I, Ann M Hemmerle, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Neuroscience/Medical Science Scholars Interdisciplinary.

It is entitled: Effects of stress-induced depression on Parkinson’s disease symptomatology

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1973
Effects of stress-induced depression on Parkinson’s disease symptomatology

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Parkinson’s disease (PD) is a chronic neurodegenerative disorder that primarily affects dopaminergic neurons of the nigrostriatal pathway resulting in debilitating motor symptoms. Parkinson’s patients also have a high risk of comorbid depression, though this aspect of the disorder is less well studied. Understanding the underlying pathology of the comorbidity is important in improving clinical treatments and the quality of life for PD patients. To address this issue, we have developed a new model combining the unilateral striatal 6-hydroxydopamine lesion model of PD with the chronic variable stress model (CVS) of depression. Dysfunction of the hypothalamic-pituitary-adrenal axis and its relationship to depression symptomatology is well established. Stress dysfunction may also have a role in the etiology of preclinical PD non-motor symptoms, and later in the course of the disease, may worsen motor symptoms. The combined model allows us to test the hypothesis that experimental depression exacerbates PD symptoms and to ascertain the mechanisms behind the increased neuronal loss.

In the first study, we examined several temporal paradigms of the combined model. Motor behavior was assessed using the forelimb asymmetry test (cylinder test) and loss of dopamine neurons was evaluated via immunohistochemical labeling of tyrosine hydroxylase and unbiased stereological cell counting. We found that animals administered CVS concomitant with the lesion exhibited worsened motor deficits and increased neurodegeneration of the dopaminergic neurons in the substantia nigra pars compacta. The second study examined potential glucocorticoid-related mechanisms behind the increased neurodegeneration. We hypothesized that glucocorticoids played a key role in the exacerbated dysfunction and neuronal loss. Animals received the glucocorticoid corticosterone (CORT) alone in a similar regimen as the flanking CVS paradigm. The administration of CORT alone did not worsen either the motor
deficits or neurodegeneration. In the second part of this study, animals were administered the glucocorticoid receptor antagonist RU486 prior to CVS concomitant with the lesion. This course of action also did not affect the behavioral deficits or neuronal loss in the combined model. Both of these experiments indicate that glucocorticoids do not have a prominent role in the worsened neuronal loss. To further explore potential mechanisms, the role of neurotrophic factors was preliminarily examined. Animals were exposed to two weeks of CVS prior to sacrifice. Messenger RNA levels of neurotrophic factors known to be neuroprotective for dopaminergic neurons were examined in brain regions involved in depression and PD. Select neurotrophic factors were altered in many regions, including the nigrostriatal pathway. The effects of chronic administration of the antidepressant desipramine on trophic factor expression were also examined. Alterations occurred in both stress and PD circuitry, though not in a complimentary fashion to the CVS changes. This study indicates possible trophic factor involvement in the increased neuronal vulnerability to injury.

Overall, the primary findings from this work indicate that CVS combined with experimental parkinsonism results in exacerbated behavioral deficits and accelerated nigral cell degeneration. These results emphasize the need for early detection of depression in PD and for development of better treatments for both the affective and motor components of the disorder.
Over the course of the several years of my dissertation research, I have had much guidance, help and support. In these few pages, I will do my best to thank all involved. First, to my advisor Kim Seroogy. Thank you so much for allowing me to join your lab and helping me through all the ups and downs of starting a new model. Thanks for your guidance, patience, and for trusting me with the “keys” of the lab on occasion. You have been a wonderful mentor, teaching me how to be a good scientist, accepting that the data are the data, and of course, the importance of controls. I have always enjoyed our conversations, both scientific and non-scientific and look forward to seeing what future discoveries are to be made.

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career turned out to be. You have always had the greatest faith in me, always telling me things 
would work out even when I was convinced they wouldn’t. Thank you for making me laugh 
when I took myself too seriously or making me laugh just because. You are my rock. I am so 
excited to start this next chapter in our lives together. All my love, always.
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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>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ACCtx</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CE</td>
<td>Coefficient of effort</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CVS</td>
<td>Chronic variable stress</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DES</td>
<td>Desipramine</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal raphe nucleus</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary adrenal</td>
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<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>SNRI</td>
<td>Selective serotonin-norepinephrine reuptake inhibitor</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
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<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNT</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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</table>
Chapter 1

Introduction

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Parkinson’s Disease

Parkinson’s disease (PD), an age-related neurodegenerative disease, was first described clinically by James Parkinson in 1817 and affects about 1-2% of the population. The most noted risk factor for the disease is aging, though a percentage of cases have strong genetic links (Thomas and Beal, 2007). Pathologically, PD is characterized primarily by the significant, progressive neurodegeneration of dopaminergic neurons within the nigrostriatal pathway and the presence of Lewy bodies in the surviving neurons. Lewy bodies are spherical cytoplasmic protein aggregates containing several proteins including α-synuclein and parkin (e.g. Schulz and Falkenburger, 2004). Cardinal symptoms manifest themselves in the form of motor impairment including, bradykinesia/akinesia, postural instability, resting tremor and rigidity. By the time motor symptoms appear, 80% of dopamine content in the striatum, which consists of the putamen and caudate, and 60% of dopaminergic neurons in the substantia nigra pars compacta (SNpc) are lost (Schulz and Falkenburger, 2004). The neuronal loss in PD is concentrated in the ventrolateral and caudal regions of the SNpc, whereas in normal aging, cell loss in concentrated in the dorsomedial portions (Schulz and Falkenburger, 2004). Loss of dopamine in the striatum appears to occur in the posterior putamen (e.g. Rodriguez-Oroz et al., 2009). A decrease in other presynaptic dopamine neuron components such as tyrosine hydroxylase (TH), vesicular monoamine transporters and dopamine transporters accompanies the loss of striatal dopamine...
content (e.g. Hornykiewicz, 2001). In PD, the disruption of dopamine signaling in the nigrostriatal system results in reduced excitation of the globus pallidus pars interna and the substantia nigra pars reticulata “direct pathway” and decreased inhibition of the globus pallidus pars externa and the subthalamic nucleus (STN) “indirect pathway” (Delong, 1990; Bartels and Leenders, 2009). The end result is increased excitation of the inhibitory (e.g. GABAergic) basal ganglia output which inhibits the activation of the thalamic nuclei projecting to the cortex, necessary for motor movement initiation (Gerfen et al., 1995; Bartels and Leenders, 2009).

The exact etiology of idiopathic PD is unknown, though it is currently thought to result from a combination of genetic and environmental risk factors. The relatively specific loss of dopaminergic neurons in the SNpc is the hypothesized result of excitotoxicity, oxidative stress, mitochondrial dysfunction, protein aggregation and inflammatory responses together with environmental insults like trauma and exposure to toxins (Howells et al., 2005; Olanow and Kordower, 2009). Familial PD, involving inherited genetic mutations, occurs in about 10% of PD cases and has an earlier age of onset and more rapid disease progression. Mutations in several genes have been observed in familial PD including α-synuclein, parkin, and leucine-rich repeat kinase (LRRK) (Stoessl, 2010). Many of these genes are involved in the ubiquitin-proteasome system and their dysfunction is thought to lead to protein misfolding and aggregation within the cells, as well as defects of the mitochondrial system (Stoessl, 2010; Schapira and Jenner, 2011). Why the nigrostriatal pathway specifically is so greatly affected is unknown, but many of the neurons vulnerable in PD have long axons which are poorly myelinated and rich in pigments such as neuromelanin and lipofuscin (Grinberg et al., 2009). Longer neurons lacking the myelin insulation require a greater amount of energy expenditure and turnover for maintenance which results in higher levels of oxidative stress (Nieuwenhuys, 1999; Braak et al.,
2003). During high levels of oxidative stress, hydrogen peroxide can interact with neuromelanin, forming free radicals and increasing cell death (González-Hernández et al., 2010), providing one possible reason for the vulnerability of nigral dopaminergic neurons.

Traditionally, PD is thought of as a motor disorder, primarily involving the dopaminergic nigrostriatal pathway. At the same time, dopamine projections from the SNpc innervate, to some extent, other regions besides the caudate-putamen and globus pallidus, including, the nucleus accumbens, olfactory bulb, piriform and entorhinal cortices, amygdala, hippocampus and locus coeruleus (LC) (Lindvall and Björklund, 1983). In the Parkinson’s disease state, it is now known that neurons are affected in multiple systems, both dopaminergic and non-dopaminergic, with more widespread neurodegeneration occurring as the disease advances. The progression of Lewy body formation during PD appears to occur in successive order or stages as research by Braak and colleagues indicates (Braak et al., 2004; Braak and Del Tredici, 2008), although not without controversy (Schulz and Falkenburger, 2004). Braak stages one and two are considered preclinical, though non-motor symptoms are often present, with Lewy bodies observed within the enteric nervous system, olfactory bulb and the lower brainstem. In Braak stages three and four, Lewy bodies are present in the SNpc and deep nuclei and it is during these stages that motor symptoms first manifest themselves. In the last stages, Lewy bodies progress to the limbic and neocortical regions and are associated with the more cognitive dysfunctions of the disease (Braak et al., 2004; Chaudhuri et al., 2006).

It is increasingly understood that many other neurotransmitter systems degenerate or are altered in PD, though generally to a lesser extent than the dopaminergic cell loss of the nigrostriatal pathway (Halliday et al., 1990). Other brain regions also affected include the LC, the dorsal nucleus of the vagus, dorsal raphe nucleus, the basal nucleus of Meynert, the reticular
nuclei, the amygdala, and the CA2 region of the hippocampal formation (Braak et al., 2004; Ferrer et al., 2010). Neurodegeneration and Lewy body formation occurs in the dopaminergic pathways outside the nigrostriatal system and noradrenergic, serotoninergic, GABAergic, and cholinergic systems, as well as the cerebral cortex (cingulate and entorhinal cortices), olfactory bulb and the autonomic nervous system (Forno, 1996; Schulz and Falkenburger, 2004; Grinberg et al., 2009). For instance, serotonin is depleted in the hypothalamus and frontal cortex of PD post-mortem brains, though not to the extent of dopamine loss in the SNpc (Fox et al., 2009). The functioning of the serotoninergic neurons also appears to be altered, as the firing rate is increased in the dorsal raphe nucleus (Zhang et al., 2007; Kaya et al., 2008). Several studies have found extensive loss of noradrenergic neurons in the LC as well, likely preceding the dopaminergic loss (Rommelfanger and Weinshenker, 2007). In animals, depletion of norepinephrine in the LC of knockout mice created motor dysfunction in many behavioral tests, whereas mice treated with MPTP do not display such severe dysfunction (Rommelfanger et al., 2007). Taking into account extra-nigral dysfunction will likely improve the development of PD animal models and lead to better clinical treatments.

Dopamine loss remains the primary concern in treating PD, as the amount of neurodegeneration within the nigrostriatal pathway is greatest and results in the most debilitating physical symptoms of the disease. No current clinical treatment for PD stops the progressive neurodegeneration, but several therapies can help improve the motor symptoms up to a point. The discovery of dopamine as a neurotransmitter in the 1950s (reviewed by Benes, 2001) led to the development of the dopamine precursor L-dopa (levodopa) as a replacement therapy that is still the gold-standard treatment of clinicians today. However, long-term use of levodopa causes side effects in many patients including dyskinesias, oscillations of effectiveness over the course
of the day, and psychological effects including hallucinations (e.g. Poewe, 2010). Other newer
drug treatments include dopamine agonists, monoamine oxidase B inhibitors and catechol-O-
methyl-transferase inhibitors (e.g. Rascol et al., 2003). In addition to pharmacological therapies,
the use of high-frequency deep brain stimulation of assorted targets (e.g. STN) has become an
increasingly common therapy and can create a prolonged improvement in motor symptoms and a
reduction in the use of dopamine replacement therapies (e.g. Romito and Albanese, 2010). As
most therapies focus on treatment of motor symptoms resulting from dopaminergic
neurodegeneration, they do not well address concerns regarding other systems affected in PD.
The use of animal models is important in evaluating treatments of PD that are more beneficial, as
well as assisting in the study of the underlying mechanisms of PD.

Animal models of PD

There are many types of animal models for PD, but generally, they fall under the
category of either genetic manipulation (normally in mice) or use of a neurotoxin (used in both
rodents and primates). No model is currently able to mimic all the pathological hallmarks of PD.
Some issues include the relatively acute nature of neurodegeneration observed in many
neurotoxin models or the lack of dopamine loss in the nigrostriatal system, as seen in many
genetic models. The most commonly used types of neurotoxins are 1-methyl-4-phenyl-1,2,3,6-
tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) which will be discussed in detail
below.

Research uncovering the genes associated with the development of PD has lead to the
generation of genetic models, either overexpressing or knocking out genes of interest. The gene
α-synuclein is utilized in many transgenic models, with some motor dysfunction and non-motor
symptoms often observed depending on the gene promoter used (Chesselet, 2008). However, these models do not result in progressive dopaminergic degeneration (Giasson et al., 2002; Kirik et al., 2002; Bezard and Przedborski, 2011), though some demonstrate striatal dopamine loss (Masliah et al., 2000). Transgenic mice with LRRK2 overexpression have age-related development of motor impairment, but once again do not produce the progressive cell loss needed to be an accurate PD model (Li et al., 2009; reviewed by Olanow and Kordower, 2009). Knockout of Pitx3 (a homeobox transcription factor expressed in dopaminergic neurons) in mice produces a large loss of dopaminergic neurons and motor deficits that are reversed by levodopa treatment (Hwang et al., 2005; Olanow and Kordower, 2009). However, the dopaminergic cell loss occurs early in development and the mice are born blind creating a large confounding factor for behavioral evaluation (reviewed by Meredith et al., 2008). In Drosophila, parkin mutations can cause mitochondrial dysfunction, a common trait of sporadic PD, but this is not observed in genetic mouse models (Dodson and Guo, 2007; Meredith et al., 2008). Still, a recently developed parkin trangenic model is able to produce motor deficits as well as age-related dopamine loss in the nigrostriatal pathway (Lu et al., 2009). Genetic models are particularly useful in understanding the underlying mechanisms of PD, but are currently not as useful in neuroprotection studies because of the lack of dopaminergic cell loss.

The discovery of MPTP as a model for PD occurred serendipitously in the early 1980s when young drug addicts began exhibiting parkinsonian symptoms. What they thought was synthetic heroin was actually the neurotoxin MPTP and resulted in them displaying the cardinal motor symptoms of PD (Davis et al., 1979; Langston and Ballard, 1983). In animals, MPTP can cross the blood brain barrier and therefore systemic administration is possible (reviewed in Schober, 2004; Meredith et al., 2008). Once in the brain, astrocytes take up MPTP and convert it
into 1-methyl-4-phenylpyridinium (MPP+). Dopamine transporters then take up MPP+ and this toxin causes impairment of mitochondrial respiration by inhibiting complex I (Schober, 2004). As rats lack the ability to convert MPTP into MPP+, this model is restricted to use in mice and primates (Meredith et al., 2008). The presence of protein inclusions similar to Lewy bodies have been detected in cortical regions after administration and the toxin has been demonstrated to affect regions outside the nigrostriatal pathway (Hallman et al., 1985; Schober, 2004; Meredith et al., 2008). However, the model is not as sensitive in uncovering motor behavior deficits in mice (Meredith and Kang, 2006; Meredith et al., 2008).

The neurotoxin 6-OHDA is a hydroxylated analogue of dopamine specific for catecholaminergic neurons originally isolated by Senoh in 1959 (Senoh and Witkop, 1959; Blum et al., 2001) and is taken up by neuronal membrane transporters (Breese and Traylor, 1971). Once inside the neuron, oxidation of 6-OHDA occurs and produces reactive oxygen species, leading to eventual cell death (Orth and Tabrizi, 2003; Meredith et al., 2008). Though 6-OHDA cannot cross the blood brain barrier, specific brain regions can be targeted through intracranial injection (Schober, 2004; Meredith et al., 2008). Unilateral injections can cause quantifiable asymmetric motor deficits, with the contralateral side able to serve as an internal control (Schober, 2004). Commonly targeted regions include the SNpc, the medial forebrain bundle (MFB), and the striatum. Injection into the SNpc causes a very acute loss of dopamine cell bodies followed shortly by the loss of dopaminergic striatal projections (Jeon et al., 1995; Schober, 2004; Meredith et al., 2008). Injection into the MFB causes a dying back of the striatal projections first before the loss of dopamine cell bodies, and while it is not as acute as direct injection into the SNpc, it does not mimic the progressive nature of PD well (Zham, 1991; Meredith et al., 2008). Injection into the striatum causes a more progressive loss of
dopaminergic neurons, with neuronal loss continuing out for up to 16 weeks after injection (Sauer and Ortel, 1994; Kirik et al., 1998; Schober, 2004). The degeneration is preceded by cellular atrophy and loss of phenotype expression (Sauer and Ortel, 1994). For the purposes of the experiments contained within this dissertation, we will utilize the unilateral striatal 6-OHDA model of PD and take advantage of the relatively progressive nature of neuronal degeneration. One of the major drawbacks for this model is that lack of \( \alpha \)-synuclein inclusion formation. The neurotoxin also does not cause any changes to the extra-nigral systems affected in PD, which are likely involved in the presence of non-motor symptoms within the disorder. Still, this model has many advantages, primarily that the progressive nature of neuronal loss allowing researchers to intervene with methods that will improve (or worsen) neurodegeneration.

