RESTORATION OF MOTOR AND NON-MOTOR FUNCTIONS BY NEUROTROPHIC FACTORS IN NONHUMAN PRIMATES WITH DOPAMINE DEPLETION

by

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Parkinson's disease (PD) is a progressive debilitating neurodegenerative disorder characterized by resting tremor, rigidity, bradykinesia and postural instability. As the disease progresses there is a loss of dopamine (DA) neurons in the substantia nigra projecting to the various forebrain and sub-cortical regions. Current treatments for PD are unable to prevent or curtail the neurodegenerative process; so rescuing remaining dopamine in the mid-brain has been the recent focus of research examining the effectiveness of neurotrophic factors (NTFs) in the treatment of PD. In this dissertation, the ability of three novel, recently discovered NTFs to restore DA neurons and motor function in a nonhuman primate model of PD was examined. The NTFs were Cerebral Dopamine Neurotrophic Factor (CDNF) and two variants of Neurturin (NRTN), N2 and N4, that have mutations that prevent binding to heparin sulfate binding sites in the brain. These studies used the unilateral low dose (0.15 ± 0.001 mg/kg) monkey 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD to cause loss of DA neurons. Six groups of monkeys were studied: vehicle-treated (negative control), Glial Cell-line Derived Neurotropic Factor (GDNF, positive control), two groups of CDNF-treated monkeys (450 µg and 150 µg), and N2 and N4-treated groups. After MPTP, monkeys developed moderate symptoms of PD (PD rating scale score=7.9±0.5 on a scale of 0-22, $p<0.001$), motor dysfunction and increased daytime sleepiness. After three months of infusions, all three NTFs (150 µg CDNF, N2 and N4) significantly increased the number of DA neurons in the substantia nigra, $p=0.03$, and improved parkinsonian symptoms measured by rating scale, $p<0.001$. Most motor functions were

significantly correlated with the number of DA neurons in the substantia nigra. N4 significantly improved daytime sleep duration, bouts and wake-latency ($p=0.02$, $p=0.06$ and $p=0.02$, respectively). In summary, CDNF, N2 and N4 trophic factors are neurorestorative to DA neurons, motor function is tightly correlated with DA neuronal number, and N4 improved the non-motor symptom of increased daytime sleepiness in this monkey PD model. These factors hold promise for clinical therapy for PD patients.

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ABBREVIATIONS

6-OHDA – 6-hydroxydopamine

AAV – recombinant Adeno-associated virus

BDNF – Brain-derived neurotrophic factor

CDNF – Cerebral dopamine neurotrophic factor

CNS – Central nervous system

CPAP – continuous positive air pressure

CSF – Cerebrospinal fluid

DA – Dopamine

DAT – Dopamine transporter

DMNC – dorsal motor nuclear complex of IX and X

DOPAC – dihydroxyphenylacetic acid

EMG – Electromyography

GABA – γ-aminobutyric acid

GDNF – Glial cell-line derived neurotrophic factor

GFL – GDNF family of ligands

GHRH – Growth hormone releasing hormone

GI -- Gastrointestinal

GPe – Globus pallidus, external segment

GPi – Globus pallidus, internal segment

HVA – Homovanillic acid

IM – Intramuscular

IRP – Iron-regulatory element-binding proteins

IV – Intravenous

LB – Lewy body

M1 – Primary motor cortex

MANF – Mesencephalic astrocyte-derived neurotrophic factor

MAO – Monoamine oxidase

MFB – Medial forebrain bundle

MPDP – 1-methyl-4-phenyl-2,3-dihydropyridinium

MPP – 1-methyl-4-phenyl-pyridinium ion

MPPP – 1-methyl-4-phenyl-4-propionoxy-piperidine

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSN – Medium spiny neurons

NGF – Nerve growth factor

NRTN – Neurturin

NTF – Neurotrophic factor

OCD – Obsessive-compulsive disorder

PD – Parkinson’s disease

REM – Rapid eye movement

ROS – Reactive oxygen species

S1 – Primary sensory cortex

SC – Subcutaneous

SMA – Supplementary motor area

SNpc – Substantia nigra, pars compacta

SNpr – Substantia nigra, pars reticulata

STN – Subthalamic nucleus

TAN – Tonically active neurons

UPDRS – Unified Parkinson's disease rating scale

VMAT – Vesicular monoamine transporter

VTA – Ventral tegmental area

1.0 GENERAL INTRODUCTION

1.1 PARKINSON'S DISEASE (PD): OVERVIEW

1.1.1 Clinical Characteristics of PD

James Parkinson in 1817 wrote "An essay on the shaking palsy" and was the first person to describe in detail the clinical symptoms of this disease that later went on to bear his name [Parkinson, 1817]. Jean-Martin Charcot, a famous neurologist at la Salpêtrière hospital in Paris who contributed significantly to our understanding of this disease through his lectures sixty years later, referred to as Charcot's lectures, distinguished this disease from other tremor-related neurological disorders and gave further details about the manifestation and time course of the progression of the disease as it was understood in the late 19^{th} century (called 'paralysis agitans') [Charcot, 1877]. Charcot gave credit to Parkinson for his pioneering descriptions by referring to this disease as "maladie de Parkinson" or Parkinson's disease (PD), as it is now called [Charcot, 1877]. However, PD existed long before James Parkinson described six such cases in detail in 1817. Galen of Pergamon observed a similar disease and wrote an Egyptian papyrus (1350-1200 BC) that described it, suggesting that parkinsonism was occurring at that time [Forno, 1996]. Similarly, characteristics of the disease were also described in Charaka Samhita (400 – 600 BC) [Prasad *et al.*, 2004; Nishteswar, 2011], one of the two foundational textbooks of Ayurveda. A

detailed description of PD was given in the ayurvedic textbook Basavarajiyam (1400 AD), and disease was called "*kampa vata*" [Nishteswar, 2011]. Leonard da Vinci also described many characteristics of the disease between 1489 and 1506 AD [Forno, 1996].

The cardinal features of PD are the movement problems of rigidity, resting tremor, and bradykinesia (i.e., abnormal slowness of movement). In later stages of the disease, loss of postural reflexes leads to postural instability and postural deformities associated with rigidity, such as flexed posture of the neck, trunk, elbows and knees are present [Hughes *et al.*, 1992, 1993; Jankovic, 2008]. Patients with PD also exhibit a variety of secondary motor symptoms that affect their functioning such as freezing of gait, blank facial expression and speech disorders [Lang *et al.*, 1998].

Non-motor features of the disease are common and are often considered the most debilitating limitations to normal daily functioning of patients [McDowell *et al.*, 2012; Videnovic *et al.*, 2012]. Roughly 90% of patients have substantial impact on their quality of life because of these non-motor symptoms, yet they are under-recognized because they are not considered part of the cardinal clinical features required for diagnosis of this disease [Chaudhuri *et al.*, 2010]. These non-motor symptoms include sleep disturbances, anosmia, autonomic dysfunction, decreased motivation, depression, anxiety and cognitive dysfunction [Chaudhuri *et al.*, 2010]. Interestingly, James Parkinson described the presence of sleep disturbances and other non-motor symptoms in his original essay [Parkinson, 1817].

PD is a progressive neurodegenerative disease of the nervous system [Lang *et al.*, 1998; Jankovic, 2008]. There are no biomarkers that are currently recognized for the early ante-mortem diagnosis of PD. The motor system manifestations of the disease are only evident after the pathology of the disease has reached an advanced stage, with typically over 60-80% of the

neurotransmitter dopamine (DA) lost in the striatum of patients before the first motor symptoms appear [Bernheimer *et al.*, 1973; Hornykiewicz, 1998; Jankovic, 2008]. At this time, the diagnosis of the disease still relies upon the presence, severity, progression of clinical motor symptoms of the disease and confirmation of diagnosis depends on post-mortem neuropathology [Hughes *et al.*, 1992, 1993; Litvan *et al.*, 2003; Braak *et al.*, 2003, 2004; Jankovic, 2008]. It is currently recognized that development of specific biomarkers for PD, allowing for earlier suspicion of the disease, would be useful to identify groups at risk of developing PD and would provide patients with an opportunity to start a neurorestorative therapy early in the disease [Airavaara *et al.*, 2011; Aron *et al.*, 2011].

1.1.2 **Etiology of PD**

Several genetic and environmental factors are now considered to play important roles in the etiology of the disease. The role of genetic factors in the etiology of PD was first described by Gowers [Gowers, 1896], who made the observation that there was an increase in PD occurrence among the relatives of PD patients. A minority of the cases, approximately 5-10% of PD patients, have a clear familial form of PD with an autosomal-dominant Mendelian form of inheritance [Olanow *et al.*, 1999; Schapira, 2006]. Mutations in ∝-synuclein, parkin, UCHL1, DJ1, PINK1, and LRRK2 are known to cause genetic forms of PD [reviewed in Moore *et al.*, 2005]. Some of them, like DJ1 and PINK1, encode for mitochondrial proteins and over-expression of others, like ∝-synuclein and parkin, induce mitochondrial deficits [Schapira, 2006]. In most cases, the proteins they encode are involved in cellular responses to oxidative stress, mitochondrial function, or affect normal functioning of protein degradation pathways in midbrain DA neurons [Schapira, 2006]. The lack of proper elimination of toxic free radicals, by-

products of oxidation reactions, and accumulation of ∝-synuclein can lead to acceleration of neuronal cell death [reviewed in Moore *et al.,* 2005; Schapira, 2006].

The concept that exposure to exogenous factors might also lead to the development of PD began to be recognized in late 1970's after drug addicts injecting a synthetic meperidine derivative resembling heroin developed remarkably similar anatomical and clinical features of PD [Davis *et al.,* 1979]. The compound, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, came to be referred to by the abbreviation, MPTP, and was an unintended by-product in the synthetic process (see Section 1.4.2 for further details). Since then, a number of epidemiological studies have examined the contribution of many environmental factors that increase the risk of developing PD. These include rural residency [Rajput *et al.,* 1986; Barbeau *et al.,* 1987], drinking well water [Tanner *et al.,* 1996], exposure to pesticides [Fleming *et al.,* 1994], industrial chemicals [Seidler *et al.,* 1996], and farming [Tanner *et al.,* 1996]. We now know that exposure to a number of exogenous toxins are associated with an increased risk of developing PD due to increased exposure of midbrain DA neurons to environmental toxins. These include trace metals, cyanide, organic solvents, carbon monoxide, carbon disulfide, rotenone, paraquat, and manganese [Olanow *et al.,* 1999; Schapira, 2006]. These chemicals lead to increased oxidative stress in the vulnerable midbrain DA neurons, that are affected in PD (See Section 1.4.3 for discussion of detailed biochemical changes).

Post-mortem brain pathology is used to definitively diagnose PD, distinguishing it from other similar disorders. The presence of two characteristic changes in brain tissue is required for this diagnosis: the presence of evidence to conclusively determine loss of midbrain DA neurons that project to basal ganglia and the presence of Lewy pathology [Fahn, 2003; Litvan *et al.,* 2003]. Lewy pathology is defined as the presence of intra-neuronal inclusions of aggregates of

misfolded proteins containing ∝-synuclein [Hawkes *et al.,* 2010]. These inclusions are detected using ∝-synuclein immunohistochemistry. Neurons containing these inclusions are typically referred to as 'Lewy bodies', that are round eosinophilic inclusions in a neuronal cell body or pleomorphic inclusions in cell processes that are dot-like, thread-shaped or spindle-shaped within axons and dendrites called as 'Lewy neurites'. It is now beginning to be accepted that there is a long dormant period of time before classic motor signs of PD begin to appear. Braak proposed a new staging system with six stages of progression of the disease from the brainstem to the neocortex based on neuro-pathological studies of post-mortem brain tissue of PD patients with varying degrees of Lewy pathology and severity of the disease [Braak *et al.,* 2003, 2006, 2008]. According to the Braak hypothesis, the progression of pathology appears to begin with Stage I, in the gut gastric myenteric plexus, followed by pathology in the olfactory bulb and dorsal motor nuclear complex of cranial nerves IX and X (DMNC). In Stage II, the Lewy pathology spreads to the pons, locus coeruleus, lower raphe and magnocellular parts of reticular formation. Only in Stage III is there involvement of the substantia nigra, and other basal mid-brain and forebrain regions including the amygdala, tegmental pedunculopontine nucleus, and tuberomamillary nucleus. The first two stages, before the spread of the pathology to midbrain and substantia nigra, are referred to as the prodromal phase of the disease, before the appearance of clinically-defined PD motor symptoms. Henceforth, these stages are referred to in this document as pre-clinical or pre-diagnostic stages of PD. In Stage IV, pathology progresses from the allocortex to the neocortex and there is presence of Lewy pathology in the thalamic intra-laminar nuclei, interstitial nucleus of stria terminalis, temporal mesocortex, and second sector of Ammon's horn, such that the pathology becomes widespread in the brain. In Stage V, there is intensification of the damage in areas already affected from stages I-IV, followed by progression to insular,

subgenual mesocortex and anterior cingulate cortex. In the final stage of progression (i.e., Stage VI) there is widespread damage affecting the entire topography of the brain including secondary sensorimotor areas like sensory association areas and pre-motor areas. In very advanced cases primary areas of the neocortex including primary auditory areas, primary motor and sensory areas (M1 and S1) are also affected. A dual-hit theory has been proposed by Hawkes, Tredici and Braak [Hawkes *et al.,* 2009] as a cause for this disease, where a pathogen enters the body via both the nasal and gut route (dual-hit) and spreads to the olfactory cortex and gut neurons (Stage I) and spreads eventually up the central nervous system to affect the entire brain.

1.2 THE BASAL GANGLIA

1.2.1 Anatomy of basal ganglia circuits

1.2.1.1 Anatomical connections within basal ganglia structures

The basal ganglia are comprised of a number of sub-cortical structures that form a complex network of connections with several nuclei in the forebrain, midbrain and thalamus (**Figure 1**). The basal ganglia were traditionally thought to be involved in motor control and execution [Alexander *et al.,* 1990]. However, with the advancement of our understanding of the different nuclei, the basal ganglia are now considered to be important in the processes that underlie the learning of both new complex motor behaviors (i.e., movement, goal-directed behaviors), as well as a number of non-motor behaviors (i.e., habits, emotions, motivation, sleep and cognitive function) [Yin *et al.,* 2006; Graybiel *et al.,* 2008; Haber *et al.,* 2009]. In this section, the different component nuclei of the basal ganglia, their anatomical location and how they are

connected to the other nuclei, as well as the nature of their output projections (excitatory or inhibitory) and the targets of their innervation are described [Alexander *et al.*, 1990; DeLong *et al.*, 2007; Haber *et al.*, 2009].

The components of the basal ganglia include **(**see **Figure 1)**:

A. The striatum, comprised of the caudate nucleus, and the putamen,
B. The subthalamic nucleus (STN),
C. The globus pallidus, comprised of the internal segment (GPi), the external segment (GPe) and the ventral pallidum,
D. The substantia nigra, comprised of the pars compacta (SNpc) and the pars reticulata (SNpr)

The major input pathways to the basal ganglia come through the striatum and STN. All inputs are excitatory. The major output pathways of basal ganglia go through GPi and SNpr. All outputs from the basal ganglia are inhibitory.

A. The striatum,

   Inputs: The striatum receives inputs from many distinct functional regions from across the entire cerebral cortex (for details, see Section 1.2.1.2). This is the pathway for the main input into the basal ganglia. All inputs to the striatum are glutamatergic and excitatory in nature.

   Outputs: The main output from the striatum is through the globus pallidus, both GPi and GPe. All outputs from striatal neurons are GABAergic and inhibitory.

B. The subthalamic nucleus (STN),

   Inputs: The STN receives both glutamatergic excitatory inputs from the cerebral cortex and inhibitory GABAergic inputs from the GPe.

   Outputs: The Globus Pallidus is the main output nucleus receiving excitatory outputs from the STN. The STN sends excitatory glutamatergic outputs to both GPe and GPi.

C. The globus pallidus, comprised of the

Globus pallidus, internal segment (GPi),

Inputs: The GPi receives inhibitory GABAergic inputs from both the striatum and the GPe, as well as excitatory glutamatergic inputs from the STN.

Outputs: GPi neurons send inhibitory GABA outputs to the thalamus and brainstem.

Globus pallidus, external segment (GPe)

Inputs: The GPe receives inhibitory GABAergic inputs from the striatum and excitatory glutamatergic inputs from the STN.

Outputs: GPe neurons send inhibitory GABAergic outputs back to the striatum, STN and the GPi.

D. The substantia nigra, comprised of

Substantia Nigra, pars compacta (SNpc)

Inputs: The SNpc receives inhibitory GABA inputs from the striatum.

Outputs: SNpc sends its output to the striatum using the neurotransmitter dopamine, DA. DA has excitatory effects on D1 receptors contained in striatal neurons that project through the direct pathway to the GPi. DA has inhibitory effects on D2 receptors contained in striatal neurons that project through the indirect pathway to the GPi via connections through the GPe-STN circuit. Thus, the SNpc dopaminergic nigrostriatal projection plays an important modulatory role in mediating basal ganglia function through both these pathways.

Substantia Nigra, pars reticulata (SNpr)

Inputs: The SNpr receives inhibitory GABAergic inputs from the striatum and the GPe, as well as excitatory glutamatergic input from STN.

Outputs: The outputs from SNpr are inhibitory GABAergic outputs to the thalamus and brainstem. These SNpr outputs specifically connect to thalamus that project to cortical and to brainstem regions that control eye movements.

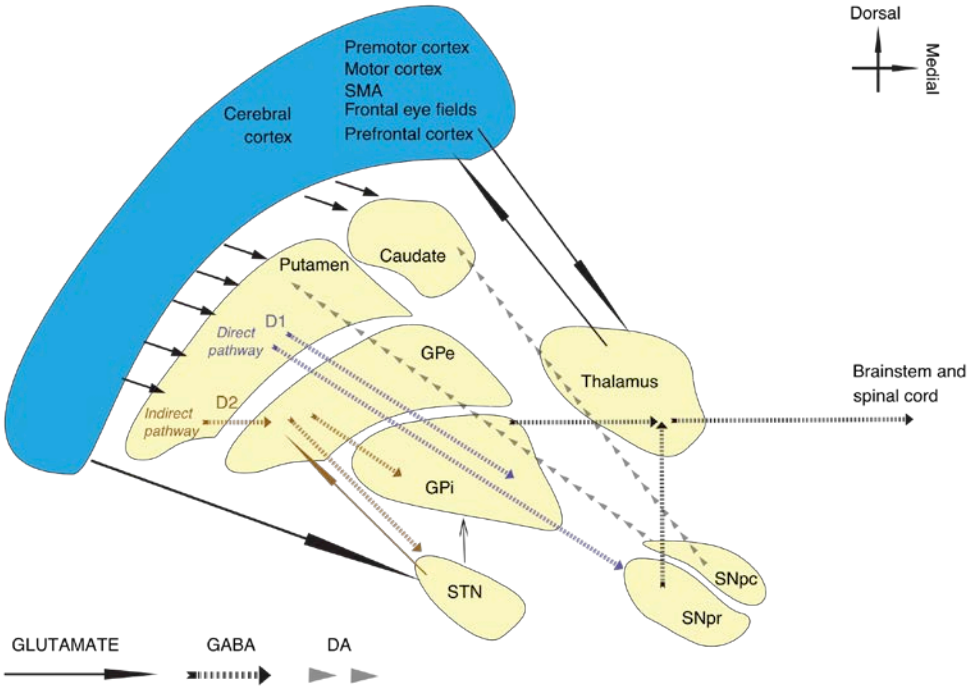


Figure 1. Basic circuit diagram of the different interconnected nuclei in basal ganglia. The three main neurotransmitter systems are indicated by three different arrowheads: glutamate, GABA and DA. Substantia Nigra, pars compacta (SNpc); Substantia Nigra, pars reticulata (SNpr); Globus pallidus, external segment (GPe); Globus pallidus, internal segment (GPi); subthalamic nucleus (STN); Supplementary motor area (SMA); [based on Figure 31.2, Fundamental Neuroscience, 2002]

1.2.1.2 Distinct parallel sub-circuits within and outside the basal ganglia

The striatum receives input to the basal ganglia from almost the entire cerebral cortex. Cortical input is glutamatergic. Projections from the cortex are made on to the dendrites of GABAergic medium spiny neurons (MSN), the major type of neuron present in the striatum. The topography of projections from the cerebral cortex are somewhat preserved within the basal ganglia through distinct 'parallel loops' that forms sub-circuits within each sub-cortical basal ganglia structure [Alexander *et al.,* 1986, 1990; Middleton *et al.,* 2000]. An example of this sub-circuit is the somatomotor circuit that is affected in PD. The neurons from the somatosensory and motor cortices project to the putamen. In PD, the putamen is generally first affected before the disease spreads to other sub-circuits [Graybiel *et al.,* 2000, 2008]. Not surprisingly then, the putamen is the target of our neurotrophic factor infusions after MPTP administration in monkeys (see Section 2.2.3). In the later stages of PD, the disease progresses the other sub-circuits including the limbic and cognitive circuits. The spread of the disease within the basal ganglia follows a dorsolateral to ventromedial path that corresponds roughly to the spread of the dysfunction from anterior putamen involved in motor functions to the posterior tail of the caudate nucleus involved in higher order functions like cognition [Graybiel *et al.,* 2000, 2008]. There are also basal ganglia circuits that are specifically oculomotor, cognitive and limbic in nature. [Middleton *et al.,* 2000].

The ventral portion of the striatum is generally not included as part of the traditional basal ganglia circuitry. However, the ventral striatum, consisting of the nucleus accumbens and the olfactory tubercle, has connections that follow a similar pattern as the traditional basal ganglia [Cardinal *et al.,* 2002; Nicola, 2007]. The DA neurons from the mid-brain ventral tegmental area (VTA) send projections to the nucleus accumbens. However, unlike the

traditional basal ganglia structures that receive glutamatergic input from neocortex, nucleus accumbens receives direct input from the amygdala, which is part of the limbic system. The limbic system is involved in the control a variety of behaviors including mood, memory, motivation and olfaction [Mogenson *et al.,* 1980; Salamone, 1992; Ikemoto *et al.,* 1999; Cardinal *et al.,* 2002; Nicola, 2007; Haluk *et al.,* 2009]. Thus, changes to motivation and mood that are seen in later stages of PD could be linked to changes in the function of the ventral striatum. Thus, increasing the activity of accumbens circuits could lead to improvement of motivation leading to better performance in motor tasks (see Section 6.4.2).

1.2.2 **Physiology of the basal ganglia**

The study of the physiology of basal ganglia has been mostly focused on the relationship of its component nuclei to behaviors associated with movement. Much of what we know about the functions of the different structures within the basal ganglia comes from lesion studies in animals, followed by subsequent examination of the firing patterns of the constituent neurons in each structure in relation to various forms of movement [Alexander *et al.,* 1990; Hamada *et al.,* 1992; Chesselet *et al.,* 1996; Middleton *et al.,* 1996; Wichmann *et al.,* 1996; Fundamental Neuroscience, 2002; Yin *et al.,* 2004; Clarke *et al.,* 2008; Inokawa *et al.,* 2010; Bostan *et al.,* 2010].

A. The striatum,

   In the striatum, 80-90% of neurons are inhibitory GABA-containing medium spiny neurons (MSN) that project outside the striatum and fire at 0.1-1 Hz. About 10-15% of the neurons in striatum are tonically active, firing at 2-10 Hz, and hence are called Tonically Active Neurons (TAN's). These neurons are differentiated from the MSN's

based on their firing frequency, and they appear to be cholinergic interneurons [Inokawa *et al.,* 2010]. An even smaller subset of the neurons within the striatum are parvalbumin-positive fast-spiking inhibitory local GABAergic interneurons [Inokawa *et al.,* 2010]. Different regions of the striatum have been linked to different behaviors. In rodents, there is just one contiguous structure, and lesions of the dorsolateral portions of this rodent striatum disrupt the formation of habits [Yin *et al.,* 2004]. In primates, lesions of the medial portions of the striatum, mainly the anterior portion of the head of the caudate along with very small lesions in the anterior and medial portions of putamen, lead to enhanced preservative behavior [Clarke *et al.,* 2008].

B. The subthalamic nucleus (STN),

The neurons in the STN contain the excitatory neurotransmitter glutamate and fire at a rate of about 20 Hz. About 90% of these neurons increase their firing rate prior to the onset of eye or limb movement. The region is somatotopically organized into different functional circuits. Thus, there are different projections from the cortex to distinct parts of the STN, that in turn project to other distinct basal ganglia nuclei [Alexander *et al.,* 1990; Bostan *et al.,* 2010]. Lesions of STN in normal monkeys induce dyskinesias in the contralateral limbs [Hamada *et al.,* 1992].

C. The globus pallidus, comprised of

Globus pallidus, internal segment (GPi),

The neurons in GPi are inhibitory and contain the neurotransmitter GABA, They are tonically active and fire at very high rates of 60-80 Hz. The neurons in this region are again somatotopically organized with leg and arm regions present in the GPi. Approximately 70% of GPi neurons increase their activity and 30% decrease their activity during arm movement. However, lesions of the GPi lead to no discernible physical changes to movement in either humans or experimental animals. Most of the

findings from lesions of the GPi result in subtle temporary changes in fine motor movement, at most [Wichmann *et al.,* 1996], so their particular role in normal behavior is still an open question.

Globus pallidus, external segment (GPe)

The neurons present in GPe also contain the inhibitory neurotransmitter GABA and can be classified into two types based on their firing rates. A majority of these neurons fire at a relatively high frequency of around 70 Hz, with their firing interrupted by long pauses. A small minority of GPe neurons fire around 10 Hz and have frequent spontaneous bursts of activity. Both types of neurons increase their activity during movement. Injections of biccuculine to inhibit neuronal activity in GPe, or lesions of GPe, induce rigidity or cataplexy [Wichmann *et al.,* 1996]. GPe is also implicated in Huntington's disease, as it is one of the main sites of neuronal degeneration. Chorea, which is comprised of dance-like movements that spread from one muscle to another, belongs to a group of neurological disorders called dyskinesias. Chorea is also common in Huntington's disease and abnormal functioning of the GPe is thought to mediate the generation of this dyskinetic movement [Chesselet *et al.*, 1996].

D. The substantia nigra, comprised of

Substantia Nigra, pars compacta (SNpc)

The DA neurons in SNpc fire at a constant rate of around 2 Hz and are not organized somatotopically. Unlike the rest of the basal ganglia, the neurons in SNpc do not fire with relation to movement. However, these DA neurons fire in response to environmentally relevant cues such as reward, motivation or salient instructions. Thus, the firing activity of these neurons has been suggested to modify striatal neuron outputs based on cortical inputs that occur related to a specific environmental context. For instance whenever a

tone is paired with the reward of juice after pressing a lever, the dopamine cells initially respond to the lever press and eventually the cells increase their firing just upon presentation of the rewarding tone. These DA neurons project to the striatum within basal ganglia 'parallel loops' that are part of sensorimotor, associative and limbic circuits (see Section 1.2.1.2). However, we do not yet fully understand all the specific roles that are played by the DA system through its projections to these basal ganglia sub-circuits.

Substantia Nigra, pars reticulata (SNpr)

SNpr has GABAergic inhibitory neurons that are tonically active and they provide the output for face and eye movement-related activity from the basal ganglia. The neurons here are again somatotopically organized. However, unlike GPi neurons the SNpr neurons decrease their firing rate during saccadic eye movements. Lesions in the medial regions of SNpr produce a characteristic visual hallucination syndrome called "peduncular hallucinosis". In peduncular hallucinosis, patients experience vivid realistic images of people and environments familiar to them. It is this characteristic that makes it hard for patients to distinguish these images from reality. Most patients experiencing "peduncular hallucinosis" also experience abnormal sleep patterns characterized by insomnia and excessive daytime sleep, both symptoms present in many PD patients also (see Section 1.3.2, Chapters 3 and 4). Similar visual hallucinations are reported in patients with other lesions of SNpr or in brainstem compression [Middleton *et al.,* 1996].

1.3 SYMPTOMS IN PD

1.3.1 Motor symptoms in PD

There are three classical motor symptoms that have been well characterized in PD: bradykinesia, rigidity and tremor [Gelb *et al.,* 1999; Fahn, 2003; Jankovic, 2008]. However, at this time there is no definitive diagnostic pre-clinical test or biomarker to predict PD before the first appearance of any of the motor signs of PD, and the motor symptoms manifest only after there has been a significant loss of striatal DA content. The pathological confirmation of nigrostriatal degeneration and presence of Lewy bodies on post-mortem analysis represent the gold standard for the definitive diagnosis of PD [Hughes *et al.,* 1992, 1993; Olanow *et al.,* 1999; Stern *et al.,* 2012; Berg *et al.,* 2013]. Currently, the presence of a combination of motor features of the disease, asymmetry, and the patient's response to levodopa, are the main clinical criteria used for diagnosing PD.

'Bradykinesia' is derived from combining the Greek word "brady", meaning slow, and "kinesia", meaning motion. Bradykinesia in patients with PD varies in degree of slowness in all aspects of automatic, habitual, voluntary and involuntary movements from mildly slowed to a complete absence of movement ("akinesia") in a minority of patients. Some examples of automatic and habitual movements that can be affected include decreased swinging of arms during walking, decreased eye blinking, less frequent swallowing of saliva leading to an increased probability of drooling [Jahanshahi *et al.,* 1993; Bagheri *et al.,* 1999; Berardelli *et al.,* 2001; Turner *et al.,* 2003; Tumilasci *et al.,* 2006]. Bradykinesia also affects tasks that require fine motor skills such as buttoning cloths and using cutlery. It is one of the most common symptoms of the disease affecting almost all PD patients. In fact, the United Kingdom

Parkinson's disease clinical criteria lists bradykinesia as a necessary condition required for the diagnosis of PD [Gelb *et al.,* 1999]. The slowness in movement can be either during the execution phase of the movement or during the initial preparatory phase before movement. In early stages of the disease, bradykinesia usually occurs in the execution phase of movement, with a decrease in electromyography signal (EMG) needed to accelerate limb movements [Hallett *et al.,* 1980]. However, in later stages of PD the preparatory phase of movement is also affected; this is especially evident when patients are asked to self-initiate movements as opposed to responding to external cues [Jahanshahi *et al.,* 1993,1995].

Rigidity is the property of being stiff and resisting the flexion, extension or rotation of a joint during passive limb movement [Berardelli *et al.,* 1983; Cantello *et al.,* 1995; Gelb *et al.,* 1999; Broussole *et al.,* 2007; Jankovic, 2008]. Although rigidity is classified as a cardinal feature of PD it does not impair the quality of life as much as the other symptoms, except on occasions when it is associated along with pain during joint movement. Normally, when muscles stretch there is a naturally-occurring suppression of antagonistic muscles. However, in PD patients there is actually an activation of the antagonist muscles during a passive movement that is thought to cause rigidity [Berardelli *et al.,* 1983; Cantello *et al.,* 1995]. The lack of suppression of antagonistic muscles is believed to result from abnormal inhibitory output of basal ganglia circuits in PD [Xia *et al.,* 2004]. In later stages of the disease the constant antagonistic muscle activation leads to postural changes such as the flexed elbows, flexed knees, flexion of the trunk and neck that leads to the classic parkinsonian posture and gait in PD (i.e., stooped posture with small steps) [Parkinson, 1817; Gelb *et al.,* 1999].

Tremor is the most prominent and noticeable motor symptom in PD. It was this symptom that led James Parkinson to initially call this disease 'Paralysis agitans', which means shaking

palsy in English [Parkinson, 1817]. The most common tremor in PD is the 'rest tremor' [Jankovic *et al.,* 1999; Shahed *et al.,* 2007; Jankovic, 2008; Hallett *et al.,* 2012]. This tremor has a characteristic frequency between 3-7 Hz. When patients perform voluntary movements or stretch their limbs the tremor is absent and hence does not affect the performance of daily activities. The pure rest tremor frequently occurs in the distal appendages (i.e., the arms and legs) and less frequently in the neck, body and head. However, most patients exhibit a range of tremors with different characteristics [Deuschl *et al.,* 1998]. Type I tremor is the classic rest tremor condition that is described above. Type II tremors are a mixture of both distal rest tremors and postural tremors. This type the postural tremor has a higher frequency (>1.5 Hz) than the distal rest tremor condition. Type III tremors refer to conditions when there is only the presence of pure postural body tremors, with no rest tremors in the distal extremities. The parkinsonian tremor arises due to pronation and supination because of an alternate activation of the agonist and antagonist muscles, and is thus characteristically different from other essential tremors that are caused by co-contraction of agonist and antagonist muscles. Oscillations in the diseased basal ganglia circuits are thought to be the primary mechanism underlying tremor development in PD [Olanow *et al.,* 1999; Jankovic, 2008; Hallett *et al.,* 2012].

