotoxin on prolonged administration or at high dosage. It acts as a substrate for the serotonin transporter (SERT) that releases serotonin from axon terminals by a nonexocytotic mechanism and blocks the reuptake of serotonin. pCA also inhibits tryptophan hydroxylase activity. It was administered at a dose of 6 mg/kg via i.p. injection four days prior to 6-OHDA i.c. neurotoxic insult. The dose of 6 mg/kg was selected to induce partial degeneration of the serotonergic nerve terminals and cell bodies. pCA was dissolved 5 mg/ml in H₂O.

2.3.4 EX-4 Intraperitoneal Injection

EX-4 was administered at a dose of 0.5 µg/kg via i.p. injection twice daily (9 a.m. and 5 p.m.) for a period of seven days one week following 6-OHDA insult. EX-4 was dissolved 2 µg/ml in H₂O.

2.4 Assessments

2.4.1 Sucrose Preference Test

Sucrose consumption was employed to measure anhedonia in rodents. Rats were first exposed to a 4-day habituation phase in which they were provided with two bottles, one containing water (designated by a white placard) and the other containing a 1% sucrose solution (designated by a black placard), attached to opposite ends of each cage. The position of the bottles was switched daily to avoid placement bias. After the habituation phase, the animals were individually housed and sucrose consumption was measured
over a two-day period by weighing the bottles daily. This measurement served as the pre-treatment value. Two weeks later, after either neurotoxins or vehicle had been administered, the rats were again individually housed and sucrose consumption was measured. This measurement served as the post-treatment value. Sucrose preference was calculated as total sucrose solution consumed divided by total fluid intake multiplied by 100.

2.4.2 Forced Swim Test

A modified forced swim test (FST) (Porsolt et al., 1978) was utilized to assess depressive behaviour. One day prior to administration of DSP-4 or pCA, rats were individually placed in plastic rectangular containers (56 cm in height and 24 cm in diameter) filled with water (40 cm water depth and temperature was maintained at 25 ± 1 °C). Total percentage of time spent immobile was recorded manually over a period of 15 minutes. Immobility was defined as stagnant floating behavior or minimal motility serving only to keep the subject’s head above the water. This test before the administration of neurotoxins served as the pre-treatment baseline measurement. Two weeks after 6-OHDA treatment, a second forced swim test was performed to assess behavioral impairment or the therapeutic value of EX-4.

2.4.3 Novel Object Recognition

Object-recognition memory was tested using a modified novel objection recognition (NOR) test (Ennaceur et al., 1988). The test apparatus was a circular arena (100 cm in diameter and 35 cm in height). Rats were exposed to the arena for two days prior to testing (for a period of 5 minutes) to habituate the animals to the testing conditions. The first test took place one day prior to DSP-4 or pCA administration. This value served as the pre-treatment measurement. The NOR test consisted of 3 phases: a habituation phase,
a familiar object phase, and finally the novel object phase. During the habituation phase, the animal was placed in the arena with no objects for a period of 10 min. The familiar object phase followed 2 minutes after the habituation phase. In this portion of the test, the rat was reintroduced into the arena which now contains two familiar objects (two cylindrical amber glass bottles measuring 17 cm in height and 5 cm in diameter) for a period of 5 min. Finally the novel object phase followed 1 hr after the familiar object phase. In this segment of the test, the rat was reintroduced to the arena which now contained one familiar and one novel object (a square transparent glass bottle with feeding nozzle that was 22 cm in height and 5.3 cm in diameter) for a period of 5 min. Familiar and novel objects were spaced approximately 12 cm apart and their position was alternated between trials to avoid location bias. The testing arena and all objects were cleaned with 70 % ethanol between phases. All test phases were recorded and percentage of time spent exploring the novel object (time spent exploring the novel object divided by the time spent exploring both objects multiplied by 100) was determined by an investigator that was blind to the treatment condition. Object exploration was defined as sniffing the novel object or making direct contact with forepaws; sitting on the object was not regarded as exploratory behavior. Animals were subjected to a second NOR test two weeks after 6-OHDA treatment to assess behavioral impairment or the therapeutic value of EX-4.

2.4.4 Open Field Test

The open field test (OFT) was employed to assess spontaneous locomotor activity. The testing apparatus consisted of a circular arena (100 cm in diameter and 35 cm in height) with a grid drawn on the floor. The grid was composed of 14 cm x 14 cm squares. Rats were exposed to the arena for two days (for 5 min each day) prior to testing to induce habituation. The first OFT was performed one day prior to DSP-4 or pCA administration.
This value served as the pre-treatment measurement. Rats were placed in the arena for a period of 5 min and the total number of squares crossed during that time period was determined. The animals were subjected to a second OFT two after 6-OHDA lesion to determine degree of motor impairment.

2.4.5 *In vivo microdialysis*

Animals were anaesthetized with isoflurane (5 % induction v/v in O₂ and 2 % maintenance) and then secured on a stereotaxic frame. CMA 11 guide cannulae were purchased from CMA microdialysis (CMA/microdialysis, Stockholm, Sweden) and inserted stereotaxically into either the striatum (from bregma in mm; AP +0.2, ML 3.0, and DV 4.2) or frontal cortex (AP +3.2, ML 1.5, and DV 3.0) then secured with dental cement (DuraLay, Reliance, Dental MFG. CO) and anchor screws. The animals were then placed in individual microdialysis cages and allowed to recover for 24 hours before dialysis commenced. Cuprophan CMA/11 microdialysis probes (4 mm membrane length, 0.24 mm o.d.) were utilized during the dialysis procedure (CMA/microdialysis, Stockholm, Sweden). During dialysis, the rats were perfused with artificial cerebrospinal fluid (aCSF) (2.5mM KCl, 125mM NaCl, 1.18mM MgCl₂·6H₂O, 1.26mM CaCl₂) pH 7.4 at a rate of 1µl/min using Harvard Apparatus model 22 syringe infusion pump. Samples were collected every 30 minutes in tubes containing 5 µl ascorbic acid (0.2µM), which served as an antioxidant. After the fourth sample, the aCSF was switched to a higher (100mM) K⁺ aCSF and Na⁺ concentration was reduced to account for the change in osmolarity. The high potassium in the aCSF causes neurotransmitter to be released from a nerve terminal and is a measure of the maximum nerve cell secretion capability. This was done for 30 minutes before returning to normal aCSF for the rest of the experiment (3 more samples, 1 every 30min for a total of 8 samples per experiment). Collected samples were frozen at once at -80 °C and analysed for DA, NA, or 5-HT.
within one week using high performance liquid chromatography (HPLC) with electrochemical detection (ECD). HPLC injection volume for microdialysis samples was always 30 μl. An example of chromatogram depicting microdialysis in a sham operated rat can be viewed in figure 2.1.

![Chromatogram](image)

*Figure 2.1: Example of chromatogram depicting microdialysis in sham rat. Image shows basal levels of neurotransmitters- noradrenaline (NA), dopamine (DA), and serotonin (5-HT).*

### 2.4.6 HPLC with ECD

The HPLC system consisted of a reservoir containing mobile phase which was circulated around by a piston pump (P580, Dionex, Germany). Flow was then directed towards a Triathlon refrigerated (4°C) autosampler (Spark-Holland, Netherlands), this led to a C18 reverse phase column maintained at 40°C (ODS 3 WM, 4.6 mm I.D. x 100 mm; Rainin Dynamax Instruments Co. INC., U.S.A.) and protected by a Microsorb guard column (C18 5 WM, 4.6 mm I.D. x 15 mm, Rainin Dynamax Instruments Co. INC., U.S.A.).
This column with guard column was used when analyzing neurotransmitter levels in both microdialysis samples and tissue homogenates. The flow then passed through to an Antec-Decade II electrochemical detector (Antec Leyden BV, Holland) fitted with a VTO3 flow cell (Vcell + 625 mV filtered to 5 abu with range set on 0.5 nA/volt). Data capture was achieved and analysed by Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Chromperfect for Windows software (Justice Innovations chromatography data systems, CA, U.S.). Chromatograms were printed out using an Epson LX-300 printer. All separations were isocratic and the mobile phase was composed of: sodium acetate (0.05 M), EDTA (0.1mM), 1-octane-sulfonic acid (ion paring reagent; 1.4 mM), 10 % methanol in deionised water and pH was adjusted to 3 using citric acid. The mobile phase was de-gassed using an in-line de-gassing unit (Jour Research) and pumped at a flow rate of 0.8 ml/min. The HPLC system was recalibrated and standards run each time the cell required cleaning or there was a need to change the mobile phase. This was done on a weekly basis. An example of a chromatogram generated by our HPLC setup can be seen in figure 2.2.
Figure 2.2: Example of chromatogram depicting monoamine and metabolite standards. Image shows a 3 pmol amount of noradrenaline (NA), dopamine (DA), and serotonin (5-HT), dihydroxy phenyl acetic acid (DOPAC), 5-hydroxy indole acetic acid (5-HIAA), homovanillic acid (HVA).

2.4.7 Internal and External Standards

3,4-dihydroxybenzylamine served as an internal standard in tissue and microdialysis samples and was added at a final concentration of 25 ng/ml to assess the loss of analyte during experimental protocols. The external standard method was used for quantifying DA, NA, and 5-HT levels measured in both tissue and microdialysis samples. Several different known concentrations of DA, NA, and 5-HT were injected into the HPLC to construct calibration curves which were then used to convert sample peak areas to monoamine concentrations. An example of a calibration curve is presented in figure 2.3.
Figure 2.3: Calibration curves for determining the amounts of DA, NA, and 5-HT in striatal/ frontal cortical homogenates and microdialysis samples. The curve was generated with DA, NA, and 5-HT as external standards. The linear equation displayed was used to calculate the amount of neurotransmitter produced using a known peak area detected.
2.4.8 Termination and Tissue Handling

Animals were always killed on completion of microdialysis. This was after a period of two weeks since the initial toxic insult. Animals were first lightly anesthetised with 5% isoflurane v/v in O₂ then quickly placed under guillotine and decapitated. Brains were immediately removed, flash frozen on dry ice, and stored at -80°C until use. On the day of the experiment brains were placed on a ceramic tile over ice for a few minutes to defrost. After the brains were defrosted, dissection was performed and the striatum and frontal cortex were removed using customized stainless steel hole punches. The dissected tissue was then placed into a microcentrifuge tube containing 500 µl ice cold PBS (pH 7.4) and 500 µl ascorbic acid (0.2µM). The sample was then immediately homogenised and frozen at -80°C until further use.

2.4.9 Tissue DA, NA, 5-HT assay

Striatal and frontal cortical tissue homogenates were treated with 0.1M perchloric acid containing ascorbic acid (0.2µM) and EDTA (0.2µM) to precipitate the cell debris (40 µl aliquot of tissue sample + 40 µl of perchloric acid mixture). These then were centrifuged at 13000xg for 10 minutes at 4°C, afterwards supernatant were passed though a syringe filter and DA, NA, and 5-HT tissue levels were estimated using HPLC with ECD (25 µl HPLC injection volume) (Biggs et al., 1992). DA, NA, and 5-HT chromatogram peaks were converted to amount values using an external standard method and expressed as nanograms per gram of tissue homogenate.
2.4.10 Transcardial perfusion-fixation

Perfuse fixation of rats was carried out when the only assessment intended was immunohistochemistry. In this procedure animals were administered a lethal dose of pentobarbital (60mg/kg; Euthatal, Merial, UK), and once animals were under deep anaesthesia, the heart was surgically exposed and a butterfly cannula (Butterfly-21, Hospira Venisystems, Ireland) was inserted into the left ventricle while the right atrium was incised. At first animals were perfused transcardially with 100 ml of heparin-PBS solution 10 units/mL (Multiparin, Wockhardt, UK) to prevent coagulation of blood, followed by another 100 ml of 4% paraformaldehyde (PFA) solution in 0.1M PBS to fix the tissue, after 30 minutes rats were decapitated and the brains carefully removed and post fixed again in 4% PFA in 0.1% PBS for 24 hours at 4°C. Brains were then cryoprotected in a 30% sucrose solution for 24 hours at 4°C then flash frozen on dry ice and stored at -80°C until use.