**Non-motor symptoms of PD**

Although dopaminergic dysfunction and the resulting motor symptoms remain the focus of most PD research, it has become common to regard PD as a multisystem disorder affecting dopaminergic, noradrenergic, and cholinergic systems resulting in both motor and non-motor symptoms (Grinberg et al., 2009). A plethora of non-motor symptoms are present in many, but not all cases of PD, including cognitive impairment, depression, sleep disorders, hallucinations, impulsive control disorder, pain, fatigue, anxiety, mood disorders, and autonomic nervous system dysfunction such as constipation, incontinence, orthostatic dysfunction, and sexual dysfunction. While many of the non-motor symptoms are associated with age, disease severity, and treatment side effects, some, such as olfactory problems, constipation, rapid eye movement disorder, and depression, can appear early in the disease process, even preceding the onset of motor symptoms (Lauterbach et al., 2004; Chaudhuri et al. 2005). Psychiatric and non-motor
symptoms can contribute greatly to the disabilities in PD, with depression and worsening cognition often having the greatest impact on the patient (Weintraub, 2004). Despite the pervasiveness and the effects on the quality of life, non-motor symptoms remain an understudied aspect of PD. To help address this issue, this dissertation will focus on developing an animal model to examine the effects of depression, one of the most common non-motor symptoms, on PD symptomatology.

**Depression within Parkinson’s disease**

The most commonly cited prevalence of depression in PD is between 40-50% of all PD cases (Cummings, 1992; Lemke, 2008; Aarsland et al., 2009). More recent clinical studies have found comorbidity anywhere from 25% (Klotsche et al., 2011) to 72% (Jasinska-Myga et al., 2010). While the elderly overall have an elevated risk of depression (about 11%) (Steffens et al., 2009) compared to the general population (about 7%) (Kessler et al., 2003), there is a considerably enhanced risk of depression in PD. Genetics may play a factor, as patients with PD had an increased risk of a relative developing depression (Arabia et al., 2007). Despite the fact that almost half of PD patients have depression, only about 26% receive medication to treat it (Richard and Kurlan, 1997; Yamamoto, 2001). This mismatch of incidence versus diagnosis is likely caused by the overlap in symptoms between the two disorders. Clinicians also often fail to notice depression or dysthymic disorder in PD (Yamamoto, 2001). Both depression and PD symptoms include motor slowing, bradyphrenia (slowness of thought), sleep and appetite disturbances, weight loss, loss of interest and concentration, reduced libido and lack of emotional response (Richard, 2007). The psychomotor retardation that commonly occurs in PD can also mask the presence of depression (Lieberman, 2006). On the other hand, depressive symptoms...
such as anergy, apathy, insomnia, excessive sleep, and weight loss can also occur in PD patients who are not considered depressed (Richard, 2007). All of the above makes diagnosis and therefore subsequent treatment difficult, and should persuade clinicians to be observant to signs of depression in PD patients.

Most of the focus in PD drug treatment remains centered on the motor symptoms. However, many factors impact the quality of life in PD, and the non-motor symptoms actually have a large impact on the everyday life of the patient, despite the lack of attention from clinicians (Schrag, 2006). Non-motor, non-levodopa responsive symptoms of PD are often considered the most disabling feature of the disease (Hely et al., 2005). Research by the Parkinson’s Study Group indicated that depression accounts for 58.2% of the impairment of quality of life in PD patients and is considered a more important factor than the severity of motor dysfunction, making it one of the more disabling aspects of the disease (Richard and Kurlan., 1997; Kuopio et al., 2000; Yamamoto, 2001; Findley et al., 2002). Patients with PD and depression represent a greater burden to their caregivers (Aarsland et al., 1999; Lauterbach, 2005). Depression is also associated with worsened motor function and increased disease severity which impacts daily living (Papapetropoulos et al., 2006). However, though the rates of suicidal ideation are elevated in depressed PD patients, actual suicide attempts are uncommon (Kummer et al., 2009).

When depression is present in PD, there are often other non-motor symptoms present. Depression in PD has significant correlation with cognitive impairment (Starkstein, 1990; Tandberg et al., 1997). Depressed PD patients also have greater memory impairments compared to non-depressed PD patients (Uekermann et al., 2003). Sleep disorders, the most common non-motor symptom, and depression are also strongly correlated (Tandberg et al., 1998; Happe et al.,
2002; van Rooden et al., 2009), though there are conflicting findings on whether this is related to disease severity (Happe et al., 2002; van Rooden et al., 2009). Anxiety disorders are also common in patients with PD. Of patients that have PD and depression, 67% also had anxiety and 97% of PD patients with anxiety were also diagnosed with depression (Cummings and Masterman, 1999). Both depression and anxiety are often present before the onset of motor symptoms (Shiba et al., 2000). It is likely the high rates of co-occurrence are the result of common underlying neuropathological changes within PD itself (Henderson et al., 1992; Cummings and Masterman, 1999).

Timecourse of depression symptoms in PD

It is argued that depression in PD is not merely a psychological reaction to being diagnosed with the disease itself, but rather a secondary symptom of the ongoing neurodegeneration (McDonald et al., 2003). The incidence of severe depression in PD is twice that which is seen in other disabled patients (Mayeux, 1981; Taylor, 1985). Depression in PD does not appear to parallel the progression of the physical symptoms of the disease, suggesting independent processes may be involved (Brown and Jakanshahi 1995). Others have found the increased risk for most neuropsychiatric symptoms occurs with increased PD severity, but does not correlate with age or age of onset (Lieberman, 2006; Klotshe et al., 2011). Some studies, though, found that depression occurred in higher rates in patients that developed PD at an early age compared to those with later onset (Giladi et al., 2000; Leentjens et al., 2002; Schrag et al., 2003). Moreover, it has been put forth that the comorbidity of PD and depression may be biphasic in nature with peaks early and late during the course of the disease (Richard, 2007).

Overall, clinical studies indicate depression is present in patients at all stages of PD.
Thus, it is not known whether depression is a preclinical manifestation of the ongoing neurodegenerative process that occurs before compensatory mechanisms no longer prevent the advent of motor symptoms, or an active component of the neurodegenerative process. One opinion contends that depression precedes the onset of motor symptoms in PD. Indeed, studies have found a higher incidence of depression in patients who were later diagnosed with PD than in a comparable control population (Leentjens et al., 2003a; Alonso et al., 2009). People with depression have a higher rate of being subsequently diagnosed with PD than those with diabetes, a chronic condition, or osteoarthritis, another disease of aging (Nilsson 2001). Emotional vulnerability may also increase the chances of PD patients developing depression, possible through overlapping involvement of the anterior cingulate cortex (Serra-Mestres and Ring, 2002). Another outlook argues that the depression in PD is actually present after disease diagnosis. More severe motor symptoms as well as longer disease duration are present in PD patients diagnosed with depression compared with non-depressed PD patients (Pålhagen et al., 2008). This could be an indication of a further advanced and widespread disease state (Pålhagen et al., 2008). There is also debate on whether depression appears in PD as natural part of the disease process or whether there is a convergence of two independent pathological processes (Leentjens, 2004).

Current treatments for depression in PD

In general, tricyclic antidepressants (TCAs), selective-serotonin reuptake inhibitors (SSRIs) and selective serotonin-norepinephrine reuptake inhibitors (SNRIs) are the first choices for treating depression in PD (Schrag, 2006). The use of SSRIs is the most common choice for PD patients with depression (Richard and Kurlan, 1997), but they are not always effective
(Menza et al., 2009). There is also some concern that SSRIs may worsen motor deficits (Richard and Kurlan., 1997), though a more recent study disputes this (Arbouw et al., 2007). An overview of clinical trials studying antidepressant usage in PD found that nortriptyline, amitriptyline, fluvoxamine, and citalopram produced positive treatment outcomes, though with side effects, such as low blood pressure (Chung et al., 2003). However, the trials were not optimal in several ways including small experimental numbers and high dropout rates (Chung et al., 2003). Another meta-analysis suggests that there are few good studies of antidepressant treatment in PD. Moreover, there do not appear to be class-specific effects when using antidepressant drugs to treat depression in PD (Weintraub et al., 2005).

Interestingly, dopamine agonists, often used to address motor dysfunction in PD, are also proposed to be an effective treatment for depression in PD. The dopamine-receptor agonists pramipexole and pergolide, have displayed the ability act as an antidepressant in patients both with and without PD in smaller studies (Corrigan et al., 2000; Reichmann et al., 2003; Barone et al., 2006; Picillo et al., 2009). Pramipexole was also able to show antidepressant effects in a recent randomized double-blinded, placebo controlled study (Barone et al., 2010), but others argue that it is not a clinically relevant improvement as the effect, while significant, is not large (Leentjens, 2011). There is still need for larger randomized controlled studies to assess the effectiveness of dopamine agonists, as well as conventional antidepressants, before any definitive conclusions can be made about optimal treatment of depression in PD.

Neurological basis for depression in PD

The primacy of the neurodegeneration of dopamine neurons in the nigrostriatal pathway in PD cannot be ignored. However, it is possible that depression in PD is a result of a
combination of elements including pathology outside of the nigrostriatal pathway, such as involvement of the serotonergic or noradrenergic systems, or environmental factors (Schrag, 2006). Other dopaminergic pathways and other neurotransmitters are affected throughout the course of PD and more than likely play a role in the development of non-motor symptoms like depression. Accordingly, the impaired function of noradrenergic and serotonergic neurons may also play a role in the development of depression in PD (Yamamoto, 2001). Norepinephrine and serotonin are the two neurotransmitters most commonly associated with depression pathogenesis. Certain personality traits present in PD patients with depression, such as harm avoidance (anxious behavior), appear to be linked to serotonergic function (Menza and Mark., 1994). In depression, there is a loss of norepinephrine signal resulting from degeneration of LC projections to the frontal cortex and limbic system (Chan-Palay and Asan, 1989a). In PD, there is also a decrease in the activity and number of neurons of the serotonergic neurons in the dorsal raphe nucleus and of the noradrenergic neurons in the LC (Lieberman, 2006). A reduction of serotonergic metabolite levels in the cerebral spinal fluid is found in depressed versus non-depressed PD patients (Mayeux et al., 1984) and there is noradrenergic cell loss both rostrally and caudally (Chan-Palay and Asan, 1989b). There is also a lower density of neurons in the dorsal raphe nucleus in depressed PD patients (Paulus and Jellinger, 1991). However, in imaging studies there is no evidence in the disruption of the dorsal raphe serotonergic system when comparing depressed and non-depressed PD patients (Kim et al., 2003). Elevated serotonin transporter levels in the striatum and dorsalateral and prefrontal cortices were correlated with depressive symptoms in PD patients, but not with disease severity (Boileau et al., 2008). This highlights the possibility of serotonergic pathology in PD with depression. However, this study did not compare the depressed PD patients to non-depressed PD patients.
(Boileau et al., 2008). On the other hand, it is unlikely that the loss of serotonin alone plays a role in depression etiology in PD, because SSRIs are not always effective in treating the depression in PD patients, despite often being the first choice for treatment (Yamamoto, 2001; Leentjens et al., 2003b). Additional imaging studies have observed differences in other neurotransmitter systems between PD patients with and without depression. For example, a decrease in cortical acetylcholinesterase activity, a marker of cholinergic innervation, occurs in the cortex of PD patients displaying depressive symptomatology, particularly those that also have dementia (Bohnen et al., 2007). A reduction of acetylcholine receptor binding in the cingulate cortex and the frontoparieto-occipital lobe is also found in depressed PD patients (Meyer et al., 2009). The inferior frontal lobe also exhibits a decrease in glucose metabolism in depressed PD patients, possibly affecting mood regulation (Mayberg et al., 1990).

Non-motor symptoms in PD cannot be considered the same as symptoms caused by non-dopaminergic pathways (Fox et al., 2009; Chaudhuri and Schapira, 2009). Depressive symptomatology in PD may also be related to dopaminergic dysfunction as dopamine agonists such as pramipexole are able to improve depressive symptoms (eg. Barone et al., 2010, but see Leentjens, 2011). Decreases in dopaminergic transporter availability in the striatum are seen in PD patients with depression (Weintraub et al., 2005; Hesse et al., 2009). This is likely secondary to the dopamine loss in the region, indicating a more extensive cell loss in depressed PD patients (Hesse et al., 2009) and increased basal ganglia impairment (Weintraub et al., 2005). In post-mortem studies, a decrease in dopamine neurons in the ventral tagmental area (VTA) was detected in depressed PD patients (Brown and Gershon, 1993). Additionally, in PET imaging study, PD patients diagnosed with depression were observed to have decreased noradrenergic and dopaminergic innervation in emotion-related circuitry including the LC, anterior cingulate
cortex, thalamus, amygdala and ventral striatum compared to both controls and PD patients without depression (Remy et al., 2005). These reductions occurred in areas believed to be involved in emotional circuits (Remy et al., 2005). While dopamine loss is observed in non-nigrostriatal pathways, and can contribute to the formation of non-motor symptoms, the nigrostriatal pathway also has dopamine connections to the ventral striatum and may play a role in reward (Wise, 2009). This has implications for the presence of depressive symptoms in PD such as anhedonia. If indeed the nigrostriatal pathway functions in the reward system, then the severe loss of dopamine neurons would almost inevitably cause dysfunction in the reward pathway as well.

**Neurobiological aspects of stress and depression**

Appropriate responses to external or internal adversity (i.e. the ‘stress response’) are critical for adaptation and survival. The stress response involves the activation of multiple bodily systems, the most prominent of which are the hypothalamic-pituitary-adrenal axis and autonomic nervous system. The HPA axis (see Fig. 1) is involved in both acute and chronic stress responses in order to return the body to homeostatic balance and activates a neuroendocrine cascade that results in the increase of glucocorticoid levels. When a stressor is perceived and disrupts homeostasis, neurons contained within the paraventricular nucleus (PVN) of the hypothalamus are stimulated to release various secretagogues including corticotrophin-releasing hormone (CRH). The CRH enters the hypoposeal portal system through the median eminence and travels to the anterior pituitary where it causes the release of adrenocorticotropic hormone (ACTH). Subsequently, ACTH enters general circulation where it stimulates glucocorticoid release from the adrenal cortex. Glucocorticoids (cortisol in humans and primarily
Figure 1. Schematic diagram of the HPA axis. During a stress response, CRH neurons within the PVN of the hypothalamus release CRH into the hypophoseal portal system through the median eminence, which produces the release of ACTH from the anterior pituitary gland. The ACTH enters the general circulatory system and elicits the release of glucocorticoids from the adrenal cortex and triggers negative feedback of HPA axis activation. Other regions of the limbic system are also believed to exert a regulatory influence on HPA axis activation, with the amygdala having an excitatory effect and the hippocampus having an inhibitory effect.
corticosterone in rats and mice) subserve important adaptive functions during stress exposure (e.g., glucose mobilization), and also provide negative feedback regulation of the HPA axis activation, thereby limiting their own secretion. Levels of excitatory amino acids, norepinephrine and serotonin are also elevated during the stress response.

While initially adaptive, the protracted activation of the stress response contributes to physiological abnormalities and may be involved in the development of disease states (Herman and Cullinan, 1997). Mood disorders are often preceded by stressful life events, whether environmental or internal (Kendler et al., 1999; Gold and Chrousos, 2002), and chronic stress is considered a risk factor for depression (McGonagle and Kessler, 1990). Notably, glucocorticoid receptors (GR) are localized to many regions involved in depressive and neurological pathologies, including the prefrontal cortex (PFC), hippocampus, striatum, limbic system, nucleus accumbens, and ventral midbrain (Herman, 1993; Van Craenebroeck et al., 2005), suggesting the potential for glucocorticoids to play a role in disease processes.