There are other changes to the motor system that also occur in later stages of PD. Freezing, a variant of akinesia, is another unique characteristic of PD, although it does not occur universally in all patients [Giladi *et al.,* 1997, 2001; Schaafsma *et al.,* 2003; Bloem *et al.,* 2004; Macht *et al.,* 2007]. It is defined as the sudden and transient inability to move due to complete loss of movement, a form of akinesia. This includes freezing of the legs during walking, and inability to move arms or eyelids. Freezing of the gait, followed by loss of postural reflexes, is one of the most common reasons for patient falls in PD, that leads to other complications such as

fractures. This is compounded by problems that normally occur with the aging process, including balance problems and a decreased ability to integrate multimodal sensory cues including speech, visual, vestibular and proprioceptive cues, [Critchley *et al.,* 1981; Rascol *et al.,* 1989; Hood *et al.,* 2007; Jankovic, 2008; Ferrer *et al.,* 2012]. Decreased facial expression ("hypomimia"), decreased amplitude of voice and a decrease in size of handwriting from normal to minute ("micrographia") are other common symptoms reported in PD.

1.3.2 **Non-motor symptoms in PD**

It is now well accepted that PD is a disease that affects multiple neuronal systems and has both motor and non-motor complications [Braak *et al.,* 2003, 2004; Pfeiffer, 2007; Berg *et al.,* 2013]. The non-motor symptoms can be broadly divided into four categories: neuropsychiatric, autonomic, sensory and sleep. Among these, some symptoms including a decline in olfactory function (anosmia), sleep disorders (e.g., rapid-eye movement (REM) sleep behavioral disorder, excessive daytime sleepiness), and constipation can occur years to decades before the appearance of clinically diagnosed PD [Abbott *et al.,* 2005; Braak *et al.,* 2006, 2008; Hawkes *et al.,* 2010; Gao *et al.,* 2011].

Neuropsychiatric symptoms can be mild to severe in nature and include anhedonia, apathy or decreased motivation (discussed in detail in Section 6.4.2.1), panic attacks, social phobias, depression, problems with controlling emotions, visual hallucinations, delusion, paranoia, mania, fatigue, mood disorders, cognitive dysfunction, loss of motivation and affect [Bayulkem *et al.,* 2010; Grinberg *et al.,* 2010; Korczyn *et al.,* 2010; Pourcher *et al.,* 2010; Jellinger *et al.,* 2011; Ferrer *et al.,* 2012]. Many of these behavioral and neuropsychiatric symptoms are known to occur with increased severity as the disease progresses. There has been

some correlation shown between these symptoms and the different stages of development of PD as classified by Braak. (for details see Section 1.1.2)

Autonomic nervous system failure is also called dysautonomia. It is experienced by about 75% of PD patients [Claassen *et al.,* 2010]. It is present as a constellation of symptoms that affect the autonomic system including: cardiovascular, gastrointestinal, urological, thermoregulatory, and respiratory disturbances. Orthostatic hypotension is known to occur in PD patients where there is a sudden massive drop in blood pressure when the patient gets up from a seated position. It could be due to the degeneration of early stage cardiac sympathetic neurons. There are reports which support this based on data that show reduced uptake of I-MIBG- (I-metaiodobezylguanidine) which measures cardiac sympathetic nervous function peripherally [Doorn *et al.,* 2012; Zeimssen *et al.,* 2010]. Additionally, some post-mortem studies have reported the presence of alpha-synuclein, beginning in the axons of the cardiac sympathetic nervous system before affecting the cell bodies and other sympathetic ganglia [Zeimssen *et al.,* 2010; Ferrer *et al.,* 2012]. A multiplicity of gastrointestinal (GI) problems including dysphagia (difficulty in swallowing), drooling, dry mouth, belching, nausea, abdominal bloating, constipation and anismus are also common [Claassen *et al.,* 2010; Jost, 2010]. Some theories suggest the GI tract as the origin of degeneration of neurons in PD [Braak *et al.,* 2006; Hawkes *et al.,* 2009]. Alpha-synuclein is present in Meissner's and Auerbach's plexuses that project axons into the gastrointestinal mucosa [Braak *et al.,* 2006; Jost, 2010]. Some of the highest densities of alpha-synuclein deposits are found in lower esophagus and submandibular gland [Wakabayashi *et al.,* 1988,1990; Braak *et al.,* 2006; Beach *et al.,* 2010; Ferrer *et al.,* 2012]. Urinary tract problems, such as increased urgency and frequency to empty, nycturia (excessive urination at night) are also present in a majority of PD patients. The disinhibition of the detrusor muscle that

contracts before the bladder is full is thought as the reason for this. A bradykinesia that affects the spinchter characterized by a delayed relaxing of the muscle also adds to the problem. Apart from the symptoms described above, tachycardia (increase in heart rate), papillary dilation and other vascular and respiratory disturbances are also reported [Ferrer *et al.*, 2012].

There are different sensory abnormalities that are present in PD. They include disturbances in olfaction, vision and pain [Pfeiffer, 2007; Bayulkem *et al.*, 2010; Korczyn *et al.*, 2010; Ferrer *et al.*, 2012]. Olfactory disturbances have been reported in the early stages of PD [Hawkes *et al.*, 1997]. Anosmia has been reported as an indicator of future risk to develop PD in epidemiological studies [Ferrer *et al.*, 2012]. This also correlates well with the proposed development of alpha-synuclein aggregates in the anterior olfactory nucleus in the first stage of PD (for details see Section 1.1.2). There are dopamine neurons present in the olfactory bulb, however whether these are the first to develop these inclusions or are responsible for the condition of inability to smell is not clear. Blurred vision is present in many patients [Archibald *et al.*, 2009]. Sometimes the loss of DA neurons in the retina also leads to impaired color vision [Archibald *et al.*, 2009]. A large number of patients have also consistently reported problems with visual acuity (i.e., visual contrast sensitivity) [Pfeiffer, 2007; Archibald *et al.*, 2009]. Some patients also experience visual hallucinations (see Section 1.2.2). Various types of pain are commonly experienced by PD patients: mucoskeletal pain, dystonia-associated pain, radicular pain, akathitic pain, and central pain [Korczyn *et al.*, 2010]. The underlying causes of pain are complex and may not be directly related to the cause of the disease. The stooped and altered posture can lead to mucoskeletal and radicular pain. The dystonic pain is often associated with the non-medicated periods of the day. The central pain is thought to be of thalamic origin and has a burning characteristic.

Sleep disturbances constitute the most commonly reported non-motor problems occurring in PD [Lees et al 1988; Tandberg et al 1999; Arnulf et al 2000; Ondo et al 2001; Hobson et al 2002; Brodsky et al 2003; Arnulf 2005; Chaudhuri *et al.,* 2010; Knie et al 2011; Schulte et al 2011; Videnovic et al 2012]. Sleep disturbances have an adverse effect on the quality of life in both the patients and their caregivers. A number of problems with sleep, ranging from a change in pattern of nighttime sleep to excessive daytime sleepiness, are observed in patients [Knie et al 2011; Schulte et al 2011; Videnovic et al 2012]. Obstructive sleep apnea can also be present in PD patients and continuous positive air pressure (CPAP) devices have been used for treatment. Rest tremor occurring during sleep can also awaken the patient. Many prospective studies have shown that excessive daytime sleepiness and REM sleep problems can occur several years before the first motor symptoms of PD and hence is an important risk factor for the disease [Abbott *et al.,* 2005; Gao *et al.,* 2011; Iranzo *et al.,* 2006; Martinez-Martin, 2011; McDowell *et al.,* 2012]. REM sleep behavioral disorder, where the patients begin to act out their dreams, is known to cause injuries to both the bedmate and the patient [Iranzo *et al.,* 2006, Knie *et al.,* 2011]. Nighttime insomnia is also observed in PD patients [Schulte *et al.,* 2011]. Possible causes of insomnia include increased sleeping during the day, inability to move in the bed at night, nycturia, anxiety and depression. The presence of excessive daytime sleepiness has also been reported many years ahead of the development of clinically recognized signs of PD (See Chapter 3 for more details on daytime sleep). Many neural systems that affect sleep in the brain stem and other lower regions in the brain (for detailed discussion see Section 3.4) are affected first according to the Braak model of PD progression (see Section 1.1.2). This may well be the reason for the appearance of these non-motor symptoms well ahead of the clinical diagnosis of the motor problems in PD.

1.4 ANIMAL MODELS OF PD

The use of animal models to investigate the pathophysiology of PD has provided valuable insights both of how PD affects the basic physiology of basal ganglia circuits, and identification of treatments that may be most efficacious for treating the motor symptoms of PD. The development of PD animal models began with the accidental discovery of the role of dopamine in reserpine-treated rats. Reserpine administration leads to akinesia and central loss of monoamines in rats [Carlsson 1957]. However, only L-dopa, the precursor to dopamine, was able to resuscitate motor function in these rats; 5-hyroxytryptophan, the precursor to serotonin, was not able to recover motor function. This discovery of the ability of L-dopa to reverse motor deficits in the reserpine-treated model paved the way for the hypothesis that dopamine deficiency played a key role in PD [Carlsson 1959]. This hypothesis was confirmed shortly thereafter by the discovery that there is indeed a loss of striatal dopamine in patients with PD [Carlsson 1959; Ehringer *et al.*, 1960], which led to the search for chemical agents that could selectively incapacitate dopamine neurons and the first such discovery was of 6-hydroxydopamine (6-OHDA) to lesion DA neurons in the rat [Porter 1963; Ungerstedt 1968].

1.4.1 The 6-OHDA model of PD

It has been five decades since the development of the rat 6-OHDA model of PD and it still continues to be the model for PD that is most routinely utilized in Parkinson's disease research. In 1968, Ungerstedt injected 6-OHDA in the SNpc and was able to demonstrate loss of nigrostriatal dopamine neurons [Ungerstedt 1968].

A. Mechanism of action of 6-OHDA

The molecular structure of 6-OHDA is very similar to the molecular structure of DA, and hence the dopamine transporter has a very high affinity for the neurotoxin and transports it into the DA cell [reviewed in Blum *et al.,* 2001]. 6-OHDA accumulates within the DA neuron and two main mechanisms have been proposed for its action leading to neurotoxicity: oxidative stress and mitochondrial deficits.

Oxidative stress has been observed after 6-OHDA administration *in vivo* [Permual *et al.,* 1989, 1992; Kumar *et al.,* 1995] and *in vitro* [Tiffany-Castiglioni *et al.,* 1982; Decker *et al.,* 1993; Abad *et al.,* 1995]. An increase in oxidative stress is due to the generation of hydrogen peroxide, due to either rapid auto-oxidation of 6-OHDA [Heikkila *et al.,* 1972; Seitz *et al.,* 2000; Soto- Otero *et al.,* 2000] or deamination of 6-OHDA by the enzyme monoamine oxidase (MAO) [Breese *et al.,* 1971; Karoum *et al.,* 1993]. Hydrogen peroxide, in the presence of iron (present in SNpc giving it the charcteristic black appearance in post-mortem tissue), leads to increased formation of reactive oxygen species by the Fenton reaction [Ben Shachar *et al.,* 1991; Borisenko *et al.,* 2000]. Moreover, increased levels of iron in the striatum and SNpc accumulate after administration of 6-OHDA [Hall *et al.,* 1992; He *et al.,* 1996; Oestreicher *et al.,* 1994]. This mechanism of action of 6-OHDA was confirmed through experiments co-administering 6-OHDA and iron chelating agents resulting in a reduced neurotoxic insult of the 6-OHDA [Ben Shachar *et al.,* 1991; Borisenko *et al.,* 2000].

6-OHDA also inhibits the respiratory chain mechanism in complex I of isolated brain mitochondria *in vitro* [Glinka *et al.,* 1995, 1996, 1998]. *In vivo,* 6-OHDA leads to pathological changes in the mitochondrial membrane potential after an increase in intracellular levels of reactive oxygen species [Lotharius *et al.,* 1999]. This can lead an increase in intracellular concentration of DA, and DA is toxic to cells when it is not

present inside vesicles [Hastings *et al.,* 1996; Zigmond *et al.,* 2002]. Both these changes can also lead to cell death [Lotharius *et al.,* 1999; Hastings *et al.,* 1996; Zigmond *et al.,* 2002].

B. Methods of delivery of 6-OHDA

There are several 6-OHDA models that have been developed (for review see Deumens *et al.,* 2002; Blandini *et al.,* 2008). 6-OHDA does not cross the blood brain barrier. 6-OHDA injected directly into the two brain hemispheres leads to a non-selective loss of neurons in all monoaminergic pathways namely, dopamine, noradrenalin and serotonin leading to a complex pathology with some similarities to PD [Deumens *et al.,* 2002; Blandini *et al.,* 2008]. However, bilaterally injected animals exhibit severe motor dysfunction and require special animal care, such as tube-feeding to overcome aphagia (deficit in swallowing) and adipsia (deficit in drinking) [Deumens *et al.,* 2002; Blandini *et al.,* 2008]. To develop a more refined PD model, 6-OHDA has been administered after pre-treatment with either desipramine (i.e., a blocker of 6-OHDA uptake by noradrenergic neurons) or citalopram (i.e., a blocker of 6-OHDA uptake by serotonin neurons). A more recently developed method of 6-OHDA delivery is a unilateral 6-OHDA injection to prevent the high mobidity and mortality caused by bilateral 6-OHDA administration. Unilateral injections have been given in the SNpc, the medial forebrain bundle (MFB) and the striatum.

Injection of 6-OHDA in the SNpc leads to a massive and almost complete loss of dopamine neurons in the midbrain [Ungerstedt 1968]. The problem with this method is the lack of specificity to target the nigrostriatal dopamine neurons, as some loss in the other midbrain dopamine structures, such as the VTA, also occurs because it is difficult

to only inject in the very small structure of SNpc. SNpc injection leads to an animal model that resembles advanced stages of PD, with close to a 90% loss of TH-positive neurons. The timecourse of dopaminergic neuronal degeneration is very rapid, with loss of SNpc neurons beginning in the first twelve hours after 6-OHDA administration and the loss of striatal dopamine terminals being detectable within two to three days after 6-OHDA administration.

Injection of 6-OHDA in the MFB also leads to a nearly complete loss of DA neurons (> 95% loss of TH-positive cells) in the SNpc, with very few neurons surviving the insult [Deumens *et al.*, 2002]. The major drawback of using this method of injection is that both VTA and SNpc send axons through this bundle and hence there is a near complete loss of midbrain TH-positive neurons. This generates a condition that has more severe loss of midbrain DA neurons than observed in PD and hence this model is used to quickly generate a severe PD-like animal useful to study the mechanisms present in the disease.

Injection of 6-OHDA in the striatum is the most commonly used model of PD, as it produces immediate loss of striatal TH-positive DA terminals and initiates a process which leads to the loss of axon terminals first with a delayed, progressive, loss of SNpc TH-positive neurons [Zigmond *et al.*, 1990; Deumens *et al.*, 2002; Blandini *et al.*, 2008]. Injections of 6-OHDA into the striatum leads to less extensive damage in both SNpc neurons (roughly 50-70% loss) and striatal TH-positive terminals (roughly a 60-80% loss). Partial lesions of the dopamine system using the striatum as the injection site and a moderate concentration of 6-OHDA produces a good model of the early to middle stages of PD. This rat model has evolved as the model of choice for testing the mechanisms of

action of new treatment paradigms in PD. The time course of 6-OHDA actions in this model is described below. The injection of a single dose of 6-OHDA (20 μg/3 μL) leads to a one-third loss of TH-positive terminals in the striatum within a day [Deumens *et al.,* 2002; Blandini *et al.,* 2008]. This is followed by a slow linear progression with a loss of about two-thirds of the DA neurons within striatum by the three weeks after the lesion [Blandini *et al.,* 2008]. In the SNpc loss of DA neurons is not evident for about a week after 6-OHDA injection and DA neuronal loss slowly increases to around 20% two weeks post-6-OHDA and then peaks and stabilizes with about a 40-50% loss of DA neurons by week four after 6-OHDA injection. There is inflammation in the striatum associated with a 6-OHDA injection that progressively reduces in severity over the first four weeks after injection. Similarly, there is a weaker inflammation process in the SNpc also reduces in severity four weeks after injection.

C. Functional changes after 6-OHDA

There are three main phases of response that occur after 6-OHDA administrations [Agid *et al.,* 1973; Hefti *et al.,* 1980; Altar *et al.,* 1987; Hudson *et al.,* 1995; Zigmond *et al.,* 1981,1990, 1997, 2002]. The first phase constitutes acute changes in the striatal dopaminergic neurotransmission as a result of the neurotoxin insult. In the second phase, there are compensatory changes to accommodate the loss of DA neurotransmission. The final third phase encompasses progressive changes to the basal ganglia circuits that occur in parallel to the degenerative changes to the SNpc neurons in the midbrain following 6-OHDA administrations.

The changes that happen in Phase II and Phase III after this insult are described below. After a large initial 6-OHDA insult to striatal DA neurotransmission there are

compensatory changes in the function of the remaining neurons to try to maintain the same level of dopaminergic influence on striatal function. First, there is an increase in the amount of DA that is released from remaining DA terminals after each depolarization to maintain the level of DA available to striatal neurons. Second, there is a decrease in the high affinity DA reuptake sites resulting in prolonged DA stimulation to the post-synaptic DA receptors. These compensatory changes sometimes continue over a long period of time leading to a behavioral adaptation to the 6-OHDA insults, so that there appears to be a spontaneous recovery of function. This recovery of function can also be partially mediated by sprouting from the remaining TH-positive fibers in the striatum. There can also be an increase in the production of DA in the remaining terminals. (A detailed review of all the biochemical changes is in Section 1.4.3)

1.4.2 **MPTP model of PD**

In the mid-1970's a new meperidine analog termed 'synthetic heroin' that was supposed to contain 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP) was developed in northern California. However, the synthesis had gone awry and when the meperidine analog was used intravenously by habitual drug addicts they began to develop severe PD-like symptoms [Davis *et al.,* 1979]. It was later discovered that this contaminated batch originated from a home-based laboratory set up by a 23-year graduate student who had missed one crucial step in the biosynthesis of MPPP, and instead had produced 1-methyl-4-phenyl-1,2,5,6-tetrahydro-pyridine (MPTP) as a by product of this reaction [Langston *et al.,* 1983]. MPTP was identified as the toxin that led to the production of PD-like symptoms in relatively healthy young adults [Langston *et al.,* 1983]. Further analysis revealed 1-methyl-4-phenyl-pyridinium ion (MPP^+) as

the metabolite of MPTP that was selectively toxic to SNpc dopaminergic neurons [Langston *et al.,* 1984]. These findings led to the development of the first monkey model of PD, in which injection of MPTP in squirrel monkeys caused degeneration of nigral dopaminergic neurons and development of symptoms similar to PD [Langston *et al.,* 1984].

The monkey MPTP model has been utilized since this time to study symptoms of PD that are related to dopaminergic neuronal degeneration [Langston *et al.,* 1984; Bankiewicz *et al.,* 1986; Bergman *et al.,* 1990; Smith *et al.,* 1993; Benazzouz *et al.,* 1993; Ovadia *et al.,* 1995; Gash *et al.,* 1996; Bezard *et al.,* 2001; Emborg, 2007; Bove *et al.,* 2012]. PD-like motor symptoms in the monkey MPTP model are responsive to levodopa. Like Parkinson's patients treated for an extended time with levodopa, MPTP-treated monkeys receiving L-dopa for prolonged periods develop levodopa-induced dyskinesias [Przedborski *et al.,* 2001; Emborg, 2007].

A. Mechanism of action of MPTP

MPTP is a highly lipophilic drug that easily crosses the blood-brain-barrier. Within the brain, MPTP is converted into its active metabolite MPP^+, primarily by glial cells [Przedborski *et al.,* 2003]. The enzyme monoamine oxidase B (MAO-B) is responsible for its biotransformation, as demonstrated by the protective ability of MAO-B inhibitors to prevent this neurotoxicity [Chiba *et al.,* 1984]. MAO-B oxidizes MPTP to form 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$), but $MPDP^+$ is unstable and immediately forms the active neurotoxin metabolite MPP^+ [Chiba *et al.,* 1985]. After the release of MPP^+ into the extracellular space it is actively transported into DA neurons through the dopamine transporter (DAT), that has high affinity for MPP^+ [Javitch *et al.,* 1985; Bezard *et al.,* 1999]. Inhibition of DAT [Javitch *et al.,* 1985], or its genetic ablation

[Bezard *et al.,* 1999], prevents MPTP toxicity in these animals. MPP^+ accumulates in cells and can form a complex with neuromelanin or be trapped within synaptic vesicles by being taken up through the vesicular monoamine transporters (VMAT) on the surface of the vesicles [d'Amato *et al.,* 1987; Takahashi *et al.,* 1997; Staal *et al.,* 2000]. MPP^+ produces toxic effects through multiple mechanisms. Free MPP^+ enters the mitochondria and inhibits mitochondrial respiration through NADH-ubiquinone oxireductase of the mitochondrial electron chain (mitochondrial complex I) activity, leading to decreased production of ATP and ultimately the death of DA neurons [Nicklas et al., 1985]. Evidence of cellular protection from MPTP in transgenic animals with an overexpression of superoxide dismutase [Przedborski *et al.,* 1992] suggests that MPTP also has toxic effects by increasing the intracellular concentration of reactive oxygen species (ROS) that are toxic to cells. This is supported by the finding of increased cell loss in mice that lack superoxide dismutase or glutathione peroxidase and are treated with MPTP [Klivenyi *et al.,* 2000; Zhang *et al.,* 2000]. MPTP can also lead to increased levels of iron in the SNpc, accelerating formation of free radicals through the Fenton reaction [Temlett *et al.,* 1994]. Iron-regulatory element-binding proteins (IRPs) are intracellular RNA-binding proteins that respond to changes in cytosolic iron levels to regulate transcription of transferring receptor (TfR) and ferritin. MPTP leads to nitration of IRP's causing it degradation through ubiquitin-proteasome pathway that ultimately results in an increase in iron levels [Mandel *et al.,* 2004]. $MPDP^+$ also is known to self-oxidize and increase formation of other superoxide radicals that are toxic to the DA neurons [Chiba *et al.,* 1985; Bove *et al.,* 2012]. Nitric oxide synthase has also been implicated in the mechanism of MPTP action, as demonstrated by neuroprotection in mice lacking the

nitric oxide synthase gene that are given MPTP [Przedborski *et al.,* 1996]. Thus, both mitochondrial deficits and oxidative stress contribute to MPTP-induced DA neuronal toxicity. Similar to patients with PD where there is progression of the disease over many years, some aspects of the toxicity seen in MPTP-treated monkeys has been shown to take a long time. For example, the inflammatory reactions in basal ganglia of monkeys receiving MPTP can continue for years suggesting that the pathological process follows a long time-course of progression even after a single MPTP administration [McGeer *et al.,* 2003].

B. Methods of delivery of MPTP

MPTP has now been used to cause PD-like symptoms in various animal models, including monkeys, mice, cats, rats, guinea pigs, dogs, sheep, frog and goldfish [reviewed in Blum *et al.,* 2001; Bove *et al.,* 2012]. MPTP can be given either systemically or unilaterally, in one or multiple doses and in varying concentrations. The models that result from these administration modes are all different. The administration of MPTP based on the method of delivery leads to either unilateral or bilateral symptoms of either early stage or late stages of the disease. Although the effects of MPTP show considerable individual variability it is possible to develop predictable, stable models of the disease. The symptoms produced depend on the careful consideration of dosing of MPTP, treatment regimen, age, weight and animal species to induce the desired PD symptoms without causing adverse health problems [reviewed in Blum *et al.,* 2001; Bove *et al.,* 2012]. As monkeys will be used in this dissertation, this section will focus on the effects of MPTP in monkeys.

Short-term systemic administration of MPTP via several dosing regimens produces bilateral signs of PD [for review see Emborg, 2007; Bove *et al.,* 2012]. Generally, short-term systemic dosing involves daily injections of 0.2 mg/kg – 2.0 mg/kg, with lower dosages used for intramuscular (IM) and intravenous (IV) administration and higher dosages used for subcutaneous (SC) administration to monkeys over a period of four days to three weeks. Short-term systemic administration of MPTP has been useful for studying the effects of MPTP on neuroinflammation in the basal ganglia and for examining the time course of cell death and strategies to prevent cell death. However, the drawback of short-term systemic administration is that behavioral recovery has often been observed over a period of time, starting from around three to five weeks after MPTP administration [Mounayar *et al.*, 2007].

A regimen of chronic systemic administration of MPTP was developed to overcome these limitations. Again, 0.2 mg/kg – 2.0 mg/kg of MPTP (IM, IV or SC) per injection is given, however this is administered once or twice a week every one to two weeks for several weeks or months until the desired level of PD symptoms are present [for review see Przedborski *et al.*, 2001; Blum *et al.*, 2001; Jenner, 2003; Smeyne *et al.*, 2005; Emborg, 2007; Bove *et al.,* 2012]. There are several advantages to using this method where a slow chronic progression of symptoms that more closely reflects the progressive nature of PD is achieved. Chronic systemic administration also provides sufficient time for the animal to recover general health between each dose, and animals are dosed until a specific severity of symptoms is reached which decreases individual variability in the final model used for studies. Animals have also been studied during the early time periods of chronic systemic MPTP dosing to examine neural changes that would reflect

the pre-diagnostic stages of PD in humans including cognitive impairments [Brownell *et al.,* 1998] and sleep disturbances [Barraud *et al.,* 2009]. This chronic systemic MPTP model is relevant for many scientific inquiries as it replicates some of the non-motor features of the disease including cognitive deficits, loss of motivation and change in sleep patterns [Emborg, 2007]. Interestingly, in these primate MPTP models it was recently observed that the non-motor symptoms like sleep problems develop earlier than motor symptoms as seen in human PD [Barraud *et al.,* 2009]. Use of this model also allows investigation of compensatory changes that take place during early stages of the disease. The age of the animal plays a crucial role in the response to MPTP susceptibility, with older animals more significantly affected by MPTP compared to younger animals [Ovadia *et al.,* 1995]. In many late stage bilateral models monkeys are so severely affected that they cannot care for themselves and need additional attention for housing and feeding [Smith *et al.,* 1993]. Also, in some MPTP injection paradigms using this method there is no active process of degeneration after cessation of MPTP administration once the loss of nigral DA neurons plateaus and hence is not an exact replication of the progressive cell loss seen in PD [Garrido-Gil et al., 2009].

A third method of MPTP administration is the unilateral, intracarotid administration of MPTP [Bankiewicz *et al.,* 1986; Ovadia *et al.,* 1995; Gash *et al.,* 1996; Zhang *et al.,* 1997; Grondin *et al.,* 2002; Gash *et al.,* 2005; Emborg, 2007; Bove *et al.,* 2012]. There are several advantages to using this model including the ability of monkeys to care for themselves after a unilateral lesion. The calculation for dosing intracarotid MPTP is based on age, weight and species of monkeys to produce a very high rate of success in inducing either early or late stage symptoms of PD that are stable for many years

[Emborg, 2007; Bove *et al.,* 2012]. In this method, surgical isolation of the internal carotid artery is made and MPTP is injected [Bankiewicz *et al.,* 1986; Bergman *et al.,* 1990; Ovadia *et al.,* 1995; Gash *et al.,* 1996; Bezard *et al.,* 2001; Emborg, 2007; Bove *et al.,* 2012]. The production of symptomatology within days makes this model useful for studying neuroprotective and neurorestorative strategies, as well as for studying the pathophysiology within basal ganglia circuits that occurs secondary to loss of dopaminergic input. For example, the unilateral intracarotid MPTP monkey model was used for testing the effectiveness of deep brain stimulation for alleviating Parkinson symptoms [Benazzouz *et al.,* 1993], which was successfully transferred from pre-clinical research to testing and use in patients very rapidly [Limousin *et al.,* 1998]. One of the concerns using this low dose unilateral model could be that it may not affect non-motor symptoms and is a mild form of lesion compared to the chronic treatment. However, in later chapters in this dissertation I have found significant changes to non-motor symptoms using this model on sleep and motivational parameters in monkeys. (see Chapter 4)

1.4.3 **Biochemical changes in PD**

A. Changes to the DA neurotransmitter systems

The role of DA in PD, ever since its first description in 1960 by Ehringer and Hornykiewicz in post mortem brain, has had a profound impact on our understanding of the disease process. The research into the dopamine deficit aspect of PD was a tipping point which led to a series of investigations into the biochemical aspects of the disease, the pathophysiology of dopamine deficit and this deficit in dopamine became the

rationale for treating PD patients with levodopa the synthetic precursor to dopamine. This successful transformation of a basic science biochemical finding into clinical practice helped establish a new branch of investigation into biochemical processes detectable of post-mortem brain tissue to learn more about the neuroscience of brain diseases.

The entire nigrostriatal dopamine neuron is affected in the severely affected PD patient [Olanow *et al.,* 1999; Dauer *et al.,* 2003]. The changes in the DA system in PD are distinct from the patterns of cell loss observed in normal aging [Fearnley *et al.,* 1991; Dauer *et al.,* 2003]. In PD, the ventrolateral and caudal portions of the SNpc are first affected, whereas in normal aging the dorsomedial aspects of the SNpc are first affected. The changes to the nigrostriatal DA system begin with reduced DA content in the striatum and this reduction is always higher than that observed in the midbrain DA neurons [Bernheimer *et al.,* 1973]. This implies that the degenerative process in this disease begins in the striatum and proceeds later to the midbrain region. This process is sometimes referred to as the 'dying back' process of nigrostriatal DA system. There is also a strong correlation between the progressive worsening of the motor problems in the disease with reduced dopamine function in striatum and midbrain. This is also supported by research in experimental models of PD that closely follow the changes observed in clinical PD, where in MPTP-treated primates there is first a reduction of TH-positive terminals in the striatum before a loss is evident in the midbrain DA neurons [Przedborski *et al.,* 1996, 2001; Schmidt *et al.,* 2001].

The presence of different concentrations of DAT, the main transporter of DA, from the terminals in striatum differ across the SNpc population in midbrain region is proposed as the reason for the differential vulnerability of DA neurons in the midbrain

[Olanow *et al.,* 1999; Przedborski *et al.,* 1996, 2001]. There is some evidence that the decrease in mRNA concentration of DAT closely parallels the decrease in striatal DA content [Bernheimer *et al.,* 1973; Uhl *et al.,* 1994].

There is around a 60-80% loss of striatal DA content before the first motor signs of PD appear [Hornykiewicz, 1998; Zigmond *et al.,* 2002; Dauer *et al.,* 2003]. This implies that there is a long period of time when the disease slowly progresses in the brain before the appearance of external symptoms. It has been proposed that during this dormant period of progression there is internal compensation in the nigrostriatal DA system that takes place [Bernheimer *et al.,* 1973; Zigmond *et al.,* 1990,1992, 1997, 2002; Bezard *et al.,* 2003; Obeso *et al.,* 2004]. The ability of the diseased nigrostriatal DA pathway to internally compensate for the increasing loss of DA from the striatum until there is around 60-80% loss shows a dynamic compensatory process. This compensation may be mediated directly by changes in the nigrostriatal DA neuron, the pathways it influences and/or other circuits that are extrinsic and do not directly influence the DA pathway.