2.4.11 Immunohistochemistry

Slide mounted 12μm cryostat sections (depicting LC, and SN) from frozen brain blocks were removed from the freezer and allowed to equilibrate to room temperature for 30 minutes before post fixation in 4% w/v PFA, containing 1% w/v gluteraldehyde in deionised water for 5 minutes at 0°C. Following rinsing in 0.01M PBS for 5 minutes, sections were dehydrated through graded alcohols (70%-90%-100%) and endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂ in methanol for 10 minutes. The sections were then rehydrated and non-specific immunoreactivity was blocked using 10% swine serum in 0.01M PBS for 10 minutes. Sections were then incubated in primary antibody (anti-TH IgG raised in rabbit at 1:1000 in 0.01M PBS (Merck Chemicals Ltd.,
United Kingdom; product number 657012-100μl reference: (Du et al., 2001)) for 15 hours at 4°C. After rinsing, the sections were incubated sequentially in biotinylated mouse anti-rabbit antibody 1:500 in 0.01M PBS (Millipore Corporation Billerica, MA; catalogue number MAB201B) and ABC (Vector Labs Ltd.) complex was applied for 30 minutes at room temperature following manufacturer’s instructions. Immunoreactivity was visualized through incubation in 0.5mg/ml 3-diaminobenzidine (DAB), containing 0.009% H₂O₂ for 2 minutes at room temperature. The sections were counterstained in Harris haematoxylin, dehydrated, cleared and mounted for microscopic examination. TH+ cell counting was performed at the level of the third cranial nerve (bregma − 5.6 mm; Paxinos and Watson 1982) in the SN and in the LC (bregma − 10.3 mm; Paxinos and Watson, 1982). Cells were counted in a blinded manner in 3 adjacent tissue sections per rat in both the left SN and left LC using a Leica DC500 system (x40 and x100) and the manufacturer’s software (Leica Microsystems Ltd., Bucks, United Kingdom).

2.4.12 Statistical analysis

The data obtained from the behavioural open field test, sucrose preference test, novel object recognition test, forced swim test, TH+ cell count, and tissue DA, NA, and 5-HT assays were all subjected to one-way analysis of variance (ANOVA) and post hoc Bonferroni’s multiple comparison tests to compare difference between selected treatments. Microdialysis data was subjected to a two-way ANOVA with post hoc Bonferroni’s multiple comparison tests. In both cases data were expressed as mean ±
standard error of the mean. GraphPad Prism 5.03 software was used for all of the statistical manipulations.

2.5 Experimental Protocol and Animal Usage

Each experimental group consisted of 35 rats (11 total experimental groups used). An experimental group was further subdivided into 5 groups of 7 rats. This was done to ensure that a subset of each group would undergo only one behavioral test to prevent confounding results due to multiple test exposure. For example, in the 6-OHDA only treatment group - 7 rats underwent sucrose preference testing (n=7), 7 rats performed the FST (n=7), 7 rats performed the NOR test (n=7), 7 rats were subjected to the OFT (n=7), and 7 rats were used for dialysis (n=7) (7x5 = 35 rats per experimental group). In addition to the behavioral testing, 15 rats from each group were used for tissue content analysis (3 rats from each behavioral testing subgroup, 3 x 5 = 15 rats, n=15) and 4 rats from each experimental group underwent perfuse fixation for future immunohistochemical processing.

All rats undergoing sucrose preference testing (SPT) were exposed to sucrose and water for a period of 4 days prior to experimental testing. Animals were individually housed for the testing procedure which occurred over a period of 2 days. The first SPT occurred in the 2 days prior to exposure to either 25 mg/kg DSP-4 and/or 6 mg/kg pCA or vehicle. This test served as the pre-treatment measurement. The second SPT occurred two weeks after 6-OHDA or vehicle exposure. This value served as the post-treatment measurement. Animals undergoing the OFT and NOR test were exposed to testing arenas for 2 days (for 5 min each day) prior to experimental testing to induce habituation and prevent test anxiety. Animals in the FST, OFT, and NOR test groups were first subjected
to experimental testing one day before exposure to either DSP-4 and/or pCA or vehicle. This test served as the pre-treatment measurement. The second FST, OFT, and NOR tests occurred two weeks after 6-OHDA or vehicle exposure. The values attained from these tests served as the post-treatment measurements.

DSP-4 and/or pCA or vehicle insult was induced 4 days before exposure to either 6-OHDA or vehicle lesion. This methodology was selected to mimic the sequential condition of neurodegeneration present in PD patients. In other words, a serotonergic and/or a noradrenergic deficit was induced prior to a dopaminergic insult. The lesions were allowed to progress for a period of seven days before treatment with EX-4 (0.5 μg/kg, twice a day) or vehicle commenced. Treatment was maintained over a period of seven days. At the conclusion of the treatment period, the animals were subjected to the second set of behavioral testing (FST, NOR test, OFT, and SPT). Animals were also subjected to microdialysis to assess extracellular monoamine content at this time period (2 weeks after 6-OHDA insult). After behavioral and microdialysis assessments were completed, animals were culled and their brains were removed to perform subsequent monoamine tissue content or immunohistochemical analysis. Figure 2.4 graphically depicts the experimental protocol employed in this study.
Train rats with option of sucrose and water (bottles switched to avoid habituation)

Individually house rats and provide sucrose and water. Rats participating in the NOR test and OFT are exposed to testing arena

Measure Fluid Intake. Rats participating in the NOR test and OFT are exposed to testing arena

Provide DSP-4 (25 mg/kg) and/or pCA (6mg/kg) or Vehicle, i.p.

Provide Ex-4, 0.5 µg/kg if required. Administer injections twice daily

Provide 6-OHDA, 15 µg, i.c.

Individually house rats and provide sucrose and water. Rats participating in the NOR test and OFT are exposed to testing arena

Measure Fluid Intake. NOR, FST, and OFT testing day

Individually house rats and provide sucrose and water. Rats participating in the NOR test and OFT are exposed to testing arena

Measure Fluid Intake. NOR, FST, and OFT testing day

Microdialysis Rats were culled at the end of testing day for future cytochemistry

Figure 2.4: Experimental Protocol

* Rats were regrouped after measurement of sucrose preference was taken; both pre- and post-experimental treatment
Chapter 3: The effect of Ex-4 on behavioural paradigms in a premotor rodent model of PD with combined noradrenergic and serotonergic lesion
3.1 Introduction and Experimental Details

In this chapter, we set out to create a premotor rodent model of PD with combined serotonergic and noradrenergic lesion. This particular paradigm is completely novel and the implications behaviourally have never been explored. 6 mg/kg of pCA and 25 mg/kg of DSP-4 were utilized to create a partial lesion of both the serotonergic and noradrenergic system (dosages optimized). Administration of this drug was done four days prior to the administration of 6-OHDA. We choose this dosing regimen to mimic the Braak staging scheme of PD progression (Braak et al., 2003). In this scheme, serotonergic and noradrenergic deficits appear prior to dopaminergic degeneration. We also chose a dose of 15 mg/kg 6-OHDA injected bilaterally into the striatum to induce a partial dopaminergic lesion (dosage optimized). We selected the bilateral model of DA lesion because this particular technique more closely resembles a clinical parkinsonian condition and the progression of lesion is more gradual. In addition, bilateral systemic deficits of monoamines would produce a more valid model of the emotional and cognitive deficits that PD patients experience. We have chosen to assess whether or not our model produces depressive symptomology and memory deficits. The sucrose preference test was utilized to measure anhedonia, a component of depression. In healthy rodents, there is a clear and distinct preference for sweetened solution. However, in chronically stressed and depressed rodents, that sucrose preference is vastly decreased. We have chosen to also evaluate depressive behaviour through the FST. The FST has been extensively employed to test the efficacy of antidepressants. In this test, immobility time is indicative of a depressive state. We have chosen to perform the NOR test to assess object-recognition memory of rats subjected to our combined NA, 5-HT, and DA lesioning paradigm. This test involves presenting two objects to a rodent: one familiar object and one novel object. Healthy rats will spend more time exploring the novel object
and will largely ignore the familiar object. A reduction of time spent exploring a novel object is indicative of a memory deficit.

We have conducted the OFT to assess spontaneous locomotor activity. In this assessment, the number of pre-measured squares an animal crosses is registered and is indicative of motor ability. This test was done to ensure that our dopaminergic lesion was not severe enough to induce gross motor deficits that would confound test results. In addition, since it was our objective to produce a premotor model we needed to evaluate whether or not our dosing scheme was adequate to produce motor symptomology. All behavioural tests (SPT, FST, OFT, and NOR test) were conducted prior to toxin treatment to generate a pre treatment baseline measurement. Two weeks after 6-OHDA treatment (11 days after pCA/DSP-4 treatment), behavioural testing was conducted a second time.

EX-4 has previously been shown to be highly neuroprotective in rodent models of PD (Harkavyi et al., 2008). It is able to recover dopaminergic cell phenotype and stimulate neurogenesis (Bertilsson et al., 2008). We sought to explore whether the therapeutic value of EX-4 could be applied to our premotor rodent model and in particular whether EX-4 could promote the recovery of other monoamine deficits. EX-4 was administered to toxin treatment groups one week after 6-OHDA lesion (11 days after DSP-4 lesion). This protocol is highly relevant to the clinical PD condition since we allow the lesion to develop before we initiate EX-4 treatment. The therapeutic effect of EX-4 was evaluated after seven days of administration (0.5 µg/kg, twice a day).
3.2 Sucrose Preference Data

Our results showed that rats treated with 6-OHDA (73.21 ± 2.43 %, P<0.001) alone showed a significant decrease in sucrose preference compared to the pre-treatment baseline measurement (98.24 ± 1.54 %). pCA + 6-OHDA (48.55 ± 3.85 %) and DSP-4 + 6-OHDA (53.35 ± 4.95 %) groups showed a significant decrease in sucrose preference compared to both their respective pre-treatment baseline measurements (92.23 ± 5.99 % and 95.50 ± 2.70% respectively; P<0.001) and the 6-OHDA only group (P<0.05).

Groups treated with pCA + DSP-4 + 6-OHDA (20.80 ± 3.44 %, P<0.001) demonstrated a significant decrease in sucrose preference compared to pre-treatment baseline measurement (87.33 ± 5.83 %) and the 6-OHDA only (P<0.001), pCA + 6-OHDA (P<0.05) and DSP-4 + 6-OHDA (P<0.05) post-treatment groups. This decrease in sucrose preference was reversed in pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (84.78 ± 3.34 %, 88.35 ± 4.55%, and 80.44 ± 5.42 % respectively, P<0.001). Groups treated with DSP-4, pCA, or EX-4 solely did not show a significant decrease in sucrose preference from baseline pre-treatment measurements. It is important to note the both Sham pre- and post- treatment values were not significantly different from pre-treatment baseline measurements from all other groups. Sucrose preference data can be viewed in figure 3.1.
Sucrose Preference Test

Figure 3.1: Effect of EX-4 (0.5 µg/kg) on sucrose preference in 6-OHDA/pCA/DSP-4 lesioned rats. Sucrose and water consumption was measured over a period of two days both prior to (pre) and after experimental treatment (post). Sucrose preference was calculated as total amount of sucrose consumed over total fluid consumption X 100 to generate a percentage. Results were analyzed using one-way ANOVA (F (21, 132) =21.22) and a post hoc Bonferroni's test to compare differences between groups. (n=7)

* Indicates pCA + 6-OHDA and DSP-4 + 6-OHDA post-treatment groups are significantly different from their respective pre-treatment groups (P < 0.001) and 6-OHDA only post treatment group (P < 0.05) using Bonferonni’s multiple comparison test post hoc (P < 0.001).

