INVESTIGATING THE ROLE OF CDNF IN PARKINSON'S DISEASE

INVESTIGATING THE ROLE OF CEREBRAL DOPAMINE NEUROTROPHIC FACTOR (CDNF) IN PARKINSON'S DISEASE

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder primarily affecting the aging population over the age of sixty. Characterized by the significant degeneration of dopaminergic (DAergic) neurons of the substantia nigra causing severe motor dysfunction. Although the exact pathogenesis of this disease is still unknown endoplasmic reticulum stress and mitochondrial dysfunction are believed to play a role. PD is diagnosed after severe DAergic neuron degeneration, and yet is still often misdiagnosed. There is a need for a definitive diagnostic test for the early detection of PD. Current therapies only relieve symptoms and do not stop disease progression. Neurotrophic factors (NTF) are naturally occurring proteins that promote the survival, differentiation and maintenance of neurons and present a promising candidate for the treatment of PD. Cerebral dopamine neurotrophic factor (CDNF) is a novel NTF that protects and rescues DAergic neurons. The present study investigated the role of DAergic activity and CDNF mRNA expression in *C. elegans,* as well as understanding how does PD affect the endogenous levels of CDNF protein and mRNA expression.

We demonstrated that of the various dopamine (DA) synthesis and transport mutants tested, the impaired synthesis of DA from levodopa is linked to the up regulation of CDNF. Also, following unilateral 6-hydroxydopamine (6-OHDA) lesioning protein and mRNA expression of CDNF was not affected implicating ER stress as inducing a possible compensatory up regulation of CDNF, thus returning

levels to normal. CDNF mRNA expression was determined to decline with age and possibly increase ones vulnerability to developing a neurodegenerative disorder. An increase mRNA expression of CDNF in the PD patient population was found to be specific to platelets. Stroke patients showed an increase in CDNF expression in whole blood. In conclusion, these findings highlight the importance of the relationship between CDNF and ER stress and warrants further investigation.

"And He has bestowed upon you all that you asked for;

And were you to count the blessings of Allah,

you would not be able to list them…"

Q. 14:34

For Ami and Daddy

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ABBREVIATIONS

6-OHDA	6-Hydroxydopamine
AADC	Aromatic amino acid decarboxylase
aCSF	Artificial cerebrospinal fluid
APS	ammonium persulfate
AS	Antisense
BDNF	Brain derived neurotrophic factor
C. elegans	*Caenorhabditis elegans*
CAF	Central animal facility
CDNF	Cerebral dopamine neurotrophic factor
CNS	Central nervous system
DA	Dopamine
Daergic	Dopaminergic
DAT	Dopamine transporter
DHBA	2,3-Dihydroxybenzylamine hydrobromide
ECD	Electrochemical detection
ER	Endoplasmic reticulum
ET	Essential tremor
GABA	γ-Aminobutyric acid
GDNF	Glial cell line derived neurotrophic factor
GPe	Globus pallidus externus
GPi	Globus pallidus internus
HPLC	High performance liquid chromatography
L-DOPA	L--3,4-dihydroxyphenylalanine
MANF	Mesencephalic astrocyte derived neurotrophic factor
MeOH	Methanol
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NaOH	Sodium hydroxide
NTF	Neurotrophic factor
ODN	oligodeoxynucleotide
PC12	Pheochromocytoma
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEB	PBS-EDTA-bovine serum albumin
PMC	Primary motor cortex
PRP	Platelet-rich plasma
PVDF	Plyvinylidene difluoride
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SQ	Subcutaneous
STN	Subthalamic nucleus
TH+	Tyrosine hydroxylase positive
UPDRS	Unified Parkinson's Disease Rating Scale
UPR	Unfolded protein response
VMAT	Vesicular monoamine transporter
α-syn	alpha-synuclein

Chapter 1

Introduction to Parkinson's Disease

1.1 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 1% of the global population over the age of 60 (Connolly & Lang, 2014). PD is known to affect all ethnic groups equally; approximately 1 million Americans, over 100,000 Canadians and roughly 6.3 million people globally are affected with PD (Connolly & Lang, 2014; Dehay & Bezard, 2011). Current demographic trends suggest these numbers will double by the year 2050, making neurodegenerative diseases (PD, dementia, and motor neuron disease) the second most common cause of death among the elderly behind heart disease (Dehay & Bezard, 2011; Schapira, 2009). The economic burden of PD, in Canada alone, is estimated to be more than $1.5 billion annually in treatment expense and loss of workplace productivity (Huse et al., 2005).

The estimated prevalence of PD in Canada is 100 to 200 per 100 000 population where the incidence rate for a population of 100 000 is 10 to 20 new diagnosis yearly (Inzelberg, Nisipeanu & Schechtman, 2002). Early onset of PD is rare, with only 4% of all PD patients developing clinical signs of the disease before the age of 50. Beyond 50 years of age, the incidence rate in the population increases to 1% over the age of 60 in all individuals, after which the rate further increases to 3-5% in people 85 years and older (Alves, Forsaa, Pedersen, Gjerstad & Larsen, 2008).

Despite extensive efforts to understand the mechanisms underlying PD, much is still unknown (Sharma et al., 2013). As a result, there is no cure or proven strategy

to slow down the progressive nature of this disease. Therapies presently available only temporarily ameliorate the symptoms and are not effective long term (Sharma et al., 2013; Sullivan & Toulouse, 2011). As worldwide life expectancy increases there is a desperate need to better understand this neurodegenerative disease and develop therapies that will halt the neurodegenerative process (Sullivan & Toulouse, 2011).

1.1.1 Symptomatology

PD was originally described in the classical work, “An Essay of Shaking Palsy”, in 1817 by the British physician, James Parkinson. He described six individuals who he had observed while walking along the streets of London. He described this condition as having "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported, with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellect being uninjured" (Parkinson, 2002). He mentioned that the disease was of prolonged duration and he believed that trauma to the cervical spine and toxins, as a result of environmental pollution in London from the industrial revolution, may have a causative role in the development of this condition (Parkinson, 2002). Decades of research have since taken place, and yet the exact etiology remains unknown. PD is a progressive neurodegenerative disorder with asymmetrical onset and is predominantly characterized by motor symptoms, although non-motor symptoms do occur as well (Brakedal, Tysnes, Skeie, Larsen & Muller, 2014; Hou & Lai, 2007). Non-motor symptoms, such as cognitive, neuropsychiatric and sleep complications,

are common and can significantly interfere with a patients quality of life (Hou & Lai, 2007). Other non-motor symptoms that significantly add to the disability caused by PD include drooling, difficulty swallowing, depression, anxiety, and dementia (Connolly & Lang, 2014). Cardinal motor symptoms of PD include resting tremor, bradykinesia, postural instability, rigidity and an inability to initiate movement.

Although each individual with PD is unique and may experience different symptoms, some are quite common amongst all individuals. Resting tremor may be the first symptom of PD noticed by the patient. The tremors, as the name suggests, occur at rest, but tend to disappear with the initiation of voluntary movements, as such, they are socially bothersome but are not disabling (Pare, Curro'Dossi & Steriade, 1990). Small handwriting, loss of smell and trouble sleeping are also common in the early stages (Haehnera et al., 2009). Due to the progressive nature of the disease, symptoms worsen over time. The patient may find that it takes more time or effort to perform daily activities such as getting dressed in the morning. Bradykinesia, the slowness of movements while performing activities of daily living, may cause significant impairments to the patient's quality of life (Berardelli, Rothwell, Thompson& Hallett, 2001). At this stage of advancing disability in the disorder may require family or home care workers to help one manage the daily challenges of living with PD. Postural instability occurs later in the disease progression, and involves the slowing of walking speed, shorter stride lengths, stooped posture and the loss of normal postural reflexes that can lead to falls (Bloem, van Vugt & Beckley, 2001). Rigidity may not be obvious in the initial stages

of PD, but later on, the patient may experience stiffness associated with aching and discomfort of the limbs. For instance, the individual may experience difficulty moving their neck side to side or experience a frozen shoulder (Nutt & Wooten, 2005). As the disease progresses and the patients activities of daily living have already deteriorated substantially the individual may also experience akinesia, the inability to begin voluntary movements such as walking, and describe themselves as "stuck" to the ground when they attempt to move (Dauer & Przedborski, 2003). The motor symptoms of PD typically arise form the degeneration of the DA system (Sullivan & Toulouse, 2011).

1.1.2 Pathophysiology

The neuropathological hallmarks of PD include the presence of Lewy bodies and the significant loss of dopamine (DA) neurons in substantia nigra (SN) (Liuqing et al., 2013). Lewy bodies are cytoplasmic protein alpha-synuclein (α-syn) aggregates in surviving SN neurons which confirm the diagnosis of PD during post-mortem autopsy (Calabresi, Castrioto, Di Filippo, & Picconi, 2013). The cardinal motor symptoms of PD stem from the loss of DAergic neurons in the nigrostriatal pathway; these neurons project from the SN in the midbrain, to the striatum (Sullivan & Toulouse, 2011). Protein misfolding and aggregation is one major hypothesis for the pathogenesis of PD and the another proposes mitochondrial dysfunction and oxidative stress, both of these proposals lead to the death of DAergic neurons of the SN (Dauer & Przedborski, 2003).

1.1.3 Nigrostriatal Pathway

As mentioned previously, the most consistent finding with respect to PD is the progressive loss of dopamine within the nigrostriatal system. The severe degeneration of DAergic neurons in the SN of the ventral midbrain area causes a significant reduction of DA in the striatum (Liuqing et al., 2013; More et al., 2013). Unfortunately, the clinical features of PD only start to become clinically apparent once 60-70% of DA producing neurons have already degenerated within this pathway (Cheng, Ulane, & Burke, 2010). The nigrostriatal pathway is part of the basal ganglia motor loop, a dopaminergic (DAergic) pathway that originates in the SN sending axonal projections to the striatum. The basal ganglia is responsible for generating movement and the processing of sensory-motor information. Disruptions in this system produce motor impairments typically seen in PD (Kandel, Schwartz & Jessell, 2000).

The basal ganglia is composed of four principal nuclei: the striatum (consisting of the caudate and the putamen), the globus pallidus, the subthalamic nucleus (STN) and the SN. The globus pallidus has two parts, the globus pallidus internus (GPi) and globus pallidus externus (GPe). The SN is also divided into two parts: the pars compacta (SNpc) and pars reticulata (SNr)(**Figure 1**)(Kandel et al., 2000).

The striatum is a major recipient of inputs to the basal ganglia from the cerebral cortex, thalamus and brain stem. The striatum projects to the two major output nuclei of the basal ganglia: the GPi and the SNr. Both outputs inhibit their

target nuclei in the thalamus and brain stem via direct and indirect pathways (**Figure 2**)(Kandel et al., 2000).

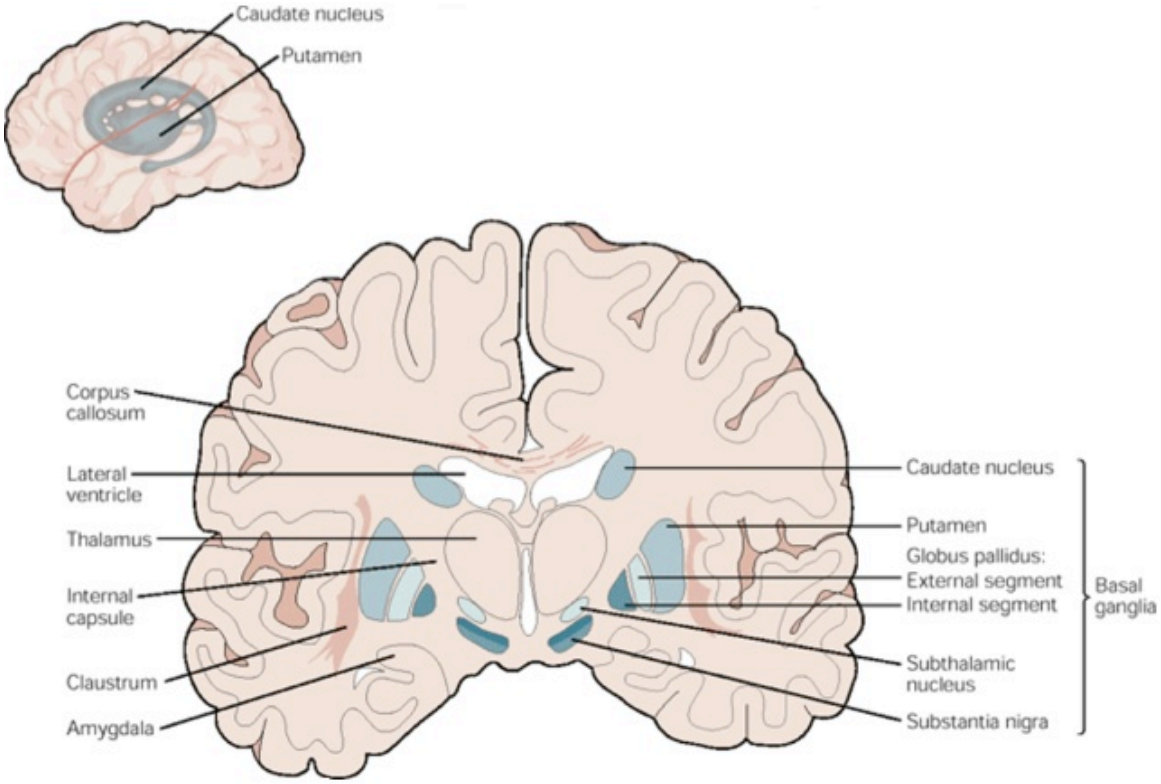

Figure 1: Coronal section of the basal ganglia (from Kandel et al., 2000)

In a healthy brain at rest, the GPi and the SNr tonically inhibit the thalamus through the release of γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter. The thalamus is unable to excite the primary motor cortex (PMC) due to the tonic inhibition from the GPi and SNr and as a result no movement is initiated, the body remains at rest (Visanji, 2014). The direct pathway of the basal ganglia removes the inhibition from the thalamus and stimulates muscle movement (**Figure 2A**). Upon the decision for voluntary movement the PMC sends an excitatory signal, via the neurotransmitter glutamate, to the putamen. Simultaneously, the putamen is also excited by DA neurotransmitter release from the SNpc, which was activated by glutamatergic input from the PMC. Excitation of the putamen increases its inhibitory GABAergic signal to the GPi and the SNr (Jung, Leem & Kim, 2014; Kandel et al., 2000). Inhibition of the GPi and SNr reduces their synaptic output, as a result the thalamus is no longer tonically inhibited allowing it to send an excitatory glutamatergic signal to the PMC and signal a muscle movement (Capper-Loup & Kaelin-Lang, 2013; Kandel et al., 2000).

Working in conjunction with the direct pathway, in the healthy brain the indirect pathway inhibits muscle movement that would conflict with the desired movement. At rest, the GPe tonically inhibits the STN through the release of the neurotransmitter GABA. This inhibition of the STN prevents its excitation of the GPi. Simultaneously the GPi and the SNr are tonically inhibiting the thalamus reducing its excitatory output to the primary motor cortex and thereby inhibiting muscle movement (Capper-Loup & Kaelin-Lang, 2013).

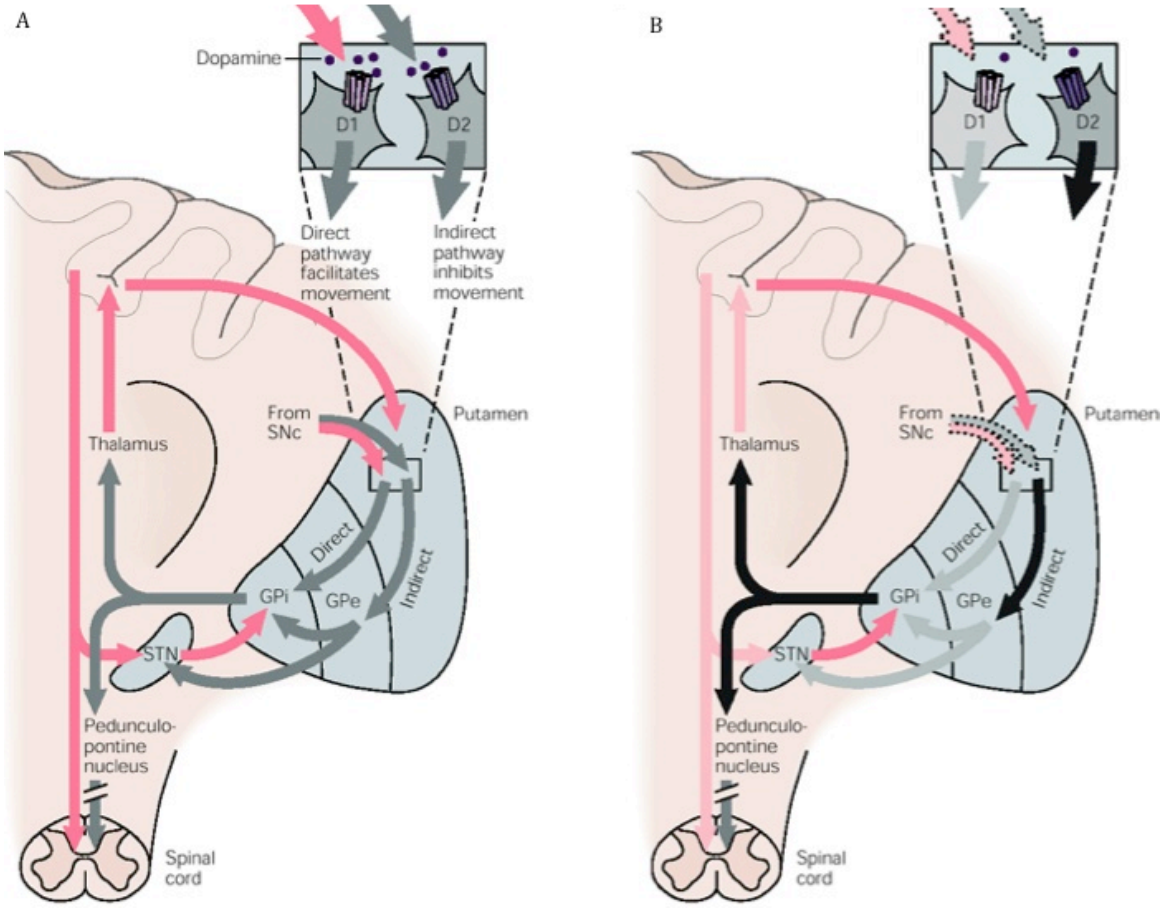

Figure 2: Basal ganglia-thalamocortical circuitry under normal conditions (A) and Parkinson's disease (B). Differential changes in activity following degeneration of nigrostriatal dopamine pathway in Parkinson's disease. Dark pink and light pink arrows indicate excitatory connections, black and gray arrows indicate inhibitory connections. Darker arrows indicate increased neuronal activity, lighter arrows indicate decreased neuronal activity (from Kandel et al., 2000).

When the decision to initiate movement is made the PMC excites the putamen via the neurotransmitter glutamate. Increase activation of the putamen increases GABAergic inhibition of the GPe. The reduced GPe activity reduces tonic inhibition of the GPi and the STN. Disinhibition of the STN allows for it to send excitatory glutamatergic signals to the GPi. Increased activation of the GPi results in an increase of its inhibitory GABAergic signal on the thalamus and thus preventing excitation of the PMC (Capper-Loup & Kaelin-Lang, 2013; Kandel et al., 2000).

Direct pathway excites areas of the cortex to activate agonist muscles and the indirect pathway further inhibits the areas of the cortex to inhibit antagonist muscles. Both work in parallel to facilitate movement (Jung et al., 2014). The SNpc modulates the direct and indirect pathway by regulating the amount of dopamine released. Striatal neurons of the direct pathway have DA D1 receptors to facilitate transmission, and for the indirect pathway DA D2 receptors of the SNpc inhibit the putamen, the net result of both is cortical activation (Jung et al., 2014; Visanji, 2014).

In PD the SNpc is the site of the selective degeneration of DAergic neurons. The loss of DA inputs from the SNpc to the striatum leads to the depletion of DA in the striatum (Sullivan & Toulouse, 2011). The reduction of striatal DA in the direct pathway results in a decreased activation of the striatum via DA D1 receptors. The reduced excitation of the striatum reduces its inhibition to the GPi. In the indirect pathway, the reduced DA levels decreases inhibition of the striatum via DA D2 receptors. Subsequently the striatum is increased in its inhibitory output to the GPe which results a reduced inhibition to the STN. This leads to an increased excitatory

output from the STN to the GPi (Capper-Loup & Kaelin-Lang, 2013; Kandel et al., 2000). The culmination of increased excitation of the GPi from the indirect pathway and the reduced inhibition of the GPi from the direct pathway significantly increases the output of the GPi. As a result there is a significant increase in the GPi's inhibitory output to the thalamus. This increased inhibition reduces that corresponding excitatory output of the thalamus to the PMC causing the decreased motor activity seen in PD (**Figure 2B**) (Kandel et al., 2000). Dopamine deficiency gives rise to a characteristic constellation of symptoms that constitute PD.

1.1.4 Diagnosis and Classification

A neurologist will make the clinical diagnosis of PD, typically defined by the presence of four cardinal features; tremor, bradykinesia, impaired postural stability, and rigidity. These symptoms have been described in detail in section 1.1.1 (Albin 2006; Gazewood, Richards & Clebak, 2013). Although there is no uniform clinical criteria established for the diagnosis of PD, the presence the cardinal features and a good response to levodopa, a DA agonist, are considered to be indicative of PD (Albin 2006). However, some of these features overlap with other neurological conditions of parkinsonism, especially in the early stages, and are often misdiagnosed as PD. Some of these include vascular parkinsonism, progressive supranuclear palsy and drug-induced parkinsonism.

