EFFECTS OF A UNILATERAL INTRASTRIATAL 6-HYDROXYDOPAMINE MODEL OF EARLY PARKINSON'S DISEASE ON MIDBRAIN DOPAMINE NEURONS IN RATS: A STEREOLOGICAL STUDY

BY

MICHELLE R. HEALY-STOFFEL

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Committee Chair: Beth Levant, M.P.A., Ph.D.

_____

John A. Stanford, Ph.D.

_____

Thomas L. Pazdernik, Ph.D.

_____

Gregory A. Reed, Ph.D.

_____

S. Omar Ahmad, O.T.D., Ph.D.

_____

Scott J. Weir, Pharm.D., Ph.D.

Date Defended: May 13, 2013

The Dissertation Committee for MICHELLE R. HEALY-STOFFEL
certifies that this is the approved version of the following dissertation:

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Committee Chair, Beth Levant, PhD

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of dopaminergic function, leading to the classical clinical signs of tremor, bradykinesia and loss of postural balance. These motor symptoms occur late in the disease, and since the treatments for late-stage PD are largely ineffective, a better understanding of the early stages of PD is needed in order to prevent and treat the disease. The early disease process is still poorly understood, however, and current difficulties in diagnosing prodromal or very early-stage PD make clinical studies challenging. Therefore, animal models of early PD are an invaluable resource in discovering the neuropathological, behavioral and biochemical features of the early stages of neurodegeneration found in PD. Early PD is associated with non-motor clinical signs such as changes to sleep patterns, olfactory functions, cognition and mood; while gross motor function is largely compensated for until the later stages of dopaminergic neurodegeneration. The nature of these early clinical signs presents a challenge when assessing PD models, however, as subtle sensory and affective changes can be difficult to quantify in animals. The goal of this work, therefore, was to investigate morphological changes to the dopamine neurons most implicated in the development of PD. Morphological endpoints, which can be robustly quantified using unbiased stereological analysis, provide information about the changes occurring to the neuronal structure during the neurodegenerative process, and offer promise as an objective method to assess the conditions which render dopamine neurons vulnerable in PD, as well as to evaluate new neuroprotectants and therapeutic interventions in early PD animal models. A deficiency in n-3 polyunsaturated fatty acids (n-3 PUFAs),

which have been shown to be neuroprotective, has been proposed as a potential factor in the vulnerability of dopamine neurons to PD. In order to determine the effects of n-3 PUFA deficiency on substantia nigra pars compacta (SNpc) dopamine neurons, morphological, behavioral and biochemical endpoints were investigated in the unilateral intrastriatal 6-hydroxydopamine (6-OHDA) model of early to moderate Parkinson's disease in Aim 1. In addition, a method was developed to use a novel staining method combining tyrosine hydroxylase (TH) staining with silver nucleolar (AgNOR) staining and stereological analysis techniques to quantify the morphological changes induced by 6-OHDA lesion in dopaminergic neurons in Aim 2. In Aim 3, this method was then expanded to investigate morphological changes to the dopaminergic nucleoli stained with AgNOR, which may lend valuable insights into the role of the nucleolus in early PD. In Aim 4 the cumulative techniques developed in the previous aims were employed to determine the differential morphological changes to the dopamine neurons and their nucleoli in the A8, A9 and A10 subpopulations of midbrain dopamine neurons, which correspond to the retrorubral field (RRF), SNpc, and ventral tegmental area (VTA), respectively. Although the functions of these different neuronal groups is complex and still not fully understood, these regions are differentially affected in PD; and in particular the A8 and A10 groups have been associated with the early non-motor effects of PD. Therefore the characterization of the differential morphological changes to these neurons and their nucleoli could provide valuable insights into the structural changes to the neurons induced by the neurodegenerative process, and may be useful in assessing early PD interventions in the future. Overall, this dissertation project contributed to a

better understanding of the morphological changes to midbrain dopaminergic neurons as determined by stereological analysis in an early model of PD.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
AA	arachidonic acid
AgNOR	silver nucleolar
ALA	α-linolenic acid
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DAT	dopamine transporter
DPA	docosapentaenoic acid
DHA	docosahexaenoic acid
EFOX	electrophile oxo-derivatives
EPA	eicosapentanoic acid
GC	gas chromatography
GDNF	glial-derived neurotrophic factor
HCl	hydrochloric acid
HED	hexanoyl dopamine
HPLC-EC	high-performance liquid chromatography-electrochemical detection
$iPLA_2$	inducible phospholipase A_2
LA	linoleic acid
LOX	lipoxygenase
LPS	lipopolysaccharide
MAO-B	monoamine oxidase B
MBF	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NE	norepinephrine
NO	nitric oxide
NOS	nitric oxide synthase

NOR	nucleolar organizing regions
NOX	NAPDH oxidase
NPD1	neuroprotectin D-1
Nurr1	nuclear receptor-related protein 1
Nur77	nerve growth factor-induced Bα
NTN	neurturin
PD	Parkinson’s disease
Pol1	ribosomal polymerase I
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RRF	retrorubral field
rRNA	ribosomal RNA
RXR	retinoic acid receptor
SN	substantia nigra
SNr	substantia nigra pars reticulata
SNpc	substantia nigra pars compacta
$sPLA_2$	secretory phospholipase A_2
TH	tyrosine hydroxylase
TIF1A	transcription initiation factor 1A
TLR	Toll-like receptor
VTA	ventral tegmental area
6-OHDA	6-hydroxydopamine

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Significance

Parkinson's disease (PD) is a progressively debilitating neurodegenerative disorder in which the neurons that produce dopamine are destroyed. Major clinical signs of PD are bradykinesia, resting tremor, rigidity and postural instability. Other motor signs of PD are the inability to blink, "masked" facial expression and impaired task coordination. Non-motor signs include autonomic dysfunctions such as constipation, hypotension, urinary frequency, sweating and impotence, as well as sleep loss, depression, dementia, and disturbances in olfactory function. Manifestation of symptoms as well as progression rate of the disease can vary greatly among patients (Zhao et al., 2010).

The neuropathology of PD is characterized by loss of melanin-pigmented neurons in the substantia nigra pars compacta, and the presence of Lewy bodies, which are eosinophilic cytoplasmic inclusions, although Lewy bodies may be absent from some familial forms of PD (Takahashi et al., 1994). Neuronal degeneration and Lewy bodies are not restricted to the substantia nigra, however, but may be found in a variety of other brain regions such as in the peduncular, raphe, and dorsal motor nuclei; the locus coeruleus, olfactory bulb, autonomic neurons and others (Levy et al., 2009). This extranigral degeneration likely contributes to many of the non-motor manifestations of PD.

1.1.1 Diagnosis and progression of early PD

PD is often clinically detected at a relatively late stage of neuronal loss, at the time that severe tremor and motor deficits manifest (Becker et al., 2002). However,

there are a number of early motor and non-motor indicators of developing PD, including olfactory deficits, visual disturbances, sleep dysregulation, and mood and cognitive disorders (Becker et al., 2002; Claassen et al., 2010).

The most commonly used clinical scale of PD progression is the Hoehn and Yahr scale introduced in 1967. Many studies have made an effort to correlate each stage with an expected time period, although results of these vary greatly (Zhao et al., 2010). This tool assigns patients a score from 1-5 based on motor function and bilateralism of symptoms. However, this scale does not address non-motor signs and functioning of the patient. Therefore, additional scales have been developed, such as the Unified Parkinson Disease Rating Scale, which monitors PD disability and impairment.

Table 1. Diagnosis of PD

Method	Features Assessed
Hoehn and Yahr Staging of Parkinson's Disease	
Stage 0	No signs of disease
Stage 1	Mild unilateral symptoms
Stage 1.5	Unilateral symptoms with axial involvement
Stage 2	Bilateral symptoms with no balance deficit
Stage 2.5	Bilateral symptoms with recovery on pull test
Stage 3	Mild to moderate symptoms, some balance disturbances, but patient remains independent
Stage 4	Severe symptoms, but patient can still walk or stand unassisted
Stage 5	Severe symptoms, and patient is usually wheelchair-bound or bedridden
Unified Parkinson Disease Rating Scale	
Part I: Non-motor Aspects of Experiences of Daily Living	Cognitive impairment, depressed mood, sleep disorders, constipation
Part II: Motor Experiences of Daily Living	Walking problems, drooling, handwriting difficulties, chewing problems
Part III: Motor Examination	Speech, rigidity, finger tapping, postural instability, resting tremor
Part IV: Motor Complications	Time spent with and functional impact of dyskinesias, pain of dystonias, functional impact and complexity of motor fluctuations

Sources: (Hoehn and Yahr, 1967; Goetz et al., 2007)

1.1.2 Treatment of PD

There is currently no cure for PD. Some patients will progress to a late stage within a few years of diagnosis, while others can continue to live independently for decades (Zhao et al., 2010). Current treatments include pharmacological management; surgical treatment, deep-brain stimulation, and physical and therapy (see Table 2). All current treatments eventually become ineffective at managing symptoms. Since diagnosis often occurs at a late stage in the disease process, current treatments aim to maintain sufficient function for independent living. With the increase in lifespan in developed countries, the burden of neurological diseases of aging may reach epidemic proportions in the near future, especially as there is a notable lack of effective treatments for these diseases (Petsko, 2006). Therefore understanding the foundations of PD, including the effects of diet on the etiology and treatment of PD, will be crucial to developing treatments to minimize suffering of PD patients.

Table 2. Current Treatments for PD

Treatment	Examples	Mechanism/Notes
Pharmacological Measures		
Amantadine		Anti-viral drug, alleviates mild symptoms
Anticholinergics	Benztropine (Cogentin), Procyclidine (Kenadrin)	Eventually limited by side effects, including dry mouth and constipation
Levidopa/Carbidopa	Sinemet	Dopamine precursor combined with DOPA decarboxylase Inhibitor to prevent Levidopa metabolism in the periphery
Dopamine Agonists	Bromocriptine, Ropinirole, Pramipexole	Act directly on dopamine receptors. Impulse control disorders are a notable side effect
Selective Monoamine Oxidase Inhibitors	Rasagaline, Selegiline	Inhibit metabolism of dopamine
COMT Inhibitors	Tolcapone, Entacapone	Blocks metabolism of levodopa to 3-*O*-methyldopa, resulting in higher plasma dopamine levels
General Measures		
Physical and/or Speech Therapy	---	Helps patients sustain independent living
Surgical Measures		
Deep Brain Stimulation	---	For patients resistant to pharmacotherapy- may relieve all major symptoms

Source: (Aminoff and Kerchner, 2011)

1.2 Etiology and pathogenesis of PD

The complex etiological processes resulting in the characteristic parkinsonian phenotype may actually be a collection of common results from various causes, making PD more accurately a syndrome than a disease. PD is more than simply loss of substantia nigra dopamine neurons, since other neuronal systems are also affected. The variability in clinical presentation, progression of disease and age of onset of the disease also suggest a multifactorial etiology.

The "multiple hit" theory encompasses the influences of perinatal anomalies and insults, genetic polymorphisms, and postnatal environmental factors to propose that initial reduction in dopamine neuron numbers or function makes an individual more susceptible to consequent stressors, and therefore more likely to develop PD (Holmäng, 2001; Carvey et al., 2006a; Barlow et al., 2007). Damage to the nigral neurons in idiopathic PD is likely accrued throughout the lifespan, until clinical signs appear when a functional threshold of dopamine neuronal loss is reached. In addition, substantia nigra neurons degenerate as a normal function of age. This process could be hastened by abnormalities in dopamine neuron development, number, or function. Disease could also result from reduced survival factors or accumulated environmental insults. The principal factors currently involved in the etiology and pathophysiology of PD are reviewed here.

1.2.1 Genetic factors in PD

As in other neurodegenerative diseases, genetics have been explored as a causative factor of PD. Although most PD cases have not been linked to genetics, a

number of studies have shown that PD is more common in relatives of PD patients than in matched controls (Payami et al., 1994; Bonifati et al., 1995; Marder et al., 1996). Several familial types of PD have been associated with autosomal dominant or recessive inheritance patterns (for review see: (Schapira, 2006; Bekris et al., 2010)). It is likely that many cases of so-called idiopathic PD are actually attributable to genetic causes, but conclusive evidence to link most PD cases to genetic causes is lacking.

Each PD gene has been assigned a "Park" name in addition to being referred to by its gene name; e.g. the *a-synuclein* gene causes Park 1 PD, the *parkin* gene causes Park 2 PD, etc. Several of the Park variants have not yet been conclusively associated with a gene. The known functions of most of the PD gene products involve the ubiquitisome and regulation of mitochondrial oxidative stress.

Park 1 PD is caused by mutations in the *α-synuclein* gene, a protein found in Lewy bodies (Polymeropoulos et al., 1997; Krüger et al., 1998). *Park 2* involves parkin, an E3 ligase which ubiquinates proteins tagged for destruction by the proteosome (Shimura et al., 2000; Zhang et al., 2000). *Park 2* mutations are associated with autosomal recessive juvenile-onset Parkinsonism (Kitada et al., 1998). *Park 6* is caused by mutations in *PINK1*, a gene associated with a familial form of recessive early-onset PD, is involved in the mitochondrial localization of parkin among other functions in protein folding regulation (Kim et al., 2008). *Park 7* is caused by *DJ1*, a C56 pepsidase thought to be involved in neuroprotection against oxidative stress (Bekris et al., 2010). *Park 8* PD is caused by mutations in *LRRK2*, a gene which encodes a multiple-domain 286-kDa cytoplasmic protein widely expressed in the brain. Although its function in the brain is not confirmed, it interacts with parkin (Smith et al.,

2005). It is also interesting to note that *LRRK2* is the most common mutation identified in association with PD, with the *LRRK2* G2019S mutation found in 2.8 to 6.6% of autosomal dominant PD families and 2 to 8% of idiopathic PD patients (Deng et al., 2005; Di Fonzo et al., 2005; Gilks et al., 2005; Nichols et al., 2005; Zabetian et al., 2005).

1.2.2 Environmental risk factors in PD

Both epidemiological and animal evidence supports the role of environmental risk factors in the development of PD. Among the various risk factors is a theme of neurotoxicity causing acute and/or progressive damage to substantia nigra neurons. Some lifestyle factors may be neuroprotective against PD. The main problem in linking environmental risk factors with idiopathic PD is that many of the models generated from these factors, while often displaying nigral neurodegeneration, rarely show the extranigral pathology of clinical PD. However, the fact that environmental factors, which are mostly oxidative stressors, have been linked to PD supports the “multiple hit” hypothesis, and undoubtedly contribute to certain cases of the disease.

1.2.3 Environmental toxins in PD

Most of the known environmental toxins involved in PD are agricultural agents. In addition, occupational exposure to metals such as aluminum and iron has been linked to PD in some studies (Gorell et al., 1999), although it is unlikely that this is the cause of most idiopathic PD. In addition, accidental exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by recreational meperidine users led to a number of cases of parkinsonism, leading to the groundbreaking MPTP model of PD pioneered by

Langston (Langston et al., 1983). In animal models, rotenone and a combination of paraquat and maneb have been shown to cause selective nigrostriatal degeneration (McCormack et al., 2002; Barlow et al., 2004; Betarbet and Greenamyre, 2004). Animal studies confirm epidemiological findings that PD risk is increased with exposure to pesticides, along with other risk factors associated with rural living such as drinking well water or living in a certain US states, suggesting that geographical factors such as soil and water pollutants may have an effect on nigral neurodegeneration. Interestingly, a recent meta-analysis on risks of rural living and PD found that among the studies analyzed, the greatest change in risk was for subjects living in rural areas for more than 40 years, implicating both the importance of early exposure and cumulative damage to substantia nigra neurons (Rajput and Uitti, 1987; Betemps and Buncher, 1993; Priyadarshi et al., 2001). While toxins have not been linked to the vast majority of PD cases, they have been an indispensable tool in better understanding the etiology of PD.

1.2.4 Infection in PD

Infection has also been implicated in the development of PD. A well-characterized cohort of encephalitis patients with parkinsonian symptoms resulted from the great influenza epidemic of 1918. Other viral infections have also been linked with PD, such as Japanese encephalitis B, and the St. Louis, West Nile and HIV viruses (Jang et al., 2009). Early-life infections may also play a role in development of PD much later in life, and lipopolysaccharide injection animal models have shown substantia nigra neurodegeneration. Epidemiological evidence for the influence of infection on PD is inconclusive, probably due to the nature of many of the clinical

studies being based on recall questionnaires and to the presence of confounding factors and post-mortem artifacts (Logroscino, 2005).

1.2.5 Diet and lifestyle in PD

Diet is an environmental factor potentially responsible for PD. A meta-analysis on nicotine and caffeine consumption showed that the risk of PD was 60% lower for smokers than non-smokers, and 30% lower for coffee drinkers than in non-coffee drinkers (Hernán et al., 2002). In addition, exposure to nicotine and caffeine in animal models showed a neuroprotective effect to dopamine neurons (Maggio et al., 1998; Chen et al., 2002). As in many diseases, dietary fat intake has also been considered as a risk factor for PD. A recent study showed that a high-fat diet enhanced dopaminergic neurodegeneration in a 6-hyrdoxydopamine (6-OHDA) rat model of PD (Morris et al., 2010). Polyunsaturated fatty acids (PUFAs) are of particular interest since they play an important role in brain composition and function, and their role in PD is discussed in detail in Section 1.7.

1.3 Pathogenesis of PD

1.3.1 Evidence of oxidative stress in PD

Oxidative stress is the central theme common to most theories of PD pathogenesis, although it is not clear whether oxidative stress is a cause or effect of PD, or both. Reactive oxygen species (ROS) are a natural product of cellular metabolism. When improperly regulated, ROS can react with cell components and initiate apoptosis in mitochondria. Oxidative stress in cells occurs due to excessive production of ROS or

decreased antioxidant mechanism functioning. Aging itself is a risk factor for oxidative stress, as more oxidized proteins are found in aging brains, and markers of oxidized DNA are detected in nuclear and mitochondrial DNA in the aged brain (Mariani et al., 2005). Brain tissue is inherently susceptible to peroxidation due to its high PUFA content. The oxidative metabolism of dopamine can yield hydrogen peroxide and other ROS which contribute to lipid peroxidation (Olanow, 1990). Increased lipid peroxidation products malonaldehyde and hydroperoxide have been found in the substantia nigra of PD patients (Dexter et al., 1989). Another cell-damaging product of lipid peroxidation, 4-hydroxynonenal, has also been detected in PD dopaminergic neurons (Yoritaka et al., 1996).

A number of cellular mechanisms have been proposed to account for the oxidative damage in PD. A decrease in the reduced form of glutathione has been found in the substantia nigra of PD patients (Sofic et al., 1992), indicating a reduced response to oxidative stressors. NAPDH oxidase (NOX) is another potential source for the oxidative damage occurring in PD. NOX metabolizes molecular oxygen, generating superoxide (Wu et al., 2003). NOX is upregulated in PD patients (Sumimoto et al., 2004; Li et al., 2005). NOX has also been implicated in the neurotoxicty of most PD models, including MPTP, lipopolysaccharide (LPS), rotenone, paraquat and α-synuclein models (Miller et al., 2009).

An additional candidate stressor is nitric oxide (NO), produced by nitric oxide synthase (NOS). There are several forms of NOS, including an inducible form activated by cytokines and LPS (Duval et al., 1996; Ebadi and Sharma, 2003). NOS is present in some neurons, including microglia (Shih et al., 2001). Along with is many signaling

roles, NO is also involved in neurotransmitter release, reuptake, synaptic plasticity, and gene activation. Increased NO levels have been found to correlate with increased mitochondrial cellular oxidation. Inhibition of NOS activity is protective in MPTP, paraquat and LPS models of PD (Miller et al., 2009).

1.3.2 Oxidative stress and mitochondrial dysfunction in PD

A discussion of cellular oxidative stress cannot exclude consideration of mitochondria, and there is considerable evidence for mitochondrial origins of PD. Mitochondria regulate a number of functions crucial to cells, including oxidative metabolism and apoptosis (Kroemer and Blomgren, 2007). Mitochondrial dysregulation of oxygen-handling processes can lead to ROS and subsequent cellular damage and death. Since the dopamine-rich substantia nigra is inherently a high-oxygen environment, nigral mitochondria may be particularly susceptible to any imbalances in oxidative regulation that develop as a function of age or pathology.

Aside from general oxidative damage to mitochondria from oxidative stress, PD has been associated particularly with Complex I dysfunction. Reduced activity of Complex I has been found in the substantia nigra of PD patients, as well as in their cerebral cortex and in blood platelets (Schapira et al., 1990; Haas et al., 1995; Keeney et al., 2006). Interestingly, neuroblastoma cells transfected with PD patients' platelet mitochondrial DNA exhibited Complex I deficiency (Swerdlow et al., 1996). These findings suggest a possible systemic deficit in Complex I activity in PD.

Models of PD can also be attributed to mitochondrial dysfunction. The most notable is the MPTP model. The active metabolite of MPTP, MPP+, is a mitochondrial

Complex I inhibitor (Nicklas et al., 1985). Additional evidence for mitochondrial involvement with PD arises from many of the known mutations associated with the disease. Parkin is a mitochondrial protection factor, and upregulates production of Complex I subunits (Darios et al., 2003). PINK 1 aids in the mitochondrial localization of parkin, and PINK1 knockout mice develop mitochondrial dysfunction (Kim et al., 2008; Wood-Kaczmar et al., 2008). DJ-1 translocates to the mitochondria in oxidative stress conditions, where is regulates antioxidant defense mechanisms (Takahashi et al., 2001; Zhang et al., 2005).

1.3.3 Oxidative stress and PUFA adduct formation in PD

The substantia nigra, even under ideal conditions, is highly prone to oxidative stress, being rich in both reactive dopamine and in peroxidation-prone PUFAs. Recently, Liu et al. (2008) synthesized four adducts of dopamine with peroxidation products derived from docosahexaenoic acid (DHA) and arachidonic acid (AA), and confirmed the presence of all four adducts in rat brain tissue. Notably, one of the AA-derived adducts, hexanoyl dopamine (HED), was found to be significantly more cytotoxic, in a dose-dependent manner, to human SH-SY5Y neuroblastoma cells expressing monoamine transporters. Cytotoxicity was not apparent in mouse fibroblast cells lacking monoamine transporters, suggesting a toxic specificity of HED for cells containing the transporters. A mitochondrial damage mechanism was proposed for HED cytoxicity, as HED led to dose-dependent increases in ROS generation, active caspase-3, and cytochrome *c* release. These findings suggest that HED is a potent stressor to mitochondria *in vitro* (Liu et al., 2008). It is likely that HED, and perhaps other uncharacterized PUFA-dopamine products, play an important role in the

pathogenesis of PD. In addition to potential scope for dietary interventions to prevent adduct formation, these detectable novel adducts may also be utilized in the future as diagnostic and/or therapeutic molecular targets.

1.3.4 Oxidative stress and protein dysfunction in PD

The main protein in the pathogenesis of PD is α-synuclein. A commonly expressed protein in neurons, it makes up to 1% of all cytosolic neuronal proteins, is highly expressed in the substantia nigra, and is a main constituent of Lewy bodies (Iwai et al., 1995). These aggregations are believed to play a role in a number of synucleinopathies, such as multiple system atrophy and dementia with Lewy bodies, and are a defining feature of nearly all forms of PD. Possible mechanisms for the function of α-synuclein include synthesis of dopamine, dopamine transporter (DAT) membrane function and synaptic regulation, chaperone functions, and a role in brain fatty acid metabolism (Souza et al., 2000; Cabin et al., 2002; Perez et al., 2002; Wersinger and Sidhu, 2003; Gorbatyuk et al., 2010). A30P and A53T mutations in α-synuclein cause a rare autosomal dominant familial form of the disease, and both mutation and overexpression of wild type α-synuclein are associated with clinical PD, as well as nigrostriatal degeneration in animal models (Levy et al., 2009).

The role that α-synuclein may play in PD pathophysiology is still unclear, but it appears that aggregation of the protein is necessary to promote its toxicity. Monomeric α-synuclein is soluble, but oligomers have been isolated from PD brains. These oligomers, called protofibrils, can form insoluble fibrils, which aggregate into Lewy bodies, along with ubiquitin and other proteins. However, the evidence indicates that

protofibrils are the more toxic species. Tyrosine nitration is a reaction product of the oxidative stressor peroxynitrite with tyrosine residues, and levels of nitrotyrosine are commonly used as a measure of peroxyntrite action in cells (Kuhn and Geddes, 2003). Tyrosine nitration of α-synuclein in PD patients blocks conversion of protofibrils to fibrils, keeping them in the more toxic state. A30P and A53T mutations *in vitro* increase the rate of protofibril formation *in vitro*. Once formed, α-synuclein protofibrils can affect the mitochondria by forming ring-like pores in the mitochondrial membrane, leading to membrane permeability and an increase in catecholamines in the cytosol. In addition, cytosolic dopamine is known to interact with α-synuclein and slow the conversion of protofibrils to fibrils, increasing their toxic potential (Conway et al., 2000; Rochet et al., 2004; Levy et al., 2009). Although its function in the pathogenesis of PD is still unclear, the fact that α-synuclein plays a role in several neurodegenerative diseases provides an intriguing opportunity to uncover commonalities in their etiology.

1.3.5 Oxidative stress and the role of iron in PD

Iron is a vital component of cellular components and enzymes, such as the heme components of cytochrome *c* and hemoglobin, but it also a dangerously reactive element that must be highly sequestered by cells. Normally free iron is tightly bound to proteins such as ferritin and transferrin, but at any time, about 5% of the iron is loosely bound to ligands which are not clearly understood, in what is known as the labile iron pool. Dysregulation of this iron has been implicated in a host of human diseases, including PD (Kruszewski, 2003; Youdim et al., 2004). Increased oxidative stress may result in free iron being released into the cytoplasm. Free iron contributes to radical formation and subsequent oxidative damage via the iron-catalyzed Fenton-like reaction,

which produces excess hydroxyl radicals under conditions of high levels of hydrogen peroxide and superoxide anions (Chinta and Andersen, 2008). It is particularly interesting to note that Fe3+ is reduced to Fe2+ in the presence of neuromelanin, possibly increasing substrate for the dangerous Fenton-like reaction in the neuromelanin-enriched substantia nigra (Mizuno et al., 1995). Other studies have found neuromelanin, which accumulates with aging, to be neuroprotective, as it binds iron and blocks the oxidation of dopamine and ascorbic acid, preserving precious antioxidant content. These iron-neuromelanin complexes are degraded by hydrogen peroxide to release free iron. Although the actions of neuromelanin and iron in PD are still unclear, it seems that they are both potentially contributory to oxidative stress in the substantia nigra (Zecca et al., 2008).

Iron content of the substantia nigra in PD patients is increased compared to age-matched controls, although whether the accumulation of iron is an early initiation or late response event is unclear. Iron infusion and high-iron diets in animals have shown increased hydroxyl radicals in the striatum and brainstems, and the use of iron chelators have attenuated MPTP toxicity in animals (Kitada et al., 1998), as well as showing promise in human therapeutics with iron chelators such as V-28 (Chinta and Andersen, 2008). Although iron obviously plays a role in the pathophysiology of PD, there is still much to be discovered about the mechanisms of its actions in the disease.

1.4 The nucleolus in the pathogenesis of PD

The nucleolus, an organelle housed within the nucleus, is the site of ribosomal RNA (rRNA) assembly, and therefore plays a crucial role in the rapid production of ribosomal units in response to cellular signaling (Olson et al., 2000; Boulon et al., 2010). The nucleolus houses many proteins that are key to neuronal growth regulation, most notably the nucleolar-specific rRNA polymerase Pol1 (Grummt, 2003). In addition to rRNA transcription, nucleolar proteins are tied to a number of functions such as cell-cycle control, DNA repair and apoptosis (Ahmad et al., 2009b). The nucleolus is also involved in directing the cellular response to stress, and is therefore implicated in determining the fate of a degenerating neuron. The nucleolar membrane is disrupted in response to a variety of cellular stressors, leading to dysregulation of a host of cellular growth and apoptotic effectors (Baltanás et al., 2011; Boyd et al., 2011). The pro-apoptotic protein Bax is bound to the nucleolar chaperone protein nucleophosmin/B23 in a mouse hypoxic model of stroke. B23 is released from the nucleolus after hypoxia, implicating nucleolar regulation of apoptosis in response to stress. The nucleolus also plays an important role in regulating the cellular proliferation factor p53 (Kerr et al., 2007). Disruption to the nucleolar membrane, which regulates p53-driven functions by nucleolar sequestration of various factors involved in its interactions (Boulon et al., 2010). The importance of basic roles of the nucleolus suggest that any dysfunction on the nucleolus will have a downstream effect on neuronal function, which could be contributing to the neurodegenerative processes found in Parkinson's disease.

The nucleolus is implicated in the etiology of a number of neurodegenerative diseases, such as Alzheimers', Huntington's, and Parkinson's diseases, although the

full extent of the relationships between the changes in the nucleoli-related factors and disease is still unclear (Hetman and Pietrzak, 2012). There is considerable evidence for nucleolar involvement in the etiology and/or effects of Parkinson's disease. Nucleolar integrity was compromised in human parkinsonian brains, and nucleolar damage was found to occur in an MPTP mouse model of Parkinson's (Rieker et al., 2011). The nucleolus houses several factors that seem to be key in proper functioning of the dopaminergic neuron. The transcription initiation factor 1A (TIF1A) regulates Pol1 under the influence of growth factors and stressors. Selective ablation of TIF1A function in adult mouse mutants led to oxidative damage, progressive dopaminergic neuron loss and motor dysfunction (Kalita et al., 2008; Rieker et al., 2011). Nucleolar damage in Parkinson's could also be precipitated by DNA damage, as was demonstrated recently when the DNA-topoisomerase-2 inhibitor etoposide inhibited Pol1 and induced nucleolar stress indicated by staining for B23/nucleophosmin (Pietrzak et al., 2011). The growing evidence for the role of the nucleolus in mature neuronal growth and regeneration makes it an attractive potential target for therapeutics in neurodegenerative disease, although much remains to be determined about the role of the nucleolus in neurodegenerative disease.

1.5 The midbrain dopamine neurons

The midbrain dopamine neurons comprise the majority of dopaminergic neurons in the brain (Mai and Paxinos, 2012). The major subgroups of these neurons include the A8, A9 and A10 groups according to the dopaminergic neuron classification scheme devised by Dahlstrom and Fuxe in 1964, and these groups roughly correspond to the retrorubral field (RRF), substantia nigra (SN) and ventral tegmental area (VTA) regions

of the midbrain, respectively (Dahlstrom and Fuxe, 1964). Apart from classification according to the location of the neurons of origin, the midbrain dopamine neurons are often classified by their projections into the mesostriatal, mesocortical and mesolimbic dopaminergic pathways (Oades and Halliday, 1987; Graybiel, 1991; Tzschentke and Schmidt, 2000) (**see Figure 1**). The A8, A9 and A10 groups all contribute to the pathways to differing degrees, and are all interconnected with each other (Jimenez-Castellanos and Graybiel, 1987; Oades and Halliday, 1987; Langer and Graybiel, 1989; Gasbarri et al., 1997; François et al., 1999). This suggests that the separate midbrain dopaminergic neuronal subpopulations are interdependent, and may therefore all contribute to disorders of dopaminergic function, such as PD (Deutch et al., 1988; Arts et al., 1996). Primates have between three to seven times more midbrain dopaminergic neurons than do rats, and the proportions of each neuron group and the topography of projections varies between primates and rodents (German and Manaye, 1993; Hosp et al., 2011), so these differences are important to consider when applying PD models to rodents.

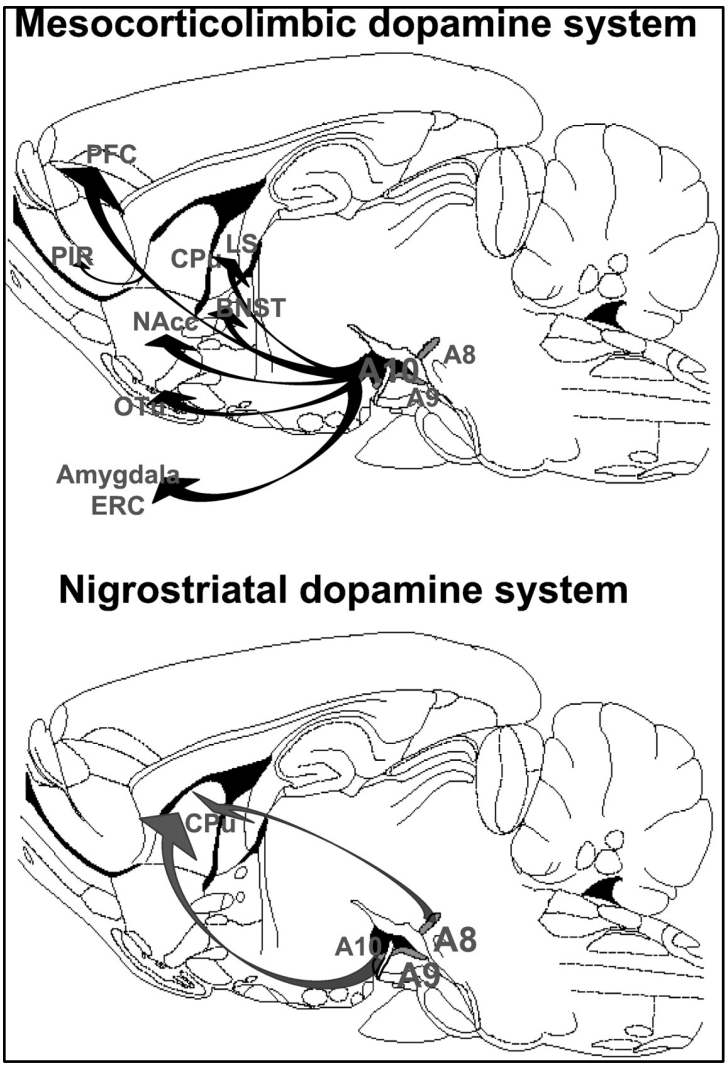

Figure 1.

Origin and major projections of the mesocorticolimbic and nigrostriatal DA systems. There are three DA midbrain nuclei: the SNc and SNl (=A9), the VTA (=A10), and the RRF (=A8). These neurons give rise to the nigrostriatal and mesocorticolimbic DA projection systems. PIR, piriform cortex; OTu, olfactory tubercles. Reprinted from *Pharmalogical Reviews* (Binder et al., 2001), with permission from The American Society of Pharmacology and Experimental Therapeutics.

1.5.1 The substantia nigra (A9) dopamine neurons

The SN contains the A9 group of midbrain dopaminergic neurons. Because the majority of these neurons project to the striatum and are involved in motor dysregulation seen in PD, the SN has been the most extensively studied dopaminergic subregion involved in the disease. The SN lies caudally to the cerebral peduncle and anterior to the red nucleus and superior cerebellar decussation (Mai and Paxinos, 2012). The SN is comprised of several subclassified nuclei, including the SN pars compacta, the SN pars reticulata (SNr), the SN medialis and the SN lateralis. The pars compacta, which is the most densely populated SN subdivision, is itself often divided into dorsal and ventral tiers, the SNCd and SNCv, respectively, and even further subdivisions of these divisions based on functional and biochemical differences in the cells (Paxinos, 1995). Approximately 300,000-550,000 pigmented SN neurons have been found in controls in human stereological studies (Pakkenberg et al., 1991; Rudow et al., 2008). In rodents, stereological analysis has determined between 6000-26,000 TH-positive neurons in the SN (Giuseppe. Giovanni, 2010). Proportions of A9 midbrain dopamine neurons to the A8 and A10 groups are much higher in primates than in rodents. It has been proposed that the A9 nuclei increases in humans correspond with the increases in the caudate and putamen relative to the volume of limbic and cortical regions (German and Manaye, 1993).