**Stress effects on neuronal plasticity**

Aberrant neuroplasticity in limbic brain regions involved in emotion and memory, such as the hippocampus, PFC and amygdala, appear to contribute to the pathophysiology of mood disorders (e.g., Duman 2002). Limbic-driven HPA axis hyperactivity is associated with major depression (Gold et al., 1988), where as hypoactivity is often seen in PTSD (Yehuda et al., 2006), suggesting that optimal regulation of glucocorticoids is required for appropriate adaption to stress. Glucocorticoids in particular can act to disrupt normal hippocampal function. *In vitro*, hippocampal cells are more susceptible to subsequent neurotoxin exposure when cells are first exposed to glucocorticoids (Sapolsky et al., 1988). With *in vivo* studies, exposure to stress
results in cell loss and neuronal atrophy in key limbic regions associated with the pathology of depression including the PFC, amygdala, and hippocampus (Magariños and McEwen, 1995a, b; Wellman, 2001; Duman, 2002). Importantly, chronic antidepressant treatment can reverse both these effects (Duman and Monteggia, 2002). The hippocampus is implicated in mood disorders as well as learning and memory and HPA axis regulation, both of which are impaired in depression (Duman and Monteggia, 2006). The hippocampus is interconnected with the PFC and amygdala, with the PFC playing an inhibitory role on the HPA axis, and the amygdala having excitatory connections (Ulrich-Lai and Herman, 2009). Therefore, effects on the hippocampus can have long-term consequences on glucocorticoid homeostasis.

As noted above, stress can cause dendritic atrophy, particularly in GR-rich regions such as the hippocampus (Magariños and McEwen, 1995a, b). Mineralocorticoids, however, do not produce cellular atrophy (Gould et al., 1991), suggesting the GR as the primary mediators of pathology. Aged rats have reduced GR expression (Sapolsky et al., 1983, Landfield and Eldridge, 1989) and signaling (Murphy et al, 2002) in the hippocampus. The density of hippocampal pyramidal neurons is also decreased in aged rats, possibly because of longtime glucocorticoid exposure (Landfield and Eldridge, 1989). It is hypothesized that the loss of feedback causes elevated corticosterone secretion via loss of negative feedback (however glucocorticoids dyshomeostasis is not observed in all aging studies) (Sapolsky, 1994). Chronic corticosterone administration and chronic stress also decrease hippocampal GR expression, suggesting a link between elevated steroid secretion, stress and the aging process (Sapolsky et al., 1985). Notably, Meaney’s group demonstrated a correlation between elevated corticosterone and memory deficits in aged rodents, suggesting that elevated glucocorticoids are linked to hippocampal pathology (Issa et al., 1990). The exact role of glucocorticoids in
neurodegeneration is complex, however, with glucocorticoids actually being neuroprotective at certain levels (Ábrahám et al., 2006).

Chronic stress and corticosterone also cause changes in neuronal morphology in other GR-rich limbic sites. Both treatments reduce dendritic complexity in the medial PFC (Wellman, 2001; Radley et al., 2004; Cerqueira et al., 2005; Dias-Ferreira et al., 2009), which is known to be involved in inhibition of stress responses and control of emotionality. Dendritic atrophy is also observed within corticostriatal circuits in animals that experienced chronic stress, including the dorsal medial striatum, whereas dendritic hypertrophy is seen in the dorsal lateral striatum (Dias-Ferreira et al., 2009). Glucocorticoids and chronic stress increase dendritic complexity in the amygdala, a region commonly linked to fear, anxiety and depression (Vyas et al., 2002; Mitra and Sapolsky, 2008). To date, neuroplasticity studies have focused on sites involved in emotion, memory and stress regulation. Due to the widespread distribution of the GR, other regions of the brain may be susceptible to deleterious effects of glucocorticoids/stress on morphology and function. These include areas such as the substantia nigra, which is central to PD pathology.

Stress dysfunction and depression pathophysiology in humans

In humans, imaging studies indicate decreased hippocampal volume in depressed subjects, indicating neuronal atrophy (Sheline et al., 1996, 2003; Duman 2004a,b). Volumetric changes also occur in the amygdala and PFC of patients with depression (Salvadore et al., 2001; Kanner, 2004; Tang et al., 2007). Subregions of the PFC (e.g., subgenual anterior cingulate cortex) are hyperactive in depression, and inactivation by deep brain stimulation causes remission of depressive symptoms, linking this stress-targeted region to the pathogenesis of the
disease (Mayberg et al., 2005). Antidepressant treatment may be effective by protecting against the loss of hippocampal (Sheline, 2003) and importantly, frontal cortical volume (Lavretsky et al., 2005).

Exposure to acute and chronic stress decreases adult hippocampal neurogenesis (Duman 2004a,b). As exposure to stress decreases new cell production in the hippocampus of animals, this may explain the decrease in total hippocampal gray matter volume observed in humans (Sheline et al., 1996; Fuchs and Flugge, 1998). In contrast, both antidepressant treatment and environmental enrichment can increase neurogenesis in the adult hippocampus and antidepressant treatment can block/reverse the decrease of neurogenesis caused by stress (Malberg et al., 2000; Brown et al., 2003; Duman 2004a).

Approximately 50% of depressed patients exhibit HPA axis dysfunction, characterized by impaired feedback regulation of the corticosteroid secretion (Kanner, 2004). The lack of dexamethasone suppression indicates dysfunction of the HPA axis, which is likely caused by increased stimulation of the pituitary gland by hypothalamic drive (Kathol et al., 1989). Patients with major depression have elevated CRH mRNA expression and cell number in the hypothalamus as well as increased levels of CRH in cerebrospinal fluid (Nemeroff et al., 1984; Swaab et al., 2005). The LC, which contains noradrenergic projections involved in stress regulation, also displays elevated levels of CRH in depressed subjects (Swaab et al., 2005). However, CRH levels do not necessarily reflect HPA axis activity (Swaab et al., 2005). The question arises whether HPA axis dysregulation causes depression or whether the emotional disturbances present in the disorder result in stress dysregulation (Van Craenenbroeck et al., 2005).
Effects of stress on dopaminergic neurons

In some suicide victims, dopamine levels are elevated in the hypothalamus (Arranz et al., 1997). After receiving the dopamine receptor agonist apomorphine, patients with both depression and elevated cortisol levels exhibit a blunted cortisol response, suggesting cortisol feedback deficits may be associated with dopamine receptor dysfunction on the hypothalamus (Duval et al., 2006). The normal physiology of dopamine in the mesolimbic and mesocortical systems is also disrupted by stress (Pani et al., 2000). In rats, acute restraint stress increases dopamine release in the nucleus accumbens and PFC (Imperato et al., 1990). Acute immobilization increases dopamine levels in the frontal cortex, whereas levels decrease in the striatum and hippocampus (Rasheed et al., 2010). Tail-shock stress caused an increase of dopamine efflux in the striatum, nucleus accumbens, and frontal cortex, though to varying degrees (Abercrombie et al., 1989). However, chronic stress generally decreases dopaminergic tone. Prefrontal cortex dopamine content is significantly reduced following CVS (Ziegler et al., 1999). Moreover, chronic unpredictable stress decreases dopamine levels in the striatum, nucleus accumbens, and frontal cortex (Rasheed et al., 2010). Chronic cold stress increases efflux of dopamine in the medial PFC, but not in the striatum and nucleus accumbens (Gresch et al., 1994). Restraint stress elevates oxidative stress products in the nigrostriatal system, potentially leading to an increased vulnerability of the dopamine neurons (Kim et al., 2005). Together, these studies suggest that the interactions of dopamine and stress are dependent on intensity and/or duration of stress exposure. Additionally, these studies indicate that although acute and chronic stress affects dopamine largely in the forebrain, there are some alterations of dopamine levels found in the striatum and mesolimbic pathway as well (Finlay and Zigmond, 1997). However, the effects of stress on the injured dopaminergic nigrostriatal system are
relatively unexplored. The dysfunction of mesotelencephalic dopaminergic pathways after exposure to stress and/or depression has implications for the pathophysiology of PD; addressing this issue is important given the comorbidity of PD and depression.

**Anti-glucocorticoids in the treatment of depression**

As noted before, abnormalities in the HPA axis occur in major depression, including elevated levels of cortisol and CRH, as well as non-suppression of the dexamethasone suppression test (Murphy, 1991; Kanner, 2004). If elevated cortisol is involved in the pathophysiology of neuropsychiatric disorders, then anti-glucocorticoid agents disrupting cortisol feedback would possibly be part of effective treatments (Murphy, 1997). Specifically, the GR antagonist mifepristone (RU486) is well tolerated by humans in several clinical studies and produces a rapid reduction of psychotic symptoms in depression (DeBattista et al., 2006).

RU486 was discovered in France at the pharmaceutical company Roussel Uclaf in 1980. It was recognized as a high affinity antagonist for progesterone and glucocorticoid receptors and possessed a low affinity for androgen receptors (Robbins and Spitz, 1996; Brogden et al., 1993). The compound does not appear to affect estrogen, monoamines, histamine, muscarine or mineralocorticoid receptors (DeBasttista et al., 2006). The ability of the compound to block GR results in the loss of negative feedback of cortisol on the HPA axis, leading to an increase in both cortisol and ACTH levels and makes RU486 useful in treating Cushing’s syndrome (DeBattista et al., 2006). This effect is dose dependent and can be eliminated with a sufficient level of glucocorticoids (DeBattista et al., 2006). The effects can be rapid, with only 4 days of treatments needed before improvement was observed in humans (Belandoff et al., 2001), but high doses are needed (Belandoff et al., 2002). It also appears to have potential benefits in treating Alzheimer’s
and bipolar disease, but produces no effect in schizophrenia treatment (Gallagher et al., 2005; DeBattista et al., 2006).

In animal studies, treatment with RU486 produced a decrease of immobility time and an increase of swimming in the forced swim test (Wulsin et al., 2010). Treatment also decreased immobility time in animals exposed to maternal separation (Aisa et al., 2008). Neuronal activity in several brain regions involved in stress regulation was affected by RU486 treatment, perhaps indicating increased stress inhibition and decreased stress excitation (Wulsin et al., 2010). Brief treatment with RU486 is also able to reverse decreased hippocampal neurogenesis caused by stress (Oomen et al., 2007). After chronic stress, RU486 can reverse the hippocampal region-specific alterations of synapsin I expression, suggesting a mechanism of action (Wu et al., 2007). These data all underscore the idea that GR antagonists may be effective in reversing glucocorticoid damage observed in depression.

**Chronic variable stress model of depression**

Animal models of depression have been employed to uncover the relationship between stress and depression symptomatology. One such model is chronic variable stress (CVS), which subjects rats (or mice) to various mild stressors in an unpredictable manner over an extended period of time (Katz et al., 1981; Herman et al., 1995; Willner, 2005). In Katz et al. (1981), chronic stress decreased open field activity and caused an increase in the circulating levels of corticosterone, which was reversed by antidepressant treatment. Chronic variable stress results in baseline hypersecretion of corticosterone and ACTH, as well as adrenal hypertrophy (Herman et al., 1995). Similarly to what is observed in humans, CRH levels are elevated in the PVN of animals exposed to CVS (Herman et al., 1995; Wang et al., 2010). In relation to glucocorticoid
receptors, CVS decreases MR mRNA throughout the hippocampus and dentate gyrus (DG), while the GR mRNA is downregulated in the CA1 region, DG and the frontoparietal cortex (Herman et al., 1995). This indicates chronic stress can impact the function that glucocorticoids play with the hippocampus (Herman et al., 1995). The CVS paradigm also caused increases of oxidative damage in several areas including, cortical regions, hippocampus, and striatum (Lucca et al., 2009), paralleling observations seen in depressed patients (Forlenza and Miller, 2006), as well as having a role in the pathology of neurodegenerative diseases such as PD (Howells et al., 2005). Chronic restraint stress, CVS and chronic administration of corticosterone all result in degeneration of apical neuronal dendrites within the hippocampus (Magariños and McEwen, 1995a,b). However, only CVS prevented habituation of the stress response and resulted in adrenal hypertrophy and thymus atrophy (Magariños and McEwan, 1995a).

The use of the CVS model results in a decrease in sucrose intake and reduced conditioned place preference (signs of anhedonia), decreases in sexual behavior and grooming, and sleep dysfunction, all of which have correlates to depression in humans (Papp et al., 1993; Willner, 2005). From some of the earliest work with the model it was known the chronic antidepressant treatment can reverse chronic stress effects (Roth and Katz, 1981). Importantly, chronic, but not acute, treatment with antidepressants results in reversal of these behaviors (Willner, 2005), another feature that has parallels in human depression symptomatology. The anhedonic behavior observed after chronic stress can also be reversed by the administration of dopamine agonists (Papp et al., 1993). Dopamine pathways are a part of the reward system, and the effects of stress on reward perception possibly occurs because of the interaction between the HPA axis and dopaminergic systems (Van Craenebroeck et al., 2005). Chronic stress can also decrease male sexual behavior and aggression (D’Aquila et al., 1994) and reference memory (Vasconcellos et
In our model of CVS, animals receive a mix of both systemic and psychogenic stressors, twice daily (once in the morning, once in the afternoon) in a randomized fashion for a 14-28 days. In this dissertation, we will be utilizing the CVS model to study the effects of stress-induced depression on the nigrostriatal system.

**Neurotrophic factor hypothesis of depression**

The neurotrophic factor hypothesis asserts that loss of neurotrophic factor support may influence neuronal pathology in depression and that antidepressant treatment mitigates depressive symptoms by reversing this loss (Nestler, 2002). Antidepressants often take several weeks to have clinical effect despite being able to influence monoamine levels acutely. Recent research provides evidence that antidepressant actions occur through synaptic and intracellular signaling changes brought about by the downstream targeting of neurotrophic factor expression (Altar, 1999; Nestler et al., 2002; Hashimoto et al., 2004). Neurotrophic factors have the ability to influence proliferation, transmitter phenotype, migration, neurite growth of early neurons, maintenance, regulation of survival and protection of toxically impaired systems (Farkas and Kriegstein, 2002). While levels of neurotrophic factors often decrease after brain development, they still play an important role in cell survival and health and many perturbations can influence their expression including brain injury, stress and physiological activity (Gall and Lauterborn, 1992; Hicks et al., 1995, 1997; Thoenen, 1995, 2000; Poo, 2001). Trophic factors also play an important role in brain plasticity, necessary in learning and memory and cognition (Black, 1999). Loss of neurotrophic levels can result in cell dysfunction, if not death (Duman and Monteggia, 2006).
Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are members of the neurotrophin family of neurotrophic factors. Their high-affinity receptors are trkB and trkC, respectively, part of tyrosine kinase receptor family. Both BDNF and NT-3 can elicit growth of serotoninergic neurons in the adult brain, as well as regrowth of serotoninergic terminals after injury (Mamounas et al., 1996; Grider et al., 2005). Tryptophan hydroxylase, the rate-limiting enzyme for serotonin, is increased in the dorsal raphe nucleus after BDNF infusion (Siuciak et al., 1998). Serotonin and BDNF also have reciprocal actions and complementary roles, such as in cell signaling (Mattson et al., 2004). Serotonin is believed to be one of the major neurotransmitters involved in depression and antidepressant treatment acutely increases serotonin levels. The increased serotonin levels may in turn affect the BDNF levels, which could facilitate the neuroplasticity changes necessary for antidepressant effectiveness (Altar, 1999).

The lack of BDNF may also be involved in depression formation. Postmortem studies in humans have detected a decrease of BDNF in the hippocampus of suicide patients and an increase in those who were receiving antidepressants (Chen et al., 2001; Karege et al., 2005). Decreased BDNF plasma levels were also detected in patients with major depression not treated with antidepressants (Shimizu et al., 2003; Angelucci et al., 2005). Both BDNF and serotonin levels appear to be affected by the aging process (Mattson et al., 2004) and depression becomes a greater risk with age (Steffens et al., 2009). In aged rats, there was a decrease in BDNF levels in the hypothalamus, but less pronounced changes of BDNF levels were observed in the hippocampus (Silhol et al., 2005). However, trkB levels were decreased in both regions in aged animals, meaning BDNF actions could be limited in the aging brain by receptor alterations (Silhol et al., 2005). The loss of trophic support in key regions could increase the risk of
maladaptive cellular plasticity and restoring neurotrophic levels may be essential for antidepressant actions.

Messenger RNA levels of BDNF and trkB are elevated in the hippocampus after chronic (but not acute) antidepressant treatment and electroconvulsive treatment (Nibuya et al., 1995). Administration of exogenous BDNF into the hippocampus and brainstem can produce antidepressant behavioral effects (Siuciak et al., 1997; Shirayama et al., 2002). After chronic antidepressant treatment, trkB is phosphorylated in the anterior cingulate cortex, prefrontal cortex and the hippocampus, all regions involved in depression (Saarelainen et al., 2003). Overexpression of truncated (inactive) trkB prevents antidepressant action (Saarelainen et al., 2003), whereas overexpression of the full-length trkB receptor enhances antidepressant effects (Koponen et al., 2005). However, knockdown of trkB in the forebrain resulted in more hyperactive than depressive behaviors (Zorner et al., 2003). The activation of trkB receptors within the limbic system appears to have regionally specific antidepressant actions.