It is important to distinguish between parkinsonism and PD. Parkinsonism is a nonspecific term characterized by motor deficits of tremor, bradykinesia, rigidity and postural instability. At least two of these features should be present to make a

diagnosis of Parkinsonism. A pathologically common feature in parkinsonism is the neurodegeneration of DAergic neurons, the cause may be due to chemical toxicity, metabolic abnormalities or structural causes. Parkinsonism can be divided into primary (idiopathic), secondary, atypical and hereditary. PD (also known as primary or idiopathic parkinsonism) makes up about 75-80% of cases of Parkinsonism. Every patient with PD is said to have parkinsonism but the converse is not true. Secondary parkinsonism, includes drug induced parkinsonism, which is a result of pharmacological agents that interfere with the effect of dopamine or with its metabolism. However, unlike idiopathic PD, drug induced parkinsonism tends to remain static and does not progress. Vascular parkinsonism is another type of secondary parkinsonism commonly misdiagnosed as PD. It involves the occurrence of lacunar strokes, the blockage of arteries supplying blood to the basal ganglia or subcortical areas of the brain. Vascular parkinsonism is manifested with bradykinesia of the limbs and gait impediments. The onset is related to the infarcts and is contralateral to the stroke. Atypical parkinsonism have clinical manifestations similar to PD that results from the neuronal loss in different components of the basal ganglia. Progressive supranucluear palsy is one such type of atypical parkinsonism that is often misdiagnosed as PD. Although behavioural symptoms appear similar to PD the response to L-DOPA is minimal at best in improving rigidity and bradykinesia. The disease progresses much faster than PD and affects the brainstem, cerebellum and basal ganglia. Hereditary parkinsonism include heredodegenerative conditions like Wilson's disease and Huntington's disease. In addition to the various

types of parkinsonisms often being misdiagnosed as PD, essential tremor (ET), the most common movement disorder, is also often mistaken for mild PD. It is commonly seen in the elderly; however, ET lacks rigidity, bradykinesia and impaired postural reflexes.

The misdiagnosis rates of PD are a major concern. A UK autopsy study found that in 100 subjects diagnosed with PD 25% of them were misdiagnosed, even in highly specialized centers the misdiagnosis rate is as high as 9% (Michell, Lewis, Foltynie & Barker, 2004). When diagnosis in primary care settings was assessed it was found that 47% of the PD diagnoses were incorrect (Pagan, 2012). Due to the rapid disease progression and heterogeneous nature of PD it is understandable why it is misdiagnosed, however progress needs to be made to better understand the disease and identify biomarkers for the early detection of PD (Pagan, 2012).

1.2 The Aging Brain

One primary risk factor for neurodegenerative disease development is advancing age (Mattson & Magnus, 2006). Aging is a physiological process that occurs asynchronously in different areas of the brain. In response to aging, cells undergo a complex biological processes in which they experience increased amounts of oxidative stress and the production of free radicals, mitochondrial dysfunction, perturbed energy homeostasis, accumulation of damaged proteins, lesions in their nucleic acids and alterations in neurotransmitter and neurotrophic factor (NTF) signalling pathways (Hindle, 2010). A dramatic increase in these cellular processes

is seen during the sixth, seventh and eighth decade of life (Mattson & Magnus, 2006). One such example of this is the depletion of DA in the SN neurons that occurs in normal aging and is exacerbated in PD, producing the hallmark pathological characteristic of PD (Mattson & Magnus, 2006; Mora, Segovia & del Arco, 2007). Aging is the single largest independent risk factor for development of PD, making aging an area of interest for researchers (Mattson, Chan, & Duan, 2002).

1.3 Neurotrophic Factors

NTFs are an important group of secreted proteins regulating the life and death of neurons. NTFs act as growth factors and play an irreplaceable role in the development, maintenance, function and plasticity of specific neuronal populations in the developing and adult vertebrate nervous system (Cheng et al., 2013; Lindolm & Saarma, 2009; Siegel & Chauhan, 2000). NTFs are very potent molecules that are capable of prompting a biological response at very low concentrations (Sullivan & Toulouse, 2011). Developing neurons compete for limited amounts of NTFs secreted by the target tissue. Those neurons which fail to obtain a sufficient quantity of the necessary NTFs are unable to survive and maintain synaptic contacts with their target tissue, as a result they die by the process of programmed cell death, referred to as apoptosis (Lindolm & Saarma, 2009; Siegel & Chauhan, 2000). In addition to promoting the differentiation and growth of developing neurons, and the maintenance and survival of adult neurons, a substantial amount of evidence suggests that under certain conditions NTFs modulate neuronal plasticity during

aging and degenerative conditions (Zhao et al, 2014). Alterations in neurotrophic levels, such as the loss of trophic support for selective neuronal populations due to age, may lead to neurodegenerative disease (Siegel & Chauhan, 2000). As PD is primarily caused by the degeneration of DAergic neurons a significant amount of research has been done on the various NTFs therapeutic effects on these neurons. The primary goal of research into NTFs is to be able to administer them to patients in a safe and targeted manner to halt or reverse the progressive degeneration of nigrostriatal dopaminergic neurons (Sullivan & Toulouse, 2011).

There are four major classes of NTFs 1) neurotrophin family; 2) the glial cell line-derived neurotrophic factor family of ligands; 3) the neurotrophic cytokines (neurokines); 4) and the novel family of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurtrophic factor (MANF).

Brain-derived neurotrophic factors (BDNF), a member of the neurotrophin family of growth factors and glial cell line-derived neurotrophic factor (GDNF) are two of the best studied neurotrophic factors with respect to PD, as they target cells in the midbrain dopaminergic neurons, respectively (de Yebenes & Mena, 2000).

1.3.1 Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a protein that is present in small amounts throughout the adult central nervous system (CNS). It supports the survival and maintenance of the retinal ganglia, sensory neurons, certain cholinergic neurons, spinal motor neurons and some DAergic neurons, including those in the SN and the striatum (Allen, Watson, Shoemark, Barua & Patel, 2013; Hyman et al., 1991). It is initially

synthesized as a glycosylated precursor, proBDNF, then cleaved to form mature BDNF intracellularly or extracellularly. Mature BDNF and proBDNF are both biologically active and have distinct biological functions (He, Zhang, Yung, Zhu, & Wang, 2013).

Reduced levels of BDNF have been associated with a number of neurodegenerative, development and neuropsychiatric conditions, including PD. In rats, BDNF is expressed in the SN and the ventral tegmental area, and is anterogradely transported from the SN to the striatum (Altar et al, 1997; Von Bartheld, Byers, Williams & Bothwell, 1996). Assessment of BDNFs therapeutic effects has utilized preclinical neurotoxic models of PD, namely 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which both selectively degenerate dopaminergic neurons. The knockdown of BDNF mRNA expression by antisense oligonucleotide infusion, a gene silencing technique, in the SN has shown to lead to the death of nigral DA neurons and mimic behavioural deficits of neurotoxic rat models of PD (Porritt, Batchelor & Howells, 2005). Additionally, with the overexpression of wild-type α-syn BDNF levels are reduced (Kohno et al., 2004; und Halbach, Minichiello & Unsicker, 2005). When BDNF was administered to rat ventral mesencephalon cultures before, during or after 6-OHDA exposure, the trophic factor was found to have protective and regenerative effects on the brain cultures (Stahl, Mylonakou, Skare, Amiry-Moghaddam & Torp, 2011). In rats, BDNF has been shown to promote neurite outgrowth in DA neurons and provide neuroprotection following MPTP (Frim et al., 1994). Furthermore, in a

nonhuman primate study by Takeda (1995), it was found that the administration of BDNF after parkinsonism was induced with MPTP significantly reduced the loss of DA neurons and ameliorated behavioural symptoms. In post mortem PD samples, reduced levels of BDNF protein and mRNA have been found in the striatum and SNpc (Howells et al., 2000). In line with these results, researchers have also found that serum BDNF concentrations are much lower in PD patients when compared to a healthy control population (Scalzo, Kummer, Bretas, Cardoso & Teixeira, 2010).

These studies demonstrate the potent effects on DAergic neuron survival and the therapeutic potential of BDNF to protect against PD or reverse its process. However, the efficacy of BDNF in the treatment of PD in humans has not yet been assessed due to safety concerns. Methods for safe and effective dosing while continuously delivering BDNF to the brain have been a major obstacle in translating to BDNF clinical trials. (Nagahara & Tuszynski, 2011)

1.3.2 Glial Cell Line-Derived Neurotrophic Factor (GDNF)

GDNF was discovered in a search for a trophic factor for the nigrostriatal DA neurons. Studies have revealed that GDNF mRNA expression is widely distributed in regions of the developing and adult brain, as well as throughout the body (Siegel & Chauhan, 2000). It has been found to be expressed in the striatum, sensory and autonomic ganglia, Purkinje cells of the cerebellum, brain stem, hippocampal neurons, and noradrenergic, serotoninergic, and cholinergic neurons (Allen et al., 2013). GDNF mRNA expression has also been found in peripheral tissues, including the kidney and testes (Allen et al., 2013; de Yebenes & Mena, 2000). Having been

shown to be an important trophic factor for midbrain dopaminergic neuron survival and stimulation of immature neuronal growth, GDNF is thought to be an attractive therapeutic target for the retardation of PD degeneration (Stayte & Vissel, 2014).

Similar to BDNF, GDNF is also known to support DAergic and motor neuron survival and a number of investigations have taken place to further understand GDNF's role in PD. The therapeutic effects of GDNFs have been studied on 6-OHDA and MPTP preclinical neurotoxic models of PD. In rat mesencephalic cell cultures, GDNF treatment following either 6-OHDA or MPTP exposure has consistently shown to be neuroprotective by promoting the survival and differentiation of DAergic neurons (Eggert et al., 1999; Hou, Lin & Mytillineou, 2002). The administration of GDNF into the SN of rats has been shown to effectively protect against retrograde cell death following intrastriatal 6-OHDA lesioning (Winkler, Sauer, Lee, & Biorklund, 1996). Also, in SN 6-OHDA lesioned rats the post-administration of GDNF into the lesion site resulted in an increased activity of tyrosine hydroxylase in that region. Tyrosine hydroxylase is the rate-limiting step in the biosynthesis of DA and because of this it indirectly estimates the ability of the brain to synthesize DA (Fine et al., 2014). The increased activity of tyrosine hydroxylase resulted in an increased release of DA, which was indicated by the improved locomotor response (Martin et al., 1996). The administration of GDNF into the striatum following a partial lesioning was associated with a significant improvement in motor function; however this improvement was diminished with the withdrawal of GDNF (Kirik, Georgievska, Rosenblad & Bjorklund, 2001).

Not only has GDNF been shown to be neuroprotective, rodent studies have also given insight into the trophic factors neurorestorative effects. When GDNF was administered to the striatum of rats prior to a neurotoxic striatal lesioning it was found to be neuroprotective of the entire nigrostratal DAergic pathway and locomotor function was preserved (Kearns, Cass, Smoot, Kryscio & Gash, 1997; Kirik, Rosenblad & Bjorklund, 2000). A series of intrastriatal injections of GDNF prior to lesioning was found to preserve DAergic neurons and reduce drug-induced rotation, a hallmark feature of unilateral 6-OHDA lesions in rats (Shults, Kimber & Martin, 1996). Research in nonhuman primate models of PD has shown that the chronic intraputamen infusion of GDNF showed increased DAergic cell count and size within the SN. Additionally, within the striatum and globus pallidus an increased fibre density was seen, elevated levels of DA as well as a significant improvement in motor performance (Grondin et al., 2010).

The promising consistent findings GDNF being neuroprotective and neurorestorative in animal models lead to the initiation of clinical trials in PD patients. GDNF however, is rapidly degraded in the human body and does not cross the blood-brain barrier (Martin et al., 1996). The direct intracerebral delivery of the NTF to the patients is the only option possible and this has added further complications to the already difficult task of transitioning to the clinical level (Collier et al., 2005; Sullivan & Toulouse, 2011).

The first clinical trial investigating GDNF began in 1996 (Nutt et al., 2003). It was a randomized controlled trial that used intraventricular delivery of GDNF to PD

patients. The study reported no clinical improvements compared to the placebo group but did shed light onto serious complications. The study had to be terminated prematurely, patients experienced implanted catheter related complications and 48 out of the 50 patients experienced adverse effects that included, but not limited to, nausea, anorexia, vomiting, dizziness, hallucinations and dyskinesia. 7 patients had such severe side effects that it led to their withdrawal from the study (Nutt et al., 2003).

Due to the lack of success of intraventricular GDNF delivery, Gill et al. (2003) delivered the trophic factor directly to the putamen. Five patients whose symptoms were poorly managed by pharmacological treatment received continuous delivery of GDNF using a catheter system. Following a 12-month period patients showed a significant reduction in their motor, dyskinesia and quality of life scores for the Unified Parkinson's Disease Rating Scale (UPDRS). Unlike the previous study, clinical side effects were limited. Only one patient experienced complications due to surgery, however this was quickly rectified and study participation was not compromised. The only consistent finding of GDNF related side effects was a mild tingling passing from the neck down to the arms (Gill et al., 2003). The same research group followed these patients over the course of a 24-month outcome study. They reported that no serious clinical side effects and found continuous GDNF infusion reduced motor dysfunction and improved ratings on the UPDRS (Patel et al., 2005). A 34 patient randomized control trial was conducted by Lang and colleagues (2006) to build upon these findings. After 6 months of intraputamenal infusion of

GDNF, no significant difference in motor symptoms was found. Additionally serious safety concerns arose as several patients experienced both device- and treatment-related adverse events and some patients even developed GDNF neutralizing antibodies to GDNF. The implications of the development of GDNF antibodies in these patients is unknown, and more preclinical research needs to be done before further human trials proceed (Lang et al., 2006).

To further support clinical development of GDNF as a therapeutic trophic factor, researchers carried out a sixth month long chronic intraputamenal infusion of GDNF in rhesus monkeys. This study resulted in the observation of a number of pathological markers of toxicity, reduction in food intake and weight loss, Purkinje cell loss and meningeal thickening (Hovland et al., 2007). In light of these serious safety issues and low clinical benefits, attention has shifted towards alternative trophic factors that could slow down or reverse the progression of neuronal degeneration while being safe to use.

1.3.3 Cerebral Dopamine Neurotrophic Factor (CDNF)

The recently discovered novel family of NTFs consists of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic-astrocyte-derived neurotrophic factor (MANF). CDNF is a vertebrate specific paralogue, derived from the same ancestral gene of human MANF but has a different function (Parkash et al., 2009). Both CDNF and MANF are expressed in different mouse, rat and human tissue. Whereas in vertebrates, including nematode *Caenorhabditis elegans*, and fruit fly, *Drosophilia melanogaster*, they have a single ancestral MANF/CDNF gene orthologue

(hereinafter referred to as CDNF), with 46% and 49% similarity, respectively, to human CDNF. Human CDNF shows a 59% amino acid identity with human MANF (**Figure 3A**)(Lindholm et al., 2007; Lindholm & Saarma, 2009). Expression of CDNF has been detected throughout the human brain, in the SN, striatum, olfactory bulb, hippocampus, thalamus, hypothalamus, midbrain, cerebellum and many other regions. Additionally, CDNF expression is also detected in non-neuronal tissues such as the heart, liver, lungs, skeletal muslces, spleen, testis and thymus (Cheng et al., 2013; Lindholm et al., 2007).

1.3.3.1 Structure of CDNF

Mature CDNF protein is 161 amino acids long (preCDNF consists of 187 amino acid residues). CDNF and MANF differ from other NTFs as they do not contain a prosequence, which suggests that their activity is not dependent on enzymatic cleavage (Lindholm et al., 2007). The CDNF protein consists of eight cysteine residues influencing its tertiary structure, it is structurally unique among other NTFs and growth factors. CDNF consists of two domains, a saposin-like N-terminal domain, stabilized by three intramolecular disulfide bonds, and a unfolded carboxy terminal domain with an intradomain cysteine bridge in a CXXC motif (**Figure 3B**)(Lindholm & Saarma, 2009; Zhao et al, 2014).

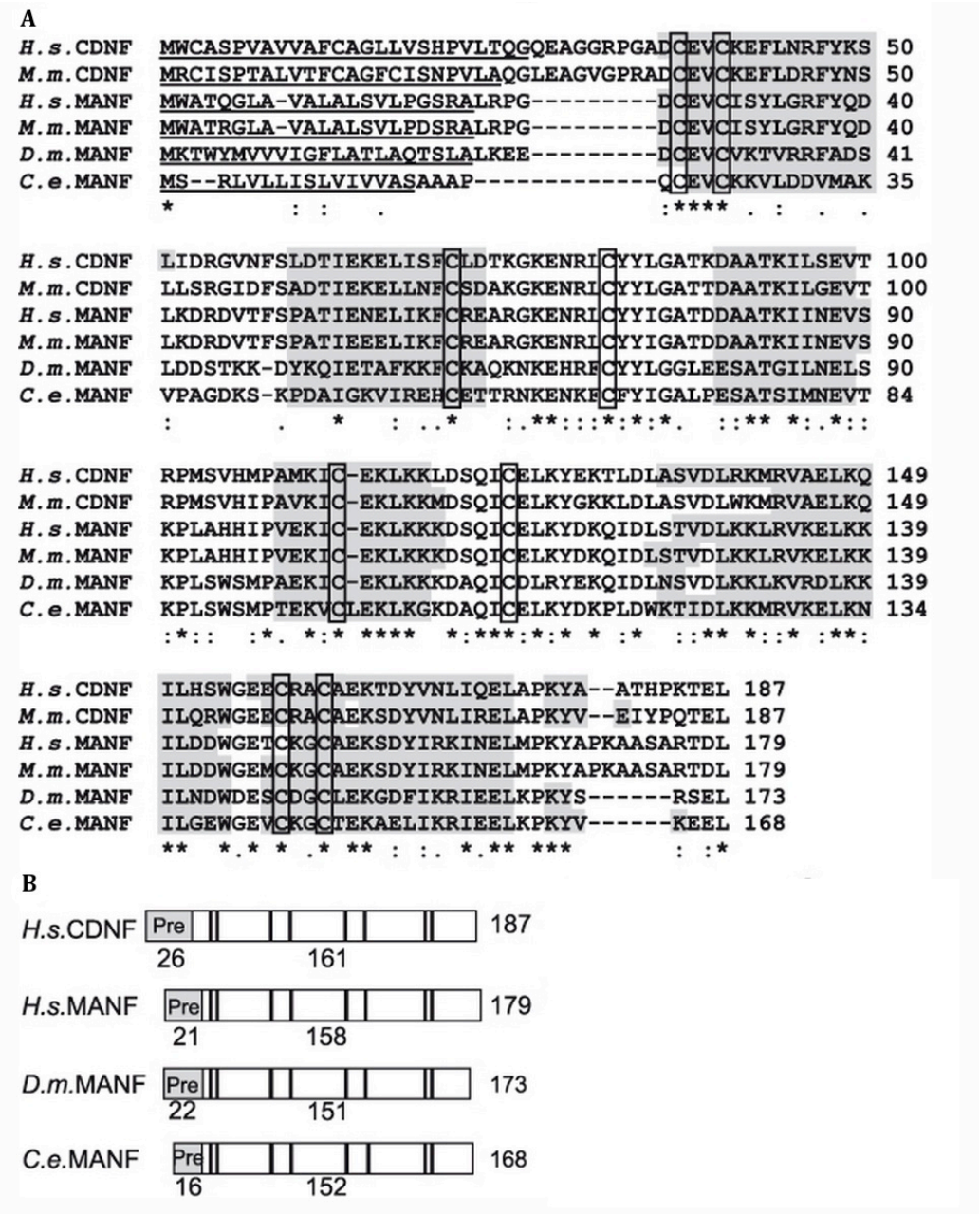

Figure 3: Evolutionary conservation of CDNF. A. Alignment of human (H.*s*), mouse (M.*m*.), *D. melanogaster* (D.*m*.) and *C.elegans* (C.*e*.) CDNF and MANF amino acid sequences. Asterisk depicts identical amino acids, semicolon depicts physiochemically highly similar and dot depicts similar amino acids. **B.** Schematic illustration of amino acid sequences of CDNF and MANF. Pre corresponds to the signal sequence, and the numbers show the length of the polypeptides in amino acids. Conserved cysteine residues are shown by vertical bars (Figure modified from Lindholm et al., 2007).

1.3.3.2 Function of CDNF

Although the underlying mechanism of CDNF function is unclear, it is known that its secretion is primarily by the classical endoplasmic reticulum (ER)-Golgi pathway (Lindholm & Saarma, 2009). Due to the lack of prosequences necessary for enzymatic activation, it is likely that CDNF is functional upon synthesis and secretion. In addition to its N-terminal domain being stabilized by disulfide bonds, this trophic factor is retained within the ER where it is thought to assist in protein folding and mitigate ER stress, thereby preventing neurodegeneration (Lindholm & Saarma, 2009; Parkash et al., 2009). The accumulation of misfolded proteins in the brain is common feature of neurodegenerative diseases like PD and research suggests the alterations in ER function is a salient feature in the disease (Liu, 2014). These disruptions in the ER folding process leads to an accumulation of misfolded/unfolded proteins, ER stress, which under normal conditions activates the cells unfolded protein response (UPR) to restore ER homeostasis. However, under conditions of chronic ER stress the UPR initiates cell death by apoptosis, eliminating the damaged cells. CDNF may facilitate protein folding in the ER and thus reducing the stress caused by misfolded and unfolded proteins proving to be a potentially fundamental in therapeutic avenue in the maintenance of dopaminergic neurons (Lindholm & Saarma, 2009).

1.3.3.3 CDNF and Parkinson's Disease

Recently, several studies have been undertaken to investigate the therapeutic role of CDNF for PD. Lindholm et al. (2007) looked at the rat neurotoxic, unilateral 6-

OHDA lesion models of PD to test the effects of CDNF on the survival of dopaminergic neurons. CDNF (10μg), GDNF (10μg) or a control vehicle was unilaterally infused into the striatum prior to 6-OHDA lesioning. They observed that the amphetamine induced turning behaviour, a hallmark feature of 6-OHDA lesions, was significantly reduced following CDNF-pre-treatment 4 weeks post-lesion in comparison to GDNF-pre treatment and control vehicle infusion. Additionally, at 4 weeks post-lesion, an almost complete rescue of dopaminergic tyrosine-hydroxylase-positive (TH+) cells in the SNpc and density of TH+ fibres in the striatum was seen in both CDNF and GDNF pre-treated rats in comparison to controls (Lindholm et al., 2007). In addition to studying the neuroprotective effects of CDNF, they also investigated the neurorestorative activity of the NTF. Both CDNF and GDNF treatment following 6-OHDA lesion resulted in a reduction of amphetamine induced rotational behaviour compared to the control group, as well as both NTFs partially recovering TH+ cells in the SNpc. Taken together, these studies concluded that CDNF is at least as efficient as GDNF in preventing and protecting against 6-OHDA induced degeneration of dopaminergic neurons (Lindholm et al., 2007). Similar findings of CDNFs neuroprotective and neurorestorative properties are found in MPTP mice models of PD. Both the pre- and post-treatment with striatal infused CDNF resulted in an improved behavioural response as well as an increased striatal DA fibre density and number of TH+ cells in the SNpc (Airavaara et al., 2012).