The SN provides relay and feedback functions to the basal ganglia, limbic structures and the cortex. The substantia nigra pars SNr largely contains GABAnergic neurons which receive input from the basal ganglia and project to the thalamus and

visual nuclei, while the SNpc contains mostly TH-positive dopamine neurons which project to the motor striatum (Mai and Paxinos, 2012). Each SNpc dopaminergic neuron receives input from about 100 striatal neurons, and itself projects back to about 5% of the striatum (Percheron et al., 1994; Matsuda et al., 2009). In addition to its sensorimotor-associated striatal connections, the SNpc also projects to areas of the striatum receiving input from the associative cortex and limbic structures, involving the SNpc in the early PD signs of cognitive and affective dysregulation as well as motor dysfunction (Becker et al., 2002; Mai and Paxinos, 2012). Nigrostriatal SN dopamine neurons are also involved in learning behaviors and responding to reward, and these processes can be affected in PD as well (Kimura, 1995; Kimura and Matsumoto, 1997; Horvitz, 2000; Da Cunha et al., 2009; Wise, 2009). In PD, the SN dopamine neurons are reduced by between 66-80% in stereological post-mortem studies and 50-90% in other studies (Hirsch et al., 1988; Pakkenberg et al., 1991; Rudow et al., 2008). Regardless of the quantitation method used, it is clear that there is massive SN dopamine neuron loss in PD. However, because most studies in humans have been done in late-stage patients and in severe PD models, further studies on the SN in early PD are needed to better understand the effects of the early disease process on this region. Evidence suggests that although the SN is heavily associated with the late-stage motor deficits occurring in PD, it is also likely also partially responsible for the visual and non-motor cognitive early signs of PD.

1.5.2 The ventral tegmental area (A10) dopamine neurons

The VTA is involved in motivation, reward, emotion, memory and addiction, and as such is heavily implicated in the non-motor signs of PD, as well as in other dopamine dysfunction associated disorders such as depression, schizophrenia, ADHD and addiction (Tzschentke, 2001; Viggiano et al., 2003; Nestler and Carlezon, 2006; Laviolette, 2007; Wise, 2009). The VTA contains the A10 group of midbrain dopaminergic neurons, and is a large and diffuse area dorsomedial to the SN and medial and both dorsal and ventral to the red nucleus in humans (McRitchie et al., 1996). The VTA neurons are smaller than those of the SN, however, and lower in degree of pigmentation. There are approximately 120,000 pigmented neurons in the VTA (about 20% of total VTA neurons) (McRitchie et al., 1997), and approximately 18,000 pigmented VTA neurons in the rat (60% of total VTA neurons) (Halliday and Törk, 1986). The proportion of A10 neurons to the total midbrain dopaminergic neuron population is much higher in rodents than in primates (German and Manaye, 1993), which is important to keep in mind when performing studies on this region.

The VTA projects to the structures in the mesocortical and mesolimbic pathways, including the prefrontal cortex, hippocampus, nucleus accumbens, amygdala, and the olfactory tubercle (Oades and Halliday, 1987; Ikemoto, 2007). In addition, it also projects to the striatum, although to a lesser degree than its cortical and limbic outputs (Ferreira et al., 2008). The VTA receives feedback input from most of the regions it innervates, and excitatory glutaminergic projections to the VTA are important in

modulating its activity, and are implicated in its role in addictive behaviors (Tzschentke, 2001). In early PD, the effects on the VTA as manifested by early loss of olfactory, cognitive and affective dysfunction may be an important indicator of the early disease (Sharpe, 1990; Aarsland et al., 2005; Lieberman, 2006; Torta and Castelli, 2008; Blonder and Slevin, 2011). In PD, the VTA is (Hirsch, 1994) similarly or less affected than A8 neurons in comparative studies of midbrain dopamine neurons (Uhl et al., 1985; Deutch et al., 1986; German et al., 1988; Hirsch et al., 1988). In rodent models of PD, the VTA is usually the least affected midbrain dopamine region, with losses of about 30-50% in moderate 6-OHDA and MPTP PD models (German et al., 1996; Rodríguez et al., 2001; Ahmad et al., 2009a). Although this dopaminergic region has been studied extensively in its relevance to other neurological disorders, its role in PD is still not well understood, although its potential relevance especially in the early stages of PD underscores the importance of better understanding the A10 VTA dopamine neurons in PD.

1.5.3 The retrorubral field (A8) neurons

The retrorubral field (RRF) is the least understood of the major midbrain dopaminergic groups, although the studies that have been performed have shown it to be an important component of several dopaminergic circuits. This region, the dopamine neurons of which correspond to the A8 dopamine group (Dahlstrom and Fuxe, 1964), is so named because it is caudolateral to the red nucleus in humans (Gibb, 1992; McRitchie et al., 1996) and rats (Paxinos, 1995). In both rodents and primates the RRF is located posterior to the SN and lateral to the VTA (Paxinos and Watson, 1998;

François et al., 1999). The RRF in humans contains a heterogeneous population of both TH-positive and TH-negative neurons (Mai and Paxinos, 2012). In both humans and rats the RRF A8 neurons make up about 10% of all the midbrain dopaminergic neurons (Paxinos, 1995). There are approximately 10,500 dopaminergic A8 neurons in humans (McRitchie et al., 1997), and are approximately 1400-6200 dopaminergic neurons on each side in rodents, depending on the quantitation method used (German and Manaye, 1993; Paxinos, 1995; German et al., 1996; Nair-Roberts et al., 2008).

Apart from its striatal projections, the RRF is also involved in the mesocortical and mesolimbic dopaminergic pathways. The RRF receives reward-pathway related noradrenergic input from the medulla and locus coeruleus, and largely projects to the striatum (Deutch et al., 1988; Mejías-Aponte et al., 2009). In both rodents and in humans, the RRF also projects to the amygdala (Deutch et al., 1986; Cho and Fudge, 2010), to the hippocampus (Gasbarri et al., 1997; François et al., 1999; Haber et al., 2000), and the prefrontal cortex in primates (Cho and Fudge, 2010). RRF neurons are depleted in PD post-mortem studies by approximately 40-60% (Uhl et al., 1985; Hirsch et al., 1988), and by approximately 30-70% in rodent 6-OHDA and MPTP models (German et al., 1988; Rodríguez et al., 2001); therefore it is likely that the loss of these dopamine neurons results in disturbances to the pathways in which these neurons normally function. The RRF is implicated in the striatal regulation of orofacial movement, and may be involved in the development of the mask-like facies typical of advanced PD (Spooren et al., 1993b). In addition, the RRF appears to be involved in the resting tremor found in PD patients, and harmaline-induced tremor in a 6-OHDA rat PD model (Hirsch et al., 1992; Kolasiewicz et al., 2012). The RRF may also be involved

with REM Sleep-Behavior Disorder (RBD), which is associated with parkinsonian disorders (Dhawan et al., 2006). Sleep disturbance, mood disorders and resting tremor are both early signs of PD, therefore, the RRF may be an important region affected early in the disease (Claassen et al., 2010).

1.6 Models of PD

Most animal PD models exhibit the destruction or degeneration (not always selective) of nigrostriatal neurons. Although these models usually represent various aspects of the clinical disease, there is still no comprehensive PD model showing the progressive destruction of nigral neurons with degeneration of other dopaminergic sites, decrease in other monoamines, aggregation of Lewy bodies, and the motor and non-motor signs of PD. There are three main types of animal PD models: neurotoxin models, inflammatory models, and genetic models.

1.6.1 The 6-hydroxydopamine (6-OHDA) model of PD

One of the most commonly used neurotoxic models of PD is a localized infusion of 6-OHDA. Structurally similar to dopamine and norepinephrine (NE), 6-OHDA is selectively transported by monoamine transporters into catecholaminergic neurons, where it is carried by retrograde transport into the neuronal bodies and oxidized into damaging hydrogen peroxide and paraquinone, leading to cell destruction (Saner and Thoenen, 1971; Kostrzewa and Jacobowitz, 1974). If desired, destruction of noradrenergic neurons can be prevented with pretreatment with dismethylimipramine, a selective blocker of the NE transporter, to produce specific dopamine neuron destruction (Breese and Traylor, 1971). 6-OHDA is the preferred method of neurotoxic PD lesion in rats, because rats are relatively resistant to MPTP toxicity

for unknown reasons, although rat's low levels of monoamine oxidase B compared to mice may play a role (Glover et al., 1986; Tsai and Lee, 1994; Blesa et al., 2012). In addition, 6-OHDA does not cross the blood-brain barrier, but must be infused directly into the brain via stereotactic surgery methods (Schober, 2004), which are easier to perform on rats than on mice. 6-OHDA causes dose-dependent striatal dopamine depletion, with measurable behavioral deficits in rodents and primates (Przedborski et al., 1995; Kirik et al., 1998; Bové et al., 2005; Blesa et al., 2012). After the 6-OHDA lesion is induced, behavioral tests are commonly performed. The most common test used is injection of apomorphine (direct agonist) or amphetamine (indirect agonist), causing an imbalanced release of dopamine from the injured and intact sides and inducing rotation to the contralateral side with dopamine agonists and the ipsilateral with dopamine reuptake inhibitors (Lane et al., 2006). Other effects of 6-OHDA have been assessed by motor tests designed to have correlates with PD, such as deficits in forelimb force, paw-reaching and stepping tests, and pole-climbing tests (Barnéoud et al., 2000; Meredith and Kang, 2006; Bethel-Brown et al., 2011); or by increased depressive behaviors and anhedonia (Santiago et al., 2010).

6-OHDA is often infused unilaterally, as bilaterally infused animals tend to have high morbidity and mortality, with severe aphagia and adipsia (Blesa et al., 2012)(cite Cenci 2002). However, bilaterally infused animals offer the most complete model of late-stage PD, which is bilateral. Unilateral lesions, on the other hand, offer the advantage of modeling the unilateral presentation found in early PD (Hoehn and Yahr, 1967). Also, in unilateral models the unlesioned side of the brain is often used as a control for detecting morphological and biological changes compared to the ipsilateral lesioned side (Schober, 2004; Blesa et al., 2012).

There are several modes of administering 6-OHDA. Injections can be made directly into the SN and the medial forebrain bundle (MBF), and these lesions generally result in a high degree of neuron loss and striatal depletion (Deumens et al., 2002). The drawback with these targets, however, is that since they are so small, it can be difficult to perform the stereotactic surgeries with enough precision to obtain consistent results across animals. In addition, the severe nature of the lesions means that they can only be used to model late-stage PD, and are therefore limited in their use for investigation of neuroprotective strategies. The intraventricular model has successfully obtained lower levels of neuronal destruction, and has the advantage of less gross destruction of dopaminergic tissue from direct needle injection (Rodríguez et al., 2001). However, this model is by nature limited to bilateral application, and therefore is also limited in its use. Perhaps the most widely used and versatile 6-OHDA model is the intrastriatal model. In this model, the 6-OHDA toxin is infused, usually unilaterally, into the striatum, where it destroys the dopaminergic terminals, and the toxin is carried back to the neuronal bodies in the SN, causing destruction there as well (Sauer and Oertel, 1994; Blesa et al., 2012). Dose-dependent effects of this model can be achieved by using multiple injections in multiple sites, to achieve a wide variety of states of neurodegeneration (Kirik et al., 1998; Deumens et al., 2002). The unilateral intrastriatal 6-OHDA infusion of 6-OHDA is a relevant model of early to moderate PD (Przedborski et al., 1995; Barnéoud et al., 2000; Deumens et al., 2002). Using this method, studies have achieved SNpc neuronal losses around the 50% thought to be lost at the appearance of clinical signs of PD (Marsden, 1990; Kirik et al., 1998; Deumens et al., 2002; Healy-Stoffel et al., 2012). This method has also been used to induce behavioral changes,

biochemical indices and biomarkers of oxidative stress that can be attenuated with various neuroprotective treatments, making this a useful model for development of treatments for early PD (Blum et al., 2001; Deumens et al., 2002; Blandini et al., 2007; Cansev et al., 2008; Blesa et al., 2012).

Like all PD models, 6-OHDA has important similarities with the disease, but does not encompass all the features of PD (Potashkin et al., 2010). There is evidence for the endogenous formation of 6-OHDA, suggesting that this toxin could be contributing to the naturally occurring disease (Jellinger et al., 1995), although the mode and dosage of the 6-OHDA model is most certainly more severe than that likely found in idiopathic PD. Most importantly, 6-OHDA causes depletion of midbrain catecholaminergic neurons, as occurs in PD, and furthermore, the pattern of loss within the major A8, A9 and A10 dopamine subregions after 6-OHDA lesion reflects the losses found in PD (Rodríguez et al., 2001; Kolasiewicz et al., 2012). The majority of cell death by striatal infusion will have taken place by one week (Meredith et al., 2008), therefore the 6-OHDA lesion method lacks the true progressive nature of idiopathic PD. The neurotoxic effects of 6-OHDA are dose-dependent (Przedborski et al., 1995; Kirik et al., 1998), however, and therefore various doses and lesion delivery methods can be used to create models of various stages of PD progression. In addition, neurotoxic lesion models lack the Lewy body formation found in idiopathic PD (Hirsch et al., 2003; Schober, 2004; Blesa et al., 2012), as well as the non-catecholaminergic neurotransmitter deficits found in PD. Although the 6-OHDA PD model is not perfect, it does recreate the most drastic and functionally relevant symptoms and pathology of PD, and therefore it is a crucial tool in investigating PD. Due to its overall ease of use and versatility, the 6-OHDA model of PD

is one of the most highly-utilized and well-established tools in the study of PD, and will likely continue to be well into the future.

1.6.2 The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD

MPTP is a synthetic opioid, an active metabolite (MPP+) of which is selectively transported into substantia nigra dopamine cells, destroying them and causing the manifestations of PD. It is metabolized by monoamine oxidase B (MAO-B) to MPP+, which is a potent Complex 1 inhibitor in nigral neurons (Glover et al., 1986; Schober, 2004). While this model may not represent the progressive manner in which most people develop the disease, it has been a useful tool to investigate the role of mitochondrial dysfunction in PD (Dauer and Przedborski, 2003). While highly toxic in primates, it is not effective in rats due to differences in monoamine oxidase expression, however, and has variable effects in mice (Hallman et al., 1985). In mice, the option of stereotactic injection is less attractive and the toxin is usually delivered systemically, which can lead to high mortality with the does needed to achieve significant dopamine neuron destruction, limiting some of the usefulness of this model in rodents (Petroske et al., 2001). MPTP may be either acutely or chronically administered, providing a more gradual destruction to mimic the progressive destruction of dopaminergic neurons found in idiopathic PD. MPP+, the toxic metabolite of MPTP can be directly infused into the rat brain, as MPP+ is a good substrate for DAT. This model is usually unilaterally employed, much like the 6-OHDA model. While not applicable across rodent species and often limited by systemic side effects, the MPTP model has proved to be a revolutionary factor in our current understanding of PD pathogenesis, and will continue to be utilized in the future.

1.6.3 Pesticide models of PD

Paraquat is a commonly used herbicide worldwide. In cells, paraquat is transported into mitochondria by a carrier-mediated process. Mitochondrial Complex I reduces it to a lethal radical species (Cochemé and Murphy, 2008), in contrast to other neurotoxins which inhibit Complex I directly. Although it is capable of producing small nigral dopamine neuron losses in rodents (Brooks et al., 1999; Fleming et al., 2004; Kuter et al., 2007), paraquat alone has not been shown to instigate progressive loss of nigral neurons by itself. Instead it is usually co-administered with maneb to increase toxicity. Maneb, is a fungicide which disrupts dopamine uptake and release (Vaccari et al., 1999). Administered together, paraquat and maneb lead to progressive loss of dopamine neurons (Thiruchelvam et al., 2003), especially in older mice and rats (Cicchetti et al., 2005). The attraction of this model lies in its progressive loss of motor neurons, motor function impairment, and induction of microgliosis, all of which are features of clinical PD. However, use of paraquat in rats can be limited by the same lung toxicity which makes it a lethal agent to humans (Saint-Pierre et al., 2006).

Rotenone is another pesticide PD model, and like MPTP, it is a Complex I inhibitor. It is notable that the progressively dying dopaminergic neurons induced by rotenone contain Lewy-like cytoplasmic inclusions immunopositive for α-synuclein and ubiquitin. However, the damage caused by rotenone can cause highly variable degrees of damage to both dopaminergic and other neuronal cells, limiting the usefulness of this model by lack of reproducibility (Meredith et al., 2008). While pesticide models have been useful in exploring the possible environmental etiology of PD, their systemic side

effects and incongruity with many of the clinical features of PD in humans may limit their usefulness in many cases.

1.6.4 Inflammatory models of PD

Inflammation is implicated in neurodegenerative disease due to effects of the ROS produced by inflammatory cascades on vulnerable neurons, notably the oxidation-prone dopamine-enriched nigral neurons. Inflammatory models of PD rely on the activation of microglia, which function as the macrophages of the brain. When activated, microglia produce pro-inflammatory cytokines, NO and ROS. This function is necessary for normal apoptosis that occurs during development and injury, but dysfunctional activation and failure to stop the inflammatory cascades are an underlying theory for many progressive diseases. Inflammatory models of PD employ injection of LPS, an endotoxic molecule which induces microglial activation. Microglia density is 4-5 times higher in the substantia nigra than in other brain regions, leading to selective degeneration of nigral neurons with an acute intracerebral dose of 5 or 10 μg of LPS (Herrera et al., 2000; Kim et al., 2000; Gao et al., 2003). Acute LPS administration in rats causes permanent lesions and behavior deficits induced by amphetamine rotation, but lack of progression, as microglia morphology returns to normal by 30 days post-injection (Iravani et al., 2005). However, progressive loss of TH-immunoreactive cells has been seen after a single insult (Qin et al., 2007). Acute systemic LPS injection in mice reveals a delayed but progressive degeneration of nigral neurons (Castaño et al., 1998).

Chronic LPS infusion by cannula has been used to create a more progressive PD model. While progressive dopaminergic neuron loss is achieved, this model has yet to establish representation of the other cardinal features of PD such as motor dysfunction, inclusions, or extranigral degeneration (Gao et al., 2003). In support of the finding that infections in the mother can lead to loss of nigral neurons in the fetus, an intrauterine LPS model has shown that prenatal exposure to LPS disrupts normal development, resulting in reduced numbers of dopamine neurons. Acute systemic LPS injection in mice reveals a delayed but progressive degeneration of nigral neurons (Castaño et al., 1998). Interestingly, rotenone given to 18-month old rats exposed to prenatal LPS enhanced the loss of dopamine neurons, supporting the "multiple hit" hypothesis of PD (Ling et al., 2004). With our increasing understanding of the role of inflammation in neurodegenerative diseases, the inflammatory model of PD will likely play an even more important role in PD studies in the future.

1.6.5 Genetic models of PD

Genetic models of PD have become an important tool in understanding the etiology of the disease. Two genetically engineered mouse models which have shown a progressive post-natal degeneration of dopamine neurons are *Pitx3*-null mice and *Engrailed*-null mice. *Pitx3* is a gene crucial to early dopaminergic neuron development, and *Pitx3*-null mice have a rapid post-natal loss of dopamine neurons and behavioral deficits alleviated by levodopa (Nunes et al., 2003; van den Munckhof et al., 2003; Hwang et al., 2005). Recent evidence has shown that a polymorphism in the *Pitx3* gene is a risk factor for sporadic PD in humans (Fuchs et al., 2009). While this model does show progressive loss of dopamine neurons, the loss is earlier in life than would

be seen in idiopathic PD. *Engrailed* knockout mice also show a progressive dopaminergic neuron loss, but also show additional cerebellar pathology which limits their use in behavioral tests (Sonnier et al., 2007).

Models of the mutations which have been linked to familial PD have also been developed. The *α-synuclein* mutant models vary in the promoter used, resulting in various mutant and wild-type over-expression models (Chesselet, 2008). These models can be useful for characterizing the effects of overabundant or mutant a-synuclein, but many of the behavioral dysfunctions they have do not correspond to PD, and many do not demonstrate a nigral neuron loss (Masliah, 2000; Fleming et al., 2005; Hwang et al., 2005; Tofaris et al., 2006). A drosophila model of PD has recently been produced to express mutant and normal forms of α-synuclein. These flies showed an age-dependent loss of TH-positive dorsomedial dopamine neurons. They also showed α-synuclein stained inclusions resembling Lewy bodies, and exhibited locomotor dysfunction with age, although it could not be shown that the deficits were a function of degenerated dopamine neurons (Feany and Bender, 2000).

Parkin is an important gene in familial PD, encoding an E3 ubiquitin ligase involved in proteosomal function. Although early mutated *parkin* lines showed mixed or absent effects on dopaminergic neuron loss and non-motor and behavioral deficits (Goldberg et al., 2003; Perez and Palmiter, 2005; Zhu et al., 2007), a mouse model transgenic for Q311X *parkin* has shown both progressive DA neuron loss and progressive motor dysfunction in late adulthood (Lu et al., 2006). *PINK1* knockout mice have decreased dopamine release (Kitada et al., 2007), and the *DJ1* mutation causes diminished resistance to oxidative stress in mice, flies and cultured cells. *DJ1* knockout

mice do not develop dopamine neuron loss, however (Dodson and Guo, 2007; Yamaguchi and Shen, 2007).

A comprehensive animal PD model has yet to be developed, although these models are useful in representing various aspects of the disease, such as extranigral neurodegeneration and Lewy body pathology. However, the increased knowledge about the etiology of PD provided by models of PD will likely lead to the continual improvement of PD models in the future.

1.7 Introduction to PUFAs

Polyunsaturated fatty acids (PUFAs) are important dietary fats containing more than one double bond in their carbon structure. The different PUFAs are named according to the number of carbons they contain, their number of double bonds, and the position of the first double bond from the methyl end of the fatty acid. Thus DHA, a 22-carbon PUFA with six double bonds and the first double bond at the third carbon from the methyl end, is designated as 22:6n-3, and is part of the n-3 class of PUFAs. PUFAs are crucial to brain composition and proper function (Willatts et al., 1998; Birch et al., 2000). DHA constitutes approximately 12-15% by weight of the total fatty acids in the human brain, while AA makes up 8-11% (Whelan, 2008). AA (22:4n-6) is distributed evenly within the brain, while DHA is highly enriched in neuronal and synaptic membranes, suggesting an important role in cell signaling (Farooqui et al., 2000).

1.7.1 Dietary PUFAs: synthesis and brain accretion

Mammals cannot synthesize essential n-3 and n-6 PUFAs *de novo*. Instead, DHA and AA must be consumed in the diet, or their precursors α-linolenic acid (ALA) and linoleic acid (LA) must be provided. ALA is metabolized by desaturases and elongase to form DHA (**see Figure 3**). The same enzymes are employed to convert LA to AA and ultimately to docosapentaenoic acid (DPA) (Dyall and Michael-Titus, 2008) (**See Figure 2**).

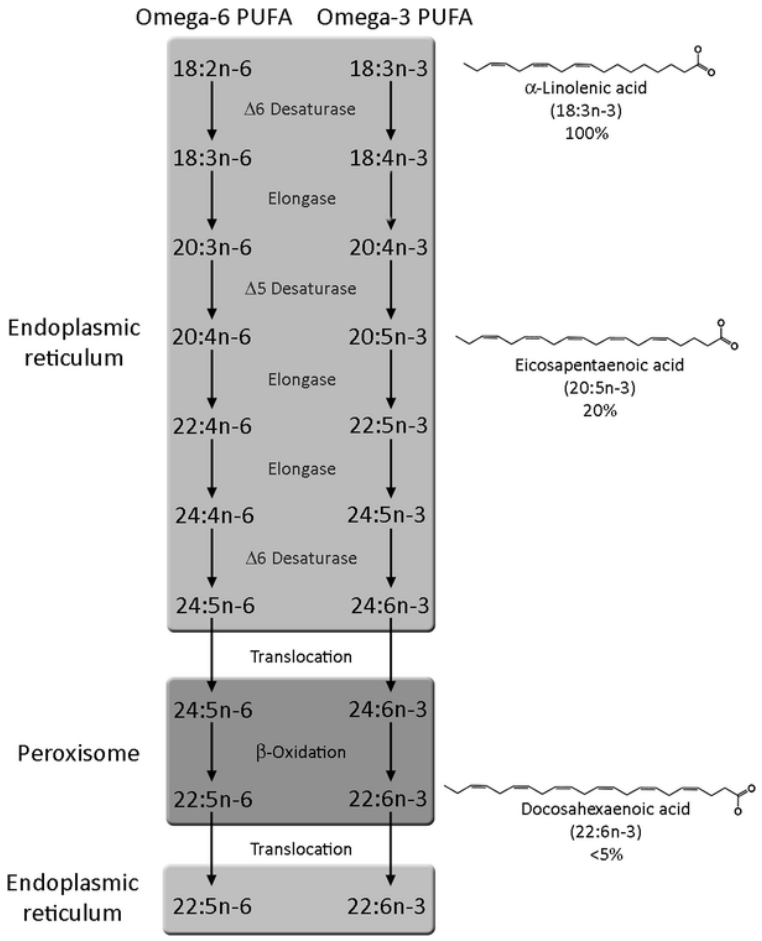

Figure 2.

Summary of omega-3 and omega-6 PUFA biosynthetic pathways. The pathways proceed through a series of desaturation and elongation steps in the endoplasmic reticulum until 24:5n − 6 and 24:6n − 3, which are translocated to the peroxisome, where the chains are shorted by C2 by one cycle of the β-oxidation pathway to form 22:5n − 6 and 22:6n − 3 (DHA), respectively. These are then translocated back to the endoplasmic reticulum for subsequent esterification into aminophospholipids. The relative efficiencies of the omega-3 PUFA conversion process are shown to the right of the pathways, for further details refer to the text. Reprinted with permission from Springer Science and Business Media: *NeuroMolecular Medicine* (Dyall and Michael-Titus, 2008)

DHA plays an important role in growth and development. DHA is supplied to the fetus by the mothers' dietary consumption and to infants in breast milk. In humans, most DHA is accumulated in late gestation and early childhood, with lifelong turnover (Clandinin et al., 1980a; Clandinin et al., 1980b; Hadley et al., 2009). Accumulation in rats increases later in gestation and most of the DHA is stored from the last three days of gestation through weaning (Kishimoto et al., 1965; Green and Yavin, 1996). Long-chain PUFAs are sometimes called "conditionally essential" in that no gross deficiency disorders are known. However, possible visual and cognitive deficits in children given a low n-3 diet and benefits bestowed by an n-3 supplementation during pregnancy have been reported (McNamara and Carlson, 2006). Dietary DHA deficiency was associated with impaired mental and visual performance in otherwise healthy term infants (Willatts et al., 1998; Birch et al., 2000). DHA activates syntaxin-3, a crucial factor in neuron growth and regeneration, and which may explain its role in optimal growth and development (Whelan, 2008).

1.7.2 Influence of dietary PUFAs

Dietary consumption of PUFAs affects tissue PUFA composition, and an n-3-PUFA deficient diet in adult rats results in decreased n-3 content of several organs, including liver, heart and testes (Bourre et al., 1992). In addition, previous breeding studies in our laboratory have shown that a decrease in n-3 fatty acids in the diet results in a decrease of brain DHA, and a concurrent increase in the n-6 fatty acid docosapentaenoic acid (DPA), by 32% and 54%, in first litter and second litter pups, respectively (Ozias et al., 2007). Thus brain DHA content is replaced in the Deficient rats with the n-6 PUFA DPA, altering the ideal n-6/n-3 PUFA ratio. Consequent

generations do not show marked decreases in percent of brain n-3 PUFAs after the second generation, so our study will be limited to two generations.

Western diets are very low in n-3 fatty acids, and have an n-6/n-3 as high as 16.7/1 (Simopoulos, 2003). Importantly, a very high n-6/n-3 ratio has been implicated in a variety of human diseases such as coronary artery disease, hypertension, diabetes, arthritis, osteoporosis, autoimmune disorders, cancer and mental health disorders (Simopoulos, 2008). Low dietary n-6/n-3 ratios have been shown to favorably alter LDL status, improve cardiovascular health, have anti-proliferation effects in colorectal cancer, decrease the risk of breast cancer, and decrease inflammation in rheumatoid arthritis and asthma (Haworth and Levy, 2007; Calder and Yaqoob, 2009; Fetterman and Zdanowicz, 2009; Hartwich et al., 2009; Lavie et al., 2009).

1.7.3 PUFA metabolism and roles in human health

PUFAs play a variety of roles in the function of the body, but one of their most important effects is their influence on plasma membrane composition. AA is an important signaling molecule in inflammatory cascades, as well as in the D_2 receptor pathway in the dopaminergic system (Lee et al., 2010). AA is preferentially cleaved from phospholipids in the brain by both cytosolic phospholipase A_2 ($cPLA_2$) and secretory phospholipase A_2 ($sPLA_2$), after which a portion of the released AA can by metabolized by cyclooxygenases (COXs) and lipoxygenases (LOXs) to form a class of compounds known as the eicosanoids. Eicosanoids include a variety of mediators of cellular activity, including prostaglandins, leukotrienes and lipoxins (**see Figure 3**). The actions of prostaglandins have a wide range of functions, from smooth muscle

contraction to vasodilatation (or vasoconstriction, depending on the particular prostaglandin and receptor) and inhibition of platelet aggregation. Leukotrienes are potent vasoconstrictors and leukocyte attractants implicated in immune responses. Lipoxins both activate macrophages and monocytes, as well as inhibiting leukocyte activation (Brunton et al., 2011). Most AA-derived metabolites have pro-inflammatory functions, although AA contributes to mediators with a broad range of functions in signaling and memory and learning modulation (Funk, 2001; Orr and Bazinet, 2008a). It is interesting to note that eicosapentanoic acid (EPA), the 20-carbon n-3 precursor to DHA, also forms eicosanoids, most of which are more anti-inflammatory than their AA-derived counterparts. EPA competes with AA for the same enzymes, so the n-6/n-3 ratio influences metabolite production. Under conditions of cell membrane damage (such as oxidative stress), abundant intracellular calcium and inflammatory stimuli, PLA_2 and COX2 transcription is upregulated, leading to increased production of AA metabolites (Orr and Bazinet, 2008a).

DHA is incorporated preferentially into phosphatidylethanolamine and phosphatidylserine on the inner membrane layer of synapses and its steric incompatibility with cholesterol drives the formation of either DHA- or cholesterol-rich lipid rafts. These rafts serve as protected micro domains and function in compartmentalization of various cell signaling molecules (Farooqui et al., 2000). DHA has also been shown to affect membrane fatty acid chain fluidity, ion permeability, elasticity, protein function, phase behavior, and fusion (Stillwell and Wassall, 2003; Wassall and Stillwell, 2008). DHA is also cleaved from the phospholipids by phospholipase, and evidence indicates that an inducible DHA-selective form of the

enzyme, $iPLA_2$, is responsible for its availability. Once unesterified, DHA goes on to perform as a ligand for a variety of receptors, such as peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor (RXR) and Toll-like receptor (TLR), as well as being metabolized by COX and LOX to form the docosanoids resolvins, neuroprotectin D1 (NPD1), and the recently discovered electrophile oxo-derivatives (EFOXs) (Lee et al., 2003; Lengqvist et al., 2004; Orr and Bazinet, 2008a; Groeger et al., 2010). Resolvins are a class of anti-inflammatory compounds produced by the COX-2 pathway in the presence of aspirin (Sharon et al., 2003; Serhan et al., 2004). Neuroprotectin D-1 (NPD1) is a peptide that is formed by phosholipase A_2 lipooxygenase on free DHA, and induces anti-apoptotic Bcl-2 proteins, inhibits pro-apoptotic Bcl-2 proteins, and suppresses inflammatory gene expression (Lukiw and Bazan, 2008). DHA and NPD1 also affect membrane structure and stability, and alter the balance of n-3 to n-6 PUFAs in the membrane, which affects the arachidonic acid cascade by suppression of COX-2. Under inflammatory conditions, macrophages were found to produce maresins, DHA-derived metabolic products with anti-inflammatory properties similar to the resolvins and NDP1 (Serhan et al., 2009). Newly discovered COX-2 EFOX metabolites act as anti-apoptotic Nrf-2 activators, PPAR-γ agonists, and inhibitors of cytokine and NO production (Groeger et al., 2010) (see Figure 3). DHA also has the ability to inhibit TLR4, an important pro-inflammatory factor, and various nuclear receptor initiators of the NfκB-mediated anti-inflammatory response (Lee et al., 2003; Weatherill et al., 2005). Several studies have documented the ability of n-3 fatty acids to decrease cytokine production, and response to COX-2 activity was decreased in a clinical study in which patients were supplemented with 15 g/day of fish oil for four weeks (Lee et al., 2003);

(Spector, 2001). Conversely, under oxidative stress, DHA is oxidized into neuroprostanes, a class of prostaglandin-like compounds formed without COX. These compounds trigger ROS formation at both sides of the phospholipid membrane (Montine et al., 2004). F4 neuroprostanes, DHA-derived lipid peroxidation products, may lead to the formation of further membrane- damaging aminophospholipids (Roberts et al., 2005).

