INVESTIGATING CDNF IN PARKINSON'S DISEASE

CEREBRAL DOPAMINE NEUROTROPHIC FACTOR (CDNF) AND PARKINSON'S DISEASE: BEHAVIOURAL AND CLINICAL INVESTIGATIONS

By

KRISTEN J. TERPSTRA

BA (Hons)

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

Master of Science (2014) McMaster University

MiNDS- Neuroscience Hamilton, Ontario

TITLE: Cerebral dopamine neurotrophic factor (CDNF) and Parkinson's disease: Behavioural and clinical investigations

AUTHOR: Kristen J. Terpstra BA (Hons), University of Western Ontario

SUPERVISOR: Dr. Ram K. Mishra

COMMITTEE: Dr. Shucui Jiang

Dr. Laurie Doering

NUMBER OF PAGES: xiii, 102

ABSTRACT

Parkinson's disease (PD) is among the most devastating neurodegenerative disorders, and affects 1% of the global population above the age of 60. Several mechanisms have been proposed to explain the dopamine degeneration exhibited in PD: mitochondrial dysfunction, and endoplasmic reticulum (ER) stress. Inaccurate diagnosis is one of the greatest challenges to treatment of PD. Currently, there is no standard diagnostic test for PD. Neurotrophic factors (NTF) are secreted proteins that promote survival and maintenance of neurons during development. The loss of NTFs for specific neuronal populations could confer susceptibility to various neurodegenerative disorders. Cerebral dopamine neurotrophic factor (CDNF) is a novel NTF selective for dopamine neurons. CDNF has demonstrated profound neuroprotective and neurorestorative effects on dopamine neurons in well established animal models of PD. Presently, there are no studies examining endogenous levels of CDNF in PD models or clinical populations of PD, prompting the present study. Findings will bring insight into the neurobiological mechanisms underlying neurodegeneration in PD. This study has determined that CDNF protein and mRNA expression is not altered following 6-OHDA lesioning, suggesting a compensatory mechanism of CDNF in response to injury. We have also determined that CDNF mRNA expression declines with age, which could confer susceptibility to developing neurodegenerative diseases such as PD. In clinical populations of PD, platelets showed a significant increase in CDNF mRNA expression that was not seen in lymphocytes or whole blood. This suggests a role of CDNF in PD, specifically for platelets; however, it is important to delineate whether this increase is the result of treatment. Incidentally, we found that CDNF mRNA expression is significantly reduced following stroke. Together, these

results stress the importance of CDNF in disorders stemming from ER stress. Future studies should examine the role of CDNF in preclinical models of stroke, as well as knockout models of PD.

I dedicate this work to my grandfather, Tadeusz Blaszczyk.
You may not be with us anymore, but I am so proud to say this work may help further research on your disease, and maybe one day there will be a cure.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Mishra for his support and guidance over the last two years. I am extremely grateful to have had the opportunity to work in your lab. Your approach to mentoring students has allowed me to develop into a competent and confident scientist, and I am forever grateful.

I would also like to thank my undergraduate supervisor, and Dr. Mishra's first student, Dr. Simon Chiu. Without your continued mentorship and dedication to my career I would not be where I am today. I cannot express in words how thankful I am to have you as a mentor. You are one of the kindest, most humble individuals I have had the pleasure of working work. I would also like to thank the other members of the Chiu lab, Dr. Bureau, Dr. Hou, Dr. Chernovsky and Hana. You all have been a tremendous influence in my life and have helped me in so many ways both academically and personally. I thank you all from the bottom of my heart.

I am so glad to have been welcomed into the Mishra lab, and to have had the pleasure of working with some of the kindest, intelligent and motivated individuals I've ever met. Asim, my lab partner, without your help I would never have been able to finish this project, nor would I have had so much fun doing so. Your continued support and open ear have made this experience one of the most remarkable thus far. Ritesh, Christal, Luke, Jay, Mattea, Dipa, Farhat, Pat, Tiffany, Sharon, Edwin, Sharnpreet, and Aaron, you all have made this experience amazing, and I will always cherish the time I spent here at McMaster. I would also like to thank Nancy Thomas, the sweetest, most humble woman. You were not only an amazing colleague, but I also consider you a great friend.

To my family, your continued support through all of these challenges is inspiring. I am so grateful to have you all in my life. I love you all. My mom Christine, you always have a way of making me feel as though everything will be okay. You never let me give up on myself and you're always there when I need a shoulder to lean on. My dad Tom, your continued interest in my project and your excitement for constructing some of the apparatus' I used was amazing. You are my rock. My brother and sister, thank you for putting up with me and always being there for me. You two are some of the brightest individuals I know and I am so glad I get to learn from both of you for the rest of my life.

To my friends, my surrogate family, I would not be who I am today if it was not for you. You all keep me sane, and are always there for me no matter what. We have been through thick and thin during these last 17 years, and I hope we get another 17 more. Thank you for spending hours troubleshooting with me, and lending me your expertise on things I needed help with. Thank you for making me laugh when I was ready to give up. And thank you for supporting all the decisions I have made. You all are my family and I love you all dearly. This experience has allowed me to make friendships that will last a lifetime. I am so glad to have met all of you and you have made this time here at McMaster wonderful.

TABLE OF CONTENTS

ABSTRACT ..... **iii**

ACKNOWLEDGEMENTS ..... **vi**

TABLE OF CONTENTS ..... **vii**

LIST OF FIGURES ..... **ix**

LIST OF TABLES ..... **xi**

ABBREVIATIONS ..... **xii**

CHAPTER 1 ..... **1**

1.1 THE AGING BRAIN 2

1.2 PARKINSON'S DISEASE 2

1.2.1 Incidence and Prevalence *3*

1.2.2 Symptomatology *4*

1.2.3 Pathophysiology *4*

1.2.4 Misdiagnosis *10*

1.3 NEUROTROPHIC FACTORS 12

1.3.1 BDNF in Parkinson's Disease *14*

1.3.2 GDNF in Parkinson's Disease *16*

1.4 CEREBRAL DOPAMINE NEUROTROPHIC FACTOR (CDNF) 18

1.4.1 CDNF Structure *20*

1.4.2 CDNF Function *20*

1.4.3 CDNF in Parkinson's Disease *21*

1.5 PERIPHERAL BLOOD AS INDICATORS OF BRAIN-BASED ILLNESS 24

1.5.1 Platelets as Markers of Dopamine Disruption *24*

1.5.2 Lymphocytes as Markers of Dopamine Disruption *25*

CHAPTER 2 ..... **27**

2.1 OBJECTIVE 1 28

2.2 METHODOLOGY 29

2.2.1 Animal *29*

2.2.2 Lesion Validation *29*

2.2.3 Sacrifice and Tissue Dissection *32*

2.2.4 Protein Quantification and Immunoblotting *32*

2.2.5 Blood Collection and Platelet Preparation *34*

2.2.6 RNA Isolation from Blood *35*

2.2.7 Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR) *36*

2.2.8 Statistical Analysis *36*

2.3 RESULTS 38

2.3.1 Effect of 6-OHDA lesioning on CDNF protein concentrations *38*

2.3.2 Effect of 6-OHDA lesioning on CDNF mRNA expression *41*

2.4 DISCUSSION 41

3.1 OBJECTIVE 2 44

3.2 METHODOLOGY 45

3.2.1 Animals *45*

3.2.2 Antisense Oligonucleotide Sequences and Infusion *45*
3.2.3 Surgical Procedures *47*
3.2.4 Behavioural Testing *49*
3.2.5 Sacrifice and Tissue Dissection *50*
3.2.6 Protein Quantification and Immunoblotting *50*
3.2.7 qRT-PCR *50*
3.2.8 Statistical Analysis *50*
3.3 RESULTS 53
3.3.1 Effect of CDNF knockdown on Locomotor Activity *53*
3.3.3 Verification of CDFN Knockdown *58*
3.3.4 Effect of CDNF Knockdown on platelet mRNA expression *58*
3.4 DISCUSSION 58

CHAPTER 3 ..... **66**
4.1 OBJECTIVE 3 67
4.2 METHODOLOGY 68
4.2.1 Participants *68*
4.2.2 Blood Collection and Preparation of Whole Blood *68*
4.2.3 qRT-PCR *68*
4.2.4 Statistical Analysis *69*
4.3 RESULTS 69
4.4 DISCUSSION 71
5.1 OBJECTIVE 4 72
5.2 METHODOLODY 73
5.2.1 Participants *73*
5.2.2 Blood Collection and Preparation of Platelets and Lymphocytes *73*
5.2.3 Preparation of Whole Blood *75*
5.2.4 RNA Isolation from Blood Samples *75*
5.2.5 qRT-PCR *75*
5.2.6 Statistical Analysis *75*
5.3 RESULTS 76
5.3.1 CDNF mRNA expression in platelets *76*
5.3.2 CDNF mRNA expression in lymphocytes *76*
5.3.3 CDNF mRNA expression in whole blood *79*
5.4 DISCUSSION 84
CONCLUSION 87
REFERENCES 89

LIST OF FIGURES

Figure 1a. Basal Ganglia Motor Circuitry - Direct and Indirect Pathways 6

Figure 1b. Basal Ganglia Motor Circuitry - Direct and Indirect Pathways in Parkinson's Disease. 8

Figure 2. Percent Sequence Homology for CDNF and MANF 19

Figure 3. 6-OHDA Lesion Site. 30

Figure 4a. CDNF Protein Expression in 6-OHDA rat Substantia Nigra. 39

Figure 4b. CDNF Protein Expression in 6-OHDA rat Striatum 40

Figure 5. Results of CDNF mRNA expression in 6-OHDA lesioned rats. 42

Figure 6a Osmotic pump cannula 48

Figure 6b. Stereotaxic coordinates of infusion site 48

Figure 7a. Results of CDNF knockdown on locomotor activity following 14 day infusion. 54

Figure 7b. Results of CDNF knockdown on locomotor activity. 55

Figure 7c. Results of aCSF infusion on locomotor activity. 56

Figure 8a. Results of CDNF knockdown on motor coordination following 14 day infusion 57

Figure 8b. Results of CDNF knockdown on motor coordination. 59

Figure 8c. Results of aCSF infusion on motor coordination. 60

Figure 9a. CDNF Protein Concentrations Following 14 day aCSF Infusion 61

Figure 9b. Verification of CDNF Knockdown following 14 day infusion. 62

Figure 10. Results of CDNF mRNA expression in CDNF knockdown rats. 63

Figure 11. Results of CDNF mRNA expression in whole blood over development. 70

Figure 12a. Results of CDNF mRNA expression in platelets. 77

Figure 12b. CDNF mRNA platelet expression is increased in Parkinson's disease. 78

Figure 13a. Results of CDNF mRNA expression in lymphocytes 80

Figure 13b. CDNF mRNA lymphocyte expression is not altered in Parkinson's disease. 81

Figure 14a. Results of CDNF mRNA expression in whole blood. 82

Figure 14b. CDNF mRNA whole blood expression is altered in Stroke patients. 83

LIST OF TABLES

Table 1. Lesion verification using Apomorphine rotations 31

Table 2. Primers used for RT-PCR analysis of CDNF mRNA tissue expression 37

Table 3. Antisense Oligonucleotide Sequence. 46

Table 4. Neurological Rating Scale for Performance on Beam Walk Apparatus 52

ABBREVIATIONS

6-OHDA	6-Hydroxydopamine
aCSF	Artificial cerebral spinal fluid
AD	Alzheimer's disease
ANOVA	Analysis of variance
APS	Ammonium persulfate
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
CAF	Central Animal Facility
CDNF	Cerebral dopamine neurotrophic factor
CNS	Central nervous system
DAT	Dopamine transporter
DJ-1	Parkinson's disease protein 1
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ET	Essential tremor
GABA	γ-Aminobutyric acid
GDNF	Glial cell-line derived neurotrophic factor
GPe	Globus pallidus externa
GPi	Globus pallidus interna
IL	Interleukin
JNK	c-Jun N-terminal kinase
L-DOPA	L-3,4-dihydroxyphenylalanine (Levodopa)
LPS	Lipopolysaccharide
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MDD	Major depressive disorder
mRNA	Messenger ribonucleic acid
MPPP	Desmethylprodine
MPTP	1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine
NGF	Nerve growth factor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
NTF	Neurotrophic factor
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cells
PD	Parkinson's disease
PEB	PBS-EDTA-Bovine Serum Albumin
PMC	Primary motor cortex
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus

SQ	Subcutaneous
TH	Tyrosine hydroxylase
UPDRS	Unified Parkinson's Disease Rating Scale
UPR	Unfolded protein response
VTA	Ventral tegmental area

CHAPTER 1

Introduction to Parkinson's Disease and Neurotrophic Factors

1.1 The Aging Brain

Aging is a complex, biological progression which increases the likelihood of developing neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD) (Yang, Huang, Gaertig, Li & Li, 2014). The probability of developing a neurodegenerative disorder dramatically increases into the sixth, seventh, and eighth decades of life (Mattson & Magnus, 2006). During normal aging, cells in the brain experience increased oxidative stress, accumulations of damaged proteins and modifications of DNA; these processes are seemingly exacerbated in pathological aging (Mattson & Magnus, 2006). Aging is postulated to be specific for each functional area of the brain, altering anatomy of neurons, tissue density, entire neurotransmitter systems, and causing synaptic dysregulation (Mora, Segovia & del Arco, 2007). Alterations in synapses and neuronal loss suggest a complex situation, for instance altered protein and neurotrophic factor (NTF) expression increases cell vulnerability during aging leading to neurodegenerative disease (Kovacs et al., 2014). Although our understanding of NTFs and how aging affects their function is in its infancy we do know the response of NTFs towards nervous system development, aging, and disease is not static (Collier et al., 2005). Deficiencies in NTF gene expression have been associated with increased vulnerability for neurodegenerative disease development, and aging alone is the single largest independent risk factor for development of neurodegenerative disorders (Mora, Segovia, & del Arco, 2007). Together, both factors are important in understanding the etiology of PD.

1.2 Parkinson's Disease

PD affects up to 1% of the global population above the age of 60, which increases to up to 4% after the age of 80 (Gazewood, Richards & Clebak, 2013). The

incidence of PD is stable across ethnicities; therefore, in an aging population such as ours, PD presents economic challenges in the years ahead. Epidemiological projections predict the incidence of PD to double by 2030 (Dodel, 2011). In Canada alone, the estimated financial impact of PD is $558.1 million per year (Parkinson Society Canada, 2003). This includes direct costs such as hospital care, drug prescriptions, care from physicians, and research, as well as indirect costs such as disability from loss of job (Mateus & Coloma, 2013). Despite the significant impact PD has on these individuals, their families, and the economy, relatively little is still understood about its etiology. Undoubtedly, the financial and economic impact of PD, exemplifies our need for future research in this area.

1.2.1 Incidence and Prevalence

In industrialized countries, the prevalence rates for PD are estimated at 0.3% of the entire population (de Lau & Breteler, 2006). Variations in prevalence rates around the world are likely due to differences in life expectancies (Hindle, 2010). PD is one of the best examples of an age-related disease as age is the largest single independent risk factor for the development of PD (Lang & Lozano, 1998). The peak incidence is found between 70 and 79 years of age (1087 per 100,000 persons), which increases to 1903 per 100,000 persons over the age of 80; the mean age of symptom onset is 60-to-65 years of age (Twelves, Perkins & Counsell, 2003; Van Den Eeden et al., 2003). Sex differences in PD are only seen in ages 50-to-59, wherein one study found that males were more likely than females (41 females and 134 males), to have PD; this rate equalized from 60 years of age onward (Pringsheim, Jette, Frolkis & Steeves, 2014).

1.2.2 Symptomatology

PD is characterized by motor, autonomic, affective and cognitive deficits (Michell, Lewis, Foltynie, & Barker, 2004). Classical or cardinal symptoms of PD primarily consist of motor impairments including resting tremor, bradykinesia (slowness of movement), akinesia (difficulty initiating movement), postural stiffness, muscular rigidity, and dystonia (muscle contractions which cause twisting and repetition of movements) (Wang, Boddapati, Emadi & Sierks, 2010; Westerlund, Hoffer & Olson, 2010; Siderowf, 2001).

Traditionally, PD was thought to be a predominantly motoric disorder; however, recent evidence has alluded to the presence of non-motor symptoms possibly preceding the motor impairments. In the early stages of PD, a decreased sense of smell is often present, followed by unemotional facial expression (Hindle, 2010). Other non-motor symptoms include cognitive decline, dementia, depression, apathy, sleep disorders, difficulty speaking as well as distortions of thought (Jankovic, 2011; Hurtig et al., 2000). Patients who received a diagnosis into the eighth decade of life have a 67.8% chance of developing dementia further exemplifying the need for early detection and research (Levy, 2007).

1.2.3 Pathophysiology

The heterogeneous nature of PD is the result of disturbances in multiple cellular processes including mitochondrial dysfunction, oxidative stress, and glutamate toxicity, all resulting in dopaminergic cell death (Blandini, Nappi, Tassorelli & Martignoni, 2000). The motor impairments have long been known to be caused by neurodegeneration in the dopamine neurons within the nigrostriatal pathway (Schapira, 2008). The non-motor symptoms arise from disturbances in other

transmitter systems, for example acetylcholine, and serotonin, which will not be discussed within the scope of this study (Lang, & Obesco, 2004).

1.2.3.1 The Basal Ganglia

PD arises from changes in the functional organization of the basal ganglia, a portion of the brain that lies between the thalamus and cerebral cortex and is responsible for voluntary movement and correct processing of sensory-motor information (Blandini et al., 2000). Collectively, the caudate nucleus, putamen, globus pallidus, substantia nigra (SN) and subthalamic nucleus (STN) are the group of nuclei that form the basal ganglia. These structures lie deep within the midbrain and are distinct anatomical regions that receive inputs from various neurotransmitter systems. The primary cortical area affected in PD is the SN which is subdivided into two distinct regions: the pars compacta (SNpc) region and the pars reticulata (SNpr) region. In particular, the SNpc region, which contains the neurotransmitter dopamine, projects to the striatum, STN and globus pallidus and modulates activity through direct and indirect basal ganglia pathways. Dopamine modulates the activity of many brain nuclei, including the putamen, nucleus accumbens, and ventral tegmental area (VTA), and plays an integral role in learning, reward- motivated behaviours, and movement (Arias-Carrion, Stamelou, Murillo-Rodriguez, Menedez-Gonzalez & Poppel, 2010). Cardinal symptoms of PD arise when up to 70% of dopamine neurons within the SNpc have degenerated (Saracchi, Fermi & Brighina, 2014).

In the healthy brain, the direct pathway of the basal ganglia stimulates muscle movements, which is illustrated in **Figure 1a**. This begins through activation of the primary motor cortex (PMC) when an individual wants to initiate a movement. The PMC sends excitatory stimulation, through the neurotransmitter glutamate, to the

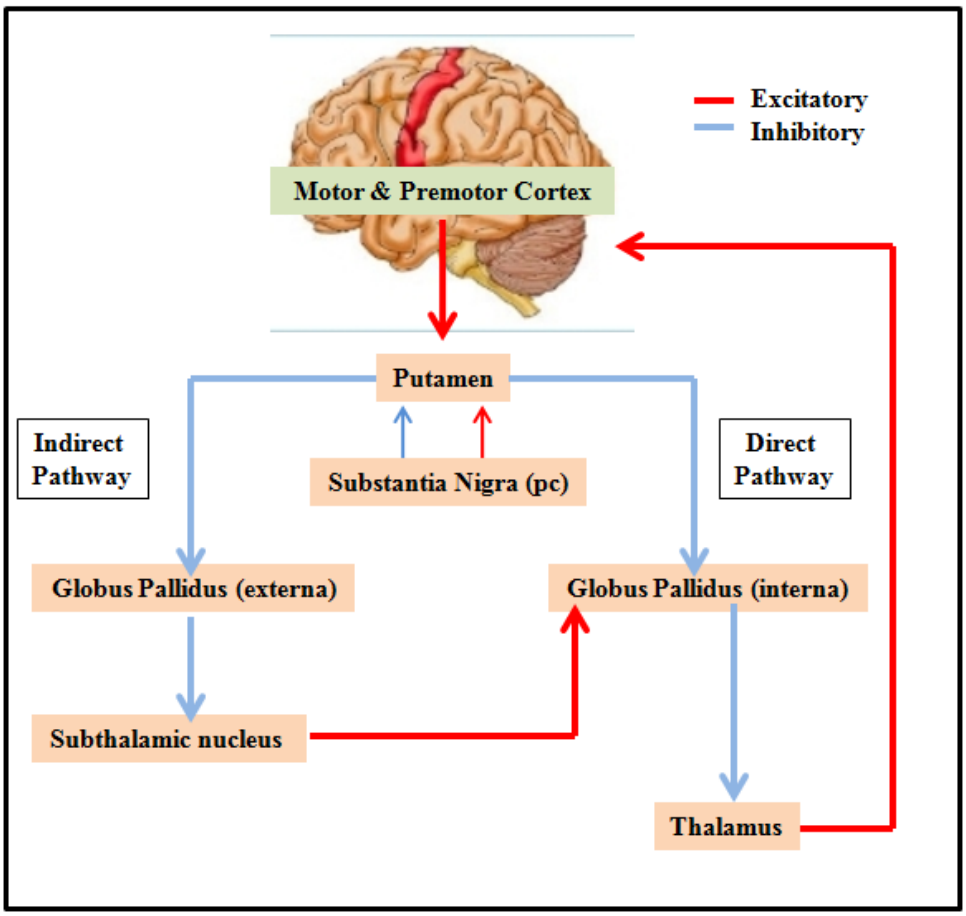

Figure 1a. Basal Ganglia Motor Circuitry - Direct and Indirect Pathways. Image depicts basal ganglia circuitry in the healthy brain.

striatum, particularly the putamen. The putamen receives input from the SNpc in the form of dopamine; dopamine has excitatory properties here. Stimulation by dopamine from the SNpc causes the stimulation of the putamen via dopamine D1 receptors. The neurons in the putamen contain γ-Aminobutyric acid (GABA), which is an inhibitory neurotransmitter. Through stimulation of the putamen, increased inhibitory GABAergic signals are sent to the globus pallidus interna (GPi). Inhibition of the GPi results in the disinhibition of the thalamus, causing increased activation and excitation of the PMC and subsequent muscle movement (Obesco et al., 2000). In the pathological PD brain, up to 70% of dopamine neurons have degenerated, because of this there is reduced excitation of the putamen (Dauer & Przedborski, 2003). This causes a reduction in inhibitory output from the putamen to the GPi, causing reduced inhibition to the thalamus which is described in **Figure 1b** (Obeso et al., 2000).