Still, the role of BDNF in depression development and treatment is not straightforward. The reward pathway, made up of the VTA projecting to the nucleus accumbens and frontal cortex, is believed to play a role in depression (D’Aquila et al., 2000; Nestler, 2002). Perhaps surprisingly, infusion of BDNF into the VTA actually caused a decrease in the latency to immobility in the forced swim test compared to control and infusion of truncated trkB into the nucleus accumbens caused antidepressant effects (Eisch et al., 2003). Both of these experiments suggest BDNF as a pro-depressant in the reward pathway (Eisch et al., 2003). Additionally, viral-mediated knockdown of BDNF in the mesolimbic pathway results in antidepressant-like activity (Berton et al., 2006). Thus, these results stand in contrast to the role BDNF appears to
play in the hippocampus and suggest, in general, that BDNF has complex actions in the regulation of depressive symptoms.

Alterations in neurotrophic factor expression may contribute to the presentation of depressive symptoms (Nestler et al., 2002). As mentioned, stress has a large role in the physiology of depression and causes an antidepressant-reversible decrease of neurogenesis in the hippocampus. After exposure to chronic stress, BDNF mRNA levels are decreased in the hippocampus whereas NT-3 mRNA levels are increased (Smith et al., 1995a; Song et al., 2006). The systemic administration of corticosterone also decreases expression of BDNF mRNA in the hippocampus, but does not affect NT-3 expression (Schaaf et al., 1997, 1998). The decrease of BDNF in the hippocampus after both acute and chronic stress can be reversed by chronic, but not acute treatment with antidepressants (Nibuya et al., 1995). Unpredictable footshock, social isolation, swim stress and maternal deprivation all decrease BDNF hippocampal expression, indicating the decrease is not a stressor-specific effect (Duman and Monteggia, 2006). Administration of corticosterone directly into the brain also results in BDNF downregulation, but adrenalectomy did not result in a complete blockage of stress effects (Smith et al., 1995a), meaning glucocorticoids are not the only factor affecting BDNF expression. Overall, loss of neurotrophic factor support in key brain regions involved in depression and HPA axis regulation may lead to increased vulnerability of neurons to maladaptive plasticity. With respect to the present work, neurotrophic factor modulation after stress or antidepressant treatment in areas involved in PD, and how that may play into the presence of depression in PD, remains to be determined and is the partial focus of this dissertation.
Parkinson’s disease and stress

Stress may play a role in the development of PD. The principle risk factor for PD is aging, which may also be associated with elevated levels of cortisol (Gould and Tanapat, 1999). Cortisol is also elevated in PD patients compared to healthy age-matched controls (Charlett et al., 1998). Acute treatment with levadopa can reduce plasma cortisol levels in PD patients (Müller and Muhlack, 2007), suggesting a connection between dopamine hypofunction and HPA axis hyperactivity. Stressful life events may also precipitate the development of PD. For example, prisoners of war had a much higher incident rate of PD development thirty-five years after release (Gibberd and Simmonds, 1980). Emotional stress can transiently increase motor symptoms (Macht et al., 2007), consistent with acute effects on nigrostriatal function. Appearance of the clinical PD symptoms during a stressful period may reflect damage to the nigrostriatal system that had been masked during the preclinical stage (Snyder et al., 1985). Loss of mesocortical dopamine neurons, which occurs in more advanced stages of PD, may result in the system becoming more vulnerable to stress, as dopamine release in the cortex inhibits stress-activated neurons in the nucleus accumbens (Finlay and Zigmond, 1997). Individuals with PD reduced hedonic responses after exposure to emotional stress, but stress did not affect the selected motor symptoms studied (Macht et al., 2007).

Glucocorticoids may have detrimental effects on PD neurodegeneration by accelerating disease progression (Kibel and Drenjančević-Perić, 2008). Brain regions involved in motor control contain GRs in neurons and glia, with double labeling studies indicating some co-localization present with dopaminergic neurons in the SNpc (Härfstrand et al., 1986; Ahima and Harlan, 1990). Stress not only affects the dopaminergic neurons in the mesocortical and mesolimbic pathways as discussed above, but the nigrostriatal pathway as well (Keefe et al.,
1990). Animals subjected to stress have increases in the motor impairments resulting from dopamine loss (Snyder et al., 1985). Acute and chronic stress, as well as chronic corticosterone administration, impaired movement during the reaching test in animals and can be reversed by administration of anxiolytic compounds, suggesting both glucocorticoids and stress-associated anxiety can influence motor function (Metz et al., 2005). After lesioning, akinetic animals are still responsive to stress and can release a relatively increased amount of extracellular dopamine, supporting the idea that stress can modulate the injured nigrostriatal system (Keefe et al., 1990). Together these studies indicate that stress can impinge on the dopaminergic control of motor movements, with glucocorticoids possibly affecting the compensatory response to the damaged motor system (Metz, 2007). Interestingly, the administration of dexamethasone diminished dopamine content in MPTP-treated animals at a high dose, but was able to protect against dopamine loss at a lower dose, demonstrating that steroids can also have potential neuroprotective properties at the right dosage (Kurkowska-Jastrzębska et al., 2004).

Other forms of stress may influence nigrostriatal structure and function in animal models. Periodic maternal separation during early development increased motor behavior deficit and tyrosine hydroxylase loss within the striatum in rats treated with 6-OHDA as adults (Pienaar et al., 2008). Maternal care disruption can have other long-term effects such as the appearance of depressive symptoms in adult animals (Matthews and Robbins, 2003). Conversely, exercise can be neuroprotective in PD in both basic and clinical studies (Mabandla and Russell, 2010). Rats subjected to maternal separation during the neonatal stress hyporesponsive period of development and later allowed to voluntarily exercise showed reduced forelimb akinesia and forelimb-use asymmetry compared to non-exercised rats (Mabandla et al., 2009). Voluntary exercise also attenuated the increased neurodegeneration seen in lesioned animals exposed to
mild prenatal stressors (Mabandla et al., 2009). However, in another study voluntarily exercised animals subjected to mild stressors as adults had a reduced protection against 6-OHDA lesions compared to non-stressed exercised animals (Howells et al., 2005). Thus, the timing and intensity of the stressors may affect the efficiency of exercise being neuroprotective.

In the striatum, stress increases the extracellular availability of glucocorticoids, glutamate, and dopamine (Smith et al., 2002). These all have the capacity to harm neurons separately and perhaps can even act in a synergistic manner to cause or exacerbate neuronal damage (Smith et al., 2002). Acute restraint stress given prior to administration of 6-OHDA increases behavioral and neurochemical deficits compared to animals that only received 6-OHDA (Smith et al., 2002). It may be that stress alone cannot cause damage to the nigrostriatal system, but can increase susceptibility to an insult (Smith et al., 2002). Acute restraint stress also elevates nigrostriatal dopamine as well as factors involved in dopamine synthesis, including tetrahydrobiopterin and tyrosine hydroxylase (Kim et al., 2005). Excitatory amino acids such as glutamate can act on receptors within the SNpc, increasing the striatal release of dopamine during stress exposure (Castro and Zigmond, 2001). As noted earlier, excitatory amino acids play a role in the neurodegeneration seen in the hippocampus after stress exposure (Magariños and McEwen, 1995b). The increase of dopamine caused by excitatory amino acids may increase the vulnerability of the neurons within the nigrostriatal pathway and produce a neurotoxic effect (Castro and Zigmond, 2001). Both chronic stress and corticosterone administration resulted in increased motor impairment in lesioned animals (Smith et al., 2008). However, although this study noted an increase in Fluoro-Jade labeled cells (indicating neurodegeneration) in the SNpc, there was no reduction in the number of tyrosine hydroxylase-positive cells (Smith et al., 2008). Interestingly, a recent report claims that lesioning of SNpc decreases cell proliferation within the
subgranular zone of the hippocampus, which chronic antidepressant treatment can attenuate (Suzuki et al., 2010). This suggests that not only can stress affect motor symptoms, but that damage to the nigrostriatal motor system could affect the control of the stress response.

**A combined depression/Parkinson’s diseases animal model**

In our own studies, we have developed an animal paradigm that combines the striatal 6-OHDA lesion model of PD with the CVS model. With this new model, we are able to examine the combined effects that depressive-like symptoms and relatively progressive dopaminergic neuron loss can have on motor symptoms and nigral cell degeneration. Dysfunction of the HPA axis is well tied to depression symptomatology and plays a part in neuropsychiatric disorder development. What is not known at this juncture is the exact role stress and affective disorders may play in the development or progression of neurodegenerative disorders such as PD. In humans, motor symptoms appear only after 70-80% of striatal dopaminergic content is lost, likely due to compensatory mechanisms in the remaining nigral neurons. The malfunction of the stress system could lead to an accelerated neurodegeneration in a positive feedback-type scenario. While acute and chronic stress do not appear to greatly affect the dopaminergic nigrostriatal pathway in healthy animals, little research has been done to determine the effects of stress on this system in the disease state of PD in both humans and animal models. We envision that use of our new animal model will help address the relationship of stress-induced depressive-like symptoms and PD dysfunction, uncover the underlying mechanisms, and hopefully generate new therapies to treat both mood and motor aspects of the disorder.
Objectives

This dissertation examines the effects of CVS on motor deficits and degeneration of the dopaminergic nigrostriatal pathway in the 6-OHDA model of PD. The overall hypothesis is that CVS exposure will worsen motor function and neurodegeneration of midbrain dopamine neurons, and that excess corticosterone and/or neurotrophic factor alterations may contribute to the increased behavioral dysfunction and neuronal loss.

Specific Aims

Aim 1A: Determine whether CVS flanking (before and after) a neurotoxin lesion exacerbates experimental parkinsonian symptomatology.

Aim 1B: Determine whether CVS preceding the neurotoxin lesion worsens experimental parkinsonian symptomatology.

Aim 1C: Determine whether CVS following the neurotoxin lesion worsens experimental parkinsonian symptomatology.

We hypothesize that the combined CVS/neurotoxin PD model will exhibit worsened motor behavior and exacerbated neurodegeneration compared to that of the 6-OHDA model alone. As the exact temporal association between PD and depression is unknown, we have designed several different temporal iterations using the combined model to study the relationship.

Aim 2A: Determine whether corticosterone administration alone will enhance the detrimental effects of experimental parkinsonism.
Aim 2B: Determine if the glucocorticoid receptor antagonist RU486 (mifepristone) will alleviate motor symptoms and neurodegeneration present in the combined chronic stress/Parkinson’s disease model.

We hypothesize that corticosterone is sufficient to cause the increased neurodegeneration observed in the combined CVS/PD model. First, we will test whether chronic administration of corticosterone alone can mediate the CVS-induced exacerbation of PD symptoms and cell degeneration. Next, we will test whether the blocking the glucocorticoid receptor, which is expressed by midbrain dopaminergic neurons, can prevent the increased parkinsonian deficits observed in the combined model.

Aim 3: Determine the effects of CVS and antidepressant treatment on neurotrophic factor expression in brain regions involved in Parkinson’s disease and depression.

We hypothesize that CVS will modify the expression of neurotrophic factors in regions involved in PD and depression. We will expose animals to the CVS regimen and then examine the mRNA expression of neurotrophic factors that are known to be neuroprotective for dopamine neurons. Finally, we hypothesize that animals receiving chronic antidepressant treatment will regulate expression of neurotrophic factors in directions opposite to those seen after CVS exposure.
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Chapter 2

Effects of chronic variable stress on dopaminergic cell degeneration and motor deficits in experimental parkinsonism

Abstract

Depression is diagnosed in about 40-50% of patients with Parkinson’s disease (PD) and greatly reduces the quality of life. Because of the high comorbidity, it is possible that there is an underlying convergence of pathological processes. We have developed a novel model by combining the chronic variable stress (CVS) regimen with a partial lesion model of PD. The CVS model can produce both physiological and behavioral aspects of depression and allows us to examine the effects of depression on PD progression. The exact temporal relationship between PD and depression is unknown at this time. To address this issue we designed several different temporal iterations using our model. Adult male Sprague Dawley rats were administered random stressors twice daily for two weeks before and after receiving a unilateral striatal 6-OHDA lesion. In a second iteration, animals received CVS only prior to the nigrostriatal lesion. In the third paradigm, animals were subjected to CVS only after the neurotoxin lesion. Examination of motor deficits using the forelimb-use asymmetry test (cylinder test) was performed two and four weeks post-lesion. The animals were then perfused and processed for tyrosine hydroxylase (TH) and neuronal nuclei (NeuN) immunoreactivity in the ventral midbrain. Analysis of the behavioral data and stereological cell counting of TH- and NeuN-positive cells in the substantia nigra pars compacta revealed that CVS concomitant with (i.e. flanking) the partial lesion exacerbated the motor deficits and the nigral neurodegeneration present in the 6-OHDA model. However, we found that exposure of the animals to CVS either prior to or following the lesion did not worsen either the motor deficits and neurodegeneration.
These data demonstrate that chronic stress-induced depression enhances behavioral dysfunction and accelerates dopaminergic neuronal death in the injured nigrostriatal system. These findings also indicate that stressors which are concurrent with the progressive nigrostriatal lesion are more critical to the exacerbation of neurodegeneration than those stressors that occur only pre- or post-lesion. The present chronic stress-induced increase of parkinsonian symptomatology underscores the importance of carefully screening PD patients for depression.
Introduction

Depression is highly correlated with Parkinson's disease (PD) and is often said to contribute more to the lowered quality of life than the debilitating motor symptoms (Cummings, 1992; Richard and Kurlan, 1997; Kuopio et al., 2000; McDonald et al., 2003). However, as most PD research focuses on the motor symptoms, this non-motor aspect of the disease remains understudied. Given its high rate of occurrence in PD compared to other chronic diseases, it is not believed that depression in PD is merely a psychological reaction to the diagnosis (Menza and Mark, 1994; Cummings, 1992; McDonald et al., 2003). Depression in PD may result from the convergence of the disease processes (Leentjens, 2004), but the exact temporal relationship between PD and depression is presently unresolved. There are two schools of thought on the comorbidity of depression and PD. One is that the onset of depression precedes and possibly leads to PD (e.g. Nilsson et al., 2000), the other that PD predisposes for depression (e.g. Remy et al., 2005). Clinically, evidence exists for both of those lines of thinking. For example, there is a high incidence of depression in patients later diagnosed with PD (Shiba et al., 2000; Leentjens et al., 2003; Ishihara and Brayne, 2006). However, more severe symptoms and longer disease duration are also associated with depressed PD patients, possibly indicating that depression occurs in a more advanced disease state (Pålhagen et al., 2008). Development of better animal models is necessary to study the comorbidity of the two disorders, as most current PD models only focus on replicating the motor symptoms of the disease.

Impairment of the hypothalamic-pituitary-adrenal (HPA) axis is observed in many depressed patients (Kanner, 2004). In depression, dysfunction of limbic system brain regions such as the hippocampus and frontal cortex are believed to be involved in the manifestation of behavioral symptoms (Nestler et al., 2002b). Many of the same brain structures involved in
neuropsychiatric diseases also have a role in stress regulation (Herman et al., 2005). Chronic stress likely results in alterations in the limbic system and possibly leads to the formation of disease states such as depression (Herman and Cullinan, 1997). For example, elevation of glucocorticoid levels can lead to alteration of dendritic plasticity in the hippocampus and the prefrontal cortex (Magariños and McEwen, 1995; Brown et al., 2005). In the present study, we made use of the chronic variable stress (CVS) procedure to produce behavioral, physiological and hormonal symptoms of depression in rats (Herman et al., 1995; Willner, 2005). This protocol has been advanced as an animal model to mirror human depression (Nestler et al., 2002a; Willner, 2002, 2005).