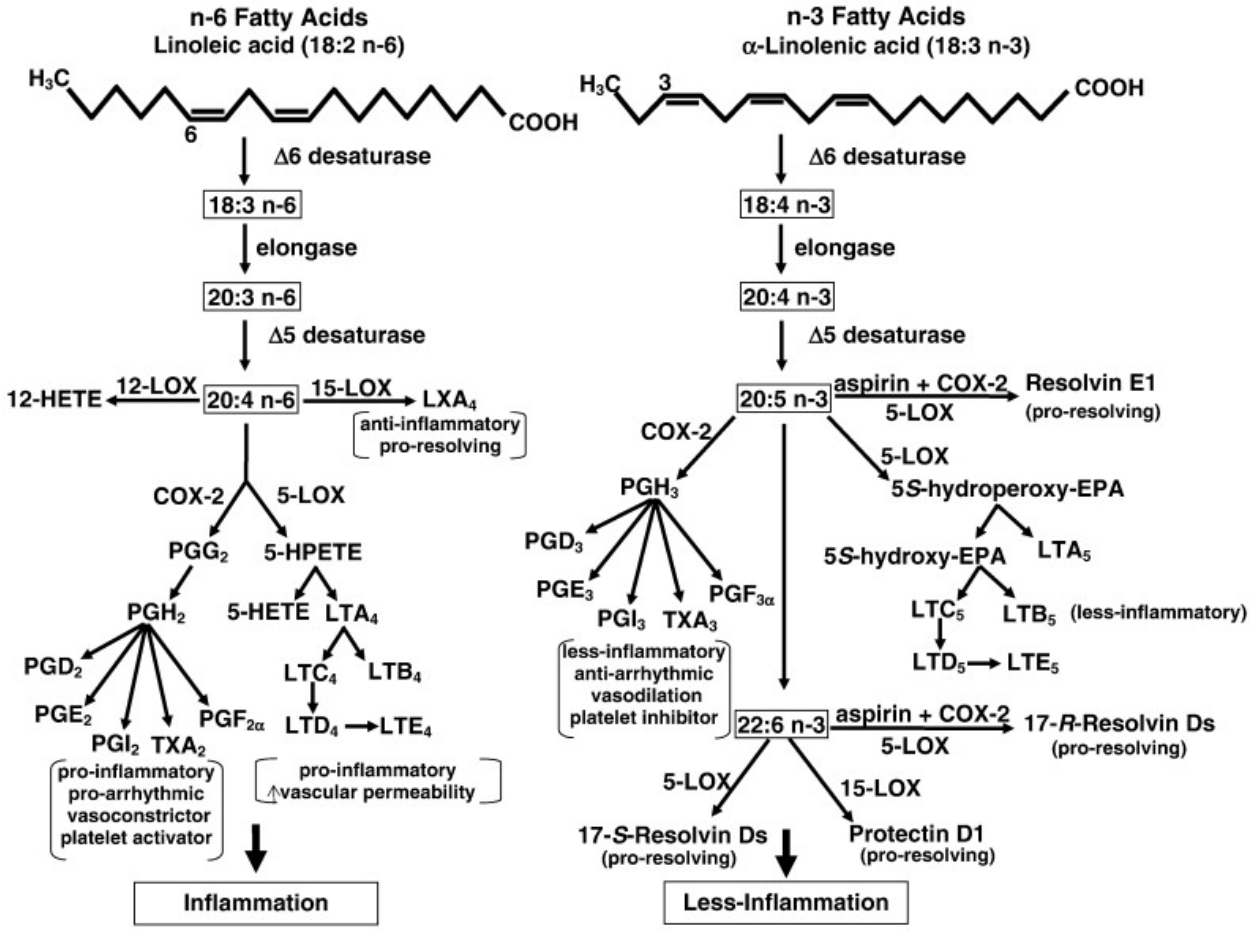

Figure 3.

The metabolism of n-3 and n-6 PUFA and the biosynthesis of their respective eicosanoid and pro-resolving mediators. N-3 PUFAs are generally less inflammatory than the n-6 PUFA. However, PGE_2 derived from n-6 PUFA can have an anti-inflammatory effect by decreasing LTB_4 production by the inhibition of 5-LOX and increasing production of LXA_4 by stimulating 15-LOX. N3 PUFA-derived eicosanoids have different physiological potencies than n-6 PUFA-derived eicosanoids. Abbreviations: HPETE, hydroperoxyeicosatetraenoic acid; LTA_4, leukotriene A_4; LXA_4, lipoxin A_4. Reprinted from the Journal of Nutritional Biochemistry (Adkins and Kelley, 2010),with permission from Elsevier.

1.7.4 N-3 PUFAs in PD

N-3 PUFAs influence a number of factors that are implicated in the development of PD. In addition, n-3 PUFAs are known to influence several factors implicated in the health and maintenance of neurons throughout the lifespan, such as RXR, nuclear receptor-related protein 1 (Nurr1) and nerve growth factor-induced Bα (Nur77) (Bousquet et al., 2008; Lukiw and Bazan, 2008; Orr and Bazinet, 2008b; Yakunin et al., 2012). Thus, dietary n-3 PUFA intake has become an area of interested with regard to preventing or slowing the progression of PD.

1.7.4.1 Dopaminergic developmental factors influenced by n-3 PUFAs

Several factors influenced by n-3 PUFAs play an important role in dopaminergic development, growth and maintenance. Thus the dysregulation of these factors may contribute to the etiology of PD. RXR is a member of the nuclear hormone family of receptors, along with steroid hormone, thyroid hormone, vitamin D receptor, and a host of others. There are three main isotypes: RXR-α, RXR-β and RXR-γ. RXR-α is expressed in liver, kidney, spleen, placenta, epidermis, and visceral tissue; RXR-β is expressed in nearly every body tissue; and RXR-γ is mostly in muscle and brain tissue. The roles of RXR are complex and not completely understood, but RXR isoforms are known to be involved in muscle metabolism, insulin resistance, atherosclerosis and cholesterol metabolism, apoptosis, and a variety of differentiation processes, including neuronal development (Szanto et al., 2004). In the dopaminergic system, RXR is a crucial developmental and survival factor, in conjunction with its *NR4A1* partners Nurr1 and Nur77 (Lévesque and Rouillard, 2007). A unique feature of RXR is its ability to

form heterodimers with many other nuclear receptors, implicating RXR in the roles of multiple transcription pathways. In particular, RXR heterodimerizes with both Nurr1 and Nur77, another member of the *NR4A1* orphan nuclear receptor family. Nurr1 is an *NR4A1* orphan nuclear receptor crucial to dopaminergic neuronal development, as well as regulation of the hypothalamic/pituitary/adrenal axis (Murphy and Conneely, 1997). Nurr1 is expressed chiefly in the substantia nigra, ventral tegmental area, midbrain and the limbic areas, all areas in which dopamine plays a vital role. Expression peaks in embryo, yet remains high in dopaminergic neurons throughout the lifespan, with Nurr1 expressed in 96% of adult substantia nigra neurons (Zetterström et al., 1997; Bäckman et al., 1999; Le et al., 1999). Nurr1 regulates the transcription of TH and DAT by binding to the NBRE sequence in the 5'-untranslated region, and binds to RXR. The resulting heterodimer plays an important role in the development of neuron cells (Schimmel et al., 1999; Sacchetti et al., 2002).

RXR, Nurr1 and Nur77 receptors act as ligand-activated transcription factors with three main structural features: (1) DNA binding domains for binding to hormone response elements, (2) ligand-binding domains specific for small lipophilic molecules, and (3) transactivation domains for activating gene transcription. There may be several endogenous ligands for RXR, but DHA is a known ligand for this receptor (Lengqvist et al., 2004), and as such dietary DHA intake may play a role in RXR regulation.

Target areas of dopaminergic innervation express both RXR-β and RXR-γ isoforms. Mice deficient in these receptors have impaired motor function, and decreased mRNA transcription of D_1 and D_2 receptors in the striatum (Krezel et al., 1998). Knockout RXR-γ animals have also been found to have abnormalities in

synaptic plasticity and learning (Szanto et al., 2004). RXR, along with dimerization partner Nur77, has been implicated in modulating a number of motor side effects of antipsychotic drugs, and decreased Nur77 has been linked to post-mortem brains from schizophrenic patients (Lévesque and Rouillard, 2007). In mice, ablation of RXR-γ resulted in depressive behaviors of despair and anhedonia, along with decreased D_2 receptor expression in the nucleus accumbens and changes in serotonin signaling (Krzyzosiak et al., 2010). Knowledge about the roles of RXR and its partners in PD are quite limited, although since RXR and Nur77 are implicated in haloperidol-induced VCMs and other neuroleptic side effects in antipsychotics, they are likely also involved in the dyskinesias associated with L-dopa treatment in PD. In one recent study, activation of RXR by synthetic RXR ligand LG100268 and RXR-Nurr1 ligand XCT0139508 in neuronal rat dopaminergic cell cultures was found to protect against oxidative stress induced by 6-OHDA and hypoxia. However, RXR expression alone could not protect the cells when treated with kainic acid and hydrogen peroxide; rather, neuroprotection against these was selective to Nurr1-expressing cells, still implicating RXR as its dimerization partner (Friling et al., 2009). The apparent complexity of the relationships among the nuclear receptors involved in the nigral neurogenesis make it difficult to isolate the effects of each, but it is apparent that these development and survival factors may play an important role in the susceptibility of adult neurons under conditions of neurodegenerative disease.

1.7.4. Roles of n-3 PUFAs in PD

Clinical studies on the role of PUFAs in the etiology of PD have generally been inconclusive, although there are several studies addressing the role of PUFAs in PD.

The prospective cohort Rotterdam study evaluated 5,289 subjects age 55 and older to monitor the association between intake of unsaturated fatty acids and the incidence of PD. In this study, a high intake of PUFAs was associated with a decreased risk of PD (de Lau et al., 2005). Since diets vary across cultures, studies could reveal whether incidence of PD is altered in populations consuming high amounts of PUFAs (particularly n-3 PUFAs). Although there is an unfortunate lack of studies addressing this question to date, one recent case-control study of a Japanese population found that n-3 PUFA intake and n-3/n-6 ratio were not associated with an increased risk of PD, although increased AA intake, interestingly, was (Miyake et al., 2010). Additionally, PUFA levels measured in post-mortem PD patients were decreased in the substantia nigra compared to other brain regions and controls (Dexter et al., 1989), although this certainly be an effect rather than a cause of PD. More clinical studies are needed to establish any potential relationships between PUFAs and PD, since these studies could reveal important insights about the cause of the disease.

The roles of n-3 PUFAs in PD have been much more extensively studied in animal models of PD, although the question of whether a high n-3 diet is beneficial in PD still remains inconclusive. However, many of the studies in animals show some benefit of n-3 PUFAs in PD. A combination of uridine and DHA given to rats for 3 days before unilateral 6-OHDA lesion increased tyrosine hydroxylase (TH), dopamine and synapsin-1 expression on the lesioned side, and decreased amphetamine-induced rotation (Cansev et al., 2008). In a bilateral MPTP rat model of PD, DHA supplementation improved motor performance and modulated Akt and Bcl-2 pathways (Hacioglu et al., 2012). In murine studies, mice were given either a control or high n-3

PUFA diet for 10 months and then treated with MPTP. The mice supplemented with n-3 PUFAs protected against MPTP-induced decrease in dopamine striatal content, TH-labeled nigral cells, and Nurr1 and DAT mRNA levels (Bousquet et al., 2008). Another MPTP mouse study found that DHA supplementation did not improve motor performance, but resulted in reduced dopaminergic neuronal loss and reduced COX2 activity compared to MPTP-intoxicated controls (Ozsoy et al., 2011a; Ozsoy et al., 2011b). DHA supplementation in an MPTP model was also found to increase glial-derived neurotrophic factor (GDNF) and neurturin (NTN), both important trophic factors involved in dopaminergic neuron health (Tanriover et al., 2010). In primates, a monkey MPTP model showed that DHA administration reduced levodopa-induced dyskinesias when given either before or after MPTP administration, inviting the prospect of its being a potentially effective preventative agent in clinical PD (Samadi et al., 2006). The evidence from these animal studies points to the ability of n-3 PUFAs to modulate factors involved in neuroprotection in PD models, suggesting that increased n-3 PUFA intake could prevent or attenuate the progress of PD in humans.

Other studies, however, have found a detrimental effect of PUFAs in PD. The double bonds in PUFAs make them susceptible to lipid peroxidation in pro-oxidative environments, and several studies have implicated high n-3 PUFAs with increased oxidative damage in PD and PD models. Neuroprostanes, DHA-derived lipid peroxidation products, are increased in the brains of advanced PD patients (Montine et al., 2004). Also, dopamine can react *in vitro* with fatty acid peroxides to form 6-OHDA, providing a potential mechanism for endogenous production of 6-OHDA in the pathogenesis of PD (Pezzella et al., 1997). In one 6-OHDA study in mice,

intraperitoneal injection of a DHA ethyl ester resulted in increased the levels of lipid peroxidation in the striatum (Kabuto et al., 2009). In A53T *α-synuclein* mutant mice, DHA supplementation increased accumulation of both soluble and insoluble α-synuclein, neuritic injury and astrocytosis, and these effects were attenuated by decreased dietary DHA content (Yakunin et al., 2012). In addition, an *in vitro* experiment with oligodendrocytes transfected with A53T *α-synuclein* supplemented with DHA for 3 days before subjection to oxidative stress showed enhanced aggregation of α-synuclein (Riedel et al., 2010). Another recent study found that PUFAs in the brain can form neurotoxic adducts with dopamine (Liu et al., 2008). Since the most toxic of the adducts was formed from an n-6 PUFA, the n-6/n-3 ratio in the diet may have an effect on development of these adducts and the damage they can cause (see Section D.2.1.3). Despite the potential adverse effects of increased lipid peroxidation and α-synuclein protein dysregulation which could accompany increased n-3 PUFA intake, the evidence for the role of n-3 PUFAs in prevention and treatment of PD remains promising, if still inconclusive. The potential for understanding the mechanisms by which n-3 PUFAs contribute to the processes involved in neuroprotection and neuronal repair, or alternately to the increased lipid peroxidation found in PD, highlights the importance of conducting high-quality clinical and preclinical studies to determine the effects of n-3 PUFAs in PD.

1.8 Stereology and staining in PD

Morphology is the study of the structure of organisms or their features. In order to accurately assess these physical features, quantitative analytical methods are needed, which can be challenging when it comes to microscopic, irregularly shaped

objects such as neurons, which are embedded among other types of cells deep within the brain. In order to study neuronal morphology, design-based stereology is the quantitative method of choice (Gundersen, 1992; Schmitz and Hof, 2005). Stereology is the study of object properties such as length, surface, area and volume, using a variety of sampling and estimation techniques. Design-based stereology refers to a set of sampling parameters that are "defined" before the study, to be independent of the size, shape, orientation and distribution of the objects; as opposed to older "method-based" stereological techniques in which estimates depended on the geometrical properties of the objects of interest (Mayhew and Gundersen, 1996; Boyce et al., 2010b). Using design-based methods that make fewer assumptions about the properties of interest reduces bias and results in more robust data than 2D counting methods (de Groot et al., 2005; Schmitz and Hof, 2005; Boyce et al., 2010a). Stereology has been particularly useful in quantifying large, irregularly shaped biological objects such as neurons, and in fact stereology is considered the gold standard in quantification of neuronal morphology (Gundersen et al., 1988b; Schmitz and Hof, 2005).

1.8.1 Sampling methods used in stereology

Design-based stereology uses systematic random sampling (SRS) to obtain unbiased results. This method is systematic because samples are selected at defined "systematic" uniform intervals. A randomized starting point is chosen, and then samples are made at uniform intervals. This principle is demonstrated in Figure 4.

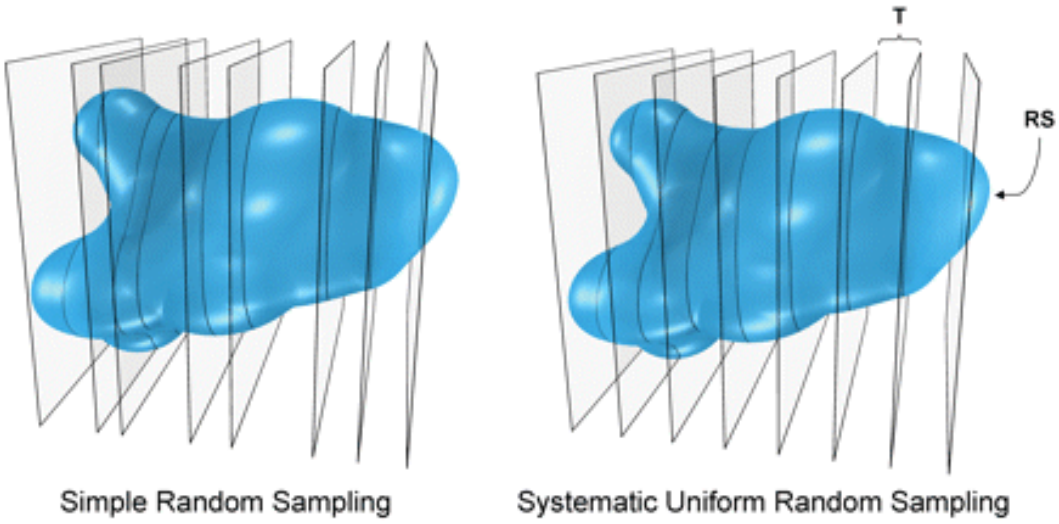

Figure 4.

Simple uniform random versus systematic uniform sampling of a structure. Simple uniform random sampling is depicted in the left figure, where the structure is cut at independent random positions along its longitudinal axis. Systematic uniform random sampling is depicted on the right. The structure is cut at a uniform constant interval (*T*) with a random start (RS), that is, the first cut is positioned uniformly random in the interval 0 to *T*, selected from a random number table or from randomly positioning of the organ in an agar block prior to sampling. Reprinted *from Toxicological Pathology* (Boyce et al., 2010b), with permission from SAGE Publications.

Counting objects is performed by placing a randomly placed counting frame which ensures that objects will not be re-counted in adjacent regions. In order to count an object, an arbitrarily defined, singly occurring marker for each object must be used. For neurons this is usually the nucleus or the nucleolus, since neurons have only one nucleus. Figure 5 demonstrates how the counting frame works:

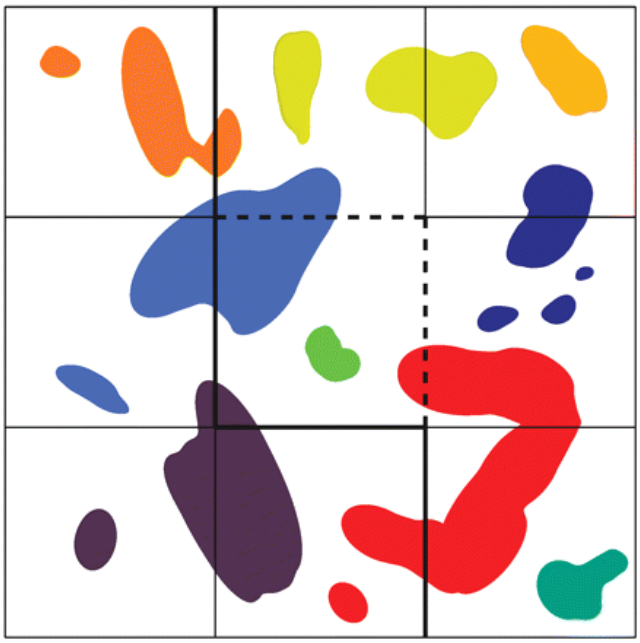

Figure 5.

The unbiased counting frame ensures that only objects that belong to the area of section sampled by the frame are counted. The figure shows a tessellation of nine potential sampling sites. The center square is sampled by an unbiased counting frame. Objects that "belong" to each of the nine possible sampling squares have a unique color, indicated by the color of the small object contained entirely in the respective square. The color assigned to the large objects reflects which sampling square they "belong to" using the counting rule of the unbiased counting frame: objects completely or partly contained in the frame (including those touching the dashed inclusion lines) and not touching the exclusion lines or their extensions (thick solid lines) are counted. The illustrated profiles represent the unique counting feature that for cells may be either nucleoli, nuclei, or cell tops. Reprinted *from Toxicological Pathology* (Boyce et al., 2010b), with permission from SAGE Publications.

1.8.2 Stereological probes

The Optical Fractionator is a commonly used probe used to count objects in stereological studies. One major potential source of bias in histological studies is that if cells or other objects are cut through, or "capped" during the sectioning process, then measuring those sliced profiles could lead to biased conclusions about their properties (Boyce et al., 2010b). The Optical Fractionator avoids this confound by using thick sections, and then sampling uniformly spaced counting sites within the X, Y and Z planes using SRS. The researcher can set "guard zones" at the top and bottom of the section to ensure that the sampling sites are within the thick section center, avoiding the potential to quantify "capped" objects (Gundersen, 1986; Gundersen et al., 1988a; Boyce et al., 2010b). Estimates of the objects sampled with this method are then extrapolated by using the fractionator principle, which is based on the assumption that by sampling a known fraction of a population, an estimate of the entire population can be derived (West et al., 1991; West, 1993).

The Nucleator probe is used to quantify the volume of objects in stereological studies. The Nucleator method has been developed to allow computer software to calculate the three-dimensional volume of objects such as cells from randomly oriented rays placed across the object, and then markers placed along the rays on boundaries of the object (Gundersen, 1988; Gundersen et al., 1988a; Mandarim-de-Lacerda, 2003).

In stereology, the coefficient of error (CE) is used as an index of accuracy of the estimation made. The CE is a computed value that takes into account various sources of sampling error, and a number of formulae have been derived to describe this error and predict the accuracy of stereological estimates (Gundersen et al., 1999; Boyce et al., 2010b). The CE arbitrarily accepted for most stereological studies is 0.15.

1.8.3 Staining for stereology

The technical demands of stereology can present issues in staining for the histologist. Whereas normal sections may be very thin (5-10 um), the sections used for the Optical Fractionator must be thick (35-60 um). In addition, it is necessary to be able to define the borders of the objects being counted, as well as to visualize the nuclei and/or nucleoli for use with the Optical Fractionator and the Nucleator probes. These needs can present a challenge, because it can be difficult to achieve complete staining of thick sections. The issue of visualization of individual structures is especially compounded in regions dense with dopaminergic neurons, such as the SN. As the name suggests, this region is already darkly pigmented with neuromelanin, and the TH stain used to stain catecholamine neurons also results in darkly stained brown neurons. In many studies a thionine or Nissl stain is used to counterstain the neurons and to

reveal non-dopaminergic glial neurons, but then this can make it very difficult to distinguish the nuclei, as it is easy for the histologist to confuse the counterstained non-dopaminergic cells with the nuclei. For these reasons, this study used a newly available commercial TH stain combined with a silver nucleolar stain (TH-AgNOR) to determine the effects of 6-OHDA partial unilateral intrastriatal lesions on cell number and morphology. AgNOR staining is a silver nitrate preparation that targets chromosomal proteins in the nucleolus known as nucleolar organizing regions (NOR). This stain, which has been extensively used by cancer pathologists to assess cell proliferation (Trerè, 2000), has recently been exploited for use as a co-stain in dopaminergic neurodegeneration studies (Switzer III et al., 2011).

In combination, the TH and AGNOR stains offer an optimal partnership for stereological analysis. TH immunostaining, which detects tyrosine hydroxylase, the rate-limiting enzyme in the pathway to dopamine synthesis, is a common method for assessing dopaminergic cells. Additionally, the AgNOR stain is used by pathologists to assess cellular proliferation in tissues. The AgNOR stain specifically pigments silver-binding proteins in the NOR's, resulting in the distinctive dark staining of the nucleoli within the cells. AgNOR staining use as an indicator of the nucleoli in neuroanatomical studies has been limited. One study used AgNOR staining to assess NORs in the hypothalamus of aged rats (Begega et al., 1999). However, our laboratory is the first to use AgNOR staining in the SNPC as a method suitable for stereological analysis of the dopaminergic neurons in a 6-OHDA model of Parkinson's disease. In particular, the stain's features lend themselves to a successful analysis of dopaminergic nucleoli, which are often obscured in TH staining alone. With the TH-AgNOR stain, the

histologist is able to clearly see the neuronal outline, dendrites, and axons, with the modified TH stain. Furthermore, the outline of the nucleolus can easily be seen with the TH-AgNOR stain, facilitating use with probes such as the Optical Fractionator, and making it possible to measure the outline of the nucleolus itself and to determine its volume using the Nucleator probe. Overall, this staining and stereology protocol offers promise as a useful tool set with which to analyze morphological changes to dopaminergic neurons in PD models.

CHAPTER 2

STATEMENT OF PURPOSE

2.1 Objectives

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder, which results in debilitating motor and non-motor symptoms, eventually leading to loss of independence and reduced quality of life for the patient. There are currently few effective treatments and no cures for PD. Since the progressive loss of dopaminergic function cannot be restored in the late stages of the disease, the best hope for future patients is prevention or early intervention. The early disease process is still poorly understood, however, especially the roles environmental or developmental factors such as n-3 polyunsaturated fatty acid (n-3 PUFA) consumption. Deficiencies in n-3 PUFAs have been implicated in dopaminergic function and in animal models of PD (Kidd, 2005; Cansev et al., 2008; Bousquet et al., 2011; Hacioglu et al., 2012; Eckert et al., 2013), although the effects of n-3 PUFAs on the number and morphology of substantia nigra pars compacta (SNpc) dopaminergic neurons is still poorly understood. Apart from the effects of diet, changes to dopaminergic morphology also occur in PD and PD models, and could be a very useful indicator of disease progression. Very little is known about dopaminergic morphology in the early stages of PD, however, since most of our knowledge about dopaminergic morphology in PD comes from post-mortem studies. Thus the long-term goals of this study were designed to increase our knowledge about the effects of n-3 PUFA deficiency and the unilateral intrastriatal 6-hydroxydopamine (6-OHDA) neurotoxic lesion on the SNpc dopamine neurons in the early stages of PD. The **OBJECTIVES** of this dissertation were to:

1. **Determine the effects of a Low n-3 PUFA diet on the unilateral intrastriatal 6-OHDA lesion, which models early to moderate PD.**
2. **Develop a method of quantitating the effects of this 6-OHDA model on dopaminergic neuronal and nucleolar morphology.**
3. **Determine the effects of this early PD model on neuronal and nucleolar morphology in midbrain dopaminergic subpopulations.**

2.2 Rationale for Models and Methods

2.2.1 Unilateral intrastriatal 6-OHDA neurotoxic lesion

The 6-OHDA neurotoxin, a well-accepted method of inducing parkinsonian lesions, is very similar in structure to dopamine; and is taken up by the monoamine transporters into the terminals, where it is transported back to and selectively destroys monoaminergic cells by oxidative mechanisms (Kostrzewa and Jacobowitz, 1974). Although the mechanism of neurodegeneration in PD is still unknown, there is evidence for the role of oxidative stress in PD (Dauer and Przedborski, 2003). The conditions created in our 6-OHDA model, including greatly decreased striatal dopamine accompanied by moderate SNpc neuronal loss and motor effects, reflect what has been found in early PD (Deumens et al., 2002), making this an appropriate early PD animal model.

This project employed the unilateral intrastriatal 6-OHDA model, using a single-site dose of 12.5 µg. The unilateral intrastriatal 6-OHDA model has been determined to be an appropriate 6-OHDA method for modeling early to moderate PD, and this model has been previously employed in our laboratory (Bethel-Brown et al., 2010). We use a

unilateral lesion so that the unlesioned hemisphere can serve as a control; and because bilateral lesions tend to result in such severe morbidity that the rats can become difficult to keep alive for the duration of the study (Schober, 2004). The single-site single-dose intrastriatal route of 6-OHDA infusion was chosen chiefly because the damage inflicted to the dopaminergic neurons is less severe than in more extensively destructive models such as the SNpc, medial forebrain bundle (MFB) or multiple-site and high-dose intrastriatal regimens (Kirik et al., 1998; Deumens et al., 2002). In addition, the striatum provides a larger target during stereotaxic surgery, which results in more consistent lesions than attempting to lesion the much smaller SNpc or MFB (Kirik et al., 1998). In addition, the direct trauma of injection into the SNpc could greatly interfere with morphological studies in this region. Overall, this method is a consistently reproducible and relevant model of early to moderate PD.

2.2.2 Changes to dopaminergic neuronal number and morphology quantified by stereology and TH-AgNOR staining

Losses in dopaminergic number and changes to dopaminergic neuronal morphology occur in PD and PD models, and are indicative of neurodegeneration-induced changes to the neurons (Jackson-Lewis et al., 1995; Gomide et al., 2005; Rudow et al., 2008). Stereological analysis uses random sampling techniques to generate unbiased estimates of three-dimensional characteristics of objects from two-dimensional slides, and is the optimal method for assessing the morphological changes induced by neurotoxic lesions and therapeutic interventions (Gundersen et al., 1988a; Schmitz and Hof, 2005). The Optical Fractionator and Nucleator probes have been shown to provide more accurate estimates of object number and volume, respectively,

than traditional histological quantitation methods (Gundersen et al., 1988a). Thus we chose the Optical Fractionator to quantify the changes to the dopaminergic neuronal number. For volumetric quantitation, the Nucleator was used to quantify neuronal volume; and in Aims 3 and 4, the Nucleator technique was adapted to a Double Nucleator technique by altering the MicroBrightField StereoInvestigator protocol to include placement of additional Nucleator markers within each neuron for simultaneous neuronal and nucleolar volume measurement.

The staining protocol chosen for this project had several advantages over traditional tyrosine hydroxylase (TH) thin-section staining with each brain on a separate slide. First, with MultiBrain® technology, up to 16 brains can be mounted on a single slide, and the brains are uniformly exposed to mounting and staining conditions. Second, with TH-staining with thiamine or Nissl counterstaining in the thick sections required by stereology (30-50μm), both over-staining and under-staining can occur. The staining can be so dark in areas dense with TH-positive neurons that counting individual cells becomes quite difficult. Furthermore, stereology probes require identification of a uniquely occurring single structure, usually the nucleus. With TH over-staining the nuclei can be obscured, and it is difficult to distinguish the nucleolus in most cells, making morphological studies of the nucleolus in the SNpc impossible. In addition, sometimes the stain will not penetrate into the middle of the thick sections, leaving the center neurons under-stained. With the modified tyrosine hydroxylase silver nucleolar (TH-AgNOR) stain, special adjustments were made to the staining protocol by NeuroScience Associates to account for the requirements of stereology and the challenges of staining dopaminergic neurons. Hydrochloric acid (HCl) was added to

improve stain penetration into the tissue, the TH antibody concentration was decreased to avoid over-staining of dense dopaminergic neuron populations, and the added AgNOR stain pigmented the nucleoli, so that they were clearly distinguishable from the neuronal body and the nucleolus in most cases. Altogether, these adjustments to the TH-staining protocol make it an ideal stain for use with stereological studies on dopaminergic neurons.

2.2.3 Rationale for Endpoints Chosen

2.2.2 Aim 1: The effects of an n-3 PUFA-deficient dietary and breeding protocol on a unilateral intrastriatal 6-OHDA lesion model in rats

Specific Aim 1 was designed to determine the effects of a dietary, and consequently tissue, n-3 PUFA deficiency on outcomes in an established animal model of early to moderate PD. We hypothesized that low dietary n-3 PUFA content influences disposition towards PD by altering the functional characteristics of nigrostriatal dopaminergic neurons, resulting in increased rotational behavior in n-3 PUFA deficient rats; and that low dietary n-3 PUFA content alters number and morphology of nigrostriatal dopaminergic neurons, rendering them vulnerable to increased damage in the unilateral intrastriatal 6-OHDA model of the disease. A previous study in our laboratory had shown a 33% decrease in SNpc neuronal number in 2nd litter n-3 PUFA deficient rats, suggesting that a chronic n-3 deficiency could lead to either decreased dopaminergic neurogenesis or to decreased ability to maintain dopaminergic neurons in adulthood; and that this process might also occur in PD (Ahmad et al., 2008). Thus, we chose to use amphetamine-induced rotational behavior assessment and stereological neuronal quantitation techniques to quantify the effects of

an n-3 PUFA deficient diet in the unilateral intrastriatal 6-OHDA model of PD. Although no change in SNpc neuronal volume was found in the rats from that study, decreased SNpc neuronal volume has been found in Parkinson's post-mortem studies, and decreased neuronal size has been found after 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in rodents (Lee et al., 1996). Therefore, we predicted that the Low n-3 diet might exacerbate the change in volume expected in the lesioned neurons due to 6-OHDA.

The Low n-3 PUFA diet protocol used in this project has been used in our laboratory to create rats with varying levels of brain phospholipid PUFA composition, while keeping all other husbandry parameters the same between the Low n-3 rats and controls. Previous breeding studies in our laboratory have shown that deficient n-3 fatty acids in the diet from conception results in a decrease of brain docosahexaenoic acid (DHA), and a concurrent increase in the n-6 fatty acid docosapentaenoic acid (DPA), by 32% and 54%, in first litter and second litter pups, respectively (Ozias et al., 2007). Thus, brain DHA content is replaced in the Low n-3 rats with the n-6 PUFA DPA, altering the ideal n-6/n-3 PUFA ratio. Consequent litters do not show marked decreases in percent of brain n-3 PUFAs after the second generation, so our study will be limited to two litters. The diet is designed to model the high n-6/n3 PUFA ratio typically found in Western diets (Simopoulos, 2008) (**Table 1**). This diet represents a clinically relevant model of n-3 deficiency, which is likely to be present in much of the aged population at risk for developing PD.

For Aim 1, rats of two subsequent litters were used with the Low n-3 PUFA diet breeding protocol to create rats with varying levels of brain n-3 PUFAs. These rats

were then given the unilateral intrastriatal 6-OHDA lesion, and then allowed to recover for seven days before d-amphetamine-induced rotational assessment. D-amphetamine-induced rotational behavior is a well-established method for validating neurotoxic PD model lesions (Schober, 2004; Potashkin et al., 2010). Since the lesioned rats do not display gross motor deficits, especially with mild to moderate neurotoxic lesions, a method is required to elicit the functional effects of the loss of striatal dopamine. Therefore amphetamine, which is an indirect dopamine agonist, is used to quantitate the functional effect of the lesion. The amphetamine-stimulated rats tend to rotate towards the side of the injury after amphetamine administration due to differing amounts of striatal dopamine in the intact and the lesioned hemispheres, (Ungerstedt and Arbuthnott, 1970). Rotations were quantified using force-plate rotometry, which provides an objective method of counting rotations (Fowler et al., 2001). Amphetamine-stimulated rotation is a good way to validate the functional presence of a lesion- however; rotational assessment is not very specific in regards to detecting the extent of neuronal loss, since rotations often do not correlate to the extent of the lesion as determined by loss of TH-positive neurons (Schwarting and Huston, 1996). However, since the purpose of our studies was to determine the extent of the SNpc lesion using stereology, this test was appropriate for our purposes of confirming a functional deficit of the lesion, and for testing the behavioral effects of an n-3 PUFA deficiency.

After an additional seven days after rotational behavior assessment, the rats were sacrificed, and brains were either immediately frozen for later dissection or perfused in 4% paraformaldehyde for sectioning and staining stereological analysis.

Frozen brains were dissected for quantitation of striatal dopamine by high-performance liquid chromatography-electrochemical detection (HPLC-EC) and frontal cortex PUFA content by gas chromatography (GC). The additional seven days was allowed between amphetamine-induced rotational assessment and sacrifice to ensure clearance of any residual amphetamine [>10 $t_{1/2}$s, tissue $t_{1/2}$ = 5-9 hrs for the elimination phase (Kuhn and Schanberg, 1978), and to allow for optimal neurodegeneration. Time- course studies have shown that the majority of damage after 6-OHDA occurs within 14 days (Przedborski et al., 1995)- thus, this was chosen as an appropriate time point for brain fixation and histology and striatal dopamine analysis.

These results are found in Chapter 4.

2.2.3 Aim 2: TH-AgNOR staining to determine the effects of a unilateral intrastriatal 6-hydroxydopamine lesion in the substantia nigra

Aim 2 was to develop a method to quantify dopaminergic neuronal morphology using stereological techniques combined with TH-AgNOR staining. Dopaminergic neuronal assessment methods are especially needed for quantifying the effects of treatments in early PD models, in which subtle changes to neurons can be difficult to detect with behavioral assessments and non-stereological neuronal quantitation techniques (Schmitz and Hof, 2005). We hypothesized that the TH-AgNOR staining method combined with stereological techniques would be a suitable method to quantify dopaminergic neuronal morphology in the rat, and that the TH-AgNOR staining method combined with stereology would reveal changes to the SNpc dopaminergic neurons induced by the unilateral intrastriatal 6-OHDA neurotoxic lesion. Thus we used this stain combined with MicroBrightField StereoInvestigator Optical Fractionator and Nucleator

software probes to quantify the SNpc planimetric volume, neuronal number and neuronal volume of SNpc dopaminergic neurons after 6-OHDA treatment.