Unlike the direct dopamine pathway, the indirect pathway inhibits muscle movements in the healthy brain. In this route, the putamen receives inhibitory input from the SNpc via dopamine. This in turn causes inhibition of the putamen, which directly acts on the globus pallidus externa (GPe) stimulating the release of GABA, causing inhibition. The output from the GPe to the STN is inhibitory. The inhibition of the STN causes decreased excitation of the GPi, thus increasing the inhibitory activation of the thalamus. The thalamus is now receiving greater inhibition than seen in the direct pathway, resulting in less stimulation of the PMC and less activation of muscles, decreasing movement (**Figure 1a**) (Bartels, & Leenders, 2009). The indirect pathway in PD, is receiving reduced inhibition to the putamen, from the SNpc because of the degeneration of dopaminergic neurons. This reduction in inhibitory output causes an increased inhibition of the GPe via the putamen. The STN is now receiving

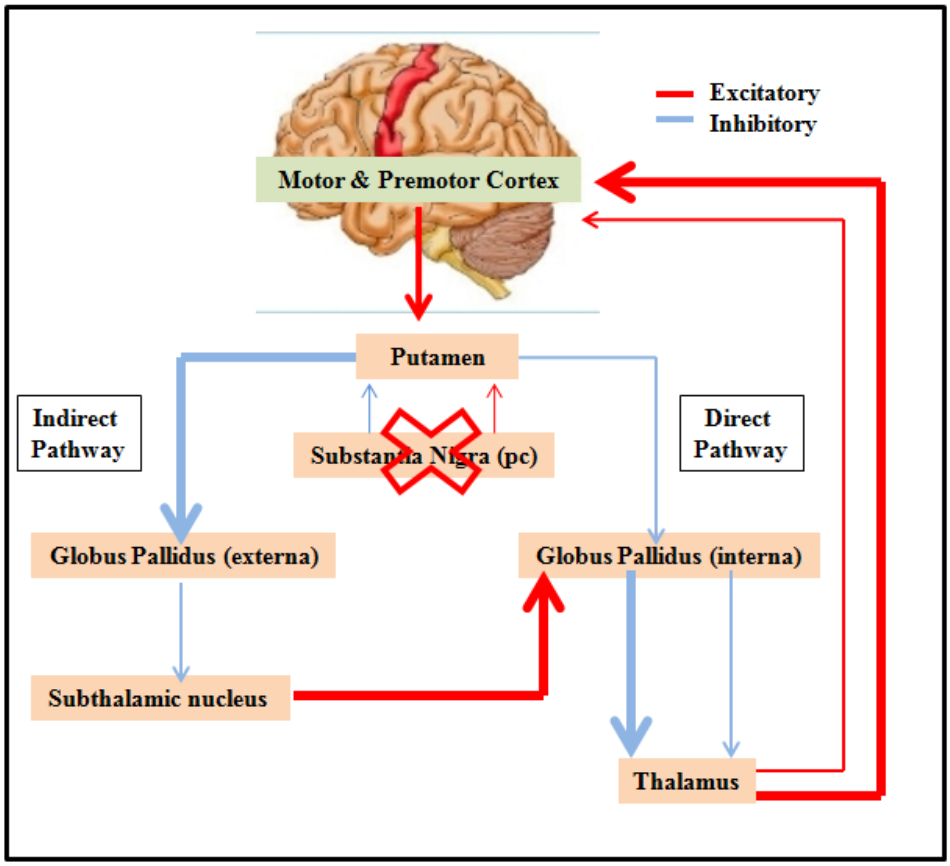

Figure 1b. Basal Ganglia Motor Circuitry - Direct and Indirect Pathways in Parkinson's. Image depicts basal ganglia in Parkinson's Disease brain. Dopamine degeneration in substantia nigra pars compacta) causes output from the globus pallidus interna to thalamus to increase via indirect pathways (indicated by thick blue arrow). Output from globus pallidus interna to thalamus is decreased via direct pathways (indicated by thin blue line). Thickness of line dictates intensity of excitatory or inhibitory output.

less inhibition, causing an increase in excitatory output to the GPi. This excitation of the GPi causes a greater inhibition of the thalamus, decreasing excitatory output to motor cortex, reducing movement (**Figure 1b**) (Obesco et al., 2000). This reduction in movement causes bradykinesia and akinesia, cardinal symptoms of PD.

1.2.3.2 Mechanisms of Dopamine Degeneration

As mentioned previously, the most consistent finding with respect to PD pathophysiology is the degeneration of dopaminergic neurons. The molecular events that underlie this loss have been difficult to ascertain. During autopsy, α-synuclein inclusions are found within the SNpc (Braak, Rub, & Del Tredici, 2006). These aggregates of misfolded proteins, referred to as Lewy bodies, are not normal concomitants of healthy aging (Selkoe, 2004). The accumulation of α-synuclein leads to the loss of synaptic proteins, loss of connectivity and ultimately programmed cell death, a process called apoptosis (Hindle, 2010). Despite dopamine neurons being relatively small compared to other neuronal populations, they have long axonal projections which require high energy for sustained function. This increases their exposure to toxins due to the large surface area, leading to higher risk of apoptosis (Mattson & Magnus, 2006).

Under certain conditions apoptosis occurs due to impairments in mitochondrial function rendering neurons vulnerable. This was first discovered to play a role in PD when Barry Kidman, a college student, attempted to synthesize desmethylprodine (MPPP), a synthetic opiod drug similar to morphine, but actually made 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and developed clinical features of PD (Coy, Balaji, Kim, & Yu, 2011). MPTP selectively destroys dopaminergic neurons in the SNpc and inhibits mitochondrial complex I of the mitochondrial electron transport

chain producing Lewy body inclusions (Lin & Beal, 2006). The accumulation of α-synuclein can lead to increased endoplasmic reticulum (ER) stress, unfolded protein response (UPR) and subsequent neurodegeneration (Bhandary, Marahatta, Kim & Chae, 2012).

The ER is an organelle that plays pivotal roles in cellular processes required for cellular survival and function (Bhandary et al., 2012). Under stress, the ER triggers a series of signal transduction events which constitute the UPR to ameliorate the accumulation of unfolded proteins (Kim, Xu & Reed, 2008). However, when these events are too severe they can induce cell death. These processes correlate to increases in oxidative stress and mitochondrial dysfunction. Oxidative stress results from an imbalance of reactive oxygen species (ROS) within the biological system, where the organism cannot readily detoxify the reactive intermediates or repair the resulting damage (Hirsch & Hunot, 2009). The 6-hydroxydopamine (6-OHDA) model, used to elicit PD-like symptoms in rodents, induces dopaminergic cell death via reactive derivatives of oxygen by inhibiting mitochondrial complex I (Belsa, Phani, Jackson-Lewis, & Przedborski, 2012). This model allows for a means of investigating potential antioxidative therapies useful for the treatment of PD. Together, these processes may provide a mechanism behind the progressive nature of PD, and emphasize the significance of the ER in the etiology of PD.

1.2.4 Misdiagnosis

As mentioned, symptoms of PD often arise after 70% of the dopamine neurons have already degenerated (Saracchi, Fermi & Brighina, 2014). Manifestation of the disease typically occurs in individuals above the age of 60; however, early onset PD can occur in persons under the age of 40. PD is typically diagnosed in a clinical

setting. Upon neurological examination, the presence of stooped posture, shuffling gait and lack of arm swing typically delineates PD from other motor disorders such as peripheral neuropathy and muscular atrophy. Despite its high prevalence and referrals to movement disorders specialists, error rates in diagnosis are as high as 24% (Tolosa, Wenning, & Poewe, 2006). These inaccuracies tend to occur in the early stages of the illness, when other movement disorders overlap, such as parkinsonism, multisystem atrophy, and progressive nuclear palsy (Tolosa et al., 2006). Moreover, 10% of patients diagnosed with PD during their life will be reclassified during post-mortem autopsy (Baumann, 2012).

Essential tremor (ET) is one disorder commonly mistaken as PD during the early phase of the illness (Nutt & Wooten, 2005). Handwriting is typically used to confirm or refute differential diagnosis. For instance, ET presents with large, tremulous writing, while PD has small and irregular writing. Some additional measures are taken to improve accuracy of diagnosis. For instance, bradykinesia, turning in a bed, opening jars, and micrographia make PD more likely (Gazewood, Richards & Clebak, 2013). On the other hand, features such as falls in the early stages, poor response to levodopa (L-DOPA) treatment, rapid progression of the disease and lack of tremor lessen the chance of a PD diagnosis. Typically, a good response to dopamine agonists is a definitive measure used to accurately diagnose PD (Hindle, 2010). Despite the various measures available, there is still a poor correlation between underlying pathology and clinical presentation, making accurate diagnosis difficult. Research has shifted its focus to investigating potential biomarkers for improved accuracy. The development of a biomarker for PD would allow for the detection of morphological changes presymptomatically to expedite the administration of

neuroprotective therapies to arrest the progressive nature of the disease upon detection (Michell et al., 2004).

1.3 Neurotrophic Factors

NTFs provide an avenue of study into the aging process and development of neurodegenerative diseases. These secreted proteins promote survival, differentiation and maintenance of neurons, as well as the establishment of synaptic connections during development (Lindholm & Saarma, 2009). Throughout development, these factors promote axonal growth and influence neuronal target finding (Siegel & Chauhan, 2000). Cells that do not receive the necessary NTFs die via apoptosis. This loss of neurotrophic support for specific neuronal populations could confer susceptibility to various neurodegenerative disorders such as AD or PD (Siegel & Chauhan, 2000).

NTF families are delineated based on structural homology, receptor types, and signal transduction pathways (Hoffer, 2011). Traditionally there are three classes of NTFs: neurotrophins, glial cell-line derived neurotrophic factor (GDNF) family ligands, and neuropoietic cytokines (Lindholm & Saarma, 2009). Neurotrophins include brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). BDNF, in particular, has potent effects on survival of dopaminergic neurons and will be discussed more thoroughly in *Section 1.3.1*. Neurotrophins have various roles in development and maintenance of the mature nervous system. During development, they are responsible for regulation of cellular fate, axonal growth, dendritic pruning, and the patterning of innervations and expression of proteins crucial for neuronal function (Huang & Reichardt, 2001). These responsibilities somewhat change in the mature nervous system, whereby they

control synaptic function, plasticity and modulate neuronal survival (Huang & Reichardt, 2001).

The GDNF family of ligands play a role in cell survival, neurite outgrowth, differentiation and migration of cells. This family consists of four neurotrophic factors: GDNF, which promotes the survival of dopaminergic neurons and will be discussed further in *Section 1.3.2*. Neurturin, and artemin, promote survival of peripheral, enteric, sympathetic and sensory neurons (Bennett et al., 2000), and persephin which, like GDNF promotes the survival of dopamine neurons, but does not support peripheral neurons (Milbrandt et al., 1998).

The third class of neurotrophic factors are neuropoietic cytokines. This family of neurotrophic factors plays a vital role in the coordination of neuronal, glial and immune responses to injury and disease (Bauer, Kerr & Patterson, 2007). Amongst this family are interleukin-6 (IL-6), IL-11, IL-27, leukaemia inhibitory factor, ciliary neurotrophic factor and cardiotrophin-like cytokine. Due to the scope of this study, this family will not be discussed further in depth.

Recently, a fourth class of NTFs has been discovered: mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) (Parkash et al., 2009). This class of NTFs shares no sequence homology or structural similarity to other NTF families suggesting a completely new mechanism of action and a potential therapeutic intervention in the treatment of neurodegenerative diseases (Lindholm & Saarma, 2009). This subclass of NTFs will be discussed further in *Section 1.4*.

In 10% of cases of PD, the disease is associated with a genetic mutation, however, in the majority of cases the cause is sporadic, or unknown (Baquet,

Bickford, & Jones, 2005). Thus, understanding the underlying mechanism of dopamine generation and survival during development and in adulthood has become a primary focus of research over the last decade. Due to of their ability to promote the survival and maintenance of specific neuronal populations, NTFs have become a budding research topic with respect to PD. NTFs are subject to retrograde and anterograde transport to and from target neurons, making them important for synthesis at local or remote sites within the brain (Siegel & Chauhan, 2000).

1.3.1 BDNF in Parkinson's Disease

As mentioned in *Section 1.3*, BDNF, NT-3, and GDNF have all been shown to promote the survival of dopaminergic neurons with varying degrees. Early studies have shown BDNF is normally expressed in the adult nigrostriatal dopamine system, particularly the striatum, SN and VTA of rat embryonic dopaminergic neurons *in vitro* (Howells et al., 2000). Primate models of PD using MPTP have shown a significant decrease in BDNF levels detected in young and middle aged primates but did not decrease further in the oldest age group (Collier et al., 2005). In post-mortem studies, BDNF mRNA and protein expression have been examined using *in situ* hybridization, immunohistochemistry, and immunoblotting all of which have demonstrated decreased levels of BDNF within the SNpc in patients with confirmed diagnosis of PD. (Baquet et al., 2005). Not only was mRNA protein expression decreased in patients with PD relative to controls, but peripheral serum levels were also decreased in this population (Ventriglia et al.,2013; Howells et al., 2000).

BDNF is hypothesized to play a role in the survival of dopamine neurons in the early stages of PD due to its ability to stimulate dopamine activity and turnover *in vitro* and *in vivo.* In rodent models, BDNF protects dopamine neurons from the toxic

effects of MPTP and 6-OHDA (Baquet et al., 2005). It has also been reported that when BDNF is injected directly into the striatum, it is taken up and retrogradely transported by dopamine neurons to the SNpc (Yoshimoto et al., 1995). The efficacy of BDNF as a therapeutic agent for PD is further strengthened by functional improvement following administration of retroviral BDNF transduced astrocytes into the striatum following 6-OHDA lesioning (Yoshimoto et al., 1995). These findings suggest that *ex vivo* gene therapy with BDNF ameliorates PD symptoms.

Despite its potential for neurorestoration in cell cultures and rodent models, the story of BDNF in PD has proven complicated. One study claims that BDNF may actually confer a susceptibility to developing PD. SNpc pigmented neurons not expressing BDNF had a greater probability of surviving than BDNF positive neurons (Siegel & Chauhan., 2000). Genetic knockout studies have attempted to shed light on whether BDNF loss causes neuronal injury to dopamine neurons, or whether neuronal injury causes the loss of BDNF, however, genetically modified animals die soon after birth (Dluzen, Story, Xu, Kucera & Walro, 1999).

In contrast to the full knockout of a gene, antisense oligonucleotides use a sequence complimentary to mRNA that inhibits its expression and induces a blockade in the transfer of genetic information from DNA to protein (Dias & Stein, 2002). This method allows for specific, non-diffuse, knockdown of the protein in the region of interest. Following antisense oligonucleotide infusion of BDNF into the SNpc, rats exhibited apomorphine induced rotational behavior, and a 40% reduction in SNpc tyrosine hydroxylase (TH)-immunoreactive neurons (Porritt, Batchelor, & Howells, 2005). This loss of BDNF leads to downregulation of dopaminergic phenotype and neuronal death which suggests BDNF mRNA expression within the SNpc may

directly contribute to the death of nigral dopamine neurons causing PD. Currently, there are no clinical studies examining the effectiveness of intracerebrally infused BDNF.

1.3.2 GDNF in Parkinson's Disease

GDNF has been well documented to protect dopaminergic neurons from neurotoxic insult *in vivo* and *in vitro* (Enciu, et al., 2011). GDNF treatment of cultured human fetal ventral mesecephalic neurons nearly doubled the number of dopamine neurons (Siegel & Chauhan, 2000). In murine models of PD, when GDNF was administered before or around the time of dopamine injury it prevented dopamine degeneration and promoted functional recovery (Enciu, et al. 2011). GDNF was able to reduce amphetamine-induced rotational behaviour and restore dopamine levels to normal following neurotoxic insult. In preclinical primate models of PD, following systemic administration of MPTP, GDNF was delivered *ex vivo* using encapsulated GDNF producing cells implanted into the lateral ventricle, which is close in proximity to the caudate nucleus, striatum (Kishima et al., 2004; Gash et al., 1996). Chronic infusion of low doses of GDNF transiently recovered motor deficits and protected striatal dopamine function while increasing the number of TH immunoreactive cells in the SNpc and was generally well tolerated. In post mortem cases of PD, GDNF has been repeatedly shown to be low in the SNpc region more so than BDNF, NGF, NT-3 or NT-4 (Chauhan, Siegel & Lee, 2001). This suggests that GDNF may be most susceptible and possibly the earliest to detect.

These promising results led to three clinical trials using GDNF. In 2003, Gill et al. delivered GDNF directly into the putamen of five PD patients. After one-year there were no serious side effects following implantation, patients showed a 39%

improvement in Unified Parkinson's Disease Rating Scale (UPDRS) and there was a significant increase in putamen dopamine storage. These results led to two additional clinical trials. A randomized, double-blind trial of GDNF administered intracerebroventricularly to patients with advanced PD using human methionyl GDNF (Nutt et al., 2003,). Primary endpoints included adverse side effects and UPDRS scores. In contrast to Gill et al.,(2003) there was no clinical improvement on the UPDRS in this study, and 100% of patients administered GDNF exhibited serious side effects including nausea/vomiting, confusion, delusions, hallucinations, hypoatremia, chest pain and aseptic meningitis. To examine dopamine levels in the striatum and SNpc, they used TH and GDNF immunoreactivity in one patient who died from an unrelated cause. They found that the infused GDNF only minimally penetrated the brain parenchyma. In 2006, the same group of researchers conducted another open label, randomized control trial. Intraputamenal infusion of human recombinant GDNF was administered to patients with moderate to severe PD (Lang et al., 2006). No significant improvement in UPDRS score was found despite improvements in ^{18}F-dopa uptake. With regards to safety of this implantation, nine patients and 17 devices (out of the total population of 34 patients) experienced serious adverse effects, particularly migration of the implanted catheter.

Studies have postulated that the modest results from these clinical trials may be due to the idea that neurotrophic factors whose receptors are widely distributed are more likely to produce severe side effects. This is further exacerbated by the need for increased dosages of these factors to bypass the fact they cannot cross the blood brain barrier (Kirik, Georgievska & Bjorklund, 2004). There are arguments that the method of delivery may be the reason, whereas others argue that the dosage needs to be

altered (Korodower & Bjorklund, 2013). Another caveat to these results lies in that GDNF seems to only exert its protective and functional effects if delivered at the same site as the lesion, indicating a site-dependent effect (Hoffer, 2011). GDNF has been determined to retrogradely protect nigral dopamine neurons when 6-OHDA is administered in the striatum; however, this is not the case when GDNF is delivered to the SNpc (Kirik et al., 2004). This is further exemplified in the MPTP murine model of PD, GDNF did not elicit functional recovery when delivered into the SN if the lesion occurred in the striatum; yet, when both GDNF and MPTP lesion occurred in the striatum functional improvement was seen (Hoffer, 2011). Regardless, the translation from preclinical models to clinical models proves challenging. Insight into additional neurotrophic factor involvement in neurodegenerative diseases could offer new therapeutic approaches.

1.4 Cerebral Dopamine Neurotrophic Factor (CDNF)

CDNF and its constituent MANF comprise a novel family of evolutionarily conserved neurotrophic factors, with specificity for dopamine neurons (Lindholm & Saarma, 2009; Lindholm et al. 2007). CDNF is expressed in vertebrates and invertabrates, as well as the fly, *Drosophila melanogaster* and nematode, *Caenorhabditis Elegans* (**Figure 2**). In the human central nervous system (CNS), CDNF is expressed in the SN, striatum, olfactory bulb, frontal cortex, caudal cortex, thalamus, hypothalamus, and is also expressed in non-neuronal tissues such as the heart, liver, lung, skeletal muscle, salivary glands, spleen, testis and thymus (Lindholm et al., 2007). Human CDNF and MANF share 59% sequence homology. Interestingly, these factors differ from all other neurotrophic factors in structure and

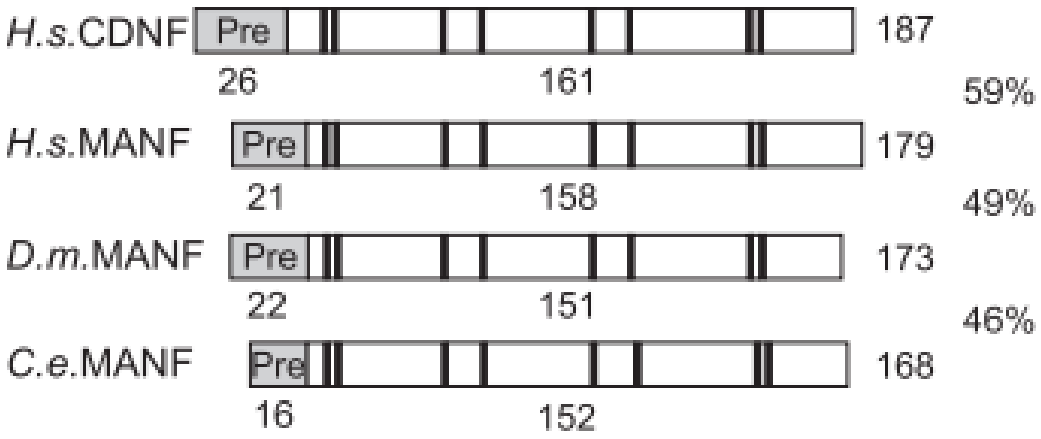

Figure 2. Percent Sequence Homology for CDNF and MANF. Taken from *Lindholm et al., (2007).* Schematic representation indicating evolutionarily conserved CDNF and MANF as percent identity among humans, *D. Melanogaster* and *C. Elegans*. Fly and worm have a single homologous CDNF/MANF gene.

location. Further, they do not promote neuronal survival in peripheral sympathetic or sensory neurons.