+ Indicates 6-OHDA only post-treatment group is significantly different from the 6-OHDA only pre-treatment group (P < 0.001) using Bonferonni’s multiple comparison test post hoc (P < 0.001). (n=7 per experimental group)

$ Indicates the DSP-4 + pCA +6-OHDA post-treatment group is significantly different from the DSP-4 + pCA + 6-OHDA pre-treatment group (P < 0.001) and both the pCA +6-OHDA and DSP-4 + 6-OHDA post treatment groups using Bonferonni’s multiple comparison test post hoc (P < 0.05).
3.3 Forced Swim Test Data

Our results showed that rats treated with 6-OHDA (29.21 ± 1.53 %, P<0.001) alone showed a significant increase in immobility time compared to the pre-treatment baseline measurement (14.44 ± 1.83 %). pCA + 6-OHDA (45.22 ± 3.72 %) and DSP-4 + 6-OHDA (42.95 ± 3.45 %) groups showed a significant increase in immobility time compared to both their respective pre-treatment baseline measurements (16.34 ± 3.11 % and 14.02 ± 2.33 % respectively; P<0.001) and the 6-OHDA only group (P<0.05). Groups treated with pCA +DSP-4+ 6-OHDA (67.89 ± 2.34 %, P<0.001) demonstrated a significant increase in immobility time compared to pre-treatment baseline measurement (17.46 ± 4.95 %) and the 6-OHDA only (P<0.001), pCA + 6-OHDA (P<0.05) and DSP-4 + 6-OHDA (P<0.05) post-treatment groups. This increase in immobility time was reversed in pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (17.48 ± 4.14 %, 20.35 ± 3.94 %, and 19.83 ± 2.57 % respectively, P<0.001). Groups treated with EX-4 alone showed a significant decrease in immobility time (4.41 ± 1.99 %, P<0.05) compared to baseline pre-treatment measurements (17.52 ± 2.11%). It is important to note the both Sham pre- and post- treatment values were not significantly different from pre-treatment baseline measurements from all other groups. Forced swim test data can be viewed in figure 3.2.
Figure 3.2: Effect of EX-4 (0.5 µg/kg) on forced swim test behavior in 6-OHDA/pCA/DSP-4 lesioned rats. Percentage of time spent immobile was measured over a 15 min period both prior to (pre) and after experimental treatment (post). Results were analyzed using one-way ANOVA (F (21, 132) =18.38) and a post hoc Bonferroni’s test to compare differences between groups. (n=7)

* Indicates 6-OHDA post-treatment group is significantly different from the 6-OHDA pre-treatment group, using Bonferroni’s multiple comparison test post hoc (P < 0.05).

+ Indicates EX-4 post-treatment group is significantly different from the EX-4 pre-treatment group, using Bonferroni’s multiple comparison test post hoc (P < 0.05).

$ Indicates pCA + 6-OHDA and DSP-4 + 6-OHDA post-treatment groups are significantly different from their respective pre-treatment groups (P < 0.001) and 6-OHDA only post treatment group (P < 0.05) using Bonferroni’s multiple comparison test post hoc (P < 0.001).

^ Indicates the DSP-4 + pCA +6-OHDA post-treatment group is significantly different from the DSP-4 + pCA +6-OHDA pre-treatment group (P < 0.001) and both the pCA +6-OHDA and DSP-4 + 6-OHDA post treatment groups using Bonferroni’s multiple comparison test post hoc (P < 0.05).
3.4 Novel Object Recognition Data

Our results showed both pCA + 6-OHDA (60.77 ± 4.41 %) and DSP-4 +6-OHDA (58.66 ± 3.52 %) groups showed a significant decrease in novel object exploration compared to their respective pre-treatment baseline measurements (84.02 ± 2.00 % and 88.23 ± 4.70 % respectively; P<0.001). Groups treated with pCA +DSP-4+ 6-OHDA (38.32 ± 4.83 %, P<0.001) demonstrated a significant decrease in novel object exploration compared to pre-treatment baseline measurement (81.64 ± 2.55 %) and the pCA + 6-OHDA (P<0.05) and DSP-4 + 6-OHDA (P<0.05) post-treatment groups. This decrease in novel object exploration was reversed in pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (77.35 ± 5.33 %, 73.89 ± 4.95 %, and 77.72 ± 4.88 % respectively, P<0.001). It is important to note the both Sham pre- and post-treatment values were not significantly different from pre-treatment baseline measurements from all other groups. NOR test data can be viewed in figure 3.3.
Figure 3.3: Effect of EX-4 (0.5 μg/kg) on novel object recognition in 6-OHDA/ pCA/DSP-4 lesioned rats. Percentage of time spent exploring the novel object was measured over a 5 min period both prior to (pre) and after experimental treatment (post). Results were analyzed using one-way ANOVA (F (21, 132) =9.88) and a post hoc Bonferroni’s test to compare differences between groups. (n=7)

* Indicates pCA + 6-OHDA and DSP-4 + 6-OHDA post-treatment groups are significantly different from their respective pre-treatment groups (P < 0.001) and 6-OHDA only post treatment group (P < 0.05) using Bonferonni’s multiple comparison test post hoc (P < 0.001).

+ Indicates the DSP-4 + pCA +6-OHDA post-treatment group is significantly different from the DSP-4 + pCA +6-OHDA pre-treatment group (P < 0.001) and both the pCA +6-OHDA and DSP-4 + 6-OHDA post treatment groups using Bonferonni’s multiple comparison test post hoc (P < 0.05).
3.5 Open Field Test Data

Our results showed no experimental group showed a significant decrease in number of squares crossed in the OFT. OFT test data can be viewed in figure 3.4.

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**Figure 3.4:** Open field test evaluation of spontaneous locomotor activity in 6-OHDA/pCA/DSP-4 lesioned rats. Number of squares (14 x 14 cm) crossed was counted over a 5 min period both prior to (pre) and after experimental treatment (post). Results were analyzed using one-way ANOVA ($F (21, 132) =2.29$) and a post hoc Bonferroni’s test to compare differences between groups. No significant differences were obtained. ($n=7$)
3.6 Chapter Summary of Results

Sucrose Preference Test

1. The 6-OHDA only group demonstrated a reduction in sucrose preference (~ 26 % reduction) compared to baseline measurement. This finding indicates that a sole bilateral dopaminergic lesion is able to produce anhedonic behaviour.

2. The DSP-4 + 6-OHDA group showed a reduction in sucrose preference (~ 44 % reduction) that was significantly lower than the 6-OHDA only group. This finding indicates that additional noradrenergic toxic insult is able to potentiate anhedonic behaviour in rats that have dopaminergic deficiency.

3. The pCA + 6-OHDA group showed a reduction in sucrose preference (~ 47 % reduction) that was significantly lower than the 6-OHDA only group. This finding indicates that additional serotonergic toxic insult is able to potentiate anhedonic behaviour in rats that have dopaminergic deficiency.

4. The DSP-4 + pCA + 6-OHDA group demonstrated a reduction in sucrose preference (~ 77 % reduction) that is significantly greater than the reduction seen in 6-OHDA, DSP-4 + 6-OHDA, and pCA + 6-OHDA treatment groups (DSP-4 + 6-OHDA and pCA + 6-OHDA groups sucrose preference was significantly lower than the 6-OHDA only group). This finding indicates that additional serotonergic and noradrenergic toxic insult is able to potentiate anhedonic behaviour in rats that have either sole dopaminergic deficiency or a dopaminergic deficiency combined with only one toxin (DSP-4 or pCA).
5. EX-4 was able to restore sucrose preference of DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups to pre-treatment baseline levels. This highlights the therapeutic value of EX-4 to treat depressive symptomology.

**Forced Swim Test**

1. The 6-OHDA only group demonstrated an increase in percentage of immobility (~102% increase) compared to baseline measurement. This finding indicates that a sole bilateral dopaminergic lesion is able to produce depressive behaviour.

2. The DSP-4 + 6-OHDA group showed an increase in percentage of immobility (~206% increase) that was significantly higher than the 6-OHDA only group. This finding indicates that additional noradrenergic toxic insult is able to potentiate depressive behaviour in rats that have dopaminergic deficiency.

3. The pCA + 6-OHDA group showed an increase in percentage of immobility (~176% increase) that was significantly higher than the 6-OHDA only group. This finding indicates that additional serotonergic toxic insult is able to potentiate depressive behaviour in rats that have dopaminergic deficiency.

4. The DSP-4 + pCA + 6-OHDA group demonstrated an increase in percentage of immobility (~288% increase) that is significantly greater than the increase seen in 6-OHDA, DSP-4 + 6-OHDA, and pCA + 6-OHDA treatment groups (DSP-4 + 6-OHDA and pCA + 6-OHDA groups immobility percentage time was significantly higher than the 6-OHDA only group). This finding indicates that additional serotonergic and noradrenergic toxic insult is able to potentiate
depressive behaviour in rats that have either sole dopaminergic deficiency or a dopaminergic deficiency combined with only one toxin (DSP-4 or pCA).

5. EX-4 was able to restore percentage of immobility time in the DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups to pre-treatment baseline levels. This highlights the therapeutic value of EX-4 to treat depressive symptomology.

6. Ex-4 alone was able to decrease percentage of immobility (~75% reduction) compared to pre-treatment baseline levels. This finding highlights the possible antidepressive properties of EX-4 stand alone therapy.

**Novel Object Recognition Test**

1. The DSP-4 + 6-OHDA treatment group demonstrated a decrease in percentage of novel object exploration (~34% reduction) compared to baseline measurement. This finding indicates that a combined noradrenergic and dopaminergic deficit is able to produce object recognition memory impairment. A sole bilateral dopaminergic lesion is not able to produce an impairment of memory.

2. The pCA + 6-OHDA treatment group demonstrated a decrease in percentage of novel object exploration (~28% reduction) compared to baseline measurement. This finding indicates that a combined serotonergic and dopaminergic deficit is able to produce object recognition memory impairment. A sole bilateral dopaminergic lesion is not able to produce an impairment of memory.
3. The DSP-4 + pCA + 6-OHDA group demonstrated a decrease in percentage of novel object exploration (~ 53 % reduction) that is significantly greater than the reduction seen in DSP-4 + 6-OHDA and pCA + 6-OHDA treatment groups. This finding indicates that additional serotonergic and noradrenergic toxic insult is able to potentiate object recognition memory impairment in rats that have a dopaminergic deficiency combined with only one toxin (DSP-4 or pCA).

4. EX-4 was able to restore percentage of novel object exploration in the DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups to pre-treatment baseline levels. This highlights the therapeutic value of EX-4 to treat cognitive impairment.

Open Field Test

1. No experimental group differed significantly in number of squares crossed from sham or pre-treatment baseline measurements. This result indicates that no group exhibited motor impairment due to toxin dosing regimen.
Chapter 4: The effect of Ex-4 on tissue and extracellular neurotransmitter levels in a premotor rodent model of PD with combined noradrenergic and serotonergic lesion
4.1 Introduction and Experimental Details

In this chapter, we set out to create a premotor rodent model of PD with selective serotonergic lesion. This particular paradigm is completely novel and the implications neurochemically have never been explored. 6 mg/kg of pCA was utilized to create a partial lesion of the serotonergic system (dosage optimized). Administration of this drug was done four days prior to the administration of 6-OHDA. We choose this dosing regimen to mimic the Braak staging scheme of PD progression (Braak et al., 2003). In this scheme, serotonergic deficit appears prior to dopaminergic degeneration. We also chose a dose of 15 mg/kg 6-OHDA injected bilaterally into the striatum to induce a partial dopaminergic lesion (dosage optimized). We selected the bilateral model of DA lesion because this particular technique more closely resembles a clinical parkinsonian condition and the progression of lesion is more gradual. In addition, bilateral systemic deficits of monoamines would produce a more valid model of the emotional and cognitive deficits that PD patients experience. In the previous chapter, behavioral testing (SPT, FST, OFT, and NOR test) demonstrated that our model produces depressive symptomology and memory deficits without hindering gross locomotor activity.

Two weeks after 6-OHDA treatment (11 days after pCA/DSP-4 treatment), 5-HT and DA tissue levels were evaluated in the FC and STR to determine the extent of damage that was produced due to our toxin administration. These particular regions were selected due to their implication in PD and various affective disorders. NA tissue levels were also evaluated to assess whether our treatment regimen had an impact on other monoamine systems. 5-HT and DA microdialysis was also undertaken two weeks after 6-OHDA insult to evaluate extracellular neurotransmitter levels in the FC and STR. NA
microdialysis was omitted in these regions because tissue levels were not significantly altered.