The changes to the nigrostriatal DA pathway could primarily result from the following mechanisms [Agid *et al.,* 1973; Hefti *et al.,* 1980; Altar *et al.,* 1987; Stachowiak *et al.,* 1987; Snyder *et al.,* 1990; Hudson *et al.,* 1995; Sherman *et al.,* 1995; Harsing *et al.,* 1996, 1997, 1998; Garris *et al.,* 1997; Zigmond *et al.,* 1981,1990, 1997, 2002]. After a loss of TH-positive fibers in the striatum there is a concurrent loss DAT. This results in an increased concentration of DA in the synaptic cleft that compensates for the loss of DA terminals. There is also a proposed increase in the synthesis of DA by the remaining DA neurons that could maintain the influence of nigrostriatal DA on its

targets. This compensatory mechanism was proposed based on the changes to the ratio of DA and its metabolite, homovanillic acid (HVA) [Zigmond *et al.,* 1990]. Similar increases in dihydroxyphenylacetic acid (DOPAC) to DA ratio provide another indicator of increased DA turnover in the diseased striatum [Zigmond, 1997]. There is also evidence of an increase in postsynaptic receptors for DA during the pre-clinical phase of PD, to also help compensate for the decrease in secreted DA [Zigmond *et al.,* 1990, 1997]. Different amounts of DA loss could lead to different levels of compensations with moderate lesions not having much of a functional change, a large lesion leading to increased DA synthesis and release, and an extensive lesion leading to rapid compensatory processes [Zigmond *et al.*, 1990,1997].

Apart from the compensatory changes described above, electrophysiological changes to the activity of the different basal ganglia nuclei can internally compensate for the changes in activity resulting from nigrostriatal DA deficit [Grace, 1991]. It has been shown that there is an increase in activity of the STN and GPi, by way of an increase in firing of these neurons before the appearance of motor symptoms during the preclinical phase in the disease [Greenamyre, 1993; Albin *et al.*, 1995]. It is proposed that the increase in firing of STN maintains the GPe physiological function and compensates for the abnormal processing of DA within the striatum in the initial stages of the disease. These changes in electrophysiological firing are found to occur after the changes to the DA receptor compositions in neurons [Zigmond *et al.*, 1990, 1997]. There is a correlation between increasing loss of DA and an increase in activity of the structures described above [Greenamyre, 1993]. These electrophysiological abnormal outputs from the basal ganglia are propagated through the output circuits to thalamus and cortex. Further

research is needed to determine if there is plasticity in these external circuits that do not directly influence the DA pathway, to maintain the homeostasis of normal function during the long pre-symptomatic period in PD, although there is a clear abnormal output from basal ganglia.

B. Lewy bodies in PD

PD, which was earlier more or less thought of as just an isolated disorder of the dopaminergic system, is becoming more complex as we learn more about the pathology of the disease [Braak *et al.,* 2000, 2003 and 2004]. It now beginning to be widely accepted that the loss of neurons in PD follows a non-random pattern and the changes involve a widespread degeneration affecting the human central, peripheral and enteric nervous systems.

Lewy bodies are composed of abnormal intra-neuronal protein aggregates that are present in cells as cytoplasmic inclusions. Lewy first described these abnormal protein aggregates in detail, within the substantia inominata and dorsal vagal nucleus in PD [Lewy, 1912]. Tretiakoff was the first to observe these inclusions in substantia nigra [Tretiakoff, 1919]. He gave these inclusions the name ‘Lewy bodies’. Since then, Lewy bodies have been consistently found in post-mortem brain tissues of PD patients. These inclusions are found in a number of regions [Braak *et al.,* 2000]. It is proposed that all the neurons that are susceptible to have Lewy body inclusions share two common properties [Braak *et al.,* 2004]. First, all the affected neurons are projection neurons that have very long and thin axons in comparison to their cell body size. Second, neurons with Lewy bodies have long, thin axons that are unmyelinated or poorly myelinated. In PD, during the long

preclinical duration of the disease, the pathological process is thought to begin in the brainstem within the dorsal motor nucleus of IX/X and progresses upward reaching the neo-cortex in the final advanced stages of the disease. Lewy bodies have been found not only within the central nervous sytem but also within the enteric nervous system [Braak *et al.,* 2006]. These changes are thought to account for the autonomic symptoms that are frequently seen in PD patients.

C. Changes to other non-DA neurotransmitter systems

There are changes to other neurotransmitter systems besides the dopaminergic system with PD. The most prominent among these are noradrenalin, serotonin, GABA and glutamate [Ohama *et al.,* 1976; Greenamyre *et al.,* 1993; Blandini *et al.,* 1996; Hornykiewicz, 1998]. However, in contrast to the consistent presence of nigrostriatal DA loss in the post-mortem brain tissue of PD patients, changes to other neurotransmitter systems are variable and have not been studied as extensively. There have been reports of inclusion of Lewy bodies in dorsal raphe nucleus and locus coeruleus neurons, along with a concurrent reduction of both serotonin and noradrenalin levels in the post-mortem brains of PD patients [Bethlem *et al.,* 1960; Ohama *et al.,* 1976]. However, only recently have these changes in other non-dopaminergic regions in PD have gained recognition [Braak *et al.,* 2000,2003,2006; Hawkes *et al.,* 2010]. Changes to GABAergic MSN neurons in the striatum have been observed, that could be due to the secondary effects of nigrostriatal DA loss [Hornykiewicz, 1998]. There is a direct correlation between the losses of DA innervation of striatum which projects to its primary target of striatal GABAergic medium spiny neurons and increase in GABA levels in the striatum [Kish *et al.,* 1986; Hornykiewicz, 1998]. Similarly, there is increased glutamatergic activity in the

striatum, which constitutes the main input to the basal ganglia, that occurs secondary to DAergic loss [Greenamyre, 1993]. An increase in the activity of STN, that contains glutamatergic connection to the basal ganglia output structures, is also observed together with changes to the glutamate receptors in these regions [Blandini *et al.,* 1996]. It is also important to note that glutamate can act as a neurotoxin in the cases of impaired cellular functioning [Blandini *et al.,* 1996; Hornykiewicz, 1998]. One of the proposed mechanism through which this might occur is that a loss of DA influence in the striatum that contains GABA inhibitory projection neurons after DA loss, that can lead to increased activity in excitatory glutamate-containing STN neurons. These changes in GABA and glutamate neurotransmission have also been observed in animal models of PD [Greenamyre, 1993; Blandini *et al.,* 1996,2000; Hornykiewicz, 1998]. The non-motor symptoms that are seen in PD before the appearance of motor symptoms could be related to changes in many of the monoaminergic neurotransmitter systems that extensively innervate the CNS before degeneration of the DA system [Braak *et al.,* 2000, 2006, 2008; Hawkes *et al.,* 2008, 2009, 2010].

1.5 NEUROTROPHIC FACTORS THAT SUPPORT DA NEURONS

Neurotrophic factors are proteins that support the growth, survival and function of specific neuronal populations during the course of development [Thoenen, 1995; Barbacid, 1995; Ibanez,1995; Saarma *et al.,* 1999, 2002, 2003]. There is interest in the use of these molecules as therapeutic agents in the treatment of a wide variety of neurological disease conditions. Stanley Cohen and Rita Levi-Montalcini discovered the first identified neurotrophic factor that affects

nerve cells [Cohen *et al.,* 1954]. They called it Nerve Growth Factor (NGF), and received the Nobel Prize in 1986 for its discovery. Since that time a number of neurotrophic factors have been identified. Neurotrophic factors can be grouped into different families based on homology of the molecules, receptors they bind to, and common transduction pathways. NGF belongs to the Neurotrophin family. There are three main families of neurotrophic factors that have been discovered, the Neurotrophin family [Cohen *et al.,* 1954], the Glial cell-line Derived Neurotrophic Factor (GDNF) family [Lin *et al.,* 1993] and the Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) family [Petrova *et al.,* 2003 and Lindholm *et al.,* 2007]. The two families that are most directly relevant to supporting dopamine neurons that degenerate in Parkinson's disease are the GDNF and MANF families [for review see Peterson *et al.,* 2008; Lindholm *et al.,* 2010; Aron *et al.,* 2011]. Specifically, GDNF and Neurturin (NRTN), that belong to the GDNF family, and Cerebral Dopamine Neurotrophic Factor (CDNF), that belongs to the MANF family, show both neuroprotection and neurorestoration of DA neurons after toxic insults in animal models of PD [Lindholm *et al.,* 2007,2008, 2010; Voutilainen *et al.,* 2011; Airavaara *et al.,* 2012].

1.5.1 **Glial Cell-line Derived Neurotrophic Factor, GDNF**

Lin et al. discovered GDNF in 1993 in a rat glial cell-line (B49), as a soluble factor released by the cell culture. GDNF is a glycosylated, disulfide bonded homodimer whose molecular weight is approximately 33–45 kDa, while the monomer has a molecular weight of 16 kDa after deglycosylation [Lin *et al.,* 1993,1994]. GDNF is synthesized as an inactive 211 amino acid pre-pro-GDNF secretory protein that is cleaved into the mature GDNF protein of 134 amino acids. The regional distribution and cellular localization of GDNF was identified using PCR techniques

in the central nervous system of rats and humans [Lin *et al.,* 1993; Schaar *et al.,* 1993, 1994; Stromberg *et al.,* 1993; Springer *et al.,* 1994]. GDNF protein was found to be present in a number of brain regions in the rat central nervous system, including the striatum, hippocampus, cortex, cerebellum and spinal cord. In the human central nervous system, GDNF transcripts were identified in the striatum, caudate, hippocampus, cortex and spinal cord. More importantly, GDNF message was expressed in the substantia nigra and basal forebrain type I astrocytes [Schaar *et al.,* 1993, 1994]. Addition of GDNF to cultured primary dopaminergic neurons *in vitro* increases DA cell number, neurite length, cell size, and dopamine uptake [Lin *et al.,* 1993,1994]. GDNF also reduces the rate of apoptosis and prolongs neuronal survival in cultured dopaminergic neurons [Clarkson *et al.,* 1995, 1997].

In PD, there is selective loss of nigrostriatal dopaminergic neurons and there is evidence that GDNF can play a therapeutic role in rescuing DA neurons in animal models of PD [Gash *et al.,* 1995; Hou *et al.,* 1996; Martin *et al.,* 1996; Opacka-Juffry *et al.,* 1995; Schults *et al.,* 1996]. *In vivo* animal models that mimic symptoms of PD are produced using selective dopaminergic neurotoxins, such as 6-hyroxy-dopamine (6-OHDA) and MPTP. GDNF has been shown to rescue injured dopaminergic neurons in these models [Hoffer *et al.,* 1994; Tomac *et al.,* 1995; Beck *et al.,* 1995; Bowenkamp *et al.,* 1995; Bjorklund *et al.,* 1997]. After intranigral administration of GDNF in the 6-OHDA-treated rat model there is neurochemical and behavioral improvement of PD-like motor symptoms [Bjorklund *et al.,* 1997]. In addition, intra-striatal injection of GDNF also prevents dopaminergic loss after 6-OHDA induced nigrostriatal lesions [Opacka-Juffry *et al.,* 1995; Sauer *et al.,* 1995; Schults *et al.,* 1996]. GDNF has also been shown to rescue dopaminergic neurons in animal models receiving the DA toxin, MPTP [Tomac *et al.,* 1995; Hou *et al.,* 1996; Gash *et al.,* 1996]. GDNF when injected in the substantia nigra or

striatum of monkeys before administration of MPTP significantly protected the dopaminergic system, while GDNF when injected after MPTP administration significantly restored dopamine levels and dopamine fiber density [Gash *et al.,* 1996; Zhang *et al.,* 1997; Connor *et al.,* 1998]. Recombinant Adeno-Associated-Viruses (AAV) that carry the gene encoding human GDNF, also have been shown to protect DA neurons from progressive neurodegeneration when injected near the substantia nigra or in the striatum of both mice and primates prior to MPTP administration [Choi-Lundberg *et al.,* 1997; Bilang-Bleuel *et al.,* 1997; Kordower *et al.,* 2000; Eslamboli *et al.,* 2005]. Thus AAV-GDNF gene therapy may also be a treatment option for PD. Thus, GDNF shows both neuroprotection and neurorestoration of DA neurons [Tomac *et al.,* 1995; Hou *et al.,* 1996; Gash *et al.,* 1996; Hoffer *et al.,* 1994; Beck *et al.,* 1995; Bowenkamp *et al.,* 1995; Bjorklund *et al.,* 1997].

There have been four clinical trials that have been conducted with GDNF [Nutt *et al.,* 2003; Gill *et al.,* 2003; Patel *et al.,* 2005; Slevin *et al.,* 2005]. There was no significant improvement in motor symptoms when GDNF was injected into the cerebrospinal fluid (CSF) in the first clinical trial [Nutt *et al.,* 2003]. The authors suggested that this may reflect poor penetration of GDNF into the brain from CSF [Nutt *et al.,* 2003]. In order to achieve better brain penetration and targeting, GDNF was directly infused in the putamen using minipumps in the subsequent trials [Gill *et al.,* 2003; Patel *et al.,* 2005; Slevin *et al.,* 2005]. The second and third open label trials led to significant clinical improvements in patients [Gill *et al.,* 2003; Patel *et al.,* 2005; Slevin *et al.,* 2005]. However, in the fourth double-blind placebo controlled trial there was no improvement seen [Lang *et al.,* 2006]. Some patients in that clinical trial developed anti-sera against GDNF, although there were no adverse consequences detected. Around the same time as the fourth study, in a parallel study in monkeys, cerebellar lesions were detected in animals that

received three to four times the dose of GDNF used in human trials [Hovland *et al.,* 2007]. The findings of no clinical improvement, coupled with development of antisera to GDNF and cerebellar lesions, led Amgen, the company that licensed the use of GDNF for the treatment of PD, to decide to stop all clinical testing of GDNF. However, there has been a lot of discussion about this decision [Barker, 2006, 2009; Evans *et al.,* 2008]. The reason for the failure in the fourth clinical trial could be due to the dose of GDNF that was used, properties of the catheter used to administer GDNF, the choice of patients included in the trials, the duration of study in the clinical trial or the inability of GDNF to reach the intended target regions in the brain [Barker , 2006, 2009; Evans *et al.,* 2008; Deierborg *et al.,* 2008]. A common failure in all the above experiments is inability to delivery bioactive GDNF that can diffuse to the entire target region in the brain [Deierborg *et al.,* 2008; Bjorklund *et al.,* 2009]. The reason for this main setback that is common to all four clinical trials could be due to the binding of GDNF to heparin-like binding sites in the brain for which GDNF has high affinity [Rider, 2006].

1.5.2 **Neurturin, NRTN**

Neurturin (NRTN), the second member of the GDNF family of neurotrophic factors, was discovered three years later in Jeffrey Milbrandt's lab [Kotzbauer *et al.,* 1996]. Mature NRTN is 100 amino acids long and shares 42% sequence homology with GDNF [Kotzbauer *et al.,* 1996]. GDNF and NRTN share intracellular signaling pathways [Kotzbauer *et al.,* 1996]. NRTN, like GDNF, promotes DA neuronal survival, and both factors have distinct functional roles in the developing and adult midbrain dopaminergic neurons [Akerud *et al.,* 1999]. GDNF and NRTN are expressed sequentially as endogenous trophic factors to the developing DA neurons. During post-natal development NRTN plays a vital role in DA innervation of the striatum. The post-

natal expression profile of NRTN is thought to sustain this dopaminergic innervation of striatum [Trupp *et al.,* 1996; Akerud *et al.,* 1999]. Thus, the selective DA neuron survival-promoting activity of NRTN is complementary to the earlier expression of GDNF that induces sprouting and increases cell-body size of dopaminergic neurons [Lin *et al.,* 1993; Bowenkamp *et al.,* 1995; Sauer *et al.,* 1995]. The combined sequential expression of GDNF and NRTN, and their distinct functional roles suggest a complimentary coordinated activity during development [Trupp *et al.,* 1996; Akerud *et al.,* 1999].

NRTN, like GDNF, also prevents the degeneration of substantia nigra dopamine neurons by being neuroprotective in 6-OHDA injected rats [Horger *et al.,* 1998; Akerud *et al.,* 1999; Rosenblad *et al.,* 1999]. NRTN has also shown neuro-restorative effects in 6-OHDA injected rats [Oiwa *et al.,* 2002]. One of the main problems, as for GDNF, is continuous delivery of NRTN to intracerebral targets in the brain over long periods of time. There have been advances made with use of safe viral vectors (recombinant Adeno-Associated Viruses rAAV and recombinant Lentivirus rLV) for transfection and delivery of neurotrophic factors *in vivo* [Bilang-Bleuel *et al.,* 1997; Lapchak *et al.,* 1997; Bensadoun *et al.,* 2000; Rosenblad *et al.,* 2000; Connor *et al.,* 2001; Kordower *et al.,* 2003]. Lentiviral delivery of NRTN to the striatum of 6-OHDA lesioned rats resulted in protection of over 90% of nigral DA cells compared to vehicle treatment [Fjord-Larsen *et al.,* 2005]. Ceregene Inc. developed an Adeno-associated virus type-2 (AAV2) vector that encodes a modified form of human NRTN, called CERE-120. When injected into 6-OHDA lesioned rats CERE-120 prevented motor deficits and loss of nigral neurons that was equipotent to GDNF and significantly improved from vehicle-treated group which had close to 70% decrease in number of TH-positive DA neurons [Gasmi *et al.,* 2007]. The above experiment was independently confirmed along with another series of experiments in

collaboration with the Kordower lab. CERE-120 was shown to provide protection to both structure and function of nigral DA neurons in both rat and monkey models of PD [Kordower *et al.,* 2006; Gasmi *et al.,* 2007; Herzog *et al.,* 2007; Herzog *et al.,* 2008; Herzog *et al.,* 2009].

There have been three clinical trials using CERE-120 [Marks *et al.,* 2008; Marks *et al.,* 2010, MJFF press release, 2013]. Intraputamenal administration of CERE-120 improved motor function in a Phase I clinical trial and was found to be safe at one-year post-administration [Marks *et al.,* 2008]. In this open label trial, 12 patients with advanced PD were injected bilaterally with CERE-120 in the putamen. This resulted in significant improvement in the Unified Parkinson's Disease Rating Scale (UPDRS) motor score when they were "off" medication [Marks *et al.,* 2008]. Importantly, none of the patients experienced dyskinesias during the trial. These initial positive results led to a Phase II double-blind randomized clinical trial with CERE-120 [Marks *et al.,* 2008, 2010]. Like GDNF, the CERE-120 phase II testing in a larger cohort of 58 PD patients failed to show any significant improvements in motor function. Post-mortem data collected from two patients from this cohort who had died of other complications, indicated that NRTN showed poor diffusion into the brain parenchyma and no NRTN was found in cell bodies of nigral DA neurons [Marks *et al.,* 2010; Vastag, 2010]. This data from post-mortem brain tissue led to refinement of the dosing of CERE-120 to both enhance and accelerate the effects of NRTN by injecting directly both into the substantia nigra and putamen at the same time. A Phase I open-label clinical trial with six PD patients was again successful [Ceregene press release 2013; MJFF press release, 2013]. This was followed by a Phase II clinical trial with fifty-one patients with PD, monitoring them for fifteen to twenty-four months to test the benefits and safety of using CERE-120 [Ceregene press release 2013; MJFF press release, 2013]. Ceregene Inc. recently announced the results from its second Phase II

clinical trial, where there was no significant improvement to the UPDRS motor scores in the absence of medication in CERE-120 administered patients compared to sham-control patients [MJFF press release, 2013]. However, CERE-120 showed improvement in other measures like the "diary-off score" (daily diaries that assess motor function throughout the day) that measures qualitative motor function throughout the day for these patients, and this trial also provided further evidence for the safety of CERE-120, the dosing and viral vector delivery methods used. The data from this trial is still being analyzed [MJFF press release, 2013]. NRTN that belongs to the same family of neurotrophic factor as GDNF is known to bind to heparin-like binding sites in the brain with high affinity. Hence, poor diffusion of NRTN within the brain could again be the reason for the statistically insignificant improvement in the above clinical trials [Marks *et al.,* 2010, Vastag, 2010; Ceregene press release 2013; MJFF press release, 2013].

Mutations in the NRTN molecule at the sites that putatively bind to these extracellular matrix components were generated and tested *in vitro* [Runeberg, Saarma and Penn, unpublished data]. Four such NTRN variants were generated, two molecules out of them i.e., N2 and N4 were found to give better yields, were more stable and easy to handle. N2 and N4 also showed improved resistance to proteolytic cleavage *in vitro*.

N2 and N4 were tested *in vivo* in a rat 6-OHDA model of the disease. N2 and N4 were treated groups improved significantly in motor function tests. In particular, N4 showed increased diffusion compared to N2 and GDNF. N4 was also significantly better than GDNF in rescuing both DA neurons and improving motor functions. This could be both due to the widespread diffusion of N4 and the increased proteolytic stability of the molecule. In a pilot study, N4 diffused the maximum with almost twice the diffusion volume of GDNF although N4 was

infused in a 10% smaller volume [Runeberg, Saarma and Penn, unpublished data]. Thus N2 and N4 have the potential to restore DA neurons and motor functions in PD.

1.5.3 **Cerebral Dopamine Neurotrophic Factor, CDNF**

In 2007, Lindholm et al. discovered a novel trophic factor, CDNF, which belongs to the newly identified MANF family of neurotrophic factors [Lindholm et al. 2007]. CDNF contains eight conserved cysteine residues and is a secreted protein with a strong homology to MANF [Lindholm et al. 2007]. In mice and humans, CDNF is encoded as a 187 amino acid protein with no 'pre' or 'pro' sequence and has a molecular weight of 18 kDa. The presence of CDNF messenger RNA and protein in the adult mouse was detected using RT-PCR and western blotting techniques respectively, in the brain, heart muscle and testis. In the brain, the CDNF message is present in embryonic, post-natal and adult stages of development [Lindholm et al. 2007]. CDNF protein has been detected in neuronal cell somas in the adult layers two to six of the cortex, hippocampus layers CA1 to CA3, and in the thalamus, striatum and non-dopaminergic solitary cells in the substantia nigra midbrain region, purkinje cells in the cerebellum and in other brain stem regions. CDNF and MANF are conserved through evolution with a 49% homology between the human peptides and those of the fruit fly (Drosophilia melanogaster) and 46% homology with worm (Caenorhabditis elegans) [Lindholm et al. 2007]. Unlike GDNF that supports motorneurons in the enteric, sensory, parasympathetic and sympathetic neurons in the peripheral nervous system, CDNF does not support the survival of these peripheral neurons [Lindholm et al. 2007]. Although CDNF is present endogenously in the adult brain it has not been found to promote neurite outgrowth or sprouting of axons in culture [Lindholm et al. 2007]. When CDNF is infused into normal mouse brain, no changes were observed in endogenous

levels of DA, DAT or TH, and no detectable changes in mouse behavior were present; there was only an increase DA metabolism in the striatum [Airavaara *et al.,* 2012].

In animal models of PD with loss of nigrostriatal DA neurons, CDNF has been shown to be both neuroprotective and neurorestorative [Lindholm et al. 2007; Airavaara *et al.,* 2012]. In a unilateral 6-OHDA rat model of PD, injection of CDNF prior to administration of the neurotoxin 6-OHDA led to significant neuroprotection of the midbrain DA neurons [Lindholm et al. 2007; Airavaara *et al.,* 2012]. This protection was comparable to that offered by GDNF, however the CDNF-treated animals continued to show significant behavioral recovery at four weeks after injection but GDNF did not have this effect [Lindholm et al. 2007]. To test if CDNF was also neurorestorative, rats were injected with 6-OHDA unilaterally and CDNF, GDNF or vehicle was infused four weeks later and the animals were tested for behavioral recovery and post-mortem measurements of recovery in nigrostriatal DA system [Lindholm et al. 2007; Airavaara *et al.,* 2012]. CDNF-treated animals showed a less than a 50% loss of DA neurons in the lesioned side of the brain and also significantly reduced the amphetamine-induced rotation behavior by about 33% [Lindholm et al. 2007; Airavaara *et al.,* 2012]. In a mouse model of PD, where MPTP was administered as a neurotoxin, CDNF again showed both neuroprotection and neurorestoration [Airavaara *et al.,* 2012]. More recently, CDNF was successfully transfected into the rat brain using AAV vector-mediated delivery [Back *et al.,* 2013]. The expression of CDNF using AAV-mediated delivery into the striatum of rats provided significant recovery of behavior and nigrostriatal DA system both when measured in a short-term study of ten weeks duration where AAV-CDNF was tested as a neuroprotective agent [Back *et al.,* 2013] and also in a neurorestorative study when AAV-CDNF offered protection fifty-four weeks after transfection in a 6-OHDA rodent model of PD [Ren *et al.,* 2013]. In a comparative study of diffusion of trophic

factors, both CDNF and MANF were shown to diffuse over a greater volume than GDNF initially after administration of the neurotrophic factors [Lindholm *et al.,* 2010; Voutilainen *et al.,* 2011]. Thus, CDNF has been shown to be neuroprotective and neurorestorative in two different rodent models of PD using different method of delivery of the trophic factor into the brain.

1.6 SPECIFIC AIMS

The main setback that has thus far prevented the translation of neurotrophic factors (i.e., GDNF and NRTN) into the clinic for the treatment of PD has been the failure of NTFs (GDNF and NRTN) in large Phase II double blind clinical trials [Barker, 2006, 2009; Evans *et al.,* 2008; Marks *et al.,* 2010, Vastag, 2010]. This failure has been attributed to several possible reasons, however there is one major common underlying theme. It is the lack of ability for these trophic factors to diffuse to reach intended targets in the brain. In order to overcome this limitation two mutant forms of NRTN that have mutations in the sites of the molecule that putatively bind to these sites in the extra cellular matrix but still maintains bioactivity for its trophic capabilities were developed [discussed in detail in Section 1.5.4], N2 and N4. Along with these two mutants, another recently discovered trophic factor, CDNF [Lindholm *et al.,* 2007], also diffuses widely in the brain. CDNF can spread easily in the brain as it belongs to a novel class of trophic factors with different structural properties. In this dissertation I address test whether these three NTFs, CDNF, N2 and N4, which readily diffuse in brain parenchyma, are effective in restoring dopaminergic function in the MPTP primate model of PD.

1.6.1 **Specific Aim 1**

To determine if CDNF and NRTN mutants that do not bind to heparin-like binding sites in the brain stimulate restoration of motor function in the unilateral MPTP monkey model of PD.

The motor aspects of PD including tremor, rigidity, bradykinesia and postural instability, were induced in rhesus monkeys unilaterally on the left side of the body by a single low-dose unilateral MPTP injection in the right carotid artery. Motor function was assessed and quantified using five separate measures of gross motor function and fine motor function, before and after MPTP administration, and in response to three neurotrophic factor/vehicle infusions into the putamen.

1.6.2 **Specific Aim 2**

To determine if CDNF and NRTN mutants that do not bind to heparin-like binding sites in the brain stimulate restoration of normal sleep patterns in monkeys receiving unilateral MPTP infusions.

Non-motor problems are present in almost all PD patients, with a very high prevalence of sleep pattern disturbances. Increased daytime sleepiness is one of the chief complaints of PD patients. The Cameron laboratory has validated actigraphic methods to noninvasively measure sleep in nonhuman primates. Actigraphy was used to assess the changes in sleep patterns before and after MPTP and after neurotrophic factor infusions. The changes in sleep patterns after MPTP were analyzed and compared with the sleep patterns after three infusions of the three NTFs (i.e., N2, N4 or CDNF). Results were compared to animals who received vehicle infusions or GDNF infusions.

1.6.3 **Specific Aim 3**

Identification of changes in the nigrostriatal dopamine system after monkeys with unilateral MPTP lesions receive intraputamenal infusions of, CDNF, N2, N4, GDNF or vehicle

Post-mortem brain tissue from unilateral MPTP-treated monkeys was collected after three months of neurotrophic factor administration and analyzed for degenerative changes in midbrain dopamine neurons. The total numbers of DA neurons present after four monthly infusions of vehicle or neurotrophic factors were counted. Changes the tissue levels of DA and DA metabolites, homovanillic acid (HVA) and DOPAC, in the terminal fields of nigrostriatal DA neurons (i.e., the caudate, putamen, and nucleus accumbens) will be measured in upcoming analyses (however, these assessments have been delayed due to the need to get permission to send the brain tissue out of the country for analysis so this information is not part of this dissertation).

2.0 EFFECTS OF CDNF, N2 AND N4 ON RECOVERY OF MOTOR FUNCTION IN MPTP-TREATED MONKEYS

2.1 INTRODUCTION

Since the first reports that GDNF could rescue dopamine neurons after toxic insult [Lin *et al.,* 1992; Schaar *et al.,* 1993, 1994; Stromberg *et al.,* 1993; Springer *et al.,* 1994; Tomac *et al.,* 1995; Hou *et al.,* 1996; Gash *et al.,* 1996] there has been considerable interest in the development of trophic factors as a treatment option for PD. GDNF has been found to be both neuroprotective [Choi-Lundberg *et al.,* 1997; Bilang-Bleuel *et al.,* 1997; Kordower *et al.,* 2000; Eslamboli *et al.,* 2005] and neurorestorative [Opacka-Juffry *et al.,* 1995; Sauer *et al.,* 1995; Schults *et al.,* 1996] in toxin-induced rodent and primate models of PD. GDNF when given either before or after toxin insult has been shown to lead to greater survival of DA neurons in the substantia nigra [Hoffer *et al.,* 1994; Beck *et al.,* 1995; Bowenkamp *et al.,* 1995; Bjorklund *et al.,* 1997], and better motor function [Tomac *et al.,* 1995; Hou *et al.,* 1996; Gash *et al.,* 1996]. However, clinical trials of GDNF have been more mixed, with the most recent Phase II clinical trials not showing significant improvement of motor function in PD patients [Patel *et al.,* 2005; Slevin *et al.,* 2005; Lang *et al.,* 2006]. One of the main reasons attributed to the failure in clinical trials is the poor diffusion of GDNF in brain tissue possibly due to its high affinity binding to

extracellular heparan sulphates [Rickard *et al.,* 2003; Saarma *et al.,* 2003; Rider, 2006; Barker, 2006, 2008; Deierborg *et al.,* 2008; Bjorklund *et al.,* 2009; Pitonen *et al.,* 2009]

Another member of the GDNF family of ligands, NRTN, has also shown potential as a therapeutic option for PD. Similar to GDNF, NRTN showed promising therapeutic efficacy in rodent and monkey models of PD [Horger *et al.,* 1998; Akerud *et al.,* 1999; Rosenblad *et al.,* 1999; Kordower *et al.,* 2006; Gasmi *et al.,* 2007; Herzog *et al.,* 2007; Herzog *et al.,* 2008; Herzog *et al.,* 2009]. An AAV vector-containing the gene for NRTN (CERE-120) was successfully tested in a small Phase I clinical trial with PD patients [Marks *et al.,* 2008; Marks *et al.,* 2010]. A larger phase II trial was carried out recently to test the effectiveness and safety of viral vector-based delivery of NRTN (CERE-120) in the brain to treat PD [Marks *et al.,* 2008; Marks *et al.,* 2010, MJFF press release, 2013]. However, in a very recent press release CERE-120 effectiveness in this Phase II clinical trial was reported to have little success [MJFF press release, 2013]. One of the main concerns that may account for the failure of the clinical trial was the inability of NRTN produced at the site of CERE-120 injection to diffuse to the various target locations that experience neurodegenerative changes in PD. GDNF and NRTN are both neurotrophic factors that belong to the GDNF family of ligands, and it is well known that both NTF's have a strong affinity to bind to heparin-like binding sites in the brain [Rider, 2006]. As a result of this binding, both GDNF and NRTN when given in the striatum are known to diffuse to fewer target regions in the large human brain compared to the much smaller brains of experimental animals [Barker, 2006, 2009; Evans *et al.,* 2008; Marks *et al.,* 2010].

The problem with diffusion of the GDNF family ligands led to the search for novel trophic factors. In 2007, CDNF was identified; a NTF that belongs to a new family of trophic factors, the MANF family [Lindholm *et al.,* 2007]. MANF family trophic factors, unlike GDNF

family trophic factors, do not bind to heparin-like binding sites, thus they have a greater distribution in the brain compared to GDNF and NRTN [Lindholm *et al.,* 2010; Voutilainen *et al.,* 2011]. The effectiveness of CDNF was tested in rodent toxin models of PD. CDNF was shown to be both neuroprotective and neurorestorative [Lindholm *et al.*, 2007; Airavaara *et al.*, 2012]. Moreover, CDNF was effective for a longer duration than GDNF in these animal models, with CDNF showing significantly improved behavioral recovery four weeks after injection, while GDNF-treated animals showed behavioral recovery for only two weeks [Lindholm *et al.*, 2007; Airavaara *et al.*, 2012]. CDNF has not been tested in animals with larger brains and one goal of the current study was to determine if CDNF is neurorestorative in the larger brain of a nonhuman primate.