1.4.1 CDNF Structure

Mature human and mouse CDNF consist of 161 amino acid residues, and is a specific paralog of MANF (Lindholm & Saarma, 2009). Human CDNF is 18kDA and does not contain a prosequence, suggesting enzymatic cleavage is not necessary for its functionality, which makes this NTF different from the others in yet another manner. The structure of CDNF has recently been discovered, indicating two domains: a saposin-like N-terminal domain and an unstructured carboxy C-terminal domain with a CXXC intradomain cystine bridge, which may explain its bifunctionality (Parkash et al., 2009). Saposin-like proteins interact with lipids suggesting CDNF may interact with membranes (Lindholm & Saarma, 2009). The CXXC motif on the C-terminus may be responsible for reducing ER stress caused by misfolding of proteins and could be the primary site of neurotrophic activity (Latge, Cabral, Almeida, & Foguel, 2013). This CXXC motif is hypothesized to be the active site for thiol-disulfide oxioreductases that catalyze the formation of intermolecular disulfide bonds. Receptors for CDNF are currently unknown (Lindholm & Saarma, 2009).

1.4.2 CDNF Function

Although the exact functional properties of CDNF are unknown, researchers speculate that CDNF is functional upon synthesis and secretion due to the lack of prosequences and consequent lack of enzymatic activity needed for activation (Lindholm & Saarma, 2009). Although secreted, CDNF and MANF are retained in the ER where they assist in protein folding and mitigation of ER stress (Aron & Klein, 2011). CDNF may help remove misfolded proteins from the ER by degradation or

enhancement of protein folding (Parkash et al., 2009). Thus, CDNF may play an important role in disorders stemming from misfolded proteins, for example PD. Studies suggest that CDNF and MANF are at least as potent as GDNF in protecting and repairing damaged dopaminergic neurons following injury (Parkash et al., 2009). MANF was found to be transported to the cortex following injection into the striatum, whilst CDNF is transported to the SN which makes this particularly important in PD.

1.4.3 CDNF in Parkinson's Disease

Lindholm et al. (2007) have been the leading investigators in CDNF research over the past decade. In 2007, they examined the effectiveness of CDNF in protecting and restoring dopamine neurons in the 6-OHDA model of PD (Lindholm et al., 2007). As a positive control, they used GDNF due to its effectiveness in this model of PD. They found that when CDNF was given prior to lesion, it was able to reduce amphetamine-induced rotational behaviour two to four weeks post lesion. This effect was greater than the effect of GDNF pre-treatment on rotational behaviour. CDNF was able to increase the number of TH positive cell bodies in the SNpc and TH positive fibres in the striatum. From this evidence, CDNF prevents 6-OHDA induced degeneration of dopamine neurons at least as effectively as GDNF. With respect to the neurorestorative effects of CDNF, when CDNF was given 4 weeks following 6-OHDA lesioning, amphetamine-induced rotational behaviour was significantly reduced and the number of TH-positive cells in the lesioned side of the brain showed a 58% recovery, similar to that of GDNF (57%). Both studies exemplify the neurorestorative and neuroprotective effects of CDNF on dopaminergic neurons after 6-OHDA lesioning. A similar study was carried out in 2011, in which CDNF, MANF and GDNF were given two weeks after of 6-OHDA lesion in a continuous infusion for

14 days (Voutilainen et al., 2011). The progressive loss of dopaminergic neurons has been known to be fully developed by four weeks, therefore, by infusing NTFs two weeks following lesioning they are postulated to be able to prevent neurodegeneration. Voutilainen et al. (2011) found similar results to Lindholm et al. (2007) in that pretreatment with CDNF almost completely prevented 6-OHDA induced rotations, when given amphetamine, and was able to protect TH-positive cell bodies in the SNpc. Notably, they also found that only CDNF was selectively retrogradely transported from the striatum to the SNpc.

Another model typically used to examine PD in non-human models, often classified as the "gold standard", is the aforementioned MPTP model. There is one study to date, that examines CDNFs protective and restorative effects in the MPTP model of PD (Airavaara, et al., 2012). Pre-treatment with bilateral injections of CDNF to the striatum improved motor behaviour and increased TH immunoreactivity in the striatum and SNpr and the number of positive TH-immunoreactive SNpc cells. Interestingly, they found this protective effect was selective for dopamine neurons, and thus was not mediated by alterative neuronal populations within the SNpc. In addition, CDNF was able to promote functional recovery one-week after MPTP injections and increase the number of TH immunoreactivity in the striatum and TH positive cells in the SNpc. These results suggest a restoration of the dopamine circuitry. Behaviourally and histochemically, CDNF has profound effects on 6-OHDA and MPTP models of PD, though the mechanism of CDNFs therapeutic effects still remains unknown.

1.4.3.1 Potential Mechanisms of Action

The mechanism of action behind CDNF's neuroprotective and neurorestorative properties is still unknown. Recently, Mei and Niu (2014) examined the role of CDNF in reversing and preventing apoptosis in pheochromocytoma cells (PC12) prior to, or following 6-OHDA lesion. They determined that compared to 6-OHDA lesion alone, pre-treatment with CDNF attenuated the effects of 6-OHDA insult in a dose-dependent manner. That is, as the dose of CDNF increased, the survival rate of the cells also increased. Similarly, post-treatment with CDNF following 6-OHDA infusion reversed the loss in cell viability also in a dose-dependent manner, compared to 6-OHDA alone. Mei and Nui (2014) went further to examine the potential underlying mechanism behind these actions, specifically focusing on the anti-apoptotic molecule Bcl-2, the pro-apoptotic molecule Bax and caspase-3 activation. They found that with 6-OHDA alone, Bcl-2 was decreased, and Bax was increased, though, this effect was attenuated when CDNF was administered pre or post-treatment. In addition, CDNF treatment reduces caspase-3 activation, a central component of the apoptotic process. These findings suggest that CDNF may exert its effects through modulation of apoptotic pathways, particularly upregulation of Bcl-Bax ratio and the downregulation of caspase 3.

Neuroinflammation is also a common pathological process among neurodegenerative disorders. The N-terminal domain of CDNF may interact with lipids, therefore providing a possible therapeutic potential. Using lipopolysaccharide (LPS), a bacteria that leads to severe inflammation, Zhao et al. (2014) examined the role of CDNF as a mediator or inhibitor of inflammation in microglia cell cultures. They found that CDNF was upregulated following exposure to LPS. To further elucidate the role of CDNF in this process, cells were pretreated with CDNF or a

vehicle prior to LPS administration. They found that CDNF remarkably reduced cytotoxicity induced by LPS. Following these findings, they examined the role of CDNF in the c-Jun N-terminal kinase (JNK) pathway. This pathway stimulates the transcription and expression of proinflammatory cytokines. As expected, phosphorylation of JNK was reduced by CDNF treatment suggesting that CDNF protects microglia against neuroinflammation by suppressing JNK pathways. The findings from these two studies suggest a potential role for CDNF in neurodegenerative diseases such as PD. Since PD is a disorder stemming from increased oxidative stress causing dopamine neuronal apoptosis, as well as neuroinflammation, CDNF may provide a new avenue into understanding the neurodegeneration exhibited in PD.

1.5 Peripheral Blood as Indicators of Brain-based Illness

Biomarkers are not a new concept. A growing body of research has been involved in finding new methods of early detection for many brain-related disorders. The blood has been thought to act as a "sentinel tissue" that reflects the state of health or disease within the body. In many cases, human brain tissue biopsy is unavailable; therefore, developing techniques to determine brain-based illness through less invasive methods is needed. Blood-based biomarkers are ideal given the accessibility, minimal invasiveness, and cost of phlebotomy.

1.5.1 Platelets as Markers of Dopamine Disruption

Platelets share several morphological characteristics with the central nervous system which make them ideal candidates. They share a common embryological origin derived from the neural crest (Plein and Berk, 2001). Platelets are easily harvested from whole blood with a very high yield; approximately 2 million platelets

can be harvested from 5ml of whole blood (Behari and Shrivastava, 2013). Many investigators have explored proteins on peripheral cells, particularly platelets, under the assumption that changes in transporter density or activity within these cells may reflect CNS neuronal changes (Frankhauser, et al., 2006). It is well known that platelets can accumulate dopamine and more readily, serotonin (Plein & Berk, 2001). Alterations in platelet uptake have been reported in Huntington's disease, schizophrenia, major depression and PD, thus platelet count variation is considered a model of dopaminergic neurons and the status of this neurotransmitter system (Ricci et al., 2001). With respect to PD, platelets have shown to be altered in the DJ-1 mutation of PD, as well as synucleins and are able to uptake L-DOPA (Behari & Shrivastava, 2013).

Platelets have been used to examine variations in mitochondrial dysfunction and apoptosis in age-related disorders (Behari & Shrivastava, 2013). Despite platelets being annucleated cells that lack the enzymatic machinery necessary for biosynthesis of monoamines, they can be considered multitransmitter storage sites like neurons, and are free from nuclear DNA contamination (Da Prada, Cesura, Launay& Richards, 1988; Behari & Shrivastava, 2013). In light of the fact that PD is a disorder of mitochondrial dysfunction, oxidative stress, and apoptosis, platelets make an excellent sentinel tissue for investigative research into biomarkers for PD.

1.5.2 Lymphocytes as Markers of Dopamine Disruption

Recent evidence suggests an impairment of the dopamine system within peripheral blood lymphocytes (PBL) (Scherzer et al., 2006). PBLs take up dopamine and express the dopamine transporters on the cell membrane which makes them ideal candidates for biomarker development, and improved accuracy in diagnosis. There is

evidence to suggest that PBLs synthesize catecholamines, which make them particularly useful in detecting changes in dopamine transmission. PBLs not only express dopamine receptors, but potentially modulate intracellular dopamine synthesis, dopamine immunoreactivity, and intracellular dopamine concentrations (Caronti, et al., 1999; Pellicano, et al., 2007). With respect to PD, it was recently found that dopamine transporter (DAT) immunoreactivity was significantly lower in drug naive, and dopamine agonist treated PD patients compared to controls (Caronti, et al., 2001). Both PD groups did not differ in dopamine transporter immunoreactivity. These results suggest that in the early stages of the disease DAT is reduced, however, therapy does not alter this disruption. Interestingly, lymphocytes may provide a promising avenue for discrimination between differential diagnoses in the early stages of the disease. Pellicano et al. (2007) found that DAT immunoreactivity was decreased in PD patients but was normal in patients with essential tremor. As mentioned previously, ET is one disorder that is commonly misdiagnosed as PD in its early stages (Nutt & Wooten, 2005). One pitfall to lymphocytes as biomarkers is that lymphocytes have a greater risk for contamination, due to the extraction technique and the possibility of collecting platelets or other blood proteins. For this reason lymphocytes are generally less pursued as biomarkers compared to platelets.

The overall objective of this project was to investigate the role of CDNF in PD using rodent models and clinical populations. The specific focus was on determining whether a reduction in CDNF is exhibited in a clinical population of PD patients using peripheral blood and post-mortem tissue. Findings will bring insight into the neurobiological mechanisms underlying neurodegeneration and impairments in PD.

CHAPTER 2

Behavioural Investigations of CDNF in Parkinson's Disease

2.1 OBJECTIVE 1

Investigating CDNF protein and mRNA concentrations in the 6-OHDA unilateral rodent model of Parkinson's disease

Research concerning PD has predominantly focused on mimicking the depletion of dopamine in the striatum and the motor impairments that follow. The 6-OHDA model is typically used to induce dopaminergic cell death and elicit PD-like symptoms in animal models (Belsa et al., 2012). This model has been beneficial in evaluating dopamine agonist treatment. The overwhelming majority of research concerning CDNF has examined its neurorestorative and neuroprotective effects in the 6-OHDA model of PD (Lindholm & Saarma, 2009; Parkash et al., 2009). Despite this evidence, what is still unknown is whether CDNF plays a role in the etiology of PD, a key component in further understanding its role in the neurodegeneration of dopamine neurons. CDNF has been found to upregulate Bcl-2 and Bax ratio while downregulating caspase 3, indicating a role in mediating apoptosis (Mei & Nui, 2014), and also played a role in suppressing neuroinflammatory pathways (Zhao et al., 2014). To date there have been no reports in the examination of CDNF concentrations in behavioural models of PD and it is unknown whether 6-OHDA treatment affects endogenous CDNF expression *in vivo.* Investigations into established models of PD could further elucidate the role of CDNF in ER stress and the UPR (Voutilainen, 2011). Therefore, the first objective of the present study was to investigate CDNF protein, and platelet mRNA concentrations in the well established 6-OHDA model of PD. It was hypothesized that following 6-OHDA lesioning: (1) CDNF protein expression will be decreased; and (2) CDNF platelet mRNA expression will be decreased.

2.2 METHODOLOGY

2.2.1 Animal

8 adult male Sprague Dawley rats (225-300g) were purchased from Charles River Laboratories International, Wilmington, MA. All animals were pre-lesioned in the SNpc using 12µg 6-OHDA. All animals were given seven days to acclimate to the animal facility, and were handled by experimenters during this time. Rats were kept under standard laboratory conditions on a reverse light/dark cycle (7am-7pm), with access to food and water *ad libitum*. All animals were housed one per cage in enriched environments. All procedures were approved by the Canadian Council on Animal Care, as well as approved by the Central Animal Facility (CAF) at McMaster University. Baseline behavioural measures were not acquired as the animals entered the facility pre-lesioned (**Figure 3**). Prior to delivery, success of the lesion was confirmed via apomorphine induced rotations 5-7 days post surgery. There were no controls involved in this study, as the non-lesioned side of the brain served as its own control.

2.2.2 Lesion Validation

Prior to sacrifice, all animals were tested using apomorphine induced rotations to confirm the lesion was still present (**Table 1**). Apomorphine is a dopamine receptor agonist, and is typically used to assess the motor integrity and asymmetry following unilateral lesion. Apomorphine binds the supersensitive dopamine receptors on the denervated side of the unilaterally lesioned brain, thus causing an imbalance in dopamine production, causing contralateral circling motor behavior (Schober, 2004). Rats received a subcutaneous (SQ) injection of 0.5 mg/kg R-(-)-apomorphine hydrochloride hemihydrate (Sigma Aldrich, Cat#A4393, Oakville, Ontario, Canada)

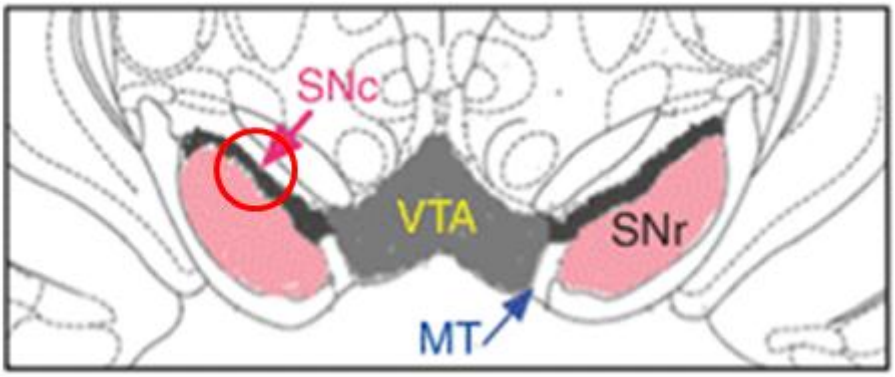

Figure 3. 6-OHDA Lesion Site. All rats received unilateral injection of 6-OHDA neurotoxin into the left substantia nigra pars compacta rgion of brain. Illustration adapted from Ciesielska et al. (2011).

Table 1.

Lesion Verification using Apomorphine Rotations.

Rat ID	Number of Apomorphine Rotations
20	417
21	586
22	279
23	416
24	426
25	423
26	231
27	229

Note: Lesion status was confirmed 24hrs prior to sacrifice to ensure depletion of dopamine neurons. Rotations were determined using number of contralateral rotations – ipsilateral rotations over a 30 minute time period (5 rotations per minute). Table indicates all lesions were successful and apparent 24hrs prior to sacrifice.

in a 0.1% ascorbic acid vehicle. Following injection, the rats were placed in a clear plastic cylinder for a 5 minute habituation period. Following habituation, rats spontaneous movement was recorded and apomorphine-induced rotations (360°) was counted for a 30 minute period. All cylinders were cleaned between each rat with 75% ethanol.

2.2.3 Sacrifice and Tissue Dissection

Following apomorphine rotations, rats were given a 24 hour washout period to ensure residual effects of apomorphine were eliminated. Rats were sacrificed two weeks after arrival. Animals were heavily anesthetized with isoflurane (Pharmaceutical Partners of Canada Inc, Richmondhill, Ontario, Canada)) and rapidly decapitated. Brains were removed and the left and right striatum and left and right SNpc regions were dissected. Tissues were stored at -80°C until further use.

2.2.4 Protein Quantification and Immunoblotting

Protein concentrations were determined using Bradford Assay, using the Bio-Rad Protein Assay reagent (Bio-Rad, Mississauga, Ontario, Canada), and a CU-640 spectrophotometer (Beckman-Coulter, Mississauga, Ontario, Canada). Optical densities of the samples were measured at 595nm in technical duplicates. Standard range, samples whose densities fell outside this range were diluted with 50µl Tris-EDTA (pH 7.4) accordingly. Samples were stored at -80°C until use.

Immunoblotting was performed to determine CDNF protein concentrations in the 6-OHDA model of PD. Sample concentrations were calculated and 15µg of protein (30µl volumes) were loaded onto a 15% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel (10 ml 1.5M Tris, pH 8.8; 8.9 ml dH_2O; 400 µl 10%

SDS, 20 ml 30% acrylamide, 400 µl 10% ammonium persulfate (APS) and 30 ul TEMED with a 4% stacking gel: 5.00 ml 0.5M Tris, pH 6.8, 12.20ml dH_2O, 200 µl 10% SDS, 2.60 ml 30% acrylamide, 100µ l 10% APS and 20ul TEMED to ensure simultaneous gel entering). Samples were loaded in random order to minimize variation between gels and samples.

Samples were loaded on gels into a Bio-Rad Mini PROTEAN III rig (Bio-Rad, Mississauga, Ontario, Canada) containing 1x running buffer (10x running buffer made of 15g/L Tris base, 72g/L Glycine, and 5g/L of SDS, diluted to 1x with distilled water). Gels were run at 60V through the stacking gel and increased to 100V through the separating gel. Precision Plus PINK ladder was used to ensure the samples did not run off the gel.

Gels were equilibrated in transfer buffer (12mM Tris base, 96mM Glycine, and 20% Methanol brought to 1 litre with distilled water) for 15 minutes. Polyvinylidene difluoride (PVDF) membranes were first washed in 100% methanol for 10 seconds to activate it, and allowed to equilibrate in transfer buffer for 15 minutes. Gels were transferred onto PVDF membranes using the BIORAD Wet Transfer Unit at 100V for 1 hour and cooled with an ice pack to avoid over-heating.

Following transfer, blots were immersed in blocking solution (5% skim milk, 50mM Tris, 150mM NaCl, 0.2% Tween-20, pH 8.5) for 1 hour at room temperature and exposed to primary antibody diluted in TBS-T (50mM Tris, 150mM NaCl, 0.2% Tween-20, pH 8.5) overnight at 4°C. Primary CDNF antibody (rabbit polyclonal) was diluted at 1:5000, in addition the housekeeping gene GAPDH was diluted to 1:10000 and added at the same time. Following overnight primary incubation, membranes were washed twice in TBS-T for 5 minutes and then for a 10 minute wash, also in

TBS-T. The membranes were then exposed to secondary antibody (anti-CDNF secondary anti-rabbit at 1:5000 dilution; anti-GAPDH secondary anti-mouse at 1:10000 dilution) for 1.5 hours at room temperature. Membranes were then washed twice in TBS-T for 5 minutes, and a last wash for 10 minutes.

After the last wash membranes were exposed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada) to visual protein bands. 1.5 ml of reagents 1 and 2 from the detection kit were added together and applied to membranes for 1 minute. Membranes were blotted dry using filter paper and wrapped in saran wrap prior to exposure. Membranes were exposed using Kodak Biomax XAR film (PerkinElmer Life Sciences, Boston, Massachusetts, USA) for 1, 10, 30, 60 and 120 seconds. Exposed films were developed in the Robert A. Smith Photographic Laboratory (McMaster University, Hamilton, ON). Protein bands were scanned onto a computer using an HP ScanJet 5590 scanner and analyzed using the ImageJ program (NIH, Bethesda, Maryland, USA).