Many PD studies on neuronal loss or changes in dopaminergic morphology in rodent models include quantitation of not only the SNpc, but also of the surrounding substantia nigra medialis, substantia nigra lateralis, substantia nigra reticularis, and ventral tegmental area (VTA). While such broad inclusion of dopaminergic regions makes for much easier region definition, especially in low-magnification quantitation methods such as TH-positive densitometry in which distinguishing the borders of the individual neuronal groups can be nearly impossible; these neuronal areas have differing functions and neuronal types (Jimenez-Castellanos and Graybiel, 1987; Da Cunha et al., 2009; Cho and Fudge, 2010). Thus grouping some or all them all together for counting and morphology assessment can lead to inconsistent results among various studies; and to inconclusive clinical relevance if it is unknown which group of neurons was affected by a particular lesion or treatment. Thus, we chose to carefully define and quantify only the SNpc for two main reasons:

1. The SNpc contains the majority of dopaminergic neurons which project to the striatum (Jimenez-Castellanos and Graybiel, 1987), and thus contains the population most likely to be affected by a striatal 6-OHDA region and most likely to reveal potentially subtle changes induced by a mild to moderate 6-OHDA lesion modeling early PD.

2. The SNpc contains more densely packed TH-positive neurons than other midbrain dopaminergic neuronal groups. Thus, for the purposes of demonstrating the optimal advantages of the modified TH-AgNOR staining technique, these neurons were the most likely in which we would be able to see a clear advantage of the better individual neuronal and nucleolar visualization provided by the TH-AgNOR stain.

These results are found in Chapter 5.

2.2.4 Aim 3: Altered neuronal and nucleolar morphology in substantia nigra dopamine neurons following 6-hydroxydopamine lesion in rats

Aim 3 was to use and expand the method developed in Aim 2 to quantify and characterize 6-OHDA-induced changes in nucleolar morphology. We hypothesized that the unilateral intrastriatal 6-OHDA neurotoxic lesion would alter SNpc dopaminergic nucleolar morphology. We chose to quantify the morphology of the nucleolus because it is a key organelle involved in the cell's response to oxidative stress, which is proposed as both a cause and an effect in PD (Dauer and Przedborski, 2003; Hartmann, 2004). Since the nucleolus in an important regulator of the cell's response to oxidative stress, and the place of rRNA production and assembly, and an important potential therapeutic target (Guarente, 1997; Hetman and Pietrzak, 2012), it is vital that this organelle in the early stages of the disease be better understood, and that quantitative methods be developed for assessing changes to the nucleolus after lesion induction or treatments. Although changes to SNpc neuronal number and neuronal volume have been investigated in PD and PD models, little is known about the changes to morphology to

the nucleolus in either animal models or in the disease, with only a single known study which quantified the volume of the nucleolus in PD brains (Mann and Yates, 1982). Using the TH-AgNOR stain and MicroBrightField StereoInvestigator software, we used the Optical Fractionator and Double Nucleator probes to quantify the SNpc planimetric volume, neuronal number and both the neuronal body and the nucleolar volume in the SNpc.

These results are found in Chapter 6.

2.2.5 Aim 4: Differentially altered neuronal number and morphology in midbrain dopaminergic subregions after a unilateral intrastriatal 6-OHDA lesion

Specific Aim 4 was to use the stereological method used in Aim 2 to determine effects of 6-OHDA on neuronal number and changes to neuronal and nucleolar volume in midbrain dopaminergic subregions. This project especially focused on the VTA and RRF, two dopaminergic subregions which are likely involved in the very early development of the disease, and could therefore lend insights into the cause and the development of a cure for PD. We hypothesized that the unilateral intrastriatal 6-OHDA lesion differentially affects neuronal number and neuronal and nucleolar morphology in the SNpc, VTA and retrorubral field (RRF). The substantia nigra (SN), VTA and RRF, corresponding to the A9, A10 and A8 groups of midbrain dopaminergic neurons as classified by Dahlstrom and Fuxe, are geographically separate groups of neurons with some overlapping projections. Notably, all are affected in PD (Dahlstrom and Fuxe, 1964; German et al., 1989; McRitchie et al., 1997; Jellinger, 1999; Rodríguez et al., 2001). Most of the mesostriatal dopamine neurons involved in the motor signs of PD originate in the SNpc (Jimenez-Castellanos and Graybiel, 1987). The VTA is the origin

of the majority of mesocortical and mesolimbic dopamine neurons, and is implicated in the non-motor dopamine-dependent aspects of Parkinson's such as depression, cognitive and affective deficits, which often present up to decades before motor signs (Marsden, 1990). The RRF neurons are known to project to both the nigrostriatal and mesolimbic systems (Jimenez-Castellanos and Graybiel, 1987). The RRF is implicated in the presence of tremor and in the early-presenting sign of sleep disturbances in Parkinson's (Lai et al., 1999; Kolasiewicz et al., 2012). There are very few studies addressing how the morphology of the dopaminergic neurons in these regions are differentially affected by PD. Furthermore, there are no known studies of nucleolar morphology in the VTA and RRF in PD models, making this a promising area of discovery.

The Optical Fractionator and Double Nucleator stereological probes used in Aim 2 were used in this aim to determine effects of 6-OHDA on neuronal number and changes to neuronal and nucleolar volume in the SNpc, VTA and RRF TH-AgNOR-stained neurons. The SNpc and RRF regions are fairly easy to outline at low magnification, but definition of the VTA has presented a challenge to neuroanatomists to define (German and Manaye, 1993). The VTA is quite difficult to define because it is a large area with irregular borders surrounded by other groups of dopaminergic neurons which can be quite difficult to differentiate from those of the VTA. Thus, we used a strictly defined anatomical protocol, defined in the Methods chapter, to outline and quantify this region.

These results are found in Chapter 7.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and methods

All experiments were performed in compliance with the NIH Guide for the Care and Use of Animals and were approved by the University of Kansas Medical Center Institutional Care and Use Committee.

3.2 Animals and husbandry

Male Long-Evans rats (90-100 days old) were bred in-house (breeding stock obtained from Harlan, Indianapolis, IN). Rats were housed in a temperature- and humidity-controlled animal facility with a 14:10-h light-dark cycle (on at 06:00 h) with *ad libitum* access to water and chow. The control diet was AIN-93G (Teklad, Indianapolis, IN), and the n-3 Deficient diet was formulated as described (**Table 1**). Adult breeders (P70) were allowed to acclimatize in the climate-and temperature controlled facility for at least one week before breeding. Dams were placed on the experimental diets at the time of initial mating. Weaned pups continued on their maternal diet throughout the study. Dams were allowed to recover for one week before being mated to produce the second litter. Litters were culled to 8 pups on postnatal day 1 and weaned on postnatal day 20. Rats were housed four per cage from weaning until lesion surgery, after which they were kept in single housing until euthanized to ensure undisturbed recovery.

3.3 Experimental Diets

Our laboratory has extensive experience studying the effects of dietary n-3 polyunsaturated fatty acid (n-3 PUFA) manipulations on behavior and neurochemistry (Levant et al., 2006b; Levant et al., 2006a; Levant et al., 2006c; Levant et al., 2007a;

Levant et al., 2007b; Ozias et al., 2007; Levant et al., 2008). The diets are 7% fat by weight and vary only in the fatty acid composition. The Control diet, AIN-93G (Teklad, Indianapolis, IN) is made from non-hydrogenated soybean oil (70 g/kg) and contains the recommended levels of ALA. The Low n-3 diet is identical to the Control, except that it is formulated with both safflower (66.5 g/kg) and soybean (3.5 g/kg) oils, and contains approximately 1% of the ALA of the Control diet (**Table 1**).

Table 1. Diet Composition and Fatty Acid Composition

Diet Fatty Acid Composition (g/kg)	Control	Low n-3	Teklad Global 2016
14:00	0.29	1.23	0.16
16:00	9.84	7.23	6.16
18:00	6.42	5.84	2.29
20:00	0.51	0.19	0.14
22:00	0.33	0.39	0.21
24:00	ND	ND	ND
16:01	ND	0.10	ND
18:01	13.39	10.99	7.38
20:1n-9	0.06	0.16	0.28
18:2n-6	28.68	37.20	17.78
18:3n-3 (ALA)	5.32	0.54	1.59
20:2n-6	0.22	0.17	0.41
20:4n-6	ND	0.62	0.11
20:5n-3 (EPA)	ND	ND	ND
22:5n-3	ND	ND	ND
22:6n-3 (DHA)	ND	ND	ND

3.4 Procedures

Rats were subjected to a partial unilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesion. D-amphetamine-stimulated rotation was assessed 7 days later. After an additional 7 days to ensure clearance of drug [>10 $t_{1/2}$s, tissue $t_{1/2}$ = 5-9 hrs for the elimination phase (Kuhn and Schanberg, 1978), rats were euthanized for dopamine quantitation or histology. Separate groups of rats were used for the histological and neurochemical end points, but all rats were evaluated for amphetamine-stimulated rotation.

3.5 6-OHDA lesions

Rats were anesthetized with isoflurane (3%) and secured in a stereotaxic frame. After creating a burr hole in the skull over the right striatum (AP+ 1.0 mm, ML+ 2.5 mm vs. bregma), a 26-gauge dome-tipped needle attached to a microliter syringe with Teflon tubing was lowered into the striatum (5.0 mm from dural surface). 6-OHDA (12.5 µg administered in a volume of 5.0 µL, at 2.5 µg/µL, in 0.9% saline with 0.1% ascorbic acid) was infused at the rate of 0.5 µL/min based on previously published methods (Bethel-Brown et al., 2010; Bethel-Brown et al., 2011). The infusion needle was left in place for an additional 5 minutes and then slowly withdrawn. Bone wax was applied to the burr hole, and the scalp closed with wound clips. Buprenex (0.05 mg/kg body weight) and ketoprofen (5 mg/kg body weight) were given post-surgery, with follow-up doses of ketoprofen for two days, and animals were allowed to recover in single housing.

3.6 D-amphetamine-stimulated rotations

Seven days after surgery, rats were injected with d-amphetamine sulfate (2.5 mg/kg, sc, in saline) and placed in a rotometer for 60 minutes. The rotometer is a 26.5 cm diameter cylinder placed atop a force-sensing actometer surface [see (Fowler et al., 2001). D-amphetamine sulfate was injected (2.5 mg/kg in 0.9% saline, S.C.) and animals were placed in a rotometer for 60 min. The rotometer is a force-sensing actometer with a 26.5 cm diameter cylindrical chamber (Bethel-Brown et al., 2010). Custom software was used to quantify the number of rotations.

3.7 Determination of striatal dopamine content

Rats were decapitated, and brains rapidly removed and frozen on dry ice. Left and right caudate-putamen were isolated by freehand dissection on ice and stored at -80° C. Concentrations of dopamine were quantified using an isocratic high-performance liquid chromatography-electrochemical detection (HPLC-EC) system (ESA Coulochem III, Chelmsford, MA) coupled to a Coulochem III dual-channel electrochemical array detector (E1 + 0.35 mV and E2 − 0.25 mV using a 5011 dual analytical cell ESA Model 5100A) as previously described (Levant et al., 2008). Tissues were extracted in 0.3 N perchloric acid. Analytes were separated using a C18 reverse phase column (ESA HR-80 C18, 4.6 mm × 80 mm, 3 µm) with a pH 4.0 citrate-acetate mobile phase containing 4.0% methanol and ~0.35 mM 1-octane-sulfonic acid at a flow rate of 1.8 ml/min. 3,4-dihydroxy-benzylamine was used as the internal standard. Protein concentrations of the extracted tissues were determined by the BCA method

(Pierce, Rockford, IL). Striatal dopamine concentrations were expressed as ng/mg protein.

3.8 Fatty Acid Content Analysis

Phospholipids were extracted from frontal cortex, a brain region not required for other endpoints according to a protocol previously used in our laboratory (Levant et al., 2004). Phospholipids were isolated by thin layer chromatography. The phospholipid band was removed and transmethylated with boron trifluoride methanol to produce fatty acid methyl esters, which were then separated on a Varian 3400 gas chromatograph (GC) with an SP-2330 capillary column, with helium used as the carrier gas. Peaks were identified by comparison to authentic standards. Data were expressed as percent of total fatty acids on the basis of peak area.

3.9 Histological analysis

3.9.1 TH-AgNOR staining

Rats were deeply anesthetized with pentobarbital and perfused with 0.1M phosphate buffered saline (PBS) (pH 7.2), followed by 4% paraformaldehyde in PBS, and decapitated. Skulls were post-fixed at 4° C for at least 7 days, and then transferred to PBS. Brains were extracted and coronal sections (50 µm) were prepared and stained by NeuroScience Associates (Knoxville, TN), using MultiBrain™ Technology. Free-floating sections were stained with a modified tyrosine hydroxylase plus silver nucleolar stain (TH-AgNOR). Sections were mounted on gelatinized (subbed) glass slides.

3.9.2 Stereology

Every sixth section containing SNpc was selected for stereological quantitation (Bregma −4.70 to −6.30mm). TH-AgNOR-stained cells were quantified using the Microbrightfield Stereoinvestigator software package combined with a Nikon Eclipse TE2000-U microscope coupled to a Heidenhein linear encoder unit and a QImaging Retiga-2000R color digital video camera. Guided by an atlas (Paxinos and Watson, 1998), the SNpc (Bregma -4.80 to -6.30) was outlined in rostrocaudal sections at 4X magnification to exclude the parabrachial pigmented nucleus (PBP), substantia nigra pars reticulata, SN pars lateralis, the accessory optic tract, SN pars medialis, and the VTA. The VTA was outlined (Bregma -5.20 to -6.80) to exclude the SNpc, SN medialis, fasciculus retroflexus, mammillotegmental tract, mammillary peduncle, interfascicular nucleus, the rostral line of the nucleus of Raphe, the medial lemniscus, the paranigral nucleus, the visual tegmental relay zone, the accessory optic tract, all portions of the interpeduncular nucleus, and the RRF. The PBP was excluded as much as it was distinguishable from the boundaries of the VTA (which is difficult in the caudal portions), but all sections were photographed and compared for consistent definition of regions. The RRF was outlined (Bregma -6.30 to -7.04) to exclude the substantia nigra reticularis and the VTA. The variations in brain positioning in the MultiBrain® embedding process provide a randomized sectioning start for each brain quantified. Cells were counted and volumes measured at 100X magnification using the simultaneous application of the optical fractionator and a double nucleator method. The nucleus was used as the unique marker for each neuron. The Double Nucleator was then employed by automatic software placement of four randomly oriented crossed rays centered at the

counting point, and four discriminately placed markers on the outside borders of each of the nucleolus and the outline of the cell body, respectively. In some neurons the nucleolus was visible within the nucleus, but dark staining of the nucleus made it impossible to accurately distinguish the borders of the nucleolus for placement of the nucleator probe. These neurons were included in the total cell count, but were not included in the volumetric analyses because it was not possible to employ the double nucleator technique. A maximum coefficient of error of 0.15 (m=1) was accepted for all results, except for the TH-positive neuronal number values for the RRF in Aim 4, in which the CEs ranged from 0.11 to 0.22 (Gundersen et al., 1999).

3.10 Data analysis

All data are presented as the mean ± S.E.M. Outliers identified by Systat on initial analysis were discarded. A significant difference was assumed if $P < 0.05$.

For the amphetamine-induced rotational analysis, rats were included in data analyses even if they had a very low number of rotations.

For Aim 1, brain phospholipid PUFA composition and rotational behavior were analyzed by two-way ANOVA with factors of diet and litter (Systat, v.12). Striatal dopamine concentration was analyzed by three-way ANOVA with factors of diet, litter and lesion (repeated measure). Post hoc comparisons were made using 1-way ANOVA and the Fisher's Least Significant Difference test. The difference in percentages of neuronal number and volume change in 2^{nd} litter rats were analyzed by Student's-t test (Instat, v. 3.0).

For Aims 2, 3, and 4, the effects of 6-OHDA between the ipsilateral and contralateral hemispheres were tested for statistical significance using paired t-tests (SigmaPlot, v.11.0). Within-subject changes in any parameter were expressed as a percentage relative to the contralateral side.

For Aim 4, the mean percentage changes among the SNpc, VTA and RRF were analyzed by repeated-measures one-way ANOVA, and post hoc comparisons were made using Student-Newman-Keuls. In the cases of neuronal number and neuronal volume, nonparametric repeated measures ANOVA followed by Dunn's multiple comparisons test was used (Instat, v.3.0).

CHAPTER 4

THE EFFECTS OF AN N-3 PUFA-DEFICIENT DIETARY AND BREEDING PROTOCOL ON A UNILATERAL INTRASTRIATAL 6-OHDA LESION MODEL IN RATS

4. 1 Abstract

Rats fed an n-3 polyunsaturated fatty acid (n-3 PUFA)-deficient diet have fewer nigrostriatal dopamine neurons (Ahmad et al., 2008), suggesting that low dietary n-3 PUFAs may increase vulnerability to Parkinson's disease (PD). In view of this potential increased vulnerability, the effects of a neurotoxic lesion PD model were examined in n-3 PUFA-deficient rats. The effects of partial unilateral striatal 6-hydroxydopamine (6-OHDA) lesions were determined in male Long-Evans rats with two levels of decrease in brain phospholipid DHA content (36% and 42%) produced using an n-3 PUFA-deficient diet and breeding protocols. A functional effect of the lesion was assessed 7 days post-surgery by measuring d-amphetamine (2.5 mg/kg, s.c.)-induced rotational behavior and striatal dopamine content. In rats with a 42% decrease in brain docosahexaenoic acid (DHA), the neuronal number and neuronal volume of tyrosine-hydroxylase silver nucleolar (TH-AgNOR)-stained substantia nigra (SN) neurons were determined by stereological analysis. Consistent with the loss of dopamine neurons after 6-OHDA infusion, amphetamine induced rotation towards the lesioned side, and striatal dopamine was depleted by more than 90% in all treatment groups ($P<0.001$), although diet had no effect on these parameters. The 6-OHDA lesion induced a decrease in TH-positive ipsilateral SN neuronal volume (51% ± 10% for Low n-3 rats and 45% ± 5% for Control rats, $P<0.001$), but no significant change in ipsilateral neuronal volume in either diet. There was no effect of diet on TH-AgNOR-stained dopaminergic neuronal number or volume. While this finding suggests that the effect of 6-OHDA was similar in both diet groups, further studies need to be pursued in order to establish whether there is a threshold effect of DHA deficiency in prevention of 6-OHDA-induced neurotoxic effects.

4.2 Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disease characterized by the loss of striatal dopamine and consequent clinical signs of tremor, rigidity and bradykinesia. Although the etiology of the majority of PD cases is still not known, evidence strongly points to several potentially modifiable contributors to neurodegenerative diseases such as PD, including oxidative stress and neuroinflammation (Jenner, 2003; Griffin, 2006; Dutta et al., 2008; Gao and Hong, 2008; Orr and Bazinet, 2008a; Amor et al., 2010; Kones, 2010). Dietary deficiency of polyunsaturated fatty acids (PUFAs) is a potential contributing factor to the development of neurodegenerative diseases, including PD. N-3 PUFAs such as docosahexaenoic acid (DHA) are crucial to brain composition and optimal function (Willatts et al., 1998; Birch et al., 2000), and have been implicated in the development and sequelae of numerous diseases in humans, including neurodegenerative diseases (Youdim et al., 2000; Orr and Bazinet, 2008a; Bazan et al., 2011).

Dietary n-3 PUFA deficiency is pervasive in Western society, and likely impacts a variety of human health outcomes. DHA and the n-6 PUFA arachidonic acid (AA) must be consumed in the diet, or their essential fatty acid precursors α-linolenic acid (ALA) and linoleic acid (LA) must be provided. In humans, most of the DHA in the brain is accumulated in late gestation and early childhood through the mother's dietary consumption and breast milk, with lifelong turnover (Clandinin et al., 1980a; Clandinin et al., 1980b; Hadley et al., 2009). In rats, accretion of brain DHA occurs primarily during the last three days of gestation through weaning (Kishimoto et al., 1965; Green and Yavin, 1996). Western diets are very low in n-3 fatty acids, and have an n-6/n-3 as high

as 16.7/1 (Simopoulos, 2003). A very high n-6/n-3 ratio has been implicated in a variety of human diseases such as coronary artery disease, hypertension, diabetes, arthritis, osteoporosis, autoimmune disorders, cancer and mental health (Simopoulos, 2008). Low dietary n-6/n-3 ratios have been shown to favorably alter LDL status, improve cardiovascular health, have anti-proliferation effects in colorectal cancer, decrease the risk of breast cancer, and decrease inflammation in rheumatoid arthritis and asthma (Haworth and Levy, 2007; Calder and Yaqoob, 2009; Fetterman and Zdanowicz, 2009; Hartwich et al., 2009; Lavie et al., 2009). Thus the high n-6/n-3 PUFA ratio found in Western diets may be the harbinger of a variety of complicated health effects that will greatly impact the aging population.

Indeed, PUFAs have already been implicated in neurodegenerative diseases, including PD. Although there is a lack of conclusive clinical evidence for the role of n-3 PUFA'S in PD, the Rotterdam study concluded that a high dietary intake of unsaturated fatty acids may be protective against PD (de Lau et al., 2005). Apart from motor improvements, n-3 PUFAs have known benefits in several affective disorders and may be especially beneficial in the non-motor aspects of PD such as depression. In one recent study, consumption of fish oil improved depressive symptoms in PD patients, when taken either with or without antidepressants (da Silva et al., 2008). Although the difficulty of completing large-scale clinical trials due to confounds and inter-study variations has not established a clear link between n-3 PUFAs and PD, PD models have shown promising evidence that n-3 PUFAs play an important role in dopaminergic neuronal maintenance and the modulation of stressors similar to those seen in PD. In PD, DHA supplementation has improved a variety of outcomes, such as decreased L-

DOPA dyskinesia (Samadi et al., 2006), decreased loss of dopaminergic neurons , and decreased striatal dopamine depletion (Bousquet et al., 2011). Overall, more studies are needed to fully understand the role of PUFAs in PD.

In this study, the effects of a dietary n-3 PUFA deficiency in the unilateral intrastriatal 6-hydroxydopamine model of PD in rats were determined using a breeding model that produced different levels of DHA deficiency with two consecutive litters from the same dams. Previous breeding studies in our laboratory have shown that a decrease in n-3 fatty acids in the diet results in a decrease of brain DHA of 32% and 54%, in first litter and second litter pups, respectively (Ozias et al., 2007). Thus brain DHA content is replaced in the Low n-3 rats with the n-6 PUFA DPA, altering the ideal n-6/n-3 PUFA ratio. Consequent litters do not show marked decreases in percent of brain n-3 PUFAs after the second litter (Levant et al., 2006a), so this study was restricted to two litters.

The unilateral intrastriatal 6-hydroxydopamine (6-OHDA) PD model employs 6-OHDA, a neurotoxin resembling dopamine, which is taken up by monoamine transporters and causes neurodegeneration by oxidative mechanisms (Kostrzewa and Jacobowitz, 1974; Glinka et al., 1997). This method is especially relevant for modeling the early stages on PD, in which neuroprotectants and dietary interventions might be especially crucial. To determine the effects of an n-3 PUFA deficiency in this model, we assessed brain PUFA composition, amphetamine-induced rotational behavior and striatal dopamine content in both 1st and 2nd litter rats. Morphological changes to substantia nigra pars compact (SNpc) neurons as quantified by stereological techniques and the tyrosine hydroxylase silver nucleolar (TH-AgNOR) staining method were

assessed only in 2nd litter rats, which exhibited the greatest diet-induced change in brain phospholipid fatty acid composition.

4.3 Results

4.3.1 Effects of diet and breeding protocol on growth

The mean weight of both the Low n-3 and Control 2nd litter rats at sacrifice (70-100 days) was increased compared to 1st litter animals (P<0.001 by ANOVA), but there was no effect of the diet on weight.

4.3.2 Effects of diet, breeding protocol and 6-OHDA lesion on brain phospholipid fatty acid composition

1st litter rats raised on the Low n-3 diet had an 36% decrease in brain DHA phospholipid content compared to those raised on the Control diet, and 2nd litter Low n-3 rats had an 42% decrease in brain DHA compared to Control rats, although the difference between 1st and 2nd litter Low n-3 brain DHA was not significant (**Fig. 1**). The loss in 1st litter rats is greater than expected with previous studies in our laboratory, in which DHA losses of approximately 32% and 55% were found in 1st and 2nd litters, respectively (Ozias et al., 2007). Furthermore, the 2nd litter rats did not achieve as high a level of DHA loss as in previous studies, which may be the reason why the morphological changes to DHA-deficient dopaminergic neurons observed in a previous study in our laboratory (Ahmad et al., 2008) were not replicated. There were compensatory increases in the Low n-3 rats of the n-6 fatty acid docosapentaenoic acid [(n-6) DPA, 22:5(n-6)] compared to the Control rats (p<0.001 by ANOVA), and DPA was increased in the 2nd litter compared to the 1st (P<0.001 by ANOVA). There was no

effect of diet or litter on levels of arachidonic acid [20:4(n-6)], in concordance with previous studies (Galli et al., 1971).

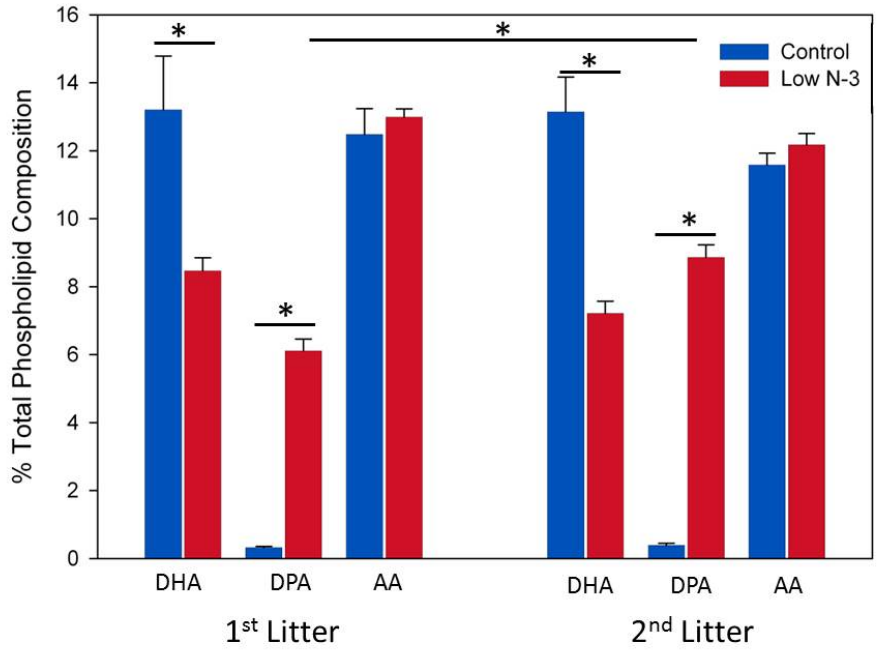

Figure 1.

Effects of diet and litter on brain total phospholipid fatty acid composition. Data are presented as the mean ± SEM (n = 6-11). *P<0.001 by ANOVA and Fisher's LSD test

4.3.3 Effects of diet, breeding protocol, and 6-OHDA lesion on striatal dopamine concentration

The dopamine levels measured in the hemispheres contralateral to the lesion were comparable to those found in previous studies (Kilts et al., 1981; Widerlöv et al., 1982; Davis et al., 2010; Levant et al., 2011). 6-OHDA lesion resulted in decreased dopamine concentrations in the ipsilateral dopamine concentrations all rats in all groups (vs. contralateral concentration, $P<0.001$ by paired t-test, n = 9-11). There was a mean striatal dopamine depletion of the ipsilateral side compared to the contralateral of 91 ± 26% in 1st Litter Control rats, 95 ± 30% in 1st Litter Low n-3 rats, 95 ± 30% in 2nd Litter Control rats, and 91 ± 26% in 2nd Litter Low n-3 rats. There was no effect of diet or litter on percentage of striatal dopamine loss, as determined by ANOVA (**Fig. 2**).

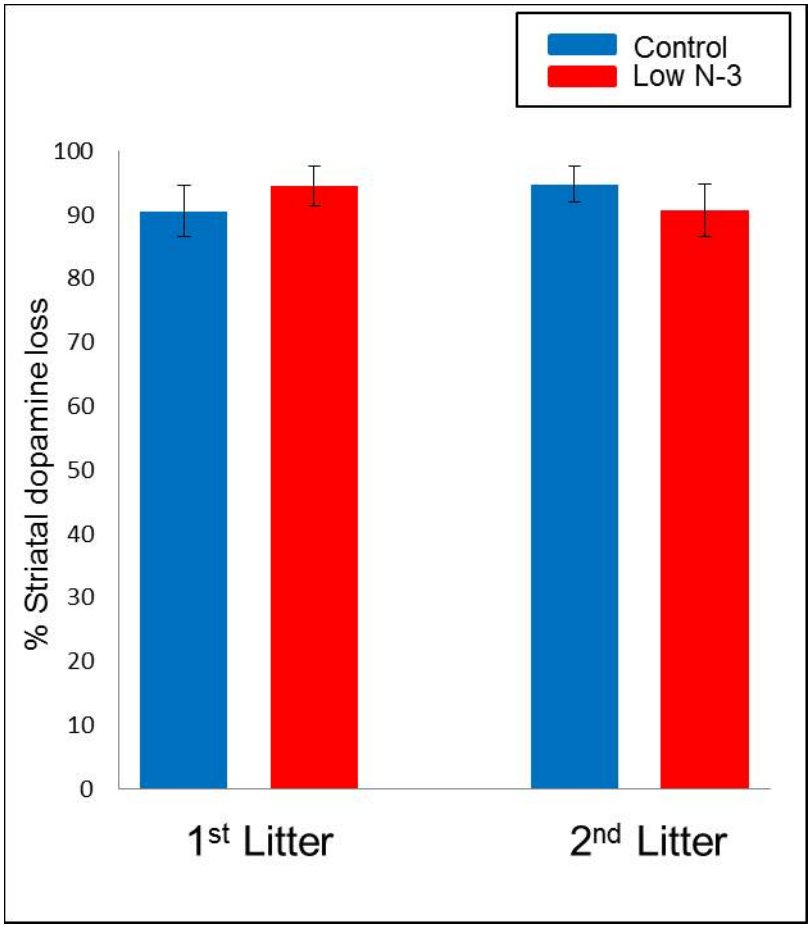

Figure 2.

Effects of diet, breeding protocol and unilateral intrastriatal 6-OHDA lesion on striatal dopamine. There was no effect as determined by ANOVA of diet or litter on percentage of striatal dopamine loss (n= 9-11).

4.3.4 Effects of diet and breeding protocol on amphetamine-induced rotational behavior

The mean total number of d-amphetamine-stimulated rotations completed by lesioned rats during the 60-minute observation period was 124 ± 26 in the 1st litter Control rats, 157 ± 32 in the 1st litter Low n-3, 153 ± 30 in the 2nd litter Control rats and 128 ± 22 in 2nd litter Low n-3 rats, indicating a functional dopaminergic deficit in the ipsilateral striatum. The numbers of recorded rotations in the 60-min recording period ranged from 2 to 641 in 1st litter Low n-3 rats, from 1 to 426 in 1st litter Control rats, from 8 to 358 in 2nd litter Low n-3 rats, and from 4 to 464 in 2nd litter Control rats. There was no effect of either diet or litter on the mean total numbers of amphetamine-stimulated rotations as determined by ANOVA (**Figure 4**).

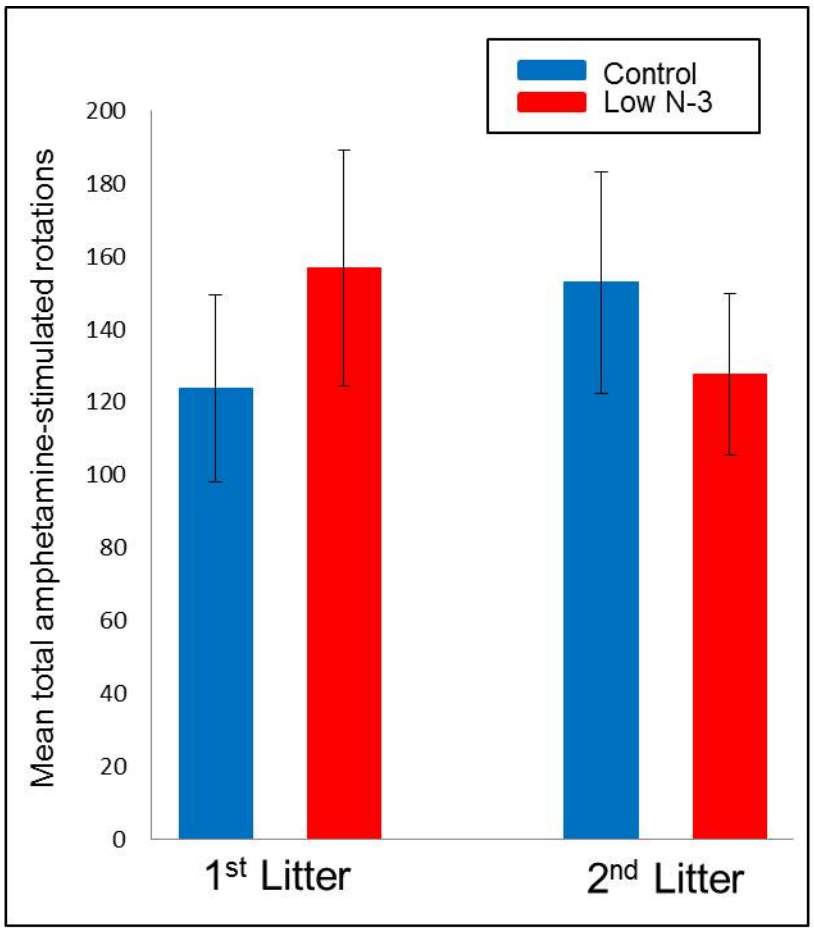

Figure 3.

Effects of diet and breeding protocol on amphetamine-induced rotational behavior. There was no effect of either diet or litter on mean total amphetamine-stimulated rotations as determined by ANOVA (n = 20-21).