Another strategy to obtain greater tissue distribution of trophic factors has been to make point mutations in NRTN in the region of the heparin-like binding site to decrease binding and thereby increase diffusion within the brain [Runeberg, Saarma and Penn, unpublished data]. Runeberg et al. made point mutations, based on the consensus sequence in a positively charged area containing arginine residues at the surface of NRTN molecule known to bind to the heparin-like binding sites. Four such mutant variants of NRTN (N1 to N4) were created [Runeberg, Saarma and Penn, unpublished data]. These four mutants were tested *in vitro* for bioactivity, ease of production, binding to heparin, and stability and effectiveness as a trophic factor to DA neurons [Runeberg, Saarma and Penn, unpublished data]. After extensive testing, two of these mutants (N2 and N4) that showed the best efficacy were tested in rodent models of PD. N2 and N4 were found to be more effective than GDNF in improving motor behaviors. In a preliminary test to check the distribution of N2 and N4 in the larger monkey brain, equimolar quantities of GDNF, N2 and N4 were injected in one monkey [Penn, unpublished data]. N4 showed the maximum

distribution followed by N2, even though the NRTN mutants were injected in smaller volumes than GDNF (N2: a 25% smaller volume, N4: a 10% smaller volume). A second goal of the current experiment was to test the effectiveness of N2 and N4 in restoring motor function and maintaining DA neurons in a primate model of PD.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Thirty female rhesus monkeys (*Macaca mulatta*) were used in this study. The animals were between the ages of 17 and 20 years, with a mean age of 18.2 ± 0.2 years. Average weight was 8.3 ± 0.2 kg. All monkeys were housed in social living pens (1.6m x 2.3m x 3.5m) that had multiple perches, toys, and a thick layer of sawdust bedding with overhead skylights that provided natural lighting, as well as artificial lighting that turned on at 0700 h and off at 1900 h. This housing facility has 20 pens in a wing, and pens have wire mesh fencing fronts so that monkeys in each pen could see and hear other monkeys in a number of other pens. Monkeys were pair-housed. Monkeys were fed Purina Monkey Chow (#5038; Ralston Purina Co., St. Louis, MO) once daily and given fresh fruit, vegetables, nuts and seeds to encourage foraging, as well as *ad libitum* access to drinking water. All monkeys were observed daily for health and menstrual status. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.2 **MPTP Administration**

Prior to MPTP administration, baseline assessments of motor and non-motor functions (for an average of 3.2±0.2 weeks) were made for each monkey. All monkeys then underwent a surgery to expose the right carotid artery and each received a right internal intracarotid injection of MPTP-HCl (Sigma Chemical Co., St. Louis MO), at a concentration of 0.14-0.16 mg/kg (0.15±0.001 mg/kg), delivered at 1 mL/min, using previously published techniques [Ovadia *et al.,* 1995; Gash *et al.,* 1996; Grondin *et al.,* 2002]. The dose was adjusted based on each animal's age and weight, as these factors have been previously shown to affect response to MPTP in macaques [Ovadia *et al.,* 1995; Ding *et al.,* 2008]. After surgery, animals were allowed to recover in quarantine for 4 days to allow for excretion of MPTP.

2.2.3 **Experimental design**

Group Size: Monkeys were assigned to six experimental groups (n=4-6/group). In the first seven monkeys tested, NTF injections were given starting at twelve weeks post-MPTP. However, in these first seven monkeys PD-like symptoms were stable from six weeks to twelve weeks post-MPTP. Hence, in all subsequent cohorts of animals in this study, NTF injections were given starting at six weeks post-MPTP. In the first seven monkeys a standard dose of 0.15 mg/kg MPTP was given. However, three monkeys receiving this dose had very little reaction to MPTP so they could not be used to test NTF's; this led to the subsequent adjustment of MPTP dosage for individual monkeys based on age and weight (as noted above in section 2.2.2). One monkey died in surgery from a toxic reaction to the anesthesia (isoflurane). Experimental groups were treated with four monthly injections of vehicle (n=4), CDNF (450 µg; n=5), CDNF (150

µg; n=6), GDNF (450 µg; n=5), N2 (337.5 µg; n=6), or N4 (280 µg; n=5), all in a 225 µL volume (Vehicle, 450 µg CDNF, 150 µg CDNF, 450 µg GDNF) and 337.5 µL volume (337.5 µg N2, 280 µg N4) of phosphate-buffered saline (CDNF, N2, N4) or citrate buffer (GDNF), pH 7.4.

Neurotrophic Factor Dosing: Many trophic factors and pharmacological agents have "inverted U" dose response curves, with lower efficacy at both lower and higher concentration levels [Gash *et al.*, 1995, 1996; Zhang *et al.*, 1997], and this was taken into consideration when choosing doses of NTFs to test in this study. The CDNF doses that were utilized for the current study were chosen based on previous work demonstrating that a 450 µg dose of GDNF, delivered intraputamenally, was effective in improving DA function in MPTP-treated monkeys [Ovadia *et al.*, 1995; Gash *et al.*, 1996; Grondin *et al.*, 2002]. We chose to test this same dose of CDNF in monkeys in one experimental group. However, CDNF had been shown to be more effective than GDNF in a rodent model of PD [Lindholm *et al.*, 2007], so there was concern that if the same was true for monkeys we may be on the diminishing slope in an "inverted U" dose response curve. Thus, a lower dose of CDNF was also tested (150 µg). A dose of N2 (337.5 µg) that was the molar equivalent of the 450 µg dose of GDNF, was tested. N4 was less soluble than N2 and hence a 280 µg dose, that could be dissolved in the same volume as the N2 solutions tested was used.

Convection Enhanced Delivery (CED) Infusion: CDNF, GDNF, N2, N4 and vehicle were given as stereotactic needle injections (using a 26 g side-port needle) into the putamen a total of four times, at 6, 10, 14 and 18 weeks post-MPTP, in MRI-guided surgeries. CED involved a slow infusion rate of 2 µL/min, that has previously been shown to distribute trophic factors in a spherical fashion within the target tissue [Gash *et al.*, 1996; Grondin *et al.*, 2002]. Each monthly

injection was made in a different location within the putamen, such that by the end of the fourth injection the entire putamen was covered, and no injection tracks crossing paths [Grondin *et al.,* 1998; Gash *et al.,* 2005]. Post-injection, the injection needle was left in place for twenty minutes, and then the needle was withdrawn from the brain at a slow rate of 1 mm/min. At the initial stages of the experiment, we used MRI to visualize the intraputamenal distribution of the infusate after each monthly injection by co-infusing Magnevist that could be detected by MRI immediately after the termination of the infusion (see **Figure 2**).

Motor function assessments: Motor function assessments were carried out throughout the duration of the study (see **Figure 3**). Motor function was evaluated using five methods: the monkey Parkinson's rating scale [Ovadia *et al.,* 1995]; measurement of minute-by-minute activity measured by omnidirectional accelerometers [Sullivan *et al.,* 2006; Hunnel *et al.,* 2006; Papailiou *et al.,* 2008; Sullivan *et al.,* 2010]; automated movement tracking using EthoVision software to assess changes in motor behaviors in a controlled environment [Grondin *et al.,* 2002; Walton *et al.,* 2006]; testing of fine motor function using the monkey Movement Assessment Panel (mMAP) [Gash *et al.,* 1999; Maswood *et al.,* 2002; Kastman *et al.,* 2012]; and detailed analysis of naturally-occurring gross and fine motor function in videotapes of monkey homepen behavior [Papillaou *et al.,* 2008]. For each measurement, raters who scored the behavior were blind to the treatment groups that monkeys were in.

A. Monkey Parkinsonian Rating Scale: The monkey Parkinson's rating scale was developed at the Kentucky Udall Center and was patterned after the human UPDRS [Ovadia *et al.,* 1995]. In the current study, behavior was videotaped while monkeys were given the opportunity to forage after seeds and nuts were scattered in the sawdust bedding in the pen. Assessments were made once a week throughout the study. Motor functions were scored from 0 (normal) to 3

(severe disability) in the following categories: rigidity, bradykinesia, posture, balance, tremor, and hand dexterity. The left side and right side were scored separately for rigidity, bradykinesia and tremor. The maximum score possible was 22. Two independent raters scored each videotaped session and assigned ratings for each video session. If variability between the two independent raters was greater than 15%, a third rater scored the session. Overall, inter-rater reliability was 88.6 $\pm$ 2.9%.

B. Activity Monitoring: Activity was measured in each animal using an omnidirectional Actical accelerometer (Respironics, Phoenix, AZ) mounted on a loose-fitting collar (Primate Products, Immokalee, FL), using previously published methods [Sullivan *et al.,* 2006; Hunnel *et al.,* 2006; Papailiou *et al.,* 2008; Sullivan *et al.,* 2010]. Monkeys adapted quickly to the collars, which did not interfere with activities such as feeding or sleeping. Activity monitors were set to collect activity counts on a per minute basis. Activity was measured for 13±0.6 days pre-MPTP. Then, monkeys did not wear collars with activity monitors for the first two weeks after MPTP surgery to allow healing of the surgical incision in the carotid region that was made for the intracarotid MPTP injection. Subsequently, activity was measured continuously for the rest of the study. For activity calculations, the start of the daytime was determined as the time of sunrise or 0700 h, depending on which occurred first; similarly the end of daytime was calculated as 1900 h or the time of sunset, depending on which occurred last. Monkeys were sedated with Ketamine HCl (0.1-0.2 mg/kg, i.m.) and activity monitors were downloaded every 2-3 weeks, calibrated and reset.

C. Automated Movement Tracking System, Ethovision: Throughout the study, monkeys were moved once a week from their homepen to a cage in a novel room and videotaped for a one-hour period, while white noise played in the

background. The Ethovision program, developed by Noldus, Inc (Wageningen, NL) was then used to track the center of gravity of each monkey over the one hour time period. Distance travelled, speed of movement and time spent with the monkey's center of gravity in the top or bottom of testing cage was quantified.

D. Monkey Movement Analysis Panel (mMAP): This apparatus and testing protocol was developed by the Kentucky Udall Center [Gash *et al.,* 1999]. Briefly, mMAP testing was performed from two to six weeks pre-MPTP (mean=3.0±0.2 weeks pre-MPTP), for 5 weeks post-MPTP (starting 10.7+0.7 days post-MPTP), and during the entire duration of the NTF infusions. For each testing session, monkeys were transferred from their homepen to a testing room and placed in a testing cage with the mMAP apparatus attached to the front. Monkeys had been acclimated to the testing cage for at least 1 week prior to the onset of testing. Each day of mMAP testing consisted of 12 trials total, 6 trials for each hand. This allowed us to compare motor function between the left hand (i.e., the side that would be affected by right intracarotid MPTP administration) and the right hand (i.e., the unaffected side). During mMAP testing, monkeys were required to reach through an opening (one opening for the left hand and another opening for right hand) to retrieve a small food reward placed on a platform the animal could see [Gash *et al.,* 1999]. The openings were each equipped with photodiodes to monitor arm/hand movements of the monkey, thereby recording with millisecond accuracy the latency to retrieve the food reward [Gash *et al.,* 1999]. Monkeys were given one minute to retrieve each treat. For each trial, on the right or left side, monkeys were given a total of five chances to respond before the trial ended. For each trial, the latency to retrieve the treat was recorded. If the monkey did not retrieve a treat at the end of five chances the trial was scored as a balk. The trials alternated between the right and left hands. The testing was

stopped if a monkey balked for three consecutive trials. Before testing, all monkeys were trained to use the mMAP apparatus until they routinely retrieved food from the receptacle with each hand. After the training period, baseline measurements were collected before MPTP administration. Monkeys were then tested twice a week after MPTP injection. Importantly, mMAP testing was carried out only twice per week so that monkeys did not become over-trained in this procedure as training, itself, leads to an improvement in motor function [Matsuzaka *et al.,* 2007].

E. Monkey Homepen Assessments: Once a week monkeys were videotaped for a seventy-five minute period in their normal homepen environment. The videotaped sessions were used to quantify the various naturally-occurring motor activities displayed by the monkeys using The Observer Program (Noldus, Inc) [Papillaou *et al.,* 2008]. Behaviors scored included eating, grasping, grooming, circling, moving, jumping, and sleeping.

Post-mortem collection of brain tissue: Eighteen weeks after unilateral MPTP injection, immediately following their fourth NTF infusion, monkeys were sacrificed and their brain tissue collected for post-mortem analysis (as indicated in **Figure 3**). Monkeys were deeply sedated with Ketamine HCl (20-25 mg/kg, SC) and sodium pentobarbital (30 mg/kg, i.v.) was administered. When the monkey completely lost reflexes, it was placed on its back and a midline incision made in the chest wall. The pericardium was opened, a cannula is inserted into the aorta through a small incision in the left ventricle, and the right auricle cut. The descending aorta was clamped to direct perfusate only to the head, and the vascular system was perfused with ice-cold physiological saline containing 5,000 IU sodium heparin and 2% sodium nitrite to remove blood from the brain. When the perfusate ran clear, the perfusion was stopped, the skull quickly opened using a bone saw, and the brain immediately removed. The brain was put in a brain mold

(immersed in ice-chilled DEPC saline), and the brain was cut every 4 mm rostral to caudal (placing the first blade just caudal to the optic chiasm), and slabs were removed and placed on glass slides. All sections containing putamen, caudate and globus pallidus were snap frozen on dry ice. Slabs containing the mid-brain dopamine regions were immediately fixed in paraformaldehyde. The fixed sections were used for post-mortem DA cell counts and the frozen sections were used for collecting tissue punches for later biochemical measurements. Each slab that had been frozen and had punches removed was wrapped in foil and all frozen slabs from each brain were placed into a single sealed plastic bag and stored at -80°C. Consistent use of these procedures provided a mean postmortem interval 48.7±2.4 min from the chest incision to collection of the last brain slab.

Staining and Counting Substantia Nigra DA neurons: 4 mm slabs containing the mid-brain dopamine regions were immersion-fixed in 4% paraformaldehyde solution at 4°C for three days. Following this, the brain slabs were transferred to a 15% sucrose solution and allowed to stay in it until the brain sunk to the bottom of the jar. The brain slabs were then transferred to 30% sucrose and the same procedure was followed. Once the brain slabs sank in the 30% solution they were wrapped in foil, snap frozen and processed so that 40 μm thick coronal sections could be cut on a sliding knife microtome through the substantia nigra. As described elsewhere [Gash *et al.,* 1996; Grondin *et al.,* 2002], SN sections were processed for immunohistochemical staining for tyrosine hydroxylase, (TH monoclonal antibody, 1:1000; Chemicon International, Temecula, CA). The number of TH+ midbrain DA neurons was measured using an optical fractionator method for unbiased stereological cell counting [Gash *et al.,* 1996; Grondin *et al.,* 2002]. Briefly, the substantia nigra is defined as consisting of all midbrain TH+ neurons except those interspersed with the oculomotor nerve rootlets. Using an

optical fractionator method for unbiased stereological cell counting, the number of TH+ neurons in the substantia nigra was estimated. On each section, a 200 μm X 200 μm grid was randomly superimposed with a 150 μm X 150 μm counting chamber placed on each fifth intersection. A 20 μm deep fraction of the counting chamber was determined by a stage encoder attached to the microscope to measure the z-axis. All neurons completely within the boundaries of the chamber or crossing the upper or right side of the chamber within the 20 μm depth were counted and their perimeter (minus neurites) measured.

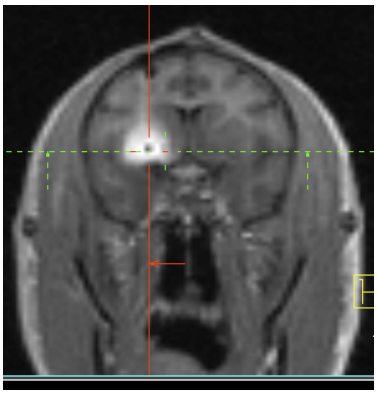


Figure 2. MRI of monkey brain after administration of 150 μg CDNF + Magnevist into the putamen.

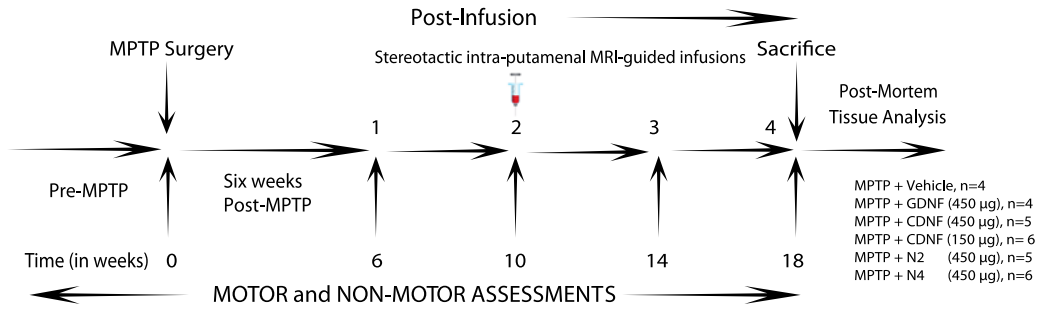


Figure 3. Experimental design. Motor and non-motor assessments were made throughout the study starting with baseline measures before MPTP administration, followed by post-MPTP, and after each infusion of NTF. The six experimental groups and sample sizes are also listed.

2.2.4 **Statistical Analysis**

For all analyses, normality and homogeneity of variance were first tested. If these criteria were met, parametric statistical tests were used. Comparisons across the five time points in the study (pre-MPTP, post-MPTP, post-infusion 1, post-infusion 2 and post-infusion 3) were made using repeated measures analysis of variance, while comparisons between two time points (pre-MPTP vs. post-MPTP, post-MPTP vs. post-infusion 3) were subsequently made using paired Student's t-tests. If normality or homogeneity of variance criteria were not met, data was transformed to normalize the data prior to statistical analyses, if possible. Cell count data was normalized by a log transformation before being analyzed using Pearson's correlations. A non-parametric statistical test, specifically the two sample Kolmogrov-Smirnov test, was used to compare the raw cell count data. Pearson's correlation coefficients, or Spearman's correlation coefficients if normality criteria were not met, were used to calculate the relationship between DA cell counts and all motor measurements made. Statistical analyses were performed using MATLAB (The MathWorks Inc., Natick, MA). Values are presented as means ± SEM. $P \leq 0.05$ was considered significant.

2.3 **RESULTS**

2.3.1 **Effect of MPTP Administration on Motor Movement**

Parkinson's Rating Scale: Monkeys at baseline had a rating scale score of 0, as the monkeys did not exhibit any PD-like symptoms. After MPTP administration the monkey Parkinson's rating scale score increased significantly to 7.9 ± 0.5, $p<0.001$ (data not shown).

Activity: There was a 57.1% decrease in the average daily activity counts, with mean activity counts decreasing from 113,472±10,055 pre-MPTP to 48,682±6,995 post-MPTP (**Figure 4**). Correspondingly, the daytime activity reduced by 58.4% from 105,097±14,854 pre-MPTP to 43,705±9,240 post-MPTP, $p<0.001$ and the nighttime activity reduced by 33.2% from 5,980±1990 pre-MPTP to 3997±996 post-MPTP, $p=0.03$ (**Figure 4**).

Ethovision: There was a significant decrease, $p<0.005$, from pre-MPTP to post-MPTP for all parameters measured using the Ethovision program [total distance moved, duration of time spent in top of cage, and distance moved in top of cage]. The total distance moved by the monkeys decreased significantly post-MPTP by 23.6% ($p=0.004$). The duration of time spent in top of cage significantly decreased from pre-MPTP to post-MPTP by 50.9% ($p=0.0004$). The corresponding decrease in the distance moved in the top of the cage was also significantly reduced by 48.6% ($p=0.0001$; **Figure 5**).

mMAP: After right-side unilateral MPTP injection there was a significant increase in time taken to retrieve a treat with the left hand from 1.17 ± 0.08 sec pre-MPTP to 2.03 ± 0.26 sec post-MPTP ($p=0.002$; $n=13$; data not shown). However, 57% of the monkeys completely stopped

using their left-hand after MPTP injection (n=17). There was no significant change in the time taken to retrieve a treat with the right hand and all of the monkeys continued to use their right hand in the task at least some of the time, 1.21 ± 0.04 sec pre-MPTP to 1.24 ± 0.09 sec post-MPTP ($p=0.32$). Balking with the left hand increased from 2.1% of the trials pre-MPTP to 69.8% of the trials post-MPTP, $p<0.001$ (**Figure 6**). Balking with the right hand increased from 1.5% pre-MPTP to 11.7% post-MPTP, $p=0.008$, but this was a significantly smaller increase in balking than occurred on the left side, $p<0.001$ (**Figure 6**).

Homepen movement: Monkeys displayed significant motor impairments naturally-occurring movement in the homepen after MPTP administration. Spontaneous circling towards the side of the lesion (circling to the right) increased significantly after MPTP, $p<0.001$ (**Figure 7 A**). There was a significant decrease in the total movement of monkeys after MPTP, $p=0.003$ (**Figure 7 B**). There was a significant decrease in fine motor movement the time to use the left hand post-MPTP, $p<0.001$ (**Figure 7 C**).

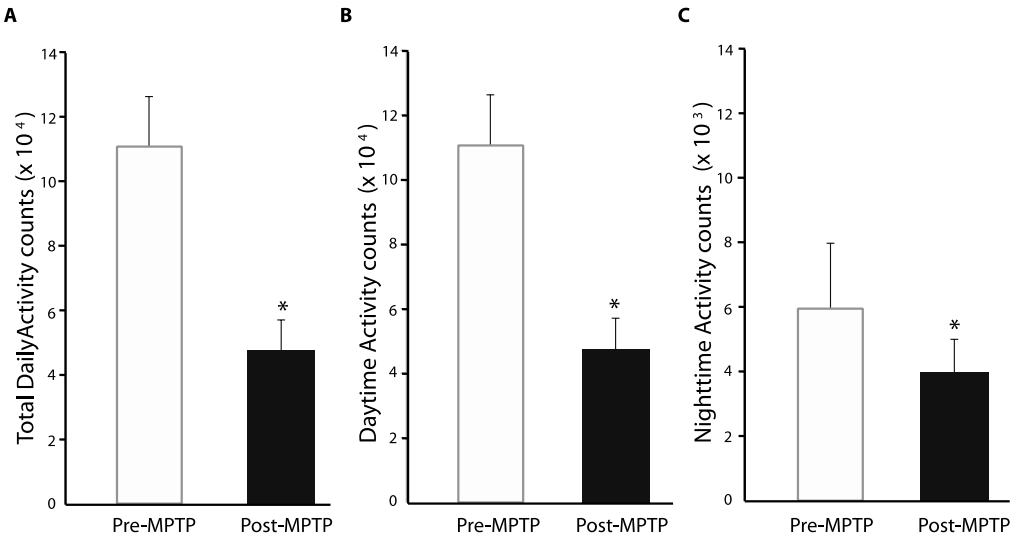


Figure 4. Mean daily activity counts before (open bars) and after (closed bars) MPTP administration, measured by accelerometer (n=30). **A.** Total Activity ($p<0.001$ compared to pre-MPTP), **B.** Daytime activity ($p<0.001$ compared to pre-MPTP), **C.** Nighttime activity ($p=0.03$ compared to pre-MPTP). Asterisks indicate a significant difference, $p<0.05$, from pre-MPTP values.

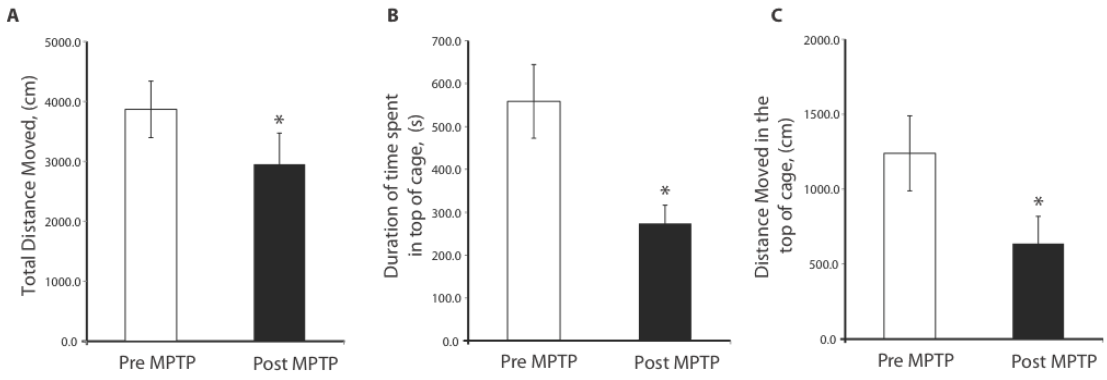


Figure 5. Movement measured by Ethovision.). **A.** Total distance moved ($p<0.005$ compared to pre-MPTP), **B.** Duration of time spent in top of cage ($p<0.005$ compared to pre-MPTP), **C.** Distance moved in top of cage ($p<0.005$ compared to pre-MPTP). Asterisks indicate a significant difference, $p<0.005$, from pre-MPTP period.

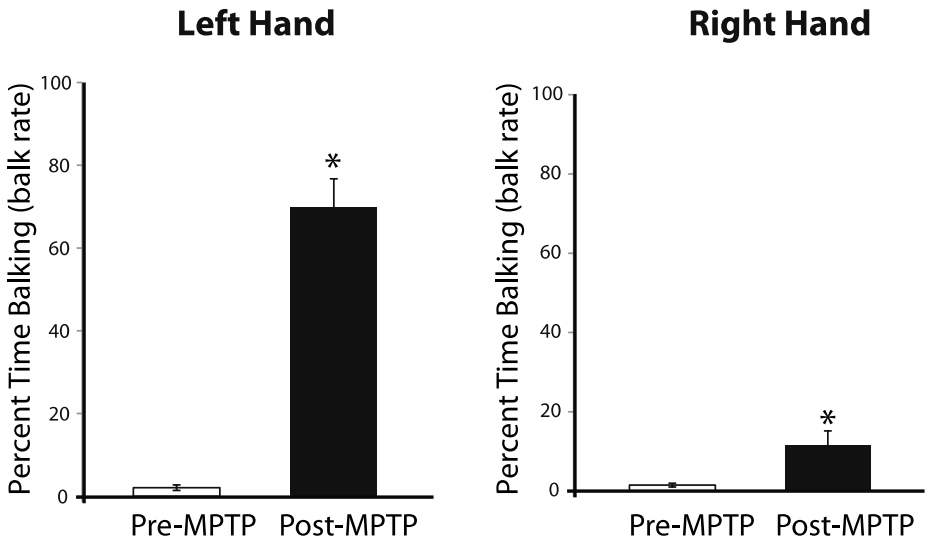


Figure 6. Balk rate during mMAP testing**.** Asterisks indicate a significant difference, $p<0.001$, from the pre-MPTP period.

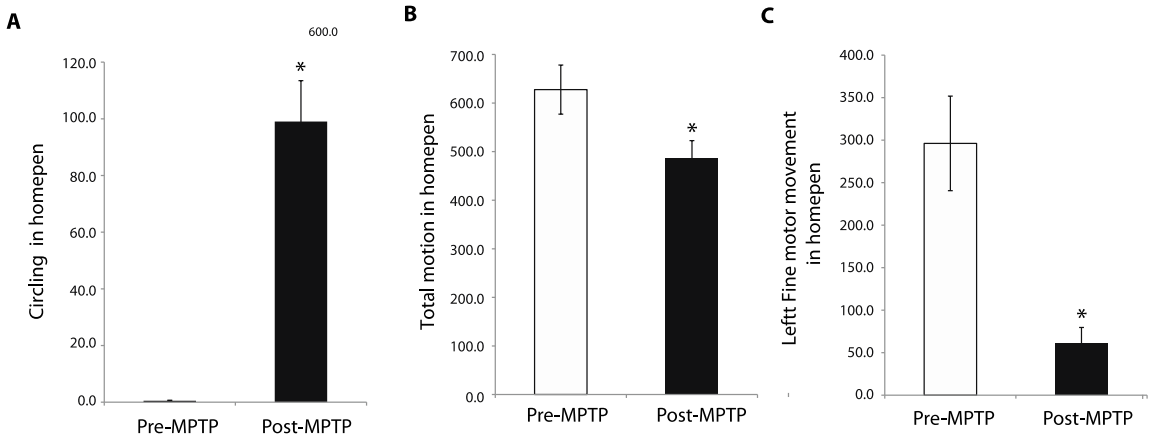


Figure 7. Changes in motor behavior by homepen assessments. Asterisks indicate a significant difference, $p<0.005$, from Pre-MPTP period.

2.3.2 **Effect of GDNF, CDNF, N2 and N4 Infusions on Motor Movement**

Monkey Parkinsonian Rating Scale: Monkeys receiving intraputamenal infusions of GDNF, 150 µg CDNF, N2 and N4 showed significant improvement in the monkey Parkinson's rating scale scores after three monthly NTF infusions (**Figure 8A**). The rating scale scores for the 150 µg CDNF, N2 and N4 groups were significantly different than the rating scale scores for the vehicle-treated group at the end of the three-month infusion period (**Figure 8B**). The 150 µg CDNF group showed progressive improvement in the rating scale score after each infusion, while the N2 and N4-treated groups had a maximal improvement in rating scale score after the first infusion and then the rating scale score remained at the same until the end of the study (**Figure 8B**).

Activity Monitoring: Monkeys activity did not recover significantly after three months of infusions in any of the six treatment groups back to the daily level of activity before MPTP administration. Similarly, both daytime and nighttime activity also did not recover back to baseline (data not shown).

Automated Movement Tracking System, Ethovision: Monkeys did not change significantly after three monthly infusions of the NTFs or vehicle compared to post-MPTP values in the different parameters [total distance moved, duration of time spent in top of cage, and distance moved in top of cage] measured using Ethovision (data not shown).

Monkey Movement Analysis Panel (mMAP):

Vehicle - Pre-MPTP the mean time taken to retrieve a treat using the left hand was 1.34 ± 0.37 sec (n=4). However, post-MPTP only one out of the four monkeys would use its left hand, i.e. 75% of the monkeys in the vehicle group did not work. The one monkey that did work was

43% slower using its left hand compared to pre-MPTP. After three months of vehicle infusions, the monkey that worked post-MPTP stopped working completely, however, there was another monkey that would use its left hand during this period, and this monkey was 41.5% slower than its pre-MPTP values. The use of the right hand was also similar from the post-MPTP period (1.46 ± 0.16 sec) to the period after the third infusion (1.00 ± 0.10 sec, p=0.14 compared to post-MPTP).

450 μg CDNF - Pre-MPTP, the mean time taken to retrieve a treat using the left hand was 1.20 ± 0.26 sec (n=3). One monkey refused to work, even in the control pre-MPTP state. Post-MPTP, two of the three monkeys continued to use their left hand, with one monkey balking. Post-MPTP, the 2 monkeys that used their left hands did so in 1.93 ± 1.03 sec (p=0.33 compared to pre-MPTP). However, after three months of 450 μg CDNF infusions only one out of the three monkeys would use its left hand with a speed of 1.22±0.28 sec that was not different from pre-MPTP (p=0.99). The use of the right hand was similar from the post-MPTP period (1.41 ± 0.39 sec) to the period after the third infusion (1.54 ± 0.44 sec, p=0.83 compared to post-MPTP).

150 μg CDNF - Pre-MPTP, the mean time taken to retrieve a treat using the left hand was 1.18 ± 0.19 sec (n=6). Post-MPTP, two of the six monkeys worked using their left hand, with four monkeys balking during left hand testing. Post-MPTP, the average time taken by the two monkeys that used their left hands was 1.65 ± 0.09 sec (p=0.15 compared to their pre-MPTP speeds). After three months of CDNF infusions four of the six monkeys used their left hand in mMAP testing. However, the two monkeys that worked throughout the study had a non-significant improvement in their total time taken with the left hand (13.5% increase in speed, p=0.35). However, the four monkeys using their left hands post-infusion no longer showed a significant decrease in speed from pre-MPTP levels, although they did show a trend toward

being slower (2.16 ± 0.54 sec, p =0.07 compared to pre-MPTP). The use of the right hand in this group was similar from the post-MPTP period (1.23 ± 0.24 sec) to the period after the third infusion (0.81 ± 0.04 sec, p=0.10).