2.2.5 Blood Collection and Platelet Preparation

10ml BD vacutainers with 1.42ml of acetate citrate dextrose were used to collect blood specimens. Once the rat was deeply anesthetized with isoflurane, an incision was made at the xiphoid process and a cut was made along the full length of the sternum to the lower abdominal cavity. Once the chest cavity was exposed a 23G needle was inserted into the right ventricle and 7-10 ml of intracardiac blood was collected.

Platelet-rich plasma (PRP) was prepared according to Hranilovic (1996). Blood was centrifuged for 35 seconds at 1250g. After centrifugation, the top 2/3 of the

plasma layer (upper pale-yellow portion) was removed using a transfer pipette and put into a 15 ml falcon tube. Cold PBS-EDTA-Bovine Serum Albumin (PEB; pH 7.0) was added at a ratio of 1:2 then the pellet was resuspended and spun for 20 minutes at 1220g. The supernatant was removed and discarded; the remaining pellet was rinsed in 7ml of PEB and recentrifuged for 5 minutes at 1220g. The supernatant was removed and the pellet resuspended in 1ml of PEB. Following resuspension, the pellet was transferred to a 1.5ml eppendorf tube where it was centrifuged for 5 minutes at 1220g in an Eppendorf 5415R small bench top centrifuge. The supernatant was carefully removes and the pellet was stored in -80°C until future use.

2.2.6 RNA Isolation from Blood

RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). Pellets were suspended in 1ml of TRIzol solution and incubated at room temperature for 5 min. 0.2ml of chloroform was added for each ml of Tri-pure isolation reagent, and shaken. Samples were incubated for 20 minutes at room temperature and then centrifuged at 12000gfor 15 minutes. The colourless RNA phase was then removed and transferred to a 1.5ml eppendorf, in which 0.5ml of iso-propanol was added. Samples were again incubated at room temperature for 10 minutes and then centrifuged for 10 minutes at 12000 g. Supernatant was then discarded, and 1ml of 75% ethanol was added to each sample, vortexed thoroughly and centrifuged at max speed for 15 minutes. Supernatant was discarded and the RNA was resuspended in 20ul of DEPC-Treated RNase-free water and heated to 55°C for 10 minutes. A DNase I kit (Invitrogen Life Technologies, Burlington, Ontario, Canada) was used to remove any contaminating DNA. RNA purity and quantity was

determined using a Beckman Spectrophotometer DU-640 measuring absorption at 260 nm and 280 nm.

2.2.7 Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Real-time quantitative RT-PCR (qRT-PCR) was used to determine absolute copy numbers of CDNF mRNA in blood samples. qRT-PCR was performed, for each sample, in triplicate using MX3000P Real-Time RT-PCR and OneStep RT-PCR kit (Stratagene, Missausaga, ON, Canada; QIAGEN Inc., Toronto, Ontario, Canada). Each reaction had a volume of 20 µl containing 60ng of total RNA. An absolute standard curve was generated using varying concentrations of cDNA: 1pg: 100fg: 10fg: 1fg: 100ag: and 10ag. Real-time RT-PCR conditions were optimized to ensure amplifications efficiencies remained constant over the course of the run. Primers were designed using OligoPerfect™ Designer software (Invitrogen Life Technologies, Burlington, Ontario, Canada) and were synthesized at MOBIX (McMaster University, Hamilton, Ontario, Canada). Components of the reaction mix were as follows: 10ul SYBR green (QIAGEN), 1.2µl each of the 500 µM CDNF primers (described in **Table 2**); 0.2 µl reverse transcriptase, and nuclease-free water to a final volume of 20 µl. Thermal profile was as follows: 50 °C for 30 min (1 cycle), 95 °C for 15 min (1 cycle), followed by 40 cycles of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 45 s, and lastly 95°C for 1 min (1 cycle). Representative qRT-PCR products showed 100% homology with rat CDNF gene regions.

2.2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Outlier detection was performed

Table 2.

Primers used for RT-PCR analysis of CDNF mRNA tissue expression.

Gene	**Primer**	**Sequence**
Rat *Cdnf*	Forward	5`- AAA GAA AAC CGC CTG TGC TA-3`
Rat *Cdnf*	Reverse	5`- TCA TTT TCC ACA GGT CCA CA-3`
Human *CDNF*	Forward	5`- AAA GAC GCA GCC ACA AAG AT-3`
Human *CDNF*	Reverse	5`- AGG ATC TGC TTC AGC TCT GC-3`

prior to analyses using GraphPad Outlier Tool. All levels of significance were defined as $p < 0.05$.

2.2.8.1 Immunoblotting

Differences in CDNF protein expression were determined using student's *t*-test. To examine whether CDNF protein expression was altered in the lesioned portion of the brain, right and left SNpc were compared. To determine whether there was retrograde transport of CDNF to the intact dopamine neurons in the striatum, right and left striatum were compared. Protein concentrations were normalized using GAPDH housekeeping antibody prior to statistical analysis.

2.2.8.2 qRT-PCR

MX3000P software performs analysis of data obtained by the real-time PCR to quantify the copy number of the target sequence in each mRNA sample. Real-Time PCR experiments were performed in triplicates which were averaged prior to analysis. Student's *t*-test was used to determine the difference in CDNF mRNA expression between 6-OHDA rats and aCSF rats.

2.3 RESULTS

2.3.1 Effect of 6-OHDA lesioning on CDNF protein concentrations

Following 6-OHDA lesioning to the left SN, CDNF protein expression was examined in left vs. right sides of the brain. Results indicated no significant difference in CDNF protein concentrations in the lesioned side (left) vs. non-lesioned side (right), *ns* (**Figure 4a**). To examine whether there was anterograde transport of CDNF from the SN to the striatum following the neurotoxic insult, right and left striatum were analyzed for CDNF protein concentrations. Results also indicated no significant difference in CDNF protein concentrations upstream in the striatum, *ns* **(Figure 4b)**.

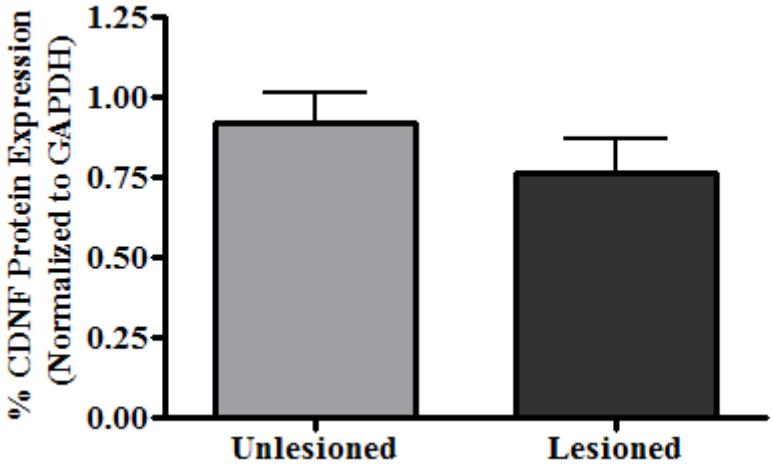

Figure 4a. CDNF Protein Expression in 6-OHDA rat Substantia Nigra. Graph depicts % protein expression in right vs. left substantia nigra. Rats received 6-OHDA lesion to the left substantia nigra, n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a two-tailed student's *t*-test was used to determine the difference in CDNF protein expression following 6-OHDA lesioning. Results indicated no significant difference in protein expression in right vs. left substantia nigra in 6-OHDA lesioned rats, *ns*.

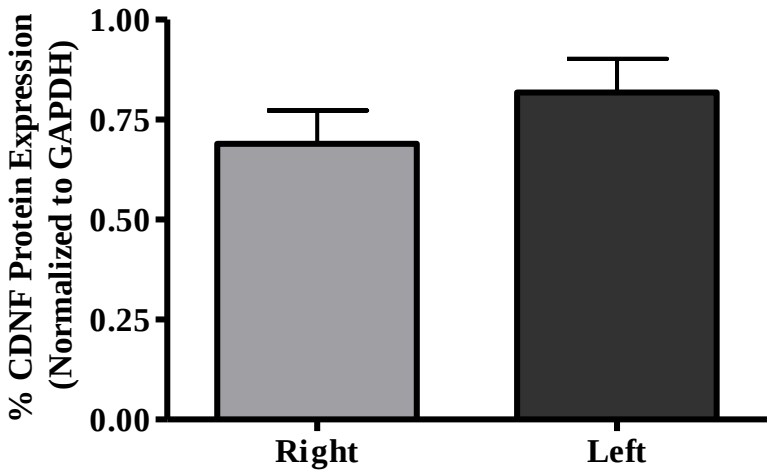

Figure 4b. CDNF Protein Expression in 6-OHDA rat Striatum. Graph depicts whole blood mRNA copy numbers (mean ± SEM). Right and Left side n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a two-tailed student's *t*-test was used to determine the difference in CDNF protein expression following 6-OHDA lesioning. Results indicated no significant difference in protein expression in right vs. left striatum in 6-OHDA lesioned rats, *ns*.

2.3.2 Effect of 6-OHDA lesioning on CDNF mRNA expression

This study did not include control animals, therefore, we included aCSF striatally infused animals from *Section 3.2.1* in order to examine peripheral blood CDNF mRNA expression in 6-OHDA lesioned rats. Following 6-OHDA lesioning, there was no significant difference in CDNF mRNA expression between experimental groups, *ns* (**Figure 5)**.

2.4 DISCUSSION

This study examined whether CDNF protein and mRNA concentrations would be reduced following 6-OHDA lesion, to determine whether a reduction in CDNF is involved in PD-like pathology. Results from this study have determined that both protein and mRNA concentrations were not reduced following 6-OHDA lesioning and remained relatively consistent across hemispheres. Since the lesion was confirmed using apomorphine 24 hours prior to sacrifice, it can assumed that the lesion was successful. These findings suggest that CDNF is unaffected by 6-OHDA lesioning and there may not be a direct role for CDNF in conferring susceptibility to developing PD. These results also suggest that the affects of the neurotoxic insult is retained within the CNS and does not affect the periphery. Results require extension and perhaps investigation into alternative models of PD, potentially the MPTP model.

The mechanisms underlying 6-OHDA induced dopaminergic cell death are well established. The dopaminergic degeneration involves the processing of hydrogen peroxidase and hydroxyl radicals in the presence of iron, and as such, 6-OHDA is easily oxidizable (Schober, 2004). 6-OHDA works quickly and dopamine neurons begin to degenerate 12 hours following injection, which results in complete dopamine depletion 2-3 days post-lesion (Schober, 2004). The CXXC motif of the C-terminal

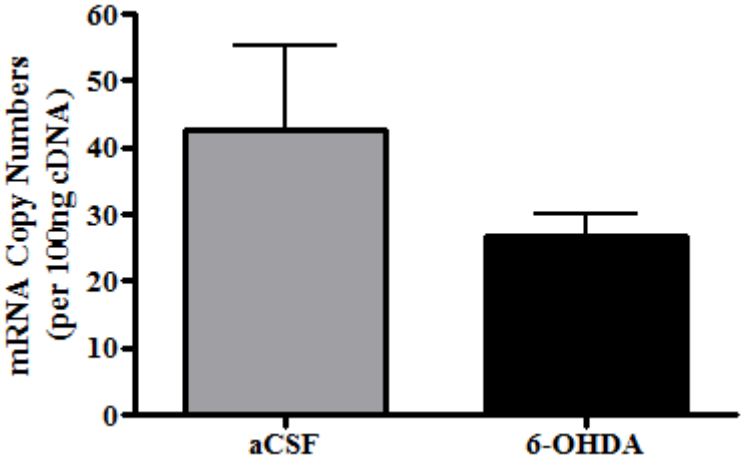

Figure 5. Results of CDNF mRNA expression in 6-OHDA lesioned rats. Graph depicts platelet mRNA copy numbers (mean ± SEM). Experimental groups were composed of n=8 for aCSF rats and n=8 for 6-OHDA rats. Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed students *t* –test indicating no significant difference in CDNF mRNA expression between aCSF and 6-OHDA lesioned rats, *ns*.

domain of CDNF is hypothesized to play a role in protecting cells against ER stress. Evidence suggests that 6-OHDA induces ER stress and the UPR in cell cultures (Ryu et al., 2002). Under prolonged ER stress, the ER upregulates gene products that induce cell death as seen in neurodegenerative diseases like PD (Yuan, 2006; Lindholm, Wootz, & Korhonen, 2006). It was anticipated that CDNF would be decreased following lesioning, due to increased dopaminergic cell death yet our results showed preserved levels of CDNF protein concentrations across lesioned versus non-lesioned hemispheres. It may possible that CDNF may have been recruited to help sustain the remaining dopamine neurons. This study did not examine TH levels in conjunction with CDNF protein expression. It would be interesting to measure whether there is a correlation between TH immunoreactive cells and CDNF protein expression to investigate whether there is an upregulation of CDNF following dopamine cell death. Future studies should also examine ER chaperones in conjunction with CDNF protein concentrations to determine whether they work concurrently to protect damaged or dying dopamine neurons following lesioning.

3.1 OBJECTIVE 2

Understanding the role of CDNF in the pathophysiology of PD through selective knockdown of CDNF *in vivo*

As mentioned previously, BDNF and GDNF have both been found to improve functionality in the 6-OHDA and MPTP models of PD, as well as restore dopamine levels to normal (Airavaara, et al., 2012; Lindholm & Saarma, 2009). Nevertheless, these models do not recapitulate the typical pathophysiological hallmarks of the disease, nor does it consider the progressive nature of PD. Animal knockout studies are commonly done in research to distinguish whether alterations in certain neuronal populations or genes result in behaviour similar to the human condition. Knockout studies have proven difficult, in that knocking out one gene may have off target genetic effects. Previously in our lab, we have had great success with antisense oligonucleotides as a means to induce knockdown in very specific regions of the brain, with little to no diffusion to surrounding areas, therefore reducing off target effects exhibited in genetic knockout animals (Dyck, Beyaert, Ferro & Mishra, 2011). Selective knockdown of synapsin II was able to induce a schizophrenia-like phenotype, with little to no adverse side effects. Therefore, we intend to use the same oligonucleotide technology to induce selective knockdown of CDNF within the rat striatum. Previous studies using antisense oligonucleotides were able to successfully knockdown BDNF, producing a PD-like phenotype (Porritt, Batchelor & Howells, 2005). Therefore, the second objective of the present study was to investigate whether the selective knockdown of CDNF in the striatum produces a progressive PD-like phenotype.

Given that selective knockdown to the striatum would result in decreased neurotrophic support, it was hypothesized that the selective knockdown of CDNF would result in: (1) decreased locomotor activity compared to aCSF animals; (2) decrease motor coordination compared to aCSF animals; and (3) decreased CDNF mRNA expression compared to aCSF animals.

3.2 METHODOLOGY

3.2.1 Animals

16 adult male Sprague Dawley rats (225-250g) were purchased from Charles River Laboratories International, Wilmington, Massachusetts, USA. All animal housing protocols were previously described in *Section 2.2.1*. Animals were subjected to behavioural testing following one-week of acclimation.

3.2.2 Antisense Oligonucleotide Sequences and Infusion

Antisense oligonucleotides were synthesized by Integrated DNA Technologies (IDT; Coralville, Iowa) (**Table 3**). All nucleotides were capped with phosphorothioates to prevent nuclease destruction (Lebedeva and Stein, 2001). The antisense sequence was formulated to target the CDNF gene in order to examine the presence of PD-like symptoms. CDNF knockdown and control animals were unilaterally infused into the striatum. Infusion into the striatum allows for retrograde-induced neuronal death and causes a progressive, slow onset of degeneration and symptoms, which bears most similarity to human patients (Schober, 2004). Although the primary area affected in PD is the SNpc, prior lesion studies in this region indicate quick onset of symptoms, and severe manifestation of symptoms, leading to continuous monitoring of health status (Schober, 2004). In addition to this, bilaterally infused animals require intensive care, thus unilateral models are more desirable. The

Table 3.

Antisense Oligonucleotide Sequence.

Antisense	**Nucleotide Sequence**
CDNF	5’T*T*T*C*C*C*T*T*G*G*T*G*T*C*C*G*C*G*C*A 3’

Note: Antisense sequences will be capped with phosphorothioates (as indicated by *) to prevent nucleotide destruction. CDNF antisense sequence was compared to the genome database to ensure a lack of homology with other genes.

intact hemisphere, in unilateral models, serves as an internal control structure. Oligonucleotides were dissolved in sterile distilled water to a final concentration of 1mM and filtered through a sterile 0.2 μm HT Tuffryn Membrane (Pall Corporation, Ann Arbor, Michigan, USA) prior to infusion. Rats were divided into two following selective knockdown of CDNF *in vivo*: Group A (n=8) served as a control for surgical procedures and was infused with artificial cerebral spinal fluid into the striatum (aCSF); Group B (n=8) was infused with CDNF antisense oligonucleotide sequences into the striatum, the primary group of interest.

14 day infusion pumps (Model 2002) were purchased from Alzet (DURECT Corporation, Cupertino, California, USA). Osmotic pumps were chosen to ensure continuous, daily infusion over manual infusion, minimizing the risk of infection. Direct cannula implantation allows for accurate administration to the brain region of interest. Each pump was filled with 5nmol of solution (or equivalent of aCSF) and was connected to a cannula via PVC60 polyvinyl cannula tubing (DURECT Cooporation) (**Figure 6a**). Each pump infused at a rate of 0.5μl/hr for 14 days.

3.2.3 Surgical Procedures

Animals were under gaseous anaesthesia during implantation of osmotic pumps and cannulas (Isofluorane, Pharmacuetical Partners of Canada Inc, Richmond Hill, ON) and were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). 28 gauge, stainless steel cannulas (Brain Infusion Kit 2, DURECT Corporation) were unilaterally implanted according to the following coordinates in reference to bregma: +0.7 mm anterior; +3.0 mm lateral; -5.0 mm ventral to the surface of the skull (**Figure 6b**). Osmotic pumps were buried along the lateral border

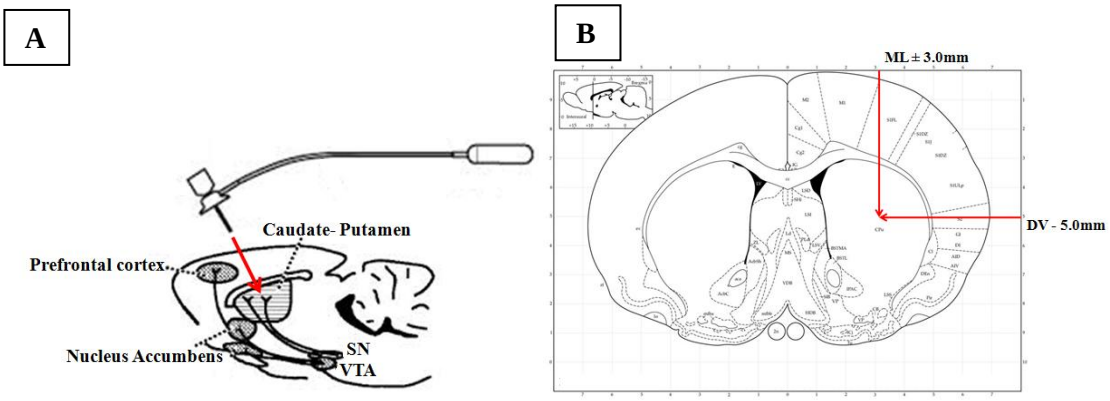

Figure 6. Stereotaxic Coordinates of Infusion Site. Image A depicts pictorial representation of cannula. Infusion will be performed using 14 day Alzet infusion pumps (Model 2002, DURECT Corp), via osmotic pump cannulas. Image B depicts left striatal site of infusion for aCSF and CDNF knockdown, at brain coordinates AP ±0.7mm, ML +3.0mm, DV -5.0mm relative to bregma (AP=anterior-posterior, ML=medial-lateral, DV=dorsal-ventral).

of the scapula and cannulas were cemented in place to avoid movement post-surgery. Animals were allowed one week for recovery before behavioural testing.

3.2.4 Behavioural Testing

All animals were tested at three different time points: prior to surgery baseline, 7 days post surgery to ensure proper recovery time, and 14 days post-surgery. This was done to ensure to monitor behavioural impairments. Animals were weighed daily to monitor general health; if an animal lost 20% of its initial body weight following surgery, the animal was said to have reached endpoint and was immediately euthanized.

3.2.4.1 Locomotor Activity

All locomotor tests were completed during the dark period of the light/dark cycle, given rats show maximum activity during those hours. Multidirectional movements were recorded using AccuScan computerized cages (AccuScan Instruments, Columbus, OH). Infrared light beams capture movements in a variety of parameters; of particular interest to us is total distance travelled (cm). Locomotor activity was recorded for a total of 180 minutes, of which, the first 30 minutes are considered habituation and the last 150 minutes are actual activity.

3.2.4.2 Beam Walk

This test assesses motor coordination of the fore and hindlimbs during precise locomotion and balance (Heuer, Smith, Lelos, Lane, & Dunnett, 2012). Animals will be placed on an 80 cm narrow beam that is elevated 60 cm from the ground. Animals will start at one end of the beam with a 20 cm x 20 cm enclosed goal box placed at the other end. Latency to traverse the beam will be measured, and the test will be repeated and averaged over two trials per test day. Animals will be trained prior to surgery for

2 days, or until the animal is able to walk the beam without assistance or falling, in under 60 seconds. On the day of testing, all rats are allotted 60 seconds to traverse the beam. A failure in completing the task constitutes exceeding the allotted time or falling off the beam at any point.

3.2.5 Sacrifice and Tissue Dissection

Rats were sacrificed using protocols described in *Section 2.2.3*.

3.2.6 Protein Quantification and Immunoblotting

All protocols for protein quantification and immunoblotting were previously described in *Section 2.2.4*. Immunoblotting was preformed to verify knockdown of CDNF in the striatum.