4.3.5 Effects of diet and lesion on TH-positive SNpc neuronal number in 2nd Litter rats

In 2nd litter rats, which had the greatest decrease in brain DHA content achieved in this study, the mean number of SNpc TH-positive neurons on the intact side (12,872 ± 469 in Control rats and 12,545 ± 371 in Low n-3 rats) was similar to findings in previous stereological studies (Kirik et al., 1998; Lindner et al., 1999; Carvalho and Nikkhah, 2001; Debeir et al., 2005; Gomide et al., 2005; Ahmad et al., 2008; Eriksen et al., 2009). The estimated numbers of contralateral TH-positive SNpc neurons in individual rats ranged from 10,336 to 13,686 in Low n-3 rats and from 11,087 to 14,500 in Control rats. The estimated numbers of ipsilateral TH-positive SNpc neurons ranged from 4,446 to 11,149 in Low n-3 rats and from 3,257 to 11,099 in Control rats. All rats had reduced numbers of TH-positive SNpc neurons after 6-OHDA lesion, and both diets showed decreased number of TH-positive cells in the ipsilateral SNpc compared to the contralateral SNpc ($P<0.001$ by paired t-test). The losses of TH-positive SNpc neurons ranged from 19% to 57% in Low n-3 rats, and from 7% to 75% in Control rats. The mean within-subject decrease in TH-positive neuron number between the ipsilateral and contralateral SNpc was 51% ± 10% for Low n-3 rats and 45% ± 5% for Control rats. There was no difference between the diets on mean percent loss of neurons on the ipsilateral side (t-test, $P>0.05$). (**Figure 4**)

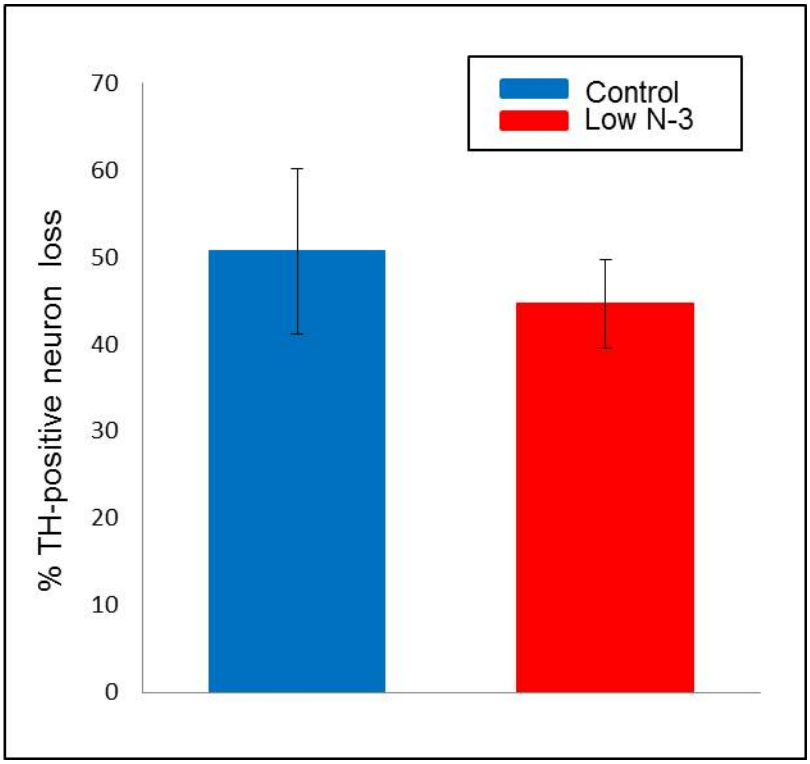

Figure 4.

Effects of diet on TH-positive SNpc neuron number. There was no effect of diet on percent neuronal loss after 6-OHDA (t-test, P>0.05, n = 7-8).

4.3.6 Effects of diet and lesion on TH-AgNOR positive neuronal volume in 2nd Litter rats

The mean neuronal volume of SNpc TH-positive neurons on the intact side (3,444 ± 118 μm^3 in Control rats and 3,037 ± 129 μm^3in Low n-3 rats) was comparable to findings in previous stereological studies (Gomide et al., 2005; Healy-Stoffel et al., 2012). Five of 7 Control and 5 of 8 Low n-3 rats exhibited decreased average neuronal body volume after 6-OHDA lesion. The estimated volumes of contralateral TH-positive SNpc neurons ranged from 2,905 to 4,103 μm^3 in Low n-3 rats and from 3,206 to 4,109 μm^3 in Control rats. The mean estimated volumes of ipsilateral TH-positive SNpc neurons ranged from 2,581 to 3169 μm^3 in Low n-3 rats and from 2,489 to 3513 μm^3 in Control rats. The change in TH-positive SNpc neuron volume ranged from +5% to -23% in Low n-3 rats, and from +3% to -39% in Control rats. There was a mean within-subject decrease in neuronal body volume of 11 ± 6% in Low n-3 rats and 7 ± 4% in Control rats, although neither decrease was significant (paired t-test, $P>0.05$). There was no difference between the diets on mean percent loss of neuronal volume on the ipsilateral side (t-test, $P>0.05$). (**Figure 5**)

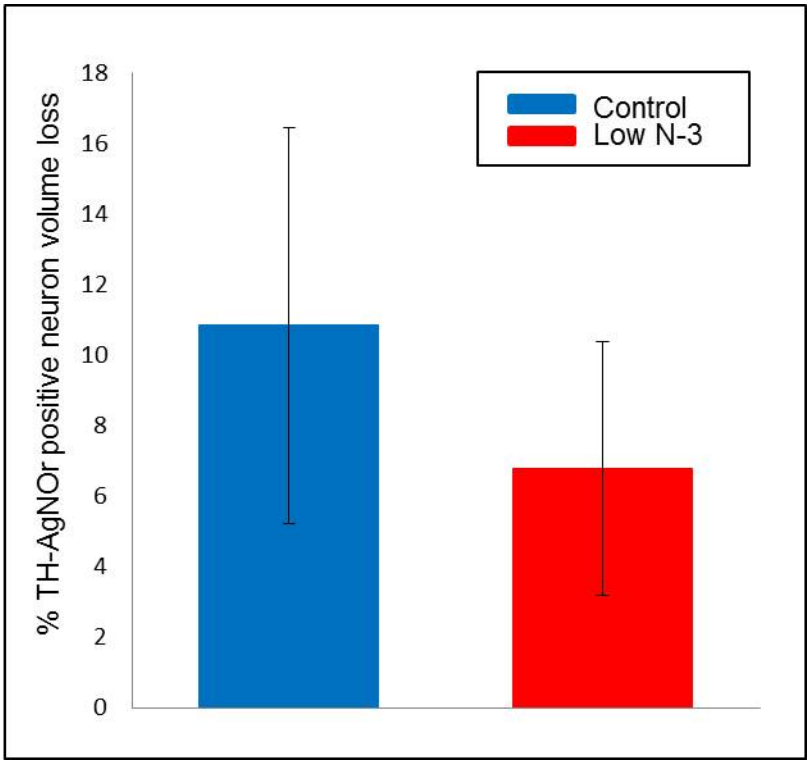

Figure 5.

Effects of diet on TH-positive SNpc neuron volume. There was no effect of diet on the percent neuronal body volume loss after 6-OHDA (t-test, P>0.05, n = 7-8).

4.4 Discussion

In this aim, a diet and breeding protocol employing a Low n-3 diet from conception was used to achieve differing levels of n-3 PUFA brain composition in rats. Although the maternal diets remain consistent throughout the study, the Low n-3 cohort's maternal DHA stores are depleted after the 1st litter and further diminished after the 2nd litter; thus the dams have less available DHA to pass on to their nursing pups (Jumpsen et al., 1997; Ozias et al., 2007). Thus, differing levels of brain DHA depletion were expected, with a greater depletion expected in the 2nd litter rats. Brain DHA was decreased 36% in 1st litter, and 45% in 2nd litter rats, although the difference between the litters was not significant. The level of DHA depletion in the 1st Litter Low n-3 rats was greater than expected. However, the 2nd litter levels of DHA depletion were comparable to, but not as great as, the 54% loss in a previous study from our laboratory (Ozias et al., 2007). Thus, the rats prepared for this study did not generate a "DHA dose-response" that would have facilitated assessment of several levels of brain DHA content on the effects of 6-OHDA lesion. Nevertheless, the study does represent a valid assessment of the effects of low n-3 PUFAs on rats with approximately 40% of the normal brain phospholipid n-3 PUFA depletion on neuronal number and volume in a mild to moderate 6-OHDA PD lesion model. As such, this study provides an informative starting point from which to design future studies in order to determine a threshold of effects for n-3 PUFA depletion in early PD models.

To validate the lesion and to determine the effects of brain PUFA content on motor behavior after striatal depletion by intrastriatal 6-OHDA, d-amphetamine-induced rotational behavior was assessed by force-plate rotometry. There was no effect of diet

or litter on d-amphetamine-induced rotations. The total numbers of rotations for a 60 min. period are comparable to previous studies (Lee et al., 1996; Roedter et al., 2001; Bethel-Brown et al., 2010). The lack of an effect of differing levels of brain and dietary n-3 PUFAs on rotational behavior may be explained in the context of a recent study in which DHA supplementation in a mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model reduced the loss of TH-positive SN neurons, but there was no improvement of motor activity (Ozsoy et al., 2011a). This suggests that even when the DHA exerts a neuroprotective effect on cells, there may be a threshold effect before functional improvements are observed. Contralateral striatal compensation could also have occurred, since projections are known to sprout from the contralateral side in lesion models (Finkelstein et al., 2000), and our relatively mild lesion may not be enough to elicit subtle effects induced by n-3 PUFA deficiency that a more severe lesion could. Overall, in our study rotational behavior was successful for lesion validation purposes, although more sensitive behavioral assessments may be needed in future studies to show differences caused by diet.

Furthermore, there was no difference in the number of neurons between the Control and Low n-3 contralateral SNpc. This is in contrast to a recent study performed in our laboratory, which found that intact rats (not 6-OHDA lesioned) fed a low n-3 PUFA diet, which produced a decrease in brain DHA of 54% in other rats using the same breeding protocol (Ozias et al., 2007), had 33% fewer SNpc TH-positive neurons (Ahmad et al., 2008), as well as another recent study which found fewer SN dopaminergic neurons in rats fed an n-3 PUFA deficient diet from conception (Cardoso et al., 2012). Although these stereology results do not support our hypothesis that there

would be differences in neuronal number between the Control and Low n-3 rats, differences in the study designs offer an explanation for these findings. The previous study in our laboratory was performed in unlesioned rats. Although using the contralateral side of the lesion as the control to compare changes in morphology is a common and well-accepted method among 6-OHDA studies (Lee et al., 1996; Kirik et al., 1998; Roedter et al., 2001; Deumens et al., 2002; Gomide et al., 2005), it could produce different results than using unlesioned rats as the control. Crossed fibers from ipsilateral striatum have been found to project to the contralateral side, and the contralateral side is slightly affected after unilateral 6-OHDA lesion (Cheng et al., 1998; Finkelstein et al., 2000). This can allow the loss of ipsilateral neurons to be underestimated when using the contralateral side as a control. However, staining conditions can vary considerably between individual animals, so using separate unlesioned animals can introduce additional bias, making within-subject comparisons between the contralateral and ipsilateral sides an attractive alternative. In addition, observing the changes that occur between the contralateral and ipsilateral sides may be most relevant to what is occurring in PD, since motor signs usually present unilaterally. In summary, although unlesioned control animals present their own biases, in future studies quantifying dopaminergic neurons from unlesioned animals would help confirm whether the neuronal deficits caused in the previous studies in unlesioned rats were replicable, or whether it is something about the lesion itself that changes the expected proportions of SNpc neurons between the Control and Low n-3 rats.

Although the 6-OHDA lesion decreased both the SNpc number and the neuronal volume in the ipsilateral SNpc in both the Control and Low n-3 rats, the change elicited

by the 6-OHDA lesion to neuronal volume was not significant in either Control or Low n-3 2nd litter rats, and there was no difference between the diet groups in the percent decrease in either neuronal number or neuronal volume. Several potential mechanisms could be contributing to the lack of an effect of diet on the SNpc neurons after a mild to moderate 6-OHDA lesion. Even if the Control diet rats initially did have more SNpc neurons than the Low n-3 rats, the DHA-sufficient Control neurons may have been more susceptible to oxidative damage than their Low n-3 counterparts, and thus any potential advantage in initial neuronal number could have been masked by increased neuronal loss in the Control rats. Despite the many studies demonstrating beneficial effects of DHA, others suggest that in high oxidation states, the increased capacity for lipid peroxidation provided by a PUFA-rich environment can lead to increased oxidative damage (Montine et al., 2004; Kabuto et al., 2009; Yakunin et al., 2012). Thus, in the high oxidative stress environment induced in the SNpc dopaminergic neurons by intrastriatal 6-OHDA infusion, a mild DHA deficiency such as that found in our rat model may in fact be beneficial in preventing the oxidative-stress-induced loss of neurons on the both the lesioned and the contralateral side, which contains a small percentage of crossed fibers from the ipsilateral side (Finkelstein et al., 2000) by providing fewer targets for lipid peroxidation (Yakunin et al., 2012). Alternatively, the fact that there was no discernible protective effect of DHA deficiency in the Low n-3 rats may support the theory that at some neurotoxic threshold, any neuroprotective benefit of n-3 PUFAs may be overwhelmed. Obviously, further studies are needed to establish the threshold of neuroprotection against 6-OHDA in our n-3 PUFA deficiency rat model.

Other studies have found decreased loss of TH-positive neurons in PD models after n-3 PUFA supplementation (Bousquet et al., 2008; Cansev et al., 2008; Ozsoy et al., 2011a). Thus it is likely that n-3 PUFA consumption or brain DHA content does play some role in the development or prevention of PD, and this hypothesis deserves the dedication of further studies. In this project, however, we created a relatively mild n-3 PUFA deficiency meant to model the Western diet, combined with a moderate loss of SNpc neurons intended to mimic early PD. Thus, our rats may not have experienced the degree of neuronal insult necessary to demonstrate any potential differences in response to 6-OHDA caused by a moderately n-3 PUFA-deficient diet. In short, this project, in attempting to best model the conditions found in pre-clinical PD and a standard Western diet, may be suffering from inconclusive results because subtle changes caused by a mild deficiency in an early disease cannot be detected by rotational behavior or stereology.

4.5 Conclusions

Overall, this study was informative, yet inconclusive. This study successfully determined the effects of low n-3 PUFAs on neuronal number and volume in a mild to moderate 6-OHDA PD lesion model. However, based on our results, it can only be concluded that low n-3 PUFAs from conception have no effect on SNpc dopaminergic number and morphology at the particular level of dietary n-3 PUFA depletion and 6-OHDA dose and route of administration employed in this study. In order to definitively say that the Low n-3 diet has no effect on the behavior or neuronal number and morphology in the unilateral intrastriatal 6-OHDA lesion model, we would need to do further studies to replicate the stereological findings previously obtained in our

laboratory on unlesioned Low n-3 rats. In addition, it would be informative to expand the lesion procedures to include a range of doses, and well as to experiment with varying doses of dietary n-3 PUFAs, to test whether our lack of findings was an indicator that there truly is no effect of the Low n-3 diet on the endpoints chosen, or whether different endpoints or if more severe neuronal stress elicited by an n-3 PUFA deficiency or a more severe 6-OHDA model is necessary to be able to discern any differences using our quantitative techniques.

CHAPTER 5

TYROSINE HYDROXYLASE-SILVER NUCLEOLAR STAINING TO DETERMINE THE EFFECTS OF A UNILATERAL INTRASTRIATAL 6-HYDROXYDOPAMINE LESION IN THE SUBSTANTIA NIGRA

5.1 Abstract

Neurotoxic lesions of the nigrostriatal pathway model the deficits found in Parkinson's disease (PD). This study used stereology and a novel staining method to examine the effects of a partial unilateral striatal 6-hydroxydopamine (6-OHDA) lesion on substantia nigra pars compacta dopamine neuron number and morphology in rats. Adult male Long-Evans rats were subjected to unilateral lesion of the substantia nigra pars compacta (SNpc) by intrastriatal microinjection of 6-OHDA (12.5 µg). Lesions were verified by d-amphetamine-stimulated rotation (2.5 mg/kg, sc) by force-plate rotometry 7 days post-surgery. Seven days after rotation testing, rats were euthanized, and brains were prepared for either histology (n = 12) or determination of striatal dopamine content by HPLC-EC (n =20). Brains prepared for histology were stained for tyrosine hydroxylase silver nucleolar (TH-AgNOR) staining using a modified protocol developed for stereological assessment. The AgNOR counterstain allowed for precise definition of the nucleolus of the cells, facilitating both counting and qualitative morphometry of TH-positive neurons. Stereological quantitation determined a 54% decrease in TH-positive neuron number ($P<0.01$), and a 14% decrease in neuron volume ($P<0.05$) on the lesioned side. Striatal dopamine concentration was decreased by 92% ($P<0.01$), suggesting that striatal dopamine analysis may overestimate the numbers of SNpc neurons lost. These findings demonstrate that combined use of TH and AgNOR staining provides improved characterization of 6-OHDA-induced pathology. Furthermore, the data suggest that decreased neuronal volume as well as number contributes to the functional deficits observed after unilateral intrastriatal 6-OHDA lesion.

5.2 Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder marked by loss of nigrostriatal dopamine neurons, resulting in clinical signs when about 80% of striatal dopamine is depleted (Calne and Langston, 1983). Current treatments are largely palliative, emphasizing the urgent need for preventative and disease-modifying treatments. However, the development of such early-stage treatments is fraught with challenges. The relationships between the causative factors of PD and the effects of neuroprotective treatments are still unclear (Ybot-Gorrin et al., 2011), and a better understanding of neuronal morphology is needed to elucidate the mechanisms of neurodegenerative disease (Przedborski et al., 2003). Thus the importance of early- to moderate-stage PD models is vital to advancing the treatment of the disease.

The partial unilateral intrastriatal 6-hydroxydopamine (6-OHDA) model is a clinically-relevant model of early-stage PD (Kirik et al., 1998; Deumens et al., 2002). This technique is widely used as an investigational tool for both screening potential therapeutics and for understanding the underlying mechanisms of early PD. Although rotational behavior and striatal dopamine concentration are routinely quantified after neurotoxic lesions to verify a functional dopaminergic deficit, advanced histological analysis is needed to assess the effects of the lesion and potential treatments on morphologic changes to the dopaminergic neurons. Histological analysis of partial 6-OHDA lesions in the substantia nigra pars compacta (SNpc) is challenging, however, because moderate degenerative changes to neuron morphology can be difficult to quantify. Stereological analysis using tyrosine hydroxylase (TH) staining of dopamine neurons has been used to study the morphological changes in several animal models of

PD, but the morphometric outcomes vary according to study. The variation in the results of PD model morphology studies has been attributed to the varying lesion, staining and quantitation methods used (Stark and Pakkenberg, 2004); and while a certain amount of inter-study variation is unavoidable, a clear understanding of the morphology of dopaminergic neurons has undoubtedly been clouded by the inconsistent findings in the literature.

Darkly pigmented TH antibody immunostaining can mask morphological markers such as the nucleolus and individual neuronal body outlines in regions thickly populated with TH-positive neurons. With the goal of better understanding the effects of 6-OHDA on dopaminergic morphology, this study used stereological methods and a newly available commercial TH stain combined with a silver nucleolar (AgNOR) stain to determine the effects of 6-OHDA partial unilateral intrastriatal lesions on cell number and morphology. AgNOR staining is a silver nitrate preparation that targets chromosomal proteins in the nucleolus known as nucleolar organizing regions (NORs). This stain, which has been extensively used by cancer pathologists to assess cell proliferation (Trerè, 2000), has recently been exploited for use as a co-stain in dopaminergic neurodegeneration studies (Switzer III et al., 2011). We will show that the combined use of TH and AgNOR staining provides improved characterization of 6-OHDA-induced pathology by facilitating the identification and quantification of neuronal structures used in stereology. In addition, our findings suggest that decreased neuronal volume as well as decreased neuronal number contributes to the functional deficits observed after unilateral 6-OHDA lesion.

5.3 Results

5.3.1. Amphetamine-stimulated rotation

The mean number of amphetamine-stimulated rotations completed by lesioned rats during the 60-minute observation period was 145 ± 24 (n = 31), indicating a functional dopaminergic deficit in the ipsilateral striatum.

5.3.2. Striatal dopamine concentrations

Dopamine concentration was decreased between the ipsilateral and contralateral striata of all rats subjected to 6-OHDA lesion, with a mean within-subject decrease in striatal dopamine of 92 ± 2% ($P<0.01$) (**Figure 2**). The dopamine levels measured in the unlesioned hemispheres were comparable to those found in previous studies (Kilts et al., 1981; Widerlöv et al., 1982; Davis et al., 2010; Levant et al., 2011).

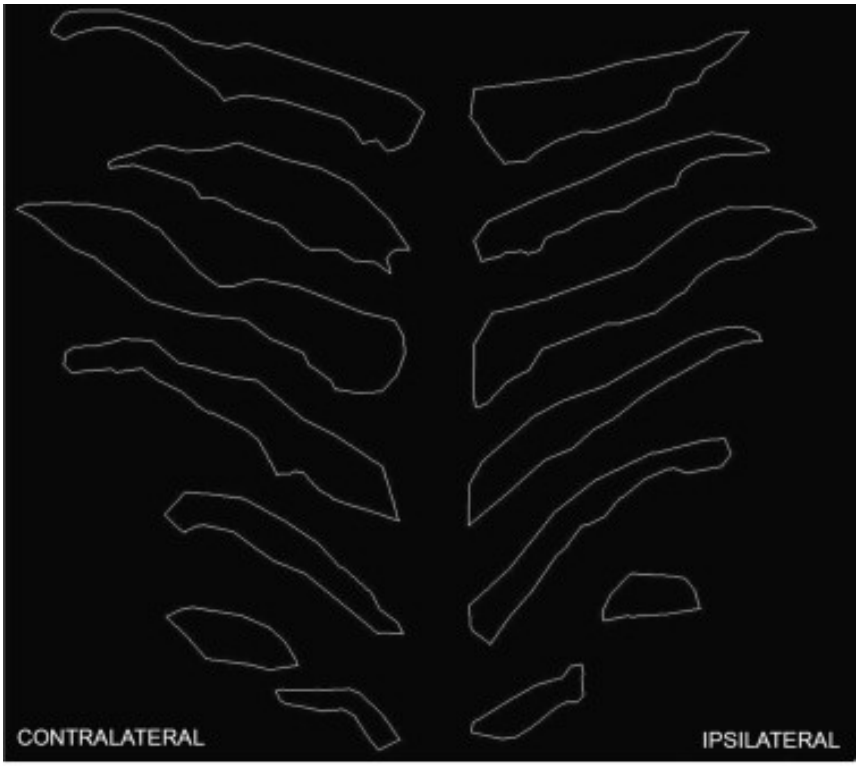

Figure 1.

Anatomical definition of regions of interest. Representative photomicrographs (4X) of digital tracings of the boundaries of the SNpc in contralateral and ipsilateral brain through 6 coronal sections of a single animal. Tracings shown are dorsal to ventral from the top to the bottom of the figure. TH-AgNOR-stained number and neuronal body volume were assessed within these boundaries. Tracings were produced using MicroBrightField's Stereoinvestigator software, and sections were aligned to facilitate comparison.

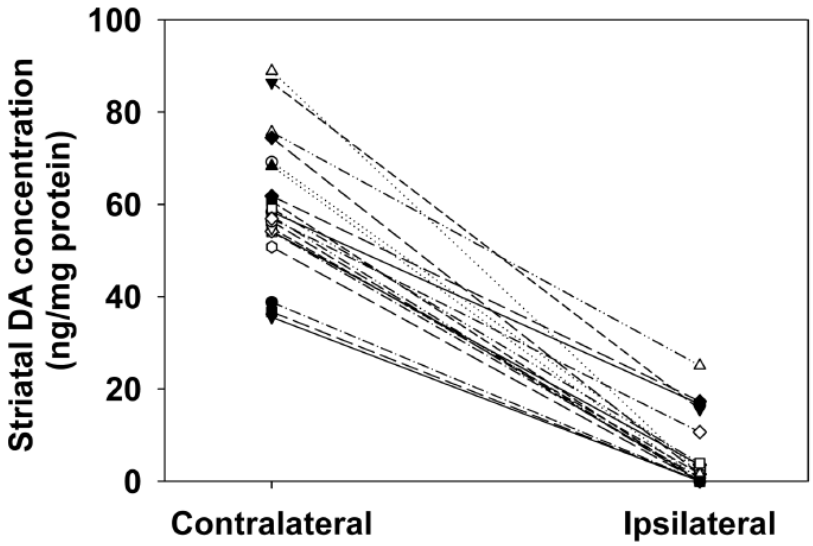

Figure 2.

Effects of unilateral intrastriatal 6-OHDA lesion on striatal dopamine. All rats had decreased striatal dopamine content in the ipsilateral striatum compared to the contralateral striatum. The mean within-subject decrease in dopamine concentration between the ipsilateral and contralateral striata was 92 ± 2%. *P<0.01 by paired t-test (n = 12).

5.3.3. Stereological analysis of SNpc TH-AgNOR-stained neurons

Low-and high-power photomicrographs demonstrate the effects of the lesion using AgNOR staining or TH-thionine counterstain (**Figure 3**). Neurodegeneration of the 6-OHDA-lesioned SNpc was evident at low magnification (**Figure 3A-3D**)**.** High-power magnification illuminates the advantages of using a combined modified TH and AgNOR staining protocol. The neuronal bodies were more clearly delineated in the AgNOR-stained sections than with the TH-thionine counterstain, allowing for more precise marking of the cell borders using the nucleator tool for volume calculations. The reduced TH antibody concentration used in the TH-AgNOR protocol results in reduced background darkening and staining of debris. In addition, the lack of thionine counterstain prevented further background clutter and facilitated a clear view of individual neuronal bodies and axons (**Figures 3G and 3H vs. 3E and 3F**). Most importantly, the addition of the AgNOR stain resulted in a darkly pigmented and clearly distinguishable nucleolus (**Figure 3G and 3H**). This selectivity in staining facilitated the use of the nucleolus within the nucleus as both a unique singly occurring cell landmark for counting with the optical fractionator, and as a center point for positioning crossed measurement rays using the Nucleator software marking tool in Stereoinvestigator. In the few instances in which the nucleus of the cell, but not the nucleolus, was visible, the rays were placed centered on the nucleus.

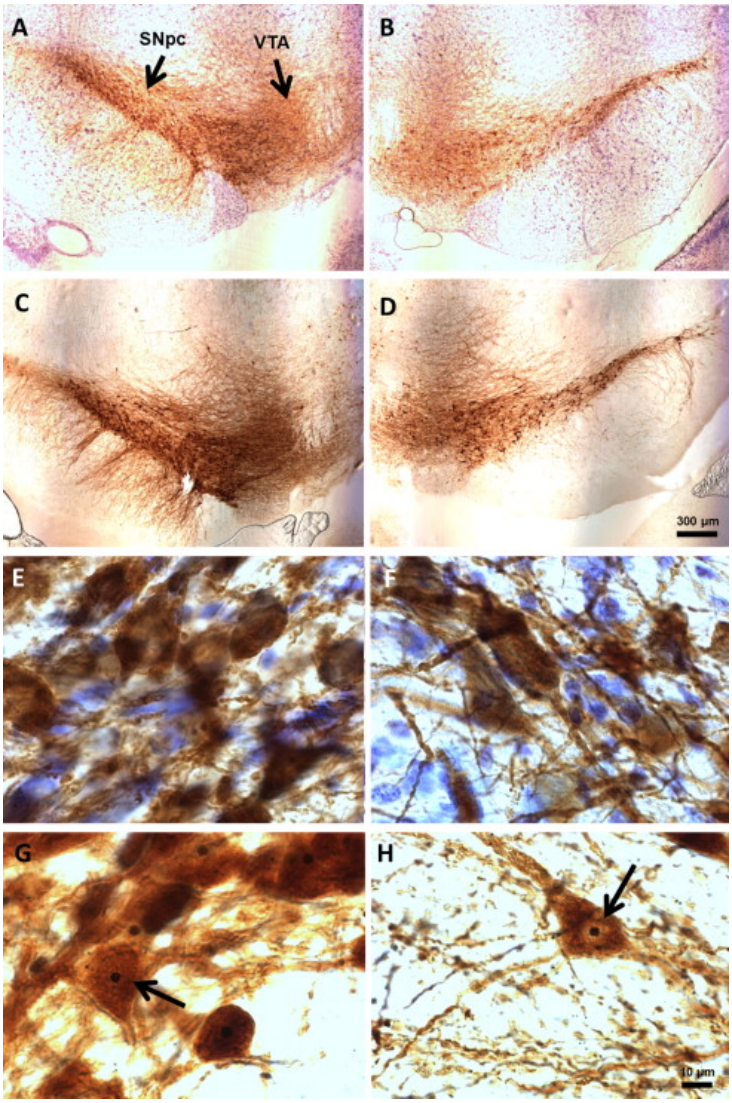

Figure 3.

Effects of unilateral intrastriatal 6-OHDA lesion on TH-thionine- and TH-AgNOR-staining in the SNpc. A. TH-thionine contralateral, 4X. B. TH-thionine ipsilateral, 4X. C. TH-AgNOR contralateral, 4X. D. TH-AgNOR ipsilateral, 4X. E. TH-thionine contralateral, 100X. F. TH-thionine ipsilateral, 100X. G. TH-AgNOR contralateral, 100X. H. TH-AgNOR ipsilateral, 100X. Representative images are shown and are all from the same animal. Scale bars are 300 µm and 10 µm for the 100X and 4X images, respectively.

5.3.3.1. TH-AgNOR-stained SNpc region volume

Eleven of 12 rats exhibited a smaller regional volume of the ipsilateral SNpc compared to the contralateral SNpc (**Fig. 4**). The regional volume of the SNpc for individual rats ranged from 3.9 to 5.3 mm^3 on the contralateral side, and from 2.6 to 4.5 mm^3 on the ipsilateral side. The within-subject percentage decrease neurons ranged from 10% to 36%. The mean within-subject decrease in volume of the SNpc region between the ipsilateral and contralateral SNpc was 20 ± 3% ($P<0.05$) as measured by planimetry.

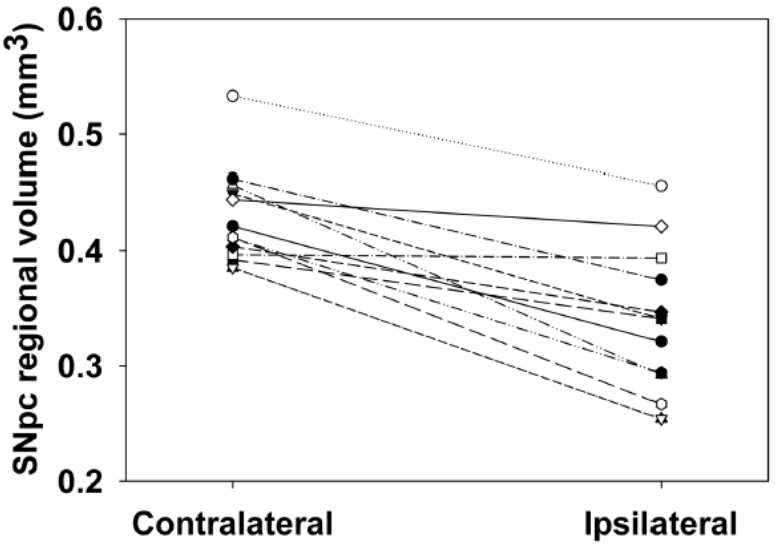

Figure 4.

Effects of unilateral intrastriatal 6-OHDA lesion on SNpc regional volume. Eleven of 12 rats exhibited a smaller regional volume in the ipsilateral SNpc compared to the contralateral SNpc assessed by planimetry. The mean within-subject decrease in regional volume between the ipsilateral and contralateral SNpc was 20 ± 3%. *P<0.05 by paired t-test (n = 12).

5.3.3.2. TH-AgNOR-stained SNpc neuron number

The mean number of SNpc TH-positive neurons on the contralateral side (13,133 ± 361) was comparable to the range of findings in previous stereological studies using traditional TH staining (Kirik et al., 1998; Lindner et al., 1999; Carvalho and Nikkhah, 2001; Debeir et al., 2005; Gomide et al., 2005; Ahmad et al., 2008; Eriksen et al., 2009). The estimated number of TH-positive SNpc neurons for each rat ranged from 11,087 to 15,389 on the contralateral side, and from 3,257 to 10,398 on the ipsilateral side. All rats showed a decrease in the number of TH-positive cells in the ipsilateral SNpc compared to the contralateral SNpc (**Fig. 5**). The within-subject percentage decrease neurons ranged from 21% to 75%. The mean within-subject decrease in TH-positive neuron number between the ipsilateral and contralateral SNpc was 54% ± 5% ($P<0.01$).

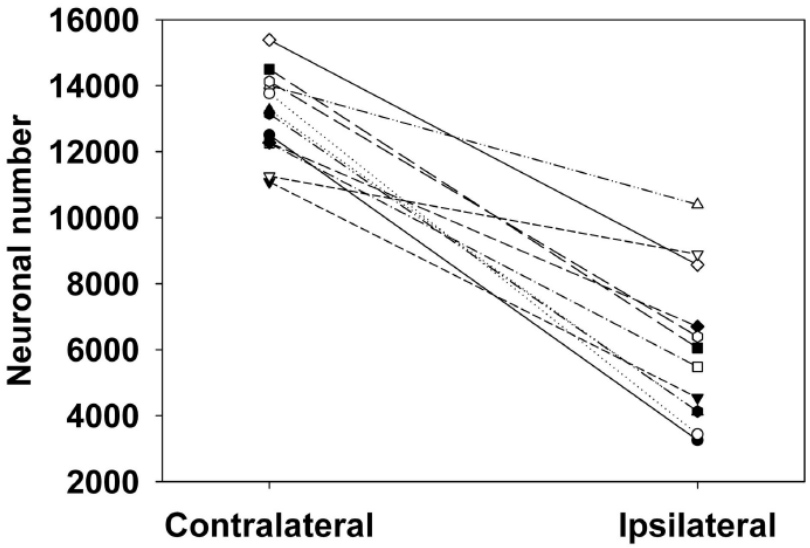

Figure 5.

Effects of unilateral intrastriatal 6-OHDA lesion on TH-AgNOR-stained neuron number in the SNpc. All rats exhibited a decrease in neuronal number in the ipsilateral SNpc compared to the contralateral SNpc with a mean within-subject decrease in neuron number between the ipsilateral and contralateral SNpc of 54 ± 5%. *P<0.01 by paired t-test (n = 12).

5.3.3.3 TH-AgNOR-stained SNpc neuron volume

Nine of 12 rats exhibited a smaller average neuronal body volume in the ipsilateral SNpc compared to the contralateral SNpc. The mean estimated neuronal volume of TH-AgNOR-positive SNpc neurons for individual rats ranged from (3,188 to 4109 μm^3) on the contralateral side, and from (2,489 to 4,110 μm^3) on the ipsilateral side. The within-subject percentage change of neuronal volume ranged from (9% increase to 39% loss). The mean within-subject decrease was 14 ± 3% ($P<0.05$) (**Figure 6A**). **Figure 6B** shows the change in the number of neurons in various size categories between ipsilateral and contralateral SNpc.

