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Aging, Stress and Inflammation in a Rat Model of Parkinson's Disease

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Abstract

Parkinson’s disease (PD) is an age-related neurodegenerative disorder that primarily targets the dopaminergic nigrostriatal pathway resulting in motor deficits. Degeneration of this pathway is also associated with α-synuclein inclusions (Lewy bodies) and neuroinflammation. In addition to the pathology and motor symptoms, there is a high association of non-motor symptoms with the disease, including depression. Aging, inflammation and depression are linked to each other independently of, and within, PD. Understanding these separate, yet interconnected, mechanisms may help influence preventative and post-diagnosis treatments. In the present work, we hypothesized that proteins related to stress-induced depression, inflammation and PD would be altered in the aged midbrain, and that inhibiting inflammation would ameliorate and increasing glucocorticoid signaling would exacerbate behavioral dysfunction and dopaminergic neuronal loss in neurotoxin models of PD.

As PD is an age-associated disease, we first explored age-dependent changes in stress-, inflammation- and PD-related proteins in male Sprague Dawley rats. Compared to young rats, we found an age-associated decrease in glucocorticoid receptor (GR) expression and an increase in α-synuclein expression in the ventral midbrain. Morphological and unbiased stereological analyses of the substantia nigra pars compacta (SNpc) revealed a significant increase in the number of microglia with age, accompanied by an inflammatory morphology. The second study examined the effect of anti-inflammatory treatment in the 6-hydroxydopamine (6-OHDA) neurotoxin model of PD. Increased inflammation is reported in PD brains and animal models, and chronic anti-inflammatory treatment is associated with a decreased risk for developing PD. Here, animals received the anti-inflammatory drug, minocycline, flanking the neurotoxin lesion. Motor behavior
was assessed using the forelimb asymmetry test and dopaminergic neuron survival in the SNpc was evaluated via immunohistochemical labeling of tyrosine hydroxylase (TH, the catecholamine biosynthetic enzyme) and unbiased stereology. Results revealed a treatment x lesion interaction trend for both behavior and TH+ cell counts, suggesting that anti-inflammatory treatment had positive effects on the outcome measures and implicating microglial activation in the mechanism of neurotoxin-mediated dopaminergic degeneration. In the third study, we expanded upon previous work showing that chronic stress combined with 6-OHDA exacerbated behavioral dysfunction and cellular degeneration in a combined chronic variable stress (CVS)/PD model. We tested the hypothesis that increased GR signaling in the SNpc, in the CVS/PD paradigm, would further exacerbate behavioral deficits and cell loss. A lentivirus-packaged GR overexpression vector was used to upregulate GR expression in nigral dopaminergic neurons prior to exposing rats to the dual model. We found that GR overexpression did not affect the behavioral deficits or neuronal degeneration in the combined model, however there was a stress x lesion interaction, supporting previous findings and suggesting that neuronal GR signaling may not be the primary mechanism of enhanced degeneration in the CVS/6-OHDA model.

Overall, the major findings from this work show an altered inflammatory environment in the aged ventral midbrain, and a role for pro-inflammatory processes in exacerbated behavioral deficits and dopaminergic cell degeneration in the 6-OHDA model of PD. The results suggest that modulation of inflammatory processes may be a promising preventative measure to reduce common age-associated neuropathologies.
In no way would I have been able to get to where I am today without the help, support and guidance of many people. I will however, try to express my gratitude in these few pages. I will begin with thanking my advisor, Kim Seroogy. Thank you for giving me the opportunity to do my dissertation work in your lab. You have taught me how to think about research, how to do research and how to write about it. Thank you so much for letting me make my mistakes, think outside of the box, and venture into some unfamiliar territory with my research. You have given me such great advice and conversation over the last five years, I really appreciate it all.

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sister who was my first friend and whose artistic talents inspired me to be just as successful in science. I’d also like to thank my grandparents who were always cheering me on and supporting me in a number of ways.
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
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<tr>
<td>CVS</td>
<td>Chronic variable stress</td>
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<td>DG</td>
<td>Dentate gyrus</td>
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<tr>
<td>DMV</td>
<td>Dorsal motor nucleus of the vagus nerve</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>ENS</td>
<td>Enteric nervous system</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary adrenal</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
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<td>NHS</td>
<td>Normal horse serum</td>
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<td>NMS</td>
<td>Non-motor symptoms</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
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<td>STN</td>
<td>Subthalamic nucleus</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter 1
Introduction

Parkinson’s Disease

Parkinson’s disease (PD) was first characterized in 1817 by Dr. James Parkinson in his work “An Essay on the Shaking Palsy”, in which he described a disease with symptoms of “involuntary tremulous motion, with lessened muscular power, in parts not in action...with a propensity to bend the trunk forwards, and to pass from walking to running pace; the senses and intellects being injured,” (Parkinson, 1817). Despite two centuries of research since this disease was first documented, both the exact etiology and a cure are still unknown.

As the second most common neurodegenerative disorder, PD mostly affects 1-2% of the population aged 65 years and older. Though a majority of the cases are age-associated, up to 10% of cases are thought to be hereditary, with familial Parkinson’s associated with a growing list of genes (Verstraeten et al., 2015). Pathologically, PD is characterized by the progressive degeneration of the nigrostriatal pathway. This pathway is composed of dopaminergic neurons containing neuromelanin that project from the substantia nigra pars compacta (SNpc) to the dorsal striatum. Additionally, the presence of Lewy bodies is found in multiple brain regions of PD patients, with research suggesting that their formation occurs in successive stages (Braak et al., 2004). Lewy bodies are a significant pathological hallmark of the disease and consist of cytoplasmic protein aggregates containing mostly α-synuclein and parkin (e.g. Shulz and Falkenburger, 2004). These pathological abnormalities manifest into what are known as the primary cardinal motor symptoms: resting tremor, rigidity, bradykinesia and postural instability.
By the time these symptoms appear, up to 80% of striatal dopamine has been diminished and 60% of dopaminergic neurons of the nigrostriatal pathway have died (Schultz and Falkenburger, 2004).

*Basal Ganglia Circuitry*

The ability to initiate and execute smooth voluntary movements is dependent on the functioning of the basal ganglia, composed of multiple subcortical nuclei within the telencephalon, diencephalon, and mesencephalon. The collaborative functioning of these nuclei is altered in PD due to the degeneration of the nigrostriatal pathway, which serves as an initiator of motor movement in the basal ganglia circuits. The nigrostriatal pathway is composed of dopaminergic neurons that originate in the pars compacta subdivision of the substantia nigra within the ventral midbrain and terminate in the forebrain striatum, another region of the basal ganglia, composed of the caudate nucleus and putamen. The dopaminergic connections from the SNpc to the striatum communicate with two pathways within the basal ganglia that control movement - the “direct pathway” which stimulates movement, and the “indirect pathway” which inhibits it (Figure 1). In the direct pathway, putamen neurons inhibit neurons in the globus pallidus (internal) (GPi). The inhibition of the GPi in turn releases neurons within the ventral anterior/ventral lateral (VA/VL) thalamus from tonic inhibition (disinhibition), allowing the thalamus to transmit signals of excitation to the motor cortex for movement initiation. In the indirect pathway, the putamen inhibits the globus pallidus (external) (GPe). The inhibition of these neurons releases neurons in subthalamic nucleus (STN) from inhibition (disinhibition). Cells from the STN subsequently activate the GPi, leading to increased inhibition of the VA/VL thalamic nuclei and, consequently, decreases movement. These two pathways are coordinated to support voluntary muscle movement (Brown, 2007; Chan et al., 2005). Ultimately, dopamine is able to excite the direct pathway for
movement initiation via dopamine D1 receptors and inhibits the indirect pathway that constrains movement via dopamine D2 receptors. The lack of dopamine in the case of PD decreases direct pathway stimulation and increases indirect pathway activation. As a result, there is decreased signaling from the VA/VL thalamus to the motor cortex. This decrease leads to the appearance of the motor symptoms.
Figure 1. Direct and indirect pathways in normal and parkinsonism conditions. The direct and indirect pathways of the basal ganglia circuits are modified in PD. In this diagram, red arrows represent inhibitory signaling and blue arrows are excitatory. The thickness of the arrows indicates an increase (thick arrow) or decrease (thin arrow) in firing-rate activity. CM, centromedial nucleus; CMA, cingulate motor area; GPe, globus pallidus, external; GPi, globus pallidus, internal; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (modified from Smith et al., 2012).
Alpha-Synuclein

The cause of the massive cellular loss in the nigrostriatal pathway that occurs in PD compared to other circuits remains unclear. A variety of factors are implicated in playing a role in the degeneration including oxidative stress, inflammation, excitotoxicity, mitochondrial dysfunction, trauma, exposure to toxins, inheritance of specific gene mutations, and protein misfolding and aggregation in Lewy bodies (Howells et al., 2005; Olanow and Kordower, 2009). Since Lewy bodies exist in both familial and idiopathic cases of PD, their formation may be a major cause of cell death, though this still somewhat controversial (Beyer et al., 2009; Marques and Outeiro, 2012).

While Lewy bodies are composed of a number of different proteins, the dominant protein in these inclusions is α-synuclein. The normal function of α-synuclein is still not completely understood, but research in non-disease models has localized the protein to the presynaptic terminal and the nuclear envelope, as well as inside the nucleus, suggesting its importance in synaptic plasticity and neurotransmitter release (Maroteaux and Scheller, 1991; George et al., 1995; Goncalves and Outeiro, 2013). In many familial forms of PD, the α-synuclein gene, SNCA, is mutated or overexpressed due to duplication or triplication of the gene coding for it (Polymeropoulos et al., 1997). In idiopathic cases, fractions of monoubiquitinated α-synuclein have been purified from the Lewy bodies, and research has suggested that these fractions play a role in Lewy body formation (Engelender, 2008). Lewy body inclusions also form in cells outside of the nigrostriatal pathway, suggesting multiple affected systems in the disease pathology (Braak et al., 2002). The Braak Staging Scheme for PD suggests that PD begins as a synucleinopathy in structures outside of the ventral midbrain, such as non-dopaminergic regions of the lower brainstem or olfactory bulb, and the gastrointestinal tract. The formed inclusions then progress
rostrally to the SNpc, at which time the cardinal motor symptoms of PD begin to develop. Cortical regions are believed to be involved with this staging hypothesis. Non-motor symptoms of PD may be a result of this staging sequence, including constipation. As a common symptom of PD, constipation supports the involvement of the enteric nervous system (ENS) in early stages of PD. Lewy body inclusions are found in projection neurons of the dorsal motor nucleus of the vagus nerve (DMV) that extend to the gut. Retrograde transport of misfolded α-synuclein to the brainstem would support the movement of the protein from periphery to the CNS via cell-to-cell transport of inclusion-forming α-synuclein (Braak et al., 2003, 2004; Pellicano et al., 2007; Burke et al., 2008; Visanji et al., 2013).

The rostral movement of Lewy body formation from the brainstem to the rest of the brain occurs via cell-to-cell transport. There is evidence of a number of different transport mechanisms at play in this movement (Visanji et al., 2013). Axonal transport of α-synuclein, shown by Freundt et al., (2012), revealed that α-synuclein fibrils could be transported down the axon of a primary neuron and released to a secondary neuron. Lee et al. (2005) reports vesicular α-synuclein, both aggregated and non-aggregated, is secreted from cells. Additionally, that these secreted forms are more prone to aggregation compared to cytosolic forms. The same groups reported the ability of cells to take up extracellular α-synuclein (Lee et al., 2008).

A cell culture study by Luk et al. (2009) showed that introduction of fibrillar α-synuclein into cells overexpressing the monomeric protein will induce formation of insoluble inclusions, similar to those seen in disease states. Later, the group showed that injection of α-synuclein fibrils in to the striatum of wildtype mice lead to presence of aggregated α-synuclein in brain regions connected to the striatum and relevant to PD pathology, specifically the SNpc. Loss of striatal dopamine and motor deficits were also reported (Luk et al., 2012). In PD patients, two groups
show that grafted dopaminergic neurons into the striatum developed Lewy body inclusions, supporting the ability of cell-to-cell transport of aggregated α-synuclein and that inclusions are able to spread to connected brain regions (Kordower et al., 2008; Li et al., 2008). In a prion-like fashion, misfolded proteins promote aggregation in previously unaffected cells and the pathology spreads (Visanji et al., 2013; Recasens and Dehay, 2014). Overall, there is abundant evidence supporting the movement of aggregated/fibrillary α-synuclein from cell-to-cell, promoting the movement of affected regions from the gut to the cortex.

Non-Nigrostriatal Systems

Alterations in dopaminergic systems beyond the nigrostriatal pathway and in non-dopaminergic circuits have also been discovered in PD (Halliday et al., 1990; Bonnet, 2000). Dopaminergic projections from the midbrain ventral tegmental area (VTA) and the medial nigral regions to the prefrontal cortex (PFC), the mesocortical pathway, degenerate in PD, although to a lesser extent than the nigrostriatal pathway. The neuronal loss in the VTA and resulting impaired dopaminergic signaling in the PFC are believed to contribute to cognitive dysfunction often seen in PD patients (Dubois and Pillon, 1995, 1997; Dymecki et al., 1996; Zgaljardic et al., 2006; Narayanan et al., 2013). The mesolimbic pathway, projecting from the VTA to nucleus accumbens, is also affected in PD; this pathway is also known as the ‘reward pathway’. Loss of dopaminergic transmission in this circuit may contribute to the common non-motor symptom of depression (Blonder and Slevin, 2011; Kousik et al., 2014; Frosini et al., 2015).

Non-dopaminergic neurotransmitter systems affected in PD include those containing noradrenaline, serotonin, glutamate, γ-aminobutyric acid (GABA) and acetylcholine (Scatton et al., 1983; Bonnet, 2000; Jellinger, 2011). Neurons in these circuits also develop Lewy bodies at
various stages of the disease (Braak et al., 2004). The dysfunction of non-dopaminergic pathways is linked to the development of certain non-motor symptoms (NMS) in PD, such as depression, anxiety, cognitive impairment, psychosis, and gastrointestinal issues (Arendt et al., 1983; Halliday et al., 1990; Bosboom et al., 2003; Braak et al., 2004; Fujita et al, 2006; Fox et al., 2009; Meyer et al., 2009; Ferrer et al., 2011; Del Tredici and Braak, 2013).

Non-motor Symptoms

Although PD is recognized most by its motor disturbances, a multitude of NMS are also associated with the disease. Patients can suffer from a range of issues including sleep disturbances, pain, cognitive deficits and dementia, incontinence, depression, and olfactory dysfunction. Over 90% of patients report the presence of NMS (Chaudhuri et al., 2015). For example, development of dementia is reported to occur in 50% of PD patients within 15 years of diagnosis and increases in prevalence with age (Hely et al., 2005; Buter et al., 2008). In addition to dementia, depression and psychosis are also highly prevalent at all disease stages, occurring in up to 50% of patients (Rickards, 2005; Chaudhuri and Schapira, 2009). Many NMS, such as olfactory dysfunction and depression, can arise prior to PD clinical diagnosis, while others are associated with a more severe disease state (Chaudhuri et al., 2015). Large-scale studies of PD patients have suggested that NMS contribute greatly to a decline in quality-of-life, sometimes more so than the motor deficits classically associated with the disease (Chaudhuri et al., 2015). While comparatively little is known about the progression of these symptoms or how they may contribute to the disease advancement and pathology, significant research on NMS in PD is ongoing (e.g. Hemmerle et al., 2014).
**Therapies**

Despite the variety of symptoms associated with PD, therapies are still limited. There are no clinical treatments to stop or slow down the neurodegeneration, but therapies have been developed that alleviate the motor symptoms for a time. The most common drug treatment is L-dopa (levodopa, a precursor for dopamine), which acts as a replacement therapy and has been commonly prescribed by physicians since the late 1960’s (Katzenschlager and Lees, 2002). Unfortunately, long-term use of the drug causes problematic side effects for patients, including dyskinesias [levodopa-induced dyskinesias (LIDs)], oscillations in effectiveness between doses, and severe psychological and cognitive effects (Marsden, 1994; Poewe, 2010). Other treatments include dopamine agonists (e.g. Mirapex, ropinirole); monoamine oxidase-B (MAO-B) inhibitors (e.g. selegiline, rasagiline) and catechol O-methyltransferase (COMT) inhibitors (e.g. entacapone), which work in conjunction with levodopa; and anticholinergics (e.g benztropine) (Farlow et al., 2004). Outside of pharmacological therapies, deep brain stimulation of assorted targets, such as the STN and SNpc, is becoming a common therapy for the treatment of motor symptoms, and for reducing the use of pharmaceuticals (Romito and Albanese, 2010). These treatments focus on the motor deficits that are caused by nigrostriatal dopamine loss, and do not address additional symptoms that arise from alterations in other neurotransmitter systems.

**Animal Models of Parkinson's Disease**

To better understand PD, its etiology and possible therapies, animal models are utilized, either taking advantage of genetic manipulations or using a neurotoxin to induce cellular degeneration. While these approaches have been instrumental in advancing knowledge in the field and providing a platform to test novel therapeutics, no model completely mimics the
neuropathological or clinical features of PD. A growing list of genes associated with PD has influenced the production of transgenic organisms, and common neurotoxins, such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), are used to induce dopaminergic neurodegeneration in rodents and non-human primates. Finally, while a variety of organisms are used to study PD (including worms and flies), discussion below will remain in the context of rodent models.

**Genetic Animal Models of PD**

A growing number of causative genes for PD has prompted the development of transgenic rodent models (Table 1). SNCA, the gene coding for α-synuclein, was the first gene mutation discovered in PD patients, and is autosomal dominant. As the table shows, this gene has either a missense mutation (i.e. A53T, A30P, E46K), or gene multiplications, resulting in protein overexpression (Pankratz and Foroud, 2007; Gasser, 2009). Animals bred with these same modifications produce several functional abnormalities in the nigrostriatal system, but there is a lack of neurodegenerative pathology (Chesselet, 2008; Dawson et al., 2010). Mice overexpressing the human A53T mutation exhibit mitochondrial dysfunction/degeneration, another pathological feature of PD (Martin et al., 2006). Delivery of α-synuclein transgenes in vivo via viral vectors is also utilized, and reveals a pathology that mimics PD. Overexpression of human wildtype α-synuclein or human A53T mutated α-synuclein in SNpc dopamine neurons induces parkinson-like pathology, to include cell loss, reduction in striatal dopamine, behavioral deficits, and importantly, α-synuclein-positive inclusions (Kirik et al., 2002; Lo Bianco et al., 2002).
A second gene leading to autosomal-dominant PD is \textit{LRRK2}. This gene has one outstanding mutation (G2019S) that is linked to both sporadic and familial PD. While the risk of developing PD with this mutation increases with age, making it an attractive gene to study (Healy et al., 2008), transgenic mouse models do not produce a robust PD phenotype, rather only altered dopamine transmission (Li et al., 2009). Of note, both of these genetic models vary in pathology depending on the promotor used (Dawson et al., 2010).