Animals subjected to CVS are given a variety of stressors in randomized fashion over a chronic period. In our model of CVS, animals receive a combination of both systemic and psychogenetic stressors. An advantage of CVS over other chronic stress models is the prevention of stress habituation by randomizing the stressors (Magariños and McEwen, 1995). The CVS regimen is known to result in the hypersecretion of adrenocorticotrophic hormone and corticosterone, increases in corticotrophin-releasing hormone mRNA in the paraventricular nucleus, as well as adrenal hypertrophy (Herman et al., 1995). The use of CVS results in many behavioral symptoms that have correlates to human depression, including anhedonia, weight loss and sleep dysfunction (Papp et al., 1993; Willner, 2005). Importantly these behavioral symptoms can be reversed by chronic, but not acute, antidepressant treatment (Roth and Katz, 1981; Willner, 2005). Glucocorticoid receptors are located throughout the brain in regions involved in both depression and PD, including the substantia nigra pars compacta (SNpc) (Härflstrand et al., 1986; Ahima and Harlan, 1990), indicating that glucocorticoids have the potential to act directly on the dopaminergic neurons involved in PD. Whereas in most
experiments stress does not appear to affect the normal nigrostriatal pathway, there is little research on the effects of stress on injured nigral dopaminergic neurons.

Stress dysfunction may be involved in the pathophysiology of PD. Stress or stress-induced depression has been hypothesized to exacerbate parkinsonian symptoms, possibly by increasing the vulnerability of midbrain dopamine cells to degeneration (Smith et al., 2002). Oxidative damage, which is associated with PD pathology, is elevated in several regions including the hippocampus, striatum and cortex after chronic stress (Howells et al., 2005; Lucca et al., 2009). In the striatum, stress can result in the elevation of dopamine, glutamate and glucocorticoids (Smith et al., 2002). In one of the few studies finding stress effects on the nigrostriatal system, acute stress results in oxidative stress sand the elevation of dopamine and tyrosine hydroxylase (TH) (Kim et al., 2005). The increase in dopamine caused by stress may create a neurotoxic environment that increases the vulnerability of nigrostriatal neurons (Castro and Zigmond, 2001). Knowing how depression affects the progression and severity of PD is essential to understanding the connection between the two diseases and, most importantly, to developing optimal treatments for depression occurring with PD.

The present study utilized a novel approach that combines animals lesioned with a striatal injection of 6-hydroxydopamine (6-OHDA), an experimental PD model, with CVS, which produces depressive-like symptoms. The method of lesioning chosen offers an advantage over other neurotoxin models by creating a more progressive lesion occurring over a several week span, with some neurodegeneration observed even 16 weeks out after the initial lesion (Sauer et al., 1994; Kirik et al., 1998). Not only does this better mirror the disease state of human PD, but it also provides a window for other factors to influence the level of neurodegeneration. We hypothesize that stress-induced depression will worsen the motor deficits and increase the
neurodegeneration present in the nigrostriatal system after the unilateral striatal injection of 6-OHDA. To help clarify the temporal aspects of depression on PD symptoms, we examined the effects of stress-induced depression on motor deficits and neurodegeneration in animals exposed to CVS only prior to the 6-OHDA lesion, only after the 6-OHDA lesion, and both prior to and after the 6-OHDA lesion.

**Methods**

*Experimental Timelines*

*Experiment 1:*

In the first iteration, animals were exposed to chronic stress both before and after (i.e. flanking) the lesion (Fig. 1A). Animals were exposed to CVS for two weeks and then unilaterally lesioned in the striatum using 6-OHDA. After allowing a few days for recovery, animals were again subjected to two weeks of CVS, with the stressors required for mobility (e.g. swimming) removed. Two and four weeks after the lesion, the forelimb asymmetry test was used to collect motor behavioral data. Animals were then perfused and processed for TH and NeuN immunohistochemistry and unbiased stereological cell counting.

*Experiment 2:*

In this experimental iteration, exposure to stress occurred prior to the experimental PD lesion (Fig. 1B). Animals were exposed to a CVS regimen for two weeks. Following CVS, the animals received a unilateral striatal 6-OHDA lesion. At two and four weeks post-lesion, behavioral data were collected using the forelimb asymmetry test. Animals were then sacrificed and processed for TH and NeuN immunohistochemistry and unbiased stereological cell counting.
**Experiment 3:**

In the final iteration, animals received the progressive lesion prior to the CVS treatment (Fig. 1C). Animals were administered a unilateral 6-OHDA lesion, and subsequently, exposed to two weeks of a CVS regimen. At two and four weeks post-lesion, the forelimb asymmetry test was used to measure motor behavior. At four weeks post-lesion, animals were sacrificed and processed for immunohistochemistry and unbiased stereological cell counting.

**Animals**

Adult male Sprague Dawley rats (230-360 g) from Harlan Laboratories (Indianapolis, IN) were used (n=6-10/group). Animals were kept two per cage in standard housing conditions (12 hr on/off light cycle) and food and water were available *ad libitum*. Animals were treated in accordance with the University of Cincinnati Institutional Animal Care and Use Committee. For each of the experimental iterations, animals were randomly divided into four weight-matched groups: two groups exposed to CVS and the other two serving as non-stressed control groups. Half of each of the CVS and control animals also received 6-OHDA, while the other half received vehicle injections (control/vehicle, control/6-OHDA, CVS/vehicle, CVS/6-OHDA).

**Partial 6-OHDA lesion**

Animals received anesthetic (87 mg/kg ketamine, 13 mg/kg xylazine; i.p.) and then were placed into a stereotaxic apparatus. Animals subsequently received two unilateral injections of the catecholamine-specific neurotoxin 6-OHDA (10 µg each in 2 µl saline + 0.2% ascorbic acid) or 6-OHDA vehicle (saline + ascorbic acid) placed into the right striatum using the following coordinates: AP:+1.6, ML:-2.4, DV:-4.2 and AP:+0.2, ML:-2.6, DV:-7.0 (Paxinos and Watson, 2007) using a 5-µl Hamilton syringe (Hamilton Company, Reno, NV). Injection of 6-OHDA into the striatum creates a relatively progressive degeneration of the dopaminergic nigrostriatal
Figure 1

A. Experiment 1: CVS flanking the lesion

B. Experiment 2: CVS prior to lesion

C. Experiment 3: CVS post-lesion only

Figure 1. Timelines of the experimental paradigms. **A:** Animals received stress for two weeks both prior to and after the lesion. Two and four weeks after the lesion behavioral data were collected and the animals were sacrificed and processed for neuroanatomical data. **B:** Animals were exposed to two weeks of stress prior to lesioning and no stress was given after the lesion. Behavioral data were obtained two and four weeks post-lesion before animals were sacrificed. **C:** Animals were exposed to two weeks of stress after lesioning (not before). Behavioral data were obtained two and four weeks post-lesion before animals were sacrificed and processed for neuroanatomical data.
pathway (Sauer et al., 1994; Kirik et al., 1998). For each injection, the needle was lowered into
the brain parenchyma for five minutes to equilibrate, the 2-µl injection was administered over ten
minutes, and the needle was then allowed to remain in the brain for an additional five minutes
before slowly being removed to prevent backflow along the needle track.

**Chronic Variable Stress Regimen:**

The CVS protocol has been extensively characterized previously (Herman et al., 1995). The following eight stressors composed the present CVS regimen: cold exposure (1h in a 4°C room), restraint (1h in plastic restraint tubes), vibration (1h on 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 (6/cage, overnight), isolation (1/cage, overnight). After surgeries, stressors requiring mobility (i.e. the swim stressors) were not administered. In order to prevent habituation, stressors were administered in a random fashion. Animals in the CVS group were exposed to two stressors each day during the light cycle, one in the morning and one in the afternoon, with the overnight stressors administered intermitantly throughout the regimen (see Table 1). The body weights of stressed and non-stressed animals were measured throughout each experiment to monitor the effectiveness of the chronic stress regimen.

**Forelimb Asymmetry Test**

Behavioral data were collected using the forelimb asymmetry test (cylinder test) to assess motor impairment as previously described (Schallert et al., 2000, 2006). This test is advantageous over other behavioral measurements such as amphetamine-induced rotations, as it is a natural, low-stress behavior and does not involve the administration of a drug. The behavioral test occurred during the dark cycle under low-light conditions. Briefly, each rat was placed individually into a transparent Plexiglas cylinder and allowed to explore the walls
### Table 1

#### CVS Regimen

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Week 2</th>
</tr>
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<tbody>
<tr>
<td><strong>Day 1</strong>: AM: Shaker (1 h)</td>
<td><strong>Day 8</strong>: AM: Cold swim (10 min)</td>
</tr>
<tr>
<td><strong>Day 1</strong>: PM: Hypoxia (30 min) + o/n crowding</td>
<td><strong>Day 8</strong>: PM: Warm swim (20 min) + o/n isolation</td>
</tr>
<tr>
<td><strong>Day 2</strong>: AM: Warm swim (20 min)</td>
<td><strong>Day 9</strong>: AM: Cold swim (10 min)</td>
</tr>
<tr>
<td><strong>Day 2</strong>: PM: Cold room (1 h)</td>
<td><strong>Day 9</strong>: PM: Cold room (1 h)</td>
</tr>
<tr>
<td><strong>Day 3</strong>: AM: Shaker (1 h)</td>
<td><strong>Day 10</strong>: AM: Warm swim (20 min)</td>
</tr>
<tr>
<td><strong>Day 3</strong>: PM: Cold swim (10 min)</td>
<td><strong>Day 10</strong>: PM: Shaker (1 h)</td>
</tr>
<tr>
<td><strong>Day 4</strong>: AM: Shaker (1 h)</td>
<td><strong>Day 11</strong>: AM: Cold room (1 h)</td>
</tr>
<tr>
<td><strong>Day 4</strong>: PM: Warm swim (20 min) + o/n isolation</td>
<td><strong>Day 11</strong>: PM: Shaker (1 h) + o/n crowding</td>
</tr>
<tr>
<td><strong>Day 5</strong>: AM: Cold room (1 h)</td>
<td><strong>Day 12</strong>: AM: Hypoxia (30 min)</td>
</tr>
<tr>
<td><strong>Day 5</strong>: PM: Cold swim (10 min)</td>
<td><strong>Day 12</strong>: PM: Warm swim (20 min)</td>
</tr>
<tr>
<td><strong>Day 6</strong>: AM: Hypoxia (30 min)</td>
<td><strong>Day 13</strong>: AM: Hypoxia (1 h)</td>
</tr>
<tr>
<td><strong>Day 6</strong>: PM: Shaker (1 h) + o/n crowding</td>
<td><strong>Day 13</strong>: PM: Cold swim (10 min) + o/n isolation</td>
</tr>
<tr>
<td><strong>Day 7</strong>: AM: Cold room (1 h)</td>
<td><strong>Day 14</strong>: AM: Cold room (1 h)</td>
</tr>
<tr>
<td><strong>Day 7</strong>: PM: Hypoxia (30 min)</td>
<td><strong>Day 14</strong>: PM: Hypoxia (30 min)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 15</strong>: AM: Restraint (1 h)</td>
<td><strong>Day 22</strong>: AM: Cold room (1 h)</td>
</tr>
<tr>
<td><strong>Day 15</strong>: PM: Shaker (1 h)</td>
<td><strong>Day 22</strong>: PM: Shaker (1 h) + o/n crowding</td>
</tr>
<tr>
<td><strong>Day 16</strong>: AM: Hypoxia (30 min)</td>
<td><strong>Day 23</strong>: AM: Shaker (1 h)</td>
</tr>
<tr>
<td><strong>Day 16</strong>: PM: Cold room (1 h)</td>
<td><strong>Day 23</strong>: PM: Hypoxia (30 min) + o/n isolation</td>
</tr>
<tr>
<td><strong>Day 17</strong>: AM: Hypoxia (30 min)</td>
<td><strong>Day 24</strong>: AM: Restraint (1 h)</td>
</tr>
<tr>
<td><strong>Day 17</strong>: PM: Cold room (1 h) + o/n crowding</td>
<td><strong>Day 24</strong>: PM: Shaker (1 h)</td>
</tr>
<tr>
<td><strong>Day 18</strong>: AM: Shaker (1 h)</td>
<td><strong>Day 25</strong>: AM: Hypoxia (30 min)</td>
</tr>
<tr>
<td><strong>Day 18</strong>: PM: Restraint (1 h)</td>
<td><strong>Day 25</strong>: PM: Cold room (1 h) + o/n crowding</td>
</tr>
<tr>
<td><strong>Day 19</strong>: AM: Cold room (1 h)</td>
<td><strong>Day 26</strong>: AM: Shaker (1 h)</td>
</tr>
<tr>
<td><strong>Day 19</strong>: PM: Hypoxia (30 min) + o/n isolation</td>
<td><strong>Day 26</strong>: PM: Restraint (1 h) + o/n isolation</td>
</tr>
<tr>
<td><strong>Day 20</strong>: AM: Restraint (1 h)</td>
<td><strong>Day 27</strong>: AM: Cold room (1 h)</td>
</tr>
<tr>
<td><strong>Day 20</strong>: PM: Hypoxia (30 min)</td>
<td><strong>Day 27</strong>: PM: Hypoxia (30 min)</td>
</tr>
<tr>
<td><strong>Day 21</strong>: AM: Cold room (1 h)</td>
<td><strong>Day 28</strong>: AM: Shaker (1 h)</td>
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<td><strong>Day 21</strong>: PM: Restraint (1 h)</td>
<td><strong>Day 28</strong>: PM: Restraint (1 h)</td>
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naturally by rearing up and using their forelimbs for weight support. A rat was considered to have completed the test when it had performed 20-25 touches. The data were recorded and analyzed by counting the number of times each rat used their right, left or both forelimbs to explore the side of the wall, as previously described (Schallert et al., 2000). A percent limb usage score was calculated from the behavioral data using the formula \[
\frac{(\text{contralateral side} + \frac{1}{2} \text{both})}{(\text{ipsilateral side} + \text{contralateral side} + \text{both})} \times 100. 
\] A score of 50% indicates equal use of both forelimbs, a score above 50% indicates a preference for using the contralateral left forelimb and a score below 50% indicates a preference for using the ipsilateral right forelimb. The cylinder test was performed two and four weeks post-lesion for all experimental iterations. Statistical analyses included one-way ANOVA followed by Neuman-Kuels post-hoc test. Data are presented as mean ± SEM. Significance is considered at \( P < 0.05 \).

**Immunohistochemistry**

One day after the final behavior test, the animals were deeply anesthetized and sacrificed via intracardial perfusion using saline and 4% paraformaldehyde (PFA). Following perfusion, the brains of the animals were collected, as well as the adrenal glands and thymus. The brains were placed in 4% PFA, post-fixed for 24 hours and subsequently placed into 30% sucrose. The thymus and adrenal glands were cleaned and weighed. Coronal sections of the brains through the ventral mesencephalon were cut (50 µm) on a sliding microtome and placed in cryoprotectant. Six sections per brain (600 µm apart) were processed for TH (catecholamine biosynthetic enzyme and marker for midbrain dopamine cells) or neuronal nuclei (NeuN; a general neuronal marker) according to our routine immunohistochemical procedures (e.g. Seroogy et al., 1994). Free-floating sections were washed in 0.1 M phosphate buffer (PB), and non-specific staining was blocked with normal horse serum (NHS) for TH or normal goat serum
(NGS) for NeuN. Sections were incubated overnight at 4°C in the primary antibody (mouse anti-TH (1:8000) or mouse anti-NeuN (1:1000) both from Chemicon International, Temecula, CA) prepared with 1% NHS or NGS and 0.2% Triton-X 100 in 0.1 M PB. The following day, sections were washed in 0.1M PB, blocked with 2% NHS (TH) or NGS (NeuN), and incubated with the anti-mouse IgG biotin-conjugated secondary antibody [horse anti-mouse (1:200) for TH and goat anti-mouse (1:400) for NeuN; both from Vector Laboratories, Inc. Burlingame, CA] for 1 hour. After washes in 0.1 PB, the tissue was placed in ABC peroxidase (Vector Laboratories) for 30 minutes and then washed in 50 mM Tris buffer (pH 7.5). Labeling was visualized using the diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories) containing 0.3% H₂O₂, buffer, and DAB reagent. Sections were given a final series of Tris buffer washes, mounted onto Superfrost plus microslides (VWR, Batavia, IL), dehydrated using a series of ethanol washes and coverslipped.

**Stereological Cell Counts**

In each group, cells immunostained for TH and NeuN were counted in the SNpc on evenly spaced sections throughout the rostrocaudal extent of the ventral mesencephalon. Cell estimates of both the lesioned and unlesioned sides of the SNpc were determined using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased stereological techniques (West, 1993). The sections were viewed on an Olympus BX-60 microscope (Melville, NY) using a CCD video camera (HV-C20, Hitachi, San Jose, CA). Contours were determined at 2X magnification and cell counting was performed at 60X using the optical fractionator. Random sample sites were determined by the software on a grid size of 170x100 for TH staining and 220x220 for NeuN staining. A guard of 2 µm was placed for each sample section. The coefficient of error was calculated using the Gundersen correction for each animal and was lower...
than 0.10. The extent of cell loss was determined by comparing the lesioned side to the contralateral unlesioned side of the SNpc. Statistical analyses included one-way ANOVA followed by Neuman-Kuels post-hoc test. Data are presented as mean ± SEM. Significance is considered at $P < 0.05$.