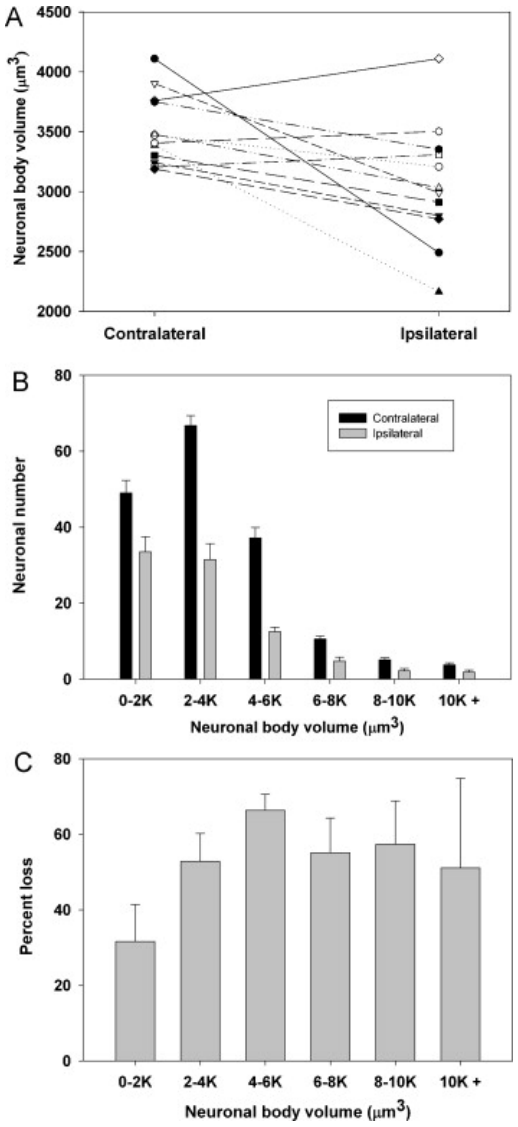

Figure 6.

Effects of unilateral intrastriatal 6-OHDA lesion on TH-AgNOR-stained neuronal body volume. (A) Effects on average neuronal cell body volume. Nine of 12 rats exhibited a smaller average neuronal body volume in the ipsilateral SNpc compared to the contralateral SNpc. The mean within-subject decrease in average neuronal body volume between the ipsilateral and contralateral SNpc was 14 ± 3%. *P<0.05 by paired t-test (n = 12). (B) Effects on frequency distribution of neuronal volume. *P<0.05 by paired t-test (n = 12), (C) Effect on percent loss in the number of cells in each size category between the ipsilateral and contralateral SNpc.

5.4 Discussion

The 6-OHDA toxin, a structural analog of dopamine, is transported by the monoamine transporter into catecholaminergic cells, leading to their destruction by oxidative mechanisms (Kostrzewa and Jacobowitz, 1974; Glinka et al., 1997). Although lacking the Lewy bodies and the progressive course of clinical PD, the 6-OHDA lesion nonetheless produces marked degeneration of targeted dopaminergic neurons, providing a functional PD model quantifiable by characteristic behavioral changes, dopamine depletion and histological observations (Ungerstedt and Arbuthnott, 1970; Kirik et al., 1998).

The partial unilateral intrastriatal 6-OHDA model, with its relatively moderate loss of SNpc TH-positive neurons, is particularly appropriate as an early-stage PD model (Kirik et al., 1998; Deumens et al., 2002; Bethel-Brown et al., 2010). The importance of early PD models is underscored by our existing knowledge about the destruction of pigmented SN neurons long before onset of the clinical disease in humans. Estimates hold that patients with the earliest detectable clinical signs have already lost about 50% of their pigmented SN neurons (Marsden, 1990). Furthermore, pigmented SN neurons are also lost as a part of natural aging (Rudow et al., 2008), and although the degree of loss is still debated, this natural attrition adds another layer of complexity to the investigation of early PD. Differences in the degree of dopaminergic degeneration among the existing studies of the partial unilateral intrastriatal 6-OHDA model have been attributed to the differences in lesion site, histology methods, toxin dose, and to biologic variability (Kirik et al., 1998; Deumens et al., 2002; Stark and Pakkenberg, 2004). Thus, the capacity for accurate, accessible morphometry of the neuronal

populations at risk in this Parkinson's model is important for the advancement of Parkinson's research.

Stereological analysis, with its use of random sampling techniques and generation of unbiased estimates of three-dimensional characteristics, is the optimal toolkit for assessing the morphological changes induced by neurotoxic lesions and therapeutic interventions (Gundersen et al., 1988a; Schmitz and Hof, 2005). Stereology comes with its own set of challenges, however, especially pertaining to its requirement of staining thick tissue sections, which can be difficult for stains to penetrate. An additional challenge is the necessary definition of specific morphological landmarks like the nucleus and nucleolus, which can be easily obscured by improper staining. This is especially important in dense, mostly homogenous populations of a darkly-staining cell type, such as TH-positive SNpc neurons. In particular, the nucleus and nucleolus can be difficult to distinguish from other darkly stained structures within the cell. Since the nucleus and the nucleolus are unique anatomical markers useful in stereological quantitation, improved staining methods such as the TH-AgNOR protocol are needed to be able to specifically identify these structures in TH-positive tissues.

AgNOR staining offers a versatile tool in specific identification of the nucleolus in TH-stained preparations. The nucleolus is localized around chromosomal segments known as NORs, which contain ribosomal RNA (rRNA)-encoding genes. NORs also contain acidic AgNOR proteins. Because the quantity of AgNOR proteins and cell proliferation rate are related, AgNOR staining has been extensively used as a tool by cancer pathologists to characterize a variety of cancer types (Derenzini et al., 2000; Trerè, 2000; Derenzini et al., 2004). The highly specific nucleolar staining achieved with

this method lends it to other applications in which visualization of the nucleolus is desirable, especially to stereological study of neural tissues.

The present findings demonstrate that the modified TH-AgNOR staining protocol offered a number of qualitative advantages observed during the histopathological analysis (**Figure 3**). Enhanced stain penetration facilitated by a hydrochloric acid (HCl) permeabilization step in the modified TH-staining protocol was combined with less background staining of fibers and debris with the reduced concentration of TH antibody. This enabled better visualization and potentially resulted in more accurate counting of both the ipsilateral and contralateral SNpc than has been possible in previous studies. The TH-AgNOR staining advantage may be more marked in lesioned tissue, where a higher degree of cell destruction and accumulation of debris makes identification of individual neurons especially challenging. Since technical variations are a likely explanation for the discrepancies between neuronal morphometry findings in the literature, this modified TH-AgNOR staining protocol should prove to be a beneficial tool in the modern characterization of classic dopaminergic neurotoxin models. It may be anticipated that its use will lead to a fuller understanding of the affected neurons, and importantly, the underlying functional changes related to morphology.

The data also clearly demonstrate that unilateral intrastriatal 6-OHDA lesions result in decreased SNpc regional volume and cell loss (**Figures 3-5**) in addition to amphetamine-stimulated rotation and the depletion of striatal dopamine (**Figure 2**)

Compared to the near-complete destruction of both SNpc neurons and striatal dopamine in medial forebrain bundle (MFB) or SNpc lesions, the degree of neuron and dopamine loss is more variable in intrastriatal 6-OHDA lesions, dependent on the site

and dose of the toxin administration (Kirik et al., 1998). However, our study found a greater disparity between percent depletion in SNpc neurons (54%) and striatal dopamine (92%) than is typical of the literature (Deumens et al., 2002). Striatal dopamine concentration is rarely measured in histological 6-OHDA studies, because it requires a separate set of animals for dopamine analysis; however, other studies have found decreases in striatal dopamine content and nigral dopamine neuron number that were more similar that those observed in this study. Of note, a stereological study using a bilateral 6-OHDA infusion (12.5µg) found a 79% dopaminergic SN neuron loss associated with a 77% striatal dopamine loss quantified by high-performance liquid chromatography-electrochemical detection (HPLC-EC) (Lindner et al., 1999). Similarly, a non-stereological study of unilateral 6-OHDA lesions (12 ug) that quantified a subsection of the SNpc, found a 47% TH-positive cell loss [SN and ventral tegmental area (VTA)] accompanied by a 54% dopamine content loss measured by radioenzymatic immunocytochemistry (Lee et al., 1996). In contrast to these studies, our lesion resulted in a larger decrease in striatal dopamine content relative to cell loss. Thus, our results suggest that even severe unilateral striatal dopamine depletion may reflect a relatively modest loss of SNpc dopamine neurons, underscoring the importance of using stereological techniques to assess the effects of the toxin on cell number and morphology.

Although only a few studies have examined both changes in dopamine cell number and striatal dopamine content, a number of studies have examined changes in SN dopaminergic cell number after 6-OHDA lesion using both non-stereological and stereological methods. Non-stereological studies indicate a loss of SNpc neurons after

6-OHDA lesions (for review, see: (Deumens et al., 2002); however, two-dimensional methods for counting cells have inherent bias that can result in inaccurate cell counts (Sterio, 1984; West, 1993). Stereological studies also demonstrate a loss of SN dopaminergic neurons after unilateral striatal 6-OHDA studies. We found a 20% decrease in SNpc regional volume and a 54% depletion of SNpc TH-positive neurons after a single-site moderate 6-OHDA dose of 12.5 µg (**Figures 4 and 5**). This finding complements two recent stereological studies that found a 33% TH-positive cell loss in the SN with an 8.75 µg dose, and a 33% loss with an 8 µg dose (Debeir et al., 2005; Gomide et al., 2005). Taken together, these stereological results support a dose-response effect of 6-OHDA dose on the number of SNpc TH-positive neurons.

Even when using stereology to reduce bias, studies must be compared carefully. Differences in lesion sites and in region definition can produce varying results. For example, Kirik et al. (Kirik et al., 1998) used a total 20 µg dose produced a loss of neurons in the SN (including the pars reticulata and lateralis) of approximately 50%, although the percentage of loss varied depending on the lesion location within the striatum and the number of sites lesioned, with as much as 70% loss with a pre-terminal three-site injection of 7 µg per site. Increasing the total terminal dose to 28-30 µg resulted in a 60-76% loss, depending on the number of sites lesioned. Similarly, another study that used a total of 28 µg over 4 striatal injection sites found a 60% loss of SNpc neurons (Carvalho and Nikkhah, 2001). In the context of the available literature overall, we observed a large loss of neurons based on the total dose of 6-OHDA. Given the high striatal dopamine loss, however, the degree of SNpc loss is less surprising.

In addition to yielding enhanced data on changes in cell number after lesion, stereological methods can quantify subtle changes in cell morphology. In this study, the addition of AgNOR staining to the standard TH staining protocol resulted in clearly defined nucleolar bodies, which could be used as foci for using the nucleator tool to determine cell body volume. We found a 14% reduction in neuronal body volume in 6-OHDA lesioned rats, indicating that volume is a quantifiable morphological characteristic related to toxicity and subsequent neurodegeneration (**Figure 6A**). Another study using stereology to quantify the effects of a unilateral striatal 6-OHDA lesion on cell volume found no difference between lesioned and intact SNpc TH-positive neurons (Gomide et al., 2005). While the time from lesion to sacrifice (14 days) was the same in both studies, the dose used by Gomide et al. (8 µg) was lower than that used in this study (12.5 µg), indicating that the observed difference in volume changes may be dose-related.

The dopaminergic cells in the rat SNpc are medium-sized neurons that are about 11 x 20 µm in size, although individual neurons vary (Paxinos, 1995). The observation of both decreased neuronal number and volume then raises the question: is the decreased volume truly due to atrophy, or is the shift in the mean volume a selective loss of the larger neurons? Both hypertrophy and atrophy of SNpc dopaminergic neurons have been observed in PD brains (Rudow et al., 2008), and atrophy of the SNpc dopaminergic neurons has been observed after intrastriatal 6-OHDA injection (Sauer and Oertel, 1994; Lee et al., 1996). The frequency histogram of the distribution of the absolute number of TH-positive SNpc neurons by size category shows a clear post-lesion shift toward smaller neuronal volumes, and there was a lower percent loss in

the smallest size category of neurons (**Figures 6B** and **6C**). Studies of the frequency distributions of the neurons in demonstrating atrophy are informative yet inconclusive, as they cannot rule out the preferential loss of large SNpc neurons. In fact, there is evidence that the subdivisions of the SNpc populated with mostly large neurons are also those most affected by neurotoxic lesions and by PD (Rodríguez et al., 2001). However, another analysis of the size frequency distribution of absolute number of neurons when a range of doses were administered showed a small increase in the numbers of smaller cells with the smallest dose of toxin, suggestive of an atrophic response rather than a strictly selective loss of large neurons (Lee et al., 1996). It is also possible that a combination of atrophic development and preferential loss of large dopaminergic neurons occurs in neurotoxic-induced degeneration, as well as in the clinical disease. Although more research is needed to better characterize the effects of 6-OHDA lesions on dopaminergic neuronal volume, our study contributes to the current understanding of toxin-induced morphology changes and offers an improved method for further investigation. Overall, the addition of the AgNOR stain to a modified TH staining protocol resulted in facilitated identification of the nucleolus and better visualization of the neuronal bodies, and thus the likelihood of improved quantitation.

5.5 Conclusions

In conclusion, we find that TH-AgNOR staining facilitates stereological analysis of the effects of 6-OHDA lesions on the SNpc of rats when compared to standard TH-thionine staining. Using this staining protocol combined with stereological analysis to determine the effects of a unilateral intrastriatal 6-OHDA lesion revealed a moderate

loss of SNpc TH-positive neurons despite severe striatal dopamine depletion. In addition, volumetric measurements confirmed SNpc TH-positive neuronal atrophy 14 days post-lesion, suggesting that 6-OHDA induces morphological changes to SN neurons relevant to those found in the PD literature (Rudow et al., 2008). These findings confirm the utility of this staining protocol, and suggest the utility of this method in further investigation of PD lesion models. Furthermore, these findings suggest that decreased neuronal volume as well as number contributes to the functional deficits observed after unilateral 6-OHDA lesion, and may also play a role in PD.

CHAPTER 6

ALTERED NUCLEOLAR MORPHOLOGY IN SUBSTANTIA NIGRA DOPAMINE NEURONS FOLLOWING 6-HYDROXYDOPAMINE LESION IN RATS

6.1 Abstract

The nucleolus, the site of ribosomal ribonucleic acid (rRNA) transcription and assembly, is an important player in the cellular response to stress. Altered nucleolar function and morphology, including decreased nucleolar volume, has been observed in Parkinson's disease (PD); thus the nucleolus represents a potential indicator of neurodegeneration in the disease. This study determined the effects of a partial unilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesion, which models the dopaminergic loss found in PD, on the nucleoli of dopaminergic cells in the substantia nigra pars compacta (SNpc). Adult male Long-Evans rats underwent unilateral intrastriatal infusion of 6-OHDA (12.5 µg). Lesions were verified by amphetamine-stimulated rotation 7 days later, and rats were euthanized 14 days after infusion. Coronal sections (50 µm) were stained with tyrosine hydroxylase silver nucleolar (TH-AgNOR) staining using MultiBrain Technology (NeuroScience Associates), which resulted in clearly defined nucleoli and neuronal outlines. Stereological methods were used to compare dopaminergic morphology between lesioned and intact hemispheres in each rat. In cells exhibiting a definable nucleolus, nucleolar volume was decreased by 16% on the ipsilateral side. The ipsilateral SNpc also exhibited an 18% decrease in SNpc planimetric volume, a 46% decrease in total TH-positive neuron number, and an 11% decrease in neuronal body volume (all $P<0.05$ by paired t-test). These findings suggest that the 6-OHDA lesion alters nucleolar morphology and that these changes are similar to those occurring in PD.

6.2 Introduction

The nucleolus is a likely participant in the neurodegenerative process of Parkinson's disease (PD). In addition to ribosomal RNA (rRNA) transcription and assembly, the nucleolus is involved in directing the cellular response to stress (Boulon et al., 2010), thus implicating it in the process of neurodegeneration. Notably, altered postmortem nucleolar size and nucleolar damage have been observed in PD, as well as in several other neurodegenerative diseases (Mann and Yates, 1982; Rieker et al., 2011; Hetman and Pietrzak, 2012). Despite the importance of the nucleolus in neuronal function, the exact mechanisms of its dysfunction in degenerating neurons are still poorly understood, justifying the need for better knowledge of the roles of this influential organelle in neurodegenerative disease.

The partial unilateral intrastriatal 6-hydroxydopamine (6-OHDA) model of PD destroys SNpc neurons by neurotoxic oxidative stress mechanisms (Kostrzewa and Jacobowitz, 1974; Glinka et al., 1997). In contrast to more extensive dopaminergic ablation models, this method causes moderate substantia nigra pars compacta (SNpc) neuronal loss resembling that found in early Parkinson's (Kostrzewa and Jacobowitz, 1974; Glinka et al., 1997; Kirik et al., 1998; Deumens et al., 2002; Bethel-Brown et al., 2010). In addition to decreases in neuronal number, morphological changes to dopaminergic neurons and their nucleoli after 6-OHDA may also reflect the processes occurring in PD (Lee et al., 1996; Gomide et al., 2005; Hetman and Pietrzak, 2012). Nucleolar function has been associated with nucleolar size; (Boulon et al., 2010) hence a decrease in nucleolar volume in the lesioned SNpc could reflect a loss of nucleolar function after 6-OHDA lesion, and thereby model the processes involved in PD.

Design-based stereology is a well-established method for obtaining robust data, and is extensively used to determine neuronal number and morphology of the SNpc in PD and in animal models (Kirik et al., 1998; Finkelstein et al., 2000; Carvalho and Nikkhah, 2001; Schmitz and Hof, 2005; Ahmad et al., 2008; Rudow et al., 2008; Eriksen et al., 2009). Accordingly, this study employed the tyrosine hydroxylase-silver nucleolar (TH-AgNOR stain), which combines tyrosine hydroxylase (TH) staining for catecholaminergic neurons with silver-binding nucleolar (AgNOR) staining (Healy-Stoffel et al., 2012), and stereological analysis techniques to determine the effects of a partial unilateral 6-OHDA model on the SNpc TH-positive neuronal and nucleolar morphology in adult male Long-Evans rats.

6.3 Results

6.3.1 Amphetamine-stimulated rotation

The mean number of amphetamine-stimulated rotations completed by lesioned rats during the 60-min observation period was 154 ± 47. The relationship between rotation and percent of SNpc neuronal was poorly correlated (r^2 = 0.135).

6.3.2 Stereological Analysis

Low-and high-power photomicrographs show the morphology of the ipsilateral and contralateral SNpc TH-positive neurons. Low (4X) magnification demonstrates the loss of TH-positive neurons typical of neurodegeneration of the lesioned SNpc (**Figure 1A and B**). High-power (100X) magnification (**Figure 1C and D**) shows the outlines of nucleolar bodies, which are heavily pigmented compared to the surrounding cytoplasm.

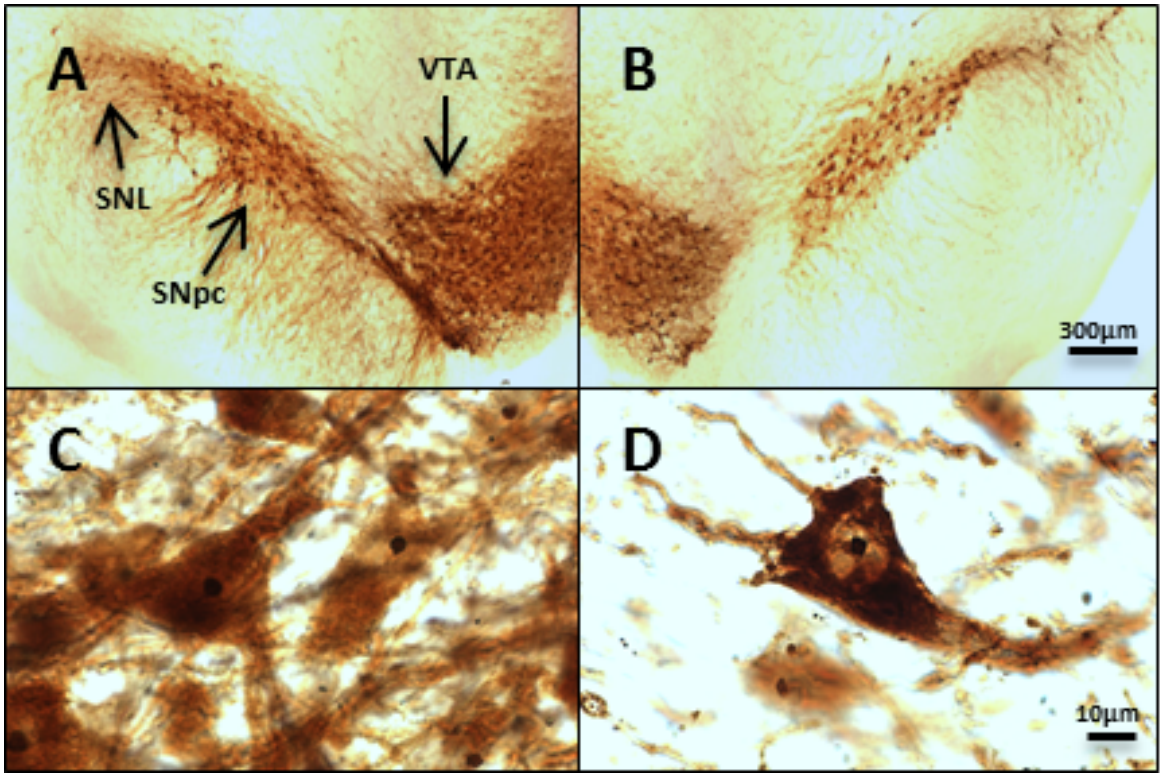

Figure 1.

High-power and low-power micrographs of TH-AgNOR stained sections of contralateral and ipsilateral substantia nigra. 4X images of TH-AgNOR-stained sections of contralateral and ipsilateral SN qualitatively show depletion of the lesioned dopaminergic regions (1A and B). 100X images show the outlines of the neurons and the AgNOR-stained nucleoli (1C and D). Representative sections from the same rat are shown. Abbreviations: SNL- substantia nigra lateralis, SNpc- substantia nigra pars compacta, VTA- ventral tegmental area

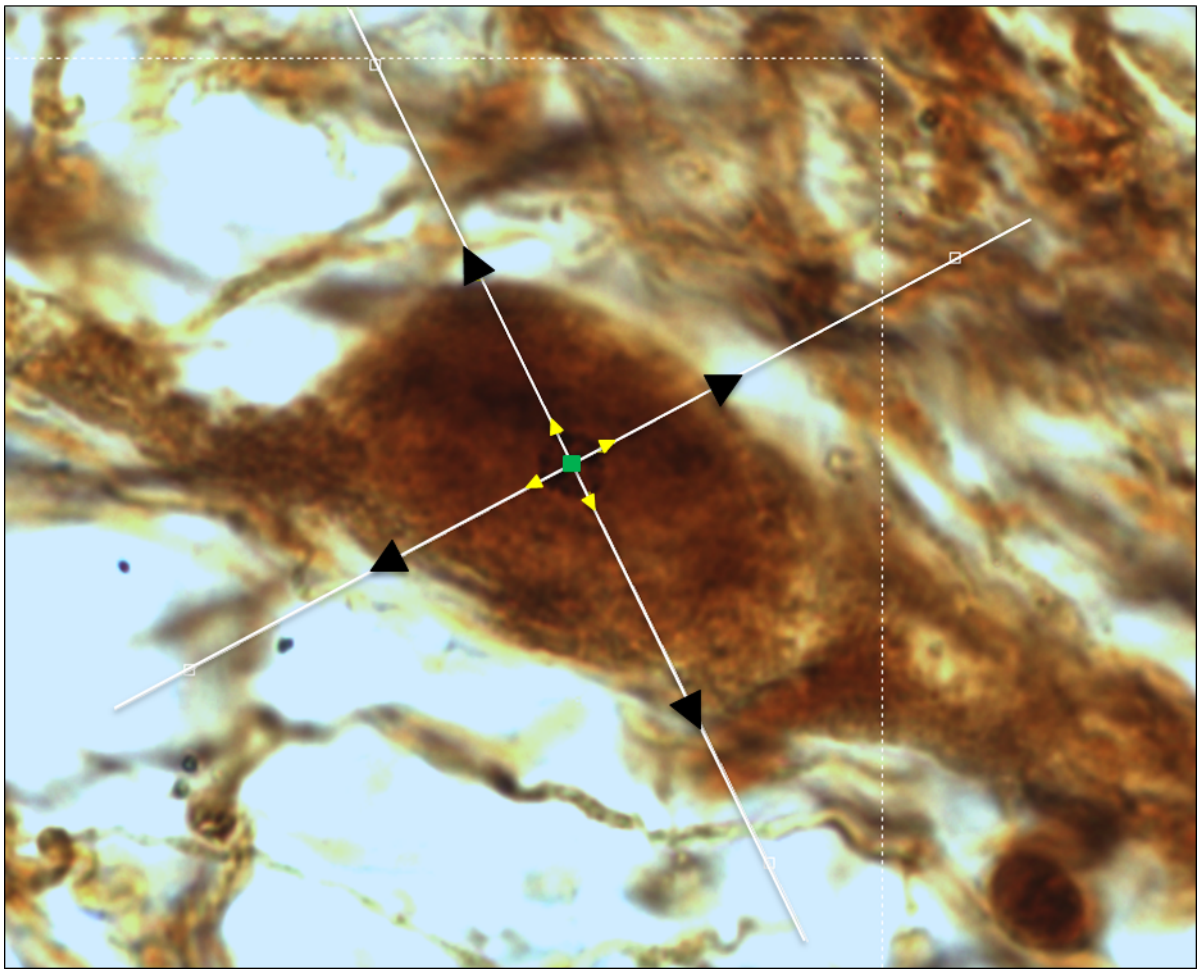

Figure 2.

The Double Nucleator technique. Markers are placed on the nucleolus center and where the nucleator rays intersect with the borders of the nucleolus and the neuronal body outline.

6.3.3 TH-AgNOR-stained SNpc region volume

Regional planimetric volume of the ipsilateral SNpc was smaller in all rats compared to the contralateral SNpc (**Figure 3A**). The planimetric volume ranged from 3.7 to 4.9 mm^3 on the contralateral side, and from 2.9 to 4.5 mm^3 on the ipsilateral side. The within-subject percentage of volume decrease ranged from 7% to 28%. The mean within-subject decrease in planimetric volume between the ipsilateral and contralateral SNpc was 18 ± 2% ($P<0.05$).

6.3.4 TH-AgNOR-stained SNpc neuron number

The mean total number of TH-positive SNpc neurons was 4631 ± 355 on the ipsilateral and 9062 ± 781 on the contralateral sides (**fig. 3B**), which is comparable with other stereological studies (Kirik et al., 1998; Carvalho and Nikkhah, 2001; Gomide et al., 2005; Ahmad et al., 2008; Eriksen et al., 2009). The number of TH-positive SNpc neurons ranged from 5,177 to 12,369 on the contralateral side, and from 3,442 to 7,289 on the ipsilateral side. All rats showed a decrease in the number of TH-positive cells in the ipsilateral SNpc compared to the contralateral SNpc. The within-subject percentage decrease neurons ranged from 18% to 68%. The mean within-subject decrease was 46% ± 5% ($P<0.05$).

6.3.5 TH-AgNOR-stained SNpc neuron volume

Average neuronal body volume was smaller in all rats in the ipsilateral SNpc compared to the contralateral SNpc (**Figure 3C**). The mean volume of SNpc TH-AgNOR positive neurons on the contralateral side (3,722 ± 60 μm^3) was comparable to

the range of findings in other stereological studies (Gomide et al., 2005; Ahmad et al., 2008). The neuronal volume of TH-AgNOR-positive SNpc neurons ranged from 3,331 to 3,961 μm^3 on the contralateral side, and from 2,263 to 3,700 μm^3 on the ipsilateral side. The within-subject percentage loss of neuronal volume ranged from 4% to 40%. There was a mean within-subject decrease of 11 ± 3% ($P<0.05$).

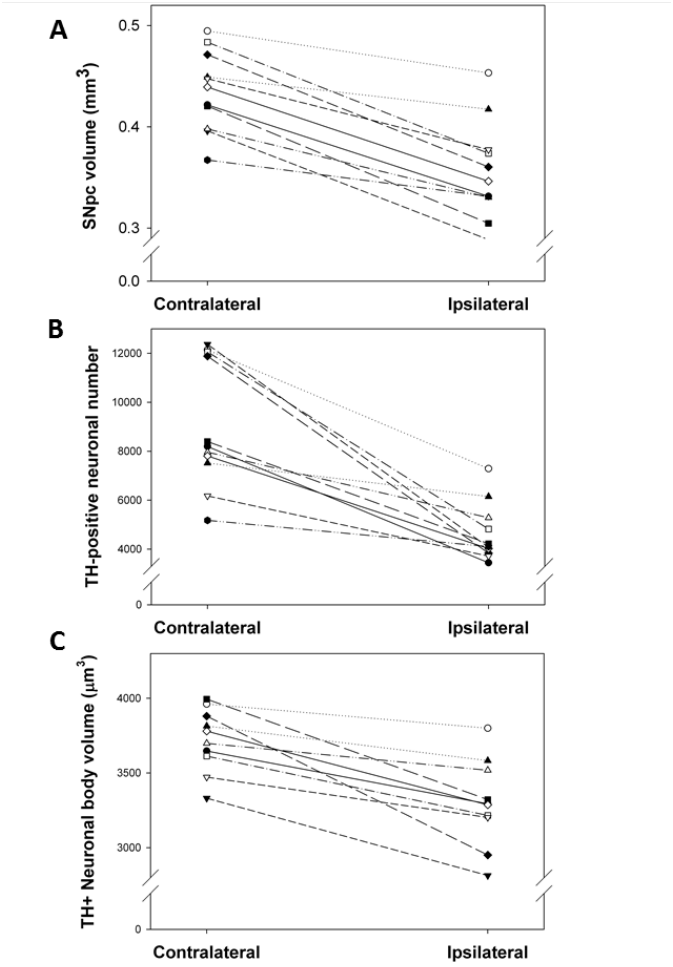

Figure 3.

Effects of unilateral intrastriatal 6-OHDA lesion on TH-AgNOR-stained planimetric volume (A), neuron number (B) and neuron volume (C) in the SNpc. All rats showed decreased SNpc planimetric volume between the ipsilateral and contralateral hemispheres. The mean within-subject decrease was 18 ± 2% ($P<0.05$ by paired t-test, n = 11). All rats showed decreased neuronal number between the ipsilateral and contralateral SNpc. The mean within-subject decrease was 46% ± 5% ($P<0.05$ by paired t-test, n = 11). All rats showed decreased neuronal volume between the ipsilateral and contralateral SNpc. The mean within-subject decrease was 11± 3% ($P<0.05$ by paired t-test, n = 11).

6.3.6 TH-AgNOR-stained SNpc nucleolar volume

Average nucleolar volume was smaller in all rats in the ipsilateral SNpc compared to the contralateral SNpc. The mean nucleolar volume of TH-AgNOR-positive SNpc neurons in individual rats ranged from 20 to 32 μm^3 on the contralateral side, and from 16 to 29 μm^3 on the ipsilateral side. The within-subject percentage decrease of nucleolar volume ranged from 5% to 32%. There was a mean within-subject decrease of 16 ± 2% ($P<0.05$) (**Figure 4A**). However, the ratio of nucleolar volume to neuronal volume between the ipsilateral and contralateral SNpc was smaller in only 6 of the 11 rats and there no significant difference between sides (**Figure 4B**).

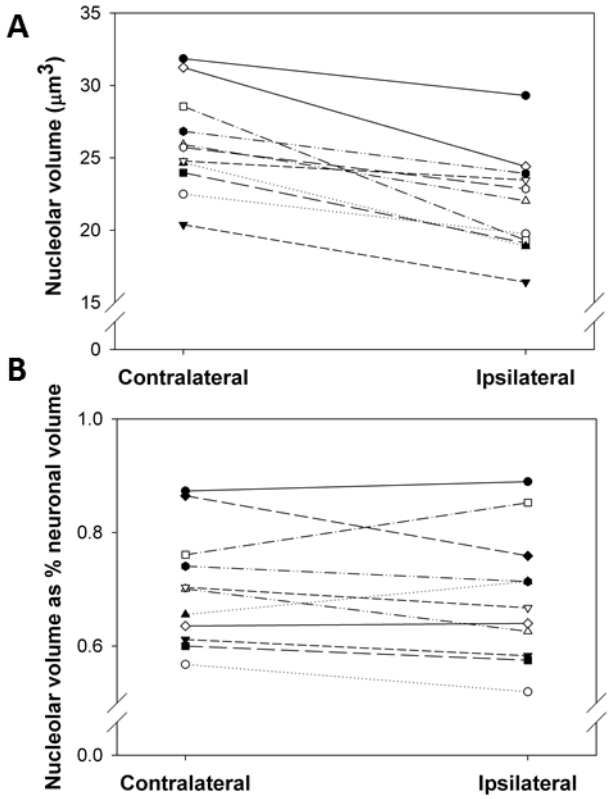

Figure 4.

Effects of unilateral intrastriatal 6-OHDA lesion on TH-AgNOR-stained nucleolar volume in the SNpc. All rats showed decreased nucleolar volume between the ipsilateral and contralateral SNpc (4A). The mean within-subject decrease was 16 ± 2% ($P<0.05$ by paired t-test, n = 11). The ratio of nucleolar volume to neuronal volume in the ipsilateral SNpc was smaller in only 6 of the 11 rats the contralateral SNpc and there was no significant difference between sides (4B).

6.4 Discussion

This study determined the effects of unilateral 6-OHDA neurotoxic lesion on SNpc neuronal number, soma volume and nucleolar morphology using stereology and TH-AgNOR staining. A functional dopaminergic deficit was confirmed in the ipsilateral striatum by mean amphetamine-stimulated rotations. The single-site injection of 6-OHDA (12.5 µg) resulted in an 18% decrease in the planimetric volume of the TH-positive ipsilateral SN and a 46% depletion of SNpc TH-positive neurons (**Figures 3A and 3B**). Furthermore, the appearance of clinical signs in PD has been associated with SNpc neuronal losses of around 50% (Marsden, 1990); thus, cell loss of this magnitude reflects a clinically relevant model of moderate PD. Neuronal volume loss was 11% with a corresponding 16% decrease in nucleolar volume on the ipsilateral side. Overall, these findings suggest that the partial unilateral 6-OHDA lesion achieved a moderate depletion of SNpc neurons comparable to the threshold findings in parkinsonian human brains.

Using the TH-AgNOR stain, the present data show a 16% decrease in nucleolar volume accompanied by an 11% decrease in neuronal body volume (**Figure 3C**). These decreases in nucleolar and neuronal size support morphological findings observed in postmortem PD brains (Mann and Yates, 1982; Rudow et al., 2008). This supports the validity of the 6-OHDA lesioned rat as model of the early stages of parkinsonian neurodegeneration, at least with respect to these parameters.