Parkin, DJ-1 and PINK1 mutations are common autosomal-recessive causes of PD; all of these involve loss-of-function mutations. Interestingly, the knockout mouse models for these proteins don’t display major abnormalities associated with PD (Lu et al., 2009; Goldberg et al., 2005). Outside of the dopaminergic alterations common to PD, Parkin knockout mice exhibit abnormalities in the nigrostriatal pathway and the noradrenergic locus coeruleus (Goldberg et al., 2005), and overexpression of human mutant forms cause progressive dopaminergic neuron degeneration (Lu et al., 2009). PINK1 knockout mice display some mitochondrial deficits (Valente et al., 2004; Kitada et al., 2007; Gautier et al., 2008). Due to lack of hallmark PD features, it is believed that these models represent very early changes that occur due to the protein mutations (Dawson et al., 2010).
Table 1

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Gene symbol</th>
<th>Protein product</th>
<th>Mode of inheritance</th>
<th>Types of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1/4</td>
<td>SNCA</td>
<td>Alpha-synuclein</td>
<td>Dominant</td>
<td>Missense, gene multiplications</td>
</tr>
<tr>
<td>PARK2</td>
<td>PRKN</td>
<td>Parkin</td>
<td>Recessive</td>
<td>Missense, nonsense, frameshift, microdeletion, insertion, exon deletion, exon duplication, splice site</td>
</tr>
<tr>
<td>PARK6</td>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
<td>Recessive</td>
<td>Missense, nonsense, frameshift, insertion, exon deletion, splice site</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>Protein DJ-1</td>
<td>Recessive</td>
<td>Missense, frameshift, exon deletion, splice site</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
<td>Dominant</td>
<td>Missense</td>
</tr>
</tbody>
</table>

Table 1. List of select genes associated with Parkinson’s disease. Modified from Pankratz and Foroud (2007).
Toxin-Based Animal Models

Toxin-based models of PD in rodents and non-human primates have been crucial to increased understanding of disease pathophysiology and to develop therapeutics (Bové and Perier, 2012). The most commonly employed neurotoxins are MPTP and 6-OHDA, which will be discussed here at length in the context of rodent models. Both are well-characterized models that mimic the critical feature of PD, neurodegeneration of the dopaminergic nigrostriatal pathway, although non-motor aspects of the disease are often absent. Additional models of toxin-induced parkinsonism include the rotenone and paraquat rodent models, pesticides that impair mitochondria dysfunction and increase oxidative stress, respectively (Bové et al., 2005; Tanner et al., 2011).

In 1976, the ability of MPTP to induce Parkinson-like symptoms was discovered by accident in a human subject (Davis et al., 1979). MPTP by itself is not toxic, but once it crosses the BBB it is metabolized into 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (MAO-B) containing cells (astrocytes and serotonergic neurons) (Heikkila et al., 1984). MPP+ has a high affinity for the plasma membrane dopamine transporter (DAT), which is why it is able to target dopaminergic neurons of the SNpc (Mayer et al., 1986). Once inside the dopamine neurons, MPP+ becomes concentrated in the mitochondria, impairing cellular respiration by inhibiting complex I, and ultimately contributing to cell death (Nicklas et al., 1987; Mizuno et al., 1987). At present, the MPTP model is predominantly used in mice and primates, as rats are resistant to MPTP, and has been successful in elucidating mechanisms of cell death and evaluating effectiveness of neuroprotective agents (Langston et al., 1983; Boyce et al., 1984; Dawson et al., 2012).
The widely used neurotoxin 6-OHDA is an attractive model of PD in mice, rats, and non-human primates, as it successfully causes degeneration of dopaminergic cells and motor deficits (Faull and Laverty, 1969; Ungerstedt and Arbuthnott, 1970; Jeon et al., 1995). Because 6-OHDA closely resembles dopamine (Figure 2), it is readily taken up by the DAT, and is able to selectively damage catecholaminergic neurons (Luthman et al., 1989). Once inside the neuron, 6-OHDA initiates the production of reactive oxygen species (ROS), which triggers oxidative stress, ultimately killing the cell (Choi et al., 1999). The nigrostriatal pathway is targeted via intracranial injections, since 6-OHDA cannot cross the BBB (Schober, 2004; Meredith et al., 2008). Injections are made into the SNpc, striatum or medial forebrain bundle (MFB), causing either acute, progressive or incomplete degeneration of the pathway (Faull and Laverty, 1969; Sauer and Oertel, 1994; Jeon et al., 1995). While this approach mimics the nigrostriatal degeneration of PD well, it does not produce α-synuclein inclusions, nor does it affect other systems. Despite these drawbacks, the ability of the neurotoxin to initiate a progressive lesion allows for evaluation of neuroprotective agents, as well as exploring the role of potentially damaging factors.
Figure 2. Comparison of the chemical structures of dopamine and 6-hydroxydopamine (6-OHDA).
Aging

Parkinson’s disease is a disease of aging, affecting 1-5% of adults over the age of 65 (de Lau and Breteler, 2006). As the human population grows and medical advances allow for longer lifespans, the incidence of PD will increase, as well as the costs and stress on both patients and their caretakers. Understanding the changes that occur as the brain ages can shed light on the vulnerability of dopamine neurons in the nigrostriatal pathway and, potentially, on the etiology of idiopathic PD. Moreover, a better understanding of brain aging could lead to novel therapeutics and preventative measures.

As the brain ages a number of changes occur such as altered gene expression, increased oxidative stress, and chronic inflammation. These alterations likely contribute to the increased susceptibility of the brain to neurodegenerative processes, ranging from stroke to cognitive impairment/Alzheimer’s disease to PD. It has become apparent that a number of changes are taking place that make the aged brain distinctly different from a “young” brain. Mitochondrial alterations result in increased reactive oxygen species (ROS) (Lee and Wei, 2012). Increased ROS is believed to contribute to the age-dependent increase in oxidized proteins and oxidized DNA, which actually serve as useful biomarkers for disease processes (Floyd and Hensley, 2002). Microarray studies in aged rodents show an overall increase inflammatory and pro-oxidant genes with a reduction in growth and anti-oxidant genes (Lee et al., 1999; Godbout et al., 2005b). The unique relationship between oxidative stress and inflammatory factors in aging has been described in the oxidation-inflammation theory of aging. This theory posits that chronic oxidative stress and inflammatory stress, as happens in an aging situation (see above), are actually the basis of the age-related changes that occur in an organism, including its brain, which is especially sensitive to oxidative stress (Butterfield, 2006; de la Fuente and Miquel, 2009). The production and release of free radicals
builds up toxins within the cell and is directly linked to inflammatory responses and neuronal damage.

Dysfunction of the HPA axis seems to occur, as increased glucocorticoids and adrenocorticotropic hormone (ACTH) levels are associated with aging, and there is decreased glucocorticoid negative feedback to the paraventricular nucleus (PVN) from the hippocampus and prefrontal cortex (Gupta and Morley, 2014). These changes are associated with a “non-resilient” recovery of the HPA axis following stressor (Seeman and Robbins, 1994). “Resilience” refers to the ability of the axis to return to baseline. In essence, after a stressor, the HPA axis takes longer to return to baseline in aged brains than in younger brains. This may be due to the decrease in glucocorticoid receptors in the hippocampus with age (Lee et al., 2012). The subsequent increase in glucocorticoids may have inflammatory consequences (discussed below).

_Aged Nigrostriatal Pathway_

The aged nigrostriatal system reveals pathological changes that are more substantial than changes in other brain regions (Reeve et al., 2014). Human postmortem studies have shown decreases in SNpc cell numbers with age (Thiessen et al., 1990; Fearnley and Lees, 1991), increases in nigral α-synuclein (Li et al., 2004) and Lewy body formation (Buchman et al., 2012). Additionally, Fearnley and Lees (1991) reported that the loss of dopaminergic cells of the SNpc displayed a regional pattern: the medial and dorsal tier of the SN lost cells at a higher rate per decade than the lateral ventral tier, whereas the loss of neurons in PD happens in an opposite pattern and at an exponentially increased rate per decade.

In the striatum, de Keyser et al. (1990) reported a steady decrease in dopamine uptake sites, specifically in the putamen, and Volkow et al. (1994) showed a loss of D2 dopamine receptors in
in vivo PET studies. The striatum also shows age-related shrinkage that parallels the rate of dopamine loss (Raz et al., 2003). In rodents, an age-dependent reduction in dopamine was also reported in the striatum (Marshall and Rosenestein, 1990). These findings differ from observations made in untreated PD patients, which revealed an increase in DA receptors as dopamine denervation increased, until treatment with L-Dopa, in which receptor levels returned to normal (Seeman and Niznik, 1990). Considering the contrast in aging- and PD-related changes in DA receptors in the striatum, it is possible that in PD the increase in receptors is compensatory to the decrease of dopaminergic projections to the striatum, and that loss of these projections occurs before the normal age-dependent decrease in DA receptors would be observed.

Oxidative stress is a general age-related threat to neurons. However, some populations are especially vulnerable, including dopamine neurons. Dopamine cells may have especially high levels of oxidative stress because 1) there is an increase in ROS due to mitochondrial dysfunction and 2) dopamine metabolism itself generates ROS (Meiser et al., 2013). Accumulated free radicals in the SNpc can result in oxidation of dopamine and the formation of 6-OHDA (Heikkila and Cohen, 1973). Interestingly, dopaminergic neurons of the SNpc are more vulnerable to oxidative stress than nearby VTA neurons (Olney et al., 1990; Jenner et al., 1992). Agrawal et al. (2010) has proposed that this differential vulnerability may be due to differences in receptor density and distribution between the two dopamine populations, and between dopaminergic neurons and non-dopaminergic cells. Overall, dopamine neurons of the nigrostriatal pathway are more vulnerable to the increased oxidative stress that comes with age compared to other dopamine populations and other cells.

Age-related increases in α-synuclein have also been reported in the SN of humans and non-human primates, showing an increase in immunostaining density within the cells (Chu and
Kordower, 2007; Xuan et al., 2011; McCormack et al., 2012), though contrasting findings were noted in mice, where a decrease in α-synuclein was reported with age (Mak et al., 2009). It has been suggested that the observed age-related increase in α-synuclein is a result of increased neuromelanin within the same cells. Xuan et al. (2011) found that only cells of the SN that contained neuromelanin displayed an increase in α-synuclein immunostaining with age in human samples, whereas non-neuromelanin cells did not have an increase in α-synuclein with age. These alterations were accompanied with a significant decrease in TH-positive cells in the aged brain. Neuromelanin usually assumes protective duties in the cells, such as scavenging free radicals and binding metals, but accumulation over a lifetime may result in vulnerability of the dopamine cells they reside in (Double and Halliday, 2006). This is consistent with previous reports by Hirsch et al. (1988) showing that neuromelanin-containing dopamine neurons degenerate to a greater extent than non-neuromelanin-containing cells in PD. The changes Xuan et al. (2011) observed were in otherwise “healthy” brains, suggesting that as the brain ages, it is constantly approaching the threshold at which PD may emerge.

The relationship between α-synuclein, neuromelanin and microglial activation has also been explored. In vitro work shows that extracellular aggregated forms of α-synuclein induce microglial activation, and extracellular neuromelanin has been reported to do the same thing in culture (Zhang et al., 2005). Zecca et al. (2008) showed that injections of human neuromelanin into the rat SN results in microglial activation and dopaminergic cell death. As neuromelanin has been found in the extracellular space of PD patients, it is hypothesized that neuromelanin is released from dying cells of the SN, and this promotes the microglial activation that is associated with the disease (Zhang et al., 2013). Overall, naturally occurring changes in the brain with age do resemble that of PD patients, and likely contribute to nigrostriatal vulnerability in the disease.
Aged Extra-Nigral Pathways

Extra-nigrostriatal dopaminergic pathways also show unique vulnerability with age. The VTA reportedly loses up to 50% of its DA neurons with age (Hirsch et al., 1987), contributing to decreased functioning of the mesocortical and mesolimbic pathways and potentially contributing to cognitive decline associated with aging (Jagust, 2013). Dopamine D1 receptors are associated with cognitive performance and decrease at a rate of 8% per decade in the PFC and occipital cortex (Suhara et al., 1991; Wang et al., 1998; Rieckmann et al., 2011). Dopamine D2 receptors are associated with a range of cognitive abilities, and these receptors are decreased by 5-10% per decade, as reported by human molecular imaging studies, in the PFC and medial temporal lobe consistent with loss of projections in the mesocortical and mesolimbic pathways (Volkow et al., 1994, 1998; Kaasinen et al., 2000; Ishibashi et al., 2009). Cognitive decline also can also be attributed to alterations in cholinergic signaling, as supported by production of cognitive issues in young patients treated with cholinergic antagonists and improvements in aged patients by cholinergic stimulation (Bartus et al., 1982). In the locus coeruleus, a 50% loss of catecholaminergic neurons by age 100 years was observed, and the cells in this region have an age-dependent accumulation of neuromelanin (Manaye et al., 1995). Overall, natural aging is a factor for degeneration in a number of PD-related regions.

Age-related inflammatory changes

Inflammatory changes also occur as the brain ages. Pro-inflammatory cytokines are increased and anti-inflammatory cytokines are decreased (Ye and Johnson, 2001; Maher et al., 2005; Nolan et al., 2005 Frank et al., 2006). Overall, the aging brain is in an increased inflammatory state. This inflammation is associated with alteration in the innate immune cells of
the brain, the microglia. Microglia are responsible for maintenance of homeostasis in the brain and for generating the initial immune response to damage or injury in the brain. Microglia in the aged brain, however, are considered “primed”, meaning that these cells express markers of inflammation and, once activated by an immune challenge, demonstrate an exaggerated and prolonged activation profile compared to “young” microglia (Wynne et al., 2009). As microglia are responsive to stimulation by glucocorticoids (Jurgens et al., 2012), it is possible that their age-related alterations may be a result of altered HPA axis functioning. Primed microglial responses have been linked to behavioral and other symptoms suffered by elderly people following illness, such as cognitive impairment, depressive behavior and increased sick behaviors (e.g., malaise, lack of energy, fatigue, and reduced appetite) (Godbout et al., 2005a; Dilger and Johnson, 2008; Corona et al., 2012). Overall, the immune system is acting aberrantly in the aged brain, and this may be due to a chronic threat to homeostasis, which would include all of the aforementioned changes that are occurring in the brain.

**Inflammation and PD**

Inflammatory processes have been implicated as playing a role in the pathophysiology of PD, beginning with Dr. Parkinson’s initial observations of inflammation associated with the disease, and further evidenced by human postmortem studies and a number of animal models (Parkinson, 1817; McGeer and McGeer, 2004). PD patients are reported to have up to a 15-fold increase in levels of cytokines, such as interleukin-1 (IL-1), interferon-γ (INF-γ), and tumor necrosis factor alpha (TNF-α), in the SN, striatum and cerebrospinal fluid (Mogi et al., 1996; Hirsch et al., 1998). Interestingly, associations between polymorphisms in inflammatory related genes (e.g. TNF-α, IL-1β) and disease development have also been identified (Hirsch and Hunot, 2009). Cerebrospinal fluid (CSF) samples from PD patients not only contained high levels of pro-
inflammatory molecules, but increased levels of inflammatory-associated proteins also correlated with more severe non-motor symptoms, such as depression and dementia (Mogi et al., 1994; Le et al., 1999; Lindqvist et al., 2013). To compliment these findings, epidemiological studies have suggested an inverse relationship between use of anti-inflammatory drugs and risk of developing PD (Hirsch and Hunot, 2009; Chen et al., 2003). It is clear that inflammatory processes are likely significant factors in the development and/or progression of PD.

A number of experimental PD paradigms also suggest that injured dopaminergic neurons have the potential to release immunogenic factors that trigger innate and adaptive responses that intensify the degeneration of the nigrostriatal system. Briefly, the innate immune response, carried out by mast cells, phagocytes, natural killer cells, etc., does not require the presence of a specific antigen to develop; rather, it is available to fight a wide range of pathogens by recognizing features common to pathogens, without leading to lasting immunity. Once a pathogen is recognized, these cells induce an inflammatory response that recruits new phagocytic cells and effector molecules to the site of infection or damage. Conversely, the adaptive immune response, carried out by T and B lymphocytes (T cells and B cells), occurs after a specific or novel antigen has been encountered, or after innate immune cells present antigens to T cells. This response will also result in lifelong protective immunity as it stimulates production of antibodies specific to the antigen. Adding to the complexity of the situation, it also appears that there is significant crosstalk between neurons and immune cells in the brain, each influencing the other as degeneration progresses (Stone et al., 2009; Sanchez-Guajardo, et al., 2013).

It is likely that stimulation of innate immunity is a secondary response to damaged and dead dopaminergic cells. When the dopamine neurons die, neuromelanin is released into the extracellular space and engulfed by microglia, suggesting that the immune system is activated once
the nigrostriatal system has already begun to degenerate. Microglia are the resident macrophages of the central nervous system (CNS). These cells monitor their local environment in the CNS with long processes, constantly checking the status of neurons and synapses around them (Perry and Holmes, 2014). In a non-inflammatory state, in which the brain is not suffering from disease or injury, microglia are surveying their microenvironment and are considered inactive, resting, or ramified. A ramified microglial cell can be identified by both morphology and gene expression, characterized by the long, thin surveying processes, the relatively small soma, and low expression of major histocompatibility complex (MHC) proteins and other antigen presenting receptors (Aloisi, 2001). The cells are in constant communication with neurons, which also present or release ligands that indicate their “health” to microglia (Streit, 2002). Once microglial cells have encountered a signal that indicates an “unhealthy” environment, such as an immunological stimulus or dying cells, they transition into an active state. This transition involves a morphological change of the cell to an amoeboid shape with shorter and thicker processes, or no processes at all. Additionally, the cells undergo alterations in gene expression and in the signaling molecules they release, to include pro- and anti-inflammatory cytokines, neurotrophic factors, reactive oxygen species (ROS) and others (Colton and Gilbert, 1987; Graeber et al., 1988; Hurley et al., 1999; Si et al., 2002) (Figure 3). These factors have been shown to work in combination to induce neurodegenerative processes (Chao et al., 1995).
Figure 3. Microglia morphology from ramified, or inactive, to activated cells. Along with morphological changes, microglia begin to release different inflammatory factors (Modified from Perry et al., 2007).
Considering the functional diversity of microglia and their high density in the ventral midbrain, they have been identified as a possible contributor to PD progression (Lawson et al., 1990; Kim et al., 2000). Postmortem brains of PD patients contain high numbers of human leukocyte antigen (HLA-DR)-positive microglia in the SN (McGeer et al., 1988). Imaging studies in PD patients show increased levels of activated microglia in multiple brain regions compared to controls (Gerhard et al., 2006). Moreover a correlation exists between levels of activated microglia and patient scores on the Unified Parkinson’s Disease Rating Scale in PD, which measures both motor and non-motor symptoms (Ouchi et al., 2005). Inflammation has also been reported in neurotoxin and genetic models of PD. Microglial activation has been associated with some α-synuclein mouse models; lines produced with the Thy1 promotor have developed activated microglia (Fleming et al., 2005), and overexpression of human wild-type α-synuclein in mice resulted in increased active microglial (CD68+), increased levels of tumor necrosis factor (TNF)-α, and increased infiltration of lymphocytes (Su et al., 2008; Theodore et al., 2008). The other common genetic mouse models of PD have not reported, or have not been characterized for, immune system involvement (Schwab et al., 2010). Additionally, observations of extracellular α-synuclein aggregates have been reported in PD models in vivo and in vitro; in vitro models show these aggregates inducing microglial activation and pro-inflammatory cytokine release, as well as neurotoxicity (Zhang et al., 2005; Lee, 2007).