The role of CDNF in preventing and reversing neurodegeneration of DAergic neurons has been further expounded upon in studies performed by Mei and Niu (2014). Using rat pheochromocytoma (PC12) cell lines, widely used cell lines for investigation of pathogenesis of PD due to its many properties similar to DA neurons, CDNF protein was administered both before and after 6-OHDA exposure. Whereas 6-OHDA treatment alone would significantly reduce cell viability, pre-treatment with CDNF significantly improved survival rates. A reversal of 6-OHDA-induced apoptosis in PC12 cells was seen when CDNF treatment followed neurotoxic exposure and significantly improved survival rates. The work by Mei and Niu (2014) is congruent with the behavioural studies undertaken by Linholm et al. (2007); CDNF protein protects and reverses the effects of 6-OHDA on dopaminergic neurons.

Although the mechanism of action of CDNF is still unknown, studies have been undertaken to elucidate its pathway of action. To do this, Mei and Niu (2014) looked at the influence of 6-OHDA on the expression of Bcl-2/Bax and caspase-3 in PC12 cells. The role of the Bcl-2 signalling pathway in apoptosis has been established; Bcl-2 is a family of proteins that regulates apoptosis and prevents cell death (Mei & Niu, 2014). Two additional measures were examined to elucidate the role of CDNF in the apoptotic pathway: Bax, a pro-apoptotic homologue of Bcl-2 that induces cell death; and caspase-3, a protease whose activation is a central step in the apoptotic process. The interplay between these anti-apoptotic and pro-apoptotic regulators is critical for cell survival. Following treatment with 6-OHDA, there was an observed decrease in expression of Bcl-2, an increase in Bax and an increased

expression of caspase-3. These effects are attenuated with both the pre- and post-treatment of CDNF. The neuroprotective and neurorestorative effects of CDNF protein on 6-OHDA treated PC12 cells is believed to be regulated through the upregulation of the Bcl-2/Bax ratio and the downregulation of caspase-3 (Mei and Niu, 2014).

Although the neuroprotective mechanism and receptors of CDNF are still unknown, a number of studies have postulated a role for CDNF in regulating neuroinflammation. Overexpression of CDNF in astrocytes gives cells the potential to resist injury and proinflammatory cytokine secretions (Cheng et al., 2013a); CDNF promotes sciatic nerve regeneration and functional motor recovery in rat transection models via CDNF lentiviral vector (Cheng et al., 2013b); CDNF protects microglia against inflammatory injuries and alleviates the production of proinflammatory cytokines (Zhao et al, 2014).

Several NTFs and growth factors are neuroprotective for dopaminergic neurons against 6-OHDA lesions. However only GDNF and neurturin have been shown to be neurorestorative. The finding of CDNF being both neuroprotective and neurorestorative is very promising for potential therapeutic protein for the treatment of PD (Sullivan & Toulouse, 2011). Despite neurorestorative and neuroprotective effects of CDNF further research is needed to identify the receptors and mechanism of action of CDNF both of which will help elucidate its role in PD (Lindholm & Saarma, 2009).

1.4 Peripheral Blood as a Biomarker for Parkinson's Disease

1.4.1 Biomarkers

Currently diagnosis of PD is made by the presence of cardinal motor features and response to medication such as L-DOPA; however, by the time motor symptoms manifest, DAergic neurons have already been significantly degenerated (Pagan, 2012). PD can be difficult to diagnose in the early stages and may be mimicked by other disease such as essential tremor or progressive supranuclear palsy as previously described. The detection of PD in individuals before the first parkinsonian motor symptoms appear (preclinical diagnosis) is very important. Early treatment strategies can slow down disease progression and help the individual maintain a higher level of functioning for a longer period of time (Spiegel, Storch & Jost, 2006). Research has focused on the development of early diagnostic biomarkers of PD in hopes of In hopes of better detecting preclinical PD. A good biomarker for PD would need to be specific for PD and be sensitive to detect susceptibility to the disease before the onset of motor symptoms (Michell, Lewis, Foltynie & Barker, 2004). The development of a successful biomarker will help reduce misdiagnosis rates in PD and identify at-risk patients and begin therapy at an earlier stage.

A number of biomarkers for PD have been investigated, which include imaging techniques, clinical tests and biochemical/genetic tests (Pagan, 2012). A number of neuroimaging techniques have been investigated for their viability in the detection of PD, including functional magnetic resonance imaging, positron emission

tomography and single photon emission computed tomography (Michell et al., 2004). Although the use of imaging is restricted due to cost, wait times and the limited number of scans an individual can receive, initial work has been promising and further studies are needed to establish diagnostic accuracy (Berg, 2008). Various clinical testing of psychological states, motor performance, olfaction and vision have also shown promise in their use to help diagnose PD. Given its heterogeneity these tests help to stratify the affected population and give insight into potential treatments (Foltynie, Brayne & Barker, 2002). Biochemical testing has been developed to identify vulnerability to PD several years prior to manifestation of symptoms. Genetic testing has not revealed a clear link between genotype and the development of PD but has shown promise in clustering the diseased population for better treatment (Sveinbjornsdottir et al., 2000). CSF has shown to be a potential biochemical marker for PD. Levels of β-phenylethylamine and orexin have been correlated with PD (Drouot et al., 2003; Zhou, Shoji, Yamada & Matsuishi, 1997). However due to the difficulty in obtaining samples, CSF is a less appealing source of a biomarker (Michell et al., 2004). Blood is an appealing candidate for peripheral biomarkers. It is easily collected, has long been used to model serotonergic and DAergic neuron behaviour and platelets exhibit similar biochemical impairments to the SN in PD patients (Schapira et al., 1990).

1.4.2 Peripheral Blood as a Biomarker

The human body has a dynamic circulatory system with cellular components that are suspended in blood and have a rapid turnover rate. Classified as a fluid

connective tissue, blood connects the entire biological system, constitutes the first line of the immune system defence and provides a protective barrier between the external and internal environments (Liew, Ma, Tang, Zheng & Dempsey, 2005). Due to the continuous interactions between blood cells and the entire body it gives rise to the possibility of subtle changes associated with diseases within the cells of the body, which may be reflected by changes in gene expression in blood cells (Liew et al., 2005). Recent studies have demonstrated that alterations of gene expression in peripheral blood are characteristic of a wide range of diseases including neuronal injuries and cancer (DePrimo et al., 2003; Tang, Lu, Aronow & Sharp, 2001). Also, psychiatric disorders such as schizophrenia and bipolar disorder have been distinguished through specific peripheral blood gene expression profiles (Tsuang et al., 2005). Blood provides a significant advantage as a potential biomarker as it is readily available in large quantities and can be accessed with minimally invasive techniques (Liew et al., 2005; Tang et al., 2001). The link between peripheral blood and the CNS has been studied extensively. Lymphocytes have been researched as a potential biomarker for CNS function and for Huntington's disease (Coccinia et al., 2005; Runne et al., 2007). Whole blood has been investigated in Alzheimer's disease and mood disorder and platelet studies of α-syn has looked for a possible link between peripheral blood and CSF in in the pathology of PD (Diamandis, Yousef, Petraki & Soosaipillai, 2000; El-Agnaf et al., 2006; Le-Niculescu et al., 2009; Michell, Lubeshi & Barker, 2005). Platelets have been used to model serotonergic and dopaminergic neuron behaviour (Da Prada, Cesura, Launay & Richards, 1988).

Oxidative stress studies on platelets have been performed, and although PD patients show some abnormalities, results have not been significant (Kikuchi et al., 2002).

Neurotrophic factors have also been studied in peripheral blood. There are conflicting reports of GDNF levels in serum showing decreased concentration in Alzheimer's disease patients whereas other research groups have shown an increase in plasma GDNF levels in the same population (Sharma et al., 2013). In depressed patients BDNF platelet levels have increased whereas treatment with selective serotonin reuptake inhibitor restored platelet BDNF levels to those of healthy controls (Serra-Millas et al., 2011). Serum and plasma BDNF levels have also been studied in mood disorders and schizophrenia (Fernandes et al., 2014). However, despite extensive efforts in peripheral blood to define molecular mechanisms underlying PD, identifying a potential biomarker for the earlier detection of PD much still much remains unknown. Peripheral blood still remains an ideal candidate for a potential biomarker as it is obtained in a minimally invasive manner and is readily available in large quantities; however, more research is needed for a biomarker that will accurately reflect normal pathological processes and be specific for PD (Liew et al., 2005; Tang et al., 2001).

Chapter 2

Caenorhabditis elegans Dopamine Quantification and CDNF mRNA Expression

2.1 *Caenorhabditis elegans*

The nematode, *Caenorhabditis elegans (C. elegans)* are one of the most widely studied and well understood organisms. Due to the complexity of neurodegenerative disorders such as PD, a number of animal models have been established to attempt to study the disease. *C elegans* are one such model along with fly, fish, rodent and non-human primate models (Li & Le, 2013). Nematodes are of great importance in the study of the molecular mechanisms underlying numerous biological and disease processes. *C. elegans* were the first muliticellular organism to have its genome completely sequenced, and it has been found that approximately 65% of the human disease genes have analogues in the worm (Baumeister, 2002; Calahorro & Ruiz-Rubio, 2011; C. elegans Sequencing Consortium, 1998). In addition to genomic similarity, this invertebrate model shares many conserved molecular pathways and cellular mechanisms with mammals and offers several advantages as an animal model for the study and development of therapeutics for human diseases (Li & Le, 2013; White, Southgate, Thomson & Brenner, 1986). They are inexpensive to work with, with over 3,000 mutant strains available for free or costing only $7 from the Caenorhabditis Genetics Center (Caenorhabditis Genetics Center, 2012) and are a very simple organism having only 959 somatic cells (Artal-Sanz et al., 2006; Kaletta & Hengartner, 2006). These nematodes have a short life cycle, rapid growth rate and high fecundity; they develop from an egg into an adult within 3 days, making it possible to study developmental processes quickly. Also, each hermaphrodite worm produces approximately 300 larvae by self-fertilization, allowing for large brood size

(Kaletta & Hengartner, 2006). *C. elegans* are a completely transparent animal allowing for them to be used with *in vivo* fluorescence markers making it easy to study neuronal activity and metabolism. This is particularly useful when studying PD as neuronal cell death can be immediately observed and quantified. The assessment of the presence or absence of specific neurons is an invaluable attribute in using this model for the study of PD (Harrington, Hamamichi, Caldwell & Caldwell, 2010).

The simplicity of the organism is one of the major advantages in working with *C. elegans*. The value of this model lies not in trying to replicate disease complexity of the human brain, but rather to study cellular events by testing various genes, pathways and small molecules (Harrington et al., 2010). As a result of their use, many laboratories have made significant discoveries in insulin signalling, synaptic proteins, and transcription factors that play a neuroprotective role in Huntington's disease (Neri, 2011). Although rodents have well over a million neurons in their brain, an adult hermaphrodite *C. elegans* has a fully mapped neuronal circuitry with exactly 302 neurons (Nass, Hall, Miller & Blakely, 2002; Nass & Blakely, 2003). Of the 302 neurons there are only 8 dopaminergic neurons. The degeneration of these 8 neurons has shown to induce PD-like symptoms similar to humans and other mammalian PD models, suggestive of a shared cellular process link between worms and humans (Langston, Ballard, Tetrud & Irwin, 1983; Nass et al., 2002; Nass & Blakely, 2003).

A major advantage over *in vitro* cellular models that *C. elegans* have in the novel therapeutic discovery process is that they allow for the modeling of not only drug-receptor interaction, but also drug ingestion, passage and transformation, distribution to the tissue and cells of interest, interaction with the primary targets as well as the cellular and tissue regulatory mechanism (Nass et al., 2002). Additionally, the use of advanced genetics and genomics technologies in *C. elegans* could be used to screen for potential neuroprotective therapeutics and study their *in vivo* mode of action. For instance, PD like symptoms in worms have been shown to be reduced following treatment with acetaminophen due to its protection of dopaminergic neurons from oxidative damage (Locke, Fox, Caldwell & Caldwell, 2008).

The pathways involved in the processing, packaging and transport of dopamine have been conserved throughout evolution (Kaletta & Hengartner, 2006). This high degree of similarity of the dopamine neuron pathways between humans and worms provides researchers with a valuable tool to understanding disease mechanisms and in drug discovery through the use of *C. elegans*. In addition to PD, *C. elegans* disease models have been developed for, Alzheimer's, depression, muscular dystrophy, pain and neuronal regeneration, diabetes and obesity (Kaletta & Hengartner, 2006). Many signalling components and neurotransmitter systems found in the mammalian nervous system are also found in the *C. elegans* system, namely glutamate, GABA, acetylcholine and DA, amongst others (Nass & Blakely,

2003). The nematode DAergic system is fundamentally very similar to vertebrates, this has been elucidated through various mutant studies.

In the *C. elegans* genome, the *cat-1* gene encodes the vesicular monoamine transporter (VMAT) that is integrated into the membrane of synaptic vesicles of the presynaptic neuron. This transport protein is required to transport monoamine neurotransmitters, such as DA, into the synapse for neuronal signalling (Duerr et al., 1999). Sulston and colleagues (1975) have found that *cat-1* animals have a dramatic increase in L-DOPA suggesting that mutants are unable to load DA into synaptic vesicles. Also its behavioural phenotype is rescued with human VMAT2 transfection supports the role of *cat-1* as a synaptic vesicle neurotransmitter (Horvitz, Chalfie, Trent, Sulston & Evans, 1982). The *cat-2* gene encodes for tyrosine hydroxylase, the enzyme responsible for catalyzing the conversion of L-tyrosine to L-DOPA in the dopamine synthesis pathway. DA levels were found to be completely depleted in these mutants when detected by formaldehyde-induced fluorescence (Sulston et al., 1975). Another conserved pathway between man and worm is shown in aromatic amino acid decarboxylase (AADC) activity. The *bas-1* gene in *C. elegans* encodes for AADC, which catalyzes the decarboxylation of L-DOPA to dopamine. It shares similar function to that which is seen in the mammalian system. Mutants containing the *bas-1* defective allele are unable to produce DA (Loer & Kenyon, 1993; Saharia et al., 2012). The dopamine transport gene, dat-1, is both structurally and functionally similar to mammalian dopamine transporters (DAT). *Dat-1* encodes for a presynaptic membrane spanning protein that clears dopamine from the synapse

back into the cytosol (Salam et al., 2013). Although the exact role of DAT in PD is unclear, DA neurons in the SN, express DAT in the highest amount, are most severely compromised, whereas mesencephalic and hypothalamic DA systems, express low levels of DAT, are less affected (Miller, Gainetdinov, Levey & Caron, 1999).

This well established model has only recently been adopted for use with a high throughput system. Worm behaviour analysis was previously limited to examining the worm on a petri dish which was both tedious and error prone. Through developments in microfluidics research, drug screening can now be done quickly and objectively with the use of the microfluidic electrotaxis device (Rezai, 2010). Drug screening, movement analysis and neuronal signalling can be identified quickly and reliably using the electrotaxis device. The worm is placed within the controlled environment of the channel where a uniform electric field can be applied along the channel's length. Once the electric field is applied, the worm reproducibly travels towards the cathode, positive electrode in the direct current electric field of the device. The worms continue to remain fertile and live normally following exposure to the electric field, thus confirming that the stimulus is not harmful (Rezai, 2012). Using this device, many behavioural phenotypes can be measured such as worm swimming speed, turning time and amplitude of the locomotion wave.. Impairments of locomotion within the electrotaxis device may be reminiscent of motor impairments found in PD (Salam et al., 2013). The electrotaxis swimming response of DA pathway mutants has helped to better understand the role of DA signalling in modulating *C. elegans* locomotion. Worms having *cat*-1 or *bas*-1 gene

mutations have been shown to exhibit a faster swimming speed in the microfluidics channel whereas *cat*-2 gene mutation have shown a normal swimming response (Salam et al., 2014). *C.elegans* with a *dat*-1 mutation show a reduced swimming speed. The altered function of DAT-1, reuptake of DA presynaptically, resulting in an change of extracellular DA levels causing the altered swimming speed (Salam et al., 2013). In addition to mutants, toxic treatments of 6-OHDA and MPTP, both degenerating DA neurons, have shown to cause defects in swimming behaviour (Salam et al., 2013). DA signalling contributes to abnormalities in electrotaxis swimming behaviour and further research into DA levels will help better our understanding of the signalling pathways role in locomotion. This microfluidics electrotaxis device provides a low cost system that allows for the high throughput screening of a number of drugs, mutations and therapies on the *C.elegans* model (Rezai, 2012).

2.1.1 Complications of Using the *C. elegans* Model

Although *C.elegans* are a very attractive model for researchers, they are not without their limitations. This model lacks certain mammalian genes and molecular pathways that are involved in human disease. Due to the simplistic nature of the model it does not always fully epitomize the pathophysiology of the human disease (Nass & Blakely, 2003). Some drugs that are effective in humans have been shown to have no effect in *C. elegans*, therefore positive results in nematode research may not always directly translate to humans. Due to the makeup of *C. elegans* it is simply not

possible for certain protein therapeutics to be taken up by worms (Pienaar, Gotz & Feany, 2010).

The simplicity of the model is one of its greatest strengths but also its greatest weakness. For instance, movement in humans is controlled by a complex network of neurons of the CNS. Impairments in these neurons lead to movement disorders such as those seen in PD. However, the 8 dopaminergic neurons of *C. elegans* can limit the model from exhibiting the complexity present in this neurodegenerative disorder (Nass et al., 2002).

2.2 Objectives

The use of novel microfluidic electrotaxis assays has provided unique insight into the involvement of dopaminergic neuronal signalling in modulating locomotion (Salam et al., 2013; Tong, Rezai, Salam, Selvaganapathy, & Gupta, 2013). DA regulation of electrotaxis behavior of *C.elegans* has been influenced by DA neuron specific neurotoxins. Exposure of the nematodes to 6-OHDA has shown a slowed response to electrotactic swimming (Salam et al., 2013). Additionally, the DA synthesis and transport mutants also exhibited an altered swimming speed in the microfluidic channel (Salam, Selvaganapathy, Mishra, & Gupta, 2014). To further elucidate the role of DA in *C. elegans* locomotion, DA levels in these mutants was quantified by high performance liquid chromatography (HPLC). Additionally, on the basis of the relationship of DAergic neuron activity and CDNF, mRNA expression of CDNF in *C. elegans* DA synthesis and transport mutants was investigated (Lindholm

et al., 2007). DA and the DAergic system plays a role in PD, to help investigate the role of CDNF and the DAergic system these DA mutants were studied. It is hypothesized that mutants will have a lower concentration of DA and this will be mirrored by their reduced CDNF mRNA expression.

2.3 Methodology

2.3.1 *C. elegans* Cultures

All worms were grown at 20°C on standard NG-agar plates and were cultured by standard methods (Brenner, 1974). The mutant strains used in this study are: N2 (wild-type), RM2702 *dat-1*(*ok157*), DY442 *cat-1*(*ok411*), CB1112 *cat-2*(*e1112*) and MT7988 *bas-1(ad446)* and were obtained from the Caenorhabditis Genetic Centre (University of Minnesota, St. Paul, Minnesota, USA). Synchronized worms were used in all experiments this was achieved by treating gravid hermaphrodites with a solution containing commercial bleach and 4N sodium hydroxide (NaOH) (3:2 ratio). Following bleach treatment, dead worms were washed with M9 buffer and incubated at room temperature for 24 h to allow fertilized embryos to hatch into L1 larvae. The nematodes were placed on NG-agar plates and grown for 69 hours until adulthood. For neurotoxic treatment of 6-OHDA, worms at the L1 stage worms were exposed to 100 µM of 6-OHDA dissolved in 1% ascorbic acid-M9 solution for 2 hours. The drug was than wash out 3 times and the nematodes were placed on NG-agar plates and grown for 69 hours until adulthood.

2.3.2 Quantification of Dopamine Levels

Dopamine was measured by a coulometric method using HPLC (Waters 2695 separations module, Waters Limited, Mississauga, Ontario Canada) and electrochemical detection (ECD) (2465 electrochemical detector, Waters Limited, Mississauga, Ontario, Canada) in series. Worms were washed three times with filtered M9 buffer to remove adhering bacteria. 290 µl of 0.1N perchloric acid and 10 µl of 2,3-Dihydroxybenzylamine hydrobromide (DHBA) (2.5x10-3mg/ml), internal standard, was added to each 100 µl sample of pelleted worms, each sample was vortexed at room temperature and sonicated 3 times on ice at 15 second intervals. Samples were then centrifuged at 4°C for 20 minutes at 14 000 rpm to remove any insoluble residue. 20 µl of the supernatant was then injected into the HPLC-ECD system and passed through an ES Industries Neptune dC18 column (5µ particle size; 4.6 mm i.d. x 150 mm) at a flow rate of 1 ml/minute (Chromsystems Catecholamines Mobile Phase; Ref 5001). The electrochemical detector was set at a potential of +0.48V with a glassy carbon working electrode, the electrochemical detector current was set to 500pA for the *c.elegans* samples. Identification and quantification of dopamine was achieved by comparing peak areas and retention times with the respective peak characteristics of the standards (**Table 1**).

2.3.3 Primer Design

*C.elegan*s CDNF mRNA transcript was obtained from *www.wormbase.com.* Using OligoPerfect™ Designer software (Invitrogen Life Technologies, Burlington, Ontario, Canada) forward and reverse primers were design and synthesized at

MOBIX (McMaster University, Hamilton, Ontario, Canada). Forward primer, 5'-CACTCGCAACAAGGAGAACA-3' and reverse primer, 5'-CAGTCGAGTGGCTTGTCGTA-3' (**Table 2**). *C.elegans* RNA was extracted and cDNA was prepared, polymerase chain reaction was used to amplify the CDNF gene and the resulting construct was sequenced to confirm its authenticity by MOBIX. PCR products showed 100% homology with the *C.elegan* CDNF gene regions.