Altered morphology is an informative tool in documenting the changes caused by the neurodegenerative process to neuronal and nucleoli volume. Quantitation of morphological changes is an important first step in establishing differences between

neurons, which can then be further explored with complementary functional or biochemical assays to determine the mechanisms of change. The volumetric changes that occurred in this study could be due to either neurodegeneration on the ipsilateral side, or to compensation-related hypertrophy on the contralateral side, since striatal sprouting after unilateral brain lesions has been proposed as a compensatory mechanism (Cheng et al., 1998; Finkelstein et al., 2000). In future studies, it would be interesting to determine the neuronal and nucleolar volume in additional unlesioned rats for comparison; however, another study using a unilateral intrastriatal 6-OHDA (8µg) and solvent-injected control animals using stereological analysis found no difference in contralateral TH-positive SNpc soma volumes between the 6-OHDA- and solvent-injected animals (Gomide et al., 2005). Nucleolar size is associated with nucleolar activity (Boulon et al., 2010), thus the 16% change in nucleolar volume in the ipsilateral hemisphere (**Figure 4A**) may reflect a change in nucleolar function and provide a valuable index of neuronal damage. Other changes to nucleolar morphology, such as nucleolar segregation and disruption, are associated with various cellular stressors, including DNA damage, hypoxia, viral infection, and nutrient stress (Boulon et al., 2010). Likewise, the stressors potentially involved in the etiology of PD could also cause altered nucleolar morphology. Altered nucleolar morphology has been reported in clinical PD, with the volume of nucleoli decreased in post-mortem PD brains by 16% (Mann and Yates, 1982). Since neuronal and nucleolar function are closely interrelated, however, it may be more relevant to consider neuronal and nucleolar volume together than separately. Indeed, in our study there was no difference in the percentage of the nucleolus to total neuronal volume in the ipsilateral and contralateral SNpc (**Figure 4B**),

suggesting a concomitant decrease of both neuronal body and nucleolus that may indicate their interdependent morphology. Although the morphological relationship between the nucleolar and neuronal body volumes in clinical PD is not currently known, stereological assessment of neuronal and nucleolar volumes in postmortem brains from Alzheimer's patients showed both neuronal and nucleolar atrophy in the CA1 region of the hippocampus (Iacono et al., 2008), implicating decreased nucleolar volume in neurodegenerative disease.

How nucleolar morphology reflects the neurodegeneration in PD is still uncertain, although there are several potential mechanisms for nucleolar damage and consequent morphology changes in PD. Greater nucleolar damage to postmortem PD neurons, assessed by loss of nucleolar integrity, has been found relative to controls (Rieker et al., 2011). Oxidative damage to RNA has been a suspected factor in a number of neurodegenerative diseases, including PD (Kong and Lin, 2010), and may play a role in nucleolar damage. For example, in mice, 1,2,3,6-tetrahydro-1-methyl-4-phenylpyridine hydrochloride (MPTP) treatment induced nucleolar damage marked by nucleophosmin staining localization to the cytoplasm and inhibited mammalian target of rapamycin (mTOR) signaling, a regulator of rRNA synthesis. (Rieker et al., 2011). The transcription initiation factor 1A (TIF-1A) regulates the nucleolus-specific RNA polymerase 1 (Pol1), and TIF-1A ablation in mouse embryonic fibroblasts results in nucleolar disruption and in upregulation of tumor-suppressing protein p53 (Yuan et al., 2005). Adult mice with selective dopaminergic neuron ablation of TIF-1A demonstrated progressive loss of SN neurons and locomotor deficits (Rieker et al., 2011). Nucleolar damage in PD could also be precipitated by DNA damage, as the DNA-topoisomerase-

2 inhibitor etoposide inhibited Pol1 and induced nucleolar stress indicated by staining for B23/nucleophosmin (Pietrzak et al., 2011). Future studies must determine specifically how nucleolar damage, and corresponding nucleolar morphology changes, contribute to the pathophysiology of PD and whether nucleolar morphology may prove useful as an indicator of disease progression.

6.5 Conclusions

In conclusion, TH-AgNOR staining combined with stereological assessment indicated altered nucleolar morphology of SNpc dopaminergic neurons in rats after a partial unilateral intrastriatal 6-OHDA. The observed decreased nucleolar volume suggests that this organelle plays a critical role in neurodegenerative processes and possibly also in the early clinical course of the PD.

CHAPTER 7

DIFFERENTIALLY ALTERED NEURONAL NUMBER AND MORPHOLOGY IN MIDBRAIN DOPAMINERGIC SUBREGIONS AFTER A UNILATERAL INTRASTRIATAL 6-OHDA LESION

7.1 Abstract

The nucleolus is an important player in the cellular response to stress, and changes in nucleolar morphology may be an indicator of neuronal response to the neurodegeneration caused by Parkinson's disease (PD). Although nucleolar morphology is altered in substantia nigra (A9 dopamine neurons) in PD, it is not known whether the nucleoli of the A8, A9 and A10 dopamine subgroups are differentially affected in PD. This study determined the effects of a partial unilateral intrastriatal 6-hydroxydopamine (6-OHDA) lesion model of PD on the nucleoli of dopaminergic cells in the A8, A9 and A10 neurons. Adult male rats underwent unilateral intrastriatal infusion of 6-OHDA (12.5 µg). Lesions were verified by amphetamine-stimulated rotation 7 days post-surgery, and rats were euthanized 14 days after infusion and brains stained with a TH-AgNOR protocol (NeuroScience Associates). Dopaminergic morphology in the lesioned and intact hemispheres in each rat was quantified with stereology. Nucleolar volume was decreased by 22% on the ipsilateral side in the A9 neurons, 24% in the A10, and 26% in the A8 (all $P<0.05$ by paired t-test). In addition, the lesion decreased dopaminergic regional planimetric volume, neuronal number and density, and neuronal body volume in the ipsilateral side in all subgroups ($P<0.05$ by paired t-test for all endpoints, except for no change in A8 density). Furthermore, dopaminergic regional planimetric volume, neuronal number and density, and neuronal body volume varied among the subgroups ($P<0.05$ by repeated-measures ANOVA). These findings suggest that although the 6-OHDA lesion did not differentially alter nucleolar morphology in the subgroups, the nucleolus of all three regions demonstrated morphological changes after 6-OHDA, which could prove useful in assessing PD treatments.

7.2 Introduction

Parkinson's disease (PD), a chronic progressive neurodegenerative disorder characterized by loss of dopamine, is best known for its motor symptoms of tremor, bradykinesia and rigidity. However, PD also features a variety of non-motor symptoms in which dopaminergic loss may play a role, such as depression, dementia, sleep disturbances and anosmia; and these non-motor signs often manifest years before the onset of motor signs (Becker et al., 2002; Claassen et al., 2010). Thus, unraveling the etiology of the early non-motor signs of PD may be the key to developing preventive treatments and early disease interventions.

The substantia nigra pars compacta (SNpc), ventral tegmental area (VTA) and retrorubral field (RRF) midbrain regions roughly corresponding to the A9, A10 and A8 groups of dopamine neurons, respectively, are responsible for the various motor and non-motor deficits in PD are differentially affected both in the disease and in PD models (Deutch et al., 1986; German et al., 1989; Hirsch et al., 1989; German et al., 1992; German and Manaye, 1993; Rodríguez et al., 2001), although the reasons for their differential susceptibility are still unclear.

The nucleolus, the site of ribosomal RNA (rRNA) synthesis, has great influence on the response of cells to stress and injury, and is a promising target for developing and assessing new treatments and therapeutics, especially in the early stages of PD. Thus, different midbrain dopamine neuron subpopulations may react differently to the processes involved in PD, and changes to nucleolar morphology may reveal new information that can lend insight into the pathophysiology of the disease.

The 6-hydroxydopamine (6-OHDA) neurotoxic lesion is a well-accepted animal model for PD, and has a demonstrated effect on the SNpc nucleolus and differential effects on different subregions. The 6-OHDA neurotoxin structurally resembles dopamine, and causes oxidative damage and cell death when taken up into the catecholamine neurons (Kostrzewa and Jacobowitz, 1974). The partial unilateral intrastriatal 6-OHDA model is relevant to model early Parkinson's, since it results in a more moderate loss of midbrain dopamine neurons than more extensive lesions (Kirik et al., 1998). Previous work from our laboratory showed that unilateral intrastriatal 6-OHDA infusion resulted in decreased SNpc neuronal number and neuronal and nucleolar volume (Healy-Stoffel et al., 2012). This study will determine the effects of a single-site intrastriatal unilateral 6-OHDA lesion on the numbers and neuronal morphology of the A8, A9 and A10 groups of dopaminergic neurons in rats, using stereological analysis and the tyrosine hydroxylase silver nucleolar (TH-AgNOR) staining method previously described (Healy-Stoffel et al., 2012).

7.3 Results

7.3.1 Stereological analysis of TH-AgNOR-stained neurons

Low-and high-power photomicrographs show the morphology of the ipsilateral and contralateral SNpc, VTA and RRF TH-AgNOR-stained neurons. Low (4X) magnification demonstrates the loss of TH-positive neurons following 6-OHDA-induced neurodegeneration (**Figure 1**). High-power (100X) magnification (**Figure 2**) shows the outlines of nucleolar bodies within neurons in the SNpc, VTA and RRF. The nucleoli are heavily pigmented with the AgNOR stain compared to the surrounding cytoplasm.

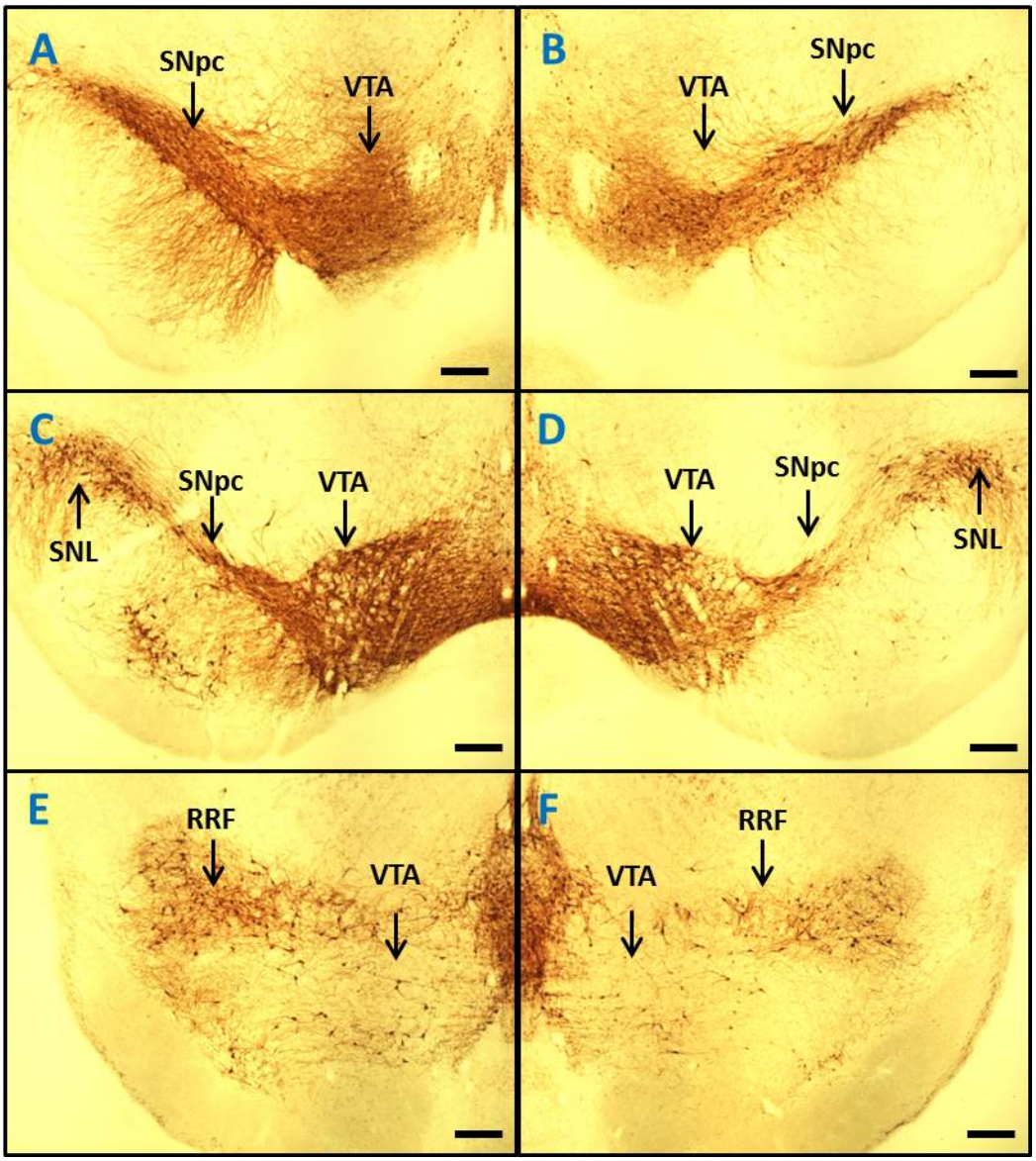

Figure 1.

Low-power micrographs of TH-AgNOR stained sections of contralateral and ipsilateral SNpc, VTA and RRF. 4X images of TH-AgNOR-stained sections of contralateral and ipsilateral SN qualitatively show depletion of the contralateral SNpc (A and C), ipsilateral SNpc (B and D), contralateral VTA (A, C and E), ipsilateral VTA (B, D and F), contralateral RRF (E) and ipsilateral RRF (F). Representative sections from the same rat are shown. Size bar = 300 µm. Abbreviations: SNL- substantia nigra lateralis, SNpc- substantia nigra pars compacta, VTA- ventral tegmental area, RRF- retrorubral field

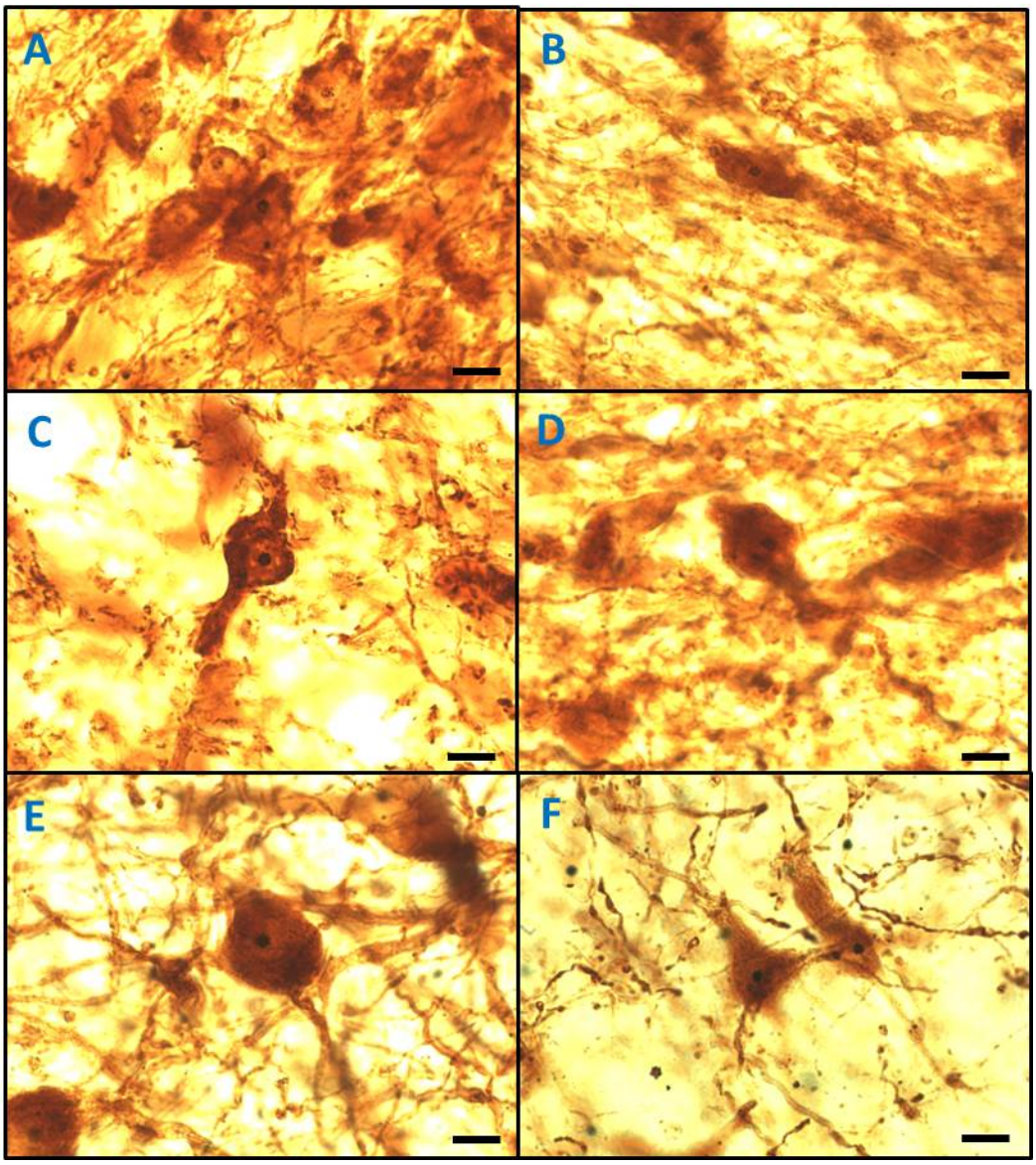

Figure 2.

High-power micrographs of TH-AgNOR stained sections of contralateral and ipsilateral SNpc, VTA and RRF. 100X images show the outlines of the neurons and the AgNOR-stained nucleoli in contralateral SNpc (A), ipsilateral SNpc (B), contralateral VTA (C), ipsilateral VTA (D), contralateral RRF (E) and ipsilateral RRF (F). Representative sections from the same rat are shown. Size bar = 10 μm. Abbreviations: SNL- substantia nigra lateralis, SNpc- substantia nigra pars compacta, VTA- ventral tegmental area, RRF- retrorubral field

7.3.2 TH-AgNOR-stained region planimetric volume

The ipsilateral SNpc was smaller in 9 out of 10 rats compared to the contralateral SNpc, the ipsilateral VTA was smaller in 8 out of 10 rats compared to the contralateral VTA, and the ipsilateral RRF was decreased in all rats compared to the contralateral RRF. The estimated planimetric volumes for individual rats ranged from 3.7 to 4.9 mm^3 in contralateral SNpc and from 2.7 to 4.5 mm^3 in ipsilateral SNpc; from 7.2 to 11.5 mm^3 in contralateral VTA and from 6.9 to 11.8 mm^3 in ipsilateral VTA; and from 3.2 to 5.5 mm^3 in contralateral RRF and from 1.9 to 4.5 mm^3 in ipsilateral RRF. The mean within-subject percent loss in planimetric volume between the ipsilateral and contralateral hemispheres was 20 ± 3% in the SNpc ($P<0.001$), 8 ± 2 % in the VTA ($P<0.01$), and 20 ± 5 % in the RRF ($P<0.05$) (**Figure 3**). The SNpc and RRF demonstrated greater planimetric volume loss than the VTA ($P<0.05$) (**Table 1**).

Table 1. Changes to TH-AgNOR-stained neurons and nucleoli in the SNpc, VTA and RRF after a unilateral intrastriatal 6-OHDA lesion

	Region	Contralateral	Ipsilateral	Within-subject % change (vs. contralateral)
Mean Planimetric volume (mm^3)	SNpc	4.3 ± 0.1	3.4 ± 0.2 ‡	-20 ± 3 *
	VTA	9.8 ± 0.6	9.1 ± 0.5 ‡	-8 ± 2
	RRF	4.4 ± 0.3	3.5 ± 0.3 ‡	-20 ± 5 *
Mean TH+ neuronal number	SNpc	10,883 ± 1042	5,546 ± 614 ‡	-46 ± 6 *
	VTA	16,614 ± 567	11,928 ± 449 ‡	-28 ± 3
	RRF	3,032 ± 321	1968 ± 204 ‡	-28 ± 11
Mean Density (Cells/mm^3)	SNpc	2,491 ± 193	1,609 ± 142 ‡	-33 ± 6 †
	VTA	1,724 ± 81	1,337 ± 53 ‡	-22 ± 3
	RRF	679 ± 56	570 ± 52	-11 ± 10
Mean TH-AgNOR+ neuronal volume (μm^3)	SNpc	3,735 ± 82	3,256 ± 175 ‡	-13 ± 4
	VTA	2,628 ± 101	2,075 ± 139 ‡	-21 ± 5 #
	RRF	4,125 ± 163	3,104 ± 152 ‡	-24 ± 4 #
Mean TH-AgNOR+ nucleolar volume (μm^3)	SNpc	25 ± 1	20 ± 1 ‡	-22 ± 3
	VTA	21 ± 2	15 ± 1 ‡	-24 ± 6
	RRF	23 ± 1	17 ± 1‡	-26 ± 4

Data are presented at the mean ± SEM.
‡ P<0.5 vs. contralateral side by paired t-test.# P<0.5 vs. SNpc by nonparametric repeated measures ANOVA followed by Dunn's multiple comparisons test.
* P<0.5 vs. VTA by repeated measures ANOVA followed by Student-Newman-Keuls or nonparametric repeated measures ANOVA followed by Dunn's multiple comparisons test.
† P<0.5 vs. RRF by repeated measures ANOVA followed by Student-Newman-Keuls.

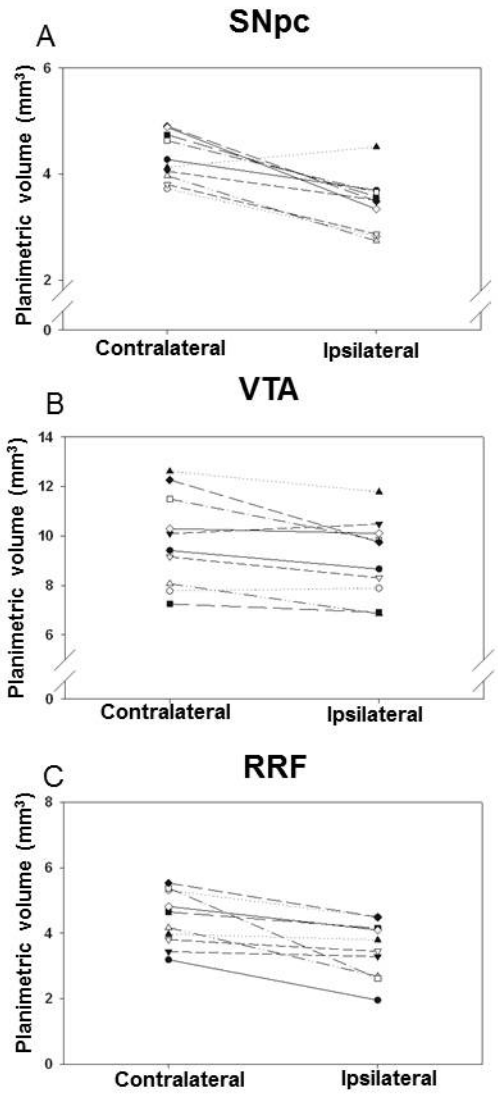

Figure 3.

Effects of unilateral intrastriatal 6-OHDA on planimetric regional volume. Nine out of 10 rats showed decreased SNpc planimetric volume between the ipsilateral and contralateral hemispheres. Eight out of 10 rats showed decreased VTA planimetric volume between the ipsilateral and contralateral hemispheres. All rats showed decreased RRF planimetric volume between the ipsilateral and contralateral hemispheres.

7.3.3 TH-AgNOR-stained neuron number

All rats had decreased ipsilateral TH-positive neuronal numbers compared to the contralateral SNpc and VTA, and 8 out of 10 rats had decreased TH-positive neuronal numbers compared to the contralateral RRF (**Figure 4**). The estimated TH-positive neuron numbers in individual rats ranged from 3,890 to 16,177 in contralateral SNpc and from 3,877 to 9,422 in ipsilateral SNpc; from 13,477 to 19,862 in contralateral VTA and from 9,494 to 13,818 in ipsilateral VTA; and from 1,205 to 4,164 in contralateral RRF and from 1,055 to 3,335 in ipsilateral RRF. The mean within-subject percent loss in TH-positive neuron number between the ipsilateral and contralateral hemispheres was 46 ± 6% in the SNpc ($P<0.001$), 28 ± 3% in the VTA ($P<0.001$), and 28 ± 11% in the RRF ($P<0.05$). The SNpc demonstrated an increased percentage of TH-positive neuron number loss compared to the VTA ($P<0.05$) (**Table 1**).

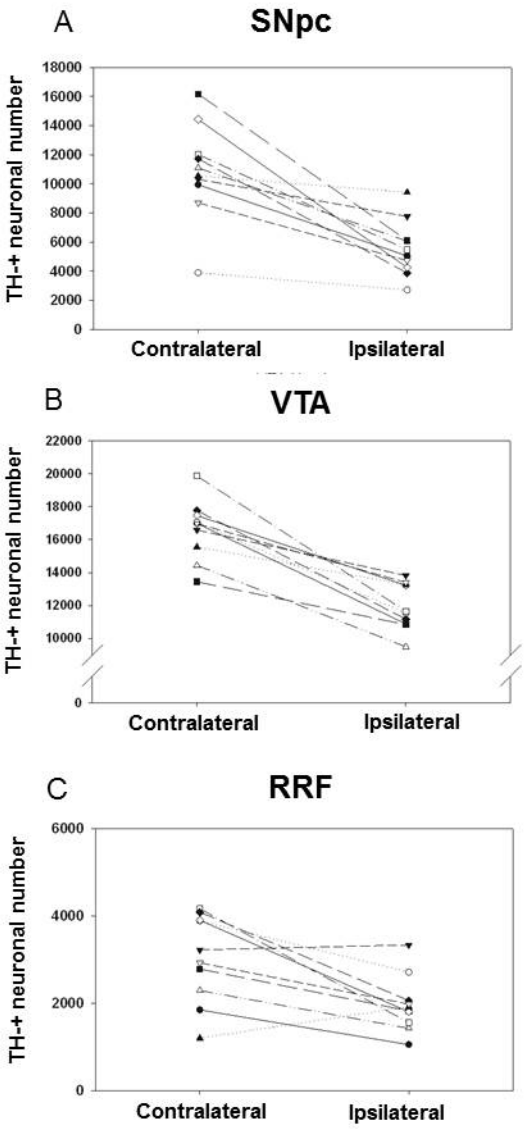

Figure 4.

Effects of unilateral intrastriatal 6-OHDA on TH-positive neuron number. All rats (n=10) showed decreased SNpc neuron number between the ipsilateral and contralateral hemispheres. All rats showed decreased VTA neuron number between the ipsilateral and contralateral hemispheres. Eight out of 10 rats showed decreased RRF neuron number between the ipsilateral and contralateral hemispheres.

7.3.4 TH-AgNOR-stained density

All rats had decreased ipsilateral TH-positive neuronal density compared to the contralateral SNpc and the contralateral VTA, and 8 out of 10 rats had decreased TH-positive neuronal density compared to the contralateral RRF (**Figure 5**). The estimated TH-positive neuron density for individual rats ranged from 1,045 to 3,412 neurons/mm^3 in contralateral SNpc and from 960 to 2,213 neurons/mm^3 in ipsilateral SNpc; from 1,233 to 2,193 neurons/mm^3 in contralateral VTA and from 1,125 to 1,613 neurons/mm^3 in ipsilateral VTA; and from 303 to 739 neurons/mm^3 in contralateral RRF and from 440 to 1,016 neurons/mm^3 in ipsilateral RRF. The mean within-subject percent loss in TH-positive neuronal density between the ipsilateral and contralateral hemispheres was 33 ± 6 % in the SNpc ($P<0.001$), 22 ± 3% in the VTA ($P<0.001$), and 11.1 ± 10.0% in the RRF. The SNpc demonstrated an increased percentage of TH-positive neuron number loss compared to the RRF ($P<0.05$). Mean TH-positive neuronal density of the ipsilateral RRF was unchanged compared to the contralateral side (**Table 1**).

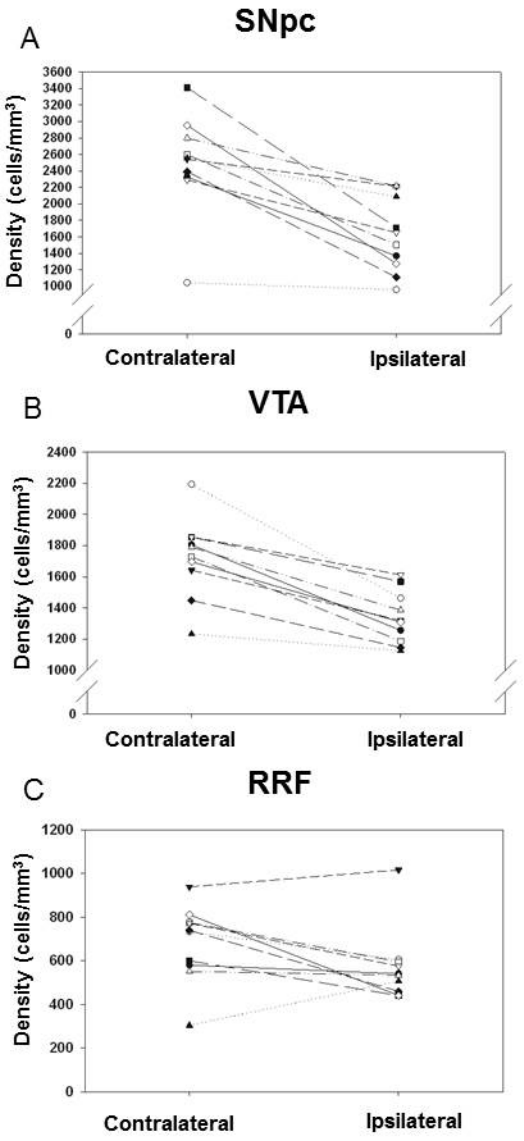

Figure 5.

Effects of unilateral intrastriatal 6-OHDA on TH-positive neuronal density. All rats (n=10) showed decreased SNpc neuronal density between the ipsilateral and contralateral hemispheres. All rats showed decreased VTA neuronal density between the ipsilateral and contralateral hemispheres. Eight out of 10 rats showed decreased RRF neuronal density between the ipsilateral and contralateral hemispheres.

7.3.5 TH-AgNOR-stained neuron volume

Eight out of 10 rats had decreased TH-positive neuronal volume compared to the contralateral SNpc, 9 out of 10 rats had decreased TH-positive neuronal volume compared to the contralateral VTA, and all rats had decreased TH-positive neuronal volume compared to the contralateral RRF (**Fig. 6**). The mean TH-positive neuron volumes for individual rats ranged from 3,327 to 4,104 μm^3 in contralateral SNpc and from 2,216 to 4,161 μm^3 in ipsilateral SNpc; from 2,206 to 3,377 μm^3 in contralateral VTA and from 1,733 to 2,985 μm^3 in ipsilateral VTA; and from 3,522 to 5,065 μm^3 in contralateral RRF and from 2,374 to 3,849 μm^3 in ipsilateral RRF. The mean within-subject percent loss in TH-positive neuronal volume between the ipsilateral and contralateral hemispheres was 13 ± 4% in the SNpc ($P<0.05$), 21 ± 5% in the VTA ($P<0.05$), and 24 ± 4% in the RRF ($P<0.001$). The VTA demonstrated an increased percentage TH-positive neuronal volume loss compared to the SNpc ($P<0.05$). The RRF demonstrated an increased TH-positive percentage neuronal volume loss compared to the SNpc ($P<0.001$) (**Table 1**).

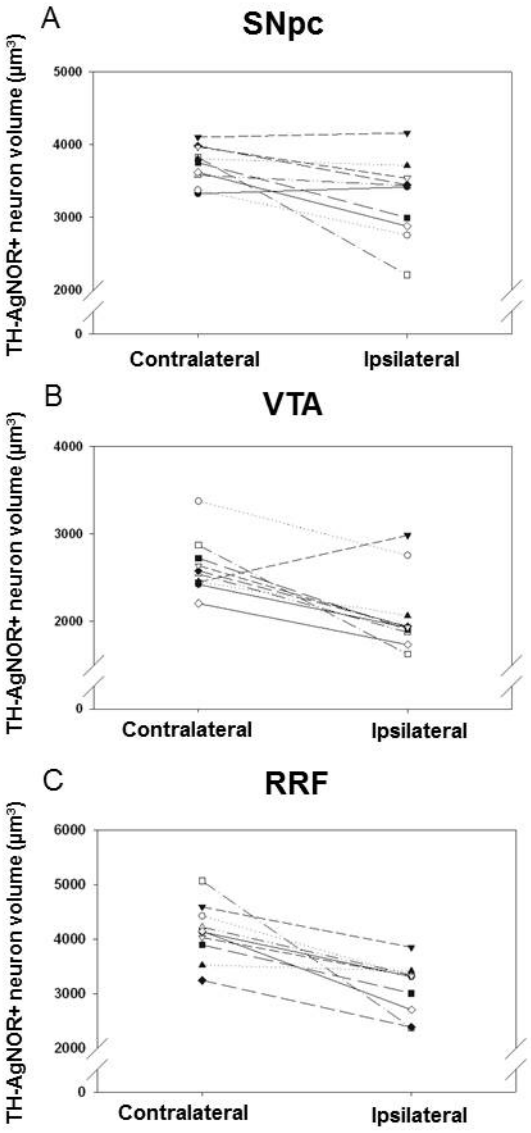

Figure 6.

Effects of unilateral intrastriatal 6-OHDA on TH-AgNOR-positive neuronal volume. Eight out of 10 rats showed decreased SNpc neuronal volume between the ipsilateral and contralateral hemispheres. Nine out of 10 showed decreased VTA neuronal volume between the ipsilateral and contralateral hemispheres. Nine out of 10 rats showed decreased RRF neuronal volume between the ipsilateral and contralateral hemispheres.

7.3.6 TH-AgNOR-stained nucleolar volume

All rats had decreased TH-positive SNpc, VTA and RRF nucleolar volume compared to the contralateral hemisphere (**Figure 7**). The mean TH-positive neuron volumes for individual rats ranged from 20 to 29 μm^3 in contralateral SNpc and from 16 to 24 μm^3 in ipsilateral SNpc; from 15 to 37 μm^3 in contralateral VTA and from 12 to 18 μm^3 in ipsilateral VTA; and from 20 to 27 μm^3 in contralateral RRF and from 13 to 20 μm^3 in ipsilateral RRF. The mean within-subject decrease in TH-positive nucleolar volume between the ipsilateral and contralateral was 22 ± 3% in the SNpc ($P<0.001$), 24 ± 6% in the VTA ($P<0.001$), and 26 ± 4% in the RRF ($P<0.001$). There was no significant difference among the percentage of loss in TH-positive nucleolar volume among the SNpc, VTA or RRF (**Table 1**).