In the 6-OHDA neurotoxin model of PD, imaging and immunohistochemical analyses of the ventral midbrain and SN showed a progressive increase in activated microglia up to 28 days after striatal or MFB lesions (Cicchetti et al., 2002; Rodrigues et al., 2004; Yasada et al., 2008; Henry et al., 2009; Sy et al., 2010). Importantly, it was also demonstrated that inflammation
precedes neurodegeneration in the 6-OHDA model, in both the striatum and SN (Cicchetti et al., 2002; Depino et al., 2003; Marinova-Mutafchieva et al., 2009). MPTP models have also revealed a role for inflammatory processes in degeneration. Postmortem analysis of MPTP exposed patients and monkeys who survived 3-16 years and 5-14 years, respectively, showed microglial activation (Langston et al., 1999; McGeer et al., 2003). The MPTP mouse model has also supported a role for the adaptive immune response in neurodegeneration as lymphocytes have been found in the SNpc in close proximity to dopaminergic neurons following i.p. injections of the compound (Brochard et al., 2009; Kurkowska-Jastrzebska et al., 1999). While neurotoxin models have highlighted microglial activation in PD-related neurodegeneration, they have not clarified whether inflammation or degeneration comes first in the human disease.

*In vivo* and *in vitro* models of microglia activation in the ventral midbrain have also shed light on their damaging role. The bacterial endotoxin lipopolysaccharide (LPS) has been used in a number of these studies as it stimulates microglial activation, as well as the secretion of neurotoxic factors and pro- and anti-inflammatory molecules (Elin and Wolff, 1976; Reed and Milton, 2001; Pålsson-McDermott and O’Neill, 2004). LPS does not directly affect neurons, therefore, neuronal death may be a secondary effect (Bronstein et al., 1995; Araki et al., 2001; Liu et al., 2002). The strong pro-inflammatory response to LPS makes it an attractive model for studying the relationship between microglial activation and dopaminergic neuron degeneration. LPS injection into or near the SN results in selective depletion of dopaminergic neurons (Arimoto et al., 2003; Gao et al., 2002; Herrera et al., 2005; Irvani et al., 2005; Hunter et al., 2007). In 6-OHDA rodent models of PD, microglia have been reported to be activated in the striatum and/or SN for up to four weeks following toxin injection (He et al., 2001; Cicchetti et al., 2002). Furthermore, in combination with PD models, such as the 6-OHDA and MPTP models, LPS
activation of microglia was shown to exacerbate dopaminergic cell loss in the SN (Koprich et al., 2008). A brief summary of direct and indirect models of inflammation is outlined in Figure 4.
Figure 4. Direct and indirect inflammatory stimulators. (Modified from Liu and Hong, 2003).
Epidemiological studies show that long-term use of anti-inflammatories, specifically non-steroidal anti-inflammatory drugs (NSAIDs), is associated with delayed or reduced risk for developing PD. While several studies were performed looking at associations between select NSAIDs and risk of PD, and many produced contrasting results, meta-analysis of the data concluded that non-aspirin NSAIDs do have a protective effect on the dopaminergic neurons of the ventral midbrain in chronic and regular users (Chen et al., 2003; Gao et al., 2011; Wahner et al., 2007; Ton et al., 2006; Bornebroek et al., 2007; Etminan et al., 2008; Samii et al., 2009; Gagne and Power, 2010). Many labs have examined the neuroprotective effects of anti-inflammatories in vivo and in vitro. In vitro cell culture of rat primary embryonic cells from the mesencephalon with ibuprofen showed neuroprotection against glutamatergic neurotoxicity and promoted dopaminergic neuron proliferation (Caster et al., 2000). Administration of compounds that target and inhibit inflammatory processes [e.g. minocycline, β-hydroxybutyric acid (BHBA)] in various PD animal models have resulted in reduced inflammation, decreased dopaminergic neuron loss and improved behavioral deficits (Du et al., 2001; He et al., 2001; Quintero et al., 2006; Whitton, 2010; Lv et al., 2015; Wang et al., 2015).

Whereas there is significant evidence for the critical role of innate immunity in PD progression, the activity of adaptive immunity should not be overlooked. There is constant communication between the CNS and the peripheral adaptive system, and they influence each other, and therefore, the neurons. Evidence for activation of the adaptive immune response comes from the presence of CD8+ and CD4+ T cell lymphocytes in the brains of PD patients (Brochard et al., 2009). The initiator of the adaptive immune response in PD models and patients is believed to be modified α-synuclein; protein overexpression, protein aggregates and the nitrated form (N-α-syn) stimulate T-cell production (Reynolds et al., 2009). These claims are supported by a number
of animal models, as well as observations of increased levels of N-α-syn in PD post-mortem brains (Giasson et al., 2000; Reynolds et al., 2009). Of note, N-α-syn’s activation of microglia increases nitric oxide, which stimulates the nitration of α-synuclein; N-α-syn also aggregates more readily than the non-nitratred form (Uversky et al., 2005). Further involvement and interaction of peripheral immune cells in the CNS is due to increased permeability of the blood-brain barrier (BBB), which allows the infiltration of these cells after pro-inflammatory molecules have been secreted by activated microglia and even astrocytes (Mosley et al., 2012). Research into the role of adaptive immunity in PD is still expanding, and while it will not be discussed at length in this document, it is important to note its relevance when it comes to alteration in α-synuclein.

**Stress/Glucocorticoids and Inflammation**

Biologically, stress can be defined as any stimulus that challenges an organism and results in a stress response. Part of the stress response is the release of glucocorticoids, which go on to exert a number of effects around the body and brain, to include maintenance of homeostasis, gluconeogenesis, and anti- and pro-inflammatory processes (Sapolsky et al., 2000). The role of glucocorticoids in inflammatory processes is complex, and factors that contribute to the outcomes the stimulus, levels of hormone, tissue and timing. Traditionally, glucocorticoids serve an anti-inflammatory purpose and have been utilized to suppress inflammatory processes (Cruz-Topete and Cidlowski, 2015). Glucocorticoids function in this way by suppressing pro-inflammatory genes through signal transduction (Barnes, 2006). However, glucocorticoid signaling, following a stressor, has been shown to induce an inflammatory state in the brain (Sorrells and Sapolsky, 2007). The mechanism by which glucocorticoids are able to exert both effects is still unclear, but
an important distinction to make when discussing their dual effects is *where* the effect is taking place, either the CNS or PNS.

In some cases, acute stress has shown to suppress inflammation in the brain. Nadeau and Rivest (2002, 2003) reported that an acute stress levels following LPS injection into the brain reduces damage and inflammatory markers. However, a significant amount of literature show the pro-inflammatory effects of stress and glucocorticoid signaling in the brain. Acute stressors (1 day/session) have resulted in increased expression of pro-inflammatory genes and microglia cell markers (Walker et al., 2013). Chronic or high levels of glucocorticoids are also shown to exacerbate inflammation, as shown by enhanced microglial activation independently or following an LPS/chronic stress paradigm (de Pablos et al., 2006; Walker et al., 2013). Nair et al. (2006) proposes that the pro-inflammatory response to glucocorticoids is actually a secondary response to glucocorticoid signaling on neurons, suggesting that it is actually signaling via the NMDA receptor that is pro-inflammatory. This is supported by other work looking explicitly at the glucocorticoid receptor (GR) on microglia and observing its anti-inflammatory response to glucocorticoids (Ros-Bernal et al., 2011). Again, the complexity of neuroinflammation and glucocorticoid signaling cannot be understated.

As mentioned previously, inflammation is believed to play a role in PD etiology and/or disease progression. Interestingly, elevated levels of glucocorticoids are consistently reported in idiopathic PD cases compared to age-matched controls (Charlett et al., 1998; Herrero et al., 2015). This elevation is likely to contribute to the increased inflammatory profile of PD brains, however it is unclear if alterations in basal glucocorticoid levels, and inflammation, are a response to or a cause for the neurodegeneration. Elevations glucocorticoids can also result from altered hypothalamic-pituitary-adrenal (HPA) axis function (discussed in more detail below). This
dysregulation is observed in depressed patients as well, and it has been shown that antidepressant treatments can reduce inflammation (reviewed by Hashioka, 2011). Regardless of initial insult, research supports a link between elevated glucocorticoid signaling, inflammation and neurodegeneration.

**Stress and Depression in PD**

Depression affects a reported 40-50% of PD patients (Rickards, 2005). It contributes significantly to a decrease in quality-of-life and may a more prominent predictor of quality-of-life than motor deficits (Schrag et al., 2000). While depression is suffered by nearly half of PD patients, only about 26% of PD patients are treated for it with pharmaceuticals (Yamamoto, 2001). A problem that is encountered is that, often, patients do not accurately self-report symptoms, either due to embarrassment or because they don’t recognize the symptoms themselves (Kuopio et al., 2000; The Global Parkinson’s Disease Survey, 2001). Depression and PD can have similar symptoms, to include bradykinesia, bradyphreia, sleep and appetite disturbances, weight loss, loss of interest, increased risk behavior and other non-motor symptoms, making it difficult for patients and clinicians to make a depression diagnosis (Rickards, 2005). Depression is also associated with worsened motor symptoms (Papapetropoulos et al., 2006). These points taken together suggest that both disorders may involve common neuropathological changes (Masterman and Cummings, 1999).

*Neurological basis for depression*

While degeneration of the nigrostriatal pathway is the primary feature of PD, other systems and pathways are also affected. These include, but are not limited to, the serotonergic pathways
originating in the raphe nuclei and extending to the hippocampus, limbic cortex and PFC, and the noradrenergic pathways originating in the locus coeruleus and extending to the PFC, among other areas (Scatton et al., 1983; Halliday, 1990; Braak et al., 2003; Lemke, 2008). Degeneration or dysfunction of serotonergic and noradrenergic pathways likely contributes to many of the non-motor symptoms of PD, and both pathways are part of the pathophysiology of major depression (Yamamoto, 2001). Some evidence exists suggesting that PD patients with depression have more alterations to these systems than PD patients without depression. Boileau et al. (2008) found that elevated levels of serotonin transporters in the striatum and PFC are correlated with depressive symptoms in PD, and Remy et al. (2005) showed an inverse relationship between noradrenaline transporter binding in locus coeruleus and regions of the limbic system and anxiety, a symptom of depression, in PD patients with vs without depression.

Evidence for non-dopaminergic pathways contributing to depression in PD is compelling, however dopaminergic signaling may play a role as well, as depressive symptomatology is improved in PD patients following dopamine agonist treatment (pramipexole) (Leentjens, 2011). As mentioned above, depression has been associated with more severe motor symptoms in PD (Papapetropoulos et al., 2006). This may be due to the fact that a correlation exists between decreased dopamine transporter availability in the striatum and affect scores for anxiety and depression (Weintraub et al., 2005). An alteration such as this will affect basal ganglia functioning, responsible for motor ability. Dopaminergic signaling is also involved in the mesolimbic, or reward, pathway. Because anhedonia, or loss of the ability to experience pleasure, is a symptom of depression, this system has also been suggested as another relevant pathway in depression pathology (Nestler and Carlezon, 2006). A handful of dysfunctional pathways are shared between PD and depression, however a clear link between the two disorders is yet to be established.
Depression has also been linked with hypothalamic-pituitary-adrenal (HPA) axis dysfunction. Hyperactivity of this circuit results in elevated cortisol levels, which is common in depressed patients (Vreesburg et al., 2009). The main function of the HPA axis is to modulate the stress response and, as such, it is involved in both acute and chronic stress responses, maintaining homeostasis in the body by activating a hormone cascade that ends in the release of glucocorticoids, such as cortisol (corticosterone in rodents) (de Kloet et al., 1998). As Figure 5 illustrates, when an organism experiences a stressor, either psychogenic or physiogenic, and homeostasis is disrupted, neurons within the paraventricular nucleus (PVN) of the hypothalamus release corticotrophin-releasing hormone (CRH). Via the hypophyseal portal system, CRH is transported to the anterior pituitary where it stimulates the release of adrenocorticotrophic hormone (ACTH) from corticotrope cells. From the pituitary, ACTH enters the systemic vasculature and travels to the adrenal cortex where it stimulates glucocorticoid secretion. Glucocorticoids elicit effects on cells and tissues all over the body, including the brain which contains its receptor, the glucocorticoid receptor (GR) in many regions (Herman, 1993; Van Craenebroeck et al., 2005). Important to the function of the HPA axis, one of those brain regions is the PFC, which, once stimulated, provides negative feedback on the circuit, ceasing its activity.

Although the release of glucocorticoids is adaptive and necessary, hyperactivity of the HPA axis, and chronic glucocorticoid release, is maladaptive and may be involved in the development of depression and other disease states (e.g., Herman and Cullinan, 1997). Chronic stimulation of this circuit can be prompted by stressful life events, which is a risk factor for depression. In addition to life stress, chronic threats to physiological homeostasis can elicit long-term stimulation of the HPA axis, and include chronic illnesses like obesity, human immunodeficiency virus (HIV) and PD (Zapanti et al., 2008; Djamshidian et al., 2011; Prpić-Križevac et al., 2012), all of which
are considered risk factors for depression and vice versa (Tandberg et al., 1997; Treisman and Angelino, 2007; Luppino et al., 2010).
Figure 5: Illustration of HPA axis circuitry. Following a stressful stimulus, PVN neurons of the hypothalamus are stimulated to release CRH. This hormone travels to the anterior pituitary gland via the hypophyseal portal vein to initiate release of ACTH into the vasculature. Once ACTH reaches the adrenal cortex of the adrenal glands, cortisol release is stimulated. Cortisol has a negative feedback effect on the circuit by acting on GR in the hypothalamus and turning HPA the axis off, ceasing cortisol release. Other regions that regulate the HPA axis are the amygdala, which stimulates it, and the hippocampus, which inhibits it.
Glucocorticoids

Glucocorticoid release is stimulated by activation of the HPA axis via a number of stimuli. The glucocorticoid receptor is found all over the body and in multiple brain regions (Herman, 1993). The receptor is a transcription factor that is found, unbound to its ligand, in the cytosol; once bound, however, receptor-ligand complexes dimerize and translocate to the nucleus where they bind to DNA (Drouin et al., 1992). At this level, the dimers modulate transcription of a variety of genes, to include those involved with gluconeogenesis and inflammation (Chantong et al., 2012; Renga et al., 2012). Signaling of GR is critical for a variety of CNS functions, such as arousal, cognition, mood and sleep, as well as to maintain homeostasis of cardiovascular tone, immune reactions, growth and reproduction (Chrousos and Kino, 2009). Also of benefit, GR signaling regulates the neurotrophin brain-derived neurotrophic factor (BDNF), which is necessary to support the neuroplastic changes that occur with learning and memory formation (Deppermann et al., 2014). Whereas GR stimulation elicits a number of beneficial effects, overstimulation, such that occurs in a chronic stress situation, can be detrimental.

While chronic stress will elevate glucocorticoids, HPA axis dysfunction can do the same. Dysfunction of the HPA axis is characterized by impaired feedback regulation, resulting in unregulated glucocorticoid release, and is seen in about 50% of depressed patients (Kanner, 2004). This dysregulation can be initiated by life stressors or by increased stimulation of the pituitary gland, evidenced by elevated levels of CRH in depressed patients (Nemeroff et al., 1984; Kathol et al., 1989; Swaab et al., 2005). Demonstrations of the negative effects of chronic stress and GR stimulation have been shown in the fields of stress, learning and memory, and inflammation, to name a few. Sapolsky et al. (1985) famously demonstrated that prolonged glucocorticoid exposure
was harmful to hippocampal neural populations. After treating mice with corticosterone for three months, there was a depletion of corticosterone receptors in the hippocampus, loss of neurons in the CA3 region, cells and increased numbers of glia cells. Interestingly, these changes are also evident in aged rodents (Sapolsky et al., 1985). In the PFC, layer II/III neurons lose dendritic elaborations (dendritic length, branching, spine density) following chronic stress (Radley et al., 2006; Holmes and Wellman, 2009), and these changes are mimicked by chronic administration of glucocorticoids (Wellman, 2001; Liu and Aghajanian, 2008). Chronic glucocorticoid treatment also reduced BDNF levels in the PFC, as does chronic stress (Gourley et al., 2009; Lin et al., 2009). Negative effects of glucocorticoid signaling have also been observed in dopaminergic neurons.

Dopaminergic signaling of the mesolimbic and mesocortical pathways is affected in PD, depression and by stress (Pani et al., 2000; Nestler and Carlezon, 2006). Acute stress has been found to increase or decrease dopaminergic signaling to a variety of pathway terminals depending on the stressor and paradigm (Abercrombie et al., 1989; Imperato et al., 1990; Rasheed et al., 2010). Chronic stress, however, generally decreases dopaminergic tone in the prefrontal cortex, striatum, and nucleus accumbens (Ziegler et al., 1999; Rasheed et al., 2010). Restraint stress has been shown to elevate levels of oxidative stress products in the nigrostriatal system, showing that stressors may actually increase cellular vulnerability (Kim et al., 2005). These findings suggest that stress may directly influence dopamine vulnerability, potentially promoting cell death. Whereas dopaminergic signaling in the mesocortical pathway is largely affected, depending on the stressor, dopamine signaling in the striatum is affected as well (Kim et al., 2005; Kim et al., 2013), implicating a role for stress in increased nigrostriatal damage in comorbid cases of PD and depression.
Chronic variable stress animal model of depression

To study the effects of chronic stress, animal models of stress and depression are utilized. As rodents cannot be diagnosed with depression, paradigms that elicit similar physiological and behavioral responses as depressed patients have been developed. One of those models is chronic variable stress (CVS), which involves subjecting rodents to various stressors in a random order over an extended period of time (Katz et al., 1981; Herman et al., 1995; Willner, 2005). This paradigm induces an elevation of baseline corticosterone and ACTH secretion, results in adrenal hypertrophy and alters outcomes in a variety of behavioral tests that measure anxiety, anhedonia and motivation behaviors. Considering the extent to which the CVS animal model produces changes that mimic patient physiology and behavior, it is a well-accepted and well-documented model for depression.