Standard	Volume Injected	mDA Injected (µg)	0.1M PCA (µl)	Dopamine (mg/ml)	DHBA (mg/ml)	Total Volume (µl)
DA1	20 µl	1.0×10^{-5}	980	10 µl of 5.0×10^{-5}	10 µl of 2.5×10^{-4}	1000
DA2	20 µl	5.0×10^{-5}	980	50 µl of 5.0×10^{-5}	10 µl of 2.5×10^{-4}	1000
DA3	20 µl	1.0×10^{-4}	980	100 µl of 5.0×10^{-5}	10 µl of 2.5×10^{-4}	1000
DA4	20 µl	2.0×10^{-4}	980	200 µl of 5.0×10^{-5}	10 µl of 2.5×10^{-4}	1000
DA5	20 µl	3.0×10^{-4}	980	300 µl of 5.0×10^{-5}	10 µl of 2.5×10^{-4}	1000
DA6	20 µl	5.0×10^{-4}	980	50 µl of 5.0×10^{-4}	10 µl of 2.5×10^{-4}	1000

Table 1: Dopamine Standards Preparation: 0.1M PCA was first prepared by diluting 428 µl PCA with 50ml HPLC grade water. Dopamine stock (5 mg/ml) was then prepared by dissolving 20 mg DA in 4 ml 0.1M PCA. A series of 1:10 dilutions with 0.1M PCA was carried out to yield a 5.0×10^{-5} mg/ml solution. The DHBA stock, internal standard, of 2.5 mg/ml was prepared by dissolving 10 mg of DHBA in 4 ml of 0.1M PCA. The stock was then diluted to 2.5×10^{-4} mg/ml by performing a 1:10 serial dilution. Six DA standards of equal volume were prepared with equal concentrations of DHBA.

Gene	**Primer**	**Sequence**
C. elegans CDNF	Forward	5'-CACTCGCAACAAGGAGAACA-3'
	Reverse	5'-CAGTCGAGTGGCTTGTCGTA-3'
Rat CDNF	Forward	5'-AAAGAAAACCGCCTGTGCTA-3'
	Reverse	5'-TCATTTTCCACAGGTCCACA-3'
Human CDNF	Forward	5'-AAAGACGCAGCCACAAAGAT-3'
	Reverse	5'-AGGATCTGCTTCAGCTCTGC-3'

Table 2: Primers used for qRT-PCR analysis of CDNF mRNA expression.

2.3.4 RNA Isolation of *C.elegans* samples

100 µl of *C.elegans* were homogenized in 1 ml of TRIzol reagent by passing the sample through a 21G needle and 1 ml syringe 20 times. Following this homogenization step RNA was extracted from the crude homogenate using the TRIzol method (Invitrogen Life Technologies, Burlington, Ontario, Canada) according to manufacturer's protocol. A DNase I kit from 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.

2.3.5 Real-Time qRT-PCR

Analysis of the CDNF gene copy number was performed by using QIAGEN OneStep RT-PCR kit (Qiagen Inc., Toronto, Ontario, Canada) according to the manufacturer's protocol. Real-time qRT-PCR reactions were performed in technical triplicates with each reaction having a volume of 20µl containing 80ng of total RNA. The following specific PCR primers (5µM) were used: forward primer, 5'-CACTCGCAACAAGGAGAACA-3' and reverse primer, 5'-CAGTCGAGTGGCTTGTCGTA-3'. In addition to the RNA loaded the reaction mix contained the following: 10 µl SYBR green (QIAGEN), 1.2 µl each of the 5µM CDNF primers; 0.2 µl reverse transcriptase, and nuclease-free water to a final volume of 20 µl. Real-time RT-PCR conditions were 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, 55 °C for 60 s, and 72 °C for 45 s, and lastly 95 °C for 1 min (1 cycle). MX3000P Real-Time RT-PCR (Stratagene, Mississauga, ON,

Canada) was used to perform all PCR reactions. Conditions were optimized to ensure amplifications were in the exponential phase and efficiencies remained constant throughout, no primer-dimers were detected. 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. Representative real-time RT-PCR products each showed 100% homology with the CDNF gene regions.

2.4 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Prior to analyses, outlier detection was performed using GraphPad Outlier Tool. Significance was defined as $p < 0.05$.

2.4.1 *C. elegans* Dopamine Levels

Dopamine levels, as determined through HPLC, were expressed in femtograms (fg) of DA per 100mg of of *C. elegans*. A one-way analysis of variance (ANOVA) was used to examine the effects of the various genetic mutants on dopamine levels between groups; followed by Tukey's post-hoc test.

2.4.2 *C. elegans* CDNF mRNA Expression

A one-way ANOVA was used to compare the differences in mRNA copy number expression between the various genetic mutants. Tukey's post-hoc tests were then employed.

2.5 Results

2.5.1 Quantification of Dopamine Levels

HPLC coupled to electrochemical detection was used to investigate the differences in DA concentrations in wild-type (*N2*), *cat-1*, *cat-2*, *dat-1* and *bas-1* strains in crude *C. elegans* homogenates. A one-way ANOVA showed a significant difference in DA concentrations, $F(4,10) = 3.396$, ****$p < 0.0001$ (**Figure 4**). Tukey's post-hoc tests determined that the elevated levels of DA in *cat-1* mutants was the only strain that showed a significant difference, ****$p < 0.0001$. *Cat-1* mutant strains are deficient in the synaptic vesicular monoamine transporter.

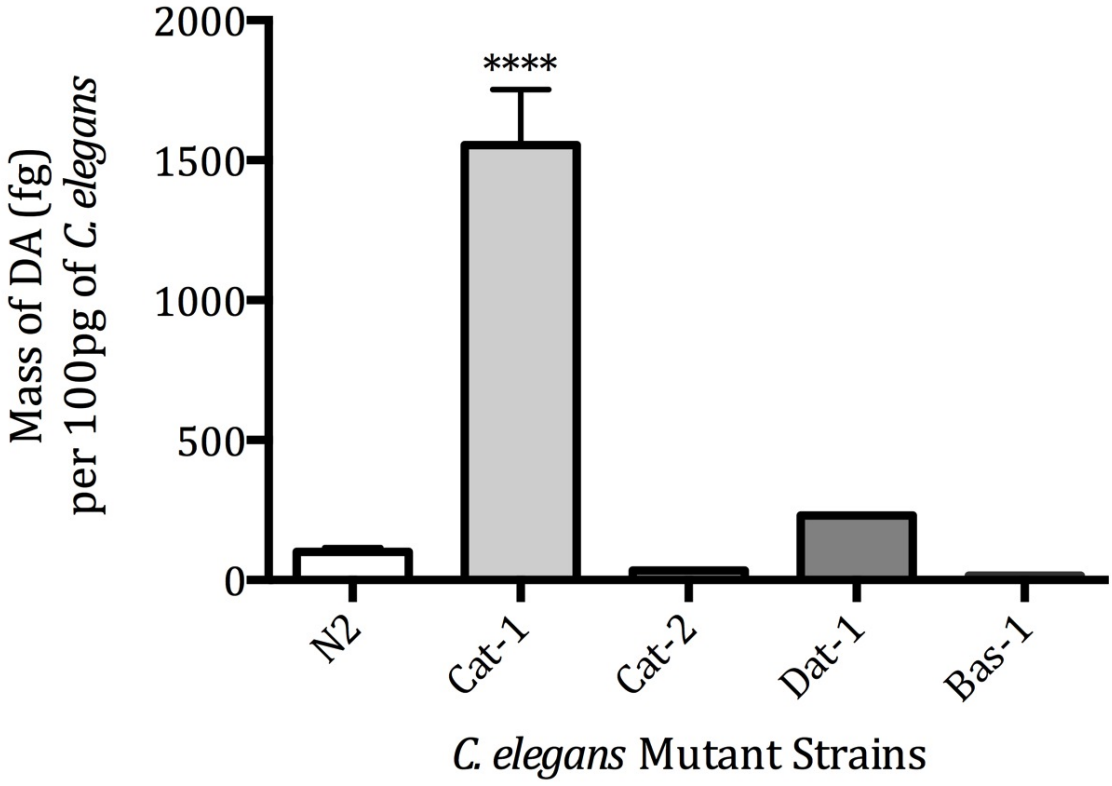

Figure 4: Dopamine quantification in *C. elegans* by high performance liquid chromatography. High performance liquid chromatography with electrochemical detection was performed on crude *C. elegans* homogenates to quantify DA concentration. Mass of dopamine was expressed in femtograms per 100 picograms of worms. ANOVA, $F(4,10) = 3.396$, ****$p < 0.0001$.

2.5.2 CDNF mRNA Expression

The differences in *C. elegans* CDNF mRNA expression in wild type, control, (N2), *cat-1*, *cat-2*, *dat-1*, *bas-1* and 6-OHDA treated wild type worms was analyzed using a one-way ANOVA and a significant difference was found, $F(5,12) = 1.068$, $^{**}p < 0.01$ (**Figure 5**). Tukey's post-hoc tests indicated a significant increase in *bas-1* CDNF mRNA expression in comparison to N2 worms, $^{*}p < 0.05$, *cat-2* worms, $^{*}p < 0.05$, and 6-OHDA treated worms, $^{***}p < 0.001$.

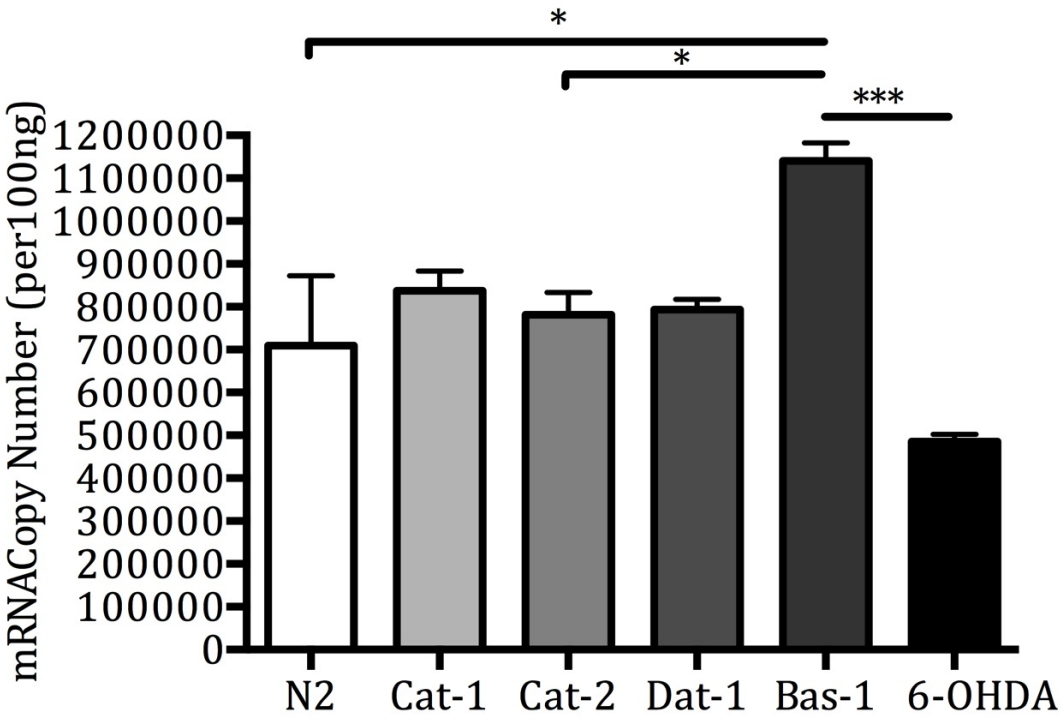

Figure 5: CDNF mRNA expression in *C. elegans*. The graph depicts *C. elegans* CDNF mRNA copy numbers for wild-type N2 worms, mutant strains *cat-1*, *cat-2*, *dat-1*, *bas-1* and 6-OHDA treated wild type worms. Real-time qRT-PCR experiments were performed and analyzed using one-way ANOVA, $F(5,12) = 1.068$, $^{**}p < 0.01$.

2.6 Discussion

Previous work has shown that various DA synthesis and transport mutants influence *C. elegans* swimming behaviour. Although DA neurotransmitter levels have been detected using by older techniques of formaldehyde-induced fluorescence they have not been thoroughly studied using the more recent, more effective techniques of HPLC. (Nass & Blakely, 2003; Sulston et al., 1975). By using HPLC coupled to electrochemical detection a significant difference in the concentrations of DA in wild-type (*N2*), *cat-1*, *cat-2*, *dat-1* and *bas-1* strains in *C. elegans* was detected. The *cat-1* gene encodes for the synaptic vesicular monoamine transporter; dopamine is loaded into synaptic vesicles encoded by the *cat-1* gene and is found in neurons at similar concentrations to those in mammalian neurons (Nass & Blakely, 2003). C*at-1* mutants were found to have significantly elevated in DA concentrations compared to all other strains. This increase in DA is in line with behaviour studies that found an increase in electrotaxis swimming speed in *cat-1* mutants (Salam et al., 2014). Also, previous studies investigating amphetamine and its affinity for VMAT-2 has shown that the prevention of vesicular uptake of DA by amphetamine may reduce DA at the synapse but also cause an increase cytoplasmic concentrations (Brown, Hanson & Fleckenstein, 2002; Gonzalez, Walther, Pazos & Uhl, 1994). This result could be attributed to the disparity between homogenate and vesicular preparations. The HPLC-ECD performed looks at DA levels in whole *C. elegans* homogenates. DA concentration in strains without the ability to encode tyrosine hydroxylase (TH), *cat-2*, were depleted, although not significantly. The reduction in DA concentration

in these mutants was consistent with findings by Sulston et al. (1975) using formaldehyde-induced fluorescence and Sanyal and colleagues (2004) study using HPLC. This indicates that majority of the *C. elegans* DA is synthesized by means involving TH. Worms deficient for *dat-1*, plasma membrane dopamine transporter showed a slight, non-significant increase in dopamine concentration. This inability of DA to be taken back up presynaptically due to *dat-1* mutation results in an elevated amount of extracellular DA. This extracellular accumulation is paired with an increase intracellular production to compensate for the lack of DA reuptake which is reflected in homogenate DA analysis with HPLC-ECD (McDonald, Hardie, Lessen, Carvelli & Matthies, 2007; Cao, Gelwix, Caldwell & Caldwell, 2005). *Bas-1* mutants, deficient in the ability to encode AADC for the synthesis of dopamine from L-DOPA also showed a non-significant reduction in dopamine concentrations in comparison to wild-type strains. Giving insight into the integral role of AADC for the synthesis of DA in *C. elegans* and not just limited to the mammalian system (Loer & Kenyon, 1993).

Our study took a novel approach to investigating the role of CDNF and the DAergic system by measuring CDNF mRNA expression in *C. elegans* DA synthesis and transport mutants. CDNF mRNA expression in *bas-1* mutants showed a significance increase in expression. The *bas-1* mutant is unable to convert intracellular L-DOPA to dopamine. As a result the excess L-DOPA is believed to have caused the increase in CDNF mRNA expression. Studies in rats of repeated treatment with L-DOPA have shown to increase BDNF mRNA expression in the SN. It is unclear

if the increase is a result of therapeutic actions of L-DOPA or to compensate for L-DOPA related toxicity (Mena, Davila & Sulzer, 1997; Zhang, Andren & Svenningsson, 2006). Also, 6-OHDA treated worms showed a slight, non-significant decrease in CDNF mRNA expression. Although it is possible that due to the short exposure time, 2 hours, and the small number of DAergic neurons, that these results were not significant, however, as it is these findings suggest CDNF is unaffected by 6-OHDA treatment. The mechanism underlying 6-OHDA toxicity are well established and may provide a possible explanation for why mRNA expression of CDNF was not significantly reduced. 6-OHDA is readily taken up via DA transporters where it causes oxidative stress (Tieu, 2011). This can interfere with normal ER functioning and trigger a UPR response leading to apoptosis (Chen et al., 2004). Despite DAergic neuron degeneration by 6-OHDA *in vitro* studies have shown that ER stress has only a minor effect on CDNF protein secretion (Apostolou et al., 2008). This minor effect could be reflected in the mRNA expression within the *C. elegans* organism. Future studies should investigate various concentrations and duration of 6-ODHA exposure as well as other neurotoxins such as MPTP.

C. elegans provide an opportunity to study the dopamine synthesis pathway and gain insight into the roles it may play in human diseases such as Parkinson's. Biochemical and behavioural studies can help better understand the pathology of the disease and be a means for therapeutic discovery.

Chapter 3

CDNF mRNA Expression and Protein Concentration in A Rat Model of Parkinson's Disease

3.1 6-Hydroxydopamine Model of Parkinson's Disease

Rodents prove to be an excellent model for studies on neurodegenerative disorders such as PD. Unilateral 6-OHDA induced degeneration of the nigrostriatal DA pathway is a classical example of one such animal model of PD currently used. It was the first PD animal model of human disease ever generated and since its introduction over 40 years ago, it has remained one of the most widely used lesions in the nigrostriatal dopaminergic system (Ungerstedt, 1968). 6-OHDA is not capable of crossing the blood brain barrier therefore it is typically stereotactically injected directly into the desired brain region causing a massive degeneration of dopaminergic neurons (Tieu, 2011). Dopamine transporters have a high affinity for 6-OHDA and it is readily taken up into the neurons where it accumulates in the cytosol. Here it is oxidized and generates reactive oxygen species causing oxidative stress damaging proteins, lipids and DNA (Haghdoost-Yadzi et al., 2009; Tieu, 2011). Additionally, 6-OHDA inhibits activity of mitochondrial complexes I and IV which leads to mitochondrial impairment and ATP deficiency. These negative effects of 6-OHDA lead to the apoptosis of dopaminergic neurons (Haghdoost-Yadzi et al., 2009).

An advantage of using the unilateral 6-OHDA model of PD in rats is that an injection of a dopamine receptor agonist, such as apomorphine or amphetamine, induces a characteristic 360° rotational motor behaviour. This motor response is a standard behavioural test to validate the lesion and is used as a means to quantify the effectiveness of dopamine therapies. In particular, apomorphine stimulates the

sensitized DAergic receptors on the lesioned side of the brain, leading to contralateral rotation, whereas amphetamine stimulates dopamine release from intact terminals leading to ispilateral rotations of the animal (Tieu, 2011). Both methods are easily quantifiable.

3.2 Objective

As described earlier Lindholm and colleagues (2007) have demonstrated in unilateral 6-OHDA lesion PD model rats that treatment with CDNF has both neuroprotective and neurorestorative effects. These results sparked further research into the novel NTF and these studies supported the earlier claim of CDNFs therapeutic effects (Lindholm & Saarma, 2009; Parkash et al., 2009). Despite this research the role of CDNF in the etiology of PD is still not understood, and no study has investigated how this neurodegenerative disease affects endogenous levels of CDNF. The purpose of this study was to fill in this gap within the literature and investigate how does a unilateral 6-OHDA lesion affect native levels of CDNF. Changes in this novel neurotrophic factors mRNA expression in platelets was investigated as well as examining the changes in CDNF protein expression in the SN and striatum. It was hypothesized that both mRNA expression and protein expression of CDNF will be decreased.

3.3 Methodology

3.3.1 Animals

Eight adult male Sprague-Dawley Parkinson's model rats (225-300g) were ordered from Charles River Laboratories (Senneville, Quebec, Canada) at 225-300g. A single injection lesion to the left SNpc was performed using 12 μg 6-OHDA. To confirm the success of the lesion an apomorphine dopamine challenge (0.2mg/kg, subcutaneous (SQ) injection) was performed 5-7 days after surgery. The rotational behavioural criteria for successful lesioning was quantified by a minimum average of 5 rotations per minute over a period of 5 minutes. Animals were shipped and delivered to the Central Animal Facility at McMaster University (Hamilton, Ontario, Canada). Before being subjected to any behavioural testing rats were allowed 7 days to adjust to the environment and were handled by experimenters during this time. Animals were housed individually in standard cages in a room at 22°C kept on a 12 hour reverse light/dark cycle (7am-7pm). Rats were given access to food and water *ad libitum*. All animals housing and testing was done in compliance with the Canadian Council on Animal Care, as well as approved by the Central Animal Facility (CAF) at McMaster University.

3.3.2 Lesion Validation

As previously mentioned (Section 3.1) an advantage of the unilateral 6-OHDA model of PD in rats is the ability to validate the lesion using a dopamine challenge. Therefore after 14 days in the animal facility, 24 hours prior to sacrificing the animals, the lesion was confirmed using an apomorphine challenge. Each rodent was

weighed and received a 0.5 mg/kg SQ injection of R-(-)-apomorphine hydrochloride hemihydrate (Sigma Aldrich, Cat#A4393, Oakville, Ontario, Canada) in a 0.1% ascorbic acid vehicle. Once injected, the animal was placed in a clear plastic cylinder and allowed to habituate for 5 minutes. After habituation the rats spontaneous movement was observed for 30 minutes and the number of apomorphine-induced rotations (360°) was recorded. Following testing each rat was returned to its home cage and the cylinder was cleaned with 75% ethanol. This protocol was repeated for each rat. The unilateral 6-OHDA lesion was deemed successful if the critera of 5 rotations per minute was reached (**Table 3**)

Rat ID	Total Number of Apomorphine Rotations in 30 Minutes	Average Rotations per Minute
20	417	13.9
21	586	19.5
22	279	9.3
23	416	13.9
24	426	14.2
25	423	14.1
26	231	7.7
27	229	7.6

Table 3: Lesion Validation. Lesion status was confirmed by challenging 6-OHDA lesioned rats with apomorphine. Complete 360 degree contralateral rotations were scored and a successful lesion was validated by a minimum of 5 rotations per minute. Testing was done 24 hours prior to sacrificing.

3.3.3 Sacrifice and Tissue Collection

24 hours were allowed to pass following the apomorphine challenge before the rats were sacrificed to ensure the residual effects of the dopamine agonist were eliminated. Animals were heavily anaesthetized with isoflurane (Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada), 7-10ml of intracardiac blood was collected (see section 3.3.5) and then the animal was quickly decapitated in accordance with McMaster University, CAF guidelines. Brains were quickly removed and the striatum and SN from each hemisphere were dissected. Tissues were stored at -80°C until further use.

3.3.4 Protein Quantification and Immunoblotting

A Bradford Assay was used to determine protein concentrations using the Bio-Rad Protein Assay reagent (Bio-Rad, Mississauga, ON, Canada) and a CU-640 spectrophotometer (Beckman-Coulter, Mississauga, ON, Canada). Sample optical densities were measured at 595 nm in duplicates. A purified protein bovine serum albumin (BSA) standard of 1.2 – 10.0 µg/ml was used to quantify the rat tissue. Samples that fell outside of the linear range were diluted with 50 nM Tris and 1 mM EDTA (pH 7.4) and reanalyzed. Samples were stored at -80°C until further use.

CDNF Protein concentrations in PD model 6-OHDA rats were determined by immunoblotting. Samples concentrations were calculated and 15µg of protein (30µl volumes) was separated on 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel (10 ml 1.5M Tris, pH 8.8; 8.9 ml dH2O; 400 µl 10% SDS, 20 ml 30% acrylamide, 400 µl 10% ammonium persulfate (APS) and 30 µl of TEMED. To

minimize variations between gels the samples were loaded in a random order a stacking gel. The use of a stacking gel ensures all the samples entered the separating gel simultaneously. The 4% stacking gel was prepared as follows: 5.00ml 0.5M Tris, pH 6.8, 12.20ml dH2O, 200µl 10% SDS, 2.60ml of 30% acrylamide, 100µl 10% APS and 20µl TEMED.