3.2.7 qRT-PCR

All protocols were previously discussed in *Section 2.2.7*.

3.2.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4.0 software. Outlier detection was performed prior to analyses using GraphPad Outlier Tool. All levels of significance were defined as $p < 0.05$.

3.2.8.1 Locomotor Activity

Locomotor activity was analyzed for each time point (0, 7, 14 days) using total distance travelled (cm) for the 150 min recording period. To examine the effect of infusion treatment on locomotor activity over the course of the 14 day period a two-way analysis of variance (ANOVA), followed by Bonferroni post hoc test. A second analysis was performed to examine the locomotor activity for each treatment group using a one-way ANOVA within each treatment group, followed by Tukey's post hoc test.

3.2.8.2 Beam Walk

Beam walk was analyzed according to Yu et al. (2013). Neurological status was determined using a 7 point Likert Scale indicated in **Table 4**. A two-way ANOVA was used to determine the effect of treatment over the course of the three time points, followed by Bonferroni post hoc tests. A second analysis was performed using a one-way ANOVA within each treatment group, followed by Tukey's post hoc test.

3.2.8.3 Immunoblotting

Immunoblotting was done to verify success of the antisense knockdown. Differences in CDNF protein expression between infused (left) vs. non-infused (right) striata of CDNF knockdown rats was examined using a two-tailed student's *t*-test. This was also done using aCSF rats to ensure the surgical process did not affect CDNF protein concentrations. To determine whether there were differences in CDNF protein concentrations between CDNF knockdown rats vs. aCSF rats, a two-tailed student's *t*-test was performed. Results are reported as percentage change in protein expression from right vs. left hemispheres, following normalization to GAPDH.

3.2.8.4 qRT-PCR

MX3000P software performs analysis of data obtained by the real-time PCR to quantify the copy number of the target sequence in each mRNA sample. Real-Time PCR experiments were performed in triplicates which were averaged prior to analysis A student's *t*-test was used to determine the difference in CDNF mRNA expression between aCSF infused rats and CDNF knockdown rats.

Table 4.

Neurological Rating Scale for Performance on Beam Walk Apparatus.

Score	Neurological Status
1	Rat is unable to place the affected hindlimb on the horizontal surface of the beam
2	Rat places affected hindlimb on the horizontal surface of the beam and maintains balance for at least 5 seconds
3	The rat traverses the beam while dragging the affected hindlimb
4	Rat traverses beam and at least once places the affected hindlimb on the horizontal surface of the beam
5	Rat crosses the beam and places the affected hindlimb on the horizontal surface to aid in less than 50% of its steps
6	The rats uses the affected hindlimb to aid more than half of its steps
7	No more than 2 footslips

3.3 RESULTS

3.3.1 Effect of CDNF knockdown on Locomotor Activity

Following 14 day infusion with CDNF antisense oligonucleotides, CDNF infused animals showed increased locomotor activity compared to aCSF infused control rats (**Figure 7a).** Two-way ANOVA results revealed a significant interaction, $F(1,39) = 3.43$, $p < 0.0002$; Bonferroni post-hoc tests determined there was a significant increase in locomotor behavior between CDNF infused and aCSF infused rats only after 14 days of infusion ***$p < 0.001$, not after 7 days, *ns*. A one-way ANOVA determined that in CDNF infused rats, locomotor activity was increased significantly (**Figure 7b**), $F(2,19)= 7.80$, $p < 0.0034$; Tukey's post-hoc tests determined that locomotor behavior was not significantly affected from baseline to day 7, *ns*, but significantly increased from baseline to day 14 $p < .01$ and day 7 to day 14, $p < 0.05$. ACSF rats showed no significant change in locomotor activity across all time points (**Figure 7c**) suggesting surgical procedure did not affect locomotion.

. **3.3.2 Effect of CDNF knockdown on Motor Coordination**

14 day infusion of CDNF antisense resulted in significantly impaired motor coordination (main effect of treatment, $F(1,42) = 13.29$, $p = 0.0007$; within group effect across date of testing, $F(2,42) = 24.40$, $p < 0.0001$) (**Figure 8a**). CDNF knockdown animals had significantly impaired motor coordination compared to aCSF animals after 7 days of infusion (*$p < 0.05$) which further declined after 14 days (**$p < 0.01$). These results suggest that surgical procedure did not affect motor coordination. A one-way ANOVA determined that neurological status in CDNF knockdown animals significantly declined (*$p < 0.05$). Tukey's post hoc tests revealed that motor impairments were seen in as little as one week (****$p < 0.0001$), which

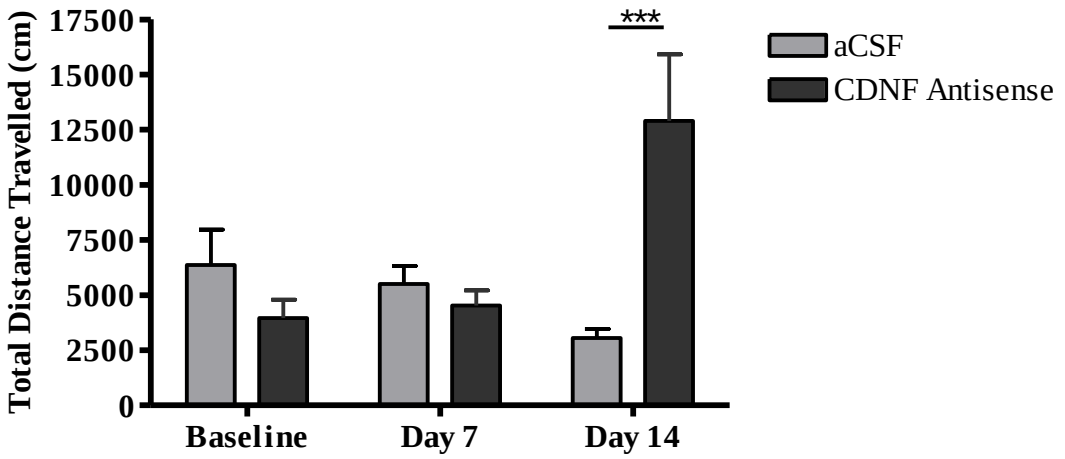

Figure 7a. Results of CDNF knockdown on locomotor activity following 14 day infusion. Graph depicts total distance travelled (cm) (mean ± SEM) for each treatment group at baseline, 7 days post surgery, and 14 days post surgery. CDNF knockdown and aCSF values are both an average of 8 rats, $F(1,39) = 3.43$, $p < 0.0002$. Baseline CDNF knockdown vs. aCSF results were not significantly different, nor were CDNF knockdown vs. aCSF after one-week of infusion. Two-weeks of infusion significantly increased locomotor activity, ***$p < 0.001$.

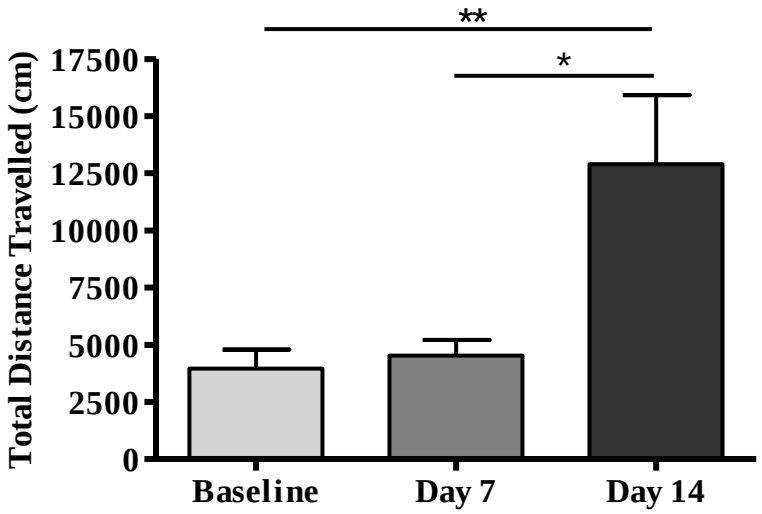

Figure 7b. Results of CDNF knockdown on locomotor activity. Graph depicts total distance travelled (cm) (mean ± SEM) following continuous infusion of CDNF antisense at baseline, 7 days post surgery, and 14 days post surgery. $F(2,19)= 7.80$, $p < 0.0034$.

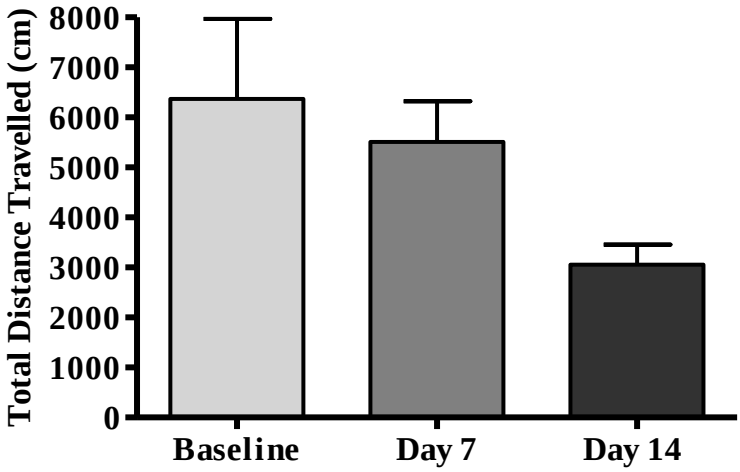

Figure 7c. Results of aCSF infusion on locomotor activity. Graph depicts total distance travelled (cm) (mean ± SEM) following continuous infusion of aCSF at baseline, 7 days post surgery, and 14 days post surgery, *ns*

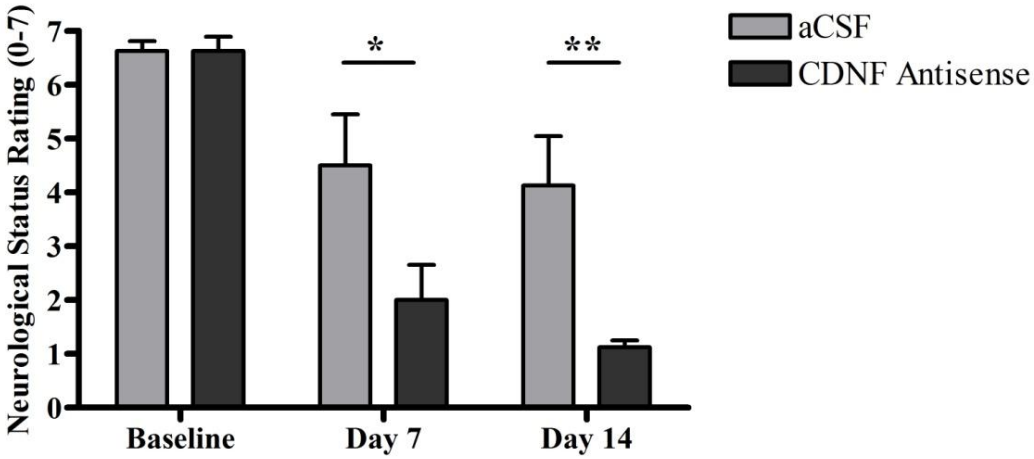

Figure 8a. Results of CDNF knockdown on motor coordination following 14 day infusion. Graph depicts neurological status on beam walk apparatus (mean ± SEM) for each treatment group at baseline, 7 days post surgery, and 14 days post surgery. $F(1,42) = 13.29$, $p = 0.0007$. CDNF knockdown and aCSF values are both an average of 8 rats. Baseline CDNF knockdown vs. aCSF results were not significantly different. At one-week of infusion CDNF knockdown significantly differed from aCSF *p 0.05; as well as at two-weeks of infusion CDNF knockdown vs. aCSF, **$p < 0.01$.

were sustained for the full 14 days (****$p < 0.0001$); however, this impairment was not significantly worsened from week one to week two, *ns* (**Figure 8b**). Control aCSF animals did not show significantly impairments in motor coordination at any time point, indicating a significant effect of knockdown, not surgical procedure (**Figure 8c**).

3.3.3 Verification of CDFN Knockdown

Following 14 days of antisense infusion, as expected, there was no significant difference between right and left striata for aCSF infused rats, *ns* (**Figure 9a**). There was also no significant difference in CDNF protein concentrations in right vs. left striata for CDNF knockdown rats, *ns*, suggesting that the knockdown was not effective (**Figure 9b**). Due to the lack of effectiveness in the knockdown, a one-way ANOVA between groups was not performed as anticipated.

3.3.4 Effect of CDNF Knockdown on platelet mRNA expression

Following 14 day infusion, peripheral blood was examined for differences in CDNF mRNA expression downstream. Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed student's *t* –test. Results indicated no significant difference in CDNF mRNA expression between experimental groups, *ns* (**Figure 10)**. These results suggest that the insult in retained within the CNS and did not affect the periphery.

3.4 DISCUSSION

It has been suggested that loss of endogenous NTFs may trigger neuronal cell death in certain neuronal populations as exhibited in PD and AD (Evans & Barker, 2008). For example, studies examining conditional knockout of GDNF causes a progressive PD-like phenotype, and almost complete dopaminergic cell death,

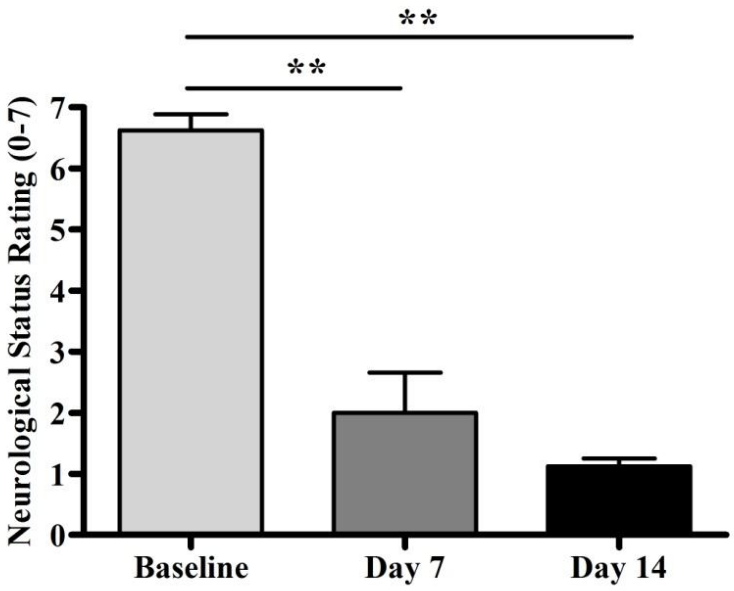

Figure 8b. Results of CDNF knockdown on motor coordination. Graph depicts neurological status on beam walk apparatus (mean ± SEM) following continuous infusion of CDNF antisense at baseline, 7 days post surgery, and 14 days post surgery. (*$p < 0.05$).

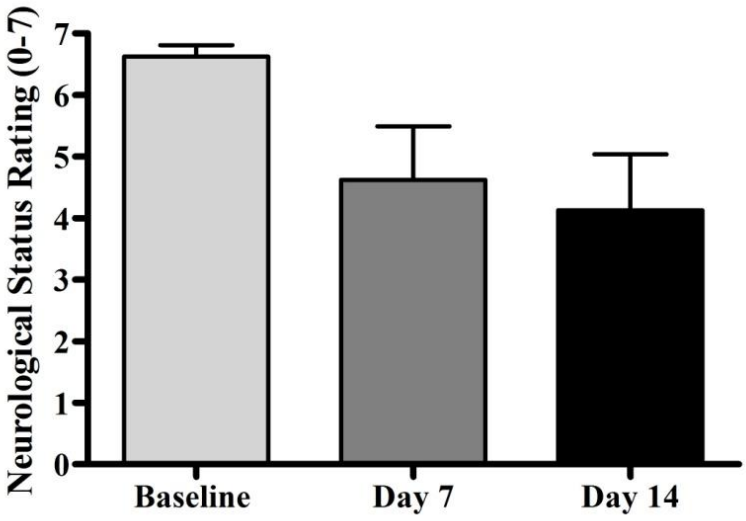

Figure 8c. Results of aCSF infusion on motor coordination. Graph depicts neurological status on beam walk apparatus (mean ± SEM) following following continuous infusion of aCSF at baseline, 7 days post surgery, and 14 days post surgery, *ns*.

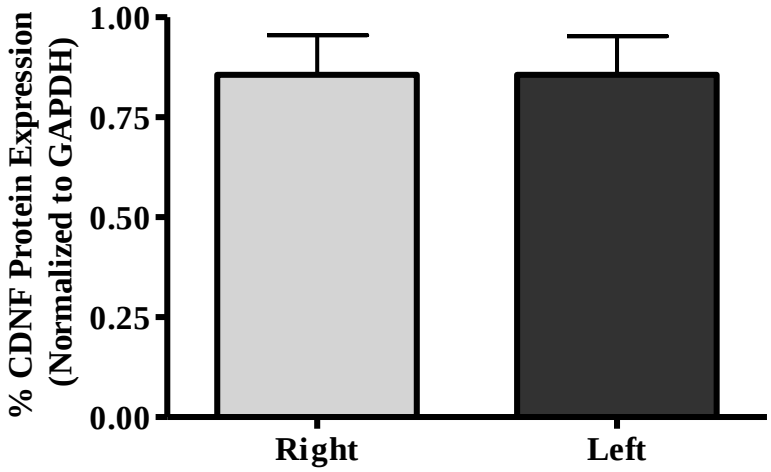

Figure 9a. CDNF Protein Concentrations Following 14 day aCSF Infusion. Graph depicts % protein expression in right vs. left striatum. Rats received 14 day infusion to left striatum, n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a two-tailed student's *t*-test was used to determine efficacy of the knockdown. As expected, there was no significant difference in CDNF protein expression in right vs. left striatum of aCSF infused rats, *ns*.

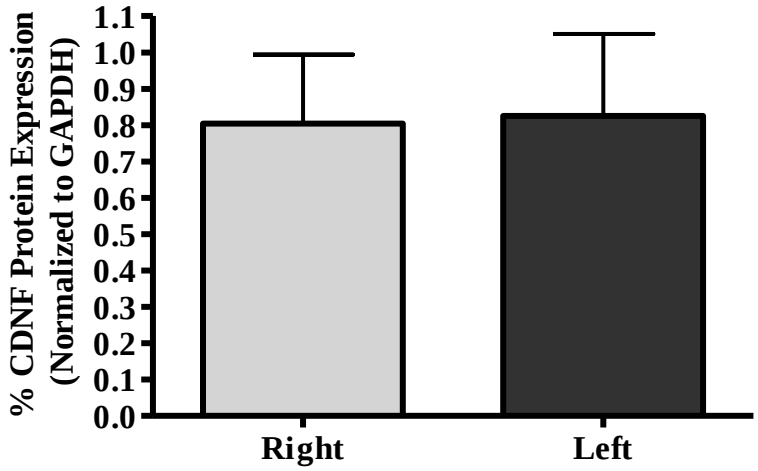

Figure 9b. Verification of CDNF Knockdown following 14 day infusion. Graph depicts % protein expression in right vs. left striatum. Rats received 14 day infusion to left striatum, n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a two-tailed student's *t*-test was used to determine efficacy of the knockdown. Results indicated no significant difference in CDNF protein expression in right vs. left striatum of CDNF knockdown rats, *ns*.

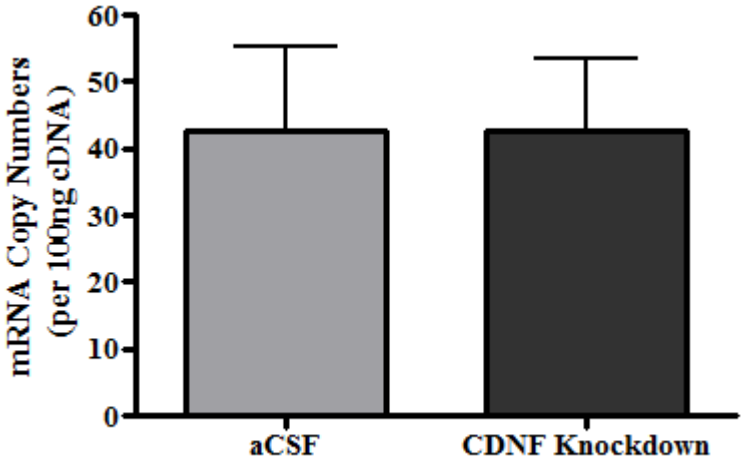

Figure 10. Results of CDNF mRNA expression in CDNF knockdown rats. Graph depicts platelet mRNA copy numbers (mean ± SEM). Experimental groups were composed of n=8 for aCSF rats and n=8 for CDNF knockdown rats. Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed students *t* –test indicating no significant difference in CDNF mRNA expression between aCSF and CDNF knockdown rats, *ns*.

indicating downregulation of a single NTF can produce massive neuronal death (Pascual, et al., 2008). Recently, antisense oligonucleotides have provided a means for temporarily and selective knock down of the region of interest. Studies examining BDNF knockdown using antisense oligonucleotides found success in producing dopaminergic neuronal death and rotational behaviour following apomorphine injection (Porritt, Batchelor, & Howells, 2005). To date there have been no studies examining a knockout model of CDNF. The overwhelming majority of studies have investigated the neuroprotective and neurorestorative effects of CDNF (Lindholm et al., 2007; Voutilainen et al., 2011); however, it is still unknown whether reductions in CDNF confer susceptibility to developing PD.

The present study examined whether selective knockdown of CDNF would produce behavioural impairments typically seen in animal models of PD, although results were difficult to interpret behaviorally. CDNF knockdown rats showed increased locomotor activity compared to aCSF rats. These results were contradictory to what was anticipated. ACSF rats showed a linear decrease in locomotion over the 14 day period suggesting habituation to the locomotor chamber and subsequent decrease in exploratory behavior. It is possible that CDNF knockdown does not allow for the same habituation process as seen in aCSF rats. With respect to motor coordination, as expected, CDNF knockdown rats had decreased motor coordination on the beam walk apparatus compared to aCSF rats.