Results

Experiment 1

Animal body weights

Animal body weights were monitored throughout the flanking experiment, to determine the efficacy of the CVS regimen. All four groups were weight-matched at the beginning of the experiment (Fig. 2). Over the next four weeks, in which animals received CVS flanking the lesion, the stressed groups weighed significantly less than the control animals ($F_{3,18} = 6.454$ (week 2), $F_{3,18} = 6.317$ (week 3); $F_{3,18} = 3.457$ (week 4), $F_{3,18} = 3.622$ (week 5); Fig. 2; $P < 0.01$). After the cessation of the stressors, the differences between the groups became insignificant. There were no body weight differences between the two control groups and the two stressed groups.

Adrenal glands and thymus weight

In this first experiment, where animals received CVS concomitant with the 6-OHDA lesion, there were no differences in the weights between the stressed and unstressed groups or the lesioned and unlesioned groups for the adrenal glands and thymus collected after sacrifice (Fig. 3).

Behavior

The cylinder test (forelimb asymmetry test) was used to evaluate any motor behavioral
**Figure 2.** Weights of control and CVS animals exposed to stress-induced depression flanking the 6-OHDA lesion. There were no differences in weight at the beginning of the study. During the first two weeks of CVS, stressed-exposed animals gained less weight compared to control animals. After the lesion, animals received an additional two weeks of CVS and continued to gain less weight. In the last two weeks, when the animals were no longer exposed to stress, the weights of CVS animals reached the same weight as the non-stressed controls. Data are presented as mean ± SEM. *P < 0.01 vs. controls. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
Figure 3. A: The thymus weights of animals exposed to the CVS regimen both prior to and after the lesion. There were no significant differences among the groups. B: Adrenal gland weights. No differences were found among any of the groups. Data are presented as mean ± SEM. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
**Figure 4.** Assessment of behavioral motor deficits in animals exposed to CVS flanking the lesion using the cylinder test. At both two and four weeks post-lesion, limb usage of the lesioned animals compared to the vehicle-treated animals was significantly impaired. At four weeks (but not two weeks) post-lesion, CVS-treated lesioned rats (CVS/6-OHDA) also exhibited a significantly exacerbated reduction in impaired forelimb usage compared to the unstressed lesioned rats (Con/6-OHDA). There were no differences between vehicle-treated animals. Data are presented as mean ± SEM. #P < 0.05 for Con/6-OHDA vs CVS/6-OHDA; *P < 0.01 for Con/Veh and CVS/Veh vs Con/6-OHDA and CVS/6-OHDA. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
Figure 5.  A-B: TH+ and NeuN+ stereological cell counts in the SNpc of animals receiving CVS flanking the lesion. Although both lesioned groups lost a significant amount of cells compared to controls, cell counts show that the TH+ cell loss was exacerbated in the CVS/6-OHDA rats vs the control/6-OHDA rats. NeuN+ cells were reduced in a similar pattern and number. There were no differences between the vehicle-treated animals. Data are presented as mean ± SEM. #P < 0.05 for Con/6-OHDA vs CVS/6-OHDA; *P < 0.001 for Con/Veh and CVS/Veh vs Con/6-
OHDA and CVS/6-OHDA). C: High power photomicrographs of TH immunostaining in the SNpc of animals receiving CVS flanking the lesion. Note that whereas the two 6-OHDA lesioned groups exhibited TH+ cell loss, there was a greater loss of TH+ cells in the CVS/6-OHDA group. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
differences between the stressed and non-stressed animals, both lesioned and unlesioned (Fig. 4). In animals exposed to CVS both prior to and after receiving 6-OHDA, behavioral data were collected at two and four weeks post-lesion. There were no behavioral differences between the stressed and unstressed vehicle-injected groups. There was a reduction in the use of the impaired contralateral limb in the lesioned animals compared to the unlesioned animals at both post-lesion time points ($F_{3,20} = 11.54$ (2 weeks post-lesion), $F_{3,19} = 23.89$ (4 weeks post-lesion); Fig. 4; $P < 0.01$). Additionally, there was a significant decrease in the asymmetry score in the CVS/6-OHDA animals compared to control/6-OHDA at 4 weeks, indicating a worsening of motor impairment in stressed lesioned animals (11% vs. 26% respectively; $P < 0.05$).

**Stereological cell counts**

Stereological cell counts were obtained to determine whether there was a difference in neurodegeneration within the SNpc between the animals receiving CVS and the control groups. Immunostaining for TH was used to label nigral dopaminergic neurons and the extent of neurodegeneration was determined by comparing the lesioned side to the unlesioned contralateral side of the SNpc (Fig. 5). There were no differences obtained in numbers of nigral TH+ cells between the two vehicle-injected groups [16,894 (control/vehicle) and 17,290 (CVS/vehicle)]. As shown in Fig. 5, animals that were exposed to CVS flanking the 6-OHDA lesion demonstrated substantially decreased numbers of TH+ cells in the SNpc compared to the vehicle-treated animals [$F_{3,19} = 69.18; P < 0.01$; 5,914 (control/6-OHDA and 4,053 (CVS/6-OHDA)]. Moreover, the animals that received CVS as well as a 6-OHDA lesion (CVS/6-OHDA) exhibited a significantly lower number of TH+ cells compared to control animals that received only 6-OHDA (control/6-OHDA group) (Fig. 5A; 18% vs. 37% respectively; $P < 0.05$). NeuN+ cell counts revealed that the pattern and numbers of cells lost were similar to that obtained for
Figure 6. Weights of control and CVS animals exposed to stress-induced depression two weeks prior to the lesion. Animals were weight-matched at the beginning of the study. During the two weeks of CVS, stressed-exposed animals gained weight at a significantly lesser rate compared to control animals. After the lesion, the animals were no longer exposed to stress and by one week post-lesion the weights of CVS animals attained the same weight as the non-stressed controls. Data are presented as mean ± SEM. *P < 0.001 vs. controls. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
**Figure 7.** 

**A:** The thymus of animals exposed to a 2 week CVS regimen prior to lesion were weighed. There was no significant difference among the groups. 

**B:** Both adrenal glands from the rats were removed at the time of sacrifice and weighed. Quantification of the average weight for each condition showed no differences among any of the groups. Data are presented as mean ± SEM. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
Figure 8. Assessment of motor deficits in animals exposed to CVS prior to the lesion using the cylinder test. At both 2 and 4 weeks post-lesion, lesioned rats exhibited a significant reduction in impaired forelimb usage compared to both stress and unstressed unlesioned rats. However, there was no significant difference between the stressed (CVS/6-OHDA) and unstressed (Con/6-OHDA) lesioned animals. Additionally, there were no differences between the vehicle-treated animals. Data are presented as mean ± SEM. *P< 0.01 for Con/Veh and CVS/Veh vs Con/6-OHDA and CVS/6-OHDA. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
Figure 9. A-B: TH+ and NeuN+ stereological cell counts in the SNpc of animals receiving CVS prior to the lesion. Cell counts show that substantial numbers of TH+ cells were loss in the 6-OHDA-lesioned rats, but no differences were found between the two lesioned groups. NeuN+ cells were reduced in a similar pattern and number. Data are presented as mean ± SEM. *P < 0.001 for Con/Veh and CVS/Veh vs Con/6-OHDA and CVS/6-OHDA). C: High power photomicrographs of TH immunostaining in the SNpc of animals receiving CVS prior to the lesion. Note that the two 6-OHDA-lesioned groups exhibited TH+ cell loss to the same extent. n = 8 for Control/Vehicle, Control/6-OHDA, CVS/Vehicle; n = 10 for CVS/6-OHDA.
TH (Fig. 5B). Notably, in animals that received CVS flanking the lesion, there was a significant difference in numbers of NeuN+ cells between the CVS lesioned animals (CVS/6-OHDA group) and the unstressed control lesioned animals (control/6-OHDA group) (Fig. 5B; $P < 0.05$), indicating that the exacerbated loss of TH+ neurons represented frank neuronal degeneration.

**Experiment 2**

**Animal body weights**

In the second experiment, where animals received stress only prior to the lesion, all four groups were weight-matched at the beginning of the experiment (Fig 6). There were no differences between the two stressed groups and the two non-stressed groups during the course of the experiment. During the two weeks of CVS, both groups of stressed animals gained weight at a reduced rate compared to the control non-stressed groups ($F_{3,30} = 14.33$ (week 2), $F_{3,30} = 10.50$ (week 3); Fig. 6; $P < 0.01$). After the lesion surgeries and cessation of CVS, stressed animals regained weight such that by one week after the cessation of CVS, there were no significant differences between the stressed and non-stressed groups for the rest of the experiment.

**Adrenal glands and thymus weight**

In animals receiving CVS before the lesion, thymus and the adrenal glands collected after sacrifice displayed no differences in weight between the stressed and unstressed groups or the lesioned and unlesioned groups (Fig. 7).

**Behavior**

In animals exposed to stress prior to the lesion, there was a significant difference between the stressed and non-stressed 6-OHDA-treated animals and their respective controls at both 2 and
4 weeks, indicating motor impairment in the lesioned groups ($F_{3,14} = 12.48$ (2 weeks post-lesion), $F_{3,14} = 8.102$ (4 weeks post-lesion); Fig. 8; $P < 0.01$). However, there were no significant differences in motor behavior between the CVS and control lesioned groups at either time point. No behavioral differences were observed between the control and CVS unlesioned groups either.

**Stereological cell counts**

In the animals that received CVS prior to the lesion, there was a significant reduction in numbers of TH+ cells in the two lesioned groups compared to the unlesioned animals ($F_{3,14} = 47.75$; Fig. 9; $P < 0.01$). However, there were no differences between the stressed and non-stressed lesioned groups (Fig. 9A). No cell count differences were seen between the two vehicle-injected groups. NeuN+ cell counts were obtained and the pattern and numbers of cells lost were similar to that obtained for TH (Fig. 9B).

**Experiment 3**

**Animal body weights**

In the third experiment, in which the animals received the CVS regimen for two weeks after the lesion, all four groups were weight-matched prior to the start of the experiment. Stressed-exposed animals gained less weight during the two weeks of CVS ($F_{3,28} = 5.643$ (week 2), $F_{3,28} = 5.093$ (week 3); Fig. 10; $P < 0.01$). Upon cessation of stress, the difference between the groups became insignificant. The non-stressed groups were not significantly different between each other, nor were the two stressed groups.

**Adrenal glands and thymus weight**
There were no differences in weight between the stressed and unstressed groups or the lesioned and unlesioned groups for the thymus and the adrenal glands collected after sacrifice in Figure 10. 

**Figure 10.** Lesion + CVS

![Animal Weight Graph](image)

**Figure 10.** Weights of animals exposed to CVS after the lesion only. Animals were placed in weight-matched groups at the beginning of the study. During the two weeks of CVS, stressed-exposed animals gained less weight compared to non-stressed animals. In the last two weeks, the animals were no longer exposed to stress and the weights of CVS animals reached the same weight as the non-stressed controls. Data are presented as mean ± SEM. *P < 0.01 vs. controls. n = 8 for Vehicle/Control, CVS/Vehicle, 6-OHDA/Control; n = 10 for CVS/6-OHDA.
Figure 11. **Lesion + CVS**

**A:** The thymus of animals exposed to two weeks of CVS after the lesion were collected and weighed. There was no significant difference among the groups. **B:** Both adrenal glands were removed and weighed. Combining the weights of both left and right adrenals, quantification revealed no differences among any of the groups. Data are presented as mean ± SEM. $n = 8$ for Vehicle/Control, CVS/Vehicle, 6-OHDA/Control; $n = 10$ for CVS/6-OHDA.
Figure 12. Assessment of motor deficits in animals exposed to CVS after the lesion using the cylinder test. At both two and four weeks post-lesion, 6-OHDA-lesioned animals exhibited a significant reduction in impaired forelimb usage compared to unlesioned rats. However, there was no significant difference between the stressed (CVS/6-OHDA) and unstressed (Con/6-OHDA) lesioned animals. Additionally, there were no differences between the vehicle-treated animals. Data are presented as mean ± SEM. *P< 0.01 for Con/Veh and CVS/Veh vs Con/6-OHDA and CVS/6-OHDA). n = 8 for Vehicle/Control, CVS/Vehicle, 6-OHDA/Control; n = 10 for CVS/6-OHDA.
**Figure 13.** A-B: TH+ and NeuN+ stereological cell counts in the SNpc of animals receiving CVS after the lesion. Quantification shows numerous TH+ cells were lost in the 6-OHDA-lesioned rats, but no differences were found between the two lesioned groups. There were also no differences with the vehicle-treated animals. NeuN+ cells were reduced in a similar pattern and number. Data are presented as mean ± SEM. *P < 0.001 for Con/Veh and CVS/Veh vs Con/6-OHDA and CVS/6-OHDA. C: High power photomicrographs of TH immunostaining in the SNpc of animals subjected to CVS after the lesion. Note that the two 6-OHDA-lesioned groups appear to have exhibited the same extent of TH cell degeneration. n = 8 for Vehicle/Control, CVS/Vehicle, 6-OHDA/Control; n = 10 for CVS/6-OHDA.
animals exposed to CVS after lesioning (Fig. 11).

**Behavior**

In the final iteration, where animals only received CVS after the lesion, there was a significant reduction in the asymmetry scores of the 6-OHDA-treated animals compared to the vehicle-treated animals at both time points ($F_{3,28} = 9.527$ (2 weeks post-lesion); $F_{3,25} = 27.19$ (4 weeks post-lesion); Fig. 12; $P < 0.01$), but there were no differences in motor deficits between the stressed and non-stressed lesioned animals. There were also no behavioral differences between the stressed and non-stressed vehicle-treated groups.

**Stereological cell counts**

In animals given CVS only after the lesion there was significant TH+ cell loss in the two 6-OHDA-lesioned groups compared to the vehicle groups ($F_{3,23} = 31.67$; Fig. 13; $P < 0.01$), but no differences were found between the CVS and control lesioned groups (Fig. 13A). There were no differences between the CVS and control unlesioned groups as well. NeuN+ cell counts demonstrated similar patterns and numbers of cells lost as that obtained for TH (Fig. 13B).

**Discussion**

The major finding from this study is that chronic unpredictable stress flanking (i.e. before and after) a nigrostriatal lesion exacerbates both motor deficits and nigral dopamine cell degeneration found in the experimental PD model. In this paradigm, we created a temporal regimen with the depressive symptoms concomitant with the dopaminergic neurodegeneration using unilateral striatal injections of the neurotoxin 6-OHDA. While CVS/lesioned animals in the flanking paradigm did not exhibit a significant worsening of motor symptoms at two weeks, they did display a significant difference at four weeks. This suggests that the increase in
degeneration occurs over a chronic period. Both pre- and post-6-OHDA lesion CVS exposure is necessary, as either alone is not sufficient to accelerate dopaminergic cell degeneration and associated behavioral dysfunction in the nigrostriatal system.

In all of the experimental paradigms, stressed animals gained weight at a lesser rate during CVS exposure and the differences in weight disappeared after cessation of stressors, indicating that some of the physiological changes caused by chronic stress are reversible. This is also supported by the lack of differences in adrenal gland and thymus weights typically observed immediately after CVS, as they were collected two weeks after the end of stress exposure.

Depression in PD is the most common neuropsychiatric symptom of the disorder, occurring in about 40-50% of patients and greatly impacting the quality of life (Cummings, 1992; Weintraub, 2004). Indeed, studies have indicated that depression is one of the most disabling characteristics of the disease, likely because it is often unrecognized and therefore untreated (Richard and Kurlan, 1997; Kuopio et al., 2000). Research indicates that only about 26% of PD patients receive antidepressant treatment, while almost half of PD patients experience depression (Richard and Kurlan, 1997; Yamamoto, 2001). Depression may precede the onset of motor symptoms (Lauterbach et al., 2004; Chaudhuri et al. 2005), but it is also associated with exacerbated motor deficits and greater disease severity (Papapetropoulos et al., 2006). Patients diagnosed with PD have an increased chance of being previously diagnosed with depression (Shiba et al., 2000; Leentjens et al., 2003; Ishihara and Brayne, 2006). On the other hand, depression is more prevalent in an advanced disease state (Pålhagen et al., 2008) and in the later stages, PD pathology spreads to regions associated with depression symptomatology (Braak et al., 2004; Braak and Del Tredici, 2008). However, depression in PD does not correlate with age or age of onset or parallel the development of physical symptoms (Brown and Jakanshahi, 1995;
Lieberman, 2006; Klotsche et al., 2011). All of this leads to a complex picture, with depression likely present, to some extent, at all stages of the PD. Our experiments in this study sought to clarify whether chronic stress resulting in depressive symptoms can worsen motor deficits and neurodegeneration in experimental parkinsonism. In addition, we investigated temporal aspects of this association by determining whether CVS only prior to or after the onset of motor symptoms was sufficient to affect PD symptomatology. It appears that the presence of stress throughout the course of the neurodegeneration is necessary for worsening of the cardinal symptoms.