Gels were loaded into a Bio-Rad Mini-PROTEAN III rig (Bio-Rad, Mississauga, Ontario, Canada) containing 1x running buffer (10x running buffer of 15 g/L Tris base, 72 g/L glycine, and 5 g/L SDS, diluted with distilled H2O) and run at 60V through stacking gel and 100V through separating gel. The gels were then removed from the rig and equilibrated in transfer buffer (12 mM Tris, 96 mM glycine, and 10% methanol (MeOH), diluted in distilled H2O) for 15 minutes. Polyvinylidene difluoride (PVDF) membranes were activated in 100% methanol for 10 seconds, and allowed to equilibrate in transfer buffer for 15 minutes. Gels were then transferred onto PVDF membranes using the Bio-Rad Wet Transfer Unit (Bio-Rad). Transfer was performed for 1 hour in transfer buffer at 100V while cooled with an ice pack to prevent over-heating.

Once transferred, the resulting blots were immersed in blocking solution (5% skim milk, 50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 8.5) for 1 hour at room temperature and exposed to primary CDNF antibody (rabbit polyclonal; diluted at 1:5000) and housekeeping gene GAPDH (diluted to 1:10000), each diluted in TBS-T (50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 8.5), overnight at 4°C. Following the overnight incubation period the 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 1:5 000 dilution; anti-GAPDH secondary anti-mouse at 1:10 000 dilution) for 1.5 hours at room temperature. Membranes were then washed twice in TBS-T for 5 minutes, and then one last TBS-T wash for 10 minutes.

Visualization of the probed protein bands was achieved using Amersham ECL Prime Western Blotting Detection reagents (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). Reagent 1 and Reagent 2 of the kit were mixed together briefly and applied to the membranes for 1 min (1.5 mL of each reagent per membrane). Membranes were then quickly blotted dry using filter paper, and wrapped in low density polyethylene wrap for 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 software(NIH, Bethesda, Maryland, USA).

3.3.5 Blood Collection and Platelet Preparation

Rats were put under deep general anaesthetized with isoflourane (Pharmaceutical Partners of Canada Inc., Richmond Hill, Ontario, Canada) and pedal reflex was checked to ensure the rodent could not feel pain. An incision at the level of the xiphoid process was made, and subsequently cut along the full length of the sternum just to the right of the rat's midline. A lateral incision through the chest wall

was cut just inferior to the diaphragm. The chest wall flaps were retracted and the heart was exposed with a blunt dissection of the pleura and pericardium. A 23G was inserted into the right ventricle and 7-10ml of intracardiac blood was collected in 10ml BD vacutainers with 1.42ml of acetate citrate dextrose.

Platelet-rich plasma (PRP) preparation was carried out by adapting the protocol proposed by Hranilovic (1996). The collected blood was centrifuged at 1250g for 35 seconds, at environmental temperature 22°C. After centrifugation a red lower fraction (red blood cell component) and an upper pale-yellow turbid fraction (blood plasma component) was observed. The top two-thirds of the blood serum component was removed using a pasture pipette and put into a 15 ml conical centrifuge tube. Cold PBS-EDTA-Bovine Serum Albumin (PEB; pH 7.0) was added at a ratio of 1:2, the sample was then resuspended and spun for 20 minutes at 1220g. The supernatant was removed and discarded; the remaining pellet was resuspended in 7ml of cold PEB and centrifuged for 5 minutes at 1220g. The supernatant was removed and the pellet was resuspended in 1ml of PEB. The resuspension 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 removed and the pellet was stored at -80°C until future use.

3.3.6 RNA Isolation

RNA isolation protocol was performed as described in section 2.3.4.

3.3.7 Real-Time qRT-PCR

Rat CDNF mRNA primers were designed similar to Section 2.3.3 for *C.elegans* CDNF primer design, however the mRNA transcript was obtained from www.ncbi.nlm.nih.gov. PCR products showed 100% homology with the rat CDNF gene regions.

Protocols for qRT-PCR was previously described in section 2.3.5; however the total RNA for each well was 60ng and the specific PCR primers (5μM) used were: forward primer, 5'-AAAGAAAACCGCCTGTGCTA-3' and reverse primer, 5'-TCATTTTCCACAGGTCCACA-3' (**Table 2**). The real-time qRT-PCR conditions were 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).

3.4 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Prior to analyses, outlier detection was performed using GraphPad Outlier Tool. Results for immunoblotting and qRT-PCR were analyzed using a two-tailed student's *t*-test were Significance was defined as $p < 0.05$. PCR results were analyzed as previously described in section 2.4.2.

3.5 Results

3.5.1 Effects of Unilateral 6-OHDA Lesion on CDNF Protein Expression

To study the effects of a 6-OHDA lesion on CDNF protein expression rats with a left SN unilateral 6-OHDA lesion were studied. **Figure 6** compares the percent expression of the CDNF protein in the SN of intact hemisphere (right) and the lesioned hemishphere (left) using a student's *t*-test. Results indicated no significant difference between the two hemispheres, *ns*. To study the anterograde effects of the lesion on CDNF protein expression the striatum was also examined (**Figure 7**). A comparison of percent expression of the CDNF protein in the right striatum and left striatum showed no significant difference after a student's *t*-test was performed, *ns*.

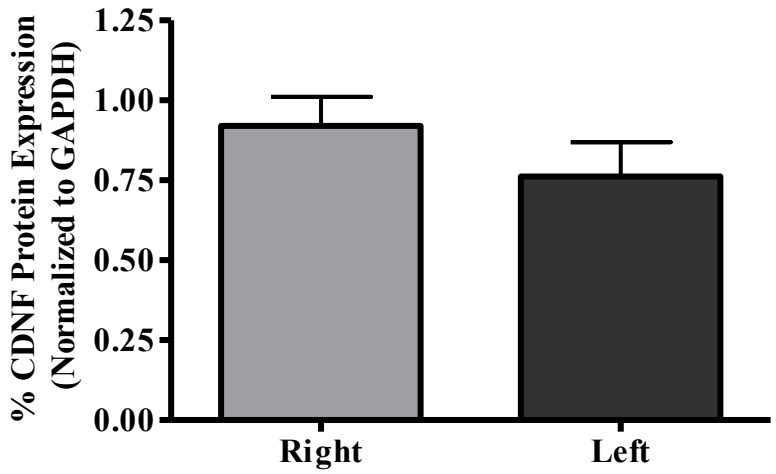

Figure 6: CDNF protein expression in 6-OHDA rat substantia nigra. Percent protein expression of CDNF in the right substantia nigra versus the left. Rats received 6-OHDA lesion in the left substantia nigra, n = 8. Protein expression was normalized to the housekeeping antibody GAPDH, stuent's *t*-test was used to determine difference in expression. Results indicated no significant difference in protein expression between the right and left substantia nigra.

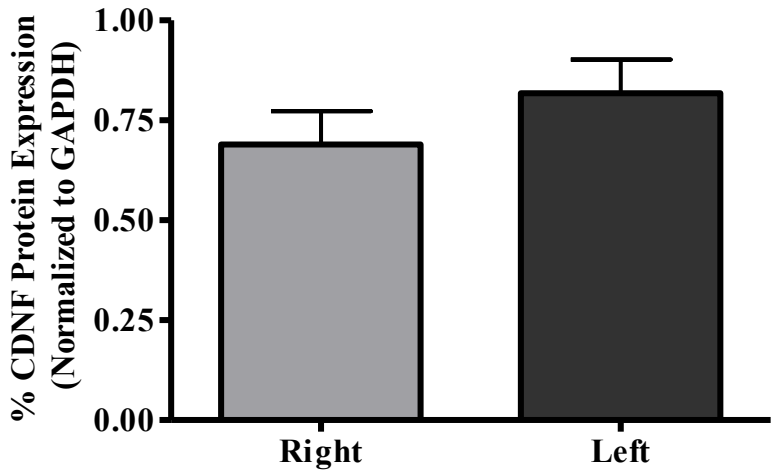

Figure 7: CDNF protein expression in 6-OHDA rat striatum. The graph depicts percent protein expression of CDNF in right versus left striatum. Rats received 6-OHDA lesion in left substantia nigra, n = 8. Protein expression was normalized to the housekeeping antibody GAPDH, stuent's *t*-test was used to determine difference in expression. Results indicated no significant difference in protein expression between the right and left striatum.

3.5.2 Effects of Unilateral 6-OHDA Lesion on CDNF mRNA Expression

Following verification of the presence of the 6-OHDA lesion using apomorphine, the expression of CDNF mRNA was analyzed in the blood. . However, since this study did not include control rats, blood from the striatally infused aCSF rats described in section 4.3.1 were used as a control. No significant difference was found between the lesioned animals in comparison to the control groups, ns (**Figure 8**).

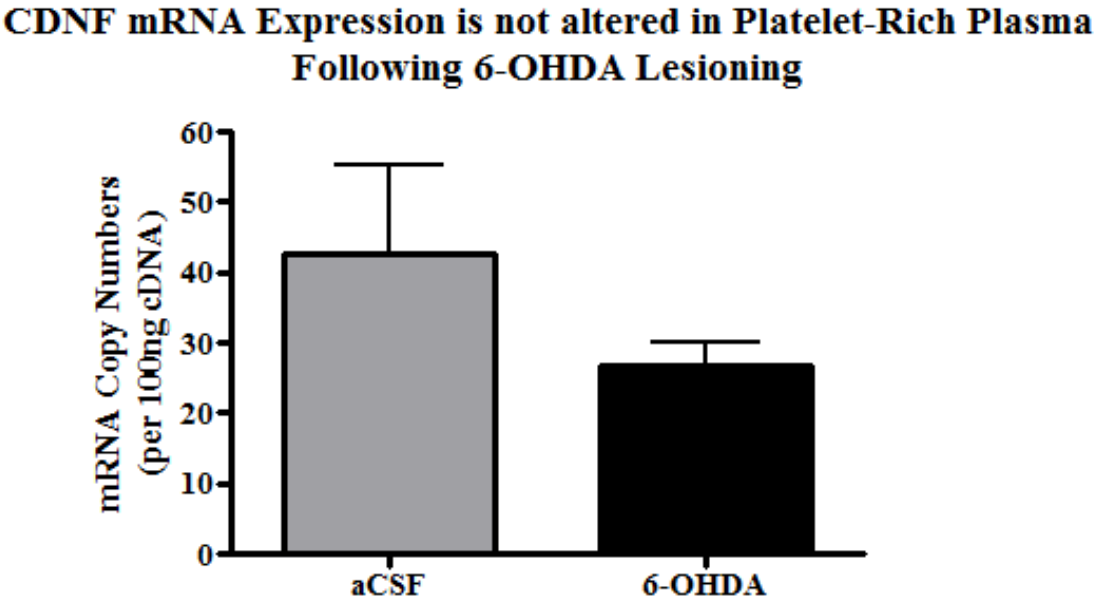

Figure 8: CDNF mRNA expression in platelet rich plasma following 6-OHDA lesion in rat SN. The graph depicts platelet CDNF mRNA copy numbers (mean ± SEM) in rats following a 6-OHDA lesion to the SN (n = 8)in comparison to control aCSF rats (n = 8) from section 4.3.1. Real-time qRT-PCR experiments were analyzed using a two-tailed stuent's *t*-test, no significant difference was found between the groups, *ns*. There was equal variance across the groups (Bartlett's test, $^{**}p < 0.01$).

3.6 Discussion

The novel evolutionary conserved protein, CDNF, has been identified in the adult rat striatum and SN and extensive amount of research has identified its neuroprotective and neurorestorative effects on DAergic neurons (Cheng et al., 2013; Lindholm et al., 2007; Mei & Niu, 2014). To date, no studies have looked at endogenous changes in CDNF protein and platelet mRNA expression levels following severe degeneration of DAergic neurons. As previously mentioned, 6-OHDA is readily taken up into neurons via dopamine transporters. Intracellularly it is oxidized and generates reactive oxygen species causing oxidative stress, also 6-OHDA inhibits mitochondrial complexes I and IV, both of which lead to apoptosis (Haghdoost-Yadzi et al., 2009; Tieu, 2011). When injected into the SN, dopaminergic neurons begin to degenerate within 24 hours and the toxin moves anterogradely to the striatum and 48-72 hours later striatal DAergic neuron degeneration begins (Faull & Laverty, 1969; Jeon, Jackson-Lewis & Burke, 1995).

The ER is responsible for the synthesis, post-translational modifications, folding, export and secretion of membrane protein (Kim, Emi, Tanabe & Murakami, 2006). Oxidative stress, such as that caused by 6-OHDA exposure, can interfere with the normal functioning of the ER, this can result in the increase and accumulation of misfolded proteins (Marciniak & Ron, 2006). This can initiate the UPR, to return the cell to homeostatic conditions or if the damage is excessive, to cause cell death (Ryu et al., 2002). 6-OHDA has also been reported to induce ER stress and the UPR causing cell death (Chen et al., 2004). The carboxy-terminal domain of CDNF, which

consists of a CXXC active site motif, may facilitate protein folding and reduce ER stress (Lindholm & Saarma, 2009).

This study examined endogenous CDNF protein and mRNA concentrations following a unilateral 6-OHDA lesion to the SN. The results concluded that there was no significant difference in protein concentration or platelet mRNA expression of CDNF. It is possible that the loss of nigrostriatal DA innervation increased the trophic activity directed to these neurons resulting an upregulation of CDNF similar to what was seen when neurotrophic support was upregulated when extracts of lesioned rat striatum was cultured with non lesioned striatum (Yurek & Fletcher-Turner, 2001). Research by Airavaara et al. (2009) in rodent ER stress studies have found that MANF, belonging to the same evolutionary conserved family as CDNF, is upregulated in response to ER stress. However it is important to note that findings from *in vitro* studies that suggest ER stress has only a minor effect on secretion of CDNF proteins (Apostolou et al., 2008). Although 6-OHDA lesion animals are widely established as a model for PD, they do not mimic all the clinical and pathological features characteristic of PD (Dauer & Przedborski, 2003). For instance, 6-OHDA does not affect other brain regions and does not result in the formation of lewy bodies that are characteristic of PD (Dauer & Przedborski, 2003). The role of CDNF in PD may function through these mechanisms as opposed to the ones that are active 6-OHDA models.

Although the degeneration of DAergic neurons by 6-OHDA lesioning was anticipated to reduce CDNF protein and mRNA expression, this was not seen. To

better understand the role of CDNF in PD animal models, if there is an upregulation or expression is not affected by ER stress further research is needed. TH-positive cell bodies and density of TH-positive fibres could be investigated further to look at the extent of the dopaminergic neuron degeneration. To further understand how PD affects endogenous levels of CDNF, MPTP or genetic models of PD should also be investigated.

Chapter 4

Understanding the Role of CDNF in the Pathophysiology of Parkinson's Disease Through the Selective CDNF Knock-Down in Rats

4.1 Antisense Oligodeoxynucleotide Technology

Antisense (AS) oligodeoxynucleotide (ODN) technology supresses the mRNA and protein levels of the target gene, consisting of chemically modified single-strand DNA complementary to the target mRNA a RNA/DNA duplex which is formed and thereby inhibits translation (Miyake, Hara, & Gleave, 2005). The most common mechanism of AS action is the occupancy activated destabilization method. RNaseH, a ubiquitous enzyme, hydrolyses the target mRNA strand of the RNA/DNA duplex. The less common occupancy only mechanism utilizes a steric blockade of splicing, arrests translation and prevents 5'- capping, inhibiting transport (Crooke, 2000; Miyake et al., 2005). AS ODN were originally proposed in 1978 and is one of the oldest gene knockdown techniques. Over the years the technology has been refined and successfully used for a variety of diseases, including cancer, insulin resistance, neurological diseases and inflammatory disease (Lee, Crosby, Baker, Graham & Crooke, 2013). When designing AS ODNs simple phosophodiester oligonucleotides cannot be used because they are too readily digested *in vivo*. Modifications need to be made to the oligonucleotide backbone rendering the oligonucleotide resistant to nuclease activity while not compromising binding affinity for target mRNA (Lee et al., 2013). One major backbone modification implements the substitution of a sulphur atom from a nonbridging oxygen at each phosphorus to produce a phosphorothioate ODN (Lebedeva & Stein, 2001). These first generation oligonucleotide analogs are much more resistant to nucleases than phophodiester oligonucleotides and are broadly distributed throughout all peripheral tissue

following intravenous administration but cannot cross the blood brain barrier (Miyake et al., 2005). Phosphorothioate ODN are not without their side effects such as acute toxicity and initiating clotting cascades; however, despite this, a number of clinical trials have successfully utilized phosphothioate ODNs (Crooke, 2000).

AS ODN can be used to knockdown selective proteins. In rodents, this can be achieved by administering AS ODN continuously through the use of osmotic mini pumps. The use of mini pumps and surgical implantation of cannulas allows for minimizing sequence degradation and transport distance as well as localizing knockdown to specific anatomical target area (Hsieh, Yang, Chu, & Kuo, 2007; Landgraf, 1996). Confirmation of knockdown can be confirmed by molecular techniques post mortem (Landgraf, 1996).

4.2 Objective

CDNF has previously been mentioned to have a neuroprotective and neurorestorative effect on DAergic neurons of the nigro-striatal pathway. Additionally, the chronic infusion of CDNF in 6-OHDA hemiparkinsonian model rats has been shown to prevent the behavioural deficits associated with PD and the accompanying loss of SN and striatal DA (Voutilainen et al., 2011). Despite these promising findings more research on CDNF is needed to better understand its role in PD as studies in other NTF have done. AS ODN studies in BDNF where its knockdown has lead to DAergic neuron death and behavioural deficits that mimicked PD model rats, however this is a major link that has not been established

in CDNF (Porritt et al., 2005). This critical gap in understanding the potentially causal role of this trophic factor in the neurodegeneration and pathogenesis of PD. Thus the aim of this project was to selectively knockdown CDNF in the rat striatum in order to better understand the role of decreased CDNF in the neurodegeneration and pathogenesis of PD. Specifically, to see if the 14 day knockdown of CDNF in the striatum will generate behavioural phenotypes similar to PD and how will it affect CDNF mRNA expression in platelets. The striatum was selected as the knockdown site based of the reasoning that an intrastriatal 6-OHDA lesion causes a slower, progressive degeneration of DAergic neurons more similar to the disease onset in human PD than intranigral regions (Sauer & Oertel, 1994).

It was hypothesized that the CDNF knockdown would result in a decrease of locomotor activity and motor coordination as well as cause a decrease in platelet mRNA expression.

4.3 Methodology

4.3.1 Animals

16 adult male Sprague Dawley rats (225-250g) were purchased from Charles Rivers Laboratories International (Senneville, Quebec, Canada). Animal housing protocol was previously described in section 3.3.1.

4.3.2 Antisense Sequence and Infusion

The antisense sequence was made to target the CDNF gene and was synthesized by Integrated DNA Technologies (IDT; Coralville, Iowa) (**Table 4**). To

prevent nuclease destruction all nucleotides were capped with phosphorothioates (Lebedeva and Stein, 2001).

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 groups: **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 for the selective knockdown of CDNF, the primary group of interest.

14 day infusion pumps (Model 2002) were purchased from Alzet (DURECT Corporation, Cupertino, California, USA). Osmotic pumps were used to ensure continuous daily infusion instead of the riskier manual infusion method. Also, direct cannula implantation with continuous infusion osmotic pumps allows for a more precise targeting of the brain region of interest and helps to minimize risk of infection. Each pump was filled with 1ml of 5nmol of solution (or equivalent volume of aCSF) and was connected to a cannula via PVC60 polyvinyl cannula tubing (DURECT Cooporation). Each pump infused at a rate of 0.5 μl/hr for 14 days. The intact hemisphere, in unilateral models, serves as an internal control structure.

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'

Table 4: Antisense Oligonucleotide Sequence. Phosphothioates are indicated with an * and were used to cap all nucleotides to prevent nuclease destruction *in vivo*.

4.3.3 Surgical Placement of Osmotic Pumps

Animals were placed in a chamber for the induction of anaesthesia using 3-5% isoflourane (Isofluorane, Pharmacuetical Partners of Canada Inc, Richmond Hill, Ontario, Canada) in an air mixture. The animal was then mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, California, USA) and a surgical plane of anaesthesia was maintained with 2.5% isofluorane by a nose cone fitted to the stereotaxic instrument. A midline incision 1.5-2cm in length was made in the scalp, from just above the eyes to the back of the head. A 28 gauge, stainless steel cannulas (Brain Infusion Kit 2, DURECT Corporation, Cupertino, California, USA) was unilaterally implanted into the left striatum according to the following Bregma coordinates: +0.7 mm anterior; +3.0 mm lateral; -5.0 mm ventral to the surface of the skull. The cannula was aligned with the coordinates and advanced into the hole in the skull. Over the lateral border of the left scapula a 5-6cm deep subcutaneous pocket was made with a blunt dissection and the osmotic pump was buried. The cannula was cemented in place and the incision was closed with staples. Animals were allowed one week for recovery before behavioural testing. Over the entire duration of the study, 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.

4.3.4 Behavioural Testing

Behavioural testing was performed prior to surgery to establish a baseline assessment. Behavioural assessments began 7 days following surgery to ensure adequate recovery time, and were repeated again at 14 days.

4.3.4.1 Locomotor Activity

Locomotor testing was completed during the dark period of the rodents light/dark cycle, since rats show maximum activity during these hours. Accuscan computerized cages (AccuScan Instruments, Columbus, Ohio, USA) were used to record total distance travelled (cm). Each rat was tested for 180 minutes, the first 30 minutes was a recorded habituation period followed by a 150 minutes period of recorded locomotor activity.

4.3.4.2 Balance Beam

Hindlimb and forelimb motor coordination during precise locomotion and balance was tested using the balance beam test (Heuer, Smith, Lelos, Lane, & Dunnett, 2012). The beam was adapted such that it was elevated 60 cm from the ground, was 2.5 cm wide and 80 cm in length. A 20 cm x 20 cm enclosed goal box was placed on one end to encourage the rat to move from the open space of the starting platform to the enclosed goal box 80 cm away. All rats were trained to traverse the balance beam prior to surgery for 2 consecutive days. Animals were first habituated to the goal box and then the starting point was moved further away from the goal box on successive trials until the rodent was able to traverse the entire distance of the apparatus. Animals were trained until they could walk the beam

unassisted, without falling, in 60 seconds or less. On the day of testing, all rats were given 3 trials, and were allotted 60 seconds to traverse the beam. A trial was considered a failure if the animal did not cross the beam within 60 seconds or fell off at any point in time.