Following CVS, a number of changes take place in the brain in regions responsible for regulation of the HPA axis. Glucocorticoid receptor mRNA is downregulated in the hippocampal CA1 region, dentate gyrus and frontoparietal cortex (Herman et al., 1995). Joëls et al. (2004) reported HPA axis regulation suppression, enhanced glutamate transmission in the dentate gyrus, diminished GABAergic signaling in the PVN, and reduced responses to serotonin in the CA1 region of the hippocampus. Oxidative damage is increased in the cortex, hippocampus and striatum following CVS, which is also observed in depression and PD patients (Forlenza and Miller, 2006; Lucca et al., 2009; Ikawa et al., 2011). Chronically stressed rodents also exhibit behaviors that mirror depressive patients. Rodents show a decrease in sucrose intake (anhedonia) following CVS, as well as altered sexual, sleep and grooming behaviors, which correlate to depressed patient behaviors (Pittenger et al., 2008). Anhedonic behavior induced by CVS in rodents can be reversed.
by treatment with dopamine agonists (Papp et al., 1993; Willner et al., 1994), suggesting a link between stress and depression and mesolimbic pathway dysfunction (Keller et al., 2013), which is also observed in PD. The effects of chronic stress can be reversed with chronic antidepressant treatment (Roth and Katz, 1981). Thus, an abundance of evidence supports the use of CVS to induce a depression-like phenotype.

Altogether, the overlap of affected pathways in PD and depression, as well as the high comorbidity, begs the question of how the two influence each other. The finding of Hemmerle et al. (2014) that CVS exacerbates effects of 6-OHDA lesions suggests that enhanced GR signaling is detrimental to the nigrostriatal pathway. To further explore the relationship of stress on nigrostriatal degeneration, we used the CVS model, in combination with the neurotoxin 6-OHDA model (discussed previously), to probe the specific role of increased neuronal GR signaling on the effects of chronic stress on nigrostriatal function and degeneration.
Objectives

This dissertation examines alterations relevant to stress and inflammation in the aged brain and in the combined stress and neurotoxin rat model of PD, CVS/6-OHDA. The overall hypothesis is that proteins related to stress-induced depression are decreased, and those related to inflammation and PD are increased in the aged midbrain; thus inhibiting inflammation will ameliorate and increasing glucocorticoid signaling in neurons will exacerbate behavioral dysfunction and dopaminergic neuronal loss in neurotoxin models of PD.

Specific Aims

Aim 1: Characterize alterations in glucocorticoid receptor, α-synuclein and microglial marker protein expression in young, middle-age and aged ventral midbrains.

Aging is the highest risk factor for developing PD. We hypothesize that the aged brain will reveal altered expression of proteins relevant to stress, inflammation and PD pathology. We expect a decrease in glucocorticoid receptor expression, an increase in microglial marker protein (Iba-1) expression, and an increase in α-synuclein expression. Examining three age groups will clarify the temporal changes of these proteins.

Aim 2: Determine if anti-inflammatory treatment will ameliorate behavioral and cellular deficits in the 6-OHDA rat model of PD.

Inflammatory processes are implicated in nigrostriatal degeneration. We hypothesize that anti-inflammatory treatment will ameliorate motor deficits and increase dopaminergic neuronal
survival in the 6-OHDA model of PD. Animals will receive minocycline, a tetracycline antibiotic, for two weeks prior to and following intrastriatal 6-OHDA lesions.

**Aim 3:** *Determine if increased neuronal expression of the glucocorticoid receptor will exacerbate behavioral and cellular outcomes in the CVS/6-OHDA rat model of PD.*

Increased glucocorticoid signaling is detrimental to neurons in previous studies using the CVS/6-OHDA model. We hypothesize that increased expression of the glucocorticoid receptor in the dopaminergic neurons of the substantia nigra will cause greater behavioral dysfunction and dopaminergic neuronal degeneration than seen in the combined CVS/6-OHDA model. Animals will receive GR overexpression lentivirus into the substantia nigra prior to undergoing CVS and intrastriatal 6-OHDA injections.
References


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delta agonist, rescue of dopaminergic neurons in the 6-OHDA parkinsonian model is associated with inhibition of microglial activation and MMP expression. J Neuroimmunol 246:69–77.


Chapter 2

Age-associated changes in Parkinson-related proteins in rat ventral midbrain

Abstract

The greatest risk factor for Parkinson’s disease (PD) is aging. Aging-associated changes of PD-related molecules, in combination with other factors such as chronic inflammation and adverse life stress, contribute to dopaminergic neuron degeneration. This is supported by idiopathic cases of PD and animal models of the disease that show increased microglial recruitment and activation in the substantia nigra, as well as a role for stress in ventral midbrain neuron vulnerability. In addition to a hyper-activated inflammatory state, the pathological hallmarks of PD, Lewy bodies composed primarily of aggregated α-synuclein, are present in the dopaminergic cell bodies. In the present study, we sought to explore the chronological time course for changes in various disease-associated proteins in the ventral midbrain of adult male Sprague Dawley rats at 3, 15 and 22 months of age. Brains were processed for immunohistochemistry and western blotting, and proteins investigated included ionized calcium-binding adaptor molecule 1 (Iba-1) for microglia, α-synuclein and glucocorticoid receptor (GR). Immunohistochemically labeled cells in the substantia nigra were counted using unbiased stereology and western blot assays were analyzed with densitometry readings. Analysis of aged (22-month-old) substantia nigra compared to young (3-month-old) demonstrated a significant increase in the number of microglia, as well as a significant increase in microglial soma area, indicating a change in activation state. Analysis of α-synuclein and GR immunoreactivity have revealed significant changes, an increase and decrease, respectively, in relative protein concentration in the ventral midbrain of the mid-age (15-month-old) and aged groups. These analyses of the age groups may clarify the chronological
timeline of microglia activation and recruitment, as well as α-synuclein accumulation and changes in GR expression, in the substantia nigra and ventral midbrain during the natural aging process.

**Introduction**

Parkinson’s disease is an age-associated disease, affecting 1-5% of adults over the age of 65 (de Lau and Breteler, 2006). The body and brain undergo a number of changes with age that are not associated with disease state, but that may promote dopaminergic vulnerability in the nigrostriatal system (Reeve et al., 2014). These includes age-related decrease in dopaminergic neurons and TH protein expression (though no change in TH+ cells) in the ventral midbrain, increased oxidative stress, increased α-synuclein, and decreased DA in the striatum (Thiessen et al., 1990; Ma et al., 1999; Li et al., 2004; Dickerson et al., 2009; Haider et al., 2011).

Inflammation is also altered in the aged brain, supported by clinical and experimental evidence. Microglial cells, the resident macrophage of the central nervous system (CNS), increase in number and develop an increased inflammatory phenotype in the aged brain (Norden and Godbout, 2013). While it is evident that microglial priming presents special challenges to the aged brain compared to the young and adult brain, it is not clear as to what precipitates this altered state.

Speculations as to the source of inflammatory response in PD have been made. *In vitro* work has linked extracellular monomeric α-synuclein, nitrated α-synuclein, and aggregated α-synuclein to microglial activation (Zhang et al., 2005; Park et al., 2008; Reynolds et al., 2008). Increases in α-synuclein expression have also been identified in aged human and non-human primate SN, potentially implicating the protein in disease etiology in the aged brain (Chu and Kordower, 2007; McCormack et al., 2012).
The glucocorticoid receptor (GR) has also been implicated in microglial regulation and activity. Loss of the receptor on microglia exacerbates inflammation and neuronal damage, observation not seen in GR-deficient neurons (Ros-Bernal et al., 2011; Carillo-de Sauvage et al., 2013). Ros-Bernal et al. (2011) also reported a decrease in GR in the human SN of PD patients. Conversely, a number of experiments have shown that microglia can be activated following stress exposure, suggesting that increased GR signaling stimulates microglia (Tynan et al., 2010; Bian et al., 2012; Farooq et al., 2012; Kopp et al., 2013).

Taken together, it is difficult to conclude which factors specifically influence microglial activity in the aged brain, and further, how these interactions may increase dopamine neuron vulnerability in the SNpc. We sought to explore the temporal alterations in PD and inflammation-related proteins in the ventral midbrain and SNpc. Using young (3 months), middle-aged (16-18 months) and aged (22-25 months) rats, we assayed for GR, α-synuclein, and Iba-1 with western blotting and immunohistochemistry. Overall, we report increases in α-synuclein protein expression and decreases in GR protein expression in the ventral midbrain. Immunohistochemical analysis of the SNpc shows significant increases in microglial cells in the aged rats, though no changes in the number of cells expression GR and α-synuclein. These data only begin to put together a potential timeline and relationship between these protein expression and cellular changes.

**Methods**

*Animals.* Male Sprague Dawley rats (Harlan) were used in this study at ages 3-4 months, 16-18 months, and 22-25 months (n=18 total). Adult rats weighed 300-850 g and were housed 2 per cage under normal conditions; 12 hr on/off light/dark cycles with free access to food and water. All procedures were conducted in compliance with the University of Cincinnati Institutional Animal Care and Use Committee.
*Western Blots*. Rats were euthanized by CO₂ asphyxiation for protein analysis. The ventral midbrain were carefully dissected out of each brain sample and stored at -80°C until analysis. The western blotting protocol was performed as described previously (Dickerson et al., 2009). To solubilize proteins within the samples, the frozen ventral mesencephalic tissue was probe-sonicated in buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The protein concentration of each sample was determined in triplicate using Bio-Rad’s DC protein assay kit (Hercules, CA). The final volume of each sample that was loaded onto a 7.5% acrylamide gel was 15 - 25 μl depending on number of wells in the gel. The loading control protein used in the assay was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For the list of antibodies used and concentrations see Table 1. Following SDS-polyacrylamide gel electrophoresis the proteins were transferred to nitrocellulose, blocked in 5% milk PBS/tween for 1 hour and then incubated at 4°C overnight in appropriate primary antibody. Following primary antibody incubation, the membranes were washed in 2% milk PBS/tween (1 hour) and then incubated in the appropriate secondary antibody diluted in 2% milk PBS/tween for an hour. After an hour PBS/tween wash, antibody binding was visualized by enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ) and exposed to Hyperfilm ECL (GE Healthcare). Films were analyzed by densitometry using Scion Image software. The mean correlated grey levels were determined by measuring the optical density of each band and subtracting background (area in same lane just below the band). The mean from the bands of young animals was set at 100% (control). Data are expressed as mean correlated grey level (percent control) ± SEM. Western blot data was analyzed using one-way ANOVA followed by the Bonferroni multiple comparison post-hoc test.
Table 1. Western blot antibodies and concentrations

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Isotype</th>
<th>Primary AB concentration</th>
<th>Secondary AB concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>Santa Cruz</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>1:2000</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>BD Biosciences</td>
<td>Mouse</td>
<td>1:1000</td>
<td>1:2000</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Wako</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>1:2000</td>
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</table>
Tissue Preparation – Immunohistochemistry. To prepare tissue for immunohistochemical analysis, rats were deeply anesthetized with sodium pentobarbital and sacrificed by intracardial perfusion using saline followed by 4% paraformaldehyde (PFA). Brains were dissected out and placed in 4% PFA for 24 hours before being transferred into 30% sucrose. Once brains were saturated with sucrose solution they were sectioned. Coronal sections through the mesencephalon were cut at 50 µm on a sliding microtome and stored in cryoprotectant. Sections were processed for ionized calcium-binding adaptor molecule 1 (Iba-1; pan-microglial marker) and α-synuclein according to our routine immunohistochemical procedures (e.g. Seroogy et al., 1994). Briefly, free-floating sections were washed in 0.1 M phosphate buffer (PB) and normal horse serum (NHS) used to block non-specific staining. Sections were incubated in primary antibody overnight at 4°C. Primary antibodies [rabbit anti-Iba-1 (1:2500), Wako Pure Chemical Industries, Ltd., Osaka, Japan; mouse anti-α-synuclein (1:250), Transduction Laboratories, Lexington, KY] were diluted in 0.1 M PB with 1% NHS and 0.2% Triton-X. Some sections to be labeled for α-synuclein were also incubated with Proteinase K (1:4000) prior to primary antibody incubation to remove soluble forms of α-synuclein from the cells; this ensured that only insoluble forms of α-synuclein would be labeled. The following day, sections were washed in 0.1M PB, blocked with 2% NHS, and incubated with the anti-mouse IgG biotin-conjugated secondary antibody [horse anti-rabbit (1:200) for Iba-1 and horse anti-mouse (1:200) for α-synuclein, both from Vector Laboratories, Inc. Burlingame, CA] for 1 hour. Following the blocking step, sections were washed again in 0.1M PB, and placed in ABC peroxidase (Vector Laboratories) for 30 minutes and then washed in 50 mM Tris buffer (pH 7.5). Labeling was visualized with diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories) containing 0.3% H2O2, buffer, and DAB reagent. Following DAB step, sections were
washed again in Tris buffer and mounted onto Superfrost plus microslides (VWR, Batavia, IL), dehydrated in a series of ethanol washes and coverslipped.

**Stereological Cell Counts.** Estimated cell counts and cell morphology analysis was performed for Iba-1 and α-synuclein immunopositive cells in all of the age groups from the SNpc. Cell count estimates were determined using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased stereological techniques (West, 1993). Analysis was performed on an Olympus BX-51 microscope (Melville, NY) using a CCD video camera (HV-20, Hitachi, San Jose, CA). Contours were drawn around the area of interest at 1.25X magnification and cell counting was performed at 60X. Random sample sites were determined by the software on a grid of 100 x 100. A 2um guard was used for each section. The change in cell number in the SNpc was determined by comparing all groups to the young control group. Once estimated cell counts were established, the average number of cells in the SNpc of the control group was set as 100%. Each cell count from every other animal in the study was divided by the control group cell count average, to provide a percentage of control.

**Microglia Morphology Analysis.** Microglial cell morphology was analyzed using Image J software (NIH). To determine relative activation states of the microglia in each condition, soma area was calculated. Images of random microglia in the SNpc were taken from three sample sections for each animal at 60X magnification on an Olympus BX-51 microscope (Melville, NY) using a CCD video camera (HV-20, Hitachi, San Jose, CA). These images were opened in the Image J software and the scale was set such that one pixel on the image was equal to 0.113 um. The freehand tool was used to trace the soma of the microglia cells, following the trace, ‘Measure’ was selected from the Analyze menu. Soma area was measured from three sample sections from each animal. The
average for each section was calculated, and then the average of the three sections was calculated to represent the average microglia soma area for that animal.

*Statistical Analysis.* Western blot, microglial soma area and stereological cell counts were analyzed using one-way ANOVAs. Data are presented as means ± SEM. Significance is considered at \( p < 0.05 \). SPSS IBM Statistics 21 was used to analyze all the data.

**Results**

*Western Blot*

Age-related modulation of Iba-1, GR, and α-synuclein was investigated in the ventral midbrain of male Sprague Dawley rats. Chronological aging showed several changes in the aging brain when compared to young animals. There was a significant decrease of GR protein expression in middle-age and aged animals compared to young, representing protein from both the cytosol and nucleus, \( F(2,12) = 14.2; \ p < 0.0007 \) (Fig.1). In contrast, protein levels of α-synuclein were elevated in middle-aged and aged animals compared to young, \( F(2,12) = 9.91; \ p = 0.0029 \) (Fig. 1). Finally, Iba-1 protein expression between age groups did not differ with age.
Figure 1. Western blot results from young, middle-age and aged ventral midbrain.

A. Quantification of western blot data in aging ventral midbrain proteins. No changes were detected in middle-aged or aged tissue for Iba-1 compared to young control. GR expression was significantly decreased in middle-aged and aged tissue. α-synuclein expression was significantly increased in middle-aged and aged tissue. There were no changes in the housekeeping protein GAPDH.

B. Representative western blot bands showing relative changes in GR and α-synuclein protein expression. Data are expressed as mean corrected grey level (percent of young) ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to young animals. n = 4-6/age group.
Stereological cell counts

Immunohistochemistry was used to detect the number of Iba-1-, α-synuclein-, and GR-positive cells in the SNpc of middle-aged and aged Sprague Dawley rats compared to young. Estimated cell counts of Iba-1 labeled microglia and α-synuclein-labeled neurons were collected using unbiased stereological cell count protocols. Analysis found that the number of Iba-1-positive cells of aged rats in the SNpc were significantly greater compared to young and middle-aged animals, $F(2,14) = 27.1; \ p < 0.0001$. There was no difference in Iba-1 cell counts between young and middle-aged animals (Fig. 2). Stereological cell counts of α-synuclein-positive cells showed no differences in cell number at any time point (Fig. 3). Sections stained for insoluble α-synuclein with Proteinase K treatment resulted in no detectable staining, and therefore were not counted (Fig. 3). Finally, our GR staining protocol was unable to detect GR labeling dense enough to be counted.
Figure 2

**Figure 2.** Iba-1+ stereological cell counts in the SNpc of young, middle-age and aged rats.

Estimated cell counts of Iba-1-positive cells. **A.** Iba-1+ stereological cell counts in the SNpc of young, middle-aged and aged rats showed a significant increase cell counts of Iba-1-positive microglia in aged rats. **B.** Representative images (4x magnification) of Iba-1 staining in the ventral midbrain of young, middle-aged and aged rats. The average cell count for each protein in young animals served as control and was set at 100%; cell counts in middle-aged and aged rats were collected and divided by the average of the control group to calculate a percent of control. Data presented as a mean ± SEM. *p < 0.001; n = 5/group.
Figure 3.

**A.** α-synuclein+ stereological cell counts in the SNpc of young, middle-aged and aged rats. α-synuclein+ stereological cell counts in the SNpc of young, middle-aged and aged rats showed no differences between age groups.

**B.** Representative images (60x magnification) of young, middle-age, and aged SN, with and without Proteinase K treatment. Data presented as a mean ± SEM. n = 4/group.
**Microglial Somal Area**

Nigral sections were processed immunohistochemically to detect Iba-1 in order to ascertain relative differences in microglia somal area, which can be indicative of microglial activation. In the aged rat, the somal area of microglia was significantly increased in the SNpc compared to both young and middle-aged animals, F(2,12) = 5.47; p = 0.021 (Fig. 4). There were no differences detected between young and middle-aged rats.
Figure 4. Microglial soma area in the SNpc of young, middle-age and aged rats. 