450 μg GDNF - Pre-MPTP, the mean time taken to retrieve a treat using the left hand was 0.81 ± 0.08 sec (n=4). Post-MPTP only one monkey used its left hand (1.11 ± 0.26 sec), with three monkeys balking. After three months of GDNF infusions three out of the four monkeys would use their left hand but continued to be slow (2.16 ± 0.54 sec, p=0.22, compared to post-MPTP). In contrast, the use of the right hand was similar from the post-MPTP period (0.92 ± 0.12 sec) to the period after the third infusion (1.17 ± 0.41 sec, p=0.38).

N2 - Pre-MPTP, the mean time taken to retrieve a treat using the left hand was 1.24 ± 0.11 sec (n=6). Post-MPTP, three of the six monkeys used their left hand and three monkeys balked. Post-MPTP, the three monkeys who used their left hands did so in 1.39 ± 0.08 sec, which was not a significant slowing from their pre-MPTP speed of 1.29 ± 0.17 sec (p=0.39). However, after three months of N2 infusions the mean time for left hand use was significantly improved for the same three monkeys from the post-MPTP speed (1.01 ± 0.10 sec, p=0.04 compared to post-MPTP). In contrast, the use of the right hand was similar from the post-MPTP period (1.38 ± 0.28 sec) to the period after the third infusion (1.27 ± 0.21 sec, p=0.18 compared to post-MPTP).

N4 - Pre-MPTP, the mean time taken to retrieve a treat using the left hand was 1.20 ± 0.11 sec (n=5). Post-MPTP, four monkeys used their left hand, with one monkey balking. There was a significant slowing of left hand use in these four monkeys from 1.20 ± 0.11 sec pre-MPTP to 2.61 ± 0.35 sec post-MPTP for three monkeys (p=0.01). However, after three months of N4 infusions the mean time for left hand use was significantly decreased to 1.60 ± 0.40 sec for the

three monkeys that continued to work (p=0.0003 compared to post-MPTP; p=0.18 compared to pre-MPTP). Similarly, the use of the right hand showed a trend towards decrease from the post-MPTP period (1.12 ± 0.12 sec) to the period after the third infusion (0.85 ± 0.04 sec, p=0.07 compared to post-MPTP).

Homepen Assessments: After infusions, there were no significant change in circling, fine motor, and stationary in homepen sessions for any of the treatment groups. However, for total movement after post-infusions of NTFs the N2 treatment group showed a trend towards a significant improvement in total movement, p=0.06 (**Figure 9**).

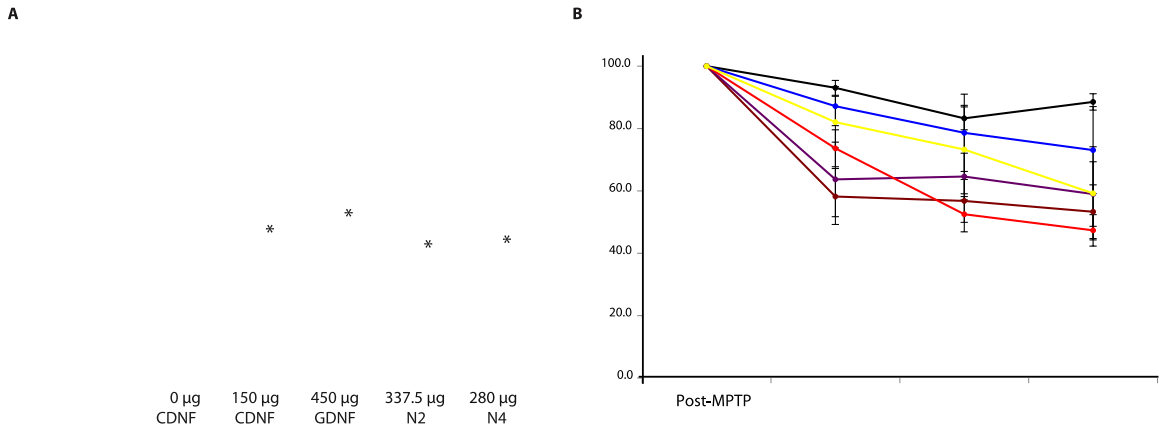


Figure 8. A. Changes in monkey Parkinson's rating scale from Post-MPTP (black bars) to Post-Infusions (grey bars) for each experimental group. Asterisks indicate a significant difference, $p<0.05$, from pre-MPTP B**.** Monkey Parkinson's rating scale scores for each experimental group, normalized to the post-MPTP mean score for each group. Vehicle: black line, $p>0.05$ post-infusion 1, 2 and 3; 450 µg CDNF: blue line, $p>0.05$ post-infusion 1, 2 and 3; 150 µg CDNF: red line, $p=0.02$ post-infusion 1, $p=0.002$ post-infusion 2, and $p=0.0002$ post-infusion 3; 450 µg GDNF: yellow line, $p>0.05$ post-infusion 1, 2 and $p=0.06$ post-infusion 3; 337.5 µg N2: brown line, $p=0.01$ post-infusion 1, 2, and 3; 280 µg N4: purple line, $p=0.03$ post-infusion 1, $p=0.04$ post-infusion 2, and $p=0.02$ post-infusion 3.

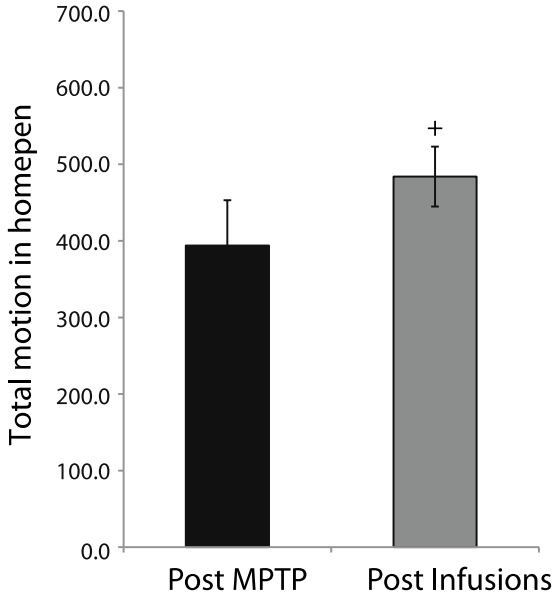


Figure 9. Changes to the total motion scored in homepen after three months of N2 (337.5 μg) infusions, p=0.06. Plus sign indicates a trend.

2.3.3 **Post-mortem cell count data and prediction of post-infusions motor measures based on cell counts measures**

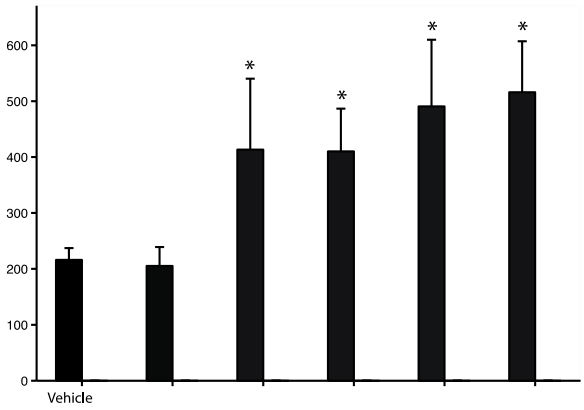


Figure 10. Number of dopamine neurons post-infusion 4. Asterisks indicate a significant difference from the vehicle-treated group, $p<0.05$.

There was a significant increase in the number of midbrain SNpc dopamine cells in monkeys receiving GDNF, 150 ug CDNF, N2, and N4 treatments compared to the vehicle group (**Figure 10**). The number of dopamine neurons was significantly correlated with a number of measures of motor function. There was a significant correlation between the Parkinson's Rating Scale score measured in the last month of the study and the number of DA neurons present at the end of the study ($r= -0.72$ $p<0.001$, **Figure 11A**). For fine motor function, the time taken to retrieve a treat with left hand in the last month of the study in mMAP testing was significantly correlated with the DA neuron cell counts ($r=-0.53$ $p=0.002$, **Figure 11B**). Another measures of gross motor activity, activity measured by accelerometer, showed a trend towards significance (Activity: $r=0.30$, $p=0.059$; **Figure 12A**). Total distance moved as measured by Ethovision analysis was significantly correlated to the DA neuron cell counts ($r= 0.45$ $p=0.009$, **Figure 12B**). Circling, a

gross motor function, at the end of the NTF infusions, was significantly correlated with the number of DA cell counts (r=-0.32 p=0.05, **Figure 13A**). And left fine motor values measured in the homepen assessments showed a trend towards significance (r=0.30 p=0.06, **Figure 13B**).

A median split of monkeys divided by the number of DA cells they had in the post-mortem analysis (**Figure 14A)** showed significant differences in monkey Parkinson's rating scale score (bottom half: 6.88 ± 0.58, top half: 3.14 ± 0.58, p<0.001, **Figure 14B**), total distance moved in Ethovision assessments (bottom half: 80.62 ± 12.47, top half: 116.27 ± 16.24, p=0.05, **Figure 14C**), and time taken to retrieve a treat with the left hand in mMAP testing (bottom half: 39.57 ± 7.60, top half: 22.22 ± 7.81, p=0.04, **Figure 14D**).

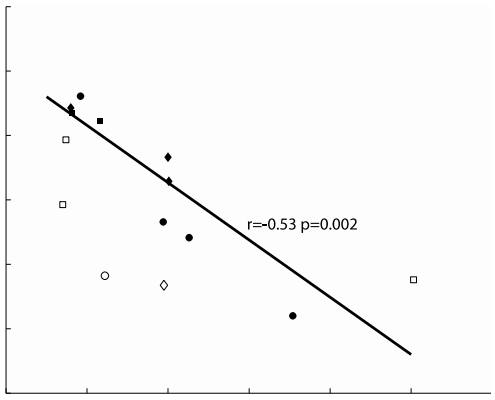
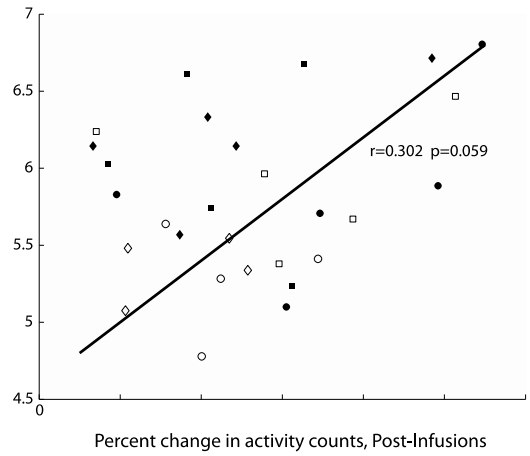


Figure 11. Correlation between DA cell counts and (**A)** Monkey Parkinson's rating scale score, and (**B)** left hand time to retrieve a treat in the mMAP. Vehicle (open diamond), 450 μg GDNF (open square), 450 μg CDNF (open circle), 150 μg CDNF (closed circle), 337.5 μg N2 (closed square), 280 μg N4 (closed diamond)



otal distance moved, Post-Infusions

Figure 12. Correlation between DA cell counts and **A.** Percent change in activity from post-MPTP to post-Infusions of NTFs, **B.** Percent change in total distance moved measured by Ethovision. Vehicle (open diamond), 450 μg GDNF (open square), 450 μg CDNF (open circle), 150 μg CDNF (closed circle), 337.5 μg N2 (closed square), 280 μg N4 (closed diamond)

A

2 p= 0.05

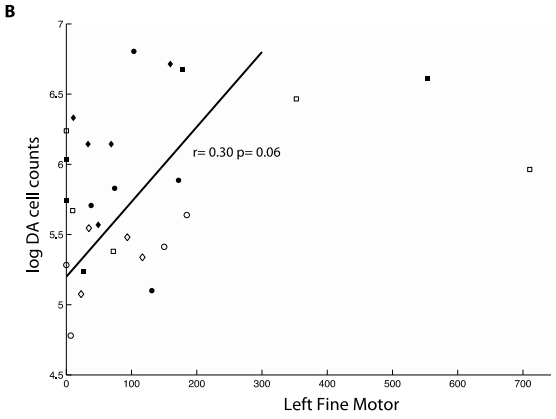


Figure 13. Correlation between DA cell counts and **A.** Circling, and **B.** Left fine motor measured in the homepen. Vehicle (open diamond), 450 μg GDNF (open square), 450 μg CDNF (open circle), 150 μg CDNF (closed circle), 337.5 μg N2 (closed square), 280 μg N4 (closed diamond)

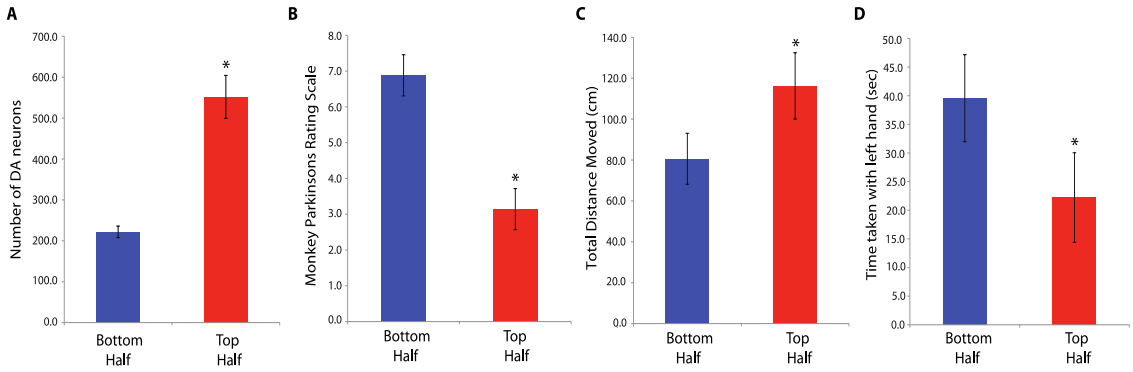


Figure 14. Comparison of **A.** Number of DA neurons, **B.** monkey Parkinson's rating scale score, **C.** Total distance moved measured in ethovision, and **D.** Time taken to retrieve treat with left hand measured in mMAP, in animals that had DA cell counts in the bottom half (Blue bars) and top half (Red bars) of the population in post-mortem analysis ($p<0.001$, $p<0.001$, $p=0.05$, $p=0.04$ respectively). Asterisks indicate a significant difference, $p<0.05$

2.4 DISCUSSION

2.4.1 Effects of unilateral low-dose MPTP on motor movement

There were significant PD-like symptoms, that represented early to mid-stage severity of the disease, in monkeys that received a single unilateral low-dose MPTP intracarotid injections (0.15±0.001 mg/kg). This study used multiple strategies to carefully define the extent of impairments that developed in gross and fine motor function. Gross motor impairments were assessed using the monkey Parkinson's rating scale, activity monitoring by accelerometer, an automated program to track movement (i.e., Ethovision), and homepen scoring of naturally-occurring motor assessments. Fine motor movements were measured using the monkey movement assessment panel (mMAP) and by homepen scoring of fine motor behaviors. Monkeys had an average Parkinson's rating scale score of 7.9 ± 0.5 out of a possible score of 0-22. This level of impairment was similar to what was previously reported by Ding *et al.* [2008], using the same animal model. Locomotion, measured by Ethovision scoring showed an approximate 50% reduction post-MPTP, comparable to the loss seen in previous studies using this monkey model [Ding *et al.*, 2008; Grondin *et al.*, 2008]. Locomotion was also measured for the first time in this monkey model using accelerometers, which similarly showed about a 50% reduction in gross motor activity. Fine motor performance, measured using mMAP, showed a significant increase in balking (~75% with the left hand) after MPTP. This was comparable to the decrease in naturally-occurring fine motor performance scored for the first time in this animal model in homepen assessments (~70%). Overall, moderate impairments in both gross motor function and fine motor function were comprehensively characterized for the first time in this dissertation, showing that this nonhuman primate model of PD reliably shows approximately a

50% impairment in total gross motor function and 70% impairment in fine motor function on the affected side.

Although the two quantitative measures of gross motor function made in this study, using accelerometers and Ethovision assessments, showed about a 50% reduction post-MPTP, this was not outside of the normal range of gross motor function seen in normal populations of rhesus monkeys. Previous studies in rhesus monkeys have shown that there can be an 8-10-fold variation in naturally-occurring activity level in monkeys in a variety of housing conditions ranging from single animal cages to large group living pens [Sullivan *et al.,* 2006; Hunnel *et al.,* 2006; Papailiou *et al.,* 2008; Sullivan *et al.,* 2010]. After MPTP, activity levels for monkeys uniformly fell close at the low end of the naturally-occurring spectrum for this species. This is the first time behavior of MPTP-treated monkeys in their normal home environments has been comprehensibly assessed. Interestingly, this comprehensive analysis also showed that post-MPTP there was a significant increase in spontaneous circling towards the side of the lesion (i.e. circling to the right side). This behavior has been characterized in detail in rodent models of PD and is regularly used to assess the effectiveness of treatment in these models [Hefti *et al.,* 1980, Lindholm *et al.,* 2007; Voutilainen *et al.,* 2011]. However, very little is known about this characteristic in primates [reviewed in detail in Blum *et al.,* 2001; Bove *et al.,* 2012]. Similarly, the Ethovision assessments measured in this study during the pre-MPTP baseline period were in the normal range of locomotion activity in adult rhesus monkeys, as previously reported [Walton *et al.,* 2006]. However, after MPTP administration the measures of locomotion in our 17-20 year old monkeys resembled that of older aged monkeys, 25-30 year old, that was significantly slower than locomotor activity in younger monkeys [Walton *et al.,* 2006; Grondin *et al.,* 2008]. Thus,

locomotor activity tracked by Ethovision also showed MPTP-treated animals lying close to the low-end of the naturally-occuring spectrum of locomotor activity [Walton *et al.,* 2006].

2.4.2 **Effects of neurotrophic factors CDNF, N2 and N4 on motor movement**

After three months of infusions, the GDNF, 150 μg CDNF, N2 and N4-treated groups of monkeys showed significant improvement in the monkey Parkinson's rating scale scores. Correspondingly, these same treatment groups showed significantly increased numbers of dopamine neurons in the substantia nigra in post-mortem analyses. Moreover, the number of dopamine neurons was significantly correlated with the rating scale score measured at the end of three monthly NTF infusions. There were also significant correlations in gross motor activity measured by accelerometer, Ethovision tracking, and in the homepen assessments of naturally-occurring activity. The number of dopamine neurons was also significantly correlated with fine motor function measured by both mMAP testing and naturally-occurring use of the left hand in the homepen. A median split of the monkeys into those with lower numbers of dopamine neurons vs. higher numbers of dopamine showed significant differences in these groups in rating scale scores, and measures of both gross and fine motor function. Interestingly, 100% of the monkeys in the control and 450 μg CDNF groups fell into the bottom half of the post-mortem DA neuron cell group, whereas nearly 70% of the monkeys in the three effective treatment groups (150 μg CDNF, N2 and N4) fell into the top half of the post-mortem DA neuron cell group. The improvements in these three treatment groups were comparable to the effects of GDNF that was previously reported (~ 30 % improvement in rating scale) [Zhang *et al.,* 1997; Grondin *et al.,* 1998, 2002; Gash *et al.,* 2005]. Together, these studies provide three lines of evidence that the low-dose CDNF treatment and the N2 and N4 treatments provide significant

therapeutic value in the monkey MPTP model of PD. First, all three NTFs significantly increased the number of surviving DA neurons in the substantia nigra. Second, all three NTFs significantly improved motor function measured using the monkey Parkinson's rating scale. Third, the findings that DA neuron count significantly correlated with a number of measures of both gross and fine motor movement, and that GDNF, 150 µg CDNF, N2 and N4-treated groups had significantly higher DA neuron counts compared to vehicle-treated animals provides strong evidence for neurorestorative properties of these four neurotrophic factors.

This study was initially designed as a pilot study undertaken to provide a first assessment of whether CDNF, N2 and N4 could provide neurorestoration in a primate model of PD. As a pilot study, the sample sizes for each treatment group were smaller than would be optimal to comprehensively assess the impact of these neurotrophic factors on motor function. Power analyses at the end of the experiment indicate that if there had been 8-10 animals/group it is likely that low dose CDNF would have shown significant improvements in both gross and fine motor function, whereas many more animals would have been needed to see significant results with N2 and N4 on gross and fine motor functions.

We conclude that 150 µg CDNF, N2 and N4 treatments rescue DA neurons from cell death and recover gross and fine motor functions in monkeys treated with MPTP. These three trophic factors are potential candidates for future clinical trials of novel therapeutic strategies for treating PD. Further studies with larger sample sizes and sufficient power would help identify the best treatment option. Moreover, as discussed in Chapter 5, testing alternative treatment strategies with combinations of these NTFs, given together or sequentially, may provide even more effective treatment for the motor symptoms of PD.

3.0 EFFECTS OF UNIRLATEAL MPTP INJECTIONS ON SLEEP

3.1 **INTRODUCTION**

Excessive daytime sleep (EDS) is common in patients with Parkinson's disease (PD) [Knie *et al.*, 2011; Schulte *et al.,* 2011; Videnovic *et al.,* 2012]. Although increased daytime sleepiness becomes more common with age [Pal *et al.,* 2001; Arnulf 2005; Rye *et al.,* 2006; Wolkove *et al.,* 2007], EDS is fifteen-fold more frequent in Parkinson's patients than in age-matched controls [Knie *et al.,* 2011; Tandberg *et al.,* 1999]. The Epworth Sleepiness Scale (ESS) is the most widely used scale to assess the tendency to fall asleep in patients [Johns *et al.,* 1991]. Epidemiological studies conducted in multiple centers have consistently found ESS scores that are about 50% higher in PD patients compared to age- and gender-matched controls [Ondo *et al.,* 2001; Hobson *et al.,* 2002; Brodsky *et al.,* 2003; Arnulf 2005].

Increased daytime sleep has also been reported in animal models of PD created by administration of dopaminergic neurotoxins [Hartmann *et al.,* 1971; Garcia *et al.,* 2005; Yi *et al.,* 2007; Friedman *et al.,* 2008; McDowell *et al.,* 2010, 2012]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a dopamine neurotoxin which primarily affects the dopamine-containing neurons of the SNpc (Substantia Nigra pars compacta) and has been used in mice and monkeys [Burns *et al.,* 1983; Heikkila *et al.,* 1984; McDowell *et al.,* 2012]. In MPTP-treated mice that experience a 65% loss of dopamine in the striatum, sleep duration during the normal waking period has been reported to increase about 50% compared to saline-treated mice, and the mean duration of these sleep episodes was also significantly longer [Monaca *et al.,* 2004; Laloux

et al., 2007, 2008]. In rats given the DA neurotoxin, rotenone, leading to a 75% decrease in number of DA neurons in SNpc, there was about a two-fold increase in percent time spent sleeping when rats are most active [Yi *et al.*, 2007]. Daytime sleep problems have also been reported in monkeys that received multiple systemic injections of the neurotoxin MPTP [Barraud *et al.*, 2009]. A five-fold increase in percent time spent in daytime sleep duration was observed in MPTP-treated monkeys with a greater than 95% loss of dopamine and dopamine metabolites [Barraud *et al.*, 2009].

Interestingly, recent clinical studies have reported increased daytime sleepiness occurring even before the motor symptoms of PD manifest [Gjerstad *et al.*, 2006, Dhawan *et al.*, 2006, Iranzo 2011]. This suggests that EDS may be able to serve as a diagnostic tool for detecting PD [Abbott *et al.*, 2005]. However, it is unknown whether decreasing levels of dopamine are causing increased daytime sleepiness in the early stages of PD, or whether there are other neural systems whose functions are altered in early PD that could be influencing sleep. Studies examining the effects of mild decreases in dopamine, similar to those occurring in early PD, have not been reported in animal models. To address this question, the current study in which a single low-dose, unilateral injection of MPTP was given to monkeys to mimic the early stages of loss of dopamine in PD [Ovadia *et al.*, 1995, Gash *et al.*, 1996, Grondin *et al.*, 2002], examined whether there were changes in daytime sleepiness.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Twenty eight female rhesus macaques (*Macaca mulatta*), 16-20 years of age (mean age: 18.3±0.2 years) were housed in social living pens (1.6m x 2.3m x 3.5m) that had multiple perches, toys, and a thick layer of sawdust bedding with overhead skylights that provided natural lighting. In addition to the natural lighting, artificial lighting turned on at 0700 h in the morning and lights turned off at 1900 h at night. For sleep calculations, the start of the daytime was determined as the time of sunrise or 0700 h, depending on which occurred first; similarly the end of daytime was calculated as 1900 h or the time of sunset, depending on which occurred last. This housing facility has 20 pens in a wing, and pens have wire mesh fencing fronts such that monkeys in each pen could see and hear other monkeys in a number of other pens. Monkeys were pair-housed. Monkeys were fed Purina Monkey Chow (#5038; Ralston Purina Co., St. Louis, MO) once daily and given fresh fruit, vegetables, nuts and seeds to encourage foraging, as well as *ad libitum* access to drinking water. All monkeys were observed daily for health and menstrual status. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.2 MPTP Administration

Prior to MPTP administration, baseline assessments of motor function and sleep were made for approximately a two-week period (13.2±0.5 days) for each monkey. All monkeys then

underwent a surgery to expose the right carotid artery and each received a right intracarotid injection of MPTP-HCl (Sigma Chemical Co., St. Louis MO), at a concentration of 0.14-0.16 mg/kg (average dose=0.15±0.001 mg/kg), delivered at 1 mL/min, using previously published techniques [Ovadia *et al.*, 1995; Gash *et al.*, 1996; Grondin *et al.*, 2002]. After surgery, animals were allowed to recover in quarantine for 4 days to allow for excretion of MPTP. Monkeys did not wear collars with activity monitors for the first two weeks after surgery to allow healing of the surgical incision that was made for the intracarotid injection. Activity was measured from 3-6 weeks post-MPTP administration.

3.2.3 **Experimental design**

Group Size: Monkeys were assigned into six experimental groups (n=4-6/group). At six weeks post-MPTP, when stable symptoms of PD were established, monkeys were assigned to an experimental group, taking care to make sure the distribution of PD-like symptoms was uniform across the six experimental groups. In the treatment groups, each monkey received four intraputamenal infusions of trophic factor or vehicle at monthly intervals (see **Figure 2**). Experimental groups were treated with four monthly injections of vehicle (n=4), GDNF (450 µg; n=5), CDNF (450µg; n=5), CDNF (150 µg; n=6), N2 (337.5 µg; n=6), or N4 (280 µg; n=5). Vehicle, GDNF and CDNF were given in a 225 µL volume and N2 and N4 were given in a 337.5 µL volume of phosphate-buffered saline (vehicle, CDNF, N2, N4) or citrate buffer (GDNF), pH 7.4.

Activity Monitoring: Activity was measured in each animal using an omnidirectional Actical accelerometer (Respironics, Phoenix, AZ) mounted on a loose-fitting collar (Primate Products, Immokalee, FL), using previously published methods [Sullivan *et al.*, 2006; Hunnel *et*

al., 2006; Papailiou *et al.,* 2008; Sullivan *et al.,* 2010]. Monkeys adapted quickly to the collars, which did not interfere with activities such as feeding or sleeping. Activity monitors were set to collect activity counts per minute. Activity was measured for approximately two-week periods in each monkey prior to MPTP (13.2±0.5 days), 3-4 weeks post-MPTP (10.6±0.7 days), and 5-6 weeks post-MPTP administration (11.0±0.4 days).

3.2.4 **Sleep Assessments**

Sleep Measurement from Actigraphic Records: Analysis parameters for defining actigraphic sleep were developed in a previous study in which sleep was measured by EEG and actigraphy and validated by infra-red videography [Herringa *et al.,* 2009]. Sleep was defined as twelve minutes of continuous zero activity counts. Various sleep parameters were calculated including: nighttime sleep latency (time from lights off to first sleep episode overlapping with or after lights off), number of nighttime awakenings, total night sleep duration, morning wake latency (time from lights on to beginning of first wake episode after or overlapping with lights on), total daytime sleep duration, and number of daytime sleep bouts.

Validation of Sleep Measurement Post-MPTP by Videography: After MPTP administration monkeys had reduced mobility and in order to ensure that our algorithm for quantifying actigraphy-defined sleep (i.e., twelve minutes of zero activity counts) was still valid post-MPTP, monkeys were videotaped for a twenty-four hour period by infra-red videography. Videotapes were manually scored for sleep behavior (i.e., monkeys sitting in a hunched sleep position not moving) in thirteen monkeys for at least thirty minutes of daytime sleep (detected by actigraphy) both before and after MPTP administration. Videographically-defined sleep was compared to actigraphy-defined sleep.

3.2.5 **Assessment of Motor Function**

To confirm previously reported findings that a single, low-dose intracarotid injection of MPTP results in stable mild motor dysfunction [Ovadia *et al.,* 1995, Ding *et al.,* 2008], we assessed monkeys on the primate version of the Unified Parkinson's Disease Rating Scale [the monkey Parkinson's rating scale; Smith *et al.,* 1993, Ovadia *et al.,* 1995] once a week for 4 weeks pre-MPTP and 6 weeks post-MPTP. Motor functions were scored from 0 (normal) to 3 (severe disability) in the following categories: rigidity, bradykinesia, posture, balance, tremor, and hand dexterity. The left side and right side were scored separately for rigidity, bradykinesia and tremor. The maximum score possible was 22. Two independent raters scored each videotaped session and assigned ratings for each video session. If variability between the two independent raters was greater than 15%, a third rater scored the session. Overall, inter-rater reliability was $88.6 \pm 2.9\%$.

3.2.6 **Statistical Analysis**

For all analyses, normality and homogeneity of variance criteria were met, and a repeated measures analysis of variance (ANOVA) was used to identify significant changes in each sleep parameter across the pre-MPTP, 3-4 weeks post-MPTP and 5-6 weeks post-MPTP periods. Post-hoc comparisons were made using paired Student's t-tests. A repeated measures analysis of variance (ANOVA) was also used to identify significant changes in each sleep parameter across the four quartiles across the day and if it was significant post-hoc t-tests, with bonferroni

corrections, were used to identify specific significant pair wise comparisons. Pearson's correlation coefficients were used to calculate the relationship between daytime sleep duration and daytime sleep bouts and the monkey Parkinson's rating scale score. Statistical analyses were performed using MATLAB (The MathWorks Inc., Natick, MA). Values are presented as means ± SEM. $P \leq 0.05$ was considered significant.

3.3 RESULTS

The specificity of actigraphy-defined sleep for the current MPTP study was plotted on the plot of the receiver-operating characteristic (ROC) analysis from our previous study in normal monkeys that defined twelve minutes of zero activity as sleep (Herringa *et al.,* 2009; **Figure 15**). The specificity for post-MPTP daytime sleep was validated using videography to determine if the criteria for identifying sleep remained reliable post-MPTP. Using the same criteria to identify sleep that had been optimal in monkeys pre-MPTP (actigraphy-defined sleep=12 min of zero activity counts), the specificity of post-MPTP sleep fell directly on the pre-MPTP ROC curve (**Figure 15**).

Pre-MPTP, the average sleep duration during the day was 91.03 ± 10.16 minutes and the mean number of daytime sleep bouts was 4.93 ± 0.44 bouts/day. Daytime sleep duration increased significantly by three to four weeks post-MPTP to 210.71 ± 21.49 minutes ($p<0.001$; **Figure 16A**). Daytime sleep duration remained stable at five to six weeks post-MPTP (200.50 ± 17.89 minutes, $p<0.001$; **Figure 16A**). The number of sleep bouts during the day also significantly increased post-MPTP to 9.97 ± 0.82 bouts/day by three to four weeks post-MPTP, and remained elevated five to six weeks post-MPTP at 9.80 ± 0.68 bouts/day ($p<0.001$; **Figure 16B**). Correspondingly, the average awake duration during the day significantly decreased from

628.97±10.16 minutes pre-MPTP to 509.29 ± 21.49 minutes ($p<0.001$) three to four weeks post-MPTP administration, and to 519.50 ± 17.89 minutes ($p<0.001$) five to six weeks post-MPTP administration (data not shown). The mean latency to wake up in the morning pre-MPTP was 4.74 ± 1.99 minutes after lights on. At three to four weeks after MPTP administration the latency to wake showed a trend toward increasing to 7.79 ± 2.55 minutes ($p=0.08$; **Figure 16C**). And there was a significant increase (8.44 ± 2.30 minutes; $p=0.05$; **Figure 16C**) at five to six weeks post-MPTP administration.