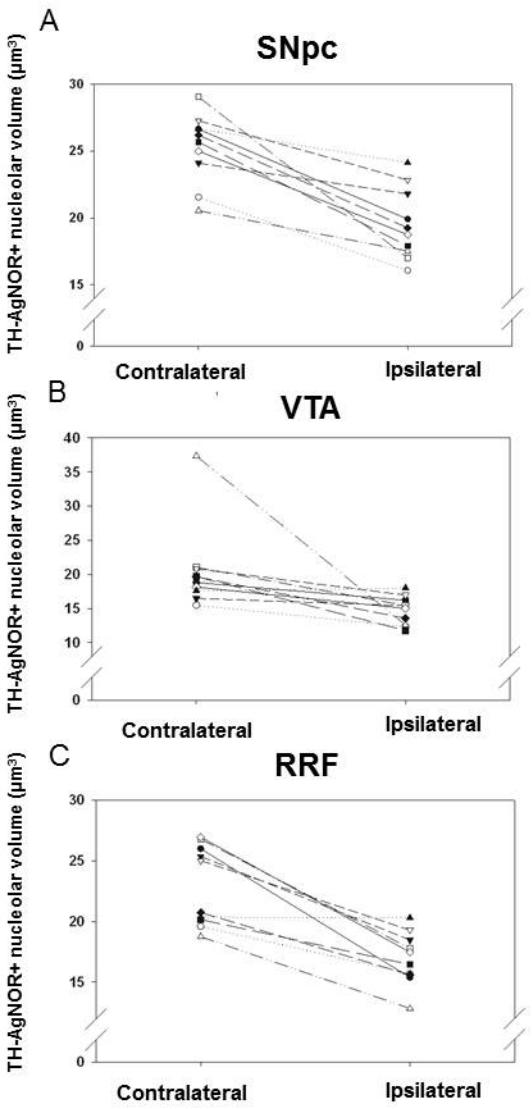

Figure 7.

Effects of unilateral intrastriatal 6-OHDA on TH-AgNOR-positive nucleolar volume. All rats (n = 10) showed decreased SNpc nucleolar volume between the ipsilateral and contralateral hemispheres. All rats showed decreased VTA nucleolar volume between the ipsilateral and contralateral hemispheres. Nine out of 10 rats showed decreased RRF nucleolar volume between the ipsilateral and contralateral hemispheres.

7.4 Discussion

This study determined the effects of a unilateral intrastriatal 6-OHDA lesion on differential susceptibility of neuronal number and morphology in the A8, A9 and A10 dopamine neuron groups in rats. The SN, VTA and RRF, corresponding to the A9, A10 and A8 groups of midbrain dopaminergic neurons, respectively, are geographically separate groups of neurons that have some overlap in their projections, and are all three regions are affected in PD. The substantia nigra, especially the pars compacta portion, is the main site for the origin of the mesostriatal dopamine neurons involved in motor disorders like PD, Huntington's disease and progressive supranuclear palsy; and its role in PD is well documented (German et al., 1989; Damier et al., 1999; Eriksen et al., 2009). The VTA is the origin of the majority of mesocortical and mesolimbic dopamine projections, and is involved in cognition, motivation, addiction, emotion and reward, and in PD, the VTA is implicated in the non-motor dopamine-dependent aspects of PD such as depression, cognitive and affective deficits (Lieberman, 2006; Sillitoe and Vogel, 2008; Da Cunha et al., 2009; Blonder and Slevin, 2011). The role of the RRF is the least understood of these regions, although its neurons are known to project to the nigrostriatal, mesocortical and mesolimbic systems (Deutch et al., 1988; Arts et al., 1996; François et al., 1999; Cho and Fudge, 2010). In rats, A8 neurons innervate the striatum, the amygdala and other regions, implicating these regions in modulation of both motor and limbic circuits (Jimenez-Castellanos and Graybiel, 1987). There is known cell loss in the RRF in both post-mortem PD brains and in and Parkinson's animal models, and the RRF is implicated in the presence of tremor, orofacial motor

dysfunction, and in sleep and visual disturbances in PD and PD models (Spooren et al., 1993a; Jellinger, 1999; Lai et al., 1999; Aarsland et al., 2005; Kolasiewicz et al., 2012). Although there have been many studies on the projections, circuitry and function in these three dopaminergic regions, there are few studies addressing how the morphology of the dopaminergic neurons in these regions are differentially affected by PD, making this a promising area of investigation using modern histological and stereology tools.

6-OHDA enters the cells by monoamine transporter uptake, and causes neurodegeneration of monoaminergic neurons by oxidative mechanisms (Kostrzewa and Jacobowitz, 1974). This model is relevant to early PD because it causes less extensive SNpc neuron loss and milder behavioral deficits than those found in more severe lesion methods (Deumens et al., 2002). Of course, intrastriatal 6-OHDA differs from PD in that the toxin is administered directly into the striatum, whereas the origins of neurodegeneration in PD are still unknown. In respect to the A8, A9 and A10 dopaminergic subregions, differential neurotoxicity could be expected simply based on the reduced numbers of projections to the striatum from the A8 and A10 regions compared to the A9 neurons of the SNpc (Jimenez-Castellanos and Graybiel, 1987; German et al., 1988), and the intrastriatal route of toxin administration could cause disproportional loss of A9 neurons to that found in PD. However, similar patterns of dopaminergic neuron loss to those in this study have been found after both intraventricular administration of 6-OHDA in the rat and intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse (German et al., 1996; Rodríguez et al., 2001), suggesting that the route of 6-OHDA infusion may not greatly

affect the proportion of cell loss. Most importantly, the pattern of dopamine cell loss induced by 6-OHDA reflects the losses found in post-mortem parkinsonian brains, reinforcing the likelihood that the same processes may be occurring in both 6-OHDA neurotoxicity and in PD; and making the intrastriatal 6-OHDA lesion a potentially useful model of all the dopaminergic neuron populations affected in PD.

The unilateral intrastriatal 6-OHDA lesion administered in this study induced a 20% loss of region volume loss in the SN, an 8% loss in the VTA, and a 20% loss in the RRF. Most studies do not report changes in the regional volume of dopaminergic subnuclei, but decreases in region volume have been reported in the SN and the VTA in PD (McRitchie et al., 1997; Kashihara et al., 2011; Ziegler et al., 2013). However, one stereological study on the volume of the RRF in PD found no change in region volume in diseased patients (McRitchie et al., 1997). Our planimetric volume findings were interesting because the degree of volume loss found in the VTA was less than that in the SN and RRF, which reflects the pattern of neuronal loss found in these regions in animal studies (German et al., 1988; German et al., 1996; Rodríguez et al., 2001). This suggests that a loss of regional volume is associated with the degree of neuronal loss in PD models; and although these findings conflict with what has been found in PD, the scarcity of robust stereological studies in this area warrant further investigation into the differential vulnerability of dopaminergic neuronal group regional volumes. Furthermore, the unilateral intrastriatal 6-OHDA lesion resulted in a neuronal density (neurons/mm^3) loss of 33% in the SN, 22% in the VTA, and 11% (not significant) in the RRF. Losses of neuronal density have been reported in PD in the SN and the VTA, although no change was found in neuronal density in the RRF (Jellinger, 1991; German

and Manaye, 1993; McRitchie et al., 1997). Our findings reflect the results found in PD studies, and furthermore are comparable to the findings in a recent stereological unilateral 6-OHDA lesion study in rats (8 µg), which found a 25% loss of neuronal density in the SN, and a 32% loss in the VTA (Gomide et al., 2005). While neuronal density is related to both the regional volume and to the neuronal number, it indicates that the regional volume loss is due to a loss of neurons, and not simply due to degeneration of TH-pigmented axons and dendrites. More anatomical studies of the midbrain dopaminergic neurons should report the region volume and neuronal density findings, in order to furnish a more complete understanding of the neuronal losses occurring in PD.

Single-site unilateral intrastriatal administration of 6-OHDA induced a 46% loss of TH-positive neurons in the SNpc, a 28% loss in the VTA, and a 28% loss in the RRF. The SNpc has the most projections to the striatum, and is the most affected in PD lesion models (German et al., 1988; Hirsch et al., 1988; German et al., 1996; Rodríguez et al., 2001). The percentage of SNpc neurons lost in this study is comparable to others' findings in moderate PD models (Lee et al., 1996; Rodríguez et al., 2001), although in more extensive lesion models the loss of SNpc dopaminergic neurons is much greater and resembles the levels of SN neuronal loss found in PD post-mortem studies (Kirik et al., 1998; Deumens et al., 2002). In post-mortem PD brains, most of which are at an advanced stage of neuronal loss, SNpc neurons are decreased by 70% or more (Hirsch et al., 1988). The VTA chiefly projects to the mesolimbic and mesocortical dopaminergic pathways (Oades and Halliday, 1987; van Domburg and ten Donkelaar, 1991), and studies in the VTA show that it is usually less vulnerable than the SN and

similarly affected to the A8 neurons in PD, and the least vulnerable region in PD models (Uhl et al., 1985; German et al., 1988; Hirsch et al., 1988; Hirsch, 1994; German et al., 1996; Rodríguez et al., 2001). Consistent with the design of our lesion as a model of early stage PD, we found a smaller loss of neurons in all of these neuronal groups than is typically found in PD post-mortem studies, which are assumed to be in the late stages of the disease. Our results complement the findings of another study of dopaminergic neuronal loss after 6-OHDA administration, in which a 300 µg intraventricular infusion induced losses of 50-80% in the A9 group, and 30-50% losses in the A8 and A10 groups (Rodríguez et al., 2001). Interestingly, even in more severe lesion models and in PD, the ultimate levels of neuronal loss in the A8 and A10 groups peak at around 70% for the A8 RRF neurons in most studies, and around 60% for the A10 VTA neurons (Uhl et al., 1985; German et al., 1988; Hirsch et al., 1988; German et al., 1996; Rodríguez et al., 2001). This could indicate that although the A8 and A10 groups may be ultimately less susceptible in end-stage loss of numbers, they may be more sensitive to early loss of neurons. Even if the VTA and RRF ultimately lose a smaller percentage of their numbers, they may still be more vulnerable to a loss of function than their SNpc counterparts, and may thus be responsible for the early non-motor signs of Parkinson's. An important thing to keep in mind when comparing rodent models with post-mortem PD studies is that rodents have a lower proportion of A9 and a higher proportion of A10 dopaminergic neurons relative to primates, although the proportions of A8 neurons are similar (German and Manaye, 1993); therefore susceptibility to PD-modeling lesions may be different. This is especially relevant for analysis of motor signs which manifest after a critical threshold of dopamine loss is reached as is thought to occur in PD

(Marsden, 1990). However, rodent studies still provide a meaningful and practical way to model the disease and assess treatments. In fact, the rodents' greater proportions of the A10 neurons implicated in the non-motor signs of Parkinson's may make them more sensitive to insults in these neurons, and thus better models for learning about the early disease process.

Changes to neuronal volume can be an indicator of altered neuronal function, and therefore potentially useful indicators of the changes induced by neurodegeneration. In this study, 6-OHDA induced a TH-positive neuronal body volume loss of 13% in the SNpc, 21% in the VTA and 24% in the RRF. The fact that the SNpc neurons in this study were relatively resistant to change in volume compared to those of the VTA and RRF is especially intriguing when considered in the context of the increased SNpc dopaminergic neuron loss compared to the VTA and RRF. This may indicate that the ipsilateral RRF and VTA neurons are either more susceptible to decreased volume in early stages of the disease, or that there is relatively greater hypertrophy on the contralateral side- either of which could be indicators that these lesser-understood dopamine subpopulations may play an important role in the early disease process. Alternatively, the ipsilateral SNpc neurons could be relatively hypertrophied in comparison to those of the VTA and RRF in order to compensate for the greater degree of neuronal loss in the SNpc. The results of studies of the effects of PD on neuronal volume in the SN have been mixed (Rudow et al., 2008); but recent studies using stereological analysis have found that neuronal volume is decreased in Parkinson's subjects, and that these findings are in contrast to hypertrophy of SN neurons found in normal aging (Rudow et al., 2008). In the VTA, dopaminergic

neuronal size was decreased in PD (McRitchie et al., 1997), as well as in a mouse chronic MPTP and probenecid model of PD. In the chronic MPTP and probenecid model, the morphological sequelae of the neuroprotective effects of exercise were determined, and the MPTP-induced losses in the volume of VTA neurons were restored after 18 weeks of exercise. This indicates that the morphology of VTA neurons is an effective indicator of neuronal response to neuroprotective treatments (Ahmad et al., 2009a). In the RRF, neuronal volume studies are lacking, but decreased A8 neuronal diameter was observed after intraventricular 6-OHDA in rats (Rodríguez et al., 2001). Because altered neuronal volume is associated with neurodegeneration (Iacono et al., 2008; Rudow et al., 2008), it is a potentially useful endpoint for assessing damage induced by PD. It has been proposed that neurons may undergo hypertrophy in initial reaction to stress, compensating for decreased function, and then may atrophy as the insult overwhelms the cell's ability to function, resulting in ultimate degeneration (Rudow et al., 2008). Of course, a limitation of post-mortem histology studies is the inability to determine whether the decreased volume is due to neuronal atrophy or to preferential loss of larger neurons. In addition, in unilateral lesion studies, as well as in early PD, which usually presents with unilateral clinical motor signs (Hoehn and Yahr, 1967); the difference in neuronal size between the ipsilateral and contralateral sides could be due to relative compensatory hypertrophy of the intact neurons as discussed in detail in Aim 3. However, the ability to quantify that volumetric changes are occurring in a model of early PD presents a very useful tool for assessing early treatments and interventions, since neuronal size changes can be a sign of cellular dysfunction (Janson et al., 1991; Smith et al., 1999; Rudow et al., 2008).

In addition to the changes to neuronal volume, the 6-OHDA lesion resulted in a nucleolar volume loss of 22% in the SNpc, a 22% loss in the VTA, and a 25% loss in the RRF. A 16% decrease in nucleolar volume has also been observed in post-mortem PD brains (Mann and Yates, 1982). Although studies on the effects of PD to the nucleolar volume are lacking in the VTA and RRF, it is interesting that in the present study, the nucleolar volume is decreased in both the VTA and the RRF, suggesting that alterations to nucleolar morphology in these regions may also be present in PD. Although the 6-OHDA lesion did not differentially alter nucleolar morphology in the SNpc, VTA and RRF, the A8 and A10 neurons, which are often less affected than the SN in PD, demonstrated similar nucleolar morphology changes to the SN. This suggests that A8 and A10 nucleolar morphology may be particularly susceptible to changes even early in PD, and could therefore be a useful assessment tool for developing and assessing early PD therapies. Although histological snapshots of nucleolar volume are subject to the same limitations that are discussed above regarding neuronal volume, the nucleolus plays important role in the cellular response to the oxidative stressors implicated in PD, and it is likely that morphological changes to the nucleolus reflect the presence of neurodegeneration. Thus nucleolar morphology studies offer promise as an important evaluation tool in early treatment assessment, in addition to their potential to lend insights to the etiology of the disease.

The metabolism and protein expression of dopaminergic neuron subpopulations are known to be different, providing an explanation for the differential susceptibility in PD. Of course, the differences in the number of striatal projections from each region likely influence each subpopulation's role in PD, but there are other potential factors

influencing subregion susceptibility. For example, the dopamine transporter (DAT) is differentially expressed in dopaminergic subpopulations; thus it has been proposed that the neuronal subgroups have differential metabolism of dopamine, which may contribute to their susceptibility in PD (Gibb and Lees, 1991; Blanchard et al., 1994). In support of this theory, the tissue content of melanin, a metabolite of tyrosine used an indicator of dopamine turnover, is differentially altered in dopaminergic subregions in Parkinson's (Gibb, 1992). In addition, the protein calbindin is differentially expressed in dopaminergic subregions, being present in the less-affected dopaminergic regions in PD and therefore may be associated with neuroprotection (Gibb, 1992). As the site of rRNA transcription, the nucleolus likely plays an important role in directing these factors in the differential susceptibility of dopaminergic neurons to PD, although further studies are needed to determine whether nucleolar changes in morphology are associated with changes in protein expression and metabolism; and whether these changes are a cause or an effect of Parkinson' pathology. In addition, the damage in PD is associated with oxidative damage, and the nucleolus is a key responder to oxidative stressors in the cell (Boulon et al., 2010; Hetman and Pietrzak, 2012). Thus the nucleoli in the midbrain dopaminergic subpopulations may play an important role in the differential response of these neurons to oxidative insult in PD. Further studies are needed to determine the roles of the nucleolus in the differential effects of PD on midbrain dopaminergic subpopulations, but determining the changes to neuronal morphology is an important part of the discovery process.

7.4 Conclusions

In conclusion, TH-AgNOR staining combined with stereological assessment indicated altered morphology of SNpc, VTA and RRF dopaminergic neurons in rats after a partial unilateral intrastriatal 6-OHDA. The observed decreased nucleolar volume suggests that this organelle is differentially affected in the dopaminergic subpopulations, and elucidating the mechanisms of the differential susceptibility of dopaminergic neurons may lead to new insights into the pathophysiology of PD, as well as contributing to the development of diagnostics and treatments.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Project Significance

8.1.1 Introduction

Although we are approaching the 200th anniversary of the 1817 publication of James Parkinson's *An Essay on the Shaking Palsy* describing the disease that would bear his name (Parkinson, 2002), there is still no cure for the progressive and debilitating neurodegenerative disorder of Parkinson's disease (PD). A major obstacle to advancements of treatments for the disease may be that much of our knowledge about PD comes from late-stage clinical assessment or post-mortem pathological studies. Likewise, many animal models of PD have also focused on reproducing a severe loss of dopamine similar to that found in clinical PD (Deumens et al., 2002). However, in severe PD and PD models, too much damage may already have occurred to be able to assess the effects of neuroprotective treatments and preventative interventions. Thus it is crucial to the advancement of the treatment of PD that we learn about the pathological processes occurring in the early stages of the neurodegeneration caused by PD.

Although the etiology of idiopathic PD is still unknown, a number of environmental factors, including n-3 polyunsaturated fatty acid (n-3 PUFA) intake have been proposed to contribute to susceptibility to PD. Thus the first way in which this dissertation aimed to increase knowledge of early PD was to determine the effects of a Low n-3 PUFA diet on functional and neuronal morphology effects in the well-established unilateral intrastriatal 6-hydroxydopamine (6-OHDA) rat model of PD. The effect of an n-3 PUFA-deficient diet has been linked to human health throughout the

lifespan, including advanced age (Swanson et al., 2012). Discoveries linking PUFA composition and the development of neurodegenerative disorders have the potential to change food regulation policies, the food industry, and standards of care in how nutrition is provided throughout life, thus this project has important long-term potential impact.

This project also elucidated the aspects of the pathobiological changes likely to be occurring in early PD was by determining the effects on dopaminergic number and neuronal and nucleolar morphology in the unilateral intrastriatal 6-OHDA model of early to moderate PD. Because neuronal structure and function are interrelated, changes to neurons determined by morphology can be informative of disease processes and effects of treatments. Changes to dopaminergic neuronal morphology occur in PD and PD models, and may be a useful index of the degree of disease progression and level of response to preventative and therapeutic treatments, as well as leading to better understanding of the processes occurring at the neuronal level in early PD.

In order to better treat and to cure PD, its etiology and the early stages of the disease need to be better understood, so that interventions take place before the damage becomes irreversible and untreatable. This dissertation contributed to the body of knowledge of the effects of an early to moderate model of PD on the dopaminergic neurons involved in early PD, and future projects can be built on the studies performed in this dissertation to further expand our knowledge of the mechanisms of PD.

8.1.2 Challenges in studying early PD

In general, early PD presents a number of inherent challenges to the clinical or basic science researcher. The most difficult problem to overcome may be the issue of

early detection of the development of the disease. The dopaminergic system is remarkable in its ability to compensate until quite a high threshold of damage has been reached. It has been estimated that PD patients may first present with motor dysfunction when approximately 80% of the striatal dopamine has already been depleted (Calne and Langston, 1983). Thus even if there is a stage at which clinicians can intervene and prevent PD, it is likely that patients presenting at such a late stage are beyond the threshold of intervention. Since the motor deficits are a relatively late manifestation of dopaminergic deficit, earlier diagnostic signs need to be pursued, such as the affective, cognitive and sleep disturbances attributed to ventral tegmental area (VTA) and retrorubral field (RRF) function, in addition to more sensitive detection of substantia nigra pars compacta (SNpc)-derived motor deficits.

Because animal studies can be designed to control for the factors of aging and comorbidity, they provide a promising resource to better understand the etiology of PD and its early effects on neurons. However, an additional challenge in detecting the early signs of PD in animal models is that they are of a nature that is often difficult to assess in animals. There is much debate on the best ways to detect and assess PD-relevant behavioral endpoints in animal models, and non-motor effects such as depression, cognitive changes and sleep disturbances are even more challenging to quantify (Potashkin et al., 2010; Rana et al., 2013). For this reason, less subjective endpoints such as assessment of neuronal morphology are highly desirable for studying the effects of early PD models on dopaminergic neurons.

8.2 The effects of an n-3 PUFA-deficient dietary and breeding protocol on a unilateral intrastriatal 6-OHDA lesion model in rats

8.2.1 Outcome of this project

In Aim 1, a diet and breeding protocol employing a Low n-3 diet from conception was used to achieve differing levels of n-3 PUFA brain composition in rats, and the effects of the diet on a unilateral intrastriatal 6-OHDA rat model of mild to moderate PD. There was no effect of diet on either amphetamine-stimulated rotational behavior or striatal dopamine depletion in either 1st or 2nd litter rats; or in SNpc dopaminergic neuronal number or morphology as quantified in 2nd litter rats by tyrosine hydroxylase silver nucleolar (TH-AgNOR) staining and stereological analysis. These findings were in contrast to a previous study from our laboratory, which found a 33% decrease in TH-positive SNpc neuronal number in unlesioned 2nd litter rats fed the Low n-3 diet. In future studies, quantifying dopaminergic neurons from unlesioned animals would help confirm whether the neuronal deficits caused in the previous studies in unlesioned rats were replicable, or whether aspects of the 6-OHDA lesion changed the expected proportions of SNpc neurons between the Control and Low n-3 rats.

8.2.2 Future studies of the effects of a Low n-3 PUFA diet and breeding protocol on the 6-OHDA PD lesion model in rats

Future studies for determining the effects of Low n-3 PUFAs on early PD involve several approaches to determine the threshold of a potential effect of n-3 PUFAs in a mild to moderate PD model. One potential reason there was no effect of diet on the

lesion is that the relatively mild lesion model we used may not have caused a threshold of neurotoxicity needed to reveal the protective effects of the n-3 PUFA deficient diet. In addition, our moderate n-3 PUFA deficiency may not have created drastic n-3 PUFA deficits that may be needed to show a preferentially detrimental effect in 6-OHDA neurotoxicity. Further studies are needed to fully understand the threshold of detectable effects caused by n-3 PUFA deficiency in models of early PD. Since there was no effect of differing dietary or brain DHA content in this study, in future studies it would be useful to use a range of DHA content in diets to establish a dose-responsive effect on the endpoints. In addition, it would be interesting to introduce several different doses of 6-OHDA, to see if there is a dose-response effect to the toxin, and whether n-3 PUFA status affects that. Furthermore, since behavioral tests are sometimes unable to detect subtle differences between neuronal damage between treatment groups, it would be interesting to perform biochemical assays to gain more knowledge of what is occurring between the Control and Low n-3 brains. Some informative endpoints to include would be measuring lipid peroxidation or DA-specific neurotoxic adducts (see Introduction), or to look at indicators of dopaminergic function like DAT, vesicular monoamine transporter, or TH expression, which serve as indirect indicators of dopamine function. In addition, even if our model created conditions too mild to find an effect in behavior or neuronal stereological analysis, the Low n-3 diet may be affecting PUFA-influenced factors implicated in the development and maintenance of dopaminergic neurons, such as Nurr1, and RXR (see Section 1.7.4.1 for more details). Assessment of these factors in future studies will yield a more comprehensive perspective of the processes occurring in our PD model.

8.3 TH-AgNOR staining to determine the effects of a unilateral intrastriatal 6-OHDA lesion in the substantia nigra

8.3.1 Outcome of this project

In Aim 2, a method was developed using stereological techniques and the TH-AgNOR staining method to quantify neuronal number and volume in the SNpc of adult Long-Evans rats after unilateral intrastriatal 6-OHDA lesion. This was accomplished by using a modified TH-AgNOR staining method combined with stereological techniques, which enabled better visualization and potentially more accurate counting of the SNpc than has been possible in previous studies. In the past, many studies have not quantified individual TH-positive neurons or quantified neuronal morphology, instead relying on biased 2D counting techniques to estimate changes to the neurons. Our novel neuronal quantitation method using a combination of TH-AgNOR staining and stereological techniques overcame several obstacles that have previously stood between the researcher and easy, efficient quantitation of TH-positive neuronal number and morphology. Stereological studies using traditional TH-only staining protocols developed for thin-section staining can result in over-staining and can obscure the nucleus, which is a necessary unique marker for the neuron as required by many stereological protocols. With the modified TH-AgNOR stain, staining modifications were implemented by NeuroScience Associates to address both the requirements of stereology and the challenges of staining dopaminergic neurons. Hydrochloric acid (HCl) was added to improve stain penetration into the tissue, the TH antibody concentration was decreased to avoid over-staining of dense dopaminergic neuron

populations, and the added AgNOR stain pigmented the nucleoli, so that they were visually distinct from the neuronal body and the nucleolus in most cases. Furthermore, this method revealed new findings about the changes to SNpc dopaminergic neuronal morphology after a unilateral intrastriatal 6-OHDA lesion. In Aim 2, a 20% decrease in SNpc regional volume and a 54% depletion of SNpc TH-positive neurons was determined after a single-site 12.5 µg 6-OHDA infusion. There was also a 11% reduction in neuronal body volume in 6-OHDA lesioned rats, indicating that volume is a quantifiable morphological characteristic related to toxicity and subsequent neurodegeneration. Our findings contributed to the SNpc morphological literature, and supported recent studies about changes to SNpc neuronal number and volume in PD (Rudow et al., 2008; Eriksen et al., 2009). This method, by making it much easier to determine individual cells and place markers on individual structures, improves the accessibility stereological studies in the SNpc for the average researcher. This could result in greater numbers of higher-quality studies in the future literature, and consequently a better understanding of the neurodegenerative process in PD.

8.4 Altered neuronal and nucleolar morphology in dopamine neurons following 6-OHDA lesion in rats

8.4.1 Outcome of this project

In this study, the unilateral intrastriatal 6-OHDA lesion caused decreased neuronal volume and nucleolar volume in the SNpc neurons in Aims 1,2 and 3, and in the SNpc, VTA and RRF in Aim 4. The changes to neuronal and nucleolar volume found in this dissertation project indicate that the 6-OHDA toxin is causing an effect to

the neurons that is likely similar to what is occurring to the neurons in PD. Although there is a lack of stereological nucleolar morphology studies in the VTA and the RRF, our findings in the 6-OHDA rat model support the findings in the SNpc in post mortem PD brains (Mann and Yates, 1982). Our findings underscore the importance of understanding the morphological effects of PD models on dopamine neurons, and pave the way for future studies in this area.

8.4.2 Determining morphological changes to dopamine neurons.

Morphology is a very useful tool that tells us about the physical characteristics of the neurons and allows us to compare them between the "normal" and diseases states. Morphological studies have been especially invaluable in neurodegenerative disease studies in humans because they allow determination of changes wrought by the disease process within the limits of only having a fixed post-mortem brain with which to study. The morphological findings in human subjects can be compared to complementary findings in animal disease models, and those morphological findings in animals can be correlated to other objectively determined behavioral, biochemical and anatomical analysis endpoints to extrapolate the mechanisms of the disease in humans from the findings in the animal models. Since this dissertation has established an effective staining and stereological analysis method for quantification of dopaminergic neurons, and validated its ability to detect differences induced in dopaminergic neurons after a mild to moderate 6-OHDA lesion, the obvious next step is to expand the use of this method to determine the dysfunction of dopamine neurons in other studies in animals and in humans.

8.4.3 General challenges in future morphology studies of dopaminergic neurons

In designing future morphological experiments on midbrain dopaminergic neurons, there are a number of challenges to be considered. First, although the A8, A9 and A10 dopamine neurons are classified by their geographical location and their proposed function based on the majority of their projections, in reality these anatomical distinctions may be fairly arbitrary, since the projections and consequent functions have some overlap (Deutch et al., 1988; German and Manaye, 1993; Ferreira et al., 2008; Da Cunha et al., 2009; Hosp et al., 2011) (see Section 1.5 for a detailed description of dopaminergic subgroups). The complexity of the connections in the midbrain dopaminergic system, paired with the fact that the borders of the regions can be very difficult to distinguish based on relative anatomical landmarks, means that accurate, standard and consistent regional definition is crucially important to meaningful and robust morphological studies in both animal models and in humans.

In addition, the dopaminergic neurons affected in PD are not exempt from the complex and potentially confounding effects of natural aging and comorbid conditions on neuronal morphology. In order to understand how the neurons are different in PD, we need to know more about what happens to neuronal morphology in natural aging, as well as in other diseases. While there have been a number of studies on SNpc neuronal morphology in aging in both humans and in animals (Carvey et al., 2006b; Rudow et al., 2008), the effects of aging on neuronal morphology in other dopaminergic subregions remain largely unknown. Also, since PD patients often have other comorbidities

common to advanced age, such as vascular disease and diabetes, these factors should be considered when designing clinical trials and conducting post-mortem studies.

8.4.4 Future studies of dopaminergic neuronal and nucleolar volume

The studies performed in this dissertation project found that a unilateral intrastriatal 6-OHDA lesion alters neuronal and nucleolar volume in the SN, VTA and in the RRF dopaminergic neurons in the rat, although the exact mechanisms causing the change remain to be determined. Changes to neuronal volume, whether hypertrophy or atrophy, signal a functional response of the neurons to a stimulus or stressor. It has been proposed that in normal aging, the dopaminergic SN neurons decrease in number but hypertrophy in size, in order to compensate for reduced numbers (Rudow et al., 2008). In PD, however, the SN neurons have been found to be smaller in volume (Iacono et al., 2009), which suggest several possible explanations. Either the larger SN neurons are selectively susceptible, or the neurons are atrophied in response to the stressors in PD overwhelming the neurons' capability to maintain their size. Likewise, changes to nucleolar size are associated with nucleolar function, including increased rRNA transcription (Boulon et al., 2010), but how the nucleolar size or volume is related to the continuum of progressive neurodegenerative changes is still unknown. In future studies, the staining and stereological methods employed here could be used to characterize the neuronal and nucleolar morphology neurons in post-mortem human studies including both mild and severe PD cases, and in animal PD model studies to characterize the effects of various doses of 6-OHDA at multiple time points. In addition, it will be important to determine stereologically assessed nucleolar morphology in unlesioned control animals as well as in normal aging, to be able to compare the normal

nucleolar morphology to the changes throughout the course of the disease. Finally, although morphology is an invaluable tool in assessing the changes that have occurred to the neurons as a result of PD or a PD model lesion, morphological studies alone cannot establish causality of the changes. Thus, in future studies it would be informative to include additional animals designated for analysis of biochemical endpoints informative of neuronal maintenance and survival status and nucleolar function. Some relevant endpoints for assessment would be dopaminergic neuronal survival and maintenance factors like brain-derived neurotrophic factor and Nurr1; or nucleolar assessment endpoints like nucleophosmin nucleolar integrity staining.

8.5 Differentially altered neuronal number and morphology in midbrain dopaminergic subregions after a unilateral intrastriatal 6-OHDA Lesion

8.5.1 Outcome and relevance of this project

This dissertation project determined that the TH-positive neuronal number and TH-AgNOR-positive neuronal volume and nucleolar volume were differentially affected in different dopaminergic subregions in a unilateral intrastriatal 6-OHDA model of the rat (see Chapter 7). Notably, this study was the first to our knowledge to determine that the neuronal and nucleolar volume of the dopaminergic neurons was decreased in the VTA and the RRF after unilateral intrastriatal 6-OHDA lesion. These novel findings are exciting because the implications of the functional differences of these regions in PD have recently come into focus as a potential source of insight into the etiology of the early and non-motor aspects of the disease. Importantly, how the morphology of these

regions is differentially affected may yield more knowledge about the pathogenesis of PD and lead to assessment methods for treatments and neuroprotective interventions.

8.5.2 General challenges in future studies of dopaminergic subregions

The current study has considerably added to our knowledge about the different dopaminergic subregions in early PD. However, in order for these studies to be confirmed as reflective of what is occurring in PD, we need to know more about the roles that these different regions play in early PD. Although the role of the SN and its impact on motor effects has been extensively characterized in PD and in animal models, little is yet known about the roles of other dopaminergic neuronal populations in PD. In general, if PD studies include any histological analysis of dopaminergic regions or neurons, it is usually in only the SN. Future studies in humans and in animal PD models need to include other dopaminergic subregions in addition to the SN, so we can understand how the morphology of the dopamine neurons is affected during the course of the disease in comparison with the SN.

8.5.3 Future directions in animal PD models

This dissertation project established the differential effects of a unilateral intrastriatal 6-OHDA lesion on the neuronal number and neuronal and nucleolar morphology in TH-AgNOR-strained neurons in the SNpc, VTA and RRF. In the future, it would be interesting to use the TH-AgNOR staining method and our PD model to determine the neuronal morphology of dopaminergic neurons in the SN, VTA and RRF in unlesioned control animals, aged controls, and with varied doses of 6-OHDA to gain a

more comprehensive understanding of the differential effects of the lesion on the dopaminergic subpopulations.

In addition, it would be interesting to establish the effects of the Low n-3 PUFA diet used in Aim 1 on other dopaminergic brain regions, such as the VTA and the RRF, after 6-OHDA lesion. Since n-3 PUFAs have been implicated in the development and maintenance of the SNpc dopaminergic neurons, it stands to reason that they may also be crucial to optimal function of other dopaminergic neuronal subtypes. A previous study in our laboratory found the VTA in unlesioned rats fed the Low n-3 diet to have a TH-positive neuronal loss of about 34% (Ahmad et al., 2008). However, few studies have determined the effects of n-3 PUFA status on the maintenance of survival of VTA and RRF neurons either in intact rats or in PD models. Since the neurons in these regions are implicated in early PD, at which point dietary interventions might be able to elicit the most benefit, it is important to know more about how PUFA status affects these neurons.

8.5.4 Future directions in post-mortem PD brains

There is currently a dearth of comparative stereological studies of the midbrain dopaminergic subpopulations in humans, and such studies in the VTA and RRF of PD patients are particularly lacking. Complementary high-quality stereological morphological studies of the neurons in dopaminergic subpopulations affected by PD are a crucial next step in validating the relevance of the progress being made our knowledge of dopaminergic morphology in animal models of PD. While the novel TH-AgNOR staining protocol used in this dissertation project will undoubtedly improve the

quality and ease of execution of stereological studies in human PD post-mortem brains, challenges may arise regarding differences in the tissue quality and preparation (i.e. perfusion in paraformaldehyde vs. immersion fixation) between the animal PD model specimens and post-mortem PD brains. In addition, in future studies of the dopaminergic subpopulations in humans, it is especially important that the stage of both clinical and neuropathological disease advancement be determined so that the morphological effects of the disease on the neurons throughout the course of PD may be determined.

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Healy-Stoffel M, Ahmad SO, Stanford JA, Levant B. (2012) A novel use of combined tyrosine hydroxylase and silver nucleolar staining to determine the effects of a unilateral intrastriatal 6-hydroxydopamine lesion in the substantia nigra: a stereological study. *J Neurosci Methods*, **210**,187-94.

Healy-Stoffel M, Ahmad SO, Stanford JA, Levant B. (2013) Altered nucleolar morphology in substantia nigra dopamine neurons following 6-hydroxydopamine lesion in rats. *Neurosci Lett*. [Epub ahead of print]

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