A. Somal area of microglia was significantly increased in the SNpc of aged rats compared to young and middle-aged. B. Representative images (60x magnification) show the clear increase in soma area in the aged SNpc. Data presented as a mean ± SEM. *p < 0.05; n = 5/group.
Discussion

This characterization study sought to clarify the timeline by which changes in protein expression and cell types relevant to PD occur within the SNpc and ventral midbrain. Using western blot analysis, we saw changes in GR and α-synuclein protein expression in the ventral midbrain, however no significant changes in Iba-1 expression. The observed changes in the western blot both occurred at the middle-age time point. To better understand the cellular expression of these proteins in the SNpc, we used unbiased stereology to count protein-positive cells. Cell counts revealed a significant 4-fold increase in Iba-1 positive cells in the SNpc, with no observed changes in GR- and α-synuclein-labeled cells. This staggered difference in protein expression and cell counts is supported by a previous work and suggests that microglia are responsive to changes that are occurring in middle-age.

Alpha-synuclein is a hallmark protein of PD, present in Lewy body inclusions within the nigrostriatal system and other pathways. Increases in α-synuclein have been observed in the human and non-human primate aged SN previously (Chu and Kordower, 2007), but not in the aged mouse brain (Mak et al., 2009). Western blot analysis showed a significant increase in α-synuclein at the middle-age and aged time points. This increase in protein expression in the ventral midbrain was not paralleled by an increase in α-synuclein+ cells in the SNpc, or cells that contain insoluble (oligomerized) α-synuclein. We were unable to assay for oligomerized α-synuclein in the western blot as we had sonicated the tissue in a buffer that contained sodium dodecyl sulfate and disrupted the non-covalent bonds that would have bound monomeric forms of the protein together. Considering the data we collected, we can speculate as to the form and location of α-synuclein upregulated with age: first, it could be increased monomeric forms of the protein within the cells. We did not analyze α-synuclein+ cell staining density, so we can’t report on this measure, but Chu
and Kordower (2007) did report increased optical density in α-synuclein-stained cells of the nigra in aged versus young human and monkey brains. Second, the increase may be due to an increase in extracellular protein. Lee et al. (2014) reviewed growing literature that indicates that an unconventional exocytosis mechanism carries α-synuclein out of the cells and these extracellular proteins may contribute to the pathology features of Lewy body diseases, such as PD. A human aging study by Mikolaenko et al. (2005) reported that otherwise healthy aged controls had α-synuclein inclusions in the SNpc and medulla. Whether these lesions represent very early stages of PD or are simply part of aging is unsettled. Considering their report, we expect that we would find an increase in α-synuclein expression in the brainstem of the aged rats well. Further analysis of aged tissue would have to be done to evaluate these options, and would be a future direction for this study.

Glucocorticoid receptor expression was also altered in the middle-age and aged ventral midbrain, showing a significant decrease. Immunohistochemical analysis, however, showed such light staining in the nigra that were unable to evaluate changes in the number of SNpc cells expressing it over time. Evaluations of GR in the aged brain are sparse and not specific to the nigra (Wang et al., 2013). Tying our observed decrease to disease pathology, Ros-Bernal et al. (2011) showed decreased GR expression in the SN of PD patients. Again, further analysis of the tissue would be required to tease apart if the decrease is specific to a cell type, however our findings do suggest that this alteration may contribute to the cellular environment that would promote neurodegeneration and/or microglial activation in an age-dependent manner. As elevated glucocorticoids have been observed in aged populations, a decrease in GR may be compensatory and contribute to glucocorticoid resistance, which is also observed in other conditions with HPA-axis dysregulation, like depression (Zunszain et al., 2011).
Despite a lack of change in protein expression of Iba-1 in the western blot assay, immunohistochemical analysis revealed a substantial 4-fold increase in Iba-1+ microglia in the SNpc in aged rats compared to young and middle-aged. Insignificant finding in the western blot analysis is likely due to the fact that the ventral midbrain is densely packed with microglia (Lawson et al., 1990) at all age time points, so changes that occur in the rest of the ventral midbrain (SNpc and VTA) may be undetectable. In addition to this increase in cell number, the morphology of the cells showed an increased somal area, suggesting a more active state. These finding are consistent with reports of “primed” microglia in the aged CVS, though these reports come primarily from the PFC and hippocampus, or full brain samples (Lawson et al., 1990; Perlman et al., 2007). While finding increased numbers of microglial cells that are morphologically distinct in the aged brain is not a novel finding, the observation specific to the SNpc is.

Taken together, we can speculate about microglial activation in the aged ventral midbrain. The inflammatory profile changed at the aged time point and GR and α-synuclein were altered at the middle-age time point. This could suggest that microglia activation state may be in response to changes that occur in the brain, such as protein expression changes, versus a dysfunction of the microglia themselves. Age-related changes in GR expression have been reported in the hippocampus (Wang et al., 2013), which are accompanied by microglial changes (Choi and Won, 2011), though the two have not been deemed a cause and effect relationship. Experimental evidence points to a role of GR expression in inflammation, Ros-Bernal et al. (2011) showed that GR-deficient microglia, and not GR-deficient neurons, caused a significantly larger lesion in the nigrostriatal system and chronic microglial activation following MPTP treatment.

As previously discussed, an age-dependent increase in α-synuclein has been reported in the nigra of humans and monkeys. A role for α-synuclein in microglial activation has also been
investigated. Zhang et al. (2005), reports that aggregated extracellular α-synuclein activated microglia in culture. Overexpression of α-synuclein has been shown to trigger microglial activation as well (Theodore et al., 2008). Clearly, there are links between GR and α-synuclein and microglial activation. These changes likely work in concert with a number of other changes to induce microglial phenotype changes as the brain ages and in PD.
References


Chapter 3
Effects of minocycline treatment in the 6-OHDA rat model of Parkinson’s disease.

Abstract
Inflammatory processes may contribute to the etiology and/or progression of both motor deficits and non-motor disturbances suffered by Parkinson’s disease (PD) patients. We have employed a partial lesion model of PD to model both physiological and behavioral aspects of PD. In conjunction with this paradigm, we treated rats with the antibiotic, minocycline, which prevents microglia from transitioning into an activated, and therefore inflammatory, state. We hypothesized that prevention of microglial activation would be neuroprotective by limiting the extent of the lesion and improving behavioral dysfunction in the 6-OHDA model. Motor deficits were evaluated using the forelimb-use asymmetry (cylinder) test two and four weeks following neurotoxin injection. Alterations in numbers of dopaminergic cells in the substantia nigra pars compacta (SNpc) were examined using unbiased stereological cell counts of tyrosine hydroxylase (TH) immunoreactive cells. Microglia somal area was measured in the SNpc to determine any relative differences in microglial activation at time of sacrifice. Although strong trends with some of the data were observed, the results did not show that minocycline had neuroprotective effects in the present 6-OHDA model of PD.
Introduction

Parkinson’s disease (PD) is an age-associated, progressive neurodegenerative disorder affecting the dopaminergic neurons of the nigrostriatal pathway. The disease prevalence is 1% of the population 65 years of age and increases to 4% of the population at age 80 (de Lau and Breteler, 2006; Toulouse and Sullivan, 2008). The etiology of PD is still unclear, however the role of inflammation in disease onset and/or progression is compelling (Dantzer et al., 2008; Panaro and Cianciulli, 2012; Tufekci et al., 2012;). Increased inflammation has been recorded in a number of postmortem studies, neurotoxic animal models of PD, and in vitro work. Neurotoxin models of PD not only show microglial activation, but initiation of inflammatory processes prior to neurotoxin administration exacerbates damage of the nigrostriatal pathway.

Additionally, anti-inflammatory treatment in PD models can ameliorate damage (Sadeghian et al., 2012), again speaking to the critical role of inflammation in disease progression and, potentially, initiation. Quintero et al. (2006) reported a significant reduction in lesion size following 6-OHDA injection into the medial forebrain bundle and IP injections of the antibiotic minocycline for only 3 days prior to lesion or for 24 hours following lesion. Minocycline was also neuroprotective for dopaminergic substantia nigra (SN) neurons in a multiple system atrophy (MSA) model in mice that expressed wild-type alpha-synuclein in oligodendrocytes (Stefanova et al., 2007). Attentuation of microglial activation has been shown to protect up to 90% of dopamine neurons in animal models of PD (Choi et al. 2005; Vijiitruth et al. 2006).

Minocycline, a semisynthetic tetracycline antibiotic, prevents the activation and proliferation of microglial cells through a number of complex mechanisms. Minocycline inhibits the p38 mitogen-activated protein kinase (p38 MAPK) pathway initiated by TLR4 activation on microglial (Chi et al., 2006). The drug also inhibits nuclear translocation of NF-κB, thereby
preventing production of inflammatory molecules from microglial cells (Kobayashi et al., 2013). Another groups reports that minocycline attenuates MHC II expression by inhibiting transcription factor class II transactivator (CIITA) expression (Nikodemova et al., 2007). Minocycline is also shown to prevent apoptosis by acting as a caspase inhibitor and by preventing activation of inducible nitric oxide synthase (iNOS) and has successfully been used in a variety of brain injury and animal models of disease to reduce degeneration caused by activated microglia (Tikka et al., 2001; He et al, 2001).

In the present study, we administered minocycline by dissolving it in the drinking water, which rats had access to *ad libum*. Minocycline treatment began two weeks before 6-OHDA and continued throughout the entire experiment. We hypothesized that inhibition of microglial activation by minocycline would prevent dopaminergic cell loss in the SNpc following 6-OHDA injection into the striatum, as well as prevent behavioral deficits.
Figure 1

<table>
<thead>
<tr>
<th>Day 0</th>
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<th>Day 44</th>
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<td>Cylinder Test &amp; Minocycline Treatment</td>
<td>6-OHDA Surgeries</td>
<td>Cylinder Test</td>
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Figure 1. Experimental timeline. Animals received two weeks of minocycline hydrochloride dissolved in drinking water prior to 6-OHDA lesion surgery. After surgery, animals received four more weeks of minocycline in their drinking water. The cylinder test assessed motor function two and four weeks post-lesion. Animals were sacrificed and processed for immunohistochemistry following the last cylinder test.
Methods

Animals. Adult male Sprague Dawley rats (250 - 400g; Harlan Laboratories (Indianapolis, IN)) were used in this study (n = 5-8/group). Animals were housed in standard shoebox cages, 2 per cage, in standard housing conditions (12 hr on/off light cycle) and had access to food and water ad libum. Treatment of animals was in accordance with the University of Cincinnati Institutional Animal Care and Use Committee Guidelines. Animals were randomly divided into four weight-matched groups: two groups receiving minocycline treatment in drinking water, two groups receiving regular drinking water as control treatment. One groups in each treatment group received 6-OHDA striatal injections, while the other half received vehicle striatal injections. The timeline of the study is provided in Figure 2.

Oral Administration of Minocycline Hydrochloride. Minocycline (Sigma-Aldrich Corp., St. Louis, MO) was administered to the rats for the entire length of the six-week study, starting on ‘Day 0’. Minocycline was dissolved in the drinking water at a dosage of 30-40 mg/kg/day/rat. This dosage is shown to be sufficient to limit microglial activation in other studies (Ekdahl et al., 2003; Raghavendra et al., 2003; Liu et al, 2007). Minocycline solution was made up each day at a concentration of 0.35-0.45 mg/ml, depending on the weekly average weight of the rats. Daily cage water intake for two rats was ~60 ml, so we proceeded with dosage assuming that each rat drank ~30 ml of minocycline solution per day, therefore this concentration ensured that the rats received a dosage of 30-40 mg/kg/day.

Partial 6-OHDA Lesion. Injection of 6-OHDA into the striatum creates a progressive degeneration of the nigrostriatal pathway (Kirik et al., 1998; Hemmerle et al., 2014). In preparation for striatal 6-OHDA injections, animals were anesthetized (4.35 mg/kg ketamine,
0.65 mg/kg xylazine) and placed in a stereotaxic apparatus. Two unilateral injections of 6-OHDA (2.5 µg each in 2 µl saline + 0.2% ascorbic acid) or vehicle (saline + ascorbic acid) were made into the right striatum at the following coordinates according to the rat brain atlas of Paxinos and Watson (2007): AP: +1.6, ML: -2.4, DV: -4.2 and AP: +0.2, ML: -2.6, DV: -7.0 (Paxinos and Watson, 2007). For each injection, the AP and ML measurements were taken from bregma and DV measurements were taken from the skull. A 5-µl Hamilton syringe (Hamilton Company, Reno, NV) was lowered into the striatum and allowed to equilibrate for 5 minutes. The 2-µl injection was administered over 10 minutes (0.2 µl/min), and the needle remained in place for another 5 minutes before being removed slowly.

Forelimb-use Asymmetry. Motor impairment was assessed in all groups via the forelimb-use asymmetry test (cylinder test). The test was performed as previously described (Schallert et al., 2006; Hemmerle et al., 2014). Briefly, rats were placed in the center of a Plexiglass cylinder and allowed to freely explore with forelimbs for up to 20-25 forelimb wall contacts. Contacts include use of a single forelimb to explore or use of both forelimbs at once. A mirror was placed behind the cylinder so that all contacts could be recorded by video camera. This test was performed during the dark cycle with low lighting. Forelimb use was scored from the video recordings and an asymmetry score was calculated by dividing usage of the impaired limb (impaired limb contact plus ½ both) by the total contacts (impaired + unimpaired + both). This test was conducted three times over the course of this experiment: once on Day 0 for baseline measurements, then twice more every two weeks following 6-OHDA injections.

Tissue Preparation – Immunohistochemistry. Following the final behavioral test, rats were deeply anesthetized with sodium pentobarbital and sacrificed by intracardial perfusion using saline followed by 4% paraformaldehyde (PFA). Brains were dissected out and placed in 4% PFA for
24 hours before being transferred into 30% sucrose. Once brains were saturated with sucrose solution they were sectioned. Coronal sections through the mesencephalon were cut at 50 µm on a sliding microtome and stored in cryoprotectant. Sections were processed for tyrosine hydroxylase (TH; catecholamine biosynthetic enzyme and marker for dopamine cells) and ionized calcium-binding adaptor molecule 1 (Iba1; pan-microglial marker) according to our routine immunohistochemical procedures (e.g. Seroogy et al., 1994; Hemmerle et al., 2014). Briefly, free-floating sections were washed in 0.1 M phosphate buffer (PB) and normal horse serum (NHS) was used to block non-specific staining. Sections were incubated in primary antibody overnight at 4°C. Primary antibodies [mouse anti-TH (1:8000), Chemicon International, Temecula, CA; or rabbit anti-Iba1 (1:2500), Wako Pure Chemical Industries, Ltd., Osaka, Japan] were diluted in 0.1 M PB with 1% NHS and 0.2% Triton-X. The following day, sections were washed in 0.1M PB, blocked with 2% NHS, and incubated with the anti-mouse IgG biotin-conjugated secondary antibody [horse anti-mouse (1:200) for TH and horse anti-rabbit (1:200) for Iba1, both from Vector Laboratories, Inc. Burlingame, CA] for 1 hour. Following the blocking step, sections were washed again in 0.1M PB, and placed in ABC peroxidase (Vector Laboratories) for 30 minutes and then washed in 50 mM Tris buffer (pH 7.5). Labeling was visualized with diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories) containing 0.3% H2O2, buffer, and DAB reagent. Following DAB step, sections were washed again in Tris buffer and mounted onto Superfrost plus microslides (VWR, Batavia, IL), dehydrated in a series of ethanol washes and coverslipped.

**Stereological Cell Counts.** Estimated cell counts and cell morphology analysis was performed for TH and Iba1 immunopositive cells in the SNpc. Cell count estimates of the lesioned SNpc were determined using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased
stereological techniques (West, 1993). Analysis was performed on an Olympus BX-51 microscope (Olympus America Inc., Melville, NY) using a CCD video camera (HV-20, Hitachi, San Jose, CA). Contours were drawn around the area of interest at 1.25X magnification and cell counting was performed at 60X. Random sample sites were determined by the software on a grid of 100 x 100. A 2-μm guard was used for each section. The extent of cell loss in the SNpc was determined by comparing all groups to the Water/Vehicle control group. Once estimated cell counts were established, the average number of cells in the right SNpc of the control group was set as 100%. Each cell count from every other animal in the study was divided by the control group cell count average, to provide a percentage of cell survival.

Microglial Morphology Analysis. Microglial cell morphology was analyzed using Image J software (NIH). To determine relative activation states of the microglia in each condition, somal area was calculated. Images of random microglia in the SNpc were taken from three sample sections for each animal at 60X magnification on an Olympus BX-51 microscope (Olympus America Inc.) using a CCD video camera (HV-20, Hitachi). These images were opened in the Image J software and the scale was set such that one pixel on the image was equal to 0.113 μm. The freehand tool was used to trace the soma of the microglial cells, following the trace, ‘Measure’ was selected from the Analyze menu. The average for each section was calculated, and then the average of the three sections was calculated to represent the average microglia soma area for that animal.

Statistical Analysis. Asymmetrical forelimb use (cylinder test) were compared for minocycline and water treated rats, receiving 6-OHDA lesion or vehicle injection using 2 x 2 (Treatment [water, minocycline]) x (Lesion [Vehicle, 6-OHDA]) repeated measure ANOVA. Microglial morphology and stereological cell counts were compared in a 2 x 2 (Treatment [water,
minocycline]) x (Lesion [Vehicle, 6-OHDA]) ANOVA. Post hoc analysis on basis of significant main effects and interactions were followed by Bonferroni post-hoc test. Data are presented as means ± SEM. Significance is considered at \( P < 0.05 \). SPSS IBM Statistics 21 was used to analyze all the data.