4.3.5 Blood Collection and Platelet Preparation

Blood collection and platelet preparation protocol was performed as described in section 3.3.5.

4.3.6 RNA Isolation

RNA isolation protocol was performed as described in section 2.3.4.

4.3.7 Real-Time qRT-PCR

Real-time qRT-PCR was performed as described in section 3.3.7.

4.3.8 Sacrifice and Tissue Collection

Protocol was performed as previously described in section 3.3.3.

4.3.9 Protein Quantification and Immunoblotting

Protein quantification and immunoblotting protocols were performed as described in section 3.3.4. Immunoblotting was preformed to verify knockdown of CDNF in the striatum.

4.4 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Prior to analyses, outlier detection

was performed using GraphPad Outlier Tool. Significance was defined as $p < 0.05$. PCR results were analyzed as previously described in section 2.4.2.

4.4.1 Locomotor Activity

Total distance travelled (cm) during the 150 min recording period was analyzed to assess locomotor activity for each rat. To study the infusion effects on locomotor activity over the course of testing (baseline, day 7 and day 14 of infusion) a two-way ANOVA followed by Bonferroni post-hoc tests was applied. Locomotor activity within each treatment group was analyzed using a one-way ANOVA followed by Tukey's post-hoc test.

4.4.2 Balance Beam

The performance of the rats was video recorded and rated according to Yu et al. (2013). Neurological status of each rat was assessed using a 7-point Likert scale as follows: 1, unable to place affected hind limb on horizontal surface of beam; 2, affected hindlimb placed on horizontal surface of beam and balance maintained for atleast 5 seconds; 3, beam is traversed while dragging affected hindlimb; 4, beam is traversed and at least once the affected hindlimb is placed on the horizontal surface of the beam; 5, beam is traversed and affected hindlimb is placed on horizontal surface of beam to aid in less than 50% of its steps; 6, affected hindlimb used in more than half of its steps; 7, no more than 2 footslips. A two-way ANOVA was used to determine treatment effects over the infusion period, followed by Bonferroni post-hoc tests. A second analysis was performed by using a one-way ANOVA within each group, followed by Tukey's post-hoc test.

4.4.3 Immunoblotting

Data from immunoblotting was analyzed using a one-way ANOVA for repeated measures followed by Tukey's post-hoc test for aCSF and CDNF infused animals. Protein expression between hemispheres within groups 14 days post-surgery was analyzed using a student's *t*-test.

4.5 Results

4.5.1 Locomotor Activity: Effects of 14 day continuous infusion of CDNF antisense sequence on locomotor activity

Following the 14 day infusion period CDNF antisense sequences induced a significant increase in locomotion compared to aCSF infused control rats as shown by a two-way ANOVA analysis; $F(1,39) = 3.43$, $p < 0.0002$ (**Figure 9**). Bonferroni post-hoc tests determined that there was a significant increase in locomotor behaviour between CDNF and aCSF groups after an infusion of 14 days, $^{***}p < 0.001$, but not after 7 days, *ns*. A one-way ANOVA showed that CDNF infused rats has a significant increase in locomotor activity; $F(2,19) = 7.80$, $^{*}p < 0.0034$ (**Figure 10**). Tukey's post-hoc tests determined that locomotor behaviour was not significantly affected from baseline to day 7, *ns*, but did significantly increase, from day 7 to day 14, $^{*}p < 0.05$, and baseline to day 14, $^{**}p < 0.01$. Control aCSF rats did not show a significant change in locomotor activity across time points suggesting that the surgical procedure did not affect locomotor activity, *ns* (**Figure 11**).

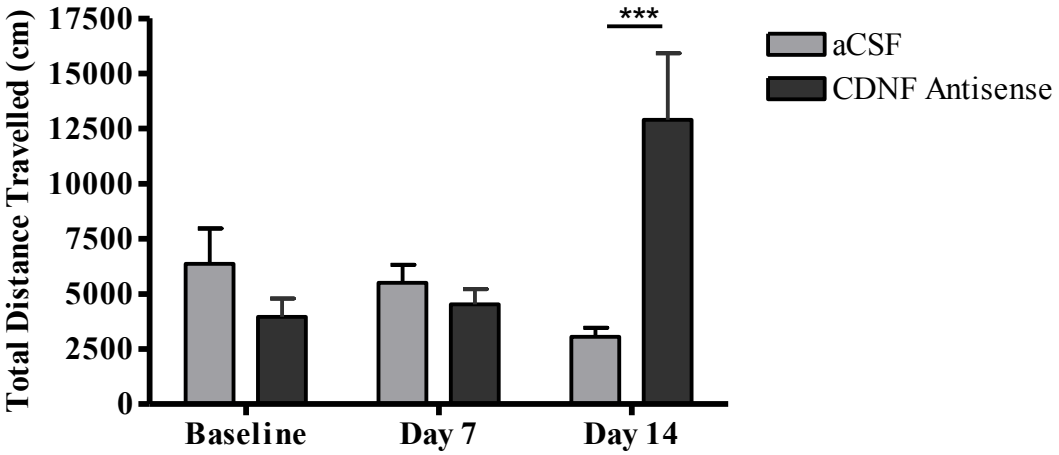

Figure 9: Locomotor activity in aCSF- and Antisense-infused rats following a 14 day infusion. The graph depicts locomotor activity as total distance travelled (cm) (mean ± SEM) for each treatment group at baseline, 7 day- and 14 day infusions. CDNF knockdown and aCSF values are each an average of 8 rats. $F(1,39) = 3.43$, $p < .0002$. CDNF knockdown vs. aCSF locomotor activity were not significantly different at baseline nor were they different after one-week of infusion. Two-weeks of infusion significantly increased CDNF knockdown locomotor activity in comparison to aCSF, $^{***}p < 0.001$.

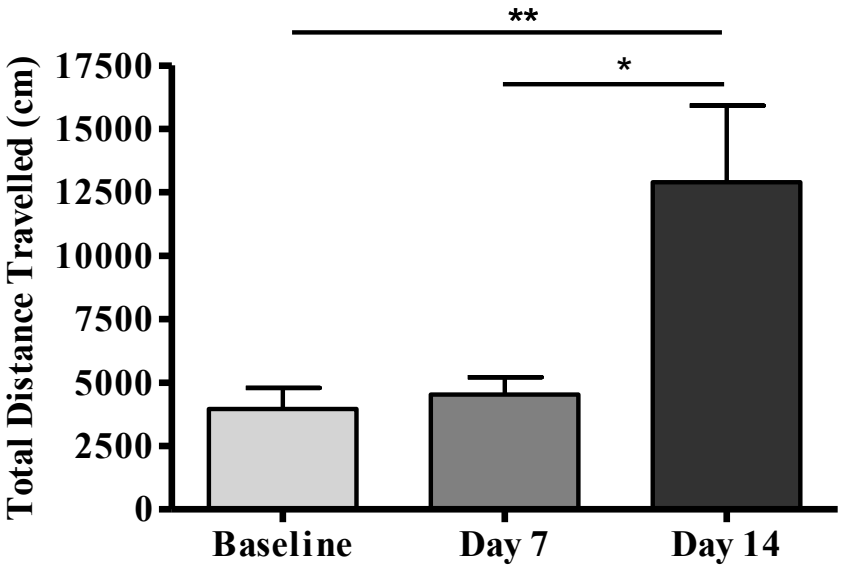

Figure 10: Locomotor activity in CDNF infused rats. The graph depicts locomotor activity as total distance travelled (cm) (mean ± SEM) following the continuous infusion of CDNF antisense at baseline, 7 day- and 14 day infusions. $F(2,19)= 7.80$, $*p < .0034$.

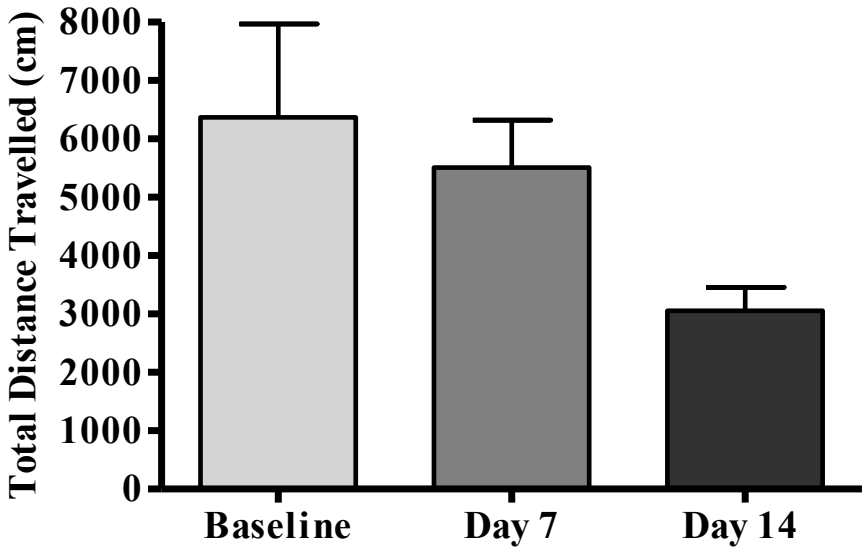

Figure 11: Locomotor activity in aCSF infused rats. The graph depicts locomotor activity as t total distance travelled (cm) (mean ± SEM) following continuous infusion of aCSF at baseline, 7 day- and 14 day infusions, *p* = *ns*.

4.5.2 Motor Coordination: Effects of 14 day continuous infusion of CDNF antisense sequence

14 day infusion of CDNF antisense significantly impaired motor coordination, main treatment effect, $F(1,42) = 13.29$, $^{***}p = 0.0007$; and within group effect across testing days, $F(2,42) = 24.40$, $^{****}p < 0.0001$) (**Figure 12**). CDNF antisense infusion progressively deteriorated motor coordination compared to aCSF animals. At 7 day infusion CDNF knockdown showed impairment compared to aCSF, $^{*}p < 0.05$, which further increased in severity after 14 days of infusion, $^{**}p < 0.01$ (**Figure 13**). The neurological status in CDNF knockdown animals showed significant decline in as little as one week when analyzed with a one-way ANOVA, $^{*}p < 0.05$. Tukey's post-hoc tests revealed that motor impairments were present between baseline and day 7 of infusion ($^{***}p <0.0001$) and these impairments were maintained between baseline and day 14 ($^{***}p < 0.0001$). Motor impairments did not worsen between the two time periods, $p = ns$ (**Figure 14**). No significant difference was found in control aCSF animals at any time, indicating an effect of treatment rather than surgical procedure.

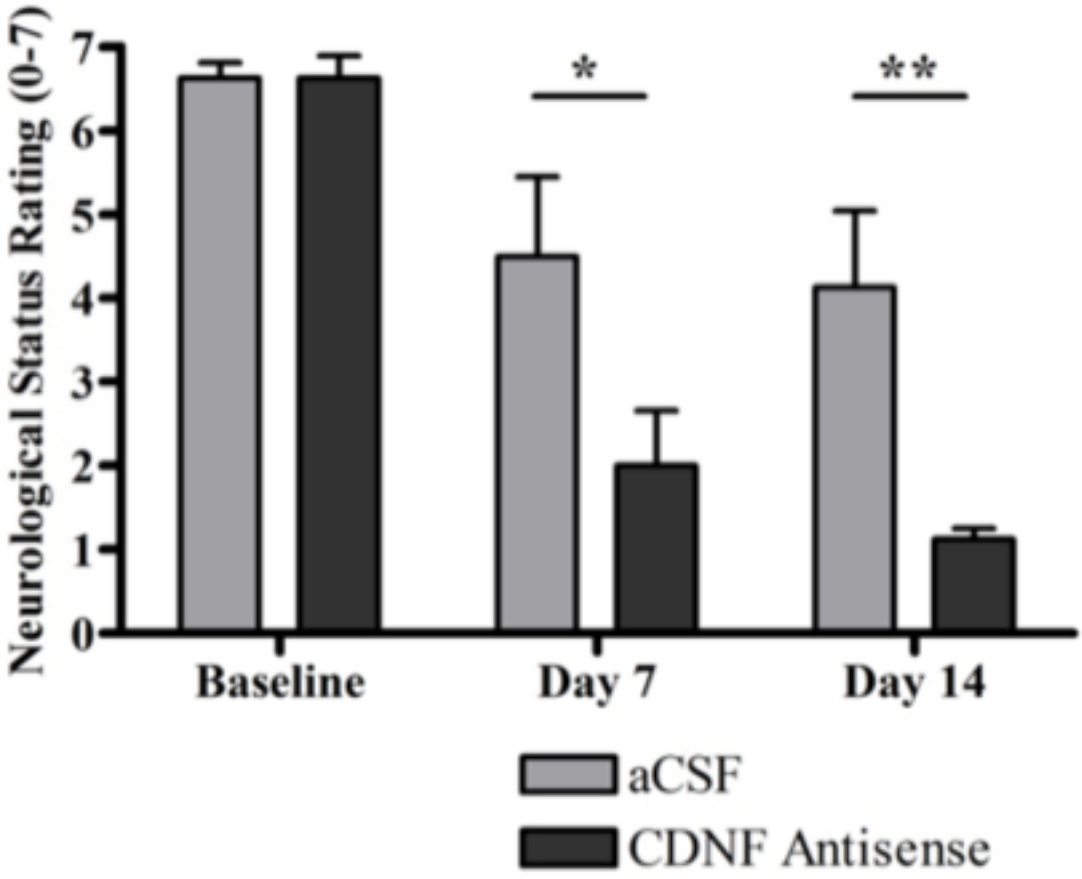

Figure 12: Motor coordination in aCSF- and Antisense-infused rats following a 14 day infusion. The graph depicts neurological status on a balance beam apparatus (mean ± SEM) for each treatment group at baseline, 7 day- and 14 day infusions. $F(1,42) = 13.29$, ***$p = 0.0007$. CDNF knockdown and aCSF values are each an average of 8 rats. At baseline CDNF knockdown vs. aCSF results were not significantly different. After 7 days of infusion CDNF knockdown motor coordination was significantly impaired in comparison to aCSF *$p < 0.05$; 14 days of infusion also showed significant decline in motor coordination, **$p < 0.01$.

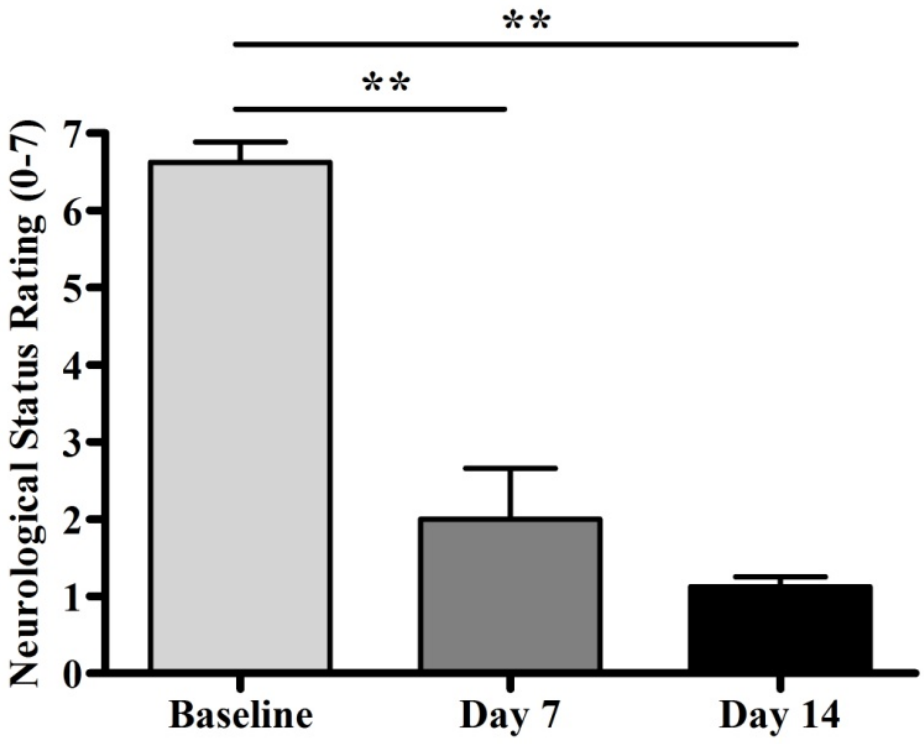

Figure 13: Motor coordination in CDNF infused rats. The graph depicts neurological status on beam walk apparatus (mean ± SEM) following the continuous infusion of CDNF antisense at baseline, 7 day- and 14 day infusions, (*$p < 0.05$).

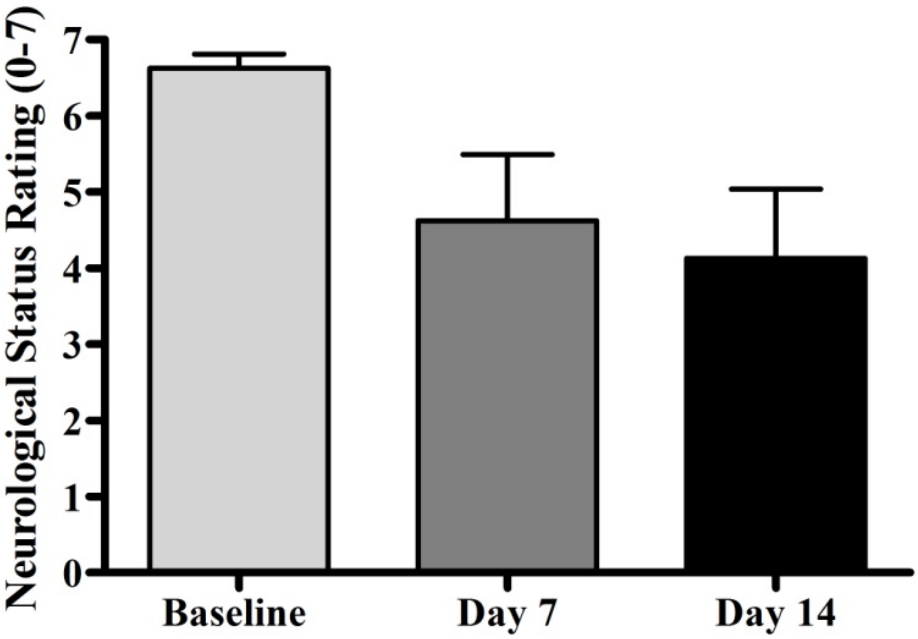

Figure 14: Motor coordination in aCSF infused rats. The graph depicts neurological status on beam walk apparatus (mean ± SEM) following continuous infusion of aCSF at baseline, 7 day- and 14 day infusions, *p* = *ns.*

4.5.3 Verification of CDNF Knockdown

The CDNF knock down was verified by measuring protein concentrations of the neurotrophic factor following the 14 day infusion period. CDNF protein concentration between the right and left striatum for aCSF infused rats showed no significant difference, *ns* (**Figure 15**). Unexpectedly, the CDNF antisense group also showed no significant difference between the two hemispheres, *ns*, indicating that the antisense knockdown was not successful in silencing gene expression (**Figure 16**). Due to these results a one-way ANOVA between groups was not performed.

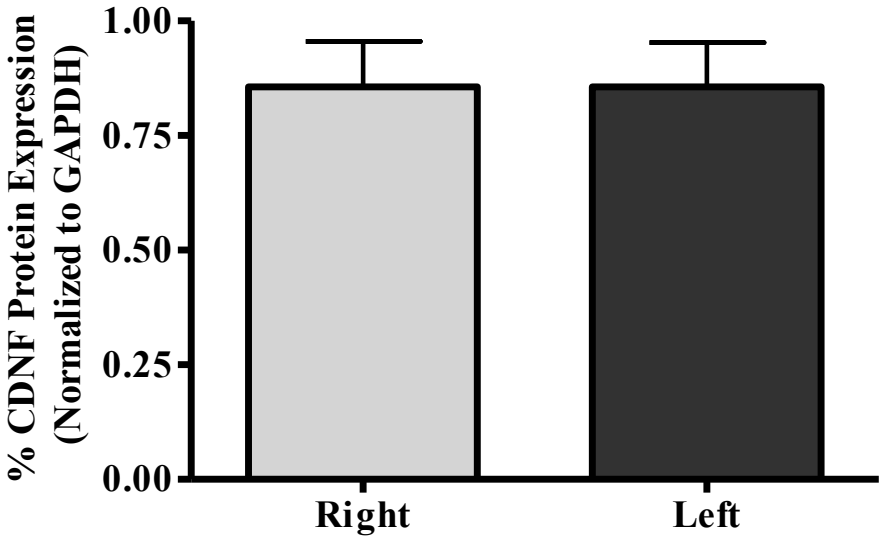

Figure 15: CDNF protein concentrations following 14 day aCSF Infusion. The graph depicts percentage of CDNF protein expression in right versus left striatum following a 14 day aCSF infusion to left striatum, n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a student's *t*-test was performed to determine any changes in CDNF protein expression. As expected, no significant difference of CDNF protein expression was found in right versus left striatum of aCSF infused rats.

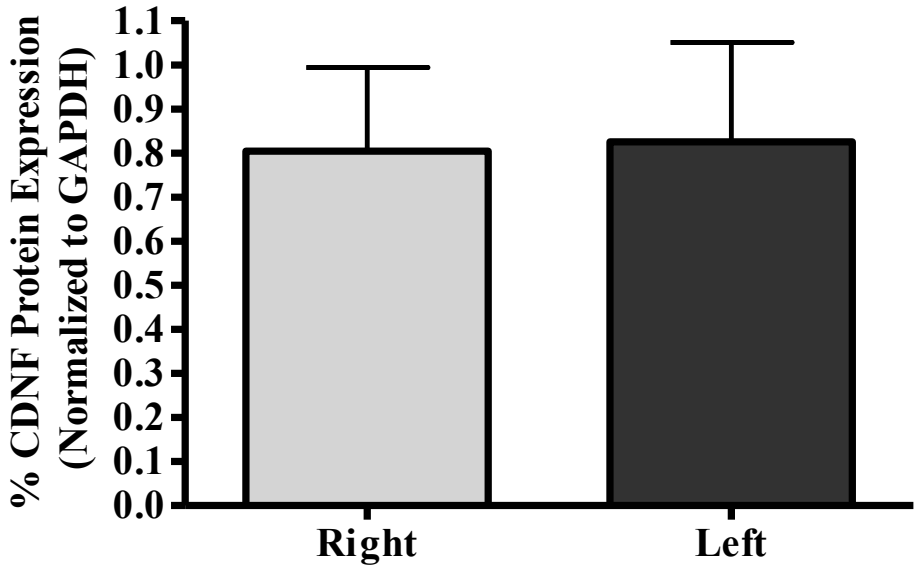

Figure 16: CDNF protein concentrations following 14 day antisense Infusion, knockdown validation. The graph depicts percentage of CDNF protein expression in right versus left striatum following a 14 day antisense infusion to left striatum, n=8. Following normalization of CDNF protein expression to housekeeping antibody GAPDH, a student's *t*-test was performed to determine efficacy of the knockdown. No significant difference of CDNF protein expression was found in right versus left striatum of antisense infused rats, *ns*.