EX-4 has previously been shown to be highly neuroprotective in rodent models of PD (Harkavyi et al., 2008). It is able to recover dopaminergic cell phenotype and stimulate neurogenesis (Bertilsson et al., 2008). We sought to explore whether the therapeutic value of EX-4 could be applied to our premotor rodent model and in particular whether EX-4 could promote the recovery of other monoamine deficits. EX-4 was administered to toxin treatment groups one week after 6-OHDA lesion (11 days after DSP-4 lesion). This protocol is highly relevant to the clinical PD condition since we allow the lesion to develop before we initiate EX-4 treatment. The therapeutic effect of EX-4 was evaluated after seven days of administration (0.5 μg/kg i.p., twice a day).

4.2 5-HT frontal cortex tissue levels

5-HT tissue levels in the frontal cortex of pCA (408.55 ± 41.54 ng/g, P<0.001), pCA + 6-OHDA (391.43 ± 24.54 ng/g, P<0.001), and DSP-4 + pCA + 6-OHDA (358.54 ± 32.55 ng/g, P<0.001) groups were significantly lower than the sham (770.74 ± 21.83 ng/g group). This decrease in 5-HT levels was reversed in pCA + 6-OHDA and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (715.53 ± 33.34 ng/g and 823.43 ± 34.65 ng/g respectively, P<0.001). 5-HT FC tissue level data test can be viewed in figure 4.1.
Figure 4.1: 5-HT tissue levels in the frontal cortex of 6-OHDA/ pCA/DSP-4 lesioned rats. 5-HT tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA (F (10, 66) =28.59) and a post hoc Bonferroni's test to compare differences between groups. (n=15)

* Indicates pCA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferroni's multiple comparison test post hoc (P < 0.001).
4.3 **DA frontal cortex tissue levels**

DA tissue levels in the frontal cortex of 6-OHDA (563.76 ± 25.53 ng/g, P<0.001), pCA + 6-OHDA (511.73 ± 34.24 ng/g, P<0.001), DSP-4 + 6-OHDA (430.70 ± 29.84 ng/g, P<0.001), and DSP-4 + pCA + 6-OHDA (440.23 ± 35.87 ng/g, P<0.001) groups were significantly lower than the sham (1000.88 ± 26.83 ng/g) group. The DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA group had a significantly lower level of DA compared to the 6-OHDA only group (P<0.05). The decrease in DA levels present in groups treated with 6-OHDA was reversed in DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (987.98 ± 11.34 ng/g, 999.12 ± 21.76 ng/g, and 987.98 ± 32.00 ng/g respectively, P<0.001). DA FC tissue level data test can be viewed in figure 4.2.
Figure 4.2: DA tissue levels in the frontal cortex of 6-OHDA/ pCA/DSP-4 lesioned rats. DA tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA ($F_{(10, 66)} = 104.90$) and a post hoc Bonferroni's test to compare differences between groups. ($n=15$)

* Indicates 6-OHDA treatment group is significantly different from the Sham group, using Bonferroni’s multiple comparison test post hoc ($P < 0.001$).

+ Indicates pCA + 6-OHDA treatment group is significantly different from the Sham group, using Bonferroni’s multiple comparison test post hoc ($P < 0.001$).

$ Indicates DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the 6-OHDA only treatment group, using Bonferroni’s multiple comparison test post hoc ($P < 0.05$).

4.4 NA frontal cortex tissue levels

NA tissue levels in the frontal cortex of DSP-4 (124.45 ± 15.67 ng/g, $P<0.001$), DSP-4 + 6-OHDA (132.70 ± 12.34 ng/g, $P<0.001$) and DSP-4 + pCA + 6-OHDA (111.33 ± 21.89 ng/g, $P<0.001$) groups were significantly lower than the sham (245.10 ± 12.85 ng/g)
group. This decrease in NA levels was reversed in DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (233.89 ± 4.95 ng/g and 238.73 ± 14.98 ng/g respectively, P<0.001). NA FC tissue level data test can be viewed in figure 4.3.

![Frontal Cortex NA Tissue levels](image)

**Figure 4.3:** NA tissue levels in the frontal cortex of 6-OHDA/ pCA/ DSP-4 lesioned rats. NA tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA (F (10, 66) =13.75) and a post hoc Bonferroni’s test to compare differences between groups. (n=15)

* Indicates DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc (P < 0.001).

### 4.5 5-HT striatal tissue levels

5-HT tissue levels in the STR of pCA (442.64 ± 40.66 ng/g, P<0.001), pCA + 6-OHDA (415.39 ± 38.87 ng/g, P<0.001), and DSP-4 + pCA + 6-OHDA (398.23 ± 51.00 ng/g,
P<0.001) groups were significantly lower than the sham (941.55 ± 32.66 ng/g) group. This decrease in 5-HT levels was reversed in pCA + 6-OHDA and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (912.36 ± 43.43 ng/g and 923.77 ± 42.99 ng/g respectively, P<0.001). 5-HT STR tissue level data test can be viewed in figure 4.4.

![Striatal 5-HT Tissue levels](image)

**Figure 4.4:** 5-HT tissue levels in the striatum of 6-OHDA/ pCA/ DSP-4 lesioned rats. 5-HT tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA (F (10, 66) =29.39) and a post hoc Bonferroni’s test to compare differences between groups. (n=15)

* Indicates pCA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc (P < 0.001).
4.6 **DA striatal tissue levels**

DA tissue levels in the STR of 6-OHDA (2177.34 ± 51.54 ng/g, P<0.001), pCA + 6-OHDA (2099.40 ± 68.66 ng/g, P<0.001), DSP-4 + 6-OHDA (1795.70 ± 66.33 ng/g, P<0.001), and DSP-4 + pCA + 6-OHDA (1700.00 ± 71.44 ng/g, P<0.001) groups were significantly lower than the sham (4532.77 ± 69.84 ng/g) group. The DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA group had a significantly lower level of DA compared to the 6-OHDA only group (P<0.05). The decrease in DA levels present in groups treated with 6-OHDA was reversed in DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA co-treated with EX-4 (4537.87 ± 67.30 ng/g, 4512.18 ± 57.34 ng/g, and 4429.99 ± 63.00 ng/g respectively, P<0.001). DA STR tissue level data test can be viewed in figure 4.5.
**Figure 4.5:** DA tissue levels in the striatum of 6-OHDA/ pCA/DSP-4 lesioned rats. DA tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA \((F (10, 66) =407.30)\) and a post hoc Bonferroni's test to compare differences between groups. \((n=15)\)

* Indicates 6-OHDA treatment group is significantly different from the Sham group, using Bonferroni’s multiple comparison test post hoc \((P < 0.001)\).

+ Indicates pCA + 6-OHDA treatment group is significantly different from the Sham group, using Bonferroni’s multiple comparison test post hoc \((P < 0.001)\).

$ Indicates DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the 6-OHDA only treatment group, using Bonferroni's multiple comparison test post hoc \((P < 0.05)\).

**4.7 NA striatal tissue levels**

NA tissue levels in the STR of DSP-4 \((367.23 \pm 43.53 \text{ ng/g, } P<0.001)\), DSP-4 + 6-OHDA \((348.32 \pm 26.88 \text{ ng/g, } P<0.001)\) and DSP-4 + pCA + 6-OHDA \((316.73 \pm 67.23 \text{ ng/g, } P<0.001)\) groups were significantly lower than the sham \((701.88 \pm 21.83 \text{ ng/g})\)
group. This decrease in NA levels was reversed in DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA groups co-treated with EX-4 (667.67 ± 19.11 ng/g and 702.22 ± 64.78 ng/g respectively, P<0.001). NA STR tissue level data test can be viewed in figure 4.6.

**Figure 4.6: NA tissue levels in the striatum of 6-OHDA/ pCA/ DSP-4 lesioned rats.** NA tissue content expressed as ng/g of tissue. Results were analyzed using one-way ANOVA (F (10, 66) =17.68) and a post hoc Bonferroni’s test to compare differences between groups. No significant differences between groups were found. (n=15)

* Indicates DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc (P < 0.001).

### 4.8 Extracellular frontal cortex 5-HT levels

Both basal and high K⁺ evoked extracellular 5-HT levels in the frontal cortex of pCA, pCA + 6-OHDA and DSP-4 + pCA + 6-OHDA groups were significantly lower than the
sham group. All sample time points from these groups were significantly lower than sham values (P<0.001). Basal sham 5-HT levels were approximately 14.52 ± 1.42 fmol/10 μl whereas pCA group levels were 119.22 ± 4.58 fmol/10 μl, pCA + 6-OHDA group levels were 122.31 ± 2.99 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 5.88 ± 1.42 fmol/10 μl (pCA and pCA + 6-OHDA values did not significantly differ from DSP-4 + pCA + 6-OHDA values). High K⁺ evoked extracellular 5-HT levels in the sham group were approximately 256.71 ± 3.46 fmol/10 μl whereas pCA group levels were 119.22 ± 4.58 fmol/10 μl, pCA + 6-OHDA group levels were 122.31 ± 2.99 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 118.88 ± 5.24 fmol/10 μl. This decrease in extracellular 5-HT levels was reversed in groups co-treated with EX-4. Basal 5-HT levels in the pCA + 6-OHDA + EX-4 group were approximately 13.93 ± 1.36 fmol/10 μl and high K⁺ evoked extracellular 5-HT levels were 251.81 ± 5.87 fmol/10 μl. Basal 5-HT levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 15.28 ± 1.03 fmol/10 μl and high K⁺ evoked extracellular 5-HT levels were 250.19 ± 4.22 fmol/10 μl (P<0.001). Extracellular 5-HT levels in the FC can be viewed in figure 4.7. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.7: Extracellular 5-HT levels in the frontal cortex of 6-OHDA/pCA/DSP-4 lesioned rats. 5-HT levels expressed as fmol/10μl of sample. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two-way ANOVA (F (6, 336) =1097.06 for treatment and F (7, 336) =14538.35 for time) and a post hoc Bonferroni’s test to compare differences between groups. (n=7)

* Indicates all time points of pCA, pCA + 6-OHDA, and DSP-4+ pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferroni’s multiple comparison test post hoc (P < 0.001).
4.9 Extracellular frontal cortex DA levels

Both basal and high K⁺ evoked extracellular DA levels in the frontal cortex of 6-OHDA, pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups were significantly lower than the sham group. All sample time points from these groups were significantly lower than sham values (P<0.001). All sample points from the DSP-4 + 6-OHDA group and the DSP-4 + pCA + 6-OHDA groups were significantly lower than 6-OHDA and pCA + 6-OHDA treatment groups (P<0.05). Basal sham DA levels were approximately 16.82 ± 1.12 fmol/10 μl whereas 6-OHDA group levels were 7.84 ± 0.64 fmol/10 μl, DSP-4 + 6-OHDA group levels were 5.71 ± 0.18 fmol/10 μl, pCA + 6-OHDA group levels were 8.31 ± 0.84 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 3.92 ± 0.71 fmol/10 μl. High K⁺ evoked extracellular DA levels in the sham group were approximately 140.47 ± 1.62 fmol/10 μl whereas 6-OHDA group levels were 76.51 ± 1.23 fmol/10 μl, DSP-4 + 6-OHDA group levels were 60.35 ± 1.77 fmol/10 μl, pCA + 6-OHDA group levels were 72.35 ± 4.77 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 54.35 ± 2.42 fmol/10 μl. This decrease in extracellular DA levels was reversed in groups co-treated with EX-4. Basal DA levels in the DSP-4 + 6-OHDA + EX-4 group were approximately 15.37 ± 1.38 fmol/10 μl and high K⁺ evoked extracellular DA levels were 141.72 ± 1.91 fmol/10 μl. Basal DA levels in the pCA + 6-OHDA + EX-4 group were approximately 16.42 ± 1.04 fmol/10 μl and high K⁺ evoked extracellular DA levels were 136.92 ± 3.32 fmol/10 μl. Basal DA levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 16.88 ± 2.01 fmol/10 μl and high K⁺ evoked extracellular DA levels were 137.44 ± 4.66 fmol/10 μl (P<0.001). Extracellular DA levels in the FC can be viewed in figure 4.8. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.8: Extracellular DA levels in the frontal cortex of 6-OHDA/ pCA/DSP-4 lesioned rats. DA levels expressed as fmol/10μl of sample. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K^+. Results were analyzed using two-way ANOVA (F (6, 336) = 1200.30 for treatment and F (7, 336) = 8432.98 for time) and a post hoc Bonferroni’s test to compare differences between groups.