**Results**

*Behavior*

To determine if minocycline treatment could prevent the motor deficits caused by 6-OHDA, motor ability was assessed using the cylinder test (forelimb asymmetry test). Baseline behavior was analyzed prior to the beginning of minocycline treatment to verify that all animals started the experiment using both forelimbs equally. Following 6-OHDA lesion, the cylinder test was used to examine deficits at two and four weeks post-lesion. We ran a 2 x 2 x 3 (treatment [minocycline, water] x lesion [6-OHDA, vehicle] x time [baseline, two week, four week]) repeated measures ANOVA of cylinder test. There was a main effect of lesion \( F(1,26) = 30.38; p < 0.001 \), a main effect of time \( F(2,52) = 13.01; p < 0.001 \) (Fig. 2), and a significant interaction of lesion and time \( F(2,52) = 6.97; p < 0.01 \). Simple effects analyses revealed that a main effect of lesion existed at two weeks post-lesion, indicating animals that received 6-OHDA had increased asymmetry compared to vehicle injected animals \( F(1,26) = 30.65, p < 0.001 \). At the four weeks post-lesion, the main effect of the lesion remained, \( F(1,26) = 18.99, p < 0.001 \), and a trending treatment and lesion interaction emerged \( F(1,26) = 4.01, p = 0.055 \). Even though the interaction was not significant, analysis of the four week post-lesion time point between minocycline-treated rats did not differ regardless of lesion condition, \( F(1,27) = 2.87, p = 0.10 \).
Stereological cell counts

Stereological cell counts were used to determine the difference in dopaminergic cell loss in the SNpc between lesioned groups compared to controls. Dopaminergic cells were labeled by immunostaining tissue for TH and histological analysis was performed using unbiased stereological cell count protocols. The average estimated TH+ cell count of the right (lesion-side) SNpc of Water/Vehicle animals was converted to 100% and experimental groups are presented as percent of control values (Fig. 3). We ran a 2 x 2 ANOVA using a log transformation (treatment [water, minocycline] x lesion [vehicle, 6-OHDA]). As expected, a main effect of lesion on TH cell counts was shown F(1,18) = 54.01; p < 0.001, with lower cells counts in 6-OHDA injected animals compared to vehicle injected. There was also a trending treatment x lesion interaction F(1,18) = 3.49; p = 0.078. Even though the interaction was not significant, analysis of the TH+ cell counts, there was a significant difference between minocycline- and water-treated rats in the lesion groups, F(1,18) = 5.86, p = 0.02.
Figure 2. Assessment of motor deficits in animals treated with minocycline or water in the 6-OHDA model. The cylinder test was used to assess motor deficits following 6-OHDA injections. At both post-lesion time points, animals injected with 6-OHDA performed significantly worse than animals that received vehicle injections. However, at the four-week time point, there was a trending interaction between drug x lesion. Data presented as a mean +/- SEM. *P<0.001 for vehicle-treated vs 6-OHDA-treated groups. n=8 for Water/6-OHDA, Minocycline/Vehicle, Minocycline/6-OHDA; n = 6 for Water/Vehicle.
Figure 3. **TH+ stereological cell counts in the SNpc of animals treated with minocycline or water in the 6-OHDA model**

**A.** TH+ stereological cell counts in the SNpc of animals receiving minocycline or regular water. Both lesion groups lost a significant number of cells compared to non-lesion groups. An interaction of drug x lesion exists. **B.** Representative (4x magnification) images of TH+ immunostaining in the SNpc. Data presented as a mean ± SEM. *P < 0.001 for vehicle-treated vs 6-OHDA treated groups; n = 7 for Minocycline/6-OHDA; n = 6 for Water/6-OHDA; n = 5 for Minocycline/Vehicle, n=4 for Water/Vehicle.
Microglia Somal Area

Ventral midbrain sections were processed immunohistochemically to detect Iba-1 in order to establish relative differences in somal area of microglia in the SNpc. Somal area did not differ in the injected side (right side) between lesioned conditions or the water and minocycline treatment groups (Fig. 4).
Figure 4. Microglial soma area in the SNpc of animals treated with minocycline or water in the 6-OHDA model. A. Microglia somal area was measured from Iba-1-labeled microglia in the SNpc. No differences were detected in somal area between groups. B. Representative images (60x magnification) of microglial cells in the SNpc. Data presented as mean ± SEM. n = 4/group.
Discussion

This experiment sought to increase understanding of inflammation in the 6-OHDA model of PD by treating rats with an anti-inflammatory agent that prevents microglial activation. To inhibit microglial activation, minocycline was administered to half of the four groups for the entirety of the experiment (6+ weeks). The drug is soluble and was dissolved in the drinking water for administration in order to reduce stress and mimic a potential clinical application. As minocycline dosage was calculated per cage (two animals) we were not able to determine the exact dosage per rat. As mentioned in the methods, we proceeded with the experiment assuming that both rats in each cage drank the same amount of minocycline solution (~30 ml/day). This volume of solution is identical to the volume consumed per cage for water treated rats. The data indicate that although very strong statistical trends exist, minocycline did not statistically lessen the severity of asymmetrical forelimb use in the cylinder behavioral assay, nor did it reduce the loss dopaminergic neuron phenotype in the SNpc. Of note, however, minocycline treated rats (both lesion and non-lesion) performed similarly in the cylinder test at four weeks post-lesion.

Microglial activation is reported in the 6-OHDA rat model of PD, though these reports of activation are recorded much closer to the time of lesioning. Maia et al. (2012) reported microglial activation in the SN starting at 14 days following 6-OHDA intrastriatal injections of 5 µg of 6-OHDA, and up to 28 days, though activation was peaked at 14 days. Additionally, activation by 28 days was significant lower than 21 days. By 56 days post-lesion, microglial activation is no longer observed (Maia et al., 2012). This work indicates that at some point between 28 and 56 days, the microglial activation subsided. We may have not been able to observe activated microglia due to the timeline of our experiment and second, due to a smaller dose of 6-ODHA (5 µg total injection vs 10 µg in Maia et al.). Further, it has been verified that microglial activation
precedes dopaminergic cell loss in the striatal 6-OHDA model, suggesting that microglia become reactive as soon as a problem is detected, not after cells begin to die (Cicchetti et al., 2002; Depino et al., 2003). To better understand the temporal changes of microglial activation in our model and paradigm, analysis of microglia at multiple time points post-lesion would be required.

To understand the role of microglial activation in the 6-OHDA PD model, we treated animals with a microglia activation inhibitor and analyzed microglia somal area as an indicator of activation state. Microglia somal area was not significantly different on the lesion side between groups at four weeks post-lesion, regardless of treatment or lesion. This parameter, however, is a relatively superficial method for analysis of inflammation. Other methods are more sensitive and are used to better evaluate the inflammatory profile in the brain, such as stereological cell counts of microglia in the SNpc, relative changes in cytokine expression and levels (eg. IL-6, TNFα), phagocytotic activity, and morphological analysis of branching complexity and branch endpoints (Nakamura et al., 1999; Jesudasan et al., 2014; Torres-Platas et al., 2014).

Minocycline is shown to be neuroprotective in many models of neurodegeneration and brain injury (Machado et al., 2011). In neurotoxic PD models, both MPTP and 6-OHDA, minocycline has been neuroprotective over the dopaminergic pathway (Du et al., 2001; He et al., 2001; Wu et al., 2002). We may not have observed any differences in activation state or seen a benefit of minocycline treatment due to delivery method. We dissolved minocycline in the drinking water at a concentration of 30-40 mg/kg, which will inhibit microglial activation (Hinwood et al., 2012), however other groups have had success by administering the drug at 45 mg/kg or better with intraperitoneal (i.p) injections or by delivering the drug locally at a low concentration (He et al., 2001; Xue et al., 2010).
Benefits from use of anti-inflammatory treatment is observed in other PD animal models as well, however in those paradigms that administered drugs orally, doses were much higher. Valera et al. (2015) just reported amelioration of dopaminergic fiber loss in the striatum, and reduction in microgliosis in the mouse AAV-α-synuclein model of PD using the anti-inflammatory Lenalidomide at a dose of 100 mg/kg. Additionally, they observed a reduction in behavioral deficits. Simvastatin, naloxone, triptolide and other anti-inflammatories have demonstrated the ability to prevent LPS-induced microglial activation and dopaminergic cell loss in the nigra following local LPS injection (Mochado et al., 2011). Delivery methods for these drugs included subcutaneous (s.c.) and i.p. injections.

Consistent with previous experiments utilizing this model, animals that received a 6-OHDA lesion demonstrated significant behavioral deficits in left forepaw use at two and four weeks post-lesion compared to vehicle controls, as well as significantly reduced TH+ cell counts in the SNpc (i.e., Hemmerle et al., 2014). Our experiment did not provide statistical evidence of minocycline’s ability to enhance use of the impaired forelimb. Considering the group sizes for behavioral testing was limited to 6-8 rats, high variability may have prevented us from drawing accurate conclusions from the test. Data supporting the ability of minocycline to prevent behavioral deficits in the 6-OHDA model do exist, but are not overwhelming; Quintero et al. (2006) used the 6-OHDA lesion model, injecting in the medial forebrain bundle, and treated animals either before or after lesion with IP injections of minocycline. Both treatments suppressed apomorphine-induced rotations at two weeks post-lesion, but by four weeks post-lesion neither treatment groups exhibited suppressed rotation, suggesting that minocycline treatment was only able to protect the nigrostriatal system temporarily. Using other anti-inflammatory substances with the 6-OHDA model, Fu et al. (2015) shows that β-Hydroxybutyric acid decreases apomorphine-induced
rotational behavior in a dose-dependent fashion. The mice in this study began daily infusions of drug three days prior to LPS injection, and for 21 days following the injection. These results suggest that a delicate balance exists between the PD model used, the dose of minocycline, the treatment paradigm, and the experiment timeline. Altering our experiment to examine the state of microglial activation and dopaminergic degeneration at different time points may provide more insight into the relationship between the two.

Overall, this experiment did not provide statistically conclusive results on the ability of minocycline to be neuroprotective in the intrastriatal 6-OHDA model of PD. Behavioral analysis via the cylinder test showed a favorable effect of minocycline treatment in 6-OHDA lesion animals, though it was only a trending interaction. Similarly, histological analysis provided some promising data, though, again, short of significance. To strengthen this study, increases in group number are necessary to boost behavioral data as well as histological. With regard to microglial activation, alterations in experimental design, primarily in regard to timeline, may be able to shed more light on this topic. Microglial activation in this model may be short-lived and unable to be detected at four weeks post-lesion. Examining the inflammatory state of the tissue at a time closer to the lesion surgery would be preferable.
References


Chapter 4

Role of increased glucocorticoid signaling in the 6-OHDA rat model of Parkinson’s disease.

Abstract

Parkinson’s disease (PD) patients not only suffer from the motor deficits associated with PD, but also with a number of non-motor issues. Depression, diagnosed in up to 50% of PD patients, is among this list of non-motor symptoms. Considering the high comorbidity of these two disorders, they may have common underlying neuropathological processes. We have employed the combined chronic variable stress (CVS)/6-OHDA model of depression and PD to examine the role of the glucocorticoid receptor (GR). CVS reliable produces both physiological and behavioral aspects of depression in rodents, to include reduced body weight and elevated levels of circulating glucocorticoids. Using a GR-expressing lentivirus, we upregulated GR in the substantia nigra pars compacta (SNpc), so as to increase GR signaling in the SNpc under chronic stress conditions. Adult male Sprague Dawley rats received an intranigral injection of either the GR-overexpressing lentivirus (pLV-GR) or a control GFP-expressing lentivirus (pLV-eGFP), then underwent a CVS-flanked 6-OHDA experimental paradigm. Motor deficits were examined using the forelimb asymmetry test (cylinder test) at two and four weeks post-lesion. Lesion (dopaminergic cell loss) was measured via immunohistochemistry for tyrosine hydroxylase (TH). Analysis of behavior revealed that, overall, lesion animals exhibited increased asymmetry compared to non-lesion animals, and that pLV-GR animals performed worse than control animals within the lesion groups. Stereological cell counts revealed that lesion animals displayed significantly less TH+ cells in the SNpc than non-lesion groups; within the lesion groups, CVS animals had a trend toward significantly TH+ cell loss, regardless of virus.
Introduction

Depression is a non-motor symptom suffered by up to 50% of PD patients, and contributes significantly to decreased quality of life (Rickards, 2005; Kuopio et al., 2000). Considering the high comorbidity between the two disorders, a common underlying pathway may be involved, though it is unclear the exact mechanism that links the two, or if/how depression actually contributes to the degeneration of the nigrostriatal pathway (Chaudhuri and Schapira, 2009; Aarsland et al., 2011; Hemmerle et al., 2012).

One characteristic of depression is elevated glucocorticoid levels (Piwowarska et al., 2011; Moreira et al., 2015). Glucocorticoids are a class of steroid hormones that manage the stress response, and the main glucocorticoid in humans is cortisol (corticosterone in rodents) (Pelt, 2011). This hypersecretion in hormone is due to hyperactivity of the HPA axis. Hyperactive dysfunction of the HPA axis can be caused by physiological or psychological stressors, as demonstrated in human and animal studies (Sapolsky, 1996; Krishnan and Nestler, 2008; Zhu et al., 2014). Chronically elevated levels of cortisol have been shown to be damaging to the hippocampus and prefrontal cortex, which play an important role in stress regulation (Sapolsky et al., 1990; Starkman et al., 1992; Brown et al., 2005). Glucocorticoids exert their effect via the glucocorticoid receptor (GR), which is found all over the body and brain, including the substantia nigra (SN) (Härfstrand et al., 1986; Ahima and Harlan, 1990).

Previously, Hemmerle et al. (2012) demonstrated in a combined chronic variable stress and 6-OHDA model of PD that chronic stress exacerbates behavioral deficits and dopaminergic cell loss in the neurotoxic model. These results suggested that elevated glucocorticoids, and glucocorticoid signaling via their receptor (GR), made the dopaminergic neurons more vulnerable to the toxic lesion. In the CVS animal model of stress and depression, animals receive a variety of
stressors in a random order over a long period of time (14 days at a time in our model). These stressors are both physiogenic (i.e. hypoxia) and psychogenic (i.e. restraint). This model reliably results in elevations of adrenocorticotropic hormone and corticosterone, and adrenal hypertrophy (Herman et al., 1995), and produces rodent behavioral symptoms that correlate to those of human depression, such as anhedonia and weight loss (Papp et al., 1993; Willner, 2005).

To determine if increased glucocorticoid signaling in the SN was the cause of lesion exacerbation in the combined CVS/6-OHDA rat model of PD, we utilized an overexpression lentivirus for the glucocorticoid receptor (GR). The construct for this receptor included a neuronal-specific promotor, so as to only upregulate GR in the neurons of the SNpc. Following viral injection into the nigra, animals were exposed to the CVS/6-OHDA model of PD.

Methods

Animals. Adult male Sprague Dawley rats [250 - 400g; Harlan Laboratories (Indianapolis, IN)] were used in this study (n = 4-8/group). Animals were housed in standard shoebox cages, 2 per cage, in standard housing conditions (12 hr on/off light cycle) and had access to food and water ad libitum. Treatment of animals was in accordance with the University of Cincinnati Institutional Animal Care and Use Committee Guidelines. Animals were randomly divided into eight weight-matched groups: four groups received intranigral injection of glucocorticoid receptor lentivirus (pLV-GR) for GR overexpression; four groups received intranigral injection of control green fluorescent protein lentivirus (pLV-eGFP). Two groups in each treatment group were exposed to CVS, and two groups served as non-stressed controls. Half of the CVS groups also received 6-OHDA striatal injections, while the other half received vehicle striatal injections. The timeline of the study is provided in Figure 1.
Figure 1. Experimental timeline. Animals completed a baseline cylinder test on Day 0. On Day 1, all animals received an intranigral injection of pLV-GR or control pLV-EGFP. Two weeks after injection, a cylinder test was performed to ensure that intranigral injections didn’t damage dopaminergic cells in the SN. The first two weeks of CVS began on Day 15 and after it was completed lesion surgeries (6-OHDA injections) were performed on Day 30. After recovery time, the second two weeks of CVS began on Day 32, ending on Day 45. The two-week post-lesion cylinder test was performed on Day 45. The four-week post-lesion cylinder test was performed on Day 59, and animals were sacrificed on Day 60.
**Lentivirus Injections.** On Day 1, intranigral injections of either PLV-GR or ePLV-EGFP were administered. PLV-GR is an overexpression vector for the glucocorticoid receptor. In preparation for nigral injections, animals were anesthetized (4.35 mg/kg ketamine, 0.65 mg/kg xylazine) and placed in a stereotaxic apparatus. A single injection of lentivirus was made into the right SN at coordinates AP: -4.9 mm, ML: -2.2 mm, and DV: -7.0 mm, according to the rat brain atlas of Paxinos and Watson (2007). For each injection, AP and ML measurements were taken from bregma and DV measurements were taken from dura. A 5-µl Hamilton syringe (Hamilton Company, Reno, NV) was lowered into the SN and allowed to equilibrate for 5 minutes. The 2-µl injection was administered over 10 minutes (0.2 µl/min), and the needle remained in place for another 5 minutes before being retracted slowly.

**GR and eGFP Lentiviral Expression Construct.** The GFP-containing plasmid (pLV-GFP) was derived from corresponding components of DNA being placed under control of elongation factor-1α (EF1α). This plasmid served as a control. The pLV-GR construct was made by insertion of full-length mouse GR cDNA (provided by L. Muglia, Cincinnati, OH) into the BamHI site of pLenti-III-EF1α (Applied Biological Materials, Richmond, BC, Canada), creating an mGR expression vector, thereby also under control of EF1α (Fig. 2A). Once plasmids were constructed, lentivirus (LV) vectors were packaged at the Hope Center Viral Vector Core (Washington University, St. Louis, MO). Packaging occurred with three helper plasmids (pMD-Lg, pCMV-G, and RSV-Rev) in 293-T cells into replication deficient virions. The lentiviral construct is a third-generation vector, containing a 5’ self-inactivating (SIN) element to prevent replication, 3’ and 5’ long-terminal repeats (LTR), and woodchuck post-transcriptional regulatory element (WPRE) to enhance protein expression. Of note, due to the size of the GR sequence, it could not be transfected within the GFP cassette.
The ability of the pLV-GR to express GR in vitro was shown by Laryea et al. (2013) using Chinese hamster ovarian cells (CHO)-K1 cells (ATCC, CCL-61). Both the pLV-GFP and the pLV-GR plasmids were gifts from Drs. Jessica McKIveen and Jim Herman, and Dr. Lou Muglia, University of Cincinnati). All experimental procedures were approved by the University of Cincinnati Institutional BioSafety Committee.
**Figure 2.** Diagram of pLV-eGFP and pLV-GR constructs with EF1α promoters. (Modified from Laryea et al., 2013)
**Chronic Variable Stress (CVS) Regimen.** To mimic stress and depression physiology, the CVS protocol was employed. The following stressors were utilized in the regimen: cold exposure (1h in a 4°C room), restraint (1h in plastic restraint tubes), vibration (1h on laboratory shaker), hypoxia (9% oxygen, 30 min), cold swim (10 min in 16-18°C water), warm swim (20 min in 31-33°C water, crowding (5-6 rats/cage, overnight), isolation (1 rat/cage, overnight). Following 6-OHDA lesion surgery, swim stressors were not administered as animal mobility was likely altered. Stressors occurred twice a day (morning and afternoon) for 14 consecutive days in a random order to prevent habituation (Table 1) (Herman et al., 1995; Willner, 2005). Overnight stressors were administered unevenly throughout the regimen. Body weights were measured on a weekly basis to monitor the effectiveness of the regimen. This model of unpredictable stressors increases baseline corticosterone (a glucocorticoid) and ACTH levels, both hormones regulate stress responses. Additionally, adrenal hypertrophy occurs and CRH levels increase (Herman et al., 1995; Wang et al., 2010). Chronic variable stress has been reported to increase anxiety-like behaviors in adult rats as well (Bondi et al., 2008). Non-stressed animals served as CVS controls.
### Table 1. Chronic Variable Stress Regimen

| Day 1 | AM: Hypoxia  
| PM: Shaker  
| ON: Crowding |
| Day 2 | AM: Warm Swim  
| PM: Cold Room |
| Day 3 | AM: Shaker  
| PM: Cold Swim |
| Day 4 | AM: Shaker  
| PM: Cold Swim  
| ON: Isolation |
| Day 5 | AM: Cold Room  
| PM: Cold Swim |
| Day 6 | AM: Shaker  
| PM: Hypoxia  
| ON: Crowding |
| Day 7 | AM: Cold Room  
| PM: Hypoxia |

| Day 8 | AM: Cold Swim  
| PM: Warm Swim  
| ON: Isolation |
| Day 9 | AM: Cold Swim  
| PM: Cold Room |
| Day 10 | AM: Warm Swim  
| PM: Shaker |
| Day 11 | AM: Cold Room  
| PM: Shaker  
| ON: Crowding |
| Day 12 | AM: Warm Swim  
| PM: Hypoxia |
| Day 13 | AM: Hypoxia  
| PM: Cold Swim  
| ON: Isolation |
| Day 14 | AM: Hypoxia  
| PM: Cold Room |
| Day 15 | AM: Shaker  
| PM: Restraint |
| Day 16 | AM: Hypoxia  
| PM: Cold Room |
| Day 17 | AM: Cold Room  
| PM: Hypoxia  
| ON: Crowding |
| Day 18 | AM: Shaker  
| PM: Restraint |
| Day 19 | AM: Hypoxia  
| PM: Cold Room  
| ON: Isolation |
| Day 20 | AM: Restraint  
| PM: Hypoxia |
| Day 21 | AM: Cold Room  
| PM: Restraint |
| Day 22 | AM: Cold Room  
| PM: Shaker  
| ON: Crowding |
| Day 23 | AM: Shaker  
| PM: Hypoxia  
| ON: Isolation |
| Day 24 | AM: Restraint  
| PM: Shaker |
| Day 25 | AM: Hypoxia  
| PM: Cold Room  
| ON: Crowding |
| Day 26 | AM: Shaker  
| PM: Restraint  
| ON: Isolation |
| Day 27 | AM: Cold Room  
| PM: Hypoxia |
| Day 28 | AM: Shaker  
| PM: Restraint |