The plasticity of many brain regions is affected by chronic stress, particularly those associated with depression. Chronic stress decreases hippocampal neurogenesis and causes atrophy of apical dendrites on pyramidal neurons (Magariños and McEwen, 1995; Duman, 2002). In the prefrontal cortex (PFC), neuronal plasticity is also altered after both chronic stress and chronic corticosterone (Wellman, 2001; Radley et al., 2004; Cerqueira et al., 2005; Dias-Ferreira et al., 2009). Both the hippocampus and the PFC have an inhibitory role in the stress response and loss of this inhibition may result in HPA axis hyperactivity. Stress may also increase neuronal vulnerability, as in hippocampal cell cultures, neurons exposed to glucocorticoids are subsequently more susceptible to cell death when given neurotoxins (Sapolsky, 1994). The widespread presence of glucocorticoid receptors throughout the brain allows the effects of stress to be observed in regions outside the limbic system. Glucocorticoids may be able to act directly on the neurons within the nigrostriatal system, as glucocorticoid receptors are colocalized with dopaminergic cells in the SNpc (Härfstrand et al., 1986; Ahima and Harlan, 1990), pointing to the ability of glucocorticoids to act directly on neurons involved in PD. Significantly, antidepressant treatment reverses many of the alterations observed in the
limbic system (Malberg et al., 2000; Duman and Monteggia, 2002), and antidepressants may be neuroprotective in patients with depression (Sheline et al., 2003; Lavretsky et al., 2005). This has implications for treating PD, with the potential of antidepressants to be neuroprotective for dopaminergic neurons in the SNpc. Indeed, a recent study found that the selective-serotonin reuptake inhibitor paroxetine can decrease neurodegeneration in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice (Chung et al., 2010). However, it remains to be determined whether antidepressant treatment in our combined model may reverse the stress-induced exacerbation of neurodegeneration.

The impairment of the stress response might be involved in the development of PD. The general environment of the aged brain is not optimal as there are increased levels of cortisol (Gould and Tanapat, 1999). Cortisol levels are also increased in PD brains compared to aged-match controls (Charlett et al., 1998). Moreover, stressful life events precede the onset of PD motor symptoms and support the multiple hit hypothesis where earlier injuries increase the vulnerability of the nigrostriatal system to later insults (Gibberd and Simmonds, 1980; Miller and O’Callaghan, 2008). The presence of PD motor symptoms after stress may actually reveal injury to the nigrostriatal system previously concealed because of compensatory mechanisms (Snyder et al., 1985). In animals, maternal separation and mild prenatal stressors increases the severity of motor deficits and striatal dopamine content loss when 6-OHDA is later administered in adults rats (Mabandla et al., 2009; Mabandla and Russell, 2010). However, clinical research has found few links between prenatal risks and PD (Gardener et al., 2010).

In PD, the loss of dopamine in the nigrostriatal pathway is the primary pathological hallmark. While the exact etiology remains unknown, the cell loss is likely the result of a combination of oxidative stress, inflammation, excitotoxicity, and mitochondrial dysfunction.
(Howells et al., 2005). The stress response can affect dopaminergic neuronal function as well. Restraint stress increases the levels of oxidative stress products within the nigrostriatal pathway (Kim et al., 2005). Chronic stress can also increase the levels of dopamine in the striatum, hippocampus and frontal cortex (Rasheed et al., 2010). The increases of extracellular levels of glutamate, glucocorticoids, and dopamine in the striatum after stress have the ability to work concurrently to cause neuronal damage (Smith et al., 2002). Chronic stress and corticosterone are able to impair animal movement during skilled motor movement tasks, suggesting that stress can affect dopaminergic control of motor function, with glucocorticoids partially mediating the effect (Metz et al., 2005, 2007). Still, while there are some alterations in the nigrostriatal pathway after stress, most of the changes in the dopaminergic systems observed in studies occur in the mesolimbic and mesocortical pathways (Finlay and Zigmond, 1997). Nevertheless, the stress response may be creating an environment that allows increased nigrostriatal damage after later injury. Acute restraint prior to lesioning is able to worsen motor deficits and the neurochemical environment of the striatum compared to unstressed animals (Smith et al., 2002). In another study that examined the effects of chronic stress on 6-OHDA lesions, increased motor dysfunction in the stressed animals was also observed, as well as an increase in Fluoro-Jade labeling, pointing towards an increase in the neurodegenerative process (Smith et al., 2008). However, no cell counts of TH+ neurons were performed, so it is unknown if this experiment resulted in actual increased nigral cell loss as seen in our study.

Although our findings show that chronic stress exacerbates neurodegeneration and motor deficits in the injured SNpc, the precise mechanisms for this effect are unresolved. It is unknown at this time whether the increase in neurodegeneration is caused by glucocorticoids acting directly through glucocorticoid receptors located in the ventral midbrain or perhaps by secondary
actions on other neurotransmitter systems. Glucocorticoids can cause excitotoxicity through increase glutamate levels, disrupt calcium homeostasis and alter cellular metabolism (Kibel and Drenjančević-Perić, 2008). In turn, glutamate may increase the release of dopamine in the substantia nigra during the stress response, leading to a more neurotoxic environment (Castro and Zigmond, 2001). Dexamethasone, a synthetic glucocorticoid, can worsen nigral neuronal loss by increasing oxidative stress levels (Argüelles et al., 2010). Intriguingly, at certain levels glucocorticoids and dexamethasone can have neuroprotective effects (Kurkowska-Jastrzębska et al., 2004; Ábrahám et al., 2006). The role glucocorticoids may play in the increased behavioral deficits and neuronal loss in our combined model will be explored in Chapter 3. Additionally, the underlying pathophysiological changes may involve both pro- and anti-apoptotic genes, which are affected by chronic stress (Kotsen et al., 2007), as well as an aberrant trophic factor environment. The effects of chronic stress (and antidepressant treatment) on neurotrophic factor expression in the nigrostriatal system are explored in Chapter 4.

The duration and modalities of the stressors are very important to the proper interpretation of any studies on stress. While it appears that the stressors need to be present both before and after the lesion, the concomitantly stressed animals also received a longer total period of stressors. It remains to be established whether it is the timing of the stressors or the duration of the exposure that leads to the worsening of the lesion effects. It is intriguing that the motor symptoms worsened two weeks after the cessation of CVS, when the animals have already recovered from some of the physiological effects observed during the CVS regimen. This suggests that continued pathological alterations contribute to the increased behavioral dysfunction and exacerbated cell death. Regardless, our finding of increased PD deficits with a model of depressive symptoms underscores the importance of carefully screening and treating
PD patients for depression to mitigate any possible worsening of motor deficits in addition to treating the affective disorder.
References


Chapter 3

Role of glucocorticoids in the increased neurodegeneration observed in lesioned animals exposed to chronic stress

Abstract

Depression is highly prevalent in Parkinson’s disease (PD) and may contribute more to the lowered quality of life than the debilitating motor symptoms. Although the etiology of depression in PD is unknown, understanding the potential pathophysiological processes and deleterious consequences of these co-morbidities is of considerable importance. We have previously determined that exposure to a stress regimen that produces physiological and behavioral correlates of depression concomitant with lesion of the nigrostriatal pathway exacerbates motor deficits and nigral cell loss. We used a well-characterized partial lesion, neurotoxin (6-hydroxydopamine; 6-OHDA) rat model of PD combined with the chronic variable stress (CVS) regimen, which mimics depression symptomatology in rodents. Depression and CVS are accompanied by glucocorticoid hypersecretion, which is known to exacerbate neurotoxicity in the hippocampus and prefrontal cortex, and is associated with aging-related neurodegeneration. The role that glucocorticoids may play in the degeneration of midbrain dopamine cells in our CVS/PD model has yet to be elucidated. First, we tested whether chronic administration of the glucocorticoid corticosterone (CORT) alone could mediate the CVS-induced exacerbation of PD symptoms and cell degeneration. Adult male Sprague Dawley rats received subcutaneous injections of CORT (3.5 mg/kg/ml in propylene glycol) or vehicle twice daily, once in the morning and once in the afternoon, to recapitulate the pattern of glucocorticoid exposure induced during the normal CVS regimen. Animals received the injections for 2 weeks prior to administering a striatal 6-OHDA lesion, and for 2 weeks thereafter. Behavioral data
were collected using the forelimb-use asymmetry test at two and four weeks post-lesion to assess motor deficits. Animals were then sacrificed, processed for tyrosine hydroxylase (TH) immunohistochemistry, and TH-positive cells were counted in the substantia nigra pars compacta using unbiased stereological techniques. Both the behavioral and morphological data revealed that while both CORT- and vehicle-treated lesioned animals exhibited motor deficits and neurodegeneration compared to the control groups, there were no significant differences between the two lesioned groups (there were also no significant differences between the two vehicle-lesioned groups). In the second experiment, animals received the glucocorticoid receptor (GR) antagonist RU486 (10 mg/kg/ml in propylene glycol) concomitant with CVS to attempt to block the increased neuronal loss observed in our combined model. Behavioral and morphological data were collected as in the previous experiment. Administration of RU486 did not result in any differences between the 6-OHDA-lesioned animals, suggesting that the GR antagonist does not protect against stress-related nigrostriatal dysfunction. These data also indicate that glucocorticoids alone are not sufficient to worsen neurotoxic cell death of midbrain dopaminergic neurons, and suggest that other stress-associated mechanisms (e.g., excitotoxicity, neuroinflammation) may be central to chronic stress-induced exacerbation of 6-OHDA neurotoxicity.
Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disease primarily affecting the nigrostriatal pathway resulting in motor dysfunction. However, it is increasingly recognized that many non-motor symptoms accompany the disorder, one of the most common being depression (Cummings, 1992; McDonald et al., 2003; Lemke, 2004). Depression in PD negatively affects the quality of life for the patients (Hely et al., 2005; Schrag, 2006) and may be associated with more severe motor symptoms (Pålhagen et al., 2008). Given the high rate of prevalence, as well as the high rate of under-treatment (Richard and Kulan, 1997; Yamamoto, 2001), it is important to understand if and how the comorbidity of depression and PD can affect the progression of PD symptoms and dopaminergic neurodegeneration. We have developed a model that combines chronic variable stress (CVS), a well-accepted model of depressive symptomatology (Herman et al., 1995; Nestler, 2002a; Wilner, 2005), with the unilateral of 6-hydroxydopamine (6-OHDA) model of PD (Sauer et al., 1994; Kirik et al., 1998). In Chapter 2, we demonstrated that stress-induced depression exacerbates both the motor deficits and dopaminergic neurodegeneration present within the 6-OHDA model. However, the mechanisms for the increased dysfunction and accelerated degeneration are presently unknown. Increased levels of glucocorticoids are observed in both the CVS model and human depression (Herman et al., 1995; Duval et al., 2006). Given the ability of glucocorticoids to modulate structural plasticity and cell survival in several brain regions, it is possible that they play a key role in dopaminergic neuronal degeneration in comorbid depression and PD.

Glucocorticoids are particularly neuroactive within the limbic system. In the hippocampus, excessive glucocorticoids can result in both neuronal loss and dendritic atrophy (Magariños and McEwen, 1995; Duman, 2002). Pre-exposure to glucocorticoids can also
increase the vulnerability of hippocampal neurons to subsequent injury (Sapolsky, 1994). Glucocorticoids decrease the expression of neurotrophins in the hippocampus and frontal cortex, likely increasing the susceptibility of the neurons to damage (Smith et al., 1995; Schaff et al., 1997, 1998; Mao et al., 2010). Both direct administration of glucocorticoids and chronic stress reduce dendritic plasticity in the prefrontal cortex (PFC) (Wellman, 2001; Radley et al., 2004; Cerqueira et al., 2005; Dias-Ferreira et al., 2009). In contrast, chronic stress causes dendritic hypertrophy in the dorsolateral striatum (Dias-Ferreira et al., 2009) and increases dendritic complexity in the amygdala (Vyas et al., 2002; Mitra and Sapolsky, 2008). In the hypothalamus, glucocorticoids elevate corticotrophin-releasing hormone mRNA levels (Swanson and Simmons, 1989), an increase also observed in suicide victims with major depression (Raadsheer, 1995). Of important relevance to PD, glucocorticoids are able to block the uptake of and elevate levels of dopamine in the striatum (Finkelstein et al., 1988; Smith et al., 2002), and it has been proposed that such increased levels of dopamine may create an environment of neuronal vulnerability (Hastings et al., 1996).

Glucocorticoid receptors (GRs) are widely distributed throughout the brain, including within the nigrostriatal system (Härfstrand et al., 1986; Ahima and Harlan, 1990). Many dopaminergic neurons of the substantia nigra co-express the GR, raising the possibility of direct glucocorticoid actions upon the dopaminergic cells under both normal and hyperglucocorticoid conditions. In addition, excess glucocorticoids could effect changes in other related and interconnected nuclei that may indirectly influence the nigrostriatal system.

Direct blocking of glucocorticoid actions results in antidepressive effects in both animal models and clinical studies. Mifepristone (RU486) is a high-affinity GR antagonist that can impede the negative feedback actions of CORT/cortisol in the hypothalamic-pituitary-adrenal
(HPA) axis and results in increased adrenocorticotrophin hormone and glucocorticoid levels (Brogden et al., 1993; Robbins and Spitz, 1996; DeBattista and Belanoff, 2006). In the PFC, RU486 can protect against neuroinflammation, which is also present within the SNpc in PD (de Pablos et al., 2006). In human studies, RU486 is capable of rapidly improving symptoms in psychotic depression (Belanoff et al., 2001; DeBattista et al., 2006). In animal models, during the forced swim test, RU486 decreases immobility time, a sign of antidepressant action (Wulsin et al., 2010; Iijima et al., 2010). Moreover, RU486 also counters long-term stress effects, as it decreases immobility time in adult rats that had previously been exposed to maternal separation (Aisa et al., 1997). If RU486 is able to act as an antidepressant and block the effects of CVS in our model, it may provide a link between glucocorticoid signaling and dopamine neuronal loss.

In this study, we tested the hypothesis that glucocorticoid actions are responsible for the exacerbated neurodegeneration found in the CVS/6-OHDA model (see Chapter 2). We examined the steroid’s potential role two-fold. First, to determine whether glucocorticoids alone are sufficient to enhance nigral cell degeneration, animals were treated with corticosterone twice daily for two weeks before and two week after the injection of 6-OHDA, to mimic the pattern of glucocorticoid exposure resulting from CVS in our previous study. In the second experiment, animals received CVS flanking the lesion as described in Chapter 2. However, prior to the administration of each stressor, the animals were given an injection of RU486 to block glucocorticoid actions, and therefore determine if the GR antagonist can prevent the detrimental effects previously observed in the combined model. In both experiments, motor behavior was assessed using the forelimb-use asymmetry test (cylinder test) at two and four weeks after the 6-OHDA lesion and neurodegeneration was measured by unbiased stereological cell counts of the dopaminergic neurons in the ventral midbrain.
Methods

Experimental Timeline

Experiment 1:

In the first experiment, animals were exposed to the glucocorticoid corticosterone (CORT) both before and after the lesion (Fig. 1A). Animals were exposed to twice-daily corticosterone injections for two weeks, after which they were unilaterally lesioned via striatal 6-OHDA injections (see below). Allowing a few days for recovery, animals were again subjected to two weeks of CORT injections. Two and four weeks after the lesion, the forelimb asymmetry test was administered to assess motor deficits. Following the final behavioral test, the animals were sacrificed and processed for TH and NeuN immunohistochemistry and unbiased stereological cell counting.

Experimental 2:

In this experiment, animals were exposed to CVS both before and after the lesion (Fig. 1B). Thirty minutes prior to each stress exposure, however, animals also received subcutaneous (s.c.) injections of the GR antagonist RU486. These animals were administered CVS and RU486 for two weeks, unilaterally lesioned via striatal 6-OHDA, and then again subjected to two weeks of CVS with continued RU486 administration (with the swim stressors were replaced with restraint to prevent complications from mobility impairment). Two and four weeks post-lesion, motor behavioral data were obtained using the forelimb asymmetry test. Animals were then sacrificed and processed for immunohistochemistry and unbiased stereological cell counting.