The increase in daytime sleep was apparent throughout the day after MPTP administration. The average daytime sleep duration during the four quarters of a day pre-MPTP was: first quartile: 34.70 ± 3.92, second quartile: 10.84 ± 2.06, third quartile: 18.39 ± 3.51, fourth quartile: 25.51 ± 3.75. Daytime sleep was greatest in the first quartile of the day, and there was a significant difference in the daytime sleep across the quartiles for both daytime sleep duration and daytime sleep bouts between the first quartile and second quartile of the day ($p<0.001$). Daytime sleep duration increased during each quartile of the day in a uniform manner by three to four weeks post-MPTP and stayed significantly elevated at five to six weeks post-MPTP ($p<0.001$, for all quartiles): first quartile: 72.60 ± 6.72 (2.1-fold increase), second quartile: 38.58 ± 6.00 (3.2-fold increase), third quartile: 36.10 ± 5.26 (2-fold increase), fourth quartile: 50.12 ± 5.93 (2-fold increase). Similarly, the daytime sleep bouts increased significantly for all four quartiles from pre-MPTP to five to six weeks post-MPTP ($p<0.001$, for all quartiles): first quartile: 1.90 ± 0.19 to 3.52 ± 0.25 bouts, second quartile: 0.70 ± 0.13 to 2.05 ±0.29 bouts, third quartile: 1.00 ± 0.15 to 1.89 ±0.24 bouts, fourth quartile: 1.02 ± 0.13 to 2.11 ± 0.18 bouts.

Average nighttime sleep duration pre-MPTP was 521.16 ± 16.22 minutes and the mean sleep bouts during the night was 18.38 ± 0.53 bouts/night. There was no change in the nighttime sleep duration of monkeys after MPTP administration (548.38 ± 16.11 minutes, five to six weeks

post-MPTP; $p>0.05$). The number of sleep bouts during the night also did not change post-MPTP (18.11 ± 0.57 bouts/night, five to six weeks post-MPTP; $p = 0.23$). Similarly, there were no significant changes to the nighttime awake duration and the number of wake bouts during the night at any time after MPTP administration.

The Monkey Parkinson's rating scale was used to determine the severity of motor impairments after MPTP administration. There was a significant correlation between daytime sleep duration 5-6 weeks post-MPTP administration and the rating scale score ($r=0.31$, $p=0.05$; **Figure 17**).

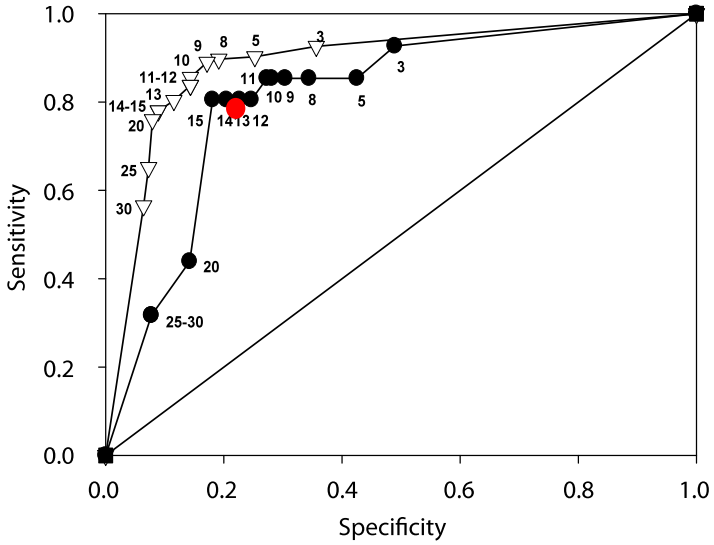


Figure 15. The receiver-operator characteristic (ROC) curves with varying duration thresholds for sleep criteria for nighttime sleep (open triangles) and daytime sleep (closed circles) as measured in a normal population of rhesus monkeys by actigraphy, EEG and validated by videography. The red circle indicates the specificity for identifying sleep from actigraphy data during the daytime in monkeys in this study post-MPTP.

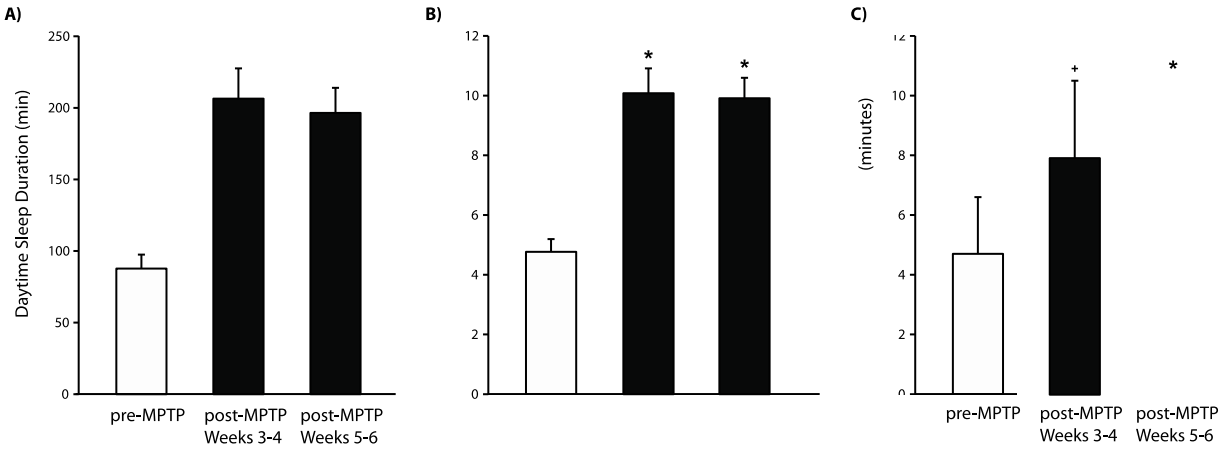


Figure 16. A. Mean daytime sleep duration, **B.** mean daytime sleep bouts, and **C.** mean latency to wake, pre-MPTP (open bar) and at 3-4 weeks and 5-6 weeks post-MPTP (closed bars). Asterisks indicate a significant difference from pre-MPTP values ($p<0.001$). Plus sign indicates a significant trend from pre-MPTP values ($p=0.08$)

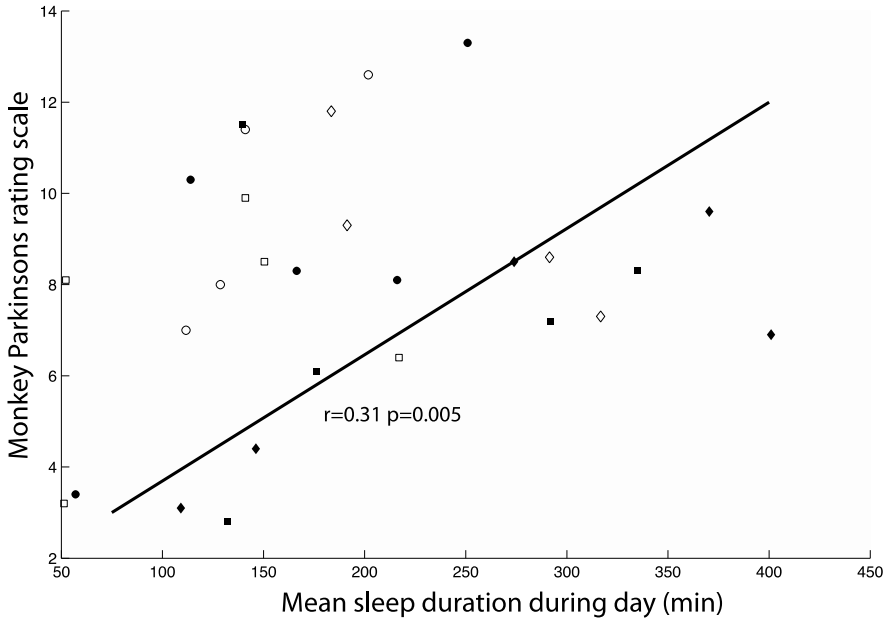


Figure 17. Correlation between, monkey Parkinson's rating scale score and daytime sleep duration post-MPTP. Vehicle (open diamond), 450 μg GDNF (open square), 450 μg CDNF (open circle), 150 μg CDNF (closed circle), 337.5 μg N2 (closed square), 280 μg N4 (closed diamond)

3.4 DISCUSSION

Monkeys that received a single low-dose unilateral MPTP injection had a significant increase in daytime sleepiness measured by actigraphy at two weeks after the DA lesion, suggesting that this primate model will be useful for future studies aimed at understanding the role of DA in the development of this prevalent non-motor symptom that occurs in the early stages of PD. This increase in daytime sleepiness occurred throughout the day. Interestingly, there was no accompanying decrease in nighttime sleep in this model. Thus, the sleep changes apparent in this low dose, unilateral MPTP nonhuman primate model are similar to what is seen clinically in early stage PD where there is a decrease in daytime sleepiness prior to impairment of nighttime sleep [Schulte *et al.,* 2011; Knie *et al.,* 2011; Videnovic *et al.,* 2012].

Actigraphy allows assessment of sleep in more natural conditions than are easily measured by EEG, such as in this study where monkeys were housed in group-living pens with a social partner. However, we were concerned that the increase in daytime sleepiness that was measured by actigraphy may be confounded by the motor problems that developed subsequent to MPTP. To address this concern, thirteen monkeys were videotaped for a twenty-four hour time period using a camera with an infrared light source (allowing visualization of monkeys during both the day and night) and the efficiency of actigraphically-defined sleep was compared to the human observer-rated sleep. Results of this analysis were also compared with the results from a previous study using EEG, actigraphy and infrared videography to define sleep in normal rhesus monkeys [Herringa *et al.,* 2009]. Receiver-operator curve (ROC) analysis was used to determine the efficiency and specificity of actigraphy-defined sleep compared to EEG-defined sleep in the previous study, and the specificity of actigraphy-defined sleep for the current MPTP study fell

right within the range of the previous study in normal monkeys (see **Figure 11**), providing strong evidence that the 2.5-fold increase in daytime sleep occurring soon after MPTP was not the result of an increase in false positive sleep measured by actigraphy.

There was a greater than two-fold increase in daytime sleep duration and number of daytime sleep bouts three to four weeks after MPTP. Similarly, the latency to wake up in the morning was also significantly increased by about two-fold at five to six weeks after MPTP. Daytime sleepiness was directly correlated with the severity of PD motor symptoms. This is strong evidence that only a moderate decrease in overall brain dopamine leads to increase in daytime sleepiness, corresponding to similar observations of increased daytime sleepiness preceding motor symptoms at PD onset [Abbott *et al.,* 2005; Gao *et al.,* 2011].

There have been reports of increased daytime sleepiness reported in animal models of PD after large, bilateral experimental lesions of the nigrostriatal dopamine pathway [Hartmann *et al.*, 1971; Garcia *et al.,* 2005; Yi *et al.,* 2007; Friedman *et al.,* 2008; McDowell *et al.*, 2010, 2012]. However, in this study, we show that a unilateral relatively mild lesion to the DA pathway is sufficient to produce about a two-fold increase in daytime sleep duration, sleep bouts and latency to wake [Subramanian *et al.,* 2012]. Not surprisingly, the increase in daytime sleepiness found in this study is lower than reported in a previous study in monkeys where a larger dose of MPTP (0.5 mg/kg MPTP intravenous injections until progressive and severe parkinsonism was established) [Barraud *et al.,* 2009] led to a > 95% loss of dopamine and dopamine metabolites was associated with a five-fold increase in daytime sleep duration.

DA plays a very complex role in the regulation of sleep that is not well characterized. Patients taking dopamine agonists have reported sudden onsets of sleepiness [Frucht *et al.,* 1999], but amphetamines that are known to increase the concentration of DA promote

wakefulness [Jankovic, 2002]. The effects of dopamine on the regulation of sleep appear to be dependent upon the type of DA drug used, the concentration of specific receptor subtypes for dopamine and the different regional distributions of these receptors. Many dopamine agonists, such as pramipexole and ropinirole, have been directly implicated in causing daytime sleepiness [Frucht *et al.,*1999; Ondo *et al.,* 2001; Montastruc *et al.,* 2001]. However, other drugs like selegiline and amantadine, that prolong the effect of dopamine, can lead to delayed sleep onset if they are taken in the latter part of the day [Videnovic *et al.,* 2012]. This dichotomy of the effects of DA on sleep could be due to their separate actions on two subtypes of DA receptors: D1-like and D2-like dopamine receptors. Low doses of dopamine may lead to increased sleepiness by acting through D2-like receptors, while high concentrations of DA might be acting through D1-like receptors to increase wakefulness [Jankovic, 2002; Monti *et al.,* 2007]. Alternatively, increased daytime sleepiness might be a characteristic of the disease itself, due to degeneration of other sleep modulating circuits in the lower brain regions [Braak *et al.,* 2002; Hawkes *et al.,* 2010; Knie *et al.,* 2011; Schulte *et al.,* 2011; Videnovic *et al.,* 2012]. However, our data suggests that there is increased daytime sleepiness after low dopamine due to MPTP lesions. This implies either that dopamine, itself, is having effects on sleep or that there are very fast compensatory changes that take place in the sleep-regulating circuits as a consequence to low dopamine. MPTP is also known to cause depletion of other monoamines, like NA [Forno, 1996; Fornai *et al.,* 2007], and serotonin [Perez-Otano *et al.,* 1991; Russ *et al.,* 1991]. A combination of lower brain neuro-modulatory systems, including the NA system, are thought to act by a common pathway to excite serotonin neurons leading to arousal [Brown *et al.,* 2002]. These changes could also partly explain the development of sleep problems that is seen in the MPTP-treated primate model

[Barraud *et al.,* 2009; Verhave *et al.,* 2011] and MPTP-treated mouse models of PD [Monaca *et al.,* 2004; Laloux *et al.,* 2007, 2008; Mc Dowell *et al.,* 2010, 2012].

PD is also associated with an increased incidence of nighttime sleep problems including insomnia, rapid eye movement (REM) sleep behavioral disorder (RBD) and sleep related breathing disorders (SRBD's) [Lees *et al.,* 1988; Arnulf *et al.,* 2000; Schulte *et al.,* 2011]. However, in our low-dose MPTP monkey model we did not observe any changes in nighttime sleep. Interestingly, clinical studies have reported no relationship between nighttime sleep and daytime sleep problems in PD [Rye *et al.,* 1999; Arnulf *et al.,* 2002; Arnulf *et al.,* 2005]. And, although some studies suggest that excessive daytime sleep may precede the diagnosis of PD [Abbott *et al.,* 2005; Gao *et al.,* 2011], nighttime sleep problems tend to occur later in the disease process [Videnovic *et al.,* 2012]. We are using an early stage model of PD, thus it may not be surprising that we see only see changes daytime sleep in this model. In monkey studies using more severe DA lesions nighttime sleep problems have been reported [Barraud *et al.,* 2009].

In this study we were not able to examine sleep during the first two weeks post-MPTP and how changes in daytime sleepiness developed relative to the development of motor impairments because monkeys could not wear collars with accelerometers during the period of time the surgical incision was healing post-MPTP. However, given that we found increased daytime sleep as soon as we were able to measure sleep post-MPTP, and the fact that previous clinical studies have reported that the incidence of longer daytime napping in a nonclinical population was associated with future risk of PD occurrence [Abbott *et al.,* 2005; Gao *et al.,* 2011], we believe that the low dose unilateral MPTP monkey model of PD will be useful for studying whether it is the moderate loss of dopamine that leads to increased daytime sleepiness that precedes the development of motor impairments in PD. To address this issue, monkeys

could wear activity monitors sewn into a pocket in a jacket, rather than housed in a collar, allowing activity monitors to be put back on monkeys immediately after administration of MPTP.

It is important to recognize that sleep was assessed in this study using an indirect measurement of changes in the pattern of motor behavior by actigraphy. Sleep assessment by actigraphy has become relatively common in clinical studies over the last 15 years [Sadeh *et al.,* 1995, 2011; Ancoli-Israel *et al.,* 2003; Herringa *et al.,* 2009]. Although there are drawbacks to measuring sleep by actigraphy rather than by continuous electroencephalography (EEG), such as an inability to discern specific sleep stages, there are also important benefits including that actigraphy is non-invasive and can easily be adapted to home environments [Ancoli-Israel *et al.,* 2003; Sadeh *et al.,* 1995, 2002, 2011]. The same is true for nonhuman primates. Sleep has been studied by EEG in freely-moving monkeys [Almirall *et al.,* 1999; Barraud *et al.,* 2009], however this required surgery to implant electrodes. Actigraphy on the other hand, provides an indirect measure of sleep without any invasive procedure, and thus monitoring the natural state of sleep in monkeys without significant manipulations that may, in of themselves, influence to the sleep behavior [Sadeh *et al.,* 1995, 2002, 2011].

In summary, we show for the first time that a mild reduction in brain dopamine levels induced by a single unilateral MPTP injection is sufficient to cause changes in sleep pattern and lead to excessive daytime sleepiness in monkeys. The neural circuits that underlie excessive daytime sleepiness are currently unknown. Future studies with this animal model can be used to identify the specific neural circuits that underlie arousal and the role of dopamine in these circuits that could lead to this sleep dysfunction. Further this animal model can also be used to test new therapies for daytime sleep disorders.

4.0 EFFECTS OF GDNF, CDNF, N2 AND N4 ON DAYTIME SLEEP IN MPTP-TREATED MONKEYS

4.1 INTRODUCTION

Non-motor symptoms in PD are under-recognized and can be an important cause of reduced quality of life, even more than the motor impairments in PD [reviewed in Knie et al., 2011; Videnovic et al., 2012]. In a recent survey, it was found that in more than 50% of cases of PD there is under-reporting of non-motor symptoms associated with PD and daytime sleepiness was the most frequently undeclared non-motor symptom (52.4% of patients) [Chaudhuri et al., 2010]. An increased incidence of accidents due to increased daytime sleepiness in PD patients has also been reported [Ondo et al., 2001; Frucht et al., 1999]. Many PD patients show evidence of increased daytime sleepiness before the motor symptoms of PD develop [Gjerstad et al., 2006; Dhawan et al., 2006; Iranzo 2011]. And, increased daytime sleepiness has been reported to be predictive of future development of PD [Abbott et al., 2005; Gao et al., 2011].

The treatment and management of increased daytime sleepiness is thus an important therapeutic goal that needs to be addressed by a comprehensive plan in order to improve the

quality of life of PD patients [Pal et al., 1999; Knie et al., 2011]. Coming up with effective treatments requires an understanding of the possible mechanisms underlying increased daytime sleepiness. The mechanisms that lead to hypersomnia during the day is of prime importance to both sleep medicine and basic research as industrial societies continue to look for ways to increase daytime productivity and reduce fatigue [Frucht et al., 1999; Brodsky 2003; Gjerstad et al., 2006; Schulte et al., 2011]. The presence of excessive daytime sleepiness in PD has triggered research to identify the role of DA in regulating sleep and to find effective treatments for it [Jankovic, 2002, see Section 3.4 for more detailed discussion on role of DA in sleep].

There are many wakefulness-promoting drugs currently available for treating daytime sleepiness. These drugs are thought to modulate dopamine release but the exact mechanisms of action are not established. However, most of these drugs have a common feature of increasing extracellular dopamine concentration in structures associated with arousal, like nucleus accumbens [Boutrel et al., 2004; Murillo-Rodríguez et al., 2007; Zolkowska et al., 2009]. Modafinil, a wakefulness-promoting drug, has been shown to increase extracellular dopamine in the nucleus accumbens and increase wakefulness in rats [Murillo-Rodríguez et al., 2007; Zolkowska et al., 2009]. Modafinil was used successfully in the treatment of excessive daytime sleepiness for PD in two small clinical trials [Hogl et al., 2002; Adler et al., 2003; Korczyn, 2006]. However, Modafinil failed in a larger double-blind study to improve daytime sleepiness [Ondo et al., 2005].

A number of secreted proteins and signaling molecules during development (growth hormone releasing hormone (GHRH), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), GDNF, and interleukin-1, (IL-1)) are thought to play a role in sleep regulation [Sassin et al., 1969; Obal et al., 1988; Kerkhofs et al., 1993; Kapas et al., 1996; Faraguna et al.,

2008; Kreuger et al., 1999; Kushikata et al., 2000]. Injection of these (GHRH, NGF, BDNF, GDNF, IL-1) is known to cause an increase of either rapid eye movement sleep or non-rapid eye movement sleep, or both. Many sleep regulatory substances like interleukins are known to enhance GDNF release. GDNF has been shown to regulate sleep in rats and rabbits [Kushikata *et al.*, 2000]. We hypothesized that NTF's may restore problems of sleep and arousal in PD and in animal models of PD. There have been many recent advances in the development and use of NTF's as treatment options for PD [Lindholm et al., 2007; Bjorklund et al., 2009; Marks et al., 2008; Marks et al., 2010; Vastag 2010; Aron et al., 2011]. However, the use of trophic factors to improve sleep problems in both patients and animal models of PD has not been investigated thus far. NRTN belongs to the GDNF family, which has been studied most extensively with regards to a potential therapeutic role in PD (see Chapter 1 for review), and CDNF is a newly discovered trophic factor that has been shown to be more effective than GDNF in rodent models of PD [Lindholm et al., 2007, 2010; Voutilainen et al., 2011]. The experiments described here were designed to test if two variants of NRTN (N2 and N4) and CDNF were effective in restoring normal daytime sleep in a primate model of PD. Reported here, for the first time, is evidence that mutant variants of NRTN, N2 and N4, are effective in restoring some aspects of sleep dysfunction in a monkey model of PD.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Twenty eight female rhesus macaques (*Macaca mulatta*), 16-20 years of age (mean age: 18.3±0.2 years) were housed in social living pens (1.6m x 2.3m x 3.5m) that had multiple perches, toys, and a thick layer of sawdust bedding with overhead skylights that provided natural lighting. In addition to the natural lighting, artificial lighting turned on at 0700 h in the morning and lights turned off at 1900 h at night. For sleep calculations, the start of the daytime was determined as the time of sunrise or 0700 h, depending on which occurred first; similarly the end of daytime was calculated as 1900 h or the time of sunset, depending on which occurred last. This housing facility has 20 pens in a wing, and pens have wire mesh fencing fronts such that monkeys in each pen could see and hear other monkeys in a number of other pens. Monkeys were pair-housed. Monkeys were fed Purina Monkey Chow (#5038; Ralston Purina Co., St. Louis, MO) once daily and given fresh fruit, vegetables, nuts and seeds to encourage foraging, as well as *ad libitum* access to drinking water. All monkeys were observed daily for health and menstrual status. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2.2 MPTP Administration

Prior to MPTP administration, baseline assessments of motor function and sleep were made for approximately a two-week period (13.2±0.5 days) for each monkey. All monkeys then

underwent a surgery to expose the right carotid artery and each received a right intracarotid injection of MPTP-HCl (Sigma Chemical Co., St. Louis MO), at a concentration of 0.14-0.16 mg/kg (average dose=0.15±0.001 mg/kg), delivered at 1 mL/min, using previously published techniques [Ovadia *et al.*, 1995; Gash *et al.*, 1996; Grondin *et al.*, 2002]. After surgery, animals were allowed to recover in quarantine for 4 days to allow for excretion of MPTP. Monkeys did not wear collars with activity monitors for the first two weeks after surgery to allow healing of the surgical incision that was made for the intracarotid injection. Activity was measured from 3-6 weeks post-MPTP administration.

4.2.3 **Experimental design**

Group Size: Monkeys were assigned into six experimental groups (n=4-6/group). At six weeks post-MPTP, when stable symptoms of PD were established, monkeys were assigned to an experimental group, taking care to make sure the distribution of PD-like symptoms was uniform across the six experimental groups. In the treatment groups, each monkey received four intraputamenal infusions of trophic factor/vehicle at monthly intervals (see **Figure 2**). Experimental groups were treated with four monthly injections of vehicle (n=4), GDNF (450 µg; n=5), CDNF (450µg; n=5), CDNF (150 µg; n=6), N2 (337.5 µg; n=6), or N4 (280 µg; n=5). Vehicle, GDNF and CDNF were given in a 225 µL volume and N2 and N4 were given in a 337.5 µL volume of phosphate-buffered saline (vehicle, CDNF, N2, N4) or citrate buffer (GDNF), pH 7.4.

Activity Monitoring: Activity was measured in each animal using an omnidirectional Actical accelerometer (Respironics, Phoenix, AZ) mounted on a loose-fitting collar (Primate Products, Immokalee, FL), using previously published methods [Sullivan *et al.*, 2006; Hunnel *et*

al., 2006; Papailiou *et al.,* 2008; Sullivan *et al.,* 2010]. Monkeys adapted quickly to the collars, which did not interfere with activities such as feeding or sleeping. Activity monitors were set to collect activity counts per minute. Activity was measured for approximately two-week periods in each monkey prior to MPTP (13.2±0.5 days), 3-4 weeks post-MPTP (10.6±0.7 days), and 5-6 weeks post-MPTP administration (11.0±0.4 days).

NTF Administration: Many trophic factors and pharmacological agents have "inverted U" dose response curves, with lower efficacy at both lower and higher concentration levels [Gash *et al.,* 1995, 1996; Zhang *et al.,* 1997], and this was taken into consideration when choosing doses of NTFs to test in this study. The CDNF doses that were utilized for the current study were chosen based on previous work demonstrating that a 450 µg dose of GDNF, delivered intraputamenally, was effective in improving DA function in MPTP-treated monkeys [Ovadia *et al.,* 1995; Gash *et al.,* 1996; Grondin *et al.,* 2002]. We chose to test this same dose of CDNF in monkeys in one experimental group. However, CDNF had been shown to be more effective than GDNF in a rodent model of PD [Lindholm *et al.,* 2007], so there was concern that if the same was true for monkeys we may be on the diminishing slope in an "inverted U" dose response curve. Thus, a lower dose of CDNF was also tested (150 µg). A dose of N2 (337.5 µg) that was the molar equivalent of the 450 µg dose of GDNF, was tested. N4 was less soluble than N2 and hence a 280 µg dose, that could be dissolved in the same volume as the N2 solutions tested was used. Monkeys were sacrificed immediately following the fourth infusion to compare the actual distribution of trophic factors within the brain and also for post infusion biochemical analysis.

4.2.4 **Sleep function assessments**

Analysis parameters for defining actigraphic sleep were developed in a previous study in which sleep was measured by EEG and actigraphy and validated by infra-red videography [Herringa *et al.*, 2009]. Sleep was defined as twelve minutes of continuous zero activity counts. Various sleep parameters were calculated including: nighttime sleep latency (time from lights off to first sleep episode overlapping with or after lights off), number of nighttime awakenings, total night sleep duration, morning wake latency (time from lights on to beginning of first wake episode after or overlapping with lights on), total daytime sleep duration, and number of daytime sleep bouts.

4.2.5 **Assessment of Motor Function**

To confirm previously reported findings that a single, low-dose intracarotid injection of MPTP results in stable mild motor dysfunction [Ovadia *et al.*, 1995, Ding *et al.*, 2008], we assessed monkeys on the primate version of the Unified Parkinson's Disease Rating Scale [the monkey Parkinson's rating scale; Smith *et al.*, 1993, Ovadia *et al.*, 1995] once a week for 4 weeks pre-MPTP and 6 weeks post-MPTP. Motor functions were scored from 0 (normal) to 3 (severe disability) in the following categories: rigidity, bradykinesia, posture, balance, tremor, and hand dexterity. The left side and right side were scored separately for rigidity, bradykinesia and tremor. The maximum score possible was 22. Two independent raters scored each videotaped session and assigned ratings for each video session. If variability between the two independent raters was greater than 15%, a third rater scored the session. Overall, inter-rater reliability was $88.6 \pm 2.9\%$.

4.2.6 **Statistical Analysis**

For all analyses, normality and homogeneity of variance criteria were met, and a paired t-test was used to identify significant changes in each sleep parameter across the post-MPTP and post-infusion three periods. Pearson's correlation coefficients were used to calculate the relationship between latency to wake and the post-mortem DA cell counts. Statistical analyses were performed using MATLAB (The MathWorks Inc., Natick, MA). Values are presented as means ± SEM. $P \leq 0.05$ was considered significant.

4.3 **RESULTS**

After three months of vehicle infusion average sleep duration during the day was 242.95 ± 29.57 minutes and the mean number of daytime sleep bouts was 11.48 ± 0.95 bouts/day, which was not significantly different from the post-MPTP values for the vehicle-treated group (daytime sleep duration: 245.76 ± 34.11 minutes; daytime sleep bouts: 11.21 ± 1.28 bouts/day). There was also no change in the daytime sleepiness after three months of NTF treatment compared to post-MPTP for the groups receiving 450 µg CDNF (Sleep duration: 145.72 ± 19.63 to 121.46 ± 39.18 minutes, p=0.17; daytime sleep bouts: 8.42 ± 1.25 to 6.96 ± 1.94 bouts/day, p=0.13), or 150 µg CDNF (Daytime sleep duration: 186.96 ± 38.57 to 199.93 ± 39.98 minutes, p=0.16; daytime sleep bouts: 9.87 ± 1.77 to 10.18 ± 1.88 bouts/day, p=0.32). For the N2 treatment group again there was a trend for reduction in daytime sleep bouts, but not for daytime sleep duration, from post-MPTP to post-infusions (daytime sleep duration: 197.28 ± 41.81 to 170.89 ± 50.66 minutes,

p=0.25; daytime sleep bouts: 9.61 ± 1.59 to 7.75 ± 1.52 bouts/day, p=0.08). However, the mean latency to wake up in the morning significantly decreased from 13.50 ± 4.80 minutes after lights on to 2.50 ± 5.20 minutes before lights on for the N2 treatment group, p=0.05. Daytime sleep duration decreased significantly after three months of N4 infusion from 272.60 ± 51.10 minutes to 229.81 ± 52.94 minutes (p=0.02 **Figure 18A**). And, daytime sleep bouts showed a trend towards reduction after three months of N4 infusion from 272.60 ± 51.10 minutes to 229.81 ± 52.94 minutes (p=0.06; **Figure 18B**). The mean latency to wake up in the morning also significantly decreased from 16.27 ± 2.26 minutes after lights on to 5.77 ± 6.21 minutes before lights on for the N4 treatment group (p=0.02; **Figure 18C**). For all monkeys, the mean time to wake up in the morning post-infusion three was significantly predictive of the number of dopamine cells present in post-mortem brain tissue (r=-0.479, p=0.005; **Figure 19**).