Although behavioural results were conflicting, immunoblotting results demonstrated the knockdown was not successful. That being said, behavioural results should be interpreted with caution. During sacrifice, some brains showed large necrotic areas around the lesion site. This could indicate a potential toxic effect of the

antisense oligonucleotides. When antisense oligonucleotides, capped with phosphorothioates, are injected into the brain, they can cause severe tissue damage, especially with chronic administration (Wahlestedt, et al., 2000). While previous studies in our lab have had great success with antisense oligonucleotides, the CDNF antisense is novel and the dosage or sequence could have been toxic and may need to be altered. It is also possible that the sequence that was used was not in the correct region to elicit full knockdown, and other regions should be targeted. It is also important to determine the appropriate dosage of antisense to elicit a behavioural response. The dosage we used was based on standard protocols from our lab (Dyck, et al., 2013). It is also possible that knockdown in the striatum does not elicit a full PD-like phenotype. The area primarily affected in PD is the SNpc, which causes subsequent depletion of dopamine in the striatum as such, future studies should examine CDNF knockdown in the SNpc. It is also important to include a mismatch sequence as a control group for the oligonucleotide technology. This is a random generation of a sequence that does not target any gene but uses the same oligonucleotide technology. This will elucidate whether the sequence itself is toxic, or the knockdown technology.

CHAPTER 3

Clinical Investigations of CDNF in Parkinson's Disease

4.1 OBJECTIVE 3

Examining CDNF mRNA concentrations over the course of development in healthy aging population

One of the primary risk factors for developing PD is advancing age, which is often over looked in animal models of PD. Compensatory mechanisms that may be present in the young brain, may no longer be functional in the aged brain. Most notably, BDNF and GDNF are actually increased in damaged striata following 6-OHDA neurotoxic insult in young rodents compared to aged (Yurek and Fletcher-Turner, 2000). These findings suggest a compensatory mechanism and neurotrophic response due to the direct injury to the striatum that may be lost over time. Aging is the single largest independent risk factor for developing neurodegenerative diseases such as AD and PD (Mora, Segovia, & del Arco, 2007). NTFs provide an avenue of study into the aging process. The loss of neurotrophic support for specific neurotransmitter populations could confer susceptibility to various neurodegenerative disorders (Collier et al., 2005). Research into CDNF is in its infancy, and therefore has not yet been investigated in the aging process. Investigating the role of CDNF in aging could provide insight into neurotrophic support over the course of development that may be reduced, which could have implications in PD. Therefore, the third objective of the present study was to measure CDNF levels in peripheral whole blood of healthy individuals, to examine CDNF mRNA expression in the human population over the course of development. It was hypothesized CDNF mRNA expression would decrease over the course of development in healthy individuals.

4.2 METHODOLOGY

4.2.1 Participants

Ethical approval for this study was obtained from the Hamilton Integrated Research Ethic Board in Hamilton, Ontario, as well as the internal Research Ethics Board at McMaster University. Participants were recruited from the Women's Health Concern's clinic, and the Mood Disorder's program at St. Josephs Healthcare, in Hamilton, Ontario, in collaboration with Dr. Benecio Frey. Participants were not recruited if they were diagnosed with a psychiatric, neurological, or neuropsychiatric disorder at any time prior to enrolment in the study. All subjects gave written informed consent. Patients were not given compensation for involvement in this study.

4.2.2 Blood Collection and Preparation of Whole Blood

2.5 ml of venous whole blood was collected using PAXgene® blood DNA tubes (PreAnalytix), per participant. RNA was extracted as per manufactures protocols and stored at -80°C until use.

4.2.3 qRT-PCR

Protocols for qRT-PCR were previously described in *Section 2.2.7*. Samples were to a final volume of 20ul, with 60ng total RNA added. Components of the reaction mix for human samples were as follows: 10ul SYBR green (QIAGEN), 1.2ul each of the 500nM CDNF primers (**Table 2**), 0.2ul reverse transcriptase, and nuclease-free water to a final volume of 20ul. Thermal profile was as follows: 50°C for 30 min (1 cycle), 95°C for 15 min (1 cycle), followed by 40 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 30 s, lastly 95°C for 1 min (1 cycle). Representative qRT-PCR products showed 100% homology with the human CDNF gene regions.

4.2.4 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4.0 software. Outlier detection was performed prior to analyses using GraphPad Outlier Tool. All levels of significance were defined as $p < 0.05$.

4.2.4.1 qRT-PCR

The MX3000P software performs analysis of data obtained by the real-time PCR to quantify the copy number of the target sequence in each mRNA sample. A one-way ANOVA will be used to determine CDNF mRNA copy number differences between the various age groups. Tukey's post-hoc tests will be used to determine whether the difference lies.

4.3 RESULTS

Participants were divided into 3 age groups; A) Children [0-18 (n=7; 3 males, 4 females; average age 12.28 years)], B) Young adults [18-50 (n=22; 6 males, 17 females; average age 34.82 years)], and C) Older adults [50+ (n=16; 5 males, 11 females; average age 63 years)]. Real-Time PCR experiments were performed in triplicates and results were analyzed using one-way ANOVA $F(2,44)= 9.29$, $p < 0.0005$, indicating a difference in CDNF mRNA expression over the course of development (**Figure 11)**. Tukey's post-hoc tests indicated a significant decrease in CDNF mRNA expression from childhood to young adulthood ($*p < 0.05$), and childhood to older adulthood ($***p < 0.001$); however, no subsequent decrease from young adulthood into older adulthood, *ns*. These results suggest that CDNF mRNA expression may decrease with aging. Reductions in CDNF over the course of development may be particularly important in PD, a disorder stemming from dopamine degeneration.

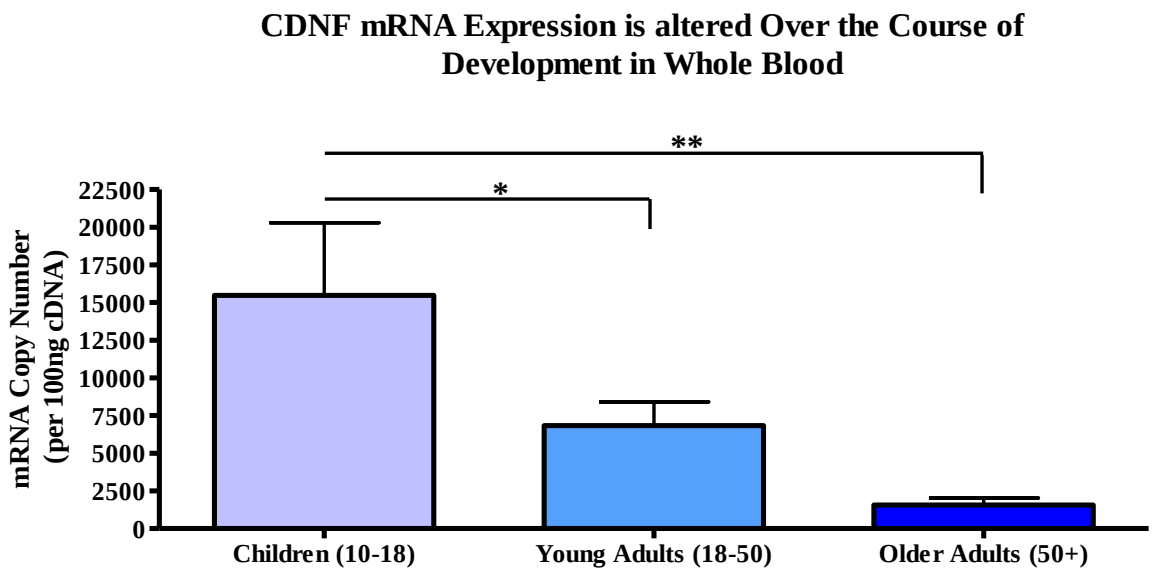

Figure 11. Results of CDNF mRNA expression in whole blood over development. Graph depicts whole blood mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using one-way ANOVA $F(2,44)= 9.29$, $p < 0.0005$, indicating a significant difference in CDNF mRNA expression over the course of development. Tukey's post-hoc tests indicate a significant decrease in CDNF mRNA expression from childhood to young adulthood ($*p < 0.05$), and childhood to older adulthood ($**p < 0.001$); however, no subsequent decrease from young adulthood into older adulthood, *ns.*

4.4 DISCUSSION

The aging process has a dramatic effect on specific neuronal populations within the brain that increases the likelihood of developing neurodegenerative diseases (Kovacs et al., 2014). Studies have shown increases in oxidized proteins and oxidized DNA lesions with age, along with enhanced neuroinflammtory processes (Floyd, & Hensley, 2002). Critical neuronal mechanisms seem to be compromised in the aging brain, in particular NTFs. For example, age related decreases in BDNF may contribute to cognitive impairments seen in AD, and decreased in GDNF may lead to PD (Mora, Segovia, & del Arco, 2007). These facts have led to numerous studies examining the therapeutic potential of NTFs in neurodegenerative disease, as increasing NTF concentrations restore neuron concentrations and promote functional improvement (Mora, Segovia, & del Arco, 2007).

CDNF is a novel NTF thus has not been examined in the aging process. The present study examined the age related changes in CDNF which has never been examined before. Our results suggest an age related decline in CDNF mRNA concentrations in whole blood from childhood to young adulthood, and childhood to older adulthood. Although there was a lack of significant decline in CDNF mRNA concentrations from young adulthood to older adulthood this could stem from the relatively small sample size we gathered from the children group (n=7). In looking at **Figure 11,** the average copy number for young controls was approximately 10,000, compared to the 2000 copies for older adults. Increasing the sample size for the child group would further elucidate this age related decline.

5.1 OBJECTIVE 4

Examine peripheral blood CDNF mRNA expression in clinical populations of Parkinson's disease

Prior research from our lab has determined that platelets and lymphocytes can be used as indicators of dysregulated dopamine signaling (Groleau et al., 2012). It is thought that if gene expression is correlated to CNS expression to a certain extent, this could drastically advance research on the mechanisms of neuropsychiatric illnesses (Sullivan et al., 2006). Given the difficulty and feasibility of obtaining post-mortem tissue, a surrogate tissue such as blood, would provide an easy and minimally invasive method of analysis (Behari and Shrivastava, 2013). While there is a plethora of research pertaining to the therapeutic potential of CDNF in animal models of PD, what still remains unknown is whether or not CDNF is reduced in human patients with PD and whether this reduction is specific to PD. Previous work in this study has determined that CDNF is unaltered in animal models of PD, suggesting a compensatory mechanism of CDNF. We have also found that there is an age-related decrease in CDNF mRNA expression, which could be important in disorders stemming from dopamine degeneration. Although this population showed a decrease in CDNF over the course of development, this does not necessarily mean these individuals will develop PD. It is unlikely that we happened to choose the individuals that will go on to develop PD. There may be a natural decline in CDNF, but there may also be a specific threshold of declination that is necessary for development of PD. Investigating clinical populations of PD would provide insight into how drastic the decline of CDNF needs to be in order to develop PD. Therefore the fourth objective was to determine: (1) whether CDNF mRNA concentrations are altered in platelets of

PD patients compared to healthy and negative controls; (2) whether CDNF mRNA concentrations are altered in lymphocytes of PD patients compared to healthy and negative controls; and (3) whether CDNF mRNA concentrations are altered in whole blood samples of PD patients compared to healthy and negative controls.

It was hypothesized that CDNF mRNA expression would be: (1) reduced in platelets, lymphocytes and whole blood samples in PD patients compared to controls; (2) this reduction in CDNF mRNA expression will be specific to PD as evidenced by comparisons to negative controls.

5.2 METHODOLODY

5.2.1 Participants

PD participants were recruited through Movement Disorders specialists in the Hamilton area. All subjects were given informed consent prior to enrolment in the study. Diagnosis was confirmed by physical examination and a second opinion was attained from a movement disorders specialist. Inclusion criteria included a primary diagnosis of Parkinson's disease with no additional movement disorder (i.e. parkinsonism, essential tremor). A group of healthy subjects were recruited at the Ambulatory care clinic at Juravinkski hospital, and at McMaster University. All patients were excluded if they received a diagnosis neuropsychiatric or neurological illness. Patients were included as healthy controls if they had diabetes, high or low blood pressure, or headaches. Participants were not given compensation for involvement in this study.

5.2.2 Blood Collection and Preparation of Platelets and Lymphocytes

30-40mL of venous blood was collected from each subject. 10ml BD vacutainers with 1.42ml of acetate citrate dextrose were used to collect blood

specimens. Platelets were prepared as follows: blood was centrifuged at 980g for 2 minutes in an Eppendorf 5810R Centrifuge, after which the top 2/3 of the top layer (pale-yellowish color) was removed using a transfer pipette and put into a 15ml falcon tube. The sample was centrifuged again at 1200g for 7 minutes. The supernatant was removed and the pellet was then washed twice in 7ml of cold PEB solution at 1200 g for 7 minutes. The supernatant was removed again, and the pellet was resuspended in 1ml of cold PEB prior to transfer to a 1.5ml eppendorf tube. Once in the 1.5ml tube, the pellet was centrifuged for 7 minutes at 1200g in an Eppendorf 5415R small bench top centrifuge. After the final centrifugation the supernatant was removed and the pellet was stored at -80°C.

Lymphocytes were prepared as follows: blood samples were emptied into a 50ml falcon tube, then 30ml of warmed (37°C) phosphate buffered saline (PBS) was added and mixed thoroughly using a pasture pipette. To isolate human lymphocytes, 15ml of ficol-paque plus (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada) was added to the blood-PBS mixture using a 9" lime glass pasture pipette. Blood was centrifuged for 30min at 400g to yield a fuzzy lymphocyte layer. Supernatant was removed and the fuzzy lymphocyte layer was transferred to another 50ml falcon tube using a transfer pipette. 30ml of PBS was added and the lymphocytes were resuspended and spun for 10 min at 110g. The supernatant was removed and the lymphocyte pellet was resuspended in 30ml of warm PBS and spun for 10 min at 110g. Supernatant was removed and the pellet was resuspended in 1ml PBS then transferred to a 1.5ml eppendorf tube. The sample was then spun at 110g for 10 min in an Eppendorf 5415R small bench top centrifuge. The supernatant was removed and the lymphocyte pellets were stored at -80°C.

5.2.3 Preparation of Whole Blood

All protocols for preparation of whole blood were previously described in *Section 4.2.2*.

5.2.4 RNA Isolation from Blood Samples

RNA extraction protocols were previously described in *Section 4.2.2*

5.2.5 qRT-PCR

Protocols for quantification of RNA were previously described in *Section 4.2.3*.

5.2.6 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4.0 software. Outlier detection was performed prior to analyses using GraphPad Outlier Tool. All levels of significance were defined as $p < 0.05$.

5.2.6.1 qRT-PCR

MX3000P software performs analysis of data obtained by the real-time PCR to quantify the copy number of the target sequence in each mRNA sample. All groups included individuals 50 years or older. All analyses utilized a one-way ANOVA, followed by Tukey's post-hoc tests to determine whether CDNF mRNA expression decreased in clinical populations of PD compared to healthy, age-matched controls. We included a negative control, stroke patients, to examine whether this reduction would be specific to PD. All analyses used Real-Time PCR experiments performed in triplicates which were averaged prior to analysis.

5.3 RESULTS

5.3.1 CDNF mRNA expression in platelets

To examine CDNF mRNA expression in platelets, experimental groups were composed of n= 15 for normal control (50+) (6 males, 10 females; average age 66.81 years), n= 7 for stroke patients (4 males, 4 females; average age 71.5 years), and n=12 PD patients (9 males, 4 females; average age 72.84 years). Results indicated a difference in CDNF mRNA expression, $F(2,33)= 4.89$, $p < 0.0142$ across experimental groups (**Figure 12a)**. Tukey's post-hoc tests revealed a significant increase in CDNF mRNA expression in PD patients compared to stroke ($*p < 0.05$), but not for and normal controls (50+), *ns*.

Since all samples were not collected at the same time, a two-tailed student's *t*-test was used to compare experimental group means. The sample population was the same as discussed above. When PD was compared to normal controls (50+), results indicated a significant increase in CDNF mRNA expression in PD patients compared to controls, $*p < 0.05$. Similarly, when PD was compared to stroke patients, there was a significant increase in CDNF mRNA expression in PD, $p < 0.05$**.** Lastly, when normal controls (50+) were compared to stroke patients, there was no significant difference in CDNF mRNA expression, *ns*. These results suggest that increased CDNF in platelets is specific to PD (all results reported in **Figure 12b**).

5.3.2 CDNF mRNA expression in lymphocytes

To examine CDNF mRNA expression in lymphocytes, experimental groups were composed of n= 22 for Normal control (50+) (12 males, 10 females; average age 66.59 years), n= 16 for stroke patients (6 males, 8 females; average age 69.64 years), and n=17 PD patients (13 males, 5 females; average age 71.72 years). Results

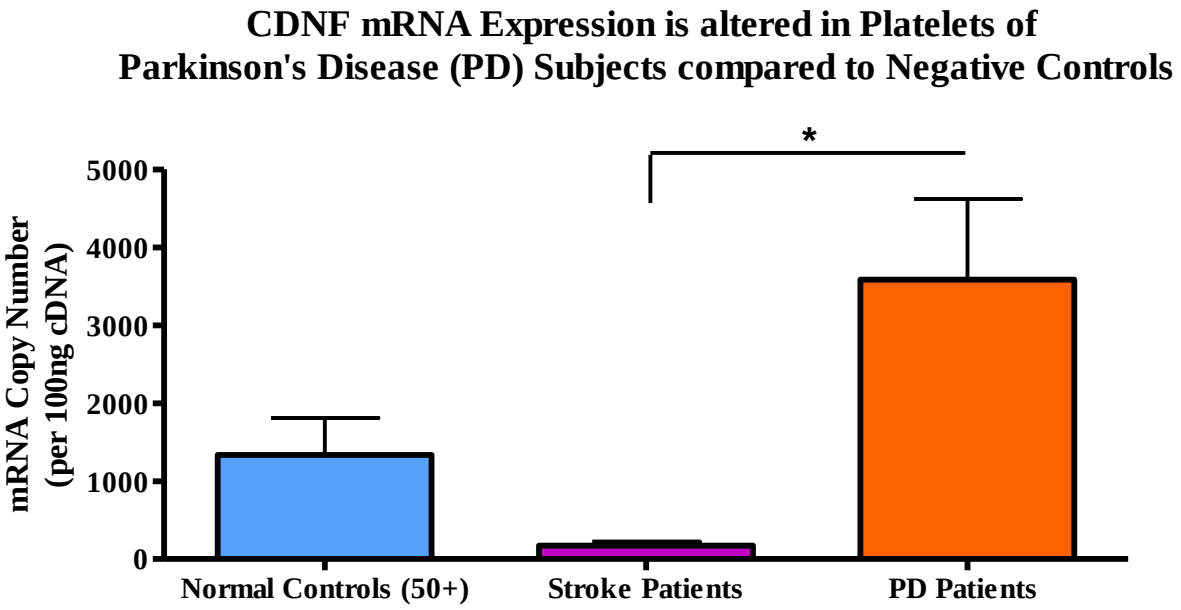

Figure 12a. Results of CDNF mRNA expression in platelets. Graph depicts platelet mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using one-way ANOVA $F(2,33)= 4.89$, $p < 0.0142$, indicating a significant increase in CDNF mRNA expression between PD patients and stroke (*$p < 0.05$), but not for and normal controls (50+), *ns*.).

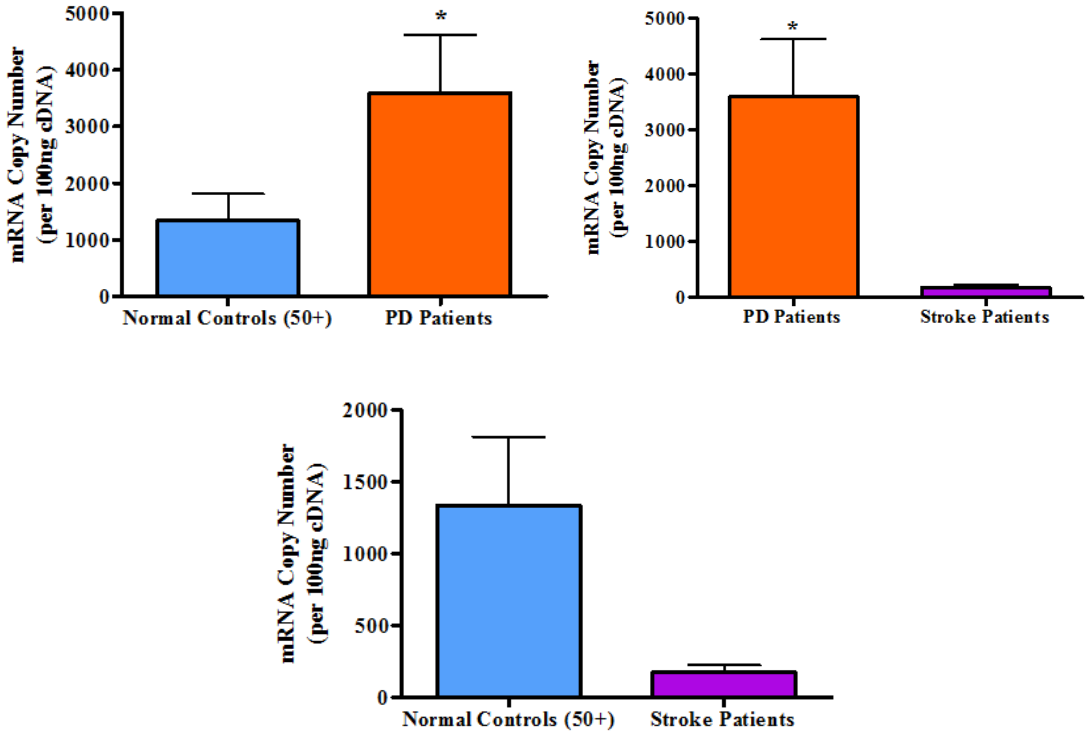

Figure 12b. CDNF mRNA platelet expression is increased in Parkinson's disease. Graph depicts platelet mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed students *t* –test indicating a significant increase in CDNF mRNA expression between PD patients and normal controls >50 (**p* < 0.05), and PD patients compared to stroke patients (**p* < 0.05), but no significant difference between normal controls and stroke patients, *ns*.

indicated no significant difference between experimental groups, *ns* (**Figure13a)**. Also, since patient samples were not collected at the same time, a two-tailed student's *t*-tests was used to compare experimental group means. Results indicated no significant difference for PD vs. normal controls (50+), *ns*, PD vs. stroke patients, *ns*, and stroke patients vs. normal controls, *ns* (**Figure 13b**).