4.5.4 CDNF mRNA platelet expression

Differences in CDNF mRNA expression in platelets were examined following the 14 day infusion period. Real-Time qRT-PCR experiments were analyzed using a two-tailed student's *t*-test. No significant difference of CDNF mRNA expression between aCSF and antisense groups was found, *ns* (**Figure 17**).

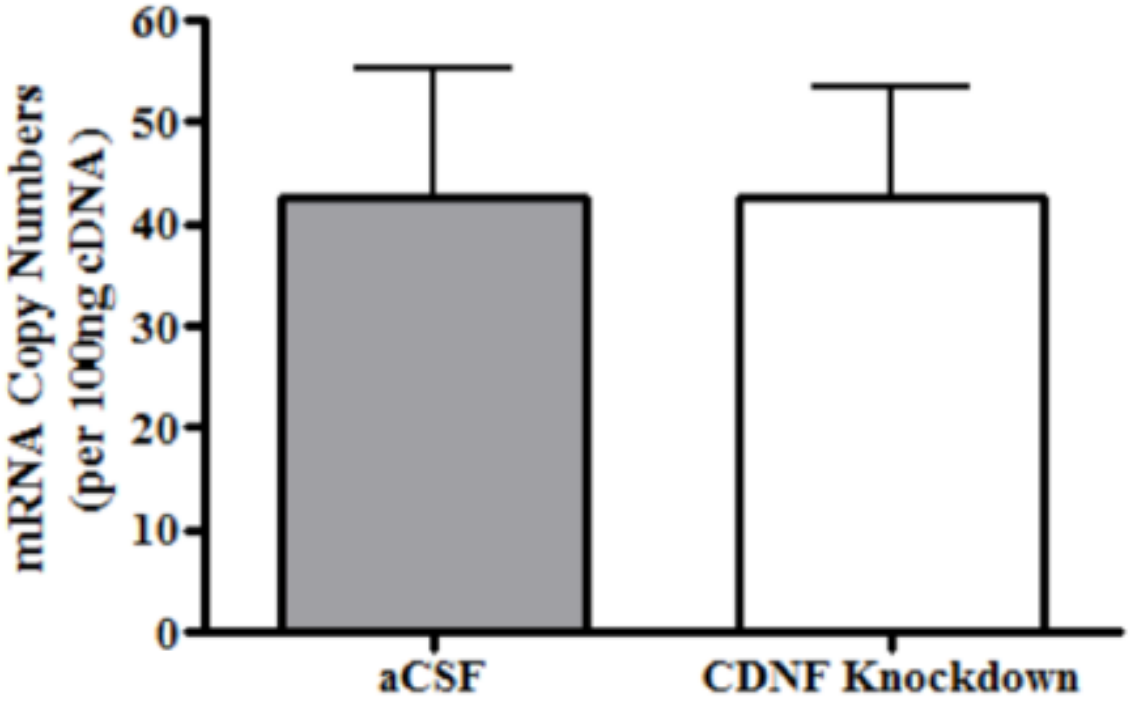

Figure 17: CDNF mRNA expression in platelet rich plasma following 14 day infusion.
The graph depicts platelet CDNF mRNA copy numbers (mean ± SEM) in rats following a 14 day infusion period of aCSF (n = 8) or CDNF antisense (n = 8). Real-time qRT-PCR experiments were analyzed using a two-tailed stuent's *t*-test, no significant difference was found between the groups, *ns*.

4.6 Discussion

Previous studies have investigated the knockdown of various neurotrophic factors to identify the causes of DAergic neuron death and to induce PD in animal models (Pascual et al., 2008; Porritt et al., 2005). BDNF antisense oligonucleotide infusion in rats has shown to induce behavioural phenotypes typical of PD model rats as well as cause the degeneration of DAergic neurons and reduce BDNF mRNA expression (Porritt et al., 2005). The suppression of GDNF expression in adult mice showed the selective decrease in TH, DAergic cell death in the nigrostriatal pathway and progressive deterioration of motor behaviours that were compatible with those seen in PD model animals (Pascual et al., 2008). This link of NTF knockdown and the DAergic degeneration and behavioural phenotypes typical of PD models has not been established in CDNF. This critical gap in understanding the potentially causal role of this trophic factor in the neurodegeneration and pathogenesis of PD.

This study sought to infuse CDNF antisense for 14 days in the striatum in hopes of reducing CDNF protein expression in the infusion site, to observe its effect on the nigrostriatal pathway and motor behaviour as well as analyze changes in peripheral mRNA expression. The results show that the selective knockdown of CDNF was not successful. Although this antisense knockdown technique has been performed in our lab several times before, CDNF was a novel gene target and thus it could have been a poor sequence or the dosage and duration of infusion may not have been correct for this application. Also, neural toxicity could have been an issue, as phosphorothioates have been shown to degrade in cells over time seriously limiting their clinical

effectiveness (Miyake et al., 2005). When sacrificing the animals, a few of the CDNF antisense rats exhibited necrotic areas around the lesion site, possibly due to phosphorothioate toxicity. Due to the lack of CDNF knockdown and neuronal toxicity, although the behavioural results do show some significance, it is difficult to accurately interpret these results as an effect of CDNF, surgical issues or otherwise. This same reasoning is applied to the mRNA expression portion of the study. All goals of this study hinged on the successful knockdown of CDNF; once immunoblotting revealed that was unsuccessful nothing can be concluded from the other experiments. Future studies producing a knockdown in CDNF knockdown should be repeated, however dosage and duration of the antisense should be carefully reconsidered, include a mismatch sequence as a negative control, and consider the use of newer siRNA gene knockdown techniques with its enhanced safety and efficiency (Dallas & Vlassov, 2006).

Chapter 5

Investigating CDNF Transcriptional Profile of Aging in *Homo Sapiens*

5.1 Objective

CDNFs neurprotective and neurorestorative properties have been extensively described earlier, this trophic factor has been studied in a variety of animals that model PD, an age related neurodegenerative disorder. Aging of the brain leads to impairments in cognition and motor skills and is a major risk factor for neurological disease (Lee, Weindruch & Prolla, 2000). Interest in CDNF and aging has been raised within our research group but no reports address the question of its expression over the course of a lifespan. This is a very important since other neurotrophic factors such as BDNF have been shown to play an important role in mammalian adults, investigation of the aging profile of CDNF is pertinent (Karega, Schwald & Cisse, 2002). The aim of this study was to assess the levels of CDNF mRNA expression in blood over the course of development in *homo sapiens*. As one ages and is at a greater risk of developing a neurodegenerative disorders such as PD, it is hypothesized that over the course of a developmental profile the levels of CDNF mRNA expression will decrease.

5.2 Methodology

5.2.1 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 Mood Disorders program and Women's Health Concerns Clinic 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 to take part in the study. Patients were not given compensation for involvement in this study.

5.2.2 Blood Collection and Whole Blood Preparation

Venous blood samples (approximately 2.5ml) were collected via phlebotomy into PAXgene® RNA Blood Tubes, per participant. RNA was isolated according to manufacturer's instructions (PreAnalytix) and stored at -80°C until use.

5.2.3 Real-Time qRT-PCR

Human CDNF mRNA primers were designed using protocols discussed in section 2.2.3 for *C.elegans* CDNF primer design, however the human mRNA transcript was obtained from www.ncbi.nlm.nih.gov. PCR products showed 100% homology with the human CDNF gene regions.

Protocols for qRT-PCR was previously described in section 2.2.5; however the total RNA for each well was 60ng and the specific PCR primers (5μM) used were: forward primer, 5'-AAAGACGCAGCCACAAAGAT-3' and reverse primer, 5'-AGGATCTGCTTCAGCTCTGC-3' (**Table 2**). The real-time qRT-PCR conditions were 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 45 s, and lastly 95 °C for 1 min (1 cycle).

5.3 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Prior to analyses, outlier detection was performed using GraphPad Outlier Tool. Significance was defined as $p < 0.05$. PCR results were analyzed as previously described in section 2.4.2.

5.4 Results

Figure 18 presents CDNF mRNA expression levels in whole blood which shows a gradual and significant decrease in humans over the course of development, following a one-way ANOVA: $F(2,44)= 9.29$, $^{***}p < 0.0005$. Tukey's post-hoc tests show a significant decrease in CDNF mRNA expression between children (ages 10-18; n=7; 3 males, 4 females; average age 12.28 years) to young adults (ages 18-50; n=22; 6 males, 17 females; average age 34.82 years), $^{*}p < 0.05$. Children and older adults (age 50 and above; n=16; 5 males, 11 females; average age 63 years) also showed a significant decrease in CDNF expression, $^{**}p < 0.01$. No significant difference between young adults and older adults was seen, *ns*.

5.5 Discussion

All cells of the nervous system are affected by aging yet not everyone who ages will develop a neurodegenerative disorder. All individuals cellular and molecular processes begin to change and render their components vulnerable to degeneration (Mattson & Magnus, 2006). NTFs such as GDNF and BDNF, have been

shown to be both neuroprotective and neurorestorative of DAergic neurons (Grondin et al., 2010; Stahl et al., 2011); however, the normal aging process has shown to lead to the declining production of these tropic factors. Comprised neurotrophic signalling has been shown to be involved in age-related neurodegeneration (Hattiangady, Rao, Shetty & Shetty, 2005; Krakora et al., 2013; Murer, Yan, & Raisman-Vozari, 2001).

This study examined the changes in CDNF mRNA expression in whole blood over the course of a lifespan. The results suggest that there is a significant decline between children and young adults, as well as between children and older adults. Although, no significant difference between young adults and older adults was found a reduction is observed. The lack of significance could be attributed to the small sample size of children, n = 7. Increasing the child group sample size could help to reduce the variance of the data. Continuing the study and increasing the sample size will help further understand the role of CDNF in aging.

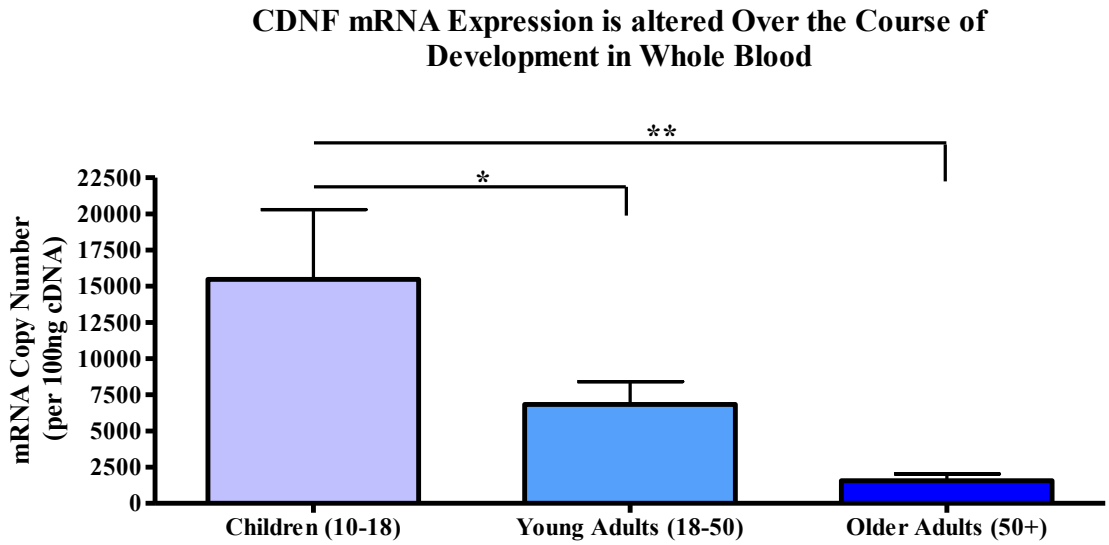

Figure 18: CDNF whole blood mRNA aging profile. The graph depicts whole blood mRNA copy numbers (mean ± SEM). Experimental groups were composed of children ages 10 to 18 (n = 7; 3 males, 4 females; average age 12.28 years), young adults ages 18 to 50 (n = 22, 6 males, 17 females; average age 34.82 years) and older adults ages 50 and above (n = 16, 5 males, 11 females; average age 63 years). Real-Time qRT-PCR experiments were analyzed using one-way ANOVA: $F(2,44)= 9.29$, $^{***}p < 0.0005$, indicating a significant difference in CDNF mRNA expression over the developmental profile. Tukey's post-hoc tests indicate a significant decrease in CDNF mRNA expression from children to young adults ($^{*}p < 0.05$), and childhood to older adults ($^{**}p < 0.01$). No decrease from young adult into older adults was found, *ns.* Bartlett's test for equal variances was also significant ($p <0.0001$).

Chapter 6

Investigating the Role of CDNF in Parkinson's Disease

6.1 Objective

Despite extensive research efforts there is currently no validated biomarker for the diagnosis of PD. Although, diagnosed clinically by the presence of cardinal features of tremor, bradykinesia, impaired postural stability, and rigidity, and a good response to L-DOPA treatment, there is a high rate of misdiagnosis with other movement disorders (Gazewood, Richards & Clebak, 2013). Behavioural symptoms manifest themselves after 60-70% of DAergic neurons have been degenerated (Albin 2006). There is an urgent need for the discovery of a sensitive and specific biomarker for the early detection of PD. Early detection of PD would allow for the therapeutic intervention to help slow down disease progression and allow the patient to maintain a higher quality of life. Many studies have investigated the role of peripheral blood components (whole blood, platelets and lymphocytes) as a potential indicator of CNS activity in various neurological diseases (see section 1.4). Blood is an ideal candidate for the development of a potential biomarker as it is readily available in large quantities and can be accessed with minimally invasive techniques (Liew et al., 2005; Tang et al., 2001).

The neuroprotective and neurorestorative effects of CDNF in animal models of PD have been extensively discussed, yet despite this it remains unknown as to how PD affects endogenous levels of CDNF. Due to this, the aim of this study was to investigate endogenous CDNF mRNA expression in the clinical PD population and investigate the role of CDNF as a potential biomarker for PD in peripheral blood. Through the investigation of blood components, namely whole blood, platelets and

lymphocyte, CDNF mRNA expression will be investigated in PD patients, stroke patients (negative control) and healthy, age matched controls, this study hopes to identify changes in CDNF mRNA expression specific to PD making it a potential biomarker for the accurate early diagnoses of PD. All subjects included in the study were 50 years of age or older to discount any age related changes in CDNF expression. The inclusion of the negative control, stroke patients, was done to identify if the expression changes were specific to PD. It is hypothesized that CDNF mRNA expression in all blood components will be specifically reduced in PD patients compared to all control groups.

6.2 Methodology

6.2.1 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. PD patients were recruited through movement disorder clinics in the Hamilton area. Physical examination and a second opinion was attained from a movement disorder specialist to confirm the diagnosis. Inclusion criteria included a primary diagnosis of Parkinson's disease with no additional movement disorder (i.e. parkinsonism, essential tremor). Patients were excluded if they received a diagnosis neuropsychiatric or neurological illness. A group of healthy subjects, matched for age and sex with the disease group were recruited at the Juravinski Hospital - Medical Ambulatory Care clinic and at

McMaster University. Patients were included as healthy controls if they had diabetes, high or low blood pressure, or headaches. All subjects gave written informed consent to take part in the study. Patients were not given compensation for involvement in this study.

6.2.2 Blood Collection and Preparation of Platelets and Lymphocytes

Venous blood samples (approximately 30-40ml) were collected via phlebotomy into 10ml BD vacutainer tubers with 1.42ml of acetate citrate dextrose.

Platelets were prepared as follows: The collected blood was centrifuged at 980g for 2 min in an Eppendorf 5810R centrifuge at environmental temperature 22°C. After centrifugation a red lower fraction (red blood cell component) and an upper pale-yellow turbid fraction (blood serum component) was observed. The top two-thirds of the blood serum component was removed using a pasture pipette and put into a 15 ml conical centrifuge tube. The sample was centrifuged again at 1200g for 7 minutes. The supernatant was removed and the pellet was resuspended in 7ml of cold PEB solution. The supernatant was removed, the pellet was resuspended in 1ml of cold PEB and transferred to a 1.5ml eppendorf tube and centrifuged at 1200g for 7 minutes in an Eppendorf 5415R small bench top centrifuge. The supernatant was carefully removed and the pellet was stored at -80°C until future use.

Lymphocytes were prepared as follows: Blood samples were emptied into a 50ml conical centrifuge tube, brought up to a volume of 30ml with warm PBS (37°C) and mixed using a transfer pipette. Human lymphocytes were isolated with a Ficoll-Paque (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada) density gradient

(15ml). The sample was centrifuged at 400g for 30min without brake. The resulting middle fuzzy lymphocyte layer was collected in a new 50ml conical tube and suspended in 30ml of warm PBS. The sample was centrifuged at 110g for 10 minutes, the supernatant was removed and the pellet was washed in another 30ml of warm PBS. After a final 10minute spin at 110g the supernatant was removed and the sample was resuspended in 1ml of warm PBS. The suspension was transferred to a 1.5ml eppendorf tube and centrifuged at 110g for 10 minutes in an Eppendorf 5415R small bench top centrifuge. The supernatant was carefully removed and the pellet was stored at -80°C until future use.

6.2.3 Preparation of Whole Blood and RNA Isolation

Protocol was performed as previously described in section 5.3.2.

6.2.4 RNA Isolation from Platelets and Lymphocytes

Protocol was performed as previously described in section 3.3.6.

6.2.5 Real-Time qRT-PCR

Protocol was performed as previously described in section 5.3.3.

6.3 Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, California, USA). Prior to analyses, outlier detection was performed using GraphPad Outlier Tool. Significance was defined as $p < 0.05$. PCR results were analyzed as previously described in section 2.4.2.

6.4 Results

6.5.1 Platelet CDNF mRNA expression in clinical populations of Parkinson's disease

A one-way ANOVA was performed to analyze the real-time qRT-PCR results; whole blood mRNA expression was analyzed for each group. In platelets the experimental groups were comprised of 15 healthy controls (n = 15; 6 males, 10 females; average age 66.81 years), 7 stroke patients (n = 7; 4 males, 4 females; average age 71.5 years), and 12 PD patients (n = 12; 9 males, 4 females; average age 72.84 years). A significant difference in CDNF mRNA expression was found, $F(2,33)= 4.89$, $^*p < 0.0142$ (**Figure 19**). Tukey's post-hoc tests showed a significant increase in CDNF mRNA expression in PD patients compared to stroke, $^*p < 0.05$, but not in comparison to healthy controls, *ns*.

A student's *t*-test was used to compare experimental group means since patient samples were not collected at the same time. PD patients show a significant increase in mRNA expression when compared to healthy controls, $^*p < 0.05$, and when compared to stroke patients, $^*p < 0.05$. Healthy controls showed no significant difference in expression when compared to stroke patients, *ns*. These results suggest that the increased CDNF mRNA expression is specific to PD patients (**Figure 20**).

6.5.2 Whole blood CDNF mRNA expression in clinical populations of Parkinson's disease

A one-way ANOVA was performed to analyze the real-time qRT-PCR results; whole blood mRNA expression was analyzed for each group. Experimental groups

comprised of healthy controls (n = 16; 5 males, 11 females; average age 63 years), stroke patients (n = 15; 7 males, 7 females; average age 71.69 years), and PD patients (n = 7; 5 males, 2 females; average age 65.28 years). Results indicated a significant difference in CDNF mRNA expression $F(2,37)= 3.45$, $*p < 0.0429$ (**Figure 21**). Tukey's post-hoc tests indicated a significant decreased in CDNF mRNA expression in stroke patients compared to both healthy controls, $*p < 0.05$, but not for PD patients, *ns*. A student's *t*-test was used to compare experimental group means since patient samples were not collected at the same time. PD compared to healthy controls indicated no significant difference in mRNA expression, *ns*. Stroke patients showed a significant different in expression when compared to healthy controls, $*p < 0.05$, and when compared to PD patients, $**p < 0.01$. These results suggest that whole blood CDNF mRNA expression is reduced in stroke patients (**Figure 22**).

6.5.3 Lymphocyte CDNF mRNA expression in clinical populations of Parkinson's disease

CDNF mRNA expression in lymphocytes was analyzed by performing a one-way ANOVA to analyze the real-time qRT-PCR results. Experimental groups were composed of healthy controls (n = 22; 12 males, 10 females; average age 66.59 years), stroke patients (n = 16; 6 males, 8 females; average age 69.64 years), and PD patients (n = 17; 13 males, 5 females; average age 71.72 years). No significant difference between experimental groups was found, *ns* (**Figure 23**).

A student's *t*-test was used to compare experimental group means since patient samples were not collected at the same time. Results indicated that no significant difference was found between experimental groups, *ns* (**Figure 24**).

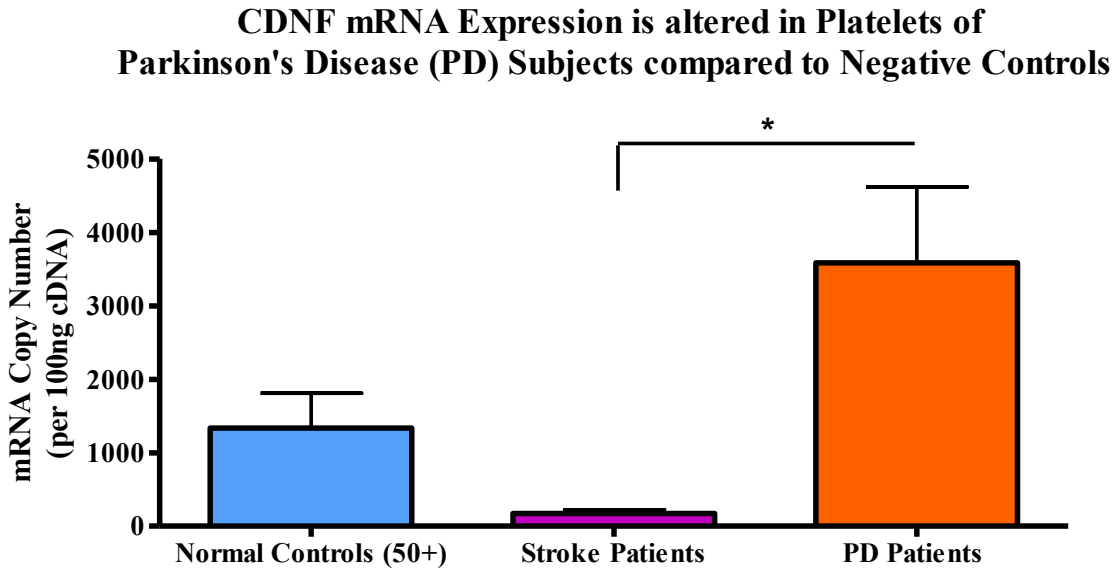

Figure 19: Platelet CDNF mRNA expression. The graph depicts platelet mRNA copy numbers (mean ± SEM). Experimental groups were composed of 15 healthy controls (n = 15; 6 males, 10 females; average age 66.81 years), 7 stroke patients (n = 7; 4 males, 4 females; average age 71.5 years), and 12 PD patients (n = 12; 9 males, 4 females; average age 72.84 years). Real-Time PCR experiments 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 PD and healthy controls, *ns*.