Schedule of CVS that was followed for this experiment. Days 1-14 were completed prior to 6-OHDA lesion; days 15-28 were began two days after 6-OHDA injections to allow for surgery recovery.
Partial 6-OHDA Lesion. Injection of 6-OHDA into the striatum creates a progressive degeneration of the nigrostriatal pathway (Kirik et al., 1998; Hemmerle et al., 2014). In preparation for striatal 6-OHDA injections, animals were anesthetized (4.35 mg/kg ketamine, 0.65 mg/kg xylazine) and placed in a stereotaxic apparatus. Two unilateral injections of 6-OHDA (2.5 µg each in 2 µl saline + 0.2% ascorbic acid) or vehicle (saline + ascorbic acid) were made into the right striatum at the following coordinates according to the rat brain atlas of Paxinos and Watson (2007): AP: +1.6, ML: -2.4, DV: -4.2 and AP: +0.2, ML: -2.6, DV: -7.0 (Paxinos and Watson, 2007). For each injection, the AP and ML measurements were taken from bregma and DV measurements were taken from the skull. A 5-µl Hamilton syringe (Hamilton Company, Reno, NV) was lowered into the striatum and allowed to equilibrate for 5 minutes. The 2-µl injection was administered over 10 minutes (0.2 µl/min), and the needle remained in place for another 5 minutes before being removed slowly.

Forelimb-use Asymmetry. Motor impairment was assessed in all groups via the forelimb-use asymmetry test (cylinder test). The test was performed as previously described (Schallert, 2006; Hemmerle et al., 2014). Briefly, rats were placed in the center of a Plexiglass cylinder and allowed to freely explore with forelimbs for up to 20-25 forelimb wall contacts. Contacts include use of a single forelimb to explore or use of both forelimbs at once. A mirror was placed behind the cylinder so that all contacts could be recorded by video camera. This test was performed during the dark cycle with low lighting. Forelimb use was scored from the video recordings and an asymmetry score was calculated by dividing usage of the impaired limb (impaired limb contact plus ½ both) by the total contacts (impaired + unimpaired + both). This test was conducted three times over the course of this experiment: once on Day 0 for baseline measurements, then twice more every two weeks following 6-OHDA injections.
Tissue Preparation – Immunohistochemistry. Following the final behavioral test, rats were deeply anesthetized with sodium pentobarbital and sacrificed by intracardial perfusion using saline followed by 4% paraformaldehyde + 0.1% Glutaraldehyde (PFA+Glu). Brains were dissected out and placed in 4% PFA for 24 hours before being transferred into 30% sucrose. Once brains were saturated with sucrose solution they were sectioned. Coronal sections through the mesencephalon were cut at 50 µm on a sliding microtome and stored in cryoprotectant. Sections were processed for tyrosine hydroxylase (TH; catecholamine biosynthetic enzyme and marker for dopamine cells) and glucocorticoid receptor (GR) according to our routine immunohistochemical procedures (e.g. Seroogy et al., 1994; Hemmerle et al., 2014). Briefly, free-floating sections were washed in 0.1 M phosphate buffer (PB) and normal horse serum (NHS) was used to block non-specific staining. Sections were incubated in primary antibody overnight at 4°C. Primary antibodies [mouse anti-TH (1:8000), Chemicon International, Temecula, CA; or rabbit anti-GR (1:1000), M-20 Santa-Cruz Biotechnology, Inc., Dallas, TX] were diluted in 0.1 M PB with 1% NHS and 0.2% Triton-X. The following day, sections were washed in 0.1M PB, blocked with 2% NHS, and incubated with the anti-mouse IgG biotin-conjugated secondary antibody [horse anti-mouse (1:200) for TH and horse anti-rabbit (1:200) for GR, both from Vector Laboratories, Inc. Burlingame, CA] for 1 hour. Following the blocking step, sections were washed again in 0.1M PB, and placed in ABC peroxidase (Vector Laboratories) for 30 minutes and then washed in 50 mM Tris buffer (pH 7.5). Labeling was visualized with diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories) containing 0.3% H₂O₂, buffer, and DAB reagent. Following DAB step, sections were washed again in Tris buffer and mounted onto Superfrost plus microslides (VWR, Batavia, IL), dehydrated in a series of ethanol washes and coverslipped.
To visualize injection of pLV-eGFP virus and TH co-localization, sections were washed in 0.1M PB, blocked in NHS and then incubated overnight with TH primary antibody were diluted in 0.1 M PB with 1% NHS and 0.2% Triton-X. The next day, section were washed with PB and incubated with anti-rabbit Alexa 594 (Invitrogen, Carlsbad, CA). Following secondary incubation, sections were washed with PB again and then mounted onto Superfrost plus microslides (VWR, Batavia, IL), coverslipped and viewed on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany) using an Evolution VF camera and Q-Cam software.

**Stereological Cell Counts.** Estimated cell counts and cell morphology analysis was performed for TH and Iba1 immunopositive cells in the SNpc. Cell count estimates of the lesioned SNpc were determined using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased stereological techniques (West, 1993). Analysis was performed on an Olympus BX-51 microscope (Olympus America Inc., Melville, NY) using a CCD video camera (HV-20, Hitachi, San Jose, CA). Contours were drawn around the area of interest at 1.25X magnification and cell counting was performed at 60X. Random sample sites were determined by the software on a grid of 100 x 100. A 2-µm guard was used for each section. The extent of cell loss in the SNpc was determined by comparing all groups to the eGFP/Control/Vehicle control group. Once estimated cell counts were established, the average number of cells in the right SNpc of the control group was set as 100%. Each cell count from every other animal in the study was divided by the control group cell count average, to provide a percentage of cell survival.

**Statistical Analysis.** Animal weights and asymmetrical forelimb use (cylinder test) were compared for pLV-GR and pLV-EGFP injected rats, receiving either CVS or not, and receiving 6-OHDA lesion or vehicle injection using 2 x 2 x 2 (Virus [pLV-EGFP, pLV-GR]) x (Stress [control, CVS]) x (Lesion [Vehicle, 6-OHDA]) three-way ANOVA. Post hoc analyses on basis of significant main
effects and interactions were followed by Bonferroni post-hoc test. Data are presented as means ± SEM. Significance is considered at $P < 0.05$. SPSS IBM Statistics 21 was used to analyze the data.

Results

Verification of viral transfection in SNpc

Successful transduction, or “hits”, of viral vectors was visually confirmed using GR immunohistochemistry. A clear increased density of DAB staining is seen in the injected SNpc compared to the non-injected side (Fig. 3A). Transduction by the pLV-eGFP into dopaminergic neurons was confirmed using double-fluorescence of TH and GFP (Fig. 3B). The TH cells are labeled in red and cells transfected with the virus are green. Both GR and GFP control constructs transduced approximately, or made hits in, 50% of test subjects by qualitative verification (Fig. 3). Using the double-fluorescence, we observed that approximately 20% of TH cells also co-localized with the virus. Only animals with positive hits were included in analysis. Evaluation of experimental outcomes in “missed” animals is not complete.
Figure 3. Verification of pLV-GR overexpression in TH+ cells of the SNpc. A. Densely labeled cells verify viral expression of pLV-GR. B. Representative image of verification of pLV-eGFP co-localization with dopaminergic cells (indicated with arrows) was observed in the SNpc of the right injected side.
Animal Weights

Animal body weights were measured each week to determine the efficacy of the CVS regimen. All eight groups were weight-matched prior to the start of the experiment. The CVS regimen was administered two weeks after the lentiviral injections and flanked the 6-OHDA surgeries for two weeks each. As expected, stress effectively attenuated weight gain of the CVS animals, as shown by a 2 x 2 x 2 x 9 repeated measure ANOVA (virus [pLV-eGFP, pLV-GR] x stress [control, CVS] x lesion [vehicle, 6-OHDA] x time [weeks 1-9]), $F(1,24) = 18.02, p < 0.001$ (Fig. 4). Simple effects analysis showed that stressed animals weighed less than non-stressed animals starting at week 4 and continuing until the end of the experiment on week 9 (Week 4 ($F(1,30) = 23.92), p < 0.001$; Week 5 ($F(1,30) = 27.17), p < 0.001$; Week 6 ($F(1,30) = 21.72), p < 0.001$; Week 7 ($F(1,30) = 22.32), p < 0.001$; Week 8 ($F(1,30) = 30.09), p < 0.001$; Week 9 ($F(1,30) = 15.45), p < 0.001$).
Figure 4. Weights of animals exposed to stress-induced depression concomitant to the 6-OHDA lesion. Weights of control and CVS animals injected with either pLV-GFP or pLV-GR, and exposed to stressors flanking the 6-OHDA lesion. No differences existed between groups until after one week of CVS. After the first week of CVS, a significant difference existed between stressed and non-stressed groups through to the end of the experiment. Data presented as a mean +/- SEM. *p < 0.001 for CVS vs non-CVS control groups. n = 7 for GR/CVS/Vehicle; n = 6 for GFP/CVS/6-OHDA; n = 5 for GFP/Con/Vehicle, GFP/Con/6-OHDA, GFP/CVS/Vehicle, GR/Con/Vehicle and GR/Con/6-OHDA; n = 4 GR/CVS/6-OHDA.
**Cylinder Test**

To determine if GR upregulation in the SN would exacerbate behavioral deficits in the combined CVS/6-OHDA model of PD, motor ability was assessed using the cylinder test (forelimb asymmetry test). Baseline behavior was recorded prior to lentivirus surgeries as well as before the start of CVS and prior to 6-OHDA injections. No groups displayed a significant difference in motor behavior at the baseline, pre-CVS and pre-6-OHDA cylinder tests (data not shown). A repeated measure $2 \times 2 \times 2 \times 5$, virus [pLV-eGFP, pLV-GR] x stress [control, CVS] x lesion [vehicle x 6-OHDA] x time [baseline, pre-CVS, pre-lesion, two weeks, four week] ANOVA of behavioral tests, there was a main effect of lesion $F(1,33) = 20.07; p < 0.001$, and a main effect of time $F(4,132) = 17.09; p < 0.001$, indicated a worsening of motor behavior in animals receiving 6-OHDA over time. There was also a significant interaction of lesion x time $F(4,132) = 13.17; p < 0.001$, and virus x lesion ($F(1,33) = 4.71; p < 0.05$) on cylinder test scores for asymmetry (Fig. 5).

Simple effects analysis showed that there was a significant difference between lesioned and unlesioned groups at both two and four weeks post-lesion (Two Week Cylinder ($F(1,37) = 27.73), p < 0.001$; Four Week Cylinder ($F(1,37)= 35.70); p < 0.001$), as 6-OHDA animals displayed increased motor deficits compared to vehicle animals (Fig. 5). A significant interaction of virus x lesion was also found at the four week cylinder test ($F(1,37) = 5.55; p < 0.05$). Further analysis revealed that while all lesion animals performed worse compared to non-lesioned animals, the deficit was much greater in pLV-GR injected rats compared to pLV-eGFP.
Figure 5. Assessment of motor deficits in animals treated with pLV-GR in the CVS/6-OHDA model. The cylinder test was used to assess motor deficits following CVS flanking 6-OHDA lesion in all groups. In both post-lesion cylinder tests, 6-OHDA-injected animals performed worse (more asymmetrical) than vehicle-injected animals. Additionally, at four weeks post-lesion, a significant interaction of virus x lesion occurred: GFP/Con/Vehicle animals performed worse than GR/Con/Vehicle animals; and GR/CVS/6-OHDA animals performed worse than GFP/CVS/6-OHDA animals. Data presented as a mean +/- SEM. *P < 0.001 for CVS vs non-CVS control groups. n = 7 for GR/CVS/Vehicle; n = 6 for GFP/CVS/6-OHDA; n = 5 for GFP/Con/Vehicle, GFP/CVS/Vehicle, and GR/Con/6-OHDA; n = 4 for GFP/Con/6-OHDA, GR/Con/Vehicle, and GR/CVS/6-OHDA.
**TH Cell Counts**

Stereological cell counts were used to determine the differences in dopaminergic cell loss in the SNpc among the groups. Dopaminergic cells were labeled by immunostaining nigral sections for TH and histological analysis was performed using unbiased stereological cell count protocols. The average estimated TH+ cell count of the right (lesion-side) SNpc of GFP/Con/Vehicle animals served as 100% and all other cell counts were divided by that value to produce a percent of control. We ran a 2 x 2 x 2 ANOVA using a log transformation (lentivirus [pLV-eGFP, pLV-GR] x stress [control, CVS] x lesion [vehicle, 6-OHDA]). Results showed a main effect of lesion $F(1,22)=47.66; p < 0.001$, as cell counts were lower in all lesioned animals compared to non-lesioned. A significant interaction of stress x lesion $F(1,22)=6.42; p < 0.05$, showing TH+ cell counts were significantly lower in animals that received a 6-OHDA lesion vs vehicle (Fig. 6). Simple effects analysis reveals that in lesioned animals, cell counts in CVS animals were significantly lower than counts in non-CVS animals ($p < 0.05$).
Figure 6. TH+ stereological cell counts in the SNpc of animals treated with pLV-GR in the CVS/6-OHDA model. A. TH+ stereological cell counts in the SNpc of animals receiving GFP control lentivirus or GR overexpression lentivirus with or without CVS flanking the lesion. All lesion groups lost a significant number of cells compared to non-lesion groups. An interaction of stress x lesion exists as well, revealing that lesion animals that received CVS had more TH+ cells loss compared to non-CVS animals, regardless of viral injection. Data presented as a mean ± SEM. *P < 0.001 for vehicle-injected vs 6-OHDA injected groups. B. Representative images of TH+ immunostaining in the SNpc for the various experimental groups. n = 5 for GFP/CVS/6-OHDA; n = 4 for GFP/Con/6-OHDA, GR/Con/6-OHDA, GR/CVS/Vehicle and GR/CVS/6-OHDA; n = 3 for GFP/Con/Vehicle, GFP/CVS/Vehicle and GR/Con/Vehicle.
Discussion

In the present study, we hypothesized that CVS, in combination with GR overexpression in the nigra and 6-OHDA would exacerbate the loss of TH+ cells in the SNpc. To verify efficacy of CVS, animals were weighed each week. After one week of CVS, there was a significant difference between CVS and non-CVS rats, as CVS rats weighed less and gained weight much slower over the rest of the experiment, even after cessation of stressors. This indicates long-term physiological changes as a result of chronic stress, attesting to the efficacy of the present CVS regimen. Additionally, after tissue was processed for immunohistochemistry, GR antibody was used to label the receptor in the ventral midbrain so upregulation of the protein could be visualized. The virus was considered to have successfully transfected when cells in the SNpc were densely labeled. Successful transfections were variable, ranging from labeled cells throughout the SN to cells more concentrated to the lateral SNpc. Overall, successful transfection could be visualized in about 50% of all injected animals, but not quantified, making for a large range of transfection efficacy within rats that were kept in the study. Though expression of green fluorescent protein (GFP) (via pLV-eGFP) can be toxic to neurons (Klein et al., 2006), we did not observe a negative effect of GFP expression in our groups. The variability and number of factors likely contributed to high variability in outcome measures.

Behavioral analysis showed that at the two-week post-lesion cylinder test, all lesioned animals displayed increased asymmetry compared to non-lesioned animals. However, at the four-week post-lesion cylinder test, an unexpected difference developed between pLV-GFP and pLV-GR treated animals in the non-lesion groups. Despite the significant difference between the averages of these groups, all non-lesion animal scores were within two standard errors of the “normal” score of 50% impaired forelimb use. Also, high variability is common in behavioral tests
and likely contributed to the differences observed. The maximum sample size of groups for the cylinder tests was an n of 7; a larger group size would probably bring the average scores of all of the non-lesion groups closer to the unaffected average score of 50%, as seen previously in this paradigm (Hemmerle et al., 2014). The results of behavior for lesion animals at the four-week post-lesion time point reveal different conclusions; within the CVS/6-OHDA groups, pLV-GR overexpressing animals had lower left forepaw use on average, but this different was not significant ($p = 0.063$). If the pLV-GR animals did perform worse than pLV-GFP animals, this could suggest that increased glucocorticoid signaling in dopamine neurons of the nigra rendered the cells more vulnerable to behavioral dysfunction in the 6-OHDA model. However, these group sizes were too small due to viral efficacy issues and ability to verify transfection to draw solid conclusions.

Dopaminergic cells counts were analyzed using unbiased stereology to calculate estimated number of TH+ cells in the SNpc at four weeks post-lesion. In non-lesion groups, CVS animals exhibited more TH+ cells in the SNpc than non-CVS animals. In lesion groups, CVS animals demonstrated less labeled cells than non-CVS groups. An explanation for the observed differences in the non-lesion groups may be due to small sample size. In the lesion groups, the CVS animals were trending toward significantly lower cell counts than the non-CVS groups, and although the GR transfection did not make a difference, this finding almost supports previous work by Hemmerle et al. (2014) showing that CVS exacerbates TH nigral degeneration in the 6-OHDA rat lesion model.