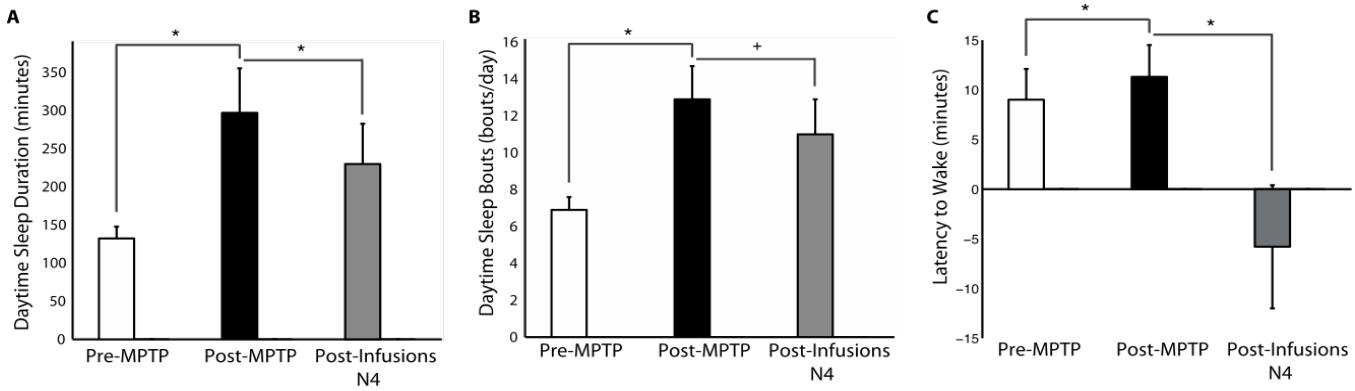


Figure 18. A. Mean daytime sleep duration, **B.** mean daytime sleep bouts, and **C.** mean latency to wake during the pre-MPTP period (open bars) ,post-MPTP (black bars) and post-infusion with N4 (grey bars). Asterisks indicate a significant difference between two time periods, as indicated by horizontal lines ($p<0.05$). A plus sign indicates significant

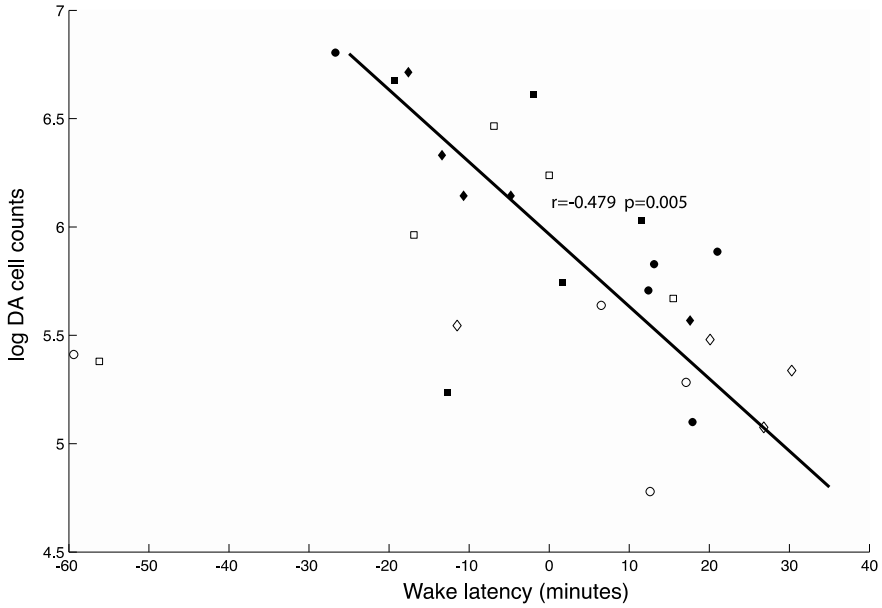


Figure 19. Correlation between, cell counts and latency to wake in the morning. Vehicle (open diamond), 450 μg GDNF (open square), 450 μg CDNF (open circle), 150 μg CDNF (closed circle), 337.5 μg N2 (closed square), 280 μg N4 (closed diamond)

4.4 DISCUSSION

Monkeys that received a single low-dose unilateral MPTP injection had a significant increase in daytime sleepiness (see Chapter 3). After treatment with neurotrophic factor infusions for three months, the daytime sleep duration in monkeys receiving three infusions of N4 decreased significantly. With both N2 and N4 there was a trend for a decrease in number of daytime sleep bouts after 3 months of treatment, and the latency to wake up in the morning decreased significantly with both N2 and N4 treatment after three monthly infusions. Thus, this study shows for the first time in an animal model of PD that NTF's can improve daytime sleep dysfunction.

The improvement in sleep seen after N2 and N4 infusions, but not CDNF and GDNF could reflect the fact that N2 and N4 have the widest distribution within the brain of the trophic factors we tested [Runeberg, Saarma and Penn, unpublished data]. In this study, all of the NTF infusions were given in the putamen, that lies in close proximity to the nucleus accumbens, a structure implicated in arousal mechanisms [Murillo-Rodríguez *et al.*, 2007]. Increased release of dopamine in the nucleus accumbens has been shown to improve wakefulness and arousal levels in rodents [Robbins *et al.*, 1997; Murillo-Rodríguez *et al.*, 2007; Monti *et al.*, 2007]. Planned analyses with the post-mortem brains from this study will look at both the tissue distribution of N2 and N4, as well as measure tissue levels of DA, TH, HVA, and DOPAC in the accumbens. A higher level of DA and DA metabolism, coupled with increased N2 and N4 staining in accumbens compared to other NTF's, would support the hypothesis that NRTN variants reached the accumbens in higher concentration than the other NTFs tested in this dissertation and that dopamine release was increased after N2 and N4 treatment.

The loss of DA-containing neurons from midbrain SNpc to the striatum is well documented after MPTP administration (see Chapter 1.4). These DA neurons receive direct projections from serotonin neurons originating in the dorsal raphe nucleus. SNpc neurons also project back to the dorsal raphe nucleus [Forno, 1996; Fornai *et al.,* 2007; Monti *et al.,* 2007]. The loss of projections from SNpc neurons to the raphe nucleus after MPTP could potentially lead to changes in the activity of serotonin neurons post-MPTP. Several studies have also shown neuronal loss and Lewy body formation in the serotonergic neurons in the dorsal raphe nucleus in PD [Ohama *et al.,* 1976; Huot *et al.,* 2011,2013]. According to the Braak staging of PD, changes to the raphe nucleus occur before DA loss in midbrain and could possibly play a role in the development of sleep problems that precede the presentation of motor symptoms in PD [Braak *et al.,* 2003, 2006; Hawkes *et al.,* 2010]. Serotonin levels in the striatum, and other regions of the brain, are indeed significantly reduced in PD [Kish *et al.,* 2008, Huot *et al.,* 2013]. Similarly, reduced serotonin levels in the striatum of MPTP-treated primates have been reported [Perez-Otano *et al.,* 1991; Russ *et al.,* 1991]. After N2 and N4 infusions, there could be restoration and recovery of function in DA neurons in SNpc that project to and receive projections from serotonergic raphe neurons. This, in turn, could restore the homeostasis of serotonin neurons that are implicated in sleep. Alternatively, N2 and N4 may have diffused far enough to directly provide trophic support to serotonin neurons [Ducray *et al.,* 2006]. Planned analyses of the post-mortem brain tissue collected in this study will measure tissue levels of serotonin and its metabolites, as well as other neurotransmitters including norepinephrine. Improved levels of serotonin correlates strongly with better response to levodopa therapy in PD patients [Huot *et al.,* 2011, 2013]; a similar change observed in these monkeys could provide evidence for improvement of sleep problems through non-dopaminergic pathways.

There is a clear loss of locus coeruleus neurons in PD patients and the loss is nearly as severe as that is seen with DA neurons [Braak *et al.,* 2000, 2003, 2004, 2006; Fornai *et al.,* 2007]. This loss is again thought to precede the DA loss according to the Braak staging of PD. In a recent study, a significant reduction in noradrenaline (NA) within motor thalamic regions in PD patients was reported [Pifl *et al.,* 2012]. These decreased levels of NA in PD patients could play a key role in the origin of sleep and arousal problems in PD. NA plays a role in the regulation of rapid eye movement sleep through central adrenergic receptors and inhibition of NA release is thought to play a role in narcolepsy, a disorder characterized by excessive daytime sleepiness [Brown *et al.,* 2002]. Also, in PD animal models, loss of NA neurons enhanced the parkinsonian symptoms and increased the loss of DA neurons in animal models [Fornai *et al.,* 2007]. Pifl *et al.,* [2012, 2013] also has reported that in monkeys after MPTP treatment only the monkeys that had significant reductions in NA in thalamic regions had parkinsonian symptoms, while the asymptomatic monkeys after MPTP did not have any changes in NA levels in thalamus. Although the reduction of NA levels and the loss of NA neurons in the locus coeruleus has been reported in PD patients, the consequences of this loss has received little attention [reviewed in, Forno, 1996; Fornai *et al.,* 2007]. There are reports that loss of locus coeruleus neurons containing NA can exacerbate the symptoms and motor dysfunction in PD [Rommelfanger *et al.,* 2007; Fornai *et al.,* 2007]. Planned analyses of post-mortem brain tissue will also examine NA levels in the locus coeruleus of the monkeys in the present study.

Some studies have reported loss of hypocretin neurons, that are involved in sleep regulation, in PD patients [Thannical *et al.,* 2007]. Moreover, a combination of orexin/hypocretin system, the NA system and the histamine system might act though a common pathway to excite

serotonin neurons leading to arousal [Brown *et al.*, 2002]. Impairment of this combined neuromodulatory system in PD could lead to an increase in daytime sleepiness.

N4 was generated from NRTN by adding the tail region of PSPN to the NRTN molecule [Runeberg, Saarma and Penn, unpublished data]. PSPN is another trophic factor that does not bind to heparin. N2 and N4 are mutant molecules about which very little is known with respect to the receptors through which they might act or the signaling cascades they might activate. Power analyses after completion of this study indicate that both CDNF and the two variants of the NRTN molecule are likely to have similar effect sizes. Thus, it is possible that N2 and N4, with slightly greater capacity to diffuse to brain regions outside the putamen, were effective in reversing daytime sleep abnormalities that developed post-MPTP, but that CDNF would be effective also if it reached the critical brain regions impacting sleep.

In summary, this is the first report of neurotrophic factor treatment leading to improvement of sleep in an animal model of PD. NTF's present a promising alternative to current treatments, like L-Dopa, to treat both PD-associated motor and non-motor symptoms, like sleep dysfunction. The mechanism through which NTF's act to restore sleep function in neural circuits should be investigated in future experiments in both animal studies and in patients. Moreover, future studies with NTF's should consider their impact on both motor and non-motor symptoms of the disease.

5.0 GENERAL DISCUSSION

5.1 DISTRIBUTION OF NTFS IN BRAIN TISSUE

There has been tremendous progress in the utilization of neurotrophic factors to test their effectiveness for treating PD since the discovery of GDNF and its DA neuron survival-promoting effects [Lin *et al.,* 1993]. The experiments using GDNF fuelled a huge number of investigations into neurotrophic factors, their mechanism of action for neuronal survival, and their role in counteracting age-related deficits that develop in the central nervous system. Other members of the GDNF family of NTFs were later discovered, including neurturin (NTRN) [Kotzbauer *et al.,* 1996]. The degeneration of DA neurons is a hallmark of PD (see Section 1.1). Most GDNF family ligands have the property of promoting DA neuron survival and led to its testing as a treatment for PD [Saarma *et al.,* 2003]. GDNF has been shown to improve PD-like symptoms in animal models of the disease both in rodents [Hoffer *et al.,* 1994; Tomac *et al.,* 1995; Beck *et al.,* 1995; Bowenkamp *et al.,* 1995; Bjorklund *et al.,* 1997] and in primates [Gash *et al.,* 1996; Zhang *et al.,* 1997; Connor *et al.,* 1998]. The beneficial effects of GDNF in animals was found to depend on a number of factors, including the dose used and tissue distribution [Zhang *et al.,* 1997; Grondin *et al.,* 1998]. New techniques for delivery of NTFs to facilitate

distribution of bioactive molecules began to be explored early [Grondin *et al.*, 1998, 2002, 2003].

The issue of poor tissue diffusion of NTF's became more apparent with the first experiments using GDNF in humans [Kordower *et al.*, 1999]. The distribution of GDNF within the brain has been a major roadblock for converting the therapeutic use of NTF's for treating PD from laboratory animals to humans in the clinic because NTF's have much further to diffuse in larger human brains. A number of phase I and phase II clinical trials have since been conducted using GDNF [Nutt *et al.*, 2003; Gill *et al.*, 2003; Patel *et al.*, 2005; Slevin *et al.*, 2005; Lang *et al.*, 2006]. In all these clinical trials, GDNF was not as efficient as when it was used in parkinsonian animal models [Hoffer *et al.*, 1994; Beck *et al.*, 1995; Bowenkamp *et al.*, 1995; Opacka-Juffry *et al.*, 1995; Tomac *et al.*, 1995; Gash *et al.*, 1995,1996; Hou *et al.*, 1996; Martin *et al.*, 1996; Schults *et al.*, 1996; Bjorklund *et al.*, 1997]. One of the main reasons for the poor effectiveness of GDNF in clinical trials was attributed to the poor distribution in the brain parenchyma [Gash *et al.*, 2005; Barker, 2006, 2009; Evans *et al.*, 2008; Deierborg *et al.*, 2008]. Many members of the GDNF family of ligands, including GDNF and NRTN, have been shown to bind with high affinity to heparin and heparan sulphate [Rickard *et al.*, 2003; Rider, 2006]. Heparan sulfate is a ubiquitous acidic polysaccharide that is present in the extracellular matrix and on cell surfaces. It acts as a receptor and regulates a number of biological actions of GDNF and NRTN, including during neuronal development [Saarma *et al.*, 2003]. There is a need for highly localized concentrations of many of these NTF's during development and binding to heparan sulfate could provide a mechanism that ensures a high concentration of NTF's at the site of developing neurons [Rider, 2006]. It has also been shown that the binding of GDNF to heparan sulfate prevents its proteolytic degradation and thus can prolong its action [Piltonen *et*

al., 2009]. Moreover, the heparin binding property of GDNF has been shown to be important in mediating the optimal neuroprotective effect of GDNF; truncated GDNF that does not bind to heparan sulfate and diffuses widely in brain parenchyma, does not have improved efficacy [Piltonen *et al.,* 2009].

Meanwhile, a search also began for a novel trophic factor that does not bind to heparan sulphate and is neurotrophic to DA neurons. CDNF was discovered using a bioinformatics approach [Lindholm *et al.,* 2007]. CDNF was found to be both neuroprotective and neurorestorative in a 6-OHDA rat model of PD [Lindholm et al. 2007]. CDNF distributes to a larger area than GDNF [Voutilainen et al. 2011]. CDNF also was shown to protect the nigrostriatal DA system in a MPTP mouse model [Airavaara *et al.,* 2012]. In this dissertation, CDNF was tested for effectiveness of NTF support to DA neurons in a primate MPTP model of PD [Langston *et al.,* 1984; Bankiewicz *et al.,* 1986; Bergman *et al.,* 1990; Smith *et al.,* 1993; Benazzouz *et al.,* 1993; Ovadia *et al.,* 1995; Gash *et al.,* 1996; Bezard *et al.,* 2001; Emborg, 2007; Bove *et al.,* 2012]. Shown in this dissertation for the first time, CDNF is effective in rescuing DA neurons and motor deficits a primate model of PD. The work undertaken here used two doses of CDNF, based on the previous effective dose of GDNF tested in primates [Zhang et al. 1997; Grondin et al. 1998] and the rodent data that suggested that CDNF was more effective than GDNF [Lindholm et al. 2007; Voutilainen et al. 2011; Airavaara *et al.,* 2012]. The low dose of CDNF (150 μg) was effective in improving the motor symptoms of PD, but not the higher dose of CDNF (450 μg) that was tested. Thus, CDNF appears to follow the classic "inverted u-shape curve" for dose effectiveness (discussed in detail in Section 5.3, 'Optimal dose and dosing regimen'). The low-dose CDNF treatment led to significant improvements in the monkey Parkinson's rating scale score after each infusion. Also, the low-dose CDNF treatment was able

to rescue fine motor function measured using mMAP, such that monkeys that had stopped working using the left hand after MPTP started working again after the low-dose CDNF infusions. With the ability of CDNF to rescue both gross motor and fine motor functions, CDNF has significant potential as a treatment option for PD patients.

More recently, the same group that discovered CDNF developed NRTN variants with point mutations in the site of the molecule that putatively binds to heparin and heparan sulphate, to test the importance of its heparin and heparan sulphate binding motifs in neuroprotection and diffusion within the brain [Runeberg, Saarma and Penn, unpublished data]. Two such NRTN mutants (N2 and N4) were found to be bioactive after mutations and also spread widely in rodent and monkey brain [Runeberg, Saarma and Penn, unpublished data]. N2 and N4 also showed successful protection of DA neurons, and were more potent than GDNF in rescuing dopamine neurons in a 6-OHDA rat model [Runeberg, Saarma and Penn, unpublished data]. Similarly, N2 and N4 also displayed significant improvement of motor performance in the monkey Parkinson's rating scale score. Both N2 and N4 were effective immediately after the first monthly infusion. The monkeys in both these groups continued to remain significantly improved until the end of the study. As shown in rodents [Runeberg, Saarma and Penn, unpublished data], it appears that N4 was more widely distributed in the brain that N2 in primates in a preliminary study [Runeberg, Saarma and Penn, unpublished data]. N4-treated monkeys displayed significant improvement in not just motor function, but also in non-motor functions of sleep and motivation. The improved efficacy of N4 in restoring non-motor functions could be due to the greater tissue distribution resulting from the small point mutations to the NRTN molecule that did not alter the neuroprotective properties of NRTN, whereas in Piltonen *et al.* [2009] a greater portion of the GDNF molecule was deleted, with the GDNF being truncated near this binding motif.

5.2 DELIVERY OF NTF'S TO THE BRAIN

At the same time that GDNF was beginning to be tested as a therapy for PD, new methods of delivery that were less invasive were beginning to be investigated, as there was concern that patients shouldn't have to undergo multiple surgeries to receive injections of NTF's [Barker, 2006, 2009]. Several novel delivery methods emerged and were tested for the delivery of bioactive compounds to the brain, including the use of catheters to pump NTF's continuously into brain tissue [Grondin *et al.,* 1998, 2002, 2003], and the use of viral-mediated gene transfer to provide a continuous supply of NTF [Bilang-Bleuel *et al.,* 1997; Lapchak *et al.,* 1997; Bensadoun *et al.,* 2000; Rosenblad *et al.,* 2000; Connor *et al.,* 2001; Kordower *et al.,* 2003]. However, development of an effective catheter system has eluded scientists so far, as all catheters tried to date form glial scars at the port of entry and thereby reduce the tissue distribution of NTF's [Deierborg *et al.,* 2008; Bjorklund *et al.,* 2009].

Retroviral-mediated gene transfer as a method of delivery was developed in the late 1980's and early 1990's and progressed from lab animals to treatment in clinics within a short span of time for use in human gene therapy trials for a number of neurological diseases [Mann *et al.,* 1983; Anderson, 1992] and was safely used *in vivo* for delivery of NTFs [Bilang-Bleuel *et al.,* 1997; Lapchak *et al.,* 1997; Bensadoun *et al.,* 2000; Rosenblad *et al.,* 2000; Connor *et al.,* 2001; Kordower *et al.,* 2003]. An Adeno-associated virus type-2 (AAV2) vector that carries a gene encoding a modified form of human NRTN, called CERE-120, was successfully developed by Ceregene Inc. and used in both rat and monkey models of PD to successfully preserve nigral

dopamine neuron loss in these animal models [Kordower *et al.,* 2006; Gasmi *et al.,* 2007; Herzog *et al.,* 2007; Herzog *et al.,* 2008; Herzog *et al.,* 2009]. Following the path of GDNF, CERE-120 was tested successfully in a small Phase I clinical trial [Marks *et al.,* 2008]. Then in larger Phase II clinical trial testing PD patients treated with CERE-120 failed to show significant improvement in parkinsonian symptoms [Marks *et al.,* 2008, 2010]. Post-mortem data from patients who died of other complications during the trial showed poor diffusion of the trophic factor in the brain parenchyma [Marks *et al.,* 2010; Vastag, 2010; Ceregene press release 2013; MJFF press release, 2013].

Thus, even using a viral vector method of delivery does not guarantee the successful diffusion of the trophic factor into the target region, as was expected. The reason for this poor diffusion using viral vector-mediated delivery could be because of the binding of AAV type-2 virus, itself, to heparan sulphate on cell surface, as it is also a viral receptor site [Summerford *et al.,* 1998]. Hybrid recombinant AAV (rAAV) is one of the many viruses that are currently available as vectors for gene transfer. Many other enveloped viruses (retrovirus, Lentivirus, herpes simplex virus: HSV-1) and non-enveloped viruses (Adenoviruses) are also used for treatment using gene therapy [Thomas *et al.,* 2003]. The binding of these viruses to extracellular matrix and their ability to safely infect and replicate only in the neurons of interest, without leading to oncogensis, in a wide array of tissues should be investigated further. There are a lot of challenges associated with using gene therapy including humoral immunity in the host that develops antibodies against the virus particles; safety issues also need to be addressed along with ethical and social implications of gene therapy [Thomas *et al.,* 2003; Masat *et al.,* 2013].

5.3 OPTIMAL NTF DOSE AND DOSING REGIMEN

Another important factor that needs to be taken into account to optimize efficacy of NTF's is the dose of the NTF used. In a unilateral MPTP-treated monkey model, GDNF was shown to have a "inverted U-shaped" response curve, with maximum effectiveness in the middle of the dosing range (~300 μg) [Zhang *et al.,* 1997]. Similarly, it was shown for GDNF that increasing the dose did not increase the efficacy of the NTF after a critical threshold for being effective was achieved [Gash *et al.,* 2005]. In this dissertation, again using the same animal model, CDNF was found to be less effective at the higher dose tested (450 μg) compared to the lower dose (150 μg). For this dissertation, the GDNF dose that was chosen had been found to be optimally effective in the unilateral MPTP-treated monkeys in a number of previous studies from the Gash lab [Gash *et al.,* 1995, 1996, 2005; Zhang *et al.,* 1997; Grondin *et al.,* 1998, 2002, 2003]. In order to compare the efficacy of CDNF, a trophic factor that had not been previously tested in primates, we used the same dose that was optimal for GDNF (450 μg). However, in a number of studies using a rodent PD model it had been shown that CDNF was more effective than GDNF at the same dose [Lindholm *et al.,* 2007, 2010; Voutilainen *et al.,* 2011; Airavaara *et al.,* 2012]. Taking this finding from rodents into account, and worrying that there may also be an "inverted U-shaped dose response curve" for CDNF, we also tested a lower dose of CDNF (150 μg) in monkeys. The studies in this dissertation show that the lower dose of CDNF (150 μg) significantly improved parkinsonian motor symptoms, while the high dose (450 μg) did not cause a significant improvement of motor symptoms.

Treatment with the 150 μg dose of CDNF, at monthly intervals, led to continued improvement in motor function measured by the monkey Parkinson's rating scale with each monthly dose of CDNF. It is possible that the time-course of improvement using low-dose CDNF may last for a longer duration than one month [Cohen *et al.,* 2011]. Hence, subsequent infusions at monthly intervals might have had additive effects so that the effectiveness of CDNF was greater after the second and third monthly CDNF infusions because a higher dose of CDNF was available in the brain. A detailed dose response curve for the effectiveness of CDNF to recover PD symptoms in rodent models of PD would be useful before further studies in nonhuman primates or humans are undertaken. A time-course analysis of the benefits after a single CDNF infusion by following the progression of improvement in a PD model would provide insight into the duration of effectiveness and the mechanisms behind the improvement seen with this NTF [Cohen *et al.,* 2011].

The planned dose for the two NRTN mutants tested (i.e., N2 and N4) was 337.5 μg. This dose was calculated for N2 as the molar equivalent of 450 μg of GDNF, and because N2 is less soluble than GDNF it was diluted in 1 μg/μl for a total of 337.5 μl infusate. N4 was even less soluble and it required 406.6 μl to dilute 337.5 μg. However, concern over infusing a significantly higher volume into the putamen led to a choice of a lower dose of N4 (280 μg) that could be prepared to be the volumetric equivalent of the N2 infusions. Surprisingly, the N4 dose (280 μg) was more effective in recovering the non-motor symptoms of PD that occurred in the MPTP model than the higher dose of N2 (337.5 μg). In contrast to CDNF, the N2- and N4-treated monkeys significantly improved motor function after the first infusion but did not show continued improvements in motor function with subsequent infusions. The N2- and N4-treated animals appeared to have reached a ceiling effect for motor improvement immediately after the

first infusion. However, this was not the case for some non-motor measures, where N4 infusions showed a progressive improvement in the latency to wake measurements after each infusion. On the other hand, for daytime sleep duration and number of daytime sleep bouts, the effects of monthly N4 infusions were variable. It would be useful for future studies in rodents using N4 to delineate a detailed dose response curve for both the motor and non-motor symptoms. It may be that the effects of N4 on latency to wake and daytime sleep might take a longer time to become apparent and stable, in contrast to the effects of N4 on motor function.

5.4 STRATEGIES FOR DEVELOPING THE MOST EFFECTIVE NTF THERAPIES

The intracellular mechanisms through which the NTF's improve function and restore the health of DA neurons is still a work in progress [Grondin et al., 1998; Airaksinen et al., 1999, 2002; Saarma et al., 1999, 2000; Sariola et al., 1999, 2003; Takahashi et al., 2001]. This is especially true for more recently discovered neurotrophic factors like CDNF, as even the receptors through which they might act are not clearly understood at this time [Lindholm *et al.,* 2007, 2008, 2010]. During the process of development, NTF's play various specific and critical roles in supporting growth, sustenance, axon guidance to targets, pruning of synapses and other functions [Saarma *et al.,* 1999, 2000; Takahashi *et al.,* 2001]. GDNF has been found to play an important role in the migration, proliferation and outgrowth of neurites from neurons in the central and peripheral nervous system. NRTN is another member of the GDNF family of neurotrophic factors and it plays an important role in target innervation, branching and terminal

formation, following axon guidance paths, and increasing cell size (trophic to the cells). CDNF is a specifically trophic to DA neurons and has been shown to be both neuroprotective and neurorestorative in a rodent PD model [Lindholm *et al.,* 2007, 2008, 2010]. CDNF is thought to play a role in protecting DA neurons against ER stress-induced cell death and facilitating protein folding in the ER. A simple combination of these trophic factors could be used to test the joint efficacy of these trophic factors in restoring function in PD. An advantage of using this combination approach is to target different aspects of this disease to produce the most beneficial outcome. A combination of NTF's could increase the benefit by rescuing neurons that are in advanced stage of degeneration by preventing or reversing the ER stress response [Palgi *et al.,* 2009, 2012; Lindholm *et al.,* 2007, 2010], while also inducing sprouting and providing trophic support to the DA terminals that are still healthy [Zigmond *et al.,* 1990, 1997, 2003; Sariola *et al.,* 1999, 2003].

The GDNF family of NTF's mediate signaling through a common receptor tyrosine kinase RET (rearranged during transformation). The four main members of the GDNF family: GDNF, NRTN, Artemin (ARTN) and Persephin (PSPN) also bind with functional specificity to four identified GDNF family receptors (GFRα 1-4) [Sariola *et al.,* 1999,2003]. Both ligand-dependent and ligand-independent RET signaling pathways have been identified. RET induces a pro-apoptotic signal dependent on caspase activation, and this is blocked when a ligand binds to RET or there is a complex formed between GFR and RET in the absence of ligands [Saarma *et al.,* 1999, 2000, Takahashi, 2001]. There has been a lot of work done *in vitro* to understand the signaling cascades through which GDNF family members mediate trophic action. In contrast, there is very little that is known about the intracellular mechanism of action for CDNF except that its binding affinity is at least an order of magnitude higher than that of GDNF binding to its

receptor [Voutilainen *et al.*, 2011]. Recently, the structure of CDNF was solved [Parkash *et al.*, 2009] and this provided insight into the possible mechanisms through which it could be signaling. At the amino terminal end of CDNF a saposin-like domain is present and saposins are trophic to cultured neurons [Parkash *et al.*, 2009; Voutilainen *et al.*, 2011]. At the carboxy-terminal end it is similar to MANF that is protective again ER-stress induced cell death. Thus, CDNF might be acting through functional domains providing protection through two distinct activities. A combination strategy, combining NTFs such as CDNF and N4, would be similar to the strategies that are now being commonly used to treat other diseases, such as cancer, where multiple drugs are used to target different aspects of the same disease to produce the most effective treatment regimen [Thompson *et al.,* 1995]. This concept of combining therapies that work on different intracellular mechanisms has received little attention in the field of neurodegenerative disorders, but deserves more attention in the future.

Another strategy that may be useful for improving effectiveness of NTF treatment would be to follow the treatment regimen for growth factor infusions that mimic their endogenous pattern of secretion during development [Saarma *et al.,* 1999, 2000; Sariola *et al.,* 1999, 2003]. During normal development there is initially an increase in concentration of GDNF signaling leading to sprouting and new neurites being formed during the embryonic stage of development [Airaksinen *et al.,* 1999, 2002]. This is followed by a sequential increase in NRTN signaling during the perinatal period that plays a crucial role in target innervation through axon guidance and terminal formation [Grondin et al., 1998; Takahashi, 2001; Airaksinen *et al.,* 1999, 2002]. This shift in pattern of signaling is also present in the enteric nervous system suggesting a common evolutionary conservation of this developmental mechanism. Thus, in order to restore the health of degenerating neurons in PD it may be necessary to use a sequential combination of

trophic factors Future experiments in animal models should consider taking into account this multifactor approach to restore function.

5.5 THE EFFECTIVENESS OF TREATING NON-MOTOR SYMPTOMS OF PD WITH NTF'S

Non-motor symptoms in PD have been described from the very beginning of the description of the disease (see review in Chapter 1) [Parkinson, 1817]. However, considerably less attention has been paid to understanding more fully the non-motor symptoms and only recently has it begun to be accepted that other non-motor systems could also be a part of this multi-factorial disease [Braak *et al.,* 2000, 2003, 2006; Hawkes *et al.,* 2010].

This dissertation reports for the first time that a low dose unilateral MPTP monkey model of PD has the non-motor symptom of daytime sleep dysfunction that can be recovered using the NTF, N4 (see Chapters 3, 4). Thus, this dissertation shows that using an unilateral low dose of MPTP that leads to a moderate lesion of dopamine neurons in the nigrostriatal pathway is sufficient to cause an imbalance in DA concentration that leads to non-motor problems like sleep. It has been known for a long time that growth factors including GHRH, NGF, BDNF and GDNF are involved in sleep regulation [Sassin *et al.,* 1969; Obal *et al.,* 1988; Kerkhofs *et al.,* 1993; Kapas *et al.,* 1996; Faraguna *et al.,* 2008; Kreuger *et al.,* 1999; Kushikata *et al.,* 2000]. However, there has been a paucity of research to test the benefits of these factors in treating sleep disorders, such as those that occur with PD. The successful use of

NTFs to treat the sleep problems associated with PD would open a wide array of treatment possibilities for sleep disorders, that would not be just limited to the sleep problems seen in PD.

5.6 FINAL CONCLUSIONS

This dissertation explored the therapeutic effectiveness of CDNF, a novel recently discovered neurotrophic factor, and two variants of the NRTN molecule (i.e., N2 and N4), to rescue nigrostriatal DA neurons and treat PD-like motor and non-motor symptoms in a low-dose MPTP unilateral non-human primate model. All three trophic factors were successful in rescuing nigrostriatal DA neurons (Chapter 2), as well as the PD-like motor symptoms measured using a monkey version of the Parkinson's rating scale (Chapter 2). We also found that there was strong dose dependence in the effects of treatment using CDNF. The lower dose of CDNF that was tested (150 μg) was effective in improving parkinsonian symptoms, but not the higher dose (450 μg) that was the molar equivalent of a known effective dose of GDNF in this same model [Zhang *et al.*, 1997; Gash *et al.*, 2005]. This suggests that there is 'an inverted U-shaped dose response curve' for the neurorestorative effects of CDNF. Gross motor functions measured by the rating scale, accelerometers and Ethovision assessment of whole body movement were all significantly correlated with the post-mortem DA cell counts ($p<0.001$, $p=0.05$, $p=0.009$, respectively). Fine motor function measured using mMAP and scoring of naturally-occurring fine motor movement in the homepen also correlated with DA cell counts ($p=0.002$ and $p=0.06$, respectively). However, with group sizes of 4-6 animals/group, CDNF, N2 and N4 did not lead to significant improvement in specific measures of gross and fine motor movement. Power analyses predict

that for the low-dose CDNF group the differences would become significant with a sample size of 8-10 monkeys.

This dissertation also presents evidence that increased daytime sleep is rapidly evident in a low-dose unilateral MPTP non-human primate model of PD (Chapter 3). As increased daytime sleepiness is a symptom that becomes apparent early in the preclinical stage of PD [Hawkes *et al.,* 2010], and is in fact a predictor of later onset of PD [Abbott *et al.,* 2005; Gao *et al.,* 2011], this finding suggests that the low-dose MPTP non-human primate model will be useful for understanding early aspects of PD. Some aspects of increased daytime sleepiness (i.e., longer latency to wake in the morning) were recovered after treatment with the NRTN variants, N2 and N4 (Chapter 4). Further evidence of a therapeutic effect of N2 and N4 on daytime sleepiness is offered by the finding that there were significant correlations between DA cell count and daytime sleep duration, number of daytime sleep bouts and latency to wake. Moreover, the N2 and N4 treatments led to a significant shortening in latency to wake. These findings offer strong support for the conclusion that N2 and N4 have significant potential to serve as new tools for the treatment of increased daytime sleepiness in PD.