5.3.3 CDNF mRNA expression in whole blood

To examine CDNF mRNA expression with respect to whole blood, experimental groups were composed of n=16 for normal controls (50+) (5 males, 11 females; average age 63 years), n= 15 for stroke patients (7 males, 7 females; average age 71.69 years), and n=7 for PD patients (5 males, 2 females; average age 65.28). Results indicated a significant difference in CDNF mRNA expression, $F(2,37)= 3.45$, $p < 0.0429$ (**Figure 14a)**. Tukey's post-hoc tests revealed a significant decreased in CDNF mRNA expression in stroke patients compared to normal controls, $*p < 0.05$, but not for PD patients, *ns*.

In addition, since patient samples were not collected at the same time, a two-tailed student's *t*-tests were used to compare experimental group means. When PD was compared to normal controls (50+) results indicated no significant different in CDNF mRNA expression, *ns*. When PD patients were compared to stroke patients, results indicated a significant decrease in CDNF mRNA expression in stroke patients, $**p < 0.01$. Similarly, this reduction in stroke patient CDNF mRNA was also seen when stroke patients were compared to normal controls (50+), $*p < 0.05$. Together, these results suggest that in whole blood, CDNF mRNA expression is reduced in stroke patients (all results shown in **Figure 14b**).

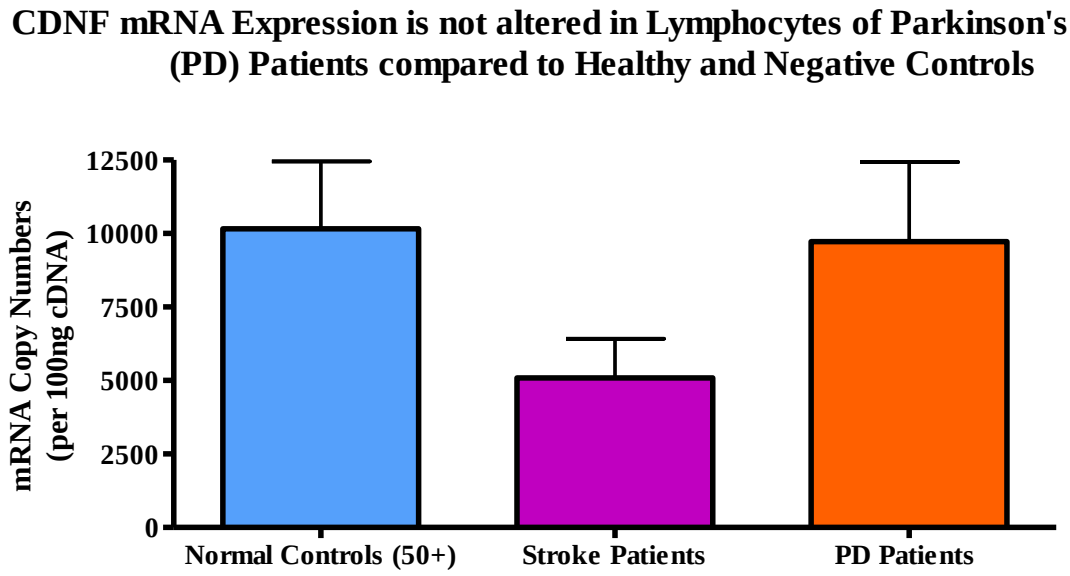

Figure 13a. Results of CDNF mRNA expression in lymphocytes. Graph depicts lymphocyte mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using one-way ANOVA. Results indicated no significant difference between experimental groups, *ns*.

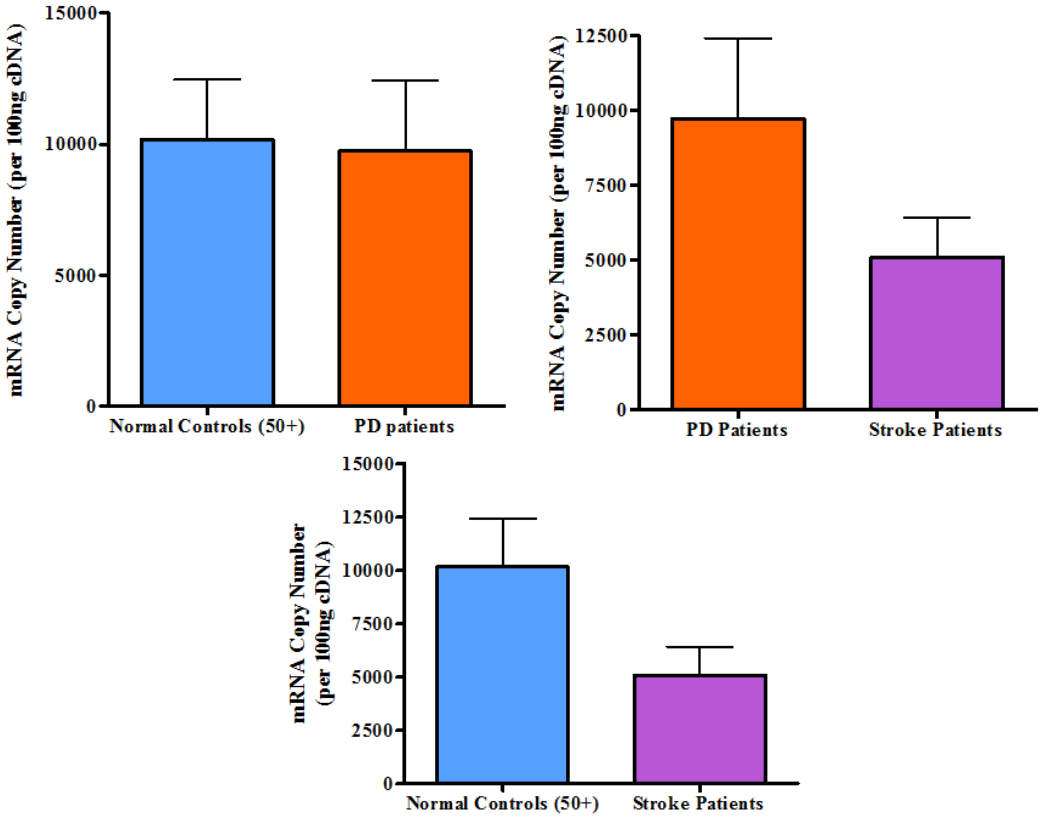

Figure 13b. CDNF mRNA lymphocyte expression is not altered in Parkinson's disease. Graph depicts platelet mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed students *t* –test indicating no significant difference in CDNF mRNA expression between PD patients and normal controls, or stroke patients, *ns*.

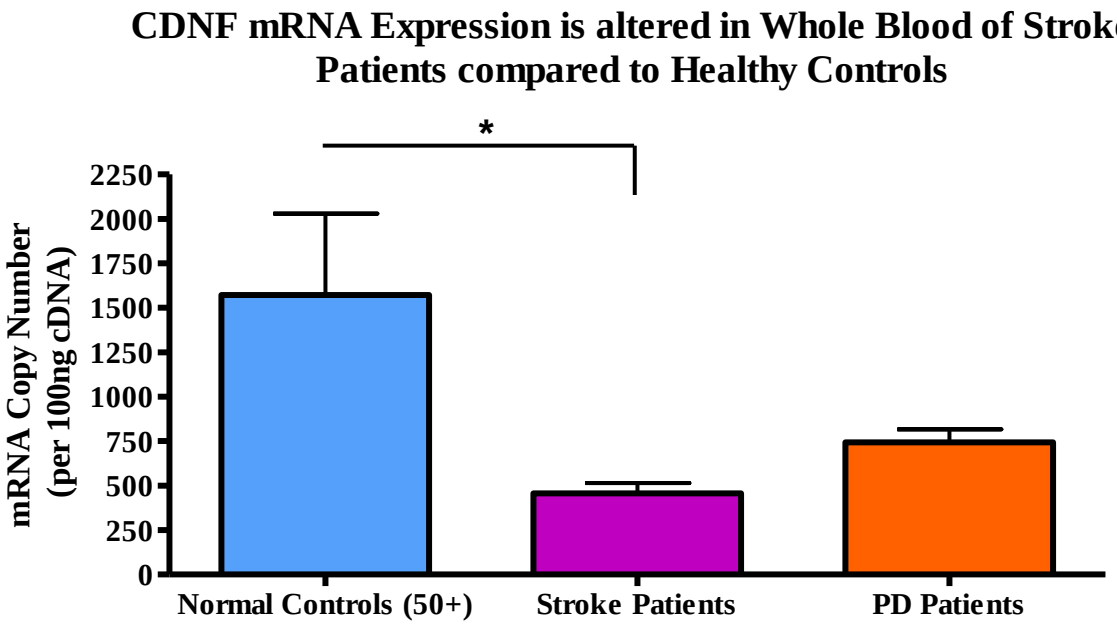

Figure 14a. Results of CDNF mRNA expression in whole blood. Graph depicts whole blood mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using a one-way ANOVA. Results indicated a significant difference in CDNF mRNA expression in stroke patients compared to normal controls, $*p < 0.05$.

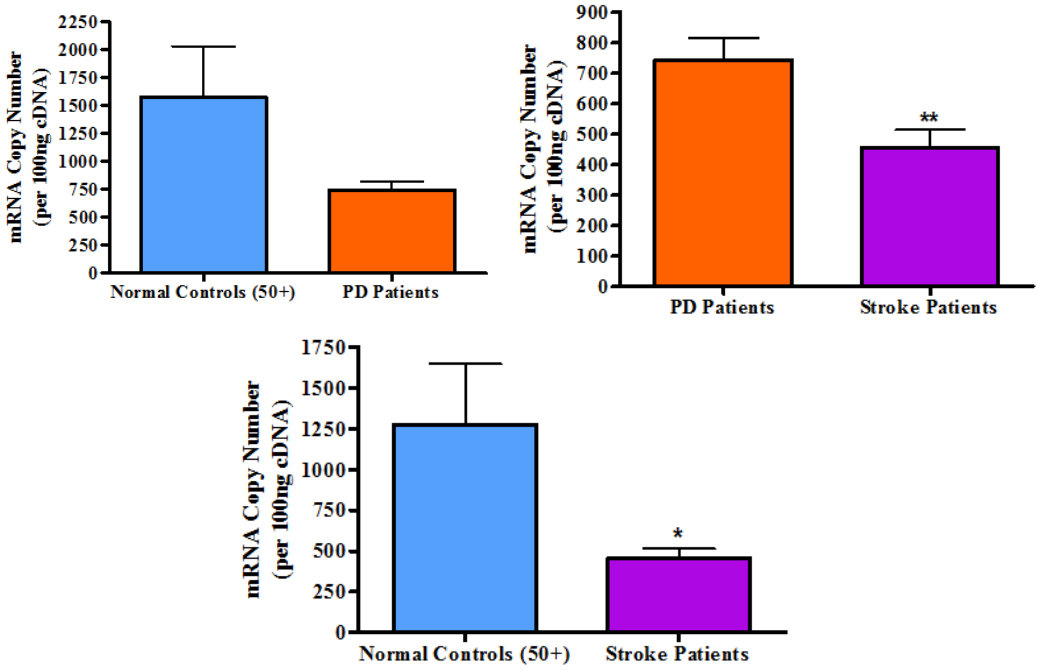

Figure 14b. CDNF mRNA whole blood expression is altered in Stroke patients. Graph depicts whole blood mRNA copy numbers (mean ± SEM). Real-Time PCR experiments were performed in triplicates and results were analyzed using a two-tailed student's *t*-test. Results indicated a significant decrease in CDNF mRNA expression in stroke patients compared to normal controls, $*p < 0.05$, and stroke patients compared to PD, $**p < 0.01$.

5.4 DISCUSSION

It is possible that reductions in CDNF over the course of development may be particularly important in PD, a disorder stemming from dopamine degeneration. While larger studies are awaited to confirm this hypothesis, the examination of CDNF levels in peripheral blood may serve as a viable biomarker in the understanding of the selective dopaminergic degeneration within this disorder. Our previous results discussed in *Section 4.4* determined that CDNF mRNA concentrations decline with age, which prompted the present study examining CDNF mRNA expression in a clinical population of patients with PD. Given the difficulty of attaining post-mortem samples for pathological disorders, blood has become a prime target for biomarker development. The ideal biomarker is easy to quantify, not subject to wide variation within the general population and is unaffected by co-morbid factors (Henley, Bates, & Tabrizi, 2005). For this reason, the feasibility and minimal cost of phlebotomy make blood an attractive candidate. Peripheral blood is comprised of multiple components, thus the present study chose to investigate whole blood, platelets and lymphocytes.

The present study is the first to report significant increases in CDNF mRNA expression in platelets of individuals with PD. Although, these results are contradictory to what was expected, it is possible that due to the oxidative damage accumulated in PD, CDNF is actually recruited, rather than depleted. CDNF is involved in mitigation of ER stress by reducing unfolded or incorrectly folded proteins (Lindholm, et al., 2007) which could explain this increase. Further studies are necessary to confirm this hypothesis.

The more likely explanation for these results lies in anti-parkinsonian treatments. All patients in this study were currently on dopamine agonist treatment; this could also explain the observed increase in expression of CDNF. Treatment with levodopa and other dopamine agonists may cause NTFs such as CDNF to increase in order to promote survival of existing neurons. Following treatment with anti-parkinsonian agents, pramipexole and ropinirole, dopamine D3 receptor agonists, mesencephalic cultures showed increased levels of BDNF and GDNF (Du, Huang, Li, & Le, 2005). It has been suggested that BDNF modulates expression of the D3 receptor, mediating dopamine agonist response (Skoloff, et al., 2006). In addition, following treatment with anti-parkinsonian drugs pergolide, and cabergoline, both D1/D2 agonists, GDNF was rapidly elevated (Onta, Kuno, Mizuta, Fujinami, Matsui, & Ohta, 2003). Since we do not know CDNFs associated receptors, these findings could provide insight into which receptors CDNF activates, causing this increase in CDNF mRNA expression. It would be interesting to see which dopamine agonist drugs cause this increase. Future research should also examine CDNF levels in drug naïve patients to elucidate whether the increase in expression is due pharmacological intervention or the pathological state of PD. This could also be accomplished using dopamine transfected cells treated with dopamine agonists to determine whether CDNF increases. Although studies on lymphocytes have shown that drug treatment did not affect DAT immunoreactivity, this may not be the case for platelets. Platelets have shown the ability to synthesize and transport dopamine which may explain this increase in the periphery (Caronti, et al., 1999; Pellicano, et al., 2007).

Lymphocytes were examined due to their ability to transport dopamine. Our results found no significant alteration in CDNF mRNA expression in lymphocytes,

suggesting platelets may be more relevant to PD. We also examined CDNF mRNA concentrations in whole blood of patients with PD compared to normal age-matched controls and stroke patients. Interestingly, we found a significant reduction in CDNF mRNA concentrations only in stroke patients, suggesting a potential role for CDNF in ischemic attacks in whole blood. Strokes are typically sudden onset and cause a cascade of apoptotic processes, damaging many neurons and regions of the brain (MacManus, Buchan, Hill, Rasquinha, & Preston, 1993). Strokes or ischemic attacks, are commonly used as a model for investigated ER stress (Yu, et al., 2010). Previous studies examining CDNFs paralog, MANF, have shown that pre-treatment with MANF induced functional improvement following induced ER stress in cultures, and a subsequent upregulation of MANF mRNA (Airavaara, et al., 2009). While research has suggested that CDNF is not upregulated following induced ER stress *in vitro*, its role in the pathological ER stress response needs to be further elucidated (Apostolou, Shen, Liang, Luo, & Fang, 2008). CDNF has never been examined in preclinical models of stroke and it is still unclear whether endogenous CDNF is secreted following injury or whether an individual is more susceptible to stroke if CDNF is reduced. An alternative explanation for these results could be that CDNF reacts to ER stress in different manners depending on the onset. PD is an example of progressive ER stress, and gradual loss of dopamine which would allow for neurotrophic support, while stroke is sudden onset, immediate ER stress that may not allow for the neurotrophic activity of CDNF. Although, the present study provides some insight into CDNFs role in stroke, whereby our results suggest a downregulation of CDNF following stroke, future studies should further elucidate the role of CDNF in preclinical models of stroke.

CONCLUSION

PD is a complex, heterogeneous disorder; therefore, understanding its underlying mechanisms is difficult and has hindered the development of efficacious therapies (Blandini et al., 2000). The vast majority of treatments available for PD merely restore motor function temporarily, but do not arrest the progressive nature of the disorder (Parkinson's Disease Society, 2003). Neurotrophic factors have become a huge research topic into PD due to their ability to promote the survival and maintenance of neuronal populations. BDNF and GDNF have received the most attention with respect to PD treatment; however, clinical response has been modest at best (Gill et al., 2003; Null et al., 2003). Both NTFs have shown promising results in preclinical and primate models of PD, but translation to the human clinical population has proven challenging. The size of these proteins and the large distribution of their receptors causes severe side effects and makes crossing the blood-brain barrier impossible (Kirik, Georgievska & Bjorklund, 2004). Therefore, research into novel neurotrophic factors is warranted.

The novel, evolutionarily conserved CDNF, has proven even more efficacious than BDNF and GDNF in restoring and protecting dopamine neurons *in vivo* (Lindholm et al., 2007). This prompted the present study. Endogenous levels of CDNF have not been measured in established animals models of PD which could give insight into the potential mechanism of degeneration of dopamine neurons, due to its specificity for this neuronal population. Our results found that CDNF protein and mRNA expression are not reduced following 6-OHDA lesioning suggesting CDNF may actually be recruited following induced dopaminergic cell death. We attempted to further elucidate the role of CDNF in the etiology of PD using antisense

oligonucleotide knockdown of CDNF. Immunoblotting results showed that the knockdown was not successful, thus future research should pursue this avenue using different means of gene elimination.

CDNF has never been measured in the human population. We found that there is a steady decline in CDNF mRNA expression over the course of development, which lends itself to the idea that decreased NTF support could confer susceptibility to neurodegenerative disease. Additional studies have found that increased CDNF mRNA expression is only seen in platelets, which is contradictory to what was anticipated. The role of CDNF in PD may not be as straight forward as one might have thought initially, and it is important to delineate whether this increase is due to pharmacological intervention or the diseased state. We also determined that CDNF was decreased in stroke patients. Together, these results shed light on the important in investigating the role for CDNF in ER stress which needs to be further elucidated, and could explain the inverse relationship between CDNF in PD and CDNF in stroke.

REFERENCES

Airavaara, M., Harvey,B. K., Voutilainen, M. H., Shen, H., Chou, J., Lindholm, P., … Hoffer, B. (2012). CDNF protects the nigrostriatal dopamine system and promotes recovery after MPTP treatment in mice. *Cell Transplant, 21*(6):1213-1223. doi:10.3727/096368911X600948.

Airavaara, M., Shen, H., Kuo, C., Peranen, J., Saarma, M., Hoffer, B., … Wang, Y. (2009). Mesencephalic astrocyte-derived neurotrophic factor reduces ischemic brain injury and promotes behavioral recovery in rats. *Journal of Comparative Neurology, 515*(1):116-124.

Apostolou, A., Shen, Y., Liang, Y., Luo, J., & Fang, S. (2008). Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. *Experimental Cell Research, 314*(13):2454-2467.

Arias-Carrion, O., Stamelou, M., Murillo-Rodriguez, E., Menendez-Gonzalez, M. & Poppel, E. (2010). Dopaminergic reward system: A short integrative review. International Archives of Medicine, 3(24):1-6.

Aron, L., & Klein, R. (2011). Repairing the parkinsonian brain with neurotrophic factors. *Trends in Neuroscience, 34*(2):88-100. doi: 10.1016/j.tins.2010.11.001

Bartels, A. L., & Leenders, K. L. (2009). Parkinson's disease: The syndrome, the pathogenesis and pathophysiology. *Cortex, 45*:915-921.

Baquet, Z.C., Bickford, P. C., & Jones, K. R. (2005). Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. *The Journal of Neuroscience, 25*(26):6251-6259. doi:10.1523/JNEUROSCI.4601-04.2005

Bauer, S., Kerr, B. J., & Patterson, P. H. (2007). The neuropoietic cytokine family in development, plasticity, disease and injury. *Nature Reviews, 8*:221-232. doi:10.1038/nrn2054

Baumann, C. R. (2012). Epidemiology, diagnosis and differential diagnosis in parkinson's disease tremor. *Parkinsonism and Related Disorders, 1851:*S90-S92.