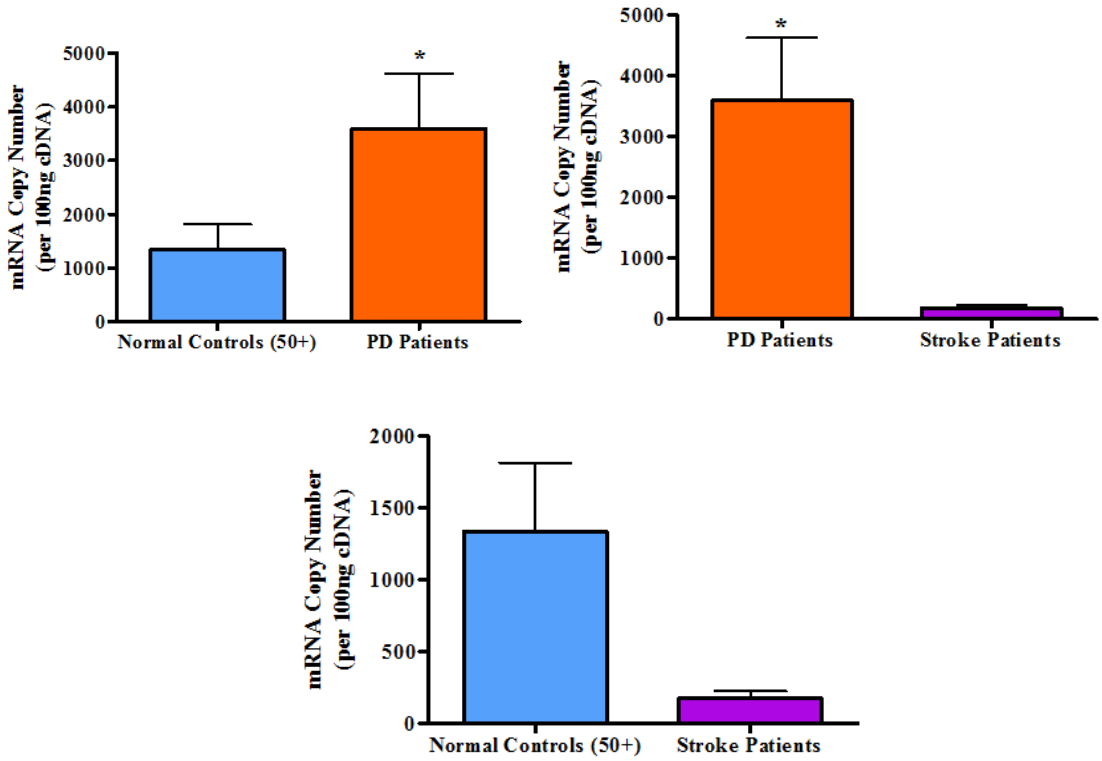

Figure 20: Increase of CDNF mRNA platelet expression specific to Parkinson's disease.
The graph depicts platelet mRNA copy numbers (mean ± SEM). Experimental groups were composed of 15 healthy controls (n = 15; 6 males, 10 females; average age 66.81 years), 7 stroke patients (n = 7; 4 males, 4 females; average age 71.5 years), and 12 PD patients (n = 12; 9 males, 4 females; average age 72.84 years). Real-Time PCR experiments were analyzed using a two-tailed students *t*-test and indicated a significant increase in CDNF mRNA expression of PD patients in comparison to healthy controls, $*p < 0.05$, and when compared to stroke patients, $*p < 0.05$), but no significant difference between normal controls and stroke patients, *ns*.

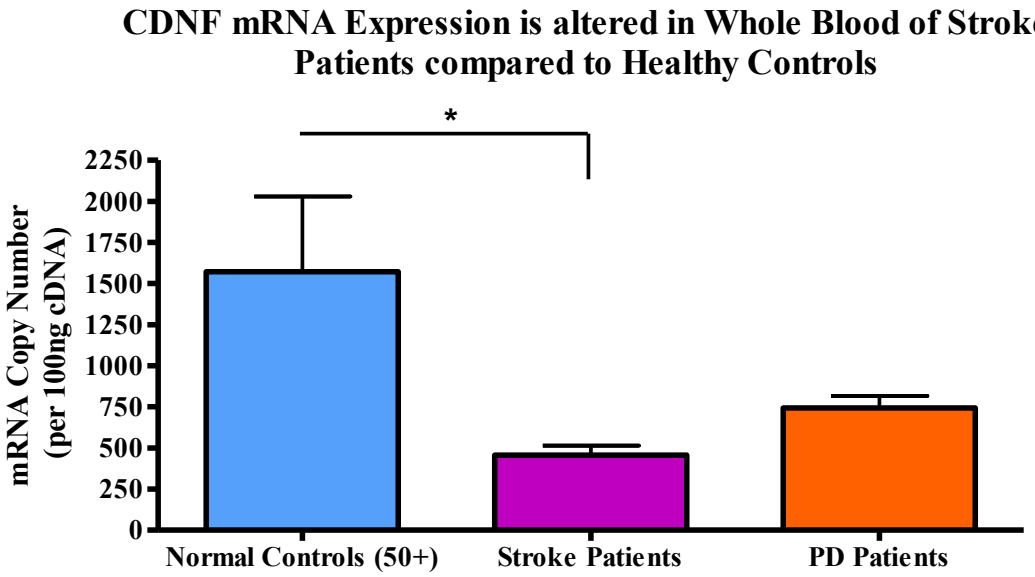

Figure 21: Whole blood CDNF mRNA expression. Graph depicts whole blood mRNA copy numbers (mean ± SEM). Experimental groups were composed of n=16 for normal controls (50+) (5 males, 11 females; average age 63 years), n= 15 for stroke patients (7 males, 7 females; average age 71.69 years), and n=7 for PD patients (5 males, 2 females; average age 65.28). Real-Time PCR experiments were analyzed using a one-way ANOVA. Results indicated a significant difference in CDNF mRNA expression in stroke patients compared to normal controls, $*p < 0.05$.

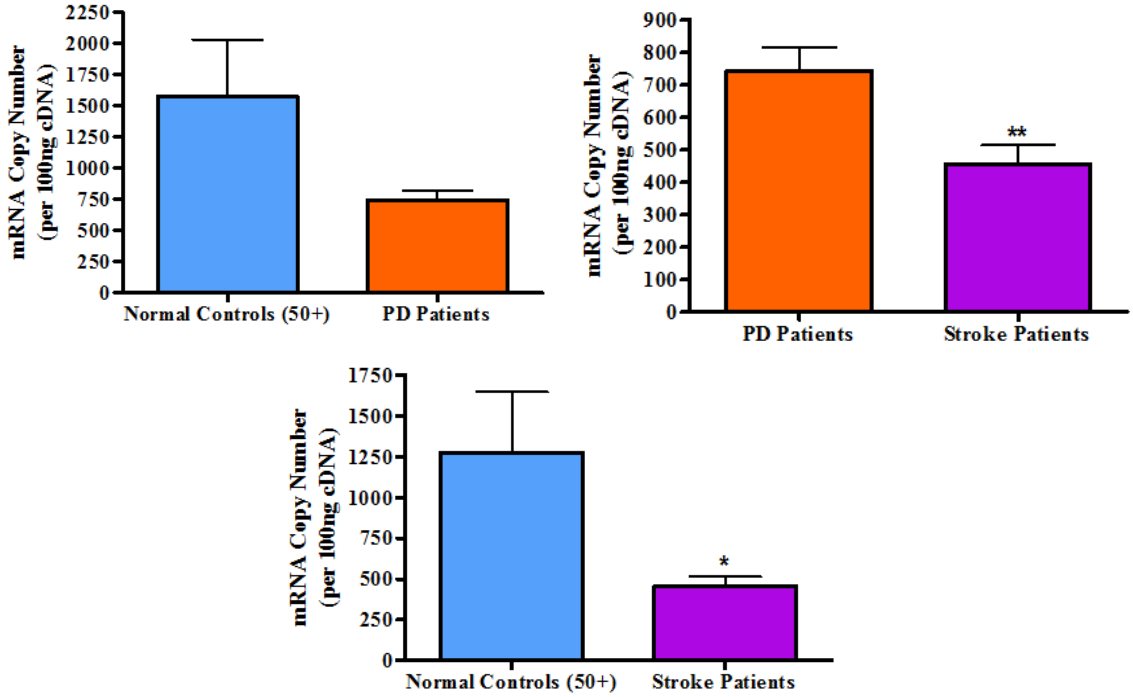

Figure 22: CDNF mRNA whole blood expression is altered in Stroke patients. The graph depicts whole blood mRNA copy numbers (mean ± SEM). Experimental groups composed of healthy controls over the age of 50 (n = 16; 5 males, 11 females; average age 63 years), stroke patients (n = 15; 7 males, 7 females; average age 71.69 years), and PD patients (n = 7; 5 males, 2 females; average age 65.28). Real-Time PCR experiments were analyzed using a one-way ANOVA. Results indicated a significant difference in CDNF mRNA expression in stroke patients compared to normal controls, $*p < 0.05$ and between stroke patients and PD patients, $**p < 0.01$.

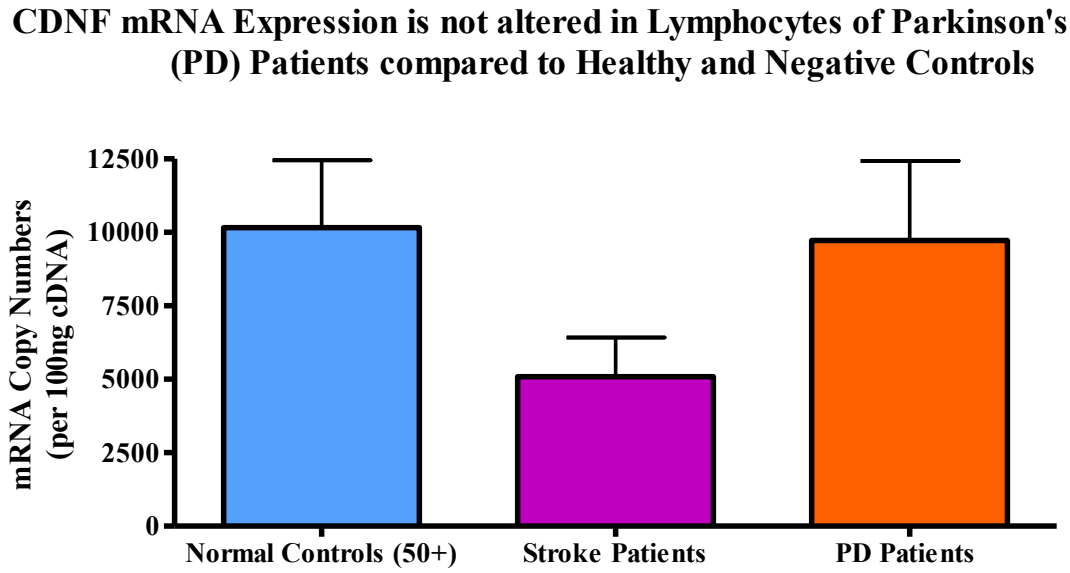

Figure 23: Lymphocyte CDNF mRNA expression. The graph depicts lymphocyte mRNA copy numbers (mean ± SEM). Experimental groups were composed of healthy controls (n = 22; 12 males, 10 females; average age 66.59 years), stroke patients (n = 16; 6 males, 8 females; average age 69.64 years), and PD patients (n = 17; 13 males, 5 females; average age 71.72 years). Real-Time PCR experiments were analyzed using a one-way ANOVA. Results indicated no significant difference between experimental groups, *ns*.

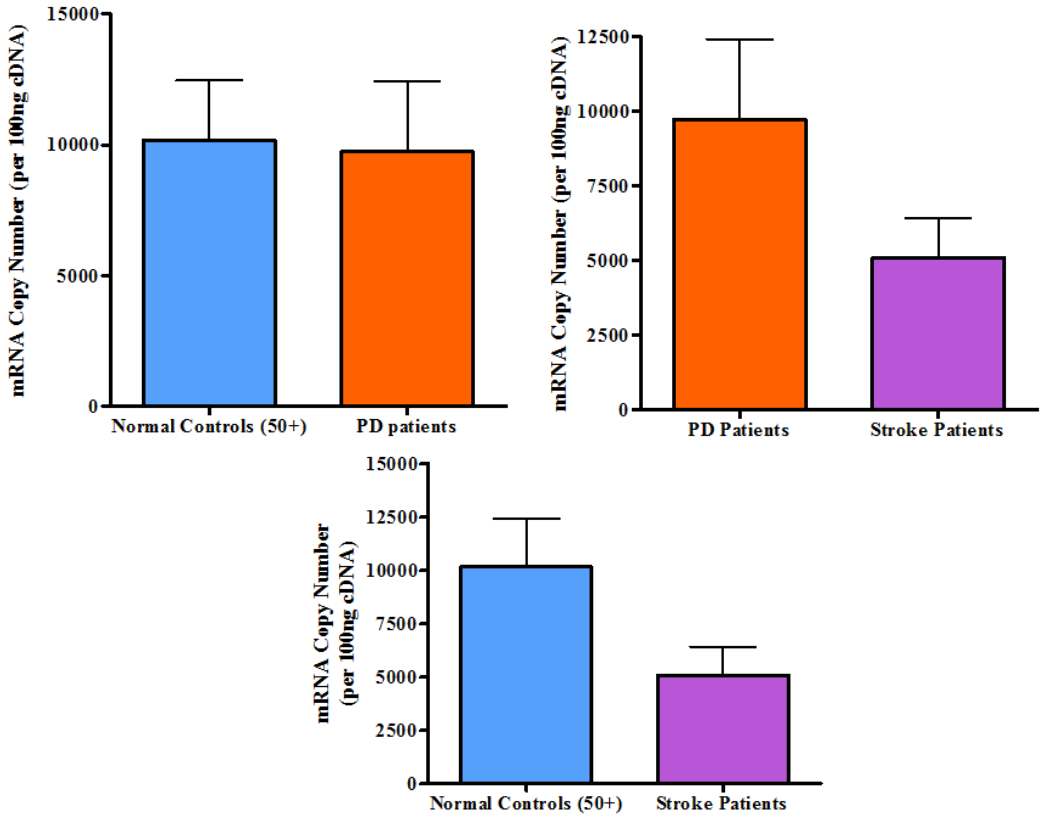

Figure 24: No alteration of CDNF mRNA lymphocyte expression. The graph depicts platelet mRNA copy numbers (mean ± SEM). Experimental groups were composed of healthy controls (n = 22; 12 males, 10 females; average age 66.59 years), stroke patients (n = 16; 6 males, 8 females; average age 69.64 years), and PD patients (n = 17; 13 males, 5 females; average age 71.72 years). Real-time PCR experiments were analyzed using a two-tailed students *t*-test, no significant difference in CDNF mRNA expression was found between healthy controls, PD patients and stroke patients, *ns*.

6.5 Discussion

PD an age related neurodegenerative disease, whose clinical symptoms appear late after the degeneration of a significant number of DAergic neurons. Identification in the early stages of the disease is imperative to help improve the lives of individuals suffering with this disease. Currently there are no validated biomarkers for the early diagnosis of PD. NTF have been show to be protective of neurons during the early developmental stages of ones life as well as over the course of aging (Hattiangady et al., 2005; Krakora et al., 2013). Also, in models of neurodegenerative disorders such as Alzheimer's disease and PD these same trophic factors have been shown to be neuroprotective and neurorestorative (Grondin et al., 2010; Stahl et al., 2011). The discovery of the novel NTF, CDNF, in both the CNS and peripheral blood provides an interesting opportunity to investigate its potential as a diagnostic tool for the early detection of PD. Blood is advantageous as a potential biomarker as it is readily available in large quantities and is accessed with minimally invasive techniques (Liew et al., 2005; Tang et al., 2001).

The previously described study in Chapter 5 has shown that over the course of the aging profile CDNF is decreasing. Future research should elaborate on these findings in a larger study to further understand the potential for CDNF as a biomarker was investigated. mRNA expression of CDNF in whole blood, platelets and lymphocytes among a population of PD patients, stroke patients and healthy, age matched controls was studied. In platelets, a significant increase in CDNF mRNA expression was observed, contrary to what was originally hypothesized. It could be

possible that although *in vitro* studies have shown ER stress to have only a minor effect on CDNF secretions that the response may be different *in vivo* when initiated by the diverse pathological processes that may be responsible for PD (Apostolou et al., 2008; Aron & Klein, 2010). CDNF has been shown to play a role in facilitating protein folding and reducing ER stress (Lindholm & Saarma, 2009). Also the progressive degeneration of neurons in PD may induce a compensatory response resulting in the up-regulation of CDNF, similar to that which is seen in NTFs when 6-OHDA lesioned striatal extracts are cultured with extracts from healthy striatum (Yurek & Flether-Turner, 2001). Additionally it is possible that the pharmacological treatments of the patients played a role in CDNF expression; all patients studied were on some form of dopamine agonist treatment at the time of blood specimen collection. Pharmacological up-regulation of endogenous neurotrophic factors has been investigated in a variety of studies(Gyarfas, Knuutila, Lindholm, Rantamaki & Castren, 2010). Anti-depressant drug treatments have been shown to increase BDNF and neurotrophin-3 levels (Altar, 199). Pramipexole and ropinirole are a new generation of anti-parkinsonian dopamine agonists that are used to treat PD. Research, both *in vivo* and *in vitro*, has shown that through the modulation of the D3 receptor these agonists are able to increase the production of endogenous GDNF and BDNF in eliciting their neuroprotective effects (Du, Li, Huang, Li & Le, 2005). Other dopamine agonists such as pergolide, cabergoline and SKF-38393 have been shown to have stimulatory effects the synthesis and secretion of GDNF and nerve growth factors through the modulation of D1/D2 receptors (Ohta et al., 2003). Unlike GDNF

and BDNF, the target molecules and signalling pathways of CDNF is still unknown (Lindholm & Saarma, 2009). Further research to reveal the mechanism of CDNFs neurotrophic activities at the molecular level is needed to help explain why the increase in CDNF mRNA expression is seen in this PD patient population. Future studies on drug naïve PD patients as well as examining the effects of DA agonists on DA receptor transfected cell lines will help provide insight into whether the changes in CDNF mRNA expression was due to pharmacological intervention or due to PD pathology.

Having identified a significant increase in platelet CDNF mRNA expression in PD patients compared to healthy controls and negative controls, stroke patients, the concentrations in lymphocytes were examined. These results indicate that no significant difference in expression was seen across the three experimental groups. For the purpose of establishing CDNF as a potential biomarker for PD this is ideal because it indicates that it is specific for a tissue type and a pathological condition. However, in interpreting the expression of CDNF mRNA in whole blood we find that stroke patients show a significant decline in concentration whereas the other two groups show no difference. This is a very interesting finding in that much is relatively unknown about CDNF and stroke. Strokes are known to induce neuron death through ER stress (Tajiri et al., 2004) and CDNFs role in mitigating ER stress has been described earlier. The correlation between this NTF and stroke has not been studied and although this study provides some insight into a possible down

regulation further research is needed in preclinical models of stroke to better understand the relationship.

6.6 Conclusion

PD is one of the most common neurodegenerative movement disorders, characterized by the progressive degeneration of DAergic neurons in the SN. Despite decades of research the etiology and pathogenesis of the disease still remain unclear (Sharma et al., 2013). Therapies today focus on treating symptoms rather than halting or retarding the progressive degeneration of dopaminergic neurons (Sullivan & Toulouse, 2011). NTF are proteins promote the survival, differentiation and maintenance of neurons and have proven to be promising therapeutic candidates for the treatment of PD (Cheng et al., 2013). GDNF and BDNF are two NTF that have been extensively studied and been shown to have neuroprotective and neurorestorative effects on DAergic neurons (Hou et al., 2002; Stahl et al., 2011). The promising results from these NTF has not translated to clinical success and as such the focus has shifted to CDNF (Lindholm et al., 2007). Validated to prevent and reverse DAergic neurodegeneration this trophic factor provides a viable alternative to the treatment of PD.

To further elucidate the interplay of CDNF and DA and DAergic neurons to better understand the etiology of PD, endogenous levels of the NTF was investigated. CDNF was measured in DA synthesis and transport mutants of *C. elegans* as well as in wild-type *C. elegans* and rats following 6-OHDA neurotoxic exposure. 6-OHDA

toxicity did not cause a change to endogenous CDNF mRNA or protein expression in these animals. Only the lack of conversion from L-DOPA to DA, *bas-1* mutants, was caused a significant increase in CDNF mRNA expression in *C. elegans* mutants. To further understand the role of CDNF in PD a knockdown of CDNF was attempted. Results from this experiment were unsuccessful as the AS ODN sequence was not effective in reducing CDNF protein expression. Future studies could investigate other techniques of silencing gene expression.

The clinical phase of our research investigated CDNF expression for the first time over the course of ones lifespan as well as in the clinical PD population. Over the course of the lifespan a steady decline in CDNF mRNA expression was found potentially leaving one vulnerable to developing a neurodegenerative disorder with the decreasing neurotrophic support. In whole blood, decreased CDNF mRNA expression was found specific to stroke patients. Also, contrary to what was expected platelet CDNF expression was increased, and this change was only specific to the PD patient population. These findings are very interesting and warrant further research to further elucidate the role of CDNF in ER stress and its relationship with the degeneration of DAergic neurons of PD.

THE END

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