* Indicates all time points of 6-OHDA and pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc (P < 0.001).

+ Indicates all time points of DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the 6-OHDA and pCA + 6-OHDA group (P < 0.001) using Bonferonni’s multiple comparison test post hoc.
4.10 Extracellular frontal cortex NA levels

Both basal and high K⁺ evoked extracellular NA levels in the frontal cortex of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups were significantly lower than the sham group. All sample time points from these groups were significantly lower than sham values (P<0.001). Basal sham NA levels were approximately 9.22 ± 1.35 fmol/10 μl whereas DSP-4 + pCA + 6-OHDA group levels were 3.98 ± 0.52 fmol/10 μl (DSP-4 and DSP-4 + 6-OHDA group levels, which did not significantly differ from DSP-4 + pCA + 6-OHDA values, can be found in chapter 3). High K⁺ evoked extracellular NA levels in the sham group were approximately 112.34 ± 1.46 fmol/10 μl whereas DSP-4 + pCA + 6-OHDA group levels were 77.30 ± 2.74 fmol/10 μl. This decrease in extracellular NA levels was reversed in groups co-treated with EX-4. Basal NA levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 11.38 ± 2.27 fmol/10 μl and high K⁺ evoked extracellular NA levels were 117.88 ± 4.27 fmol/10 μl (P<0.001).

Extracellular NA levels in the FC can be viewed in figure 4.9. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.9: Extracellular NA levels in the frontal cortex of 6-OHDA/ pCA/DSP-4 lesioned rats. NA levels expressed as fmol/10μl of sample. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two-way ANOVA (F (6, 336) =1562.13 for treatment and F (7, 336) =20461.57 for time) and a post hoc Bonferroni's test to compare differences between groups. (n=7)

* Indicates all time points of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni's multiple comparison test post hoc (P < 0.001).
4.11  Extracellular striatal 5-HT levels

Both basal and high K⁺ evoked extracellular 5-HT levels in the STR of pCA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups were significantly lower than the sham group. All sample time points from these groups were significantly lower than sham values (P<0.001). Basal sham 5-HT levels were approximately 2.55 ± 0.85 fmol/10 μl whereas pCA group levels were 1.22 ± 0.44 fmol/10 μl, pCA + 6-OHDA group levels were 0.95 ± 0.27 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 0.87 ± 0.54 fmol/10 μl (pCA and pCA + 6-OHDA values did not significantly differ from DSP-4 + pCA + 6-OHDA values). High K⁺ evoked extracellular 5-HT levels in the sham group were approximately 43.23 ± 1.36 fmol/10 μl whereas pCA group levels were 28.15 ± 2.01 fmol/10 μl, pCA + 6-OHDA group levels were 31.73 ± 2.52 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 26.23 ± 2.86 fmol/10 μl. This decrease in extracellular 5-HT levels was reversed in groups co-treated with EX-4. Basal 5-HT levels in the pCA + 6-OHDA + EX-4 group were approximately 2.52 ± 0.27 fmol/10 μl and high K⁺ evoked extracellular 5-HT levels were 41.74 ± 2.83 fmol/10 μl. Basal 5-HT levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 2.64 ± 0.35 fmol/10 μl and high K⁺ evoked extracellular 5-HT levels were 38.12 ± 3.92 fmol/10 μl (P<0.001). Extracellular 5-HT levels in the STR can be viewed in figure 4.10. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.10: Extracellular 5-HT levels in the striatum of 6-OHDA/ pCA/DSP-4 lesioned rats. 5-HT levels expressed as fmol/10μl of sample. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two-way ANOVA (F (6, 336) =183.06 for treatment and F (7, 336) = 4186.00 for time) and a post hoc Bonferroni's test to compare differences between groups. (n=7)

* Indicates all time points of pCA, pCA + 6-OHDA, and DSP-4+ pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferroni's multiple comparison test post hoc (P < 0.001).

4.12 Extracellular striatal DA levels

Both basal and high K⁺ evoked extracellular DA levels in the STR of 6-OHDA, pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups were significantly lower than the sham group. All sample time points from these groups were significantly
lower than sham values (P<0.001). All sample points from the DSP-4 + 6-OHDA group and the DSP-4 + pCA + 6-OHDA groups were significantly lower than 6-OHDA and pCA + 6-OHDA treatment groups (P<0.05). Basal sham DA levels were approximately 25.14 ± 1.61 fmol/10 µl whereas 6-OHDA group levels were 12.62 ± 2.04 fmol/10 µl, DSP-4 + 6-OHDA group levels were 8.43 ± 2.58 fmol/10 µl, pCA + 6-OHDA group levels were 10.87 ± 2.55 fmol/10 µl, and DSP-4 + pCA + 6-OHDA group levels were 7.72 ± 3.84 fmol/10 µl. High K⁺ evoked extracellular DA levels in the sham group were approximately 487.50 ± 2.74 fmol/10 µl whereas 6-OHDA group levels were 232.31 ± 3.84 fmol/10 µl, DSP-4 + 6-OHDA group levels were 177.41 ± 2.66 fmol/10 µl, pCA + 6-OHDA group levels were 227.47 ± 4.25 fmol/10 µl, and DSP-4 + pCA + 6-OHDA group levels were 162.24 ± 4.05 fmol/10 µl. This decrease in extracellular DA levels was reversed in groups co-treated with EX-4. Basal DA levels in the DSP-4 + 6-OHDA + EX-4 group were approximately 24.16 ± 2.31 fmol/10 µl and high K⁺ evoked extracellular DA levels were 482.29 ± 4.41 fmol/10 µl. Basal DA levels in the pCA + 6-OHDA + EX-4 group were approximately 24.87 ± 1.74 fmol/10 µl and high K⁺ evoked extracellular DA levels were 482.63 ± 6.72 fmol/10 µl. Basal DA levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 23.31 ± 2.47 fmol/10 µl and high K⁺ evoked extracellular DA levels were 481.16 ± 7.48 fmol/10 µl (P<0.001). Extracellular DA levels in the STR can be viewed in figure 4.11. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.11: Extracellular DA levels in the striatum of 6-OHDA/ pCA/DSP-4 lesioned rats. DA levels expressed as fmol/10μl of sample. Perfusion rate was 1μl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K+. Results were analyzed using two-way ANOVA (F (7, 384) =5106.55 for treatment and F (7, 384) =57033.39 for time) and a post hoc Bonferroni’s test to compare differences between groups. (n=7)

* Indicates all time points of 6-OHDA and pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc (P < 0.001).

+ Indicates all time points of DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment group are significantly different from the 6-OHDA and pCA+ 6-OHDA group (P<0.001) using Bonferonni’s multiple comparison test post hoc.
4.13 **Extracellular striatal NA levels**

Both basal and high K⁺ evoked extracellular NA levels in the STR of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA groups were significantly lower than the sham group. All sample time points from these groups were significantly lower than sham values (P<0.001). Basal sham NA levels were approximately 0.48 ± 0.08 fmol/10 μl whereas DSP-4 group levels were 0.21 ± 0.07 fmol/10 μl, DSP-4 + 6-OHDA group levels were 0.25 ± 0.09 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 0.19 ± 0.06 fmol/10 μl (DSP-4 and DSP-4 + 6-OHDA group levels did not significantly differ from DSP-4 + pCA + 6-OHDA values). High K⁺ evoked extracellular NA levels in the sham group were approximately 12.04 ± 0.21 fmol/10 μl whereas DSP-4 group levels were 7.50 ± 0.11 fmol/10 μl, DSP-4 + 6-OHDA group levels were 7.43 ± 0.31 fmol/10 μl, and DSP-4 + pCA + 6-OHDA group levels were 5.99 ± 1.77 fmol/10 μl. This decrease in extracellular NA levels was reversed in groups co-treated with EX-4. Basal NA levels in the DSP-4 + 6-OHDA + EX-4 group were approximately 0.54 ± 0.06 fmol/10 μl and high K⁺ evoked extracellular NA levels were 11.82 ± 0.17 fmol/10 μl. Basal NA levels in the DSP-4 + pCA + 6-OHDA + EX-4 group were approximately 0.56 ± 0.04 fmol/10 μl and high K⁺ evoked extracellular NA levels were 12.13 ± 0.84 fmol/10 μl (P<0.001). Extracellular NA levels in the STR can be viewed in figure 4.12. It is important to note that error bars are present in the figure; however, due to the range of the graph, the bars are hidden by the symbols indicating time points.
Figure 4.12: Extracellular NA levels in the striatum of 6-OHDA/pCA/ DSP-4 lesioned rats. NA levels expressed as fmol/10µl of sample. Perfusion rate was 1µl/min. Samples were collected every 30min. Bar at 90-120min indicates infusion of 100mM K⁺. Results were analyzed using two-way ANOVA ($F (6, 336) = 1937402.25$ for treatment and $F (7, 336) = 35386988.00$ for time) and a post hoc Bonferroni’s test to compare differences between groups. (n=7)

* Indicates all time points of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc ($P < 0.001$).
4.14 Chapter Summary of Results

DA, NA, and 5-HT tissue levels in the FC and STR

1. 5-HT tissue levels were reduced in the FC and STR of pCA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA (~ 47-56% reduction) treatment groups. These results highlight the damage done to the serotonergic system due to our toxin administration (pCA). Dopaminergic and additional noradrenergic insult had no effect on 5-HT levels.

2. DA tissue levels were reduced in the FC and STR of 6-OHDA (~ 45-52% reduction), pCA + 6-OHDA (~ 49-54% reduction), DSP-4 + 6-OHDA (~ 57-60% reduction), and DSP-4 + pCA + 6-OHDA (~ 57-60% reduction) treatment groups (DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment groups had significantly lower DA levels than 6-OHDA only and pCA + 6-OHDA treatment groups). These results highlight the damage done to the dopaminergic system due to our toxin administration. Additional noradrenergic lesion (DSP-4) was able to potentiate DA content loss in a rodent model with DA deficit whereas serotonergic toxin (pCA) administration had no significant effect.

3. NA tissue levels were reduced in the FC and STR of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA (~ 47-51% reduction) treatment groups. These results highlight the damage done to the noradrenergic system due to our toxin administration (DSP-4). Dopaminergic and additional serotonergic insult had no effect on NA levels.
4. EX-4 was able to restore NA, 5-HT, and DA tissue content in both the FC and STR of affected treatment groups to sham group levels. This finding highlights the ability of EX-4 to preserve the functional integrity of noradrenergic, serotonergic, and dopaminergic systems subjected to toxin exposure.

DA, NA, and 5-HT microdialysis levels in the FC and STR

1. Both basal and high K+ evoked 5-HT extracellular levels were reduced in the FC and STR of pCA, pCA + 6-OHDA, and DSP-4 + pCA + 6-OHDA (~53 -57 % reduction) treatment groups. These results highlight the damage done to the serotonergic system due to our toxin administration (pCA). Dopaminergic and additional noradrenergic insult had no effect on 5-HT levels.

2. Both basal and high K+ evoked DA extracellular levels were reduced in the FC and STR of 6-OHDA (~ 50-53% reduction), pCA + 6-OHDA (~ 55-57% reduction), DSP-4 +6-OHDA (~ 66-67% reduction), and DSP-4 + pCA +6-OHDA treatment groups (~ 64-67% reduction). (DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA treatment groups had significantly lower DA levels than 6-OHDA only and pCA + 6-OHDA treatment groups). These results highlight the damage done to the dopaminergic system due to our toxin administration. Additional noradrenergic lesion (DSP-4) was able to potentiate DA content loss in a rodent model with DA deficit whereas serotonergic toxin (pCA) administration had no significant effect.