Behari, M., & Shrivastava, M. (2013). Role of platelets in neurodegenerative disease: a universal pathophysiology. *International Journal of Neuroscience, 123*(5):287-299. doi: 10.3109/00207454.2012.751534.

Belsa, J., Phani, S., Jackson-Lewis, V., & Przedborski, S. (2012). Classic and new animal models of Parkinson's disease. *Journal of Biomedicine and Biochemistry*.

Bennett, D. L. H., Boucher, T. J., Armanini, M. P., Poulsen, K. T., Micheal, G. J., Priestley, J. V.,…Shelton, D. L. (2000). The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. *The Journal of Neuroscience, 20*(1):427-437.

Bhandary,B., Marahatta,A., Kim,H.R. & Chae,H.J.(2012). An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *Int. J. Mol. Sci. 14*, 434-456.

Blandini, F., Nappi, G., Tassorelli, C., & Martignoni, E. (2000). Functional changes of the basal ganglia circuitry in parkinson's disease. *Progress in Neurobiology, 62*:63-88.

Braak, H., Rub, U., & Tredici, K. D. (2006). Cognitive decline correlates with neuropathological stage in parkinson's disease. *Journal of the Neurological Sciences, 248*:255-258. doi: 10.1016/j.jns.2006.05.011.

Caronti, B., Antonini, G., Calderaro, C., Ruggieri, S., Palladini, G., Pontieri, F. E., …Colosimo, C. (2001). Dopamine transporter immunoreactivity in peripheral blood lymphocytes in parkinson's disease. *Journal of Neural Transmission, 108*:803-807.

Caronti, B., Tanda, G., Colosimo, C., Ruggieri, S., Calderaro, C., Pallandini, G., …Di Chiara, C. (1999). Reduced dopamine in peripheral blood lymphocytes in parkinson's disease. *NeuroReport, 10*(4)*:*2901-2910.

Chauhan, N. B., Siegel, G. J., & Lee, J. M. (2001). Depletion of glial cell line-derived neurotrophic factor in substantia nigra neurons of parkinson's disease brain. *Journal of Chemical Neuroanatomy, 21*(4):277-288. doi: 10.1016/S0891-0618(01)00115-6

Ciesielska, A., Mittermeyer, G., Hadaczek, P., Kells, A. P., Forsayeth, J., & Bankiewicz, K. S. (2011). Anterograde axonal transport of AAV2-GDNF in rat basal ganglia. *Molecular Therapy, 19*(5):922-927.

Collier, T. J., Ling, Z. D., Carvey, P. M., Fletcher-Turner, A., Yurek, D. M., Sladek, J. R.,… Kordower, J. H. (2005). Striatal trophic factor activity in aging monkeys with unilateral MPTP-induced parkinsonism. *Journal of Experimental Neurology, 191*:S60-S67. doi:10.1016/jexpneurol.2004.08.018.

Coy, A. F., Balaji, B., Kim, D., & Yu, X. (2011). Deep Brain Stimulation.

Da Prada, M., Cesura, A. M., Launay, J. M., & Richards, J. G. (1988). Platelets as a model for neurons. *Experientia, 44*:115-126.

Dauer,W. & Przedborski,S. (2003). Parkinson's disease: mechanisms and models. *Neuron 39*, 889-909.

De Lau, L. M. L., & Breteler, M. M. B. (2006). Epidemiology of parkinson's disease. *Lancet Neurology, 5:*525-35.

Dias, N., & Stein, C. A. (2002). Antisense oligonucleotides: Basic concepts and mechanisms, *Molecular Cancer Therapy, 1*:347-355.

Dluzen, D. E., Story, G. M., Xu, K., Kucera, J., & Walro, J. M. (1999). Alterations in nigrostriatal dopaminergic function within BDNF mutant mice. *Experimental Neurology, 160*(2):500-507.

Dodel, R. (2011). Interpreting health economic data in parkinson's disease. *European Neurological Review Supplment*, p 13-16.

Du, F., Li, R., Huang, Y., Li, X. and Le, W. (2005), Dopamine D3 receptor-preferring agonists induce neurotrophic effects on mesencephalic dopamine neurons. *European Journal of Neuroscience, 22*: 2422–2430. doi: 10.1111/j.1460-9568.2005.04438.x

Dyck, B. A., Beyaert, M. G. R., Ferro, M. A., & Mishra, R. K. (2011). Medial prefrontal cortical synapsin II knock-down induces behavioral abnormalities in the rat: Examining synapsin II in the pathophysiology of schizophrenia. *Schizophrenia Research, 130*(1-3):250-259. Doi: 10.1016/j.schres.2011.05.017

Enciu, A. M., Nicolescu, M., Manole, C.G., Muresanu, D.F., Popescu, L. M., & Popescu, B. O. (2011). Neuroregeneration in neurodegenerative disorders. *BioMed Central, 11*(75):1-7.

Evans, J. R., & Barker, R. A. (2008). Neurotophic factors as a therapeutic target for parkinson's disease. *Central & Peripheral Nervous System, 12*(4):437-447.

Floyd, R. A., &Hensley, K. (2002). Oxidative stress in brain aging: Implications for therapeutics of neurodegenerative disease. *Neurobiology of Aging, 23*(5):795-807.

Frankhauser, P., Grimmer, Y., Burgert, P., Deuschle, M., Schmidt, M., & Schloss, P. (2006). Characterization of the neuronal dopamine transporter DAT in human blood platelets. *Neuroscience Letters, 399*:197-201. doi:10.1016/j.neulet.2006.01.062.

Gash, D. M., Zhang, Z., Ovadia, A., Cass, W. A., Yi, A., Simmerman, L.,…Gerhardt, G. A. (1996). Functional recovery in parkinsonian monkeys treated with GDNF. *Letters to Nature, 380*(21):252-256.

Gazewood, J. D., Richards, D. R., & Clebak, K. (2013). Parkinson's disease: An update. *American Family Physician, 87*(4):267-273.

Gill, S. S., Patel, N. K., Hotton, G. R. O'Sullivan, K., McCarter, R., Bunnage, M., … Heywood, P. (2003). Direct brain infusion of glial cell line–derived neurotrophic factor in parkinson disease. *Nature Medicine, 9*(5):589-595. doi:10.1038/nm850

Groleau, S. E., Lubarda, J., Thomas, N., Ferro, M. A., Pristupa, Z., Mishra, R. K., & Gabriele, J. P. (2013). Human blood analysis reveals differences in gene expression of catecholamine-regulated protein 40 (CRP40) in schizophrenia. *Schizophrenia Research, 143*(1):203-206. doi: 10.1016/j.schres.2012.10.034

Henley, S., Bates, Gillian, & Tabrizi, S. J. (2005). Biomarkers for neurodegenerative diseases. *Degenerative and Cognitive Disease, 18*(6):698-705.

Heuer, A., Smith, G. A., Lelos, M. J., Lane, E. L., & Dunnett, S. B. (2012). Unilateral nigrostriatal 6-hydroxydopamine lesions in mice I: Motor impairments identify

extent of dopamine depletion at three different lesion sites. *Behavioral Brain Research, 228*(1):30-43. Doi: 10.1016/j.bbr.2011.11.027

Hindle, J. V. (2010). Ageing, neurodegeneration and parkinson's disease. *Age and Ageing, 39*:156-161. doi: 10.1093/ageing/afp223.

Hirsch, E., & Hunot, S. (2009). Neuroinflammation in parkinson's disease: A target for neuroprotection. *The Lancet Neurology, 8*(4):382-397. doi: 10.1016/S1474-4422(09)70062-6

Hoffer, B. J. (2011). Commentary on chronic infusion of CDNF prevents 6-OHDA-induced deficits in a rat model of Parkinson's disease. Merja H. Voutilainen et al. *Journal of Experimental Neurology, 230:*162-166. doi:10.1016/j.expneurol.2011.04.015.

Howells, D. W., Porritt, M. J., Wong, J. Y. F., Batchelor, P.E., Kalnins, R., Hughes, A. J.,… Donnan, G. A. (2000). Reduced BDNF mRNA expression in the parkinson's disease substantia nigra. *Journal of Experimental Neurology, 166*:127-135. doi:10.1006/exnr.2000.7483

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: Role in neuronal development and function. *Annual Reviews in Neuroscience, 24*:677-736.

Hurtig,H.I., Trojanowski, J. Q., Galvin, J., Ewbank, D., Schmidt, M. L., Lee, M. Y., ... Arnold, S.E. (2000). Alpha-synuclein cortical Lewy bodies correlate with dementia in Parkinson's disease. *Neurology 54*, 1916-1921.

Jankovic, J. (2011). Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry, 79,* 368-376. doi:10.1136/jnnp.2007.131045

Kim, I., Xu, W., & Reed, J. C. (2008). Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nature Reviews, 7*:1013-1030.

Kirik, D., Georgievska, B., & Bjorkland, A. (2004). Localized striatal delivery of GDNF as a treatment for parkinson's disease. *Nature Neuroscience, 7*(2):105-110.

Kishima, H., Poyot, T., Bloch, J., Dauguet, J., Conde, F., Dolle, F., ... Hantraye, P. (2004). Encapsulated GDNF-producing C2C12 cells for parkinson's disease: A pre-clinical study in chronic MPTP-treated baboons. *Neurobiology of Disease, 16*(2):428-439. doi: 10.1016/j.nbd.2004.03.012

Kordower, J. H., & Bjorklund, A. (2013). Trophic factor gene therapy for parkinson's disease. *Movement Disorders, 28*(1):96-109. doi: 10.1002/mds.25344

Kovacs, G.G., Adle-Biassette, H., Milenkovic, I., Cipriani, S., Van Scheppingenm, J., & Aronica, E. (2014). Linking pathways in the developing and aging brain with neurodegeneration. *Neuroscience, 269*(6):152-172. DOI: 10.1016/j.neuroscience.2014.03.045

Lang, A. E., & Lozana, A. M. (1998). Parkinson's disease. *The New England Journal of Medicine, 339*(15):1044-1053.

Lang, A. E., Gill, S., Patel, N. K., Lozano,A., Nutt, J. G., Penn, R., …Traub, M.(2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in parkinson's disease. *Annals of Neurology, 59*(3)*:*459-466.

Lang, A. E., & Obesco, J. A. (2004). Challenges in parkinson's disease: Restoration of the nigrostriatal dopamine system is not enough. *The Lancet Neurology, 3*(5):309-316. doi: 10.1016/S1474-4422(04)00740-9

Latge, C., Cabral, K. M. S., Almeida, M. S., & Foguel, D. (2013). ^{1}H-, ^{13}C- and ^{15}N-NMR assignment of the N-terminal domain of human cerebral dopamine neurotrophic factor (CDNF). *Biomolecular NMR Assignments, 7*(1):101-103.

Lebedeva, R., & Stein, C. A. (2001). Antisense oligonucleotides: Promise and Reality. *Annual Review of Pharmacology and Toxicity, 41*:403-419. doi: 10.1146/annurev.pharmtox.41.1.403

Levy, G. (2007). The relationship of Parkinson disease with aging. *Archives of Neurology, 64*(9):1242-1246.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature, 443*(19):787-795. doi:10.1038/nature05292

Lindholm, D., Wootz, H., & Korhonen, L. (2006). ER stress and neurodegenerative diseases. *Cell Death and Differentiation, 13*:385-392. doi:10.1038/sj.cdd.4401778

Lindholm, P., & Saarma, M. (2009). Novel CDNF/MANF family of neurotrophic factors. *Developmental Neurobiology, 70:*360-371. doi: 10.1002/dneu.20760.

Lindholm, P., Voutilainen, M. H., Lauren, J., Peranen, J., Lappanen, V. M., Andressoo, J. O.,…Saarma, M. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons *in vivo*. *Nature, 448*(5):73-78. doi: 10.1038/nature05957.

Mateus, C.,& Coloma, J. (2013). Health and cost of illness in parkinson's disease. *European Neurological Review, 8*(1):6-9.

Mattson, M. P., & Magnus, T.(2006). Ageing and neuronal vulnerability. *Nature Reviews, 7:*278-294. doi: 10.1038.nrn1886

MacManus, J. P., Buchan, A. M., Hill, I. E., Rasquinha, I., & Preston, E. (1993). Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neuroscience letters*, *164*(1), 89-92.

Mei, J. M., & Niu, C. S. (2014). Effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells via modulation of bcl-2/bax and caspase-3 activation. *Neurological Science.*

Michell, A. W., Lewis, S. J. G., Foltynie, T., & Barker, R. A. (2004). Biomarkers and parkinson's disease. *Brain, 127*(8):1693-1705. doi: 10.1093/brain/awh198.

Milbrandt, J., de Sauvage, F.J., Fahmer, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., … Johnson Jr., E. M. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron, 20*:245-253.

Mora, F., Segovia, G., & del Arco, A. (2007). Aging, plasticity and environmental enrichment: Structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Research Reviews, 55*:78-88. doi: 10.1016/j.brainresrev.2007.03.011.

Nutt, J. G., & Wooten, G. F. (2005). Diagnosis and initial management of Parkinson's disease. *The New England Journal of Medicine, 353*:1021-1027.

Nutt, J. G., Burchiel, K. J., Comella, C. L., Jankovic, J. Lang, A. E., Laws,E. R.,… ICV GDNF Study Group. (2003). Randomized, double-blind trial of glial cell-line-derived neurotrophic factor (GDNF) in PD. *Neurology, 60:*69-74.

Ohta, K., Kuno, S., Mizuta, I., Fujinami, A., Matsui, H., & Ohta, M. (2003). Effects of dopamine agonists bromocriptine, pergolide, cabergoline, and SKF-38393 on GDNF, NGF, and BDNF synthesis in cultured mouse astrocytes. *Life sciences*, *73*(5), 617-626.

Obesco, J. A., Rodriguez-Oroz, M. C., Rodriguez, M., Lanciego, J. L., Artieda, J., Gonzalo, N., ... Olanow, C.W. (2000). Pathophysiology of the basal ganglia in parkinson's disease. *Trends in Neuroscience, 23*(Suppl.):S8-S19.

Parkash, V., Lindholm, P., Peranen, J., Kalkkinen, N., Oksanen, E., Saarma, M., … Goldman, A. (2009). The structure of the conserved neurotrophic factors MANF and CDNF explains why they are bifunctional. *Protein Engineering Design & Selection, 22*(4):233-241. doi: 10.1093/protein/gzn080.

Parkinson's Society Canada. (2003). Parkinson's disease: Social and Economic impact. Pg 1-6.

Pascual, A., Hidalgo-Fiqueroa, M., Piruatm J. I., Pintado, C. O., Gomez-Diaz, R., & Lopez-Barneo, J. (2008). Absolute requirement of GDNF for adult catecholaminergic neuron survival. *Nature Neuroscience, 11*:755-761.

Pellicano, C., Buttarelli, F.R., Circella, A., Tiple, D., Giovannelli, M., Benincasa, D., … Pontieri, F. E. (2007). Dopamine transporter immunoreactivity in peripheral blood lymphocytes discriminates Parkinson's disease from essential tremor. *Journal of Neural Transmission,114*:935-938.

Plein, H., & Berk, M. (2001). The platelet as a peripheral marker in psychiatric illness. *Human Psychopharmacology, 16*:229-236.

Porritt, M. J., Batchelor, P. E., & Howells, D. W. (2005). Inhibiting BDNF expression by antisense oligonucleotide infusion causes loss of nigral dopaminergic neurons. *Experimental Neurology, 192*(1):226-234. doi: 10.1016/j.expneurol.2004.11.030

Pringsheim, T., Jette, N., Frokis, A., & Steeves, T. D. L. (2014). The prevalence of parkinson's disease: A systematic review and meta-analysis. *Movement Disorders, 00*(00):1-8. doi: 10.1002/mds.25945

Ricci, A., Bronzetti, E., Mannino, F., Mignini, F., Morosetto, C., Tayebati, S. K.,... Amenta, F. (2001). Dopamine receptors in human platelets. *Naunyn-Schmiedeberg's Archives of Pharmacology, 363*(4):376-382.

Ryu, E. J., Harding, H. P., Angelastro, J. M., Vitolo, O. V., Ron, D., & Greene, L. A. (2002). Endoplasmic reticulum stress and the unfolded protein response in cellular models of parkinson's disease. *The Journal of Neuroscience, 22*(24):10690-10698.

Saracchi, E., Fermi, S., & Brighina, L. (2014). Emerging candidate biomarkers for parkinson's disease: A review. *Aging and Disease, 5*(1): 27-34.

Schapira, A. H. V. (2008). Neurobiology and treatment of parkinson's disease. *Trends in Pharmacological Sciences, 30*(1):41-47.

Scherzer, C.R., Eklund, A. C., Morse, L. J., Liao, Z., Locascio, J. J., Fefer, D., … Gullans, S. R. (2006). Molecular markers of early Parkinson's disease based on gene expression in blood. *PNAS, 104*(3):955-960.

Schober, A. (2004). Classic toxin-induced animal models of parkinson's disease:6-OHDA and MPTP. *Cell Tissue Research, 318*:215-224. doi: 10.1007/s00441- 004-0938-y

Selkoe, D. J. (2004). Cell biology of protein misfolding:The examples of alzheimer's and parkinson's diseases. *Nature Cell Biology, 6*(11):1054-1061.

Siderowf, A. (2001). Parkinson's disease: Clinical features, epidemiology, and genetics. *Movement Disorders, 19*(3):565-578.

Siegel, G. J., & Chauhan, N. B. (2000). Neurotrophic factors in alzheimer's and parkinson's disease brain. *Brain Research Reviews, 33*:199-227.

Sokoloff, P., Diaz, J., Foll, B. L., Guillin, O., Leriche, L., Bezard, E., & Gross, C. (2006). The dopamine D3 receptor: a therapeutic target for the treatment of neuropsychiatric disorders. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders), 5*(1), 25-43.

Sullivan, P. F., Fan, C.,& Perou, C. M. (2006). Evaluating the comparability of gene expression in blood and brain. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics):141B:*261-268. doi 10.1002/ajmg.b.30272

Tolosa, E., Wenning, G., & Poewe, W. (2006). The diagnosis of Parkinson's disease. *Lancet Neurology, 5*:75-86.

Twelves, D., Perkins, K. S. M., & Counsell, C. (2003).Systematic review of incidence studies of parkinson's disease. *Movement Disorders, 18*(1):19-31.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., … Nelson, L. M. (2003). Incidence of parkinson's disease: Variation by age, gender, and race/ethnicity. *American Journal of Epidemiology, 157*(11):1015-1022.
Doi: 10.1093/aje/kwg068

Ventriglia, M., Zanardini, R., Bonomini, C., Zanetti, O., Volpe, D., Pasqualetti, P.,…Chlavetto., L. B. (2013). Serum brain-derived neurotrophic factor levels in different neurological diseases. *BioMed Research International, p 1-7.*

Voutilainen, M. H., Back, S., Peranen, J., Lindholm, P., Raasmaja, A., Mannisto, M.,…Touminen, R. K. (2011). Chronic infusion of CDNF prevents 6-OHDA-

induced deficits in a rat model of Parkinson's disease. *Journal of Experimental Neurology, 228*:99-108.doi: 10.1016/j.expneurol.2010.12.2013.

Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hokfelt, T., … Wengel, J. (2000). Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *PNAS, 97*(10):5633-5638.

Wang, M. S., Boddapati, S., Emandi, S., & Sierks, M. R. (2010). Curcumin reduces α-synuclein induced cytotoxicity in parkinson's disease cell model. *BMC Neuroscience, 11*(57):1-10. doi:10.1186/1471-2202-11-57

Westerlund, M., Hoffer, B., & Olson, L. (2010). Parkinson's disease: Exit toxins, enter genetics. *Progress in Neurobiology, 90*(2):146-156. DOI: 10.1016/j.pneurobio.2009.11.001

Yang, S., Huang, S., Gaertig, M. A., Li, X. J, & Li, S. (2014). Age-dependent decrease in chaperone activity impairs MANF expression, leading to purkinje cell degeneration in inducible SCA17 mice. *Neuron, 81*(2):349-365. doi: 10.1016/j.neuron.2013.12.002.

Yoshimoto, Y., Lin, Q., Collier, T.J., Frim, D. M., Breakefield, X. O., & Bohn, M. C. (1995). Astrocytes retrovirally transduced with BDNF elciti behavioural improvement in rat model of parkinson's disease. *Brain Research, 691*(1-2):25-36. doi: 10.1016/0006-8993(95)00596-I

Yu, Y., Liu, L., Wang, F., Liang, Y., Cha, D., Zhang, J., … Shen, Y. (2010). Induction profile of MANF/ARMET by cerebral ischemia and its implication for neuron protection. *Journal of Cerebral Blood Flow & Mechanism, 30*:79-91.

Yu, K., Wu, Y., Hu, Y., Zhang, Q., Xie, H., Liu, G., … Jia, J. (2013). Neuroprotective effects of prior exposure to enriched environment on cerebral

ischemia/reperfusion injury in rats: The possible molecular mechanism. Brain Research, http://dx.doi.org/10.1016/j. brainres.2013.09.036.

Yurek, D. M., & Fletcher-Turner, A. (2001). Differential expression of GDNF, BDNF, and NT-3in the aging nigrostriatal system following a neurotoxic lesion. *Brain Research, 891:*228-235.

Zhao, H., Cheng, L., Liu, Y., Zhang, W., Mahaarjan, S., Cui, Z.,… Nie, L. (2014). Mechanisms of anti-inflammatory property of conserved dopamine neurotrophic factor: Inhibition of JNK signaling in lipopolysaccharide-induced microglia. *Journal of Molecular Neuroscience, 52*:186-192. doi: 10.1007/s12031-013-0120-7.