In conclusion, the research presented in this dissertation shows that these novel neurotrophic factors have significant potential for treating PD early in the disease process by either halting or slowing the progression of DA neuronal loss, motor and sleep problems inherent in this disease. This is the first report of daytime sleep dysfunctions occurring early in the process of DA neuronal degeneration and improvement of PD-associated sleep problems after NTF therapy. Future experiments should address both the mechanisms of action of these novel NTFs, as well as modes of NTF treatment that optimally recover functions in both the motor and non-motor domains of this complex disease that affects multiple systems.

APPENDIX A

EFFECTS OF UNILATERAL MPTP INJETCIONS ON MOTIVATION AND THE EFFECTS OF MONTHLY TROPHIC FACTOR INJECTION OF CDNF, N2 AND N4

A.1 INTRODUCTION

Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by the progressive loss of dopamine (DA) neurons in the substantia nigra (SN) and their projections to the striatum. PD is clinically characterized by the presence of resting tremor, bradykinesia, rigidity, and postural imbalance (for details see Section 1.3). Despite the clinical diagnosis of these motor symptoms, many non-motor symptoms (NMS), including sleep disturbances, cognitive decline, greater apathy or loss of motivation, depressive symptoms, and loss of smell, among others, co-occur or even precede the onset of the motor symptoms [Pfeiffer, 2007; Bayulkem *et al.,* 2010; Korczyn *et al.,* 2010; Ferrer *et al.,* 2012]. PD patients most regularly cite the NMS, especially reduced motivation, sleep and other depressive symptoms, as most disruptive to quality of life [McDowell *et al.,* 2012; Videnovic *et al.,* 2012] and yet, animal models of PD routinely only focus on the motor symptoms of the disease.

The nonhuman primate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD has been studied as a reliable model of the motor symptoms of PD for more than two

decades [Bankiewicz *et al.,* 1986; Przedborski *et al.*, 2001]. Although the monkey MPTP model of PD does have its limitations it remains the "gold standard for preclinical testing" of PD therapies [Tieu *et al.,* 2011; Porras *et al.,* 2012]. The monkey MPTP model reliably produces a stable lesion of striatal DA neurons and concomitant motor deficits observed in PD [Bankiewicz *et al.,* 1986; Ding *et al.,* 2008; Gash *et al.,* 1996]. Like PD patients, monkeys given MPTP respond to typical anti-parkinsonism drugs and exhibit the same motor complications that result from their long-term use [Ding *et al.,* 2008]. Thus, the monkey MPTP model of PD best suited to answer the question of NMS and the treatments for the same using trophic factors.

Despite the ubiquity of the monkey MPTP model of PD for studying the motor symptoms and associated pathologies, only a few studies have examined the NMS in this model. These studies have focused on cognitive decline [Taylor *et al.,* 1990; Schneider *et al.,* 1993; Vezoli *et al.,* 2011] or sleep [Barraud et al., 2009; Verhave *et al.,* 2011; Vezoli *et al.,* 2011], but only one study [Brown et al., 2012] to date has focused on reduced motivation in the monkey model of PD.

We use a reliable model [Bankiewicz *et al.,* 1986; Ding *et al.,* 2008] of objectively measured motivation in the rhesus monkey model of PD. The Progressive Ratio (PR) task that is a "gold standard" for assessing motivation was used to objectively measure motivation [Arnold *et al.,* 1997; Paterson *et al.,* 2003; Roane, 2008; Zhang *et al.,* 2003]. This is a task used to assess motivation to seek a drug in a number of addiction as well as other studies. The objective measures of motivation that were tested in this study are, namely, the number of times a freely behaving monkey refuses to participate (i.e., number of balks) in both motor tasks and non-motor tasks.

Reduced motivation was assessed in monkeys treated with a single, unilateral right intracarotid dose of MPTP. The monkeys were then tested for the recovery of this loss of motivation after three monthly trophic factor infusions.

A.2 MATERIALS AND METHODS

A.2.1 Animals

Twenty-six female rhesus monkeys (15-20yrs, 5-8 kg) living in social pens (4m x 4m x 4.7m) that had perches, toys, and a thick layer of sawdust bedding were used in this study. Monkeys were pair housed, and could see and hear other monkeys in several other pens. Monkeys were fed Purina Monkey Chow (#5038; Ralston Purina Co., St. Louis, MO) once daily and given fruit/vegetables, seeds, and nuts daily to encourage foraging. Monkeys had ad libitum access to drinking water. Monkeys were observed multiple times daily for health and menstrual status. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

A.2.2 MPTP administration

Monkeys had baseline assessments for motor function and motivation made for 6 weeks before they received MPTP, as described below. All monkeys then underwent a surgery to expose the

right carotid artery and each received a right intracarotid injection of MPTP-HCl (Sigma Chemical Co., St. Louis MO), at a dose of 0.14-0.16 mg/kg (average dose = 0.15±0.001 mg/kg), delivered at 1 mL/min, using previously published techniques (Ding et al., 2008). Monkeys were then allowed to recover in quarantine for 3 days to allow for excretion of MPTP and they were subsequently moved back into the pen living environment. Starting 10.7±0.7 days and 20.04±1.41 days after MPTP administration, assessments of motor function and motivation, respectively, were continued for an additional four weeks.

A.2.3 Experimental design

Group Size: Monkeys were assigned into six groups (n=6/group). At six weeks post-MPTP, when stable symptoms of PD are established, monkeys were assigned in each group such that the distribution of PD-like symptoms was uniform and had homogeneity across these six groups. Each monkey received four intraputamenal infusions of trophic factor/vehicle at monthly intervals (see, **Figure 2**). Groups were treated as follows: Group 1: MPTP + vehicle; Group 2: MPTP + CDNF (450µg); Group 3: MPTP + CDNF (150 µg); Group 4: MPTP + GDNF (450 µg)); Group 5: MPTP + N2 (280 µg)); Group 6: MPTP + N4 (280 µg)

Neurotrophic Factor Dosing: Many trophic factors and pharmacological agents have "inverted U" dose response curves with lower efficacy at both lower and higher concentration levels [Gash *et al.*, 1995, 1996; Zhang *et al.*, 1997], which was taken into consideration when considering doses of NTFs to test in this study. The CDNF doses that were utilized for the current study were chosen based on previous work demonstrating that a 450 µg dose of GDNF, delivered intraputamenally, was effective in improving DA function in MPTP-treated monkeys

[Ovadia *et al.,* 1995; Gash *et al.,* 1996; Grondin *et al.,* 2002]. We chose to test this same dose of CDNF in monkeys in one group. However, CDNF had been shown to be more effective than GDNF in a rodent model of PD [Lindholm et al. 2007], so there was concern that if the same was true for monkeys as we may be on the diminishing slope in an "inverted U" dose response curve. Thus, a lower dose of CDNF was also tested (150 µg). A molar equivalent dose of N2 (337.5 µg), of the 450 µg dose of GDNF, was tested. N4 was less soluble than N2 and hence a 280 µg that corresponds to the same volume as that of the N2 dose that was administered was used.

A.2.4 Motivation assessments

Motivation was assessed in three ways: 1) by performance on a Progressive Ratio (PR) task, 2) by performance on a cognitive task of object recognition, and 3) during performance on a motor assessment task (the mMAP test).

Progressive Ratio (PR) task: The PR and object recognition tasks were performed using the Wisconsin General Testing Appartus [WGTA; Meunier et al., 1996] and previously published techniques [Rhyu et al., 2010]. Monkeys were transferred to a testing room and placed in a cage to which the WGTA was attached, with a solid sliding access door separating the monkey from the WGTA test tray. Monkeys were allowed to adapt to the testing cage for at least 1 week prior to the onset of testing. Monkeys were trained to move aside a toy that completely covered a treat in a well on a tray in front of them each time the sliding door was raised.

After training, monkeys then received PR testing. The PR test is commonly used to assess motivation in animal models of drug addition [Arnold *et al.,* 1997; Paterson *et al.,* 2003; Roane, 2008; Zhang *et al.,* 2003]. We based our PR task on previously published procedures in

monkeys [Cilia *et al.*, 2001], modifying it for use with the WGTA. During PR testing monkeys were required to move a toy aside to retrieve a treat and in each trial they had to move the toy progressively more times to retrieve the reward. For example, on the first trial, the monkey had to move the toy aside once after the screen was raised, and a treat was present under the toy. On the second trial, the monkey had to move aside the toy the first time the screen was raised (but no reward was present under the toy), the screen was briefly lowered and the toy was replaced with a treat under it, and the screen was raised a second time allowing the monkey to push aside the toy a second time to retrieve the treat. On the third trial, the treat was given the third time the screen was raised, and this pattern progressed with each trial. Monkeys were given 60 seconds to respond to each toy presentation. If a monkey did not respond within 60 seconds at any time, PR testing ceased for the day. The PR task was given for 3 days, and the number of trials a monkey completed each day was recorded. For each monkey a break point (BP), i.e, the total number of completed trials before the monkey refused to work, was calculated for each of the 3 days, and was averaged across the 3 days to yield a mean BP [Cilia *et al.*, 2001]. This 3-day test was given at 29.81$\pm$2.42 days pre-MPTP and at 20.04$\pm$1.41 days post-MPTP.

Object discrimination Task: Object discrimination testing was started the day after PR testing was completed using previously described methods [Rhyu *et al.*, 2010]. Briefly, two easily discernable objects were placed over the lateral wells of the testing tray and the position of the objects varied from trial to trial according to a random sequence. The monkey's access door was lowered, and the experimenter placed a treat under the designated object. The access door was raised and the monkey could retrieve the treat by displacing the designated object. Monkeys were tested until they reached 90% criterion (18/20 trials correct object displacement in a single testing session) with the designated object. Assessments of motivation during the object

discrimination task were made by recording each animal's balk rate for each day of testing: that is, the percentage of trials in a day that the monkey refused to work (monkeys were allowed 60 sec to move the toy in each trial). Testing ended for the day if a monkey balked for 5 trials in a row. A mean balk rate was calculated across all testing days in each phase of the experiment (i.e., pre-MPTP, post-MPTP and post-Infusions).

Assessment of motivation during a motor function task: To confirm previously reported findings that a single, low-dose intracarotid injection of MPTP results in stable mild motor dysfunction [Ding *et al.,* 2008], we assessed monkeys on the primate version of the Unified Parkinson's Disease Rating Scale [Ovadia *et al.,* 1995] once a week for 4 weeks pre-MPTP and 6 weeks post-MPTP. Monkeys were rated on bradykinesia, rigidity, tremor, balance, and posture during a 5-min, videotaped session in which seeds, nuts, raisins and other small treats were thrown into the sawdust bedding of their home pen. Two independent raters scored each videotaped session and assigned ratings. If variability was greater than 15%, a third rater scored the session. Inter-rater reliability of greater than 90% agreement was achieved.

Fine motor function of hands was measured throughout the study using the monkey Motor Assessment Panel (mMAP), an apparatus that has been shown to accurately assess motor function in both human and non-human primates [Gash *et al.,* 1999; Maswood *et al.,* 2002] as well as age-related motor decline in non-human primates [Zhang *et al.,* 2000]. mMAP testing was performed from two to six weeks pre-MPTP (Mean=3.0±0.2 weeks pre-MPTP) and for 5 weeks post-MPTP (starting 10.7±0.7 days post-MPTP). Monkeys were transferred from their home pen to a testing room and placed in a testing cage with the mMAP apparatus attached to the front. Monkeys had been acclimated to the testing cage for at least 1 week prior to the onset of testing. Each day of mMAP testing consisted of 12 trials total, 6 trials for each hand. This

allowed us to compare motor function between the left hand (i.e., the side that would be affected by right intracarotid MPTP) and the right hand (i.e., the unaffected side). During mMAP testing, monkeys were required to reach through two openings to retrieve a small food reward on an elevated platform [Gash *et al.,* 1999]. The openings were each equipped with photodiodes to monitor arm/hand movements of the subject, thereby recording with millisecond accuracy the latency to retrieve the food reward [Gash et al., 1999]. Monkeys were given 5 minutes to respond per trial before the trial ended. At each trial the latency to retrieve the treat was recorded. If the monkey did not retrieve a treat at the end of 5 minutes the trial was scored as a balk. The trials alternated between the right and left hands. The testing was stopped if the monkey did not retrieve the treat for three consecutive trials. The assessment of motivation in this task was by calculating the balk rate in the mMAP task. For these calculations mean balk rate across all sessions of mMAP testing was calculated pre-MPTP, post-MPTP and post-Infusions for each monkey.

Statistical Analysis: Prior to analyses, all dependent variables (monkey Parkinson's rating scale, mean BP, mean balks during object discrimination testing, and mean balks during mMAP testing both pre- and post-MPTP) were examined for normality and homoscedacity. As no monkeys showed impairments in the monkey Parkinson's rating scale during the baseline (pre-MPTP) period, all monkeys received a score of 0 (lowest possible score) for the baseline value, as has been reported in previous studies with monkeys using this experimental model [Brown et al., 2012]. Rating Scale data collected pre-MPTP was not used in the analyses in this study. The post-MPTP rating scale data were normally distributed. Pre- and post-MPTP BP values were normalized using a reciprocal root transformation ($-1/\sqrt{Y}$). Pre- and post-WGTA balk rate values were normalized using a square root transformation ($\sqrt{Y}$). Paired-samples t-tests were

then used to analyze the changes in BP and WGTA balk rate from pre- to post-MPTP. The pre- and post-MPTP mMAP balk data could not be normalized using standard methods, so the nonparametric Wilcoxon signed rank test was used to analyze these data from pre- to post-MPTP. Spearman correlations were employed to examine the relationship between post-MPTP BP and balks during object discrimination and mMAP testing, as well as the relationship between these measures of motivation and loss of motor function as assessed by post-MPTP monkey Parkinson's rating scale scores. Data are reported as mean±SEM. SPSS v21.0 was used for all analyses, and $p < 0.05$ was considered significant.

A.3 RESULTS

A.3.1 MPTP reduces both motor function and motivation

Compared to baseline, monkeys showed a significant decline in motor function across the 6 weeks post-MPTP as evidenced by poorer scores on the monkey Parkinson's rating scale ($t(25)=-13.30$, $p<0.001$). Monkeys also exhibited impairment in their left hand during mMAP testing post-MPTP, as evidenced by a significant increase in balk rate with this hand (pre-MPTP: 2.08±0.66%, post-MPTP: 69.81±6.92%, =4.23, $p<0.001$). Balking with the left hand was significantly increased compared to balking with the right hand post-MPTP ($t(25)=7.44$, $p<0.001$). However, monkeys also exhibited a significant increase in balks post-MPTP during mMAP testing in the right hand ($Z=3.01$, $p=0.003$; **Figure 20**).

Monkeys showed a significant decrease in motivation as assessed by BP from pre- to post-MPTP ($t(25)=3.055$, $p=0.005$), averaging 3.22+0.39 trials pre-MPTP and 2.60+0.39 trials post-MPTP (**Figure 20**). However, balk rate during object discrimination testing was not significantly increased post-MPTP ($t(24)=0.109$, $p=0.914$). Nevertheless, measures of motivation were consistent across tasks (Table 1), such that monkeys exhibiting a lower post-MPTP BP (i.e., less motivation) also exhibited more balks during object discrimination testing (i.e., less motivation; $r(s)=-0.681$, $p<0.001$) as well as more balks with the right hand during mMAP testing (i.e., less motivation; $r(s)=-0.599$, $p=0.001$). Additionally, monkeys who balked more during post-MPTP object discrimination testing also balked more with their right hands during post-MPTP mMAP testing ($r(s)=0.693$, $p<0.001$).

A.3.2 Decline in motivation predicts motor dysfunction after MPTP

Balk rate during post-MPTP mMAP testing positively predicted the post-MPTP score on the rating scale ($r(s)=0.559$, $p=0.003$; **Figure** 2**1**; **Table 1**), such that monkeys exhibiting reduced motivation after MPTP during mMAP testing (higher balk rate) also had higher scores (more impairment) on the monkey Parkinson's rating scale. BP during the post-MPTP testing period negatively predicted the post-MPTP score on the monkey Parkinson's rating scale ($r(s)=-0.442$, $p=0.024$; **Figure** 2**1**; **Table 1**) such that monkeys exhibiting reduced motivation after MPTP during PR testing (lower BP) also had higher scores (more impairment) on the rating scale.

A.3.3 Effect of CDNF, N2 and N4 on motivation after MPTP administration

In the 150 μg CDNF group there was significant improvement in motivation to work with the left affected hand as assessed by balk rate from post-MPTP to post-Infusions from 79.2±14.0 to 29.0±18.0 percent (p=0.02). Similarly the right hand balk rate which is a direct measurement of the monkey's motivation in the MPTP unaffected right hand was significantly reduced from 7.2±3.0 to 0.5±0.5 percent balking rate from post-MPTP to after three months of 150 μg CDNF trophic factor infusions (p=0.04). There was no significant change in any of the other treatment groups in these measures.

Compared to post-MPTP values in the BP during progressive ratio testing there was an increase in motivation indicated by increased BP values at the end of three monthly infusions in the treatment groups: monkeys in the 450 μg CDNF changed their BP from 1.7±0.3 post-MPTP to 3.1±0.4 post-infusions (p=0.02), 150 μg CDNF changed from 2.5±0.6 post-MPTP to 4.1±0.7 post-infusions (p=0.10), N4 changed from 1.8±0.5 post-MPTP to 4.5±1.1 post-infusions (p=0.04). There were no changes in the other treatment groups in this measure and no other measures of motivation had any significant changes in their values after infusions with trophic factors.

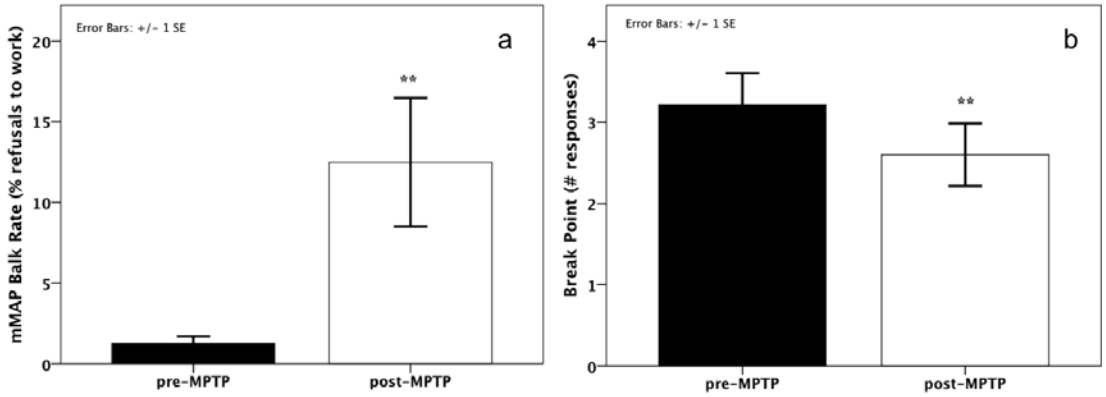


Figure 20. Monkeys showed reduced motivation after MPTP as evidenced by (a) increased balk rate with the right hand during mMAP testing, and (b) reduced Break Point in the progressive ratio task. **p<0.01

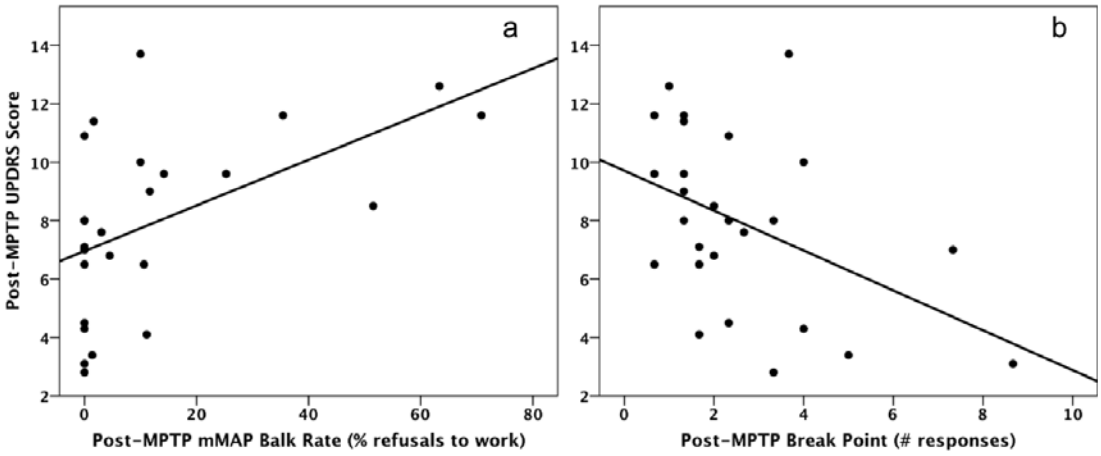


Figure 21. Reduced motivation was associated with impaired motor function after MPTP. (a) Monkeys with a higher balk rate with the right hand during mMAP testing ($r_{(s)}$ = 0.559, $p<0.01$, and (b) lower Break Point in progressive ratio testing ($r_{(s)}$= -0.442, $p<0.01$) had higher scores on the UPDRS post-MPTP.

Table 1. Correlations between measures of motivation and motor function after MPTP.

	PostMPTP Break Point	PostMPTP WGTA Balk	mMAP Balk (right hand)	PostMPTP UPDRS Score
PostMPTP Break Point	1	**-.681****	**-.599****	**-.442***
PostMPTP WGTA Balk		1	**.693****	0.345
PostMPTP mMAP Balk (right hand)			1	**.559****
PostMPTP UPDRS Score				1

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 1. Correlations between measures of motivation and motor function after MPTP.

A.4 DISCUSSION

A.4.1 Effect of unilateral low-dose MPTP administration on motivation

We found that the unilateral MPTP-lesioned monkey can be used as a reliable model for motivational decline accompanying the motor symptoms after MPTP. Similar to previously published reports, the monkeys in our study exhibited motor impairments as evidenced by poor scores on the UPDRS and poor performance on mMAP on the affected (left) hand, indicating stable lesions to the nigrostriatal pathway after a single unilateral dose of MPTP [Ovadia *et al.*, 1995; Ding *et al.*, 2008; Brown *et al.*, 2012].

To our knowledge, this is the first comprehensive, objective study of motivation in a nonhuman primate model of PD. Other studies have examined "apathy," defined as a lack of motivation [Brown *et al.*, 2012], and "no-response errors," or errors of omission [Roeltgen *et al.*, 1994] in MPTP-treated rhesus monkeys, but these assessments have limitations as reliable measures of motivation. In the Brown et al. study (2012), the measures of "apathy" were subjective measures of the monkeys' willingness to attempt tasks (i.e., walking and turning in circles using the common pole-and-collar method, and reaching tasks while restrained in a primate chair). These tasks were given while the monkeys were under physical restraint (pole-and-collar and primate chair), which may not accurately reflect their willingness to attempt particular behaviors as physical restraint has been shown to affect behavior in primates [Reinhardt *et al.*, 1995]. Roeltgen *et al.*, study (1994) used a more objective measurement (errors of omission, which reflect lack of persistence and thus are a proxy of attentiveness or motivation), but many of their subjects were also tested in a primate chair, thus confounding the reliability of the monkeys' willingness to perform. In contrast, we tested monkeys in a single

cage, which did not limit their movement, and monkeys were free to choose to participate in the task or not. In addition to recording the number of balks in the cognitive and mMAP tasks, which is similar to recording errors of omission or lack of persistence, we also used the well-established and reliable PR task to quantify motivation in our monkeys. The reliability of these methods is evidenced by the fact that each of the measures of motivation was strongly correlated with each other (**Table 1**), confirming the comprehensive nature of motivational decline in unilaterally MPTP-treated monkeys.

We further found that these monkeys' loss of motor function after MPTP may in part be governed by their loss of motivation, as evidenced by the strong correlations between measures of motivation and motor function. These findings corroborate similar reports in humans [Shiner *et al.*, 2012], and further validate the unilateral MPTP nonhuman primate model as a reliable model for both motor and non-motor symptoms in PD. The performance on the PR task was completed before the UPDRS measurements were done. However, PR performance negatively predicted UPDRS score. This further suggests that decreases in motivation after MPTP may precede motor dysfunction. Recent models on the development of PD have now begun to consider the development of other non-motor symptoms before the clinical diagnosis of motor symptoms (See Section 1.3.2 and Section 1.1.2).

That monkeys did not show an increase in balk rate during WGTA testing on a cognitive task after MPTP suggests that reduction in motivation may be specific to tasks which require the use of a particular hand (i.e., the affected side). This is supported by the fact that most monkeys failed to use their left hand during mMAP testing after MPTP, but worked better with their right hand. Although they did balk significantly more with the right hand after MPTP compared to pre-MPTP. The monkey's balk rate with the left hand was significantly higher than that with the

right hand. This suggests to us that the monkey's motivation to work for the reward on the affected side was reduced. As monkeys were not required to use one hand or the other in WGTA testing, it is likely that they relied more on using their right hands after MPTP and as such motivation on this task, as indicated by balk rates, was not significantly affected. Thus it appears that while decreased motivation and decreased motor function may go hand in hand, decreased motivation is not necessarily co morbid with decreased cognitive function in PD. Future studies should focus on a levodopa challenge test to determine if dopamine replacement therapy can improve motor function as well as motivation.

A possible mechanism for the concurrent loss of motor function and motivation in these monkeys is that dopaminergic neurons in the substantia nigra-ventral tegmental area (SN-VTA) complex were also possibly mildly affected after a unilateral MPTP injection. Whereas the SN sends dopaminergic projections to the striatum and is implicated in motor control, dopaminergic neurons in the VTA ascend to the limbic system via the nucleus accumbens (NAcc) and are associated with reward and motivation [Cardinal *et al.,* 2002]. In fact, the NAcc has long been proposed as a functional interface between the limbic system and the motor system [Mogenson *et al.,* 1980; Salamone, 1992]. It is likely that the unilateral intracarotid MPTP administration was not selective solely to neurons in the SN (i.e., the nigrostriatal pathway). Although the VTA has been shown to be less susceptible to the ameliorative effects of MPTP [Hung *et al.,* 1996; 1998], it is not immune, as studies have shown a loss of up to 20-40% of dopaminergic neurons in the VTA after MPTP [Schneider *et al.*, 1987]. This loss is likely accountable, in part, to the loss of motivation observed in our monkeys, owing to a reduction of dopaminergic function in the other dopaminergic pathway.

Taken together, our findings represent a comprehensive study of motivational decline after MPTP in the rhesus monkey, and suggest that there is a loss of motivation after MPTP injection. These findings highlight the utility of the nonhuman primate MPTP model for studying both motor and non-motor symptoms of PD. Future studies will be able to take advantage of this model for analyzing the effectiveness of treatments targeted toward the different symptoms seen in PD. This model has the potential to positively impact and find treatments for motivational decline and the reduced quality of life that PD patients face as a result of it.

A.4.2 Effect of neurotrophic factors infusion on motivation

a. Decreased Motivation in PD patients

The lack of motivation or apathy is not attributable to cognitive impairment or emotional distress or depression in PD [Levy *et al.,* 1998]. However, apathy is defined as lack of motivation that manifests itself as diminished goal-directed behavior [Pederson *et al.,* 2009]. Apathy is associated with reduced social [Brown *et al.,* 2002], and functional impairment [Gerritsen *et al.,* 2005], reduced quality of life in patients and caregivers [Aarsland *et al.,* 2007], poor illness outcome and response to treatment [Starkstein *et al.,* 2006]. In terms of the pathophysiology, the cause of apathy is dysfunction in the frontal lobes following lesion of the frontal cortex or damage to regions tightly connected to its function like basal ganglia [Dujardin *et al.,* 2007]. This frontal-sub-cortical circuit is often involved in pathological cases of apathy [Levy *et al.,* 2006]. Thus apathy is commonly seen in PD where there is reduction of DA that disrupts the normal functioning of the frontal-sub-cortical circuits. Although apathy and depression may have

overlapping symptoms in PD such as, lack of interest or pleasure many studies indicate that they are discrete syndromes in patients [Starkstein *et al.,* 1992; Levy *et al.,* 1998; Aarsland *et al.,* 1999; Isella *et al.,* 2002; Kirsch-Darrow *et al.,* 2006].

b. Changes to measures of motivation after neurotrophic factors infusions

We found that the 150 μg CDNF group, 450 μg CDNF group and the 280 μg N4 neurotrophic factor treatment groups were all effective in significantly changing measures of motivation after MPTP administration. The balk rate which is a measure of the monkey's motivation to work in a task was significantly reduced in the 150 μg CDNF group from post-MPTP to post-infusions in both the left and right hands. The break point (BP) which measures the motivation of the monkey to retrieve a treat, is a standard test used in drug addiction studies was shown to increase significantly after treatment with three months of CDNF and N4 infusions. Thus the increase in BP which is a measure of increased motivation in monkeys was significantly different from post-MPTP measurements in the 450 μg CDNF group and the 280 μg N4 group and showed a trend towards significance in the 150 μg CDNF group.

This is the first time any changes to direct measurements of motivation was shown to improve in the monkey MPTP model of PD. There has been an increased recognition of the role of non-motor symptoms in PD and the adverse impacts on the quality of life that it leads to [Chaudhuri *et al.,* 2010]. Hence recent advances in treatment options for PD patients are increasingly focused on improving their quality of life. There has been a lot of progress in using neurotrophic factors for the treatment of PD [Nutt *et al.,* 2003; Gill *et al.,* 2003; Patel *et al.,* 2005; Slevin *et al.,* 2005; Marks *et al.,* 2008; Marks *et al.,* 2010]. However these trophic factor

treatments have so far only focused on the improvement of motor symptoms. It is not known if the patients had improvements to their non-motors symptoms after being treated with trophic factors. Thus using these novel trophic factors could provide benefit to patients in both the domains of motor and non-motor symptoms.

The distribution of N4 trophic factor in the brain parenchyma was found to be the largest compared to CDNF and GDNF [Runeberg, Saarma and Penn, unpublished data]. The improvements in motivation as measured by BP are the highest for this group compared to any other trophic factor treatment. All our injections were directed only to the putamen that is involved in aspects of motor functions in basal ganglia. However the nucleus accumbens that is implicated in motivation lies just below the putamen and the larger distribution within the brain that is characteristic of N4 trophic factor could mean that improvements to motivation in this treatment group could be directly attributed to changes in the ventral striatum [Ikemoto *et al.*, 1999]. Future analysis will consider the spread of the N4 trophic factor and other neural and biochemical changes in the ventral striatum of the post-mortem monkey brains.

The effective concentration of drugs that could lead to improvement in a particular aspect of behavioral function might work effectively in specific neural sub-circuit and structure. But, the same concentration of drug might be ineffective in a neighboring neural structure that has a different function. This could be due to the inherent properties of the different neurons present in these different circuits, the physiological and anatomical organization of the inputs and outputs of this circuit. The 450 μg CDNF group significantly improved the aspects of motivation, as it could be within the range of effectiveness at this concentration in the motivational circuits that govern the progressive ratio task, but could be ineffective in improving motor behaviors. Some evidence for this is also supported by the trend in improvement ($p=0.10$) in motivation seen the

measurements of BP with the 150 μg CDNF group which was tested at a lower concentration. A more detailed dose response analysis of CDNF to improving the different aspects of symptoms of PD in animal models needs to be carried out.

Thus we show for the first time significant improvements in direct measures of objective motivation in a monkey model of PD. These trophic factors, i.e. the N4 and CDNF neurotrophic factors could be used for treatment of both motor and non-motor functions in this primate model. Future experiments should also explore infusions of these factors directly into other non-motor circuits of the basal ganglia that are also affected in PD patients. An appropriate dose, location and method of infusion that best improves both motor and non-motor functions should be considered before translation of neurotrophic factors to the clinic. Finally, clinical trials with these novel trophic factors should also comprehensively record the changes to all aspects of non-motor function including motivation along with the improvements to motor functions.

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