3. Both basal and high K+ evoked NA extracellular levels were reduced in the FC and STR of DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA +6-OHDA (~53 -57 %

120
reduction) treatment groups. These results highlight the damage done to the noradrenergic system due to our toxin administration (DSP-4). Dopaminergic and additional serotonergic insult had no effect on NA levels.

4. EX-4 was able to restore both basal and high \( K^+ \) evoked 5-HT, NA, and DA tissue content in both the FC and STR of affected treatment groups to sham group levels. This finding highlights the ability of EX-4 to preserve the functional integrity of noradrenergic, serotonergic, and dopaminergic systems subjected to toxin exposure.

5. Microdialysis results mimic tissue content data
Chapter 5: The effect of Ex-4 on TH+ cell counts in a premotor rodent model of PD with combined noradrenergic and serotonergic lesion
5.1 Introduction and Experimental Details

In this chapter, we set out to assess TH+ cell number in our premotor rodent model of PD with combined serotonergic and noradrenergic lesion. This particular paradigm is completely novel and the immunohistological implications have never been explored. 6 mg/kg of pCA and 25 mg/kg of DSP-4 were utilized to create a partial lesion of both the serotonergic and noradrenergic system (dosages optimized). Administration of this drug was done four days prior to the administration of 6-OHDA. We choose this dosing regimen to mimic the Braak staging scheme of PD progression (Braak et al., 2003). In this scheme, serotonergic and noradrenergic deficits appear prior to dopaminergic degeneration. We also chose a dose of 15 mg/kg 6-OHDA injected bilaterally into the striatum to induce a partial dopaminergic lesion (dosage optimized). We selected the bilateral model of DA lesion because this particular technique more closely resembles a clinical parkinsonian condition and the progression of lesion is more gradual. In addition, bilateral systemic deficits of monoamines would produce a more valid model of the emotional and cognitive deficits that PD patients experience. In chapter 3, we assessed whether or not our model produces depressive symptomology and memory deficits through various behavioural paradigms. All behavioural tests (SPT, FST, OFT, and NOR test) were conducted prior to toxin treatment to generate a pre treatment baseline measurement. Two weeks after 6-OHDA treatment (11 days after pCA/DSP-4 treatment), behavioural testing was conducted a second time. The values obtained from these tests served as the post treatment measurements. At this time period, 5-HT, NA, and DA tissue levels were evaluated in the FC and STR to determine the extent of damage that was produced due to our toxin administration (data presented in chapter 4). These particular regions were selected due to their implication in PD and various affective disorders. 5-HT, NA, and DA microdialysis was also undertaken two weeks
after 6-OHDA insult to evaluate extracellular neurotransmitter levels in the FC and STR (data presented in chapter 4). In this chapter, TH immunohistochemistry was performed in the SN and LC to assess possible damage to both noradrenergic and dopaminergic cell bodies. TH+ cell counting was performed at the level of the third cranial nerve (bregma – 5.6 mm; Paxinos and Watson 1982) in the SN and in the LC (bregma – 10.3 mm; Paxinos and Watson, 1982). Cells were counted in a blinded manner in 3 adjacent tissue sections per rat in both the left SN and left LC using a Leica DC500 system (x40 and x100) and the manufacturer’s software (Leica Microsystems Ltd., Bucks, United Kingdom). TH+ staining is indicative of a functional dopaminergic/noradrenergic phenotype. EX-4 has previously been shown to be highly neuroprotective in rodent models of PD (Harkavyi et al., 2008). It is able to recover dopaminergic cell phenotype and stimulate neurogenesis (Bertilsson et al., 2008). We sought to explore whether the therapeutic value of EX-4 could be applied to our premotor rodent model and in particular whether EX-4 could promote the recovery of other monoamine deficits. EX-4 was administered to toxin treatment groups one week after 6-OHDA lesion (11 days after DSP-4 lesion). This protocol is highly relevant to the clinical PD condition since we allow the lesion to develop before we initiate EX-4 treatment. The therapeutic effect of EX-4 was evaluated after seven days of administration (0.5 μg/kg, twice a day).

5.2 TH Immunohistochemistry in the LC

TH immunohistochemistry was performed in the LC of experimental groups cotreated with DSP-4 to assess damage to noradrenergic neurons. TH+ cell counts were reduced in DSP-4 only 4 (28.50 ± 1.60), DSP-4 + 6-OHDA (26.20 ± 2.67), and DSP-4 + pCA + 6-OHDA (28.35 ± 3.25) groups compared to shams (41.70 ± 1.43). This decrease in TH+
Figure 5.2: Tyrosine Hydroxylase (TH) + cell counts in the locus coeruleus (LC). Results were analyzed using one-way ANOVA ($F (5, 18) =7.669$) and a post hoc Bonferroni's test to compare differences between groups. ($n=4$)

* Indicates DSP-4, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferroni's multiple comparison test post hoc ($P < 0.05$).

5.3 **TH Immunohistochemistry in the SN**

TH immunohistochemistry was performed in the SN of experimental groups cotreated with 6-OHDA to assess damage to dopaminergic neurons. TH+ cell counts were reduced in the 6-OHDA (54.55 ± 1.30), DSP-4 + 6-OHDA (48.44 ± 1.65), pCA +6-OHDA (56.35 ± 2.11), and DSP-4 + pCA + 6-OHDA (45.23 ± 3.21) groups compared to shams.
TH+ cell counts in the DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA were lower than those in the 6-OHDA and pCA + 6-OHDA groups; however, it did not reach a level of statistical significance. The decrease in TH+ staining was reduced in groups co-treated with EX-4. The DSP-4 + 6-OHDA + EX-4 (71.32 ± 3.54), pCA + 6-OHDA + EX-4 (74.74 ± 4.21), and DSP-4 + pCA + 6-OHDA + EX-4 (70.32 ± 2.78) groups were not statistically different from sham values. Representative TH+ binding images from multiple rats can be viewed in figure 5.3. TH+ cell counts are shown in figure 5.4.
Figure 5.3: Representative Tyrosine Hydroxylase (TH) Immunostaining in the substantia nigra (SN) - Each slice is 12 μm in thickness and the scale bar represents a length of 100 μm. Rat 1 (R1), Rat 2 (R2), and Rat 3 (R3). Dopaminergic cell bodies appear dark brown in colour visualised using DAB staining.
Figure 5.4: Tyrosine Hydroxylase (TH) + cell counts in the substantia nigra (SN). Results were analyzed using one-way ANOVA ($F(7, 24) = 22.88$) and a post hoc Bonferroni’s test to compare differences between groups. ($n=4$)

* Indicates 6-OHDA, pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA treatment groups are significantly different from the Sham group, using Bonferonni’s multiple comparison test post hoc ($P < 0.05$).
5.3 Chapter Summary

TH immunohistochemistry in the LC and SN

1. TH+ cells were reduced (~31-37% reduction) in the LC of DSP-4, DSP-4 + 6-OHDA, and DSP-4 +pCA+6-OHDA treatment groups. These results highlight a change in functional phenotype in noradrenergic neuronal cell bodies due to our toxin administration.

2. TH+ cells were reduced (~31-45% reduction) in the SN of 6-OHDA, DSP-4 + 6-OHDA, pCA + 6-OHDA, and DSP-4 +pCA+6-OHDA treatment groups. These results highlight a change in functional phenotype in dopaminergic neuronal cell bodies due to our toxin administration.

3. EX-4 was able to recover TH immunoreactivity in the LC and SN of affected treatment groups. This finding highlights the ability of EX-4 to preserve the functional integrity of both noradrenergic and dopaminergic cell bodies subjected to toxin exposure.
Chapter 6: Discussion and Future Work
6.1 Discussion

The "classic" pathology of PD is degeneration of the dopaminergic nigrostriatal pathway. This degeneration produces a loss of DA within the striatum which results in subsequent impaired motor function, which is the cardinal manifestation of the disease. Current pharmacological therapies are palliative, have detrimental side effects, and progressively diminish in efficacy over time. In addition, they merely aim to augment DA transmission and so would not resolve issues arising directly from a deficit in noradrenergic and serotonergic transmission. However, substantial evidence has shown that these other transmitter systems are similarly extensively compromised in PD (Dauer et al., 2003). Both systems show extensive damage in post-mortem brain tissue from PD patients. In fact, this process is now thought to precede degeneration of dopaminergic nigrostriatal neurons. Such degeneration of the NA and 5-HT systems may contribute to non-motor symptoms of PD, particularly deficits in mood, motivation, and cognition. Collectively, although not as overt as the motor symptoms, these non-motor effects significantly decrease quality of life for many PD patients (Tolosa et al., 2007; Ziemssen et al., 2007). In addition, damage to the NA or 5-HT systems could aggravate nigrostriatal degeneration (Chaudhuri et al., 2006; Srinivasan et al., 2003). Yet compared with DA, these systems have received relatively little attention, although their involvement could well explain why the efficacy of anxiolytics and antidepressants is eventually compromised in PD patients (Park et al., 2009).

The aim of this project was first to develop and validate a premotor rodent model of PD. This was accomplished by partially degenerating DA, NA, and 5-HT systems through the use of the selective neurotoxins DSP-4, pCA, and 6-OHDA. The behavioral impairments exhibited by our pre-motor model were assessed using the sucrose preference test, forced swim test, open field test, and novel object recognition. Neurochemical deficits were assessed via in vivo microdialysis, tissue content assays, and immunohistochemistry. The second goal
of the study was to test the efficacy of EX-4 in reversing the deficits exhibited by our premotor paradigm. EX-4 has demonstrated neuroprotective properties both in vitro and in vivo (Bertilsson et al., 2008; During et al., 2003; Kim et al., 2009; Perry et al., 2002a; Perry et al., 2007; Vaillancourt et al., 2009). In particular, recent experiments strongly suggest that stimulation of GLP-1 receptors by EX-4 is efficacious in the treatment of neurodegenerative disorders. EX-4 has been shown to reverse DA deficits and stimulate neurogenesis in late-stage rodent models of PD. The work carried out in this thesis indicates that EX-4 is able to preserve the functional integrity of not only the DA system but also the NA and 5-HT systems. In addition, all alterations in behavioral activity were reversed by co-treatment with EX-4.