Depression is a common psychiatric symptom in PD patients, sometimes preceding PD diagnosis, but more prevalent in an advanced disease state, and contributing to the overall decrease in quality of life (Weintraub, 2004; Chaundhuri et al., 2005; Pålhagen et al., 2008). Unfortunately,
depression often goes untreated due to not being recognized or being underreported, as patients may be embarrassed to report depression-like symptoms (Richard and Kurlan, 1997; Kuopio et al., 2000). This results in a mere 26% of PD patients being treated with antidepressants compared to almost 50% of patients that may actually suffer (Yamamoto, 2001). This may contribute to the association between depression and greater PD severity and motor deficits (Papapetropoulos et al., 2006). Ultimately, depression is not associated with age of onset or development of motor deficits (Klotsche et al., 2011), and therefore a clear understanding of the relationship between depression and PD has not been developed. Our results trend toward suggesting the role of stress in exacerbating lesion size in the 6-OHDA model of PD, however it does not suggest that the central mechanism is glucocorticoid signaling in the dopamine neurons. This, as mentioned above, may be due to small samples sizes, which were a result of unpredictable viral transfection – only 50% of animals showed any transfection; within transfected animals it was not possible to ensure consistent efficiency of transfection across groups. As tissue was analyzed at the end of the experiment, lesion animals may have lost cells that had been transfected, and those could not be visualized at four weeks post-lesion.

Another mechanism to consider would be GR signaling in microglia in the SNpc. Microglia also express GR, which has been shown to exert both anti- and pro-inflammatory effects in the CNS. Morale et al. (2004) showed that GR-deficient mice (in both neurons and microglia) have exacerbated dopaminergic cell loss following MPTP treatment. Additionally, these transgenic mice also had three- to four-fold higher nitrite levels compared to wild-type mice (Morale et al., 2004). However, chronic stress, which stimulates GR signaling has resulted in activate, or pro-inflammatory, microglia in several stress paradigms (reviewed in Walkera et al., 2013). Contrasting finding such as these highlight the complex role microglia play, both “good”
neurorestorative roles and “bad” neurotoxic roles, depending on their microenvironment and the stimulus they are receiving (Tang and Le, 2014). These studies also show the complex role of GR on microglia, though it seems that chronic stress, and thus chronic GR stimulation, will induce a pro-inflammatory state. Interestingly, the idea that moderate levels of stress are good and chronic and high levels of stress are bad is well accepted. Yerkes and Dodson performed a number of experiments that showed, in an inverted U curve, this principle of “arousal”, or stress (Yerkes and Dodson, 1908). While their demonstration applied primarily to the ability to learn under stress, it may have been indirectly applying to microglia as well, as Tanaka et al. (2006) showed that LPS-induced activation of microglia caused learning deficits, but not cell death, in rats.

Considering the complex role of glucocorticoid signaling in inflammation and neurodegeneration, and previous findings showing concomitant inflammation and decreased GR expression, we did attempt to run this experimental paradigm while knocking down GR expression in the SNpc. The approach attempted to utilize a virally mediated knockdown technique using a short hairpin RNA targeting GR (McKlveen et al., 2013). Unfortunately, we were unable to transduce cells of the SNpc and knockdown GR expression to test the role of GR in the CVS/6-OHDA paradigm.

Overall, we were unable to implicate neuronal GR of a neurodegenerative role in the CVS/6-OHDA model of stress and PD. A strong trend, however, supports previous finding that chronic stress can exacerbate 6-ODHA lesioning, though it is unclear if this mechanism is through GR signaling on the neurons, microglia, or another process completely. Additional exploration of this topic is necessary to tease apart the effect of chronic stress and depression in PD models and PD. Moving forward, increasing group sizes and finding methods for better and consistent pLV-GR transfection may clarify some of these findings and discoveries of more.
References


Chapter 5

General Discussion

Parkinson’s disease (PD) is an age-associated disease that involves progressive degeneration of the nigrostriatal pathway, causing motor symptoms that have become the focus of clinical and basic research. A number of non-motor symptoms are also associated with PD, such as depression. The common underlying pathway between PD and depression is still unclear; however, inflammation presents itself as a potential link. The goal of this dissertation was to identify age-related changes in the rat ventral midbrain that may contribute to nigrostriatal vulnerability in PD, and to manipulate some of these factors in the 6-hydroxydopamine (6-OHDA) neurotoxin rat model of PD and the combined chronic variable stress (CVS)/6-OHDA model to evaluate their role in the progressive loss of dopaminergic neurons. In Chapter 2, we identified alterations in the inflammatory profile of the ventral midbrain, identifying an increase in the number of microglial cells and a change in their morphology to a more active state. A decrease in glucocorticoid receptor (GR) protein expression occurred at middle-age and aged time points, paralleled by an increase in α-synuclein protein expression. In Chapter 3, we evaluated the ability of an anti-inflammatory drug, minocycline, to prevent degeneration of the nigrostriatal pathway in the 6-OHDA model. Finally, in Chapter 4, we tested the hypothesis that neuronal GR signaling mediates exacerbated nigrostriatal degeneration observed previously in the CVS/6-OHDA model, in an effort to explain chronic stress and increased degeneration.

Microglial reactivity in aging

This work began with a characterization of the ventral midbrain in young, middle-aged and aged male Sprague Dawley rats (Chapter 2). We examined proteins relevant to stress (GR),
inflammation (Iba-1) and PD (α-synuclein), and our findings are consistent with alterations found in the ventral midbrain and substantia nigra pars compacta (SNpc) of PD patients (McGeer et al., 1988; Chartier-Harlin et al., 2004; Ros-Bernal et al., 2011). A decrease in GR protein expression was observed in the middle-age and aged rats, and an increase in α-synuclein was also observed at these same time points. Finally, an increase in the number of microglial cells in the aged SNpc was identified, as well as a change in their morphology to a more active state. While these changes could be occurring independently of one another in a pre-programmed age-dependent manner, there is evidence that these changes are related to, and influence, each other.

Microglial cells are the innate immune cells of the central nervous system (CNS) and are responsive to alterations in their environment (Lehnardt, 2010). In non-aged models of microglial function, both GR and α-synuclein have been examined for their roles in microglial activation. Microglial GR is typically immunosuppressive, but can, however, exert inflammatory effects. Lim et al. (2007) showed that low levels of corticosterone promote mRNA expression of pro-inflammatory cytokines, and high levels suppressed these effects. Tanaka et al. (1997) reported that GR signaling in microglia is inhibitory, preventing microglial activation and proliferation in culture. Moreover, Carrillo-de Savauge et al. (2013) reported exacerbated activation and proliferation of GR-deficient microglia following LPS treatment. The same group also showed that stress and aging produced “primed” microglia that were hyper-reactive to LPS treatment, and this hyperactivity was exacerbated in GR-deficient stress and aged mice (Carrillo-de Savauge et al., 2013). Chronic stress was shown to prime microglia by upregulating gene expression of pro-inflammatory markers (Frank et al., 2014). While these findings indicate an important role for GR signaling in microglia alone, the stress and age-related alterations in vivo imply that some other glucocorticoid-mediated mechanism could be at play, likely in the neurons. This contribution of
neuronal signaling cannot be ignored, as microglia are responsive cells. A better understanding of neuronal-microglial communication is necessary to understand how stress and aging influence these cells.

Nair and Bonneau (2006) explored this neuron-microglia communication. They hypothesized that microglial activation in response to stress (increased GR activation) is a secondary response to neuronal GR signaling, and is mediated by N-methyl-D-aspartate (NMDA) receptor activation. Glucocorticoid signaling in neurons causes an accumulation of glutamate in the extracellular space (Ioannou et al., 2003). This accumulation leads to microglial NMDA receptor activation, and thus, production of pro-inflammatory mediators (e.g. nitric oxide, cytokines) and microglial activation. Nair and Bonneau supported their claim by blocking stress-induced microglial activation with the NMDA receptor antagonist, MK-801. Overall, this indicates that GR is immunosuppressive, and may explain the inflammatory response in the aged ventral midbrain following a decrease in GR expression.

Interestingly, enhanced inflammation and cortisol hypersecretion are observed in the elderly, depressed and PD patients (de Kloet et al., 2005; Choi et al., 2008). Glucocorticoid receptor expression may have something to do with these common symptoms. Regions of the brain relevant to stress signaling, such as the hippocampus, have shown decreases in GR with age (Peiffer et al., 1991). As the hippocampus provides negative feedback to the HPA axis, this specific regional alteration in GR may contribute to cortisol hypersecretion. Hyperactivity of the HPA axis can lead to glucocorticoid resistance and enhanced inflammation. Glucocorticoid resistance is the inability of cells and tissues to respond adequately to glucocorticoids, likely due to a decrease in receptor expression (Silverman and Sternberg, 2012). As discussed, a loss of GR on resident immune cells, as a compensatory mechanism to HPA axis hyperactivity, could promote enhanced
inflammation, such as that observed in the aged ventral midbrain (see results in Chapter 2). This alteration in inflammatory profile may not be a response to injury, but an unfortunate result of glucocorticoid resistance.

Elevated levels of α-synuclein in the SNpc are also associated with aging, and extracellular α-synuclein can stimulate microglia. However, extracellular α-synuclein may not be present unless the dopamine cells have begun to break down, releasing the protein. Within the cells, α-synuclein has reportedly been implicated in mitochondria dysfunction and elevated oxidative stress (Parihar et al., 2008). But, oxidative stress has also been shown to increase α-synuclein expression (Prasad, 2010). In human brains, elevated α-synuclein has been associated with accumulating neuromelanin (Xuan et al., 2011), which increases over time, and leads to increased oxidative stress (Shamot-Nagai et al., 2006). Regardless of the mechanism, increased oxidative stress is a pathology of aging, and it damages cells, which signals microglial activation (Lull and Block, 2010). Overall, there is evidence that microglial priming and activation in the aged ventral midbrain is due to alterations in the neurons and potentially, a decrease in GR expression.

**Inflammation and stress modulate neurodegeneration in the SNpc**

Chronic stress and inflammatory processes are directly implicated in increasing neuronal vulnerability in many models, and they have been shown to influence each other (refs, see above). While we were unable to achieve statistical significance explicitly supporting these claims, we did achieve strong trends toward significance. In Chapter 3, treatment with minocycline, an anti-inflammatory agent, showed a strong trend toward preventing nigrostriatal damage in the 6-OHDA model of PD, as reported by behavioral and cellular data. Results from Chapter 4 indicated a strong trend toward CVS inducing greater TH+ cell loss. The findings for CVS worsening nigral
degeneration supports previous findings (Hemmerle et al., 2014), however this study did not implicate a role for increased neuronal GR signaling in the mechanism.

Chronic anti-inflammatory treatment is linked to decreased risk of developing PD (Gagne and Power, 2010). Animal models of PD show that induction of inflammatory processes, creating “primed” microglia, results in increased lesion size (Koprich et al., 2008) and prevention of inflammation has been shown to result in a smaller lesion (Lin et al., 2003). In Chapter 3, we showed that anti-inflammatory treatment with minocycline prior to, and following, neurotoxic lesion likely prevented nigrostriatal degeneration, in that a strong trend was detected in a drug x lesion interaction. While minocycline has been used previously in PD models to prevent nigrostriatal damage, our study was the first to administer the drug via drinking water and for such an extended period of time. The lack of statistical significance is likely due to small groups sizes. An n of 8 was the largest group size in the behavioral testing. Behavioral testing, including the cylinder test used here, typically results in substantial variability, so larger groups sizes would be more indicative of results from each experimental group. As described in the chapter, minocycline interrupts microglial activation and release of TNF-α by inhibiting p38 mitogen-activated protein kinase (p38 MAPK). It is important to mention that minocycline not only prevents microglial activation in this way, but also inhibits oxidative stress in neurons (Morimoto et al., 2005). Since the 6-OHDA model induces cell death due to increased oxidative stress (Kostrzewa and Jacobowitz, 1974), minocycline may act directly on neurons in a neuroprotective manner. If this were the case, and neurons were protected by minocycline, then the microglia may not have been stimulated to begin with.

In addition to behavioral and neuronal survival analyses completed in Chapter 3, we examined the activation state of microglia in the SNpc by measuring somal area. As microglial
cells become activated, their morphology changes from “ramified”, with small soma and long thin processes, to “amoeboid”, with a larger perikarya and shorter, thicker processes, if any. Somal area measurements did not reveal a difference in activation states between groups. We believe that we may have missed observing microglial activation due to the timeline of our experiment, looking at the cells four weeks following lesion. Previous studies report microglial activation following 6-OHDA, but their paradigms examined the cells at multiple timepoints, showing, coincidentally, the least amount of activation four weeks post-lesion (Maia et al., 2012).

Glucocorticoids have been implicated in neurodegenerative and neuroinflammatory processes. In the hippocampus, elevated glucocorticoids decrease neurogenesis (Reagan and McEwen, 1997), however a lack of glucocorticoid signaling has also been shown to be damaging (Morale et al., 2004). Hemmerle et al. (2014) previously reported that chronic stress, thus with presumably elevated glucocorticoids, had a detrimental effect on TH+ neuron survival in the 6-OHDA model of PD. While a trend was detected showing that CVS did have an effect on TH+ cell survival, it did not achieve significance. Additionally, viral overexpression of GR in neurons did not influence cell survival. These results suggest that increased GR signaling in neurons is not the mechanism by which chronic stress induces cell death. However, a caveat is that only about 20% of dopaminergic neurons were actually transduced with virus (date not shown). This may not have been a high enough transduction rate to draw conclusions on neuronal GR signaling.

Overall, this dissertation research supports the notion that altered glucocorticoid receptor expression on microglia may contribute to dopaminergic cell loss in PD. Glucocorticoid receptor signaling in microglia is likely anti-inflammatory in all (or most) instances though reports show that corticosterone injections exacerbate inflammation and RU486 inhibits it (Frank et al., 2012). There is a complex conversation occurring in the brain between microglia and neurons, and the
mentioned effects may actually be secondary to what is occurring via GR in the neurons. *In vitro* experiments and transgenic models that allow targeting of microglia are much more telling, and supportive, of the immunosuppressive role of GR on immune cells. As GR decreases with age in the ventral midbrain, and an even greater decrease of the receptor is found in PD patients, it may be that this loss is specific to immune cells, and this ultimately results in increased inflammation and an even greater vulnerability of dopaminergic cells. Perhaps, extreme downregulation of GR on glial cells is part of the etiology of idiopathic PD. Depression is a common comorbid condition with PD, and it could be that the elevated glucocorticoids associated with depression in combination with an extreme loss of GR on microglia present an even more volatile environment for the dopamine cells of the nigrostriatal system and contributing to their demise.

**Future Directions/Conclusions**

This work offers a promising start to understanding the specific role of GR signaling in the 6-OHDA model of PD. Altering GR functioning in the SNpc may be able to not only clarify the role of comorbid depression in PD, but also the role of the immune system. Before such studies can commence, however, a number of issues and caveats that have arisen over the course of the present experiments must be discussed.

Studying the processes of natural aging offers insight into alterations in the aged SNpc that may contribute to dopaminergic vulnerability in PD. As we have shown, GR expression is decreased in the aged ventral midbrain. If this alteration is found to be restricted to or predominant on microglia, this would suggest a role for HPA axis and stress signaling alterations in inflammation. Since cortisol levels are elevated in the elderly (Krishnan et al., 2002) and GR-deficit microglia are more prone to activation (Tanaka et al., 1997), the environment would favor a chronic pro-inflammatory response. Taking a closer look at the activation state of the microglia
in our model would also clarify the inflammatory microenvironment of the ventral midbrain. We observed that the microglia had increased in number and their morphology had changed, however use of antibodies that are specific to fully activated microglia would show how many of these cells, if any, are actually in a pro-inflammatory state. Our findings also reported increased α-synuclein in the aged ventral midbrain. Though we were unable to identify an aggregated protein in the cells, nor were we able to assay for any nitrated forms of the protein, Choi et al. (2010) reports that aged rats have increased α-synuclein nitration, which seems to contribute to an exaggerated inflammatory response. It is likely that many factors contribute to chronic inflammation in the aged brain and in the SNpc specifically. Further evaluation and modification of these factors, individually, in the aged model could clarify which factor contributes the most, and the relationship between them.

Chapter 3 showed that anti-inflammatory treatment may be able to ameliorate nigrostriatal damage in the 6-OHDA model. Epidemiological studies have supported the role of anti-inflammatory therapies to decrease risk of developing PD (Gagne and Power, 2010). Considering the overall conclusions from this work, evaluating the ability of an anti-inflammatory agent to protect the nigrostriatal pathway in the combined CVS/6-OHDA model would be insightful with respect to to chronic stress mechanisms of inflammation and depression and PD. Previous work in the field has suggested that while microglial GR is anti-inflammatory, increased neuronal GR may induce inflammation in microglia via NMDA receptor activation (Nair and Bonneau, 2006).

Altering GR expression in the SNpc would be a good way to investigate its role in nigrostriatal degeneration and inflammation in the region. We were able to upregulate GR in a small percentage of dopaminergic neurons in the SNpc prior to employing the CVS/6-OHDA model. While this alteration did not have an impact on the results, it could be due to the low
transduction of the virus delivery mechanism used. As discussed above, GR signaling in neurons may be the link between chronic stress and microglial activation, and this model of overexpression may be able to support that further. However, before that can occur, better transduction methods need to be developed to ensure there is an equal and more robust overexpression of GR in dopaminergic SNpc cells. To compliment this work, it would be interesting to overexpress GR specifically in microglial cells to verify GR’s immunosuppressive role.

Because PD is an age-related disease, it is important to understand the changes that are occurring as the brain ages and which changes ultimately contribute to disease onset and progression. Some of the changes that we and other investigators have observed may also contribute to dopaminergic neuron vulnerability and inflammation. Importantly, neuropathological alterations associated with depression, which is highly comorbid with PD, may exacerbate the negative effects of these alterations on the nigrostriatal system. Considering the important role of aging in the processes studies, running these experiments with aged rats (15- and 24-month-old) would provide a better understanding of the effect of age on microglial activation and dopaminergic neuron vulnerability in the 6-OHDA model with, and without, CVS. Tamás et al. (2005) showed a clear age-dependent difference in behavioral assessment following intranigral 6-OHDA injections, however, there was not a significant difference in loss of TH-positive cells in the SNpc. This difference in behavioral deficits may be due to the inflammatory changes that are well-known to occur in aged rats, as discussed earlier, to induce behavioral abnormalities, however, the inflammatory profile was not within the scope of their study. Overall, the present work suggests an important role for GR signaling in the progression of nigrostriatal degeneration and possibly the inflammation associated with PD.
References