Sucrose preference was utilized to assess anhedonic behavior in our premotor rodent model of PD. Anhedonia is a component of depression. In healthy rodents, there is a clear and distinct preference for sweetened solution. However, in chronically stressed and depressed rodents, that sucrose preference is vastly decreased (Bambico et al., 2009; Wang et al., 2009). 6-OHDA only treated rats exhibited an approximately 26% reduction in sucrose preference compared to baseline measurement. DSP-4 + 6-OHDA (~44% reduction) and pCA + 6-OHDA (~47% reduction) groups demonstrated a significantly greater reduction in sucrose preference compared to the 6-OHDA only group. Groups treated with all three toxins exhibited an even greater reduction in sucrose preference (~77% reduction) compared to groups treated with 6-OHDA and one additional toxin. These results mirrored the data obtained from the forced swim test. The forced swim test was employed to measure depressive behaviour present in our treatment groups (Porsolt et al., 1978). The FST has been extensively employed to test the efficacy of antidepressants (Kim et al., 2010; Schmidt et al., 2010). In this test, immobility time is indicative of a depressive state. 6-OHDA only treated rats exhibited
an approximately 102 % increase in immobility time compared to baseline measurement. DSP-4 + 6-OHDA (~206% increase) and pCA + 6-OHDA (~176% increase) groups demonstrated a significantly greater increase in immobility time compared to the 6-OHDA only group. Groups treated with all three toxins exhibited an even greater increase in percentage of immobility time (~288 % increase) compared to groups treated with 6-OHDA and one additional toxin. The results of the SPT and FST indicate that a sole dopaminergic lesion is adequate to produce depressive behaviour. This observation has been found previously by groups utilizing a premotor bilateral 6-OHDA or MPTP model of PD (Santiago et al., 2010; Tadaiesky et al., 2008). This condition could be explained by the loss of dopamine in the basal ganglia and the ever growing evidence of the nigrostriatal pathway in mesolimbic function (Frisina et al., 2009; Lieberman, 2006; Mayberg, 1994; Mayberg et al., 1995; Mayeux, 1990). It has been suggested that neuronal cell bodies in the SN and VTA are not as functionally distinct as has previously been thought. In fact, bilateral 6-OHDA lesioning of the SN and VTA independently produced depressive-like behaviors which were reduced by treatment with citalopram which indicates an influence of the 5-HT system on DA induced depression (Winter et al., 2007). In addition, it cannot be ruled out that our injection diffused into the ventral striatum, an area of the brain highly involved in motivational behavior and reward pathways. Partial denervation of the afferents in this area and their source in the ventral tegmental area (VTA) is common in PD. This denervation, although not as great as seen in the caudate/putamen, could partially explain the depressive/anhedonic symptomology observed in PD patients. Our results also show that an additional noradrenergic and/or serotonergic lesion further potentiated anhedonic/depressive behavior. This observation supports the work of several groups that have shown there is decreased NA transporter binding in depressed patients with PD (Remy et al., 2005). This lack of transporter binding occurs in locus coeruleus and several limbic regions which receive dense...
noradrenergic innervation including the thalamus, amygdala, anterior cingulate, and ventral striatum. These regions have been implicated in various depressive disorders and anxiety (LeDoux, 2000; Ressler et al., 1999). A reduction in LC pigmentation and NA transporter binding has also been observed in suicidal patients and patients with major depression (Arango et al., 1996; Klimek et al., 1997). 5-HT depletion has also been implicated in the pathology of depression (Kish, 2003; Leentjens et al., 2003; Lieberman, 2006). Numerous studies demonstrate a reduction of 5-HT and 5-HIAA in depressed patients with PD. In addition, a decreased SERT binding has been observed in early PD patients utilizing $[^{11}\text{C}]$McN5652 and $[^{11}\text{C}]$-3-Amino-4-(2-dimethylaminomethylphenylsulfonyl)-benzonitrile (DASB) positron emission tomography (PET) (Albin et al., 2008; Kerenyi et al., 2003). In addition, several studies utilizing tryptophan depletion have resulted in the patients experiencing depressive symptoms. Abnormal 5-HT and NA levels have been implicated in the pathophysiology of depression and several effective antidepressants on the market inhibit either 5-HT and/or NA reuptake. Although the exact mechanism of action of these drugs is largely unknown, it is assumed that the initial acute increase in synaptic transmitter induces long-lasting downstream changes in the brain after chronic antidepressant treatment (Lee et al., 2010). It is our assumption that these three transmitter systems work synergistically to produce a positive emotional state. Additional lesioning of either the NA and/or 5-HT systems produces a potentiation of depressive behavior. It is assumed that NA and 5-HT functionally compensate for DA loss in different ways. Our tissue level assay data, which will be discussed later, shows that NA loss is able to potentiate DA damage in both the FC and STR. In addition, DA transmission may functionally compensate for NA and 5-HT loss. Our tissue level data indicates that there is an increase in DA levels in DSP-4 only and pCA only treatment groups; however, this increase did not reach statistical significance. Studies involving vesicular monoamine
transporter 2 (VMAT2)-deficient mice which have reduced levels of DA, NA, and 5-HT demonstrate progressive deficits in olfactory discrimination, delayed gastric emptying, altered sleep latency, anxiety-like behavior, and age-dependent depressive behavior. These results support our data and suggest that general monoamine deficiency can be responsible for the several of the nonmotor symptoms of PD (Taylor et al., 2009).

In addition to depressive symptomology, we wanted to observe whether our model produced deficits in cognitive processes which are also experienced by PD patients. These deficits which are evident early on in the condition, take the form of bradyphrenia in respect to visuospatial, attentional, and executive processing (Lees et al., 1983). We have chosen to perform the NOR test to assess object-recognition memory of rats subjected to our combined NA, 5-HT, and DA lesioning paradigm. This test involves presenting two objects to a rodent: one familiar object and one novel object. Healthy rats will spend more time exploring the novel object and will largely ignore the familiar object. A reduction of time spent exploring a novel object is indicative of a memory deficit (Botton et al., 2010; Wong et al., 2010). Our results indicate that a sole dopaminergic lesion is not able to reduce percentage of time spent exploring the novel object; however when combined with either a NA and/or 5-HT lesion, memory impairments emerge. DSP+ 6-OHDA (~34 % reduction) and pCA + 6-OHDA (~28 % reduction) groups demonstrated a significant reduction in novel object exploration. Groups treated with all three toxins (~53% reduction) exhibited an even greater reduction in novel object exploration. Object-recognition memory has never been assessed in a 6-OHDA premotor model of PD. Other groups have shown cognitive alterations in the cued and spatial memory versions of the water maze in premotor rodent models of PD (Da Cunha et al., 2001; Da Cunha et al., 2007; Ferro et al., 2005; Reksidler et al., 2007). These alterations are hypothesized to occur due to DA deficit in the STR and FC, which
has been implicated in both procedural and working memory (Packard et al., 2002; Saint-Cyr et al., 1988; Torriero et al., 2007). Our results indicate that object memory is not strictly governed by DA reduction but requires additional NA or 5-HT deficit to produce impairments. It has previously been shown that tryptophan depletion has produced deficits in the NOR paradigm (Lieben et al., 2004). These deficits are thought to be caused by alterations in 5-HT transmission in regions within the medial temporal lobe such as parahippocampal cortices, particularly the perirhinal cortex, which is important for memory encoding, consolidation, and retrieval processes (Jenkins et al., 2010). It is important to note that these studies produced a more extensive depletion of 5-HT levels than our toxin administration protocol. NA has also been implicated in memory consolidation in rats exposed to the object recognition task (Roozendaal et al., 2008). LC degeneration has been shown to be an early indicator of Alzheimer’s pathology and increases both neuroinflammatory response and amyloid deposition (Jardanhazi-Kurutz et al., 2010). In addition, it cannot be ruled out that inflammation due to our 6-OHDA administration may be affecting various brain regions implicated in object recognition processing including the FC, STR, and hippocampus (Na et al., 2010; Owen, 2004). It has been shown that 6-OHDA lesion of the mesocortical and mesolimbic pathways, which are partially affected in PD, can cause a decrease in novel object exploratory behaviour (Fink et al., 1980). We hypothesize that the NA, DA, and 5-HT systems may each play a role in the production of object memory formation and consolidation which would explain our observed results.

We conducted the open field test to assess spontaneous locomotor activity. In this assessment, the number of pre-measured squares an animal crosses is registered and is indicative of motor ability. This test was done to ensure that our dopaminergic lesion was not severe enough to induce gross motor deficits that would confound test results. All
treatment groups did not differ significantly from their respective pre-treatment baseline levels.

5-HT, NA, and DA tissue levels were evaluated in the FC and STR to determine the extent of damage that was produced due to our toxin administration. 5-HT levels were only reduced in groups treated with pCA (pCA, pCA + 6-OHDA, DSP-4 + 6-OHDA). In the FC and STR, there was an approximately 47-56% reduction in 5-HT levels. There were no significant differences in 5-HT levels between groups treated with pCA. pCA treatment did not have a significant effect on DA or NA tissue levels. NA levels were only reduced in groups treated with DSP-4 (DSP-4, DSP-4 + 6-OHDA, DSP-4 + pCA + 6-OHDA). In the FC and STR, there was an approximately 47-51% reduction in NA levels. There were no significant differences in NA levels between groups treated with pCA. DA levels were reduced in the FC and STR of all groups treated with 6-OHDA (6-OHDA, pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA). DA levels were further significantly reduced in 6-OHDA groups cotreated with DSP-4 (DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA). 6-OHDA and pCA + 6-OHDA groups displayed an approximately 45-54% reduction in DA levels whereas DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA groups displayed a 57-60% reduction. These results indicate DSP-4 treatment potentiated DA loss in groups treated with 6-OHDA. The potentiation of dopaminergic deficit in PD models as a result of additional NA lesioning has been observed by several other groups (Lategan et al., 1990; Mavridis et al., 1991; Srinivasan et al., 2003). Both Mavridis et al. and Fornai et al. noticed that the MPTP-induced damage to nigrostriatal DA neurons was potentiated by pretreatment with DSP-4 (Fornai et al., 1995; Fornai et al., 2005; Mavridis et al., 1991). Recently Wang et al. observed increased apomorphine-induced circling behavior after dual 6-OHDA lesions into the LC and SN, respectively (Wang et al., 2010). Stimulation of the LC facilitates burst firing of
SNc neurons (Grenhoff et al., 1988). NA appears to act as a compensatory mechanism for dopaminergic loss through its ability to modify the firing rate of SNc neurons and thus the release of DA. In addition, it has been shown that extracellular NA exhibits antioxidant and anti-inflammatory properties (Rommelfanger et al., 2007). Release of co-factors such as galanin and BDNF may also aid in the neuroprotection of surviving DA neurons (Castren et al., 1995; O'Meara et al., 2000).

Analysis of extracellular NA, DA, and 5-HT content was accomplished through in vivo microdialysis. The results of microdialysis mirrored the results obtained from tissue content analysis. Both basal and high K⁺ evoked 5-HT levels were only reduced in groups treated with pCA (pCA, pCA + 6-OHDA, DSP-4 + 6-OHDA). In the FC and STR, there was an approximately 53-57% reduction in 5-HT levels. There were no significant differences in 5-HT levels between groups treated with pCA. pCA treatment did not have a significant effect on extracellular DA or NA levels. Both basal and high K⁺ evoked NA levels were only reduced in groups treated with DSP-4 (DSP-4, DSP-4 + 6-OHDA, DSP-4 + pCA + 6-OHDA). In the FC and STR, there was an approximately 53-57% reduction in NA levels. There were no significant differences in NA levels between groups treated with pCA. Both basal and high K⁺ evoked DA levels were reduced in the FC and STR of all groups treated with 6-OHDA (6-OHDA, pCA + 6-OHDA, DSP-4 + 6-OHDA, and DSP-4 + pCA + 6-OHDA). DA levels were further significantly reduced in 6-OHDA groups cotreated with DSP-4 (DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA). 6-OHDA and pCA + 6-OHDA groups displayed an approximately 50-57% reduction in DA levels whereas DSP-4 + 6-OHDA and DSP-4 + pCA + 6-OHDA groups displayed a 64-67% reduction. These results again indicate DSP-4 treatment potentiated DA loss in groups treated with 6-OHDA. The decreased levels of NA, DA, and 5-HT we observed mimics clinical data obtained from PD patients experiencing non-motor symptomology.
TH immunohistochemistry was also qualitatively performed in the SN and LC to assess damage to both noradrenergic and dopaminergic cell bodies. The results showed decreased TH+ cell number (~31-37% reduction) in the LC of groups treated with DSP-4. DSP-4 treatment has previously been shown to reduce dopamine β hydroxylase (DBH) staining in the LC indicating loss of functional neuronal phenotype (Ross et al., 1974). We assume that the reduction in TH observed also indicates a loss of function in the NA cell bodies. Reduced TH staining was also observed in the SN of all groups treated with 6-OHDA (~31-45% reduction). This result was expected since 6-OHDA treatment has long been known to cause degeneration of DA neurons in the SN. We; however, speculate that the decreases in TH+ staining observed in both the LC and SN represent a phenotypical shift in protein expression rather than cell loss. Other groups have observed a loss of TH+ expression without the concomitant loss of neurons (Bowenkamp et al., 1996; Cohen et al., 2011; Sauer et al., 1994). It is important to note that reductions in immunoreactivity observed in all these groups were partial and not complete, verifying our experimental paradigm objective. One interesting finding that we observed was that DSP-4 treatment along with 6-OHDA administration did not significantly reduce TH+ staining in the SNc compared to 6-OHDA only or pCA + 6-OHDA treatment groups although cell numbers appeared reduced. We hypothesize that DSP-4 may be altering the firing rate of SNc neurons and thus reducing the release of DA at this particular time point (Rommelfanger et al., 2007). However, it cannot be ruled out that DSP-4 can promote the degeneration of SNc neurons over a longer time course or higher dosage. In addition, a larger sample size might reveal significant differences.

EX-4 co-treatment was able to reverse all neurochemical and behavioral deficits produced by our premotor model of PD. EX-4 restored sucrose preference, immobility
time in the FST, and novel object exploration in the NOR test to pretreatment baseline levels. An important finding that we observed in the FST was the ability of EX-4 stand-alone treatment to significantly decrease percentage of immobility time. This finding is important since it highlights the ability of EX-4 to exert anti-depressive properties as a sole treatment. Very recently, Isacson et al. demonstrated that chronic standalone EX-4 treatment in rodents was able to reduce immobility time in the FST. They attributed this finding to an increase in hippocampal neurogenesis due to EX-4 administration (Isacson et al., 2010).

EX-4 was also able to restore tissue content and extracellular DA, NA, and 5-HT levels. TH binding was also recovered in groups co-treated with EX-4. All these results indicate EX-4 was able to recover functional DA, NA, and 5-HT phenotype in groups treated with toxins. The mechanism of action whereby EX-4 is able to exert its neuroprotective effects is not yet known.

It is hypothesized that the mechanism of action of EX-4 is not simply the activation of one or two receptor subtypes. It is most likely complex and multimodal. Since GLP-1Rs have been found in raphe nuclei (RN), SN, LC, STR, and FC, it is possible that EX-4 is exhibiting anti-inflammatory, anti-apoptotic and neurotrophic properties similar to GLP1-R activation in pancreatic β cells and neuronal cell lines (Merchenthaler et al., 1999; Perry et al., 2002a; Perry et al., 2002b; Perry et al., 2004). GLP-1R activation also appears to promote neuronal restoration/protection in vivo (Bertilsson et al., 2008; Harkavyi et al., 2008; Kim et al., 2009; Li et al., 2009). Preliminary data completed in our lab suggest that the mechanism of action of EX-4 may also involve activation of corticotrophin-releasing factor-1 receptor (CRF-R1) since administration of selective antagonist NBI-27914 to EX-4 co-treated groups reduced the neuroprotective effect of EX-4. This data is relevant due to recent findings that urocortin (UCN), an endogenous
peptide which is a non-selective CRF receptor agonist, was neuroprotective in the two rodent models of PD and its mechanism was CRF-R₁ receptor dependent (Abuirmeileh et al., 2007; Harkavyi et al., 2008). These results suggest that both EX-4 and UCN may utilise a common mechanism involving CRF-R₁ receptors.

GLP-1Rs are expressed in both rodent and human CNS, but are especially abundant in the hypothalamus where they are thought to regulate food intake (During et al., 2003). Taking into account the current findings, it is possible that EX-4 might work by activating GLP-1Rs in the hypothalamus which in turn would cause a release of CRF activating CRF-R₁ receptors on DA, NA, and 5-HT neurons promoting their survival. CRF₁ receptor activation has been shown to promote neural growth and arborization of noradrenergic neurones in the locus coeruleus (Swinny et al., 2006). In addition, CRF₂ receptor activation has been shown to increase TH activity via the cAMP/PKA pathway (Nanmoku et al., 2005). The release of CRF may also cause stress-induced DA release due to the stimulation of hypothalamic-pituitary-adrenal axis (Sarkar et al., 2003) which might lead to increased DA receptor stimulation including the D₃-type which is involved in neuroprotection/neurogenesis (Borta et al., 2007; Goetz et al., 1998; Van Kampen et al., 2006; Van Kampen et al., 2004). As mentioned previously, EX-4 was shown to reduce food intake via hypothalamic GLP-1R stimulation. This is interesting because there is accumulating evidence in the literature suggesting that diet restriction in laboratory animals is neuroprotective (Perry et al., 2002a; Plunet et al., 2008; Sharma et al., 2005). Authors report an upregulation of oxyradical defences such as superoxide dismutase, glutathione peroxidase, and increased expression of heath shock protein 70 in animals kept on restricted diets and also an increase in brain derived neurotrophic factor (BDNF) expression which promotes neuronal survival via TrkB receptor activation (Hurtig et al., 2000; Mattson, 2010; Mattson, 2000). It is thus possible that another part
of the neuroprotective mechanism of EX-4 is through reducing food intake in animals leading to metabolic changes similar to animals kept on restricted diets. We hypothesize that the ability of EX-4 to increase BDNF expression through dietary restriction may be partly responsible for the anti-depressive properties of the drug observed in this study (Lee et al., 2010). In addition, BDNF increase has been shown to stimulate neurogenesis which may aid in the regenerative process over time.

Recent literature has suggested that EX-4 might work through GLP-1Rs present in the SVZ and subgranular zone (SGZ), where it was proposed to stimulate neurogenesis (Bertilsson et al., 2008; Isacson et al., 2010). It is possible that EX-4 could cause neural stem cell (NSC) migration from SVZ or SGZ, possibly to repopulate the damaged SN, LC, and RN. However, this process most likely would not account for the recovery seen in this study due to the short time frame of the experiment. We hypothesize that EX-4 is rescuing neurons that have lost their dopaminergic phenotype through its anti-apoptotic and anti-inflammatory properties (Bowenkamp et al., 1996; Cohen et al., 2011; Sauer et al., 1994). Previous research has shown that cAMP-mediated pathways regulate the anti-apoptotic actions of GLP-1 in β-cells (Buteau et al., 2004; Li et al., 2005; Tews et al., 2009). GLP-1R activation increases the expression of anti-apoptotic genes such as Bc12 and Bclxl (Buteau et al., 2004). The neuroprotective effects of substances that increase cAMP have been demonstrated in many neuronal cells (D'Mello et al., 1993; Hanson et al., 1998; Mena et al., 1995). GLP-1R stimulation has also been shown to be anti-inflammatory. GLP-1 has been shown to inhibit LPS-induced production of IL-1β by cultured rat astrocytes (Iwai et al., 2006). GLP-1 also decreased expression of interleukin-6 and inducible nitric oxide synthase mRNA (Iwai et al., 2006). Li et al. demonstrated that EX-4 protected against cytokine-induced apoptosis by hindering cytokine-evoked inhibition of protein kinase B phosphorylation (Li et al., 2005). More
recently, Kim et al. found that EX-4 prevented the expression of matrix metalloproteinase-3 and subsequent MPTP-induced microglial activation in the SNC and striatum (Kim et al., 2009). They also showed a reduction of MPTP-induced expression of pro-inflammatory molecules and tumor necrosis factor α and interleukin-1β in groups treated with EX-4 (Kim et al., 2009; Kim et al., 2007; Kim et al., 2005).

Another hypothesis regarding EX-4’s mechanism of action is possible axonal sprouting (Filbin, 2003). GLP-1R activation in the SN, LC, or RN would increase intracellular cAMP (Li et al., 2003). cAMP administration in the neuronal soma has been previously shown to hinder the inhibitory effect of myelin-associated inhibitors and myelin-associated glycoprotein on axon growth in vitro (Neumann et al., 1999; Qiu et al., 2002). cAMP stimulation of the soma has also been shown to promote sensory axonal sprouting (Neumann et al., 1999; Qiu et al., 2002). It is possible that EX-4 treatment may be eliciting axonal sprouting from surviving terminals, thereby, restoring innervation to effected regions of the brain. GLP-1R stimulation can also initiate activation of the PKA/cAMP pathway (DeCastro et al., 2005; Mally et al., 2004). This pathway has been shown to regulate both TH and TPH gene expression (Foguet et al., 1993; Kim et al., 1994; Piech-Dumas et al., 2001). Increased stimulation of the PKA/cAMP pathway could lead to enhanced TH/TPH expression and subsequent restoration of DA/NA/5-HT storage and release (Chen et al., 2008; Dunkley et al., 2004; Foguet et al., 1993; Gueorguiev et al., 2006; Yamamoto et al., 2003). Possible mechanisms of EX-4 are summarised in Figure 6.1.
Figure 6.1: Proposed mechanism of action of exendin-4 in the adult rat brain. EX-4 can directly activate GLP-1Rs on the cell bodies of dopaminergic, serotonergic, and noradrenergic neurons. Direct GLP-1R activation would elicit anti-apoptotic and anti-inflammatory effects. It could also upregulate TH/TPH expression. EX-4 can also activate GLP-1Rs in the SVZ and SGZ promoting neurogenesis. EX-4 may also cause CRF release from paraventricular nucleus (PVN) of the hypothalamus (HT) via GLP-1R; CRF offers neuroprotection via the CRF-R1. EX-4 reduces food intake via hypothalamus GLP-1R activation. This dietary restriction (DR), leads to upregulated BDNF expression. BDNF promotes neuronal growth and survival via TrkB receptor activation which has also been implicated in neurogenesis.
3.2 Future Work

There are numerous questions that arise from the findings of our study. First and foremost, it would be important to assess whether the neuroprotective effects of EX-4 are truly governed by the GLP-1R. Administration of a selective GLP-1R antagonist into the brain of groups co-treated with EX-4 would demonstrate whether or not the therapeutic effects of EX-4 are governed by central GLP-1R stimulation. Alternatively, nucleus specific deletion of GLP-1R using adeno-associated virus expressing anti-sense mRNA for the GLP-1R could be employed. It would also be interesting to address the possible issue of neurogenesis. Markers of cell proliferation and immature neurons such as proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) can be used to examine whether there are new cells present in the SN, LC, and RN of experimental groups co-treated with EX-4. NAT, DAT, and SERT immunohistochemistry could be performed to qualitatively assess levels of these relevant proteins in our pre-motor paradigm (we attempted to gather preliminary data; however, our protocols were not optimized at the time). Alternatively, Western blot analysis can be conducted in the future to determine protein levels.

In addition, our paradigm needs to be tested using other behavioral models such as chronic variable stress or the Morris water maze to ensure reliable and consistent results. Our paradigm could also be assessed for anxiety related behaviors through exposure to such tests as the light/dark box and elevated plus maze. Variation of the dosage of neurotoxins could also be employed to decipher changes in behavior due to neurochemical manipulation. It is also crucial to determine changes in pre and post synaptic receptor expression to understand what is occurring at a cellular level due to our toxic intervention. Tissue content analysis and microdialysis could also be performed in
other brain regions such as the hippocampus, which also has been implicated in cognitive and affective dysfunction (Owen, 2004; Schrag, 2004).

The use of other drugs (such as 6-OHDA, or 5,7 dihydroxytryptamine) to selectively damage noradrenergic and serotonergic neurons directly could be employed to verify our findings and ensure that neurons are indeed damaged. Finally, in vitro work needs to be carried out in neuronal cell lines to understand which cell signaling pathways are activated specifically upon GLP -1R activation.

3.3 Final Remarks

We have successfully generated a novel pre-motor model of PD that recapitulates the emotional and cognitive deficits that PD patients experience. Our model not only addresses dopaminergic deficit but also noradrenergic and serotonergic system compromise. This is important, as recent literature has suggested that PD affects not only the DA system but also the NA and 5-HT pathways. Although no animal model can fully recapitulate the human PD condition, our model addresses certain prominent symptomology. This model is a useful tool in testing the efficacy of novel compounds to treat the nonmotor symptoms of PD that greatly impairs the patient’s quality of life. In addition, our model allows for the progression of neurotoxic lesions before therapeutic treatment is commenced which more accurately mimics the clinical condition. Although genetic models may offer a more valid paradigm of the pathology, these models require extensive resources and maintenance. Our premotor toxin model offers a fast and reliable means to produce some of the prominent non-motor symptomology experienced by PD patients. We have demonstrated that EX-4 is able to recover both neurochemical and behavioral deficits produced by our model. These results suggest that EX-4 could be
used as an early treatment option for the premotor symptoms of the disorder. In addition, due to the ability of EX-4 to preserve the functional integrity of the DA, NA, and 5-HT systems, it would be highly beneficial to start treatment as soon as possible. Although the exact mechanism of action of EX-4 is not known, this does not distract from its neuroprotective abilities as an endogenous peptide. Finally, results presented in this thesis combined with studies of Harkavyi et al., 2008, Bertilsson et al., 2008, Perry et al., and Kim et al., 2009 have led to the first EX-4 clinical trial with support of The Cure Parkinson’s Trust, UK.
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