Animals

Adult male Sprague Dawley rats (230-360 g) from Harlan Laboratories (Indianapolis, IN) were used (n=8-10/group). Animals were housed in standard conditions (12 hr on/off light
Figure 1

A. Experiment 1: CORT flanking the lesion

![Timeline for Experiment 1](image)

B. Experiment 2: RU486 + CVS flanking the lesion

![Timeline for Experiment 2](image)

**Figure 1.** Timelines for the experiments. **A: Experiment 1:** Animals received two weeks of corticosterone (CORT) twice daily prior to receiving 6-OHDA. After the lesion, animals received two more weeks of CORT injections. Behavioral data assessing motor function (cylinder test) were collected two and four weeks post-lesion and then animals were sacrificed and processed for immunohistochemistry. **B: Experiment 2:** For two weeks prior to the lesion, animals received a RU486 injection prior to the twice daily stressors of the CVS regimen. After receiving 6-OHDA, animals received two more weeks of RU486 injections along with CVS. Motor behavior was measured two and four weeks after the lesion using the cylinder test and the animals were subsequently sacrificed and processed immunohistochemically.
cycle), two per cage, with food and water available *ad libitum*. Animals were treated in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee. Animals were allowed to acclimate to the facilities for one week before use. It should be noted that in the second half of the RU486 experiment, animals were switched to a diet containing fenbendazole as a cautionary measure to prevent the spread of pinworm in the animal vivarium. For the first experiment, animals were divided into four weight-matched groups: vehicle/vehicle, vehicle/6-OHDA, CORT/vehicle, CORT/6-OHDA. In the second experiment animals were divided into eight weight-matched groups: control/vehicle/vehicle, control/RU486/vehicle, control/vehicle/6-OHDA, control/RU486/6-OHDA, CVS/vehicle/vehicle, CVS/RU486/vehicle, CVS/vehicle/6-OHDA, CVS/RU486/6-OHDA.

**Partial 6-OHDA lesion**

Animals were given anesthetic (87 mg/kg ketamine, 13 mg/kg xylazine; i.p.) and then placed into a stereotaxic apparatus. Animals subsequently received two unilateral injections of the catecholamine-specific neurotoxin 6-hydroxydopamine (6-OHDA; 10 µg each in 2 µl saline + 0.2% ascorbic acid) or 6-OHDA vehicle (saline + ascorbic acid) placed into the right striatum [AP:+1.6mm, ML:-2.4, DV:-4.2 and AP:+0.2, ML:-2.6, DV:-7.0 (Paxinos and Watson, 2007)] using a 5-µl Hamilton syringe (Hamilton Company, Reno, NV) (Sauer et al., 1994; Kirik et al., 1998). For each injection, the needle was slowly lowered into the brain parenchyma for five minutes to equilibrate, the 2-µl injection was administered over ten minutes, and the needle was then allowed to remain in the brain for an additional five minute before being slowly removed.

**Injections**
In the first experiment, animals received s.c. injections of CORT (Sigma-Aldrich; 3.5 mg/kg/ml in propylene glycol) or vehicle (propylene glycol). The dosage chosen was previously shown to mimic the effects of CVS (Zhang et al., 2010). The injections occurred twice daily, once in the morning and once in the afternoon to mirror the normal CVS regimen.

In the second experiment, animals received s.c. injections of RU486 (Sigma-Aldrich; 10 mg/kg/ml in propylene glycol) or vehicle (propylene glycol) 30 minutes prior to each stressor. The dose of RU486 was chosen because of previously observed antidepressant effects (Aisa et al., 2007, 2008; Wulsin et al., 2010).

**Chronic Variable Stress Regimen:**

The CVS protocol used has been characterized previously (Herman et al., 1995). The CVS regimen used the following eight stressors: cold exposure (1h in a 4°C room), restraint (1h in plastic restraint tubes, vibration (1h on 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 (6/cage, overnight), isolation (1/cage, overnight). After surgeries, stressors requiring mobility (i.e. the swim stressors) were not administered and replaced with restraint. To prevent habituation, stressors were administered in a random fashion. Animals in the CVS group were exposed to two stressors each day during the light cycle, one in the morning and one in the afternoon. The body weights of stressed and non-stressed animals were collected throughout each experiment to monitor the effectiveness of the chronic stress regimen.

**Forelimb Asymmetry Test**

Behavioral data were collected using the forelimb asymmetry test (cylinder test) to assess the differences in motor impairment as previously described (Schallert et al., 2000, Schallert, 2006). The cylinder test occurred during the beginning of the dark cycle under low-light
conditions. Briefly, each rat was placed individually into a transparent Plexiglas cylinder and
allowed to explore the walls naturally by rearing up and using their forelimbs for weight support.
A rat was considered to have completed the test when it had performed 20-25 touches. The data
were recorded and analyzed by counting the number of times the rats used their right, left or both
forelimbs to explore the side of the wall as previously described (Schallert et al., 2000). A
percent limb usage score was calculated from the behavioral data collected using the formula
\[
\frac{(\text{contralateral side} + \frac{1}{2} \text{both})}{(\text{ipsilateral side} + \text{contralateral side} + \text{both})} \times 100
\]
The cylinder test was administered two and four weeks post-lesion for both experiments. Baseline behavior
was also determined the day before the animals received the lesion. Statistical analyses included
one-way ANOVA followed by Neuman-Kuels post-hoc test. Data are presented as mean ± SEM.
Significance is considered at \(P < 0.05\).

**Immunohistochemistry**

One day after the final cylinder test, the animals were deeply anesthetized and sacrificed
via intracardial perfusion using saline and 4% paraformaldehyde (PFA). Following perfusion, the
brains of the animals were collected, as well as the adrenal glands and thymus. The brains were
placed in 4% PFA and post-fixed for 24 hours and subsequently placed into 30% sucrose. The
thymus and adrenal glands were cleaned and weighed. Using a sliding microtome, coronal
sections of the brains through the ventral mesencephalon were cut (at 50 µm). Six sections per
brain (600 µm apart) were processed for tyrosine hydroxylase (TH; catecholamine biosynthetic
enzyme and marker for midbrain dopamine cells) or neuronal nuclei (NeuN; a general neuronal
marker) according to our routine immunohistochemical procedures (e.g. Seroogy et al., 1994).
Free-floating sections were washed in 0.1 M phosphate buffer (PB), and with normal horse
serum (NHS) for TH or normal goat serum (NGS) for NeuN. Sections were incubated overnight
at 4°C in the primary antibody (mouse anti-TH (1:8000) or mouse anti-NeuN (1:1000) both from Chemicon International, Temecula, CA) made with 1% normal serum and 0.2% Triton-X 100 in 0.1 M PB. The following day, sections were washed in 0.1M PB, blocked with 2% NHS (TH) or NGS (NeuN), and incubated with the anti-mouse IgG biotin-conjugated secondary antibody [horse anti-mouse (1:200) for TH and goat anti-mouse (1:400) for NeuN, both from Vector Laboratories, Inc. Burlingame, CA] for 1 hour. After washes in 0.1 PB, the tissue was placed in ABC peroxidase (Vector Laboratories) for 30 minutes and then washed in 50 mM Tris buffer (pH 7.5). Labeling was visualized using the diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories). Sections were given a final series of 50 mM Tris buffer washes and mounted onto Superfrost plus microslides (VWR, Batavia, IL). After drying, tissue was dehydrated using a series of ethanol washes, placed in a clearing reagent and coverslipped.

**Stereological Cell Counts**

For each group, cells stained for TH and NeuN were counted in the SNpc on evenly spaced sections throughout the rostrocaudal extent of the ventral mesencephalon to determine the extent of neurodegeneration among the lesioned groups. Cell estimates of both the lesioned and unlesioned sides of the SNpc were determined using Stereo Investigator 5.05 (MicroBrightfield, Williston, VT) utilizing unbiased stereological techniques (West, 1993). The sections were viewed on an Olympus BX-60 microscope (Melville, NY) using a CCD video camera (HV-C20, Hitachi, San Jose, CA). Contours were determined at 2X magnification and cell counting was performed at 60X using the optical fractionator. A guard of 2 µm was placed for each sample section. Random sample areas were determined by the software on a grid size of 170x100 for TH staining and 220x220 for NeuN staining. The coefficient of error was calculated using the Gundersen correction for each animal and was lower than 0.10. Neuronal degeneration was
Statistical analyses included one-way ANOVA followed by Neuman-Kuels post-hoc test. Data are presented as mean ± SEM. Significance is considered at $P < 0.05$.

Results

Experiment 1

Animal Body Weights

Animal body weights were monitored throughout this CORT experiment. All four groups were weight-matched prior to the start of the experiment (Fig. 2). No significant weight differences were detected among the groups after the first week of injections, but over the next three weeks of injections, both groups of CORT animals gained weight to a significantly less degree than the vehicle-treated animals ($F_{3,13} = 5.285$ (Week 3); $F_{3,13} = 9.508$ (Week 4), $F_{3,13} = 13.34$ (Week 5); Fig. 2; $P < 0.01$). After cessation of CORT administration, there were no longer any significant differences among the group weights. No body weight differences between the two vehicle-treated groups or the two CORT-treated groups were observed throughout the course of the experiment.

Adrenal glands and thymus weights

No significant differences in the weights of either adrenal glands or thymus were observed among any of the experimental groups (Figs. 3).

Behavior

To determine whether CORT administration could exacerbate the motor deficits caused by 6-OHDA compared to animals only receiving the 6-OHDA lesion, the cylinder test was
Figure 2. Weights of animals that received corticosterone (CORT) for two weeks before and after the lesion. Animals were placed in weight-matched groups at the beginning of the study. By the second week of injections, CORT-injected animals gained less weight compared to vehicle-injected animals, a pattern which continued throughout the period of CORT treatment. In the final two weeks, animals were no longer exposed to CORT and the weights of CORT-treated animals were not significantly different from the vehicle-treated animals. Data are presented as mean ± SEM. *P < 0.05 vs. respective control. n = 8 for Vehicle/Vehicle, Vehicle/6-OHDA, CORT/6-OHDA; n = 10 for CORT/6-OHDA.
Figure 3. A: The thymus of animals treated with corticosterone (CORT) flanking the lesion were collected and weighed. There was no significant differences among the groups. B: Adrenal glands were also removed and weighed. Combining the weights of both left and right adrenals, no differences were found in the CORT-injected animals compared to the controls. Data are presented as mean ± SEM. n = 8 for Vehicle/Vehicle, Vehicle/6-OHDA, CORT/6-OHDA; n = 10 for CORT/6-OHDA.
Figure 4. Assessment of motor deficits (forelimb asymmetry) in animals via the cylinder test in animals treated with corticosterone (CORT) or vehicle flanking the lesion. Baseline behavior was collected the day before animals received the lesion; no differences were found among any of the groups. At both two and four weeks post-lesion, both groups of 6-OHDA-lesioned animals exhibited a significant reduction in usage of the impaired forelimb compared to the two groups of unlesioned rats. However, there was no significant difference between the CORT/6-OHDA and vehicle/6-OHDA animals. Additionally, there were no differences between the vehicle-treated animals. Data are presented as mean ± SEM. *P< 0.01 for Veh/Veh and CORT/Veh vs Veh/6-OHDA and CORT/6-OHDA. n = 8 for Vehicle/Vehicle, Vehicle/6-OHDA, CORT/6-OHDA; n = 10 for CORT/6-OHDA.
Figure 5. A-B: TH+ and NeuN+ stereological cell counts in the SNpc of animals receiving corticosterone (CORT) flanking the lesion. Whereas cell counts demonstrated that substantial TH+ cell loss was observed in the 6-OHDA-lesioned rats, no differences were found between the 6-OHDA-lesioned groups. There were also no differences noted between the vehicle-treated groups. NeuN+ cells were reduced in similar patterns and numbers. Data are presented as mean ± SEM. *$P < 0.001$ for Veh/Veh and CORT/Veh vs Veh/6-OHDA and CORT/6-OHDA. C: High power photomicrographs of TH immunostaining in the SNpc of animals receiving CORT flanking the lesion. Note that the two 6-OHDA-lesioned groups appear to have exhibited the same extent of TH+ cell loss. n = 8 for Vehicle/Vehicle, Vehicle/6-OHDA, CORT/6-OHDA; n = 10 for CORT/6-OHDA.
used. Prior to administration of 6-OHDA, baseline behavior was obtained for all four experimental groups. At this stage, there were no differences among experimental groups and all animals displayed normal use of both forelimbs (Fig. 4). At both two and four weeks after the lesion, animals that had received 6-OHDA displayed motor deficits by using their impaired limb significantly less than those animals that received intrastriatal vehicle-injections ($F_{3,27} = 11.65$ (2 weeks post-lesion), $F_{3,25} = 7.613$ (4 weeks post-lesion); Fig. 4; $P < 0.001$). However, there were no differences between the CORT/6-OHDA animals and the Vehicle/6-OHDA animals at either time point, nor where there any differences between the Vehicle/Vehicle and CORT/Vehicle groups.

**Stereological Cell Counts**

To determine if there were any differences in the degeneration of nigral dopaminergic cells, stereological cell counts were obtained for TH+ neurons. Both lesioned groups demonstrated a significant loss of TH+ cell bodies compared to the vehicle-injected control ($F_{3,25} = 14.54$; Fig. 5; $P < 0.001$). However, no differences in neuronal loss were measured between the two lesioned groups or between the two unlesioned groups.

**Experiment 2**

**Animal Weights**

In this second experiment, where animals received RU486 prior to the administration of CVS, all eight animals groups were weight-matched prior to the beginning of the experiment (Fig. 6). All stressed animals exhibited a significant reduction in the rate of weight gain compared to the non-stressed animals during the CVS regimen ($F_{7,58} = 4.376$ (week 3); $F_{7,56} = 5.386$ (week 4); $F_{7,56} = 7.650$ (week 5); $F_{7,56} = 6.193$ (week 6); $P < 0.05$). Interestingly, after the
Figure 6. Weights of control and CVS animals exposed to RU486 injections and CVS flanking the 6-OHDA lesion. There were no differences in weight at the beginning of the study. During the first two weeks of CVS, stressed-exposed animals gained less weight compared to control animals. After the lesion, animals received an additional two weeks of CVS and continued to gain less weight. Additionally, control animals receiving RU486 weighed significantly less than the control vehicle injected animals. In the last week, when the animals were no longer exposed to stress, the weights of CVS animals reached the same weight as the non-stressed controls. Data are presented as mean ± SEM. *$P<0.05$ CVS vs. Control. #$P<0.05$ Control/RU486 vs. Control/Vehicle. n = 8 for Control/Vehicle/Vehicle, Control/Vehicle/6-OHDA, Control/RU486/Vehicle, Control/RU486/6-OHDA, CVS/Vehicle/Vehicle, CVS/RU486/Vehicle; n = 10 for CVS/Vehicle/6-OHDA, CVS/RU486/6-OHDA.
Figure 7. A: The thymus weights of animals exposed to two weeks of CVS regimen and RU486 injections both prior to and after the lesion. There were no significant differences among the groups. B: The adrenal gland weights from the rats also revealed no significant differences among the groups. Data are presented as mean ± SEM. n = 8 for Control/Vehicle/Vehicle, Control/Vehicle/6-OHDA, Control/RU486/Vehicle, Control/RU486/6-OHDA, CVS/Vehicle/Vehicle, CVS/RU486/Vehicle; n = 10 for CVS/Vehicle/6-OHDA, CVS/RU486/6-OHDA.
**Figure 8.** Assessment of motor deficits (forelimb asymmetry) in animals receiving RU486 injections and exposed to CVS flanking the lesion. Baseline behavior was collected the day before animals received lesion surgery, and at this stage there were no differences among any of the groups. At both two and four weeks post-lesion, the impaired limb usage of the lesioned animal groups compared to the vehicle-treated animal groups was significantly diminished. However, there were no differences among any of the lesioned groups themselves. There were also no differences between the groups of vehicle-treated animals. Data are presented as mean ± SEM. *P < 0.01 for vehicle-treated vs 6-OHDA treated groups. n = 8 for Control/Vehicle/Vehicle, Control/Vehicle/6-OHDA, Control/RU486/Vehicle, Control/RU486/6-OHDA, CVS/Vehicle/Vehicle, CVS/RU486/Vehicle; n = 10 for CVS/Vehicle/6-OHDA, CVS/RU486/6-OHDA.
Figure 9. A-B: TH+ and NeuN+ stereological cell counts in the SNpc of animals receiving CVS and RU486 flanking the lesion. Although lesioned groups lost a significant number of cells compared to control groups, there were no differences between the lesioned groups. NeuN+ cells were reduced in similar patterns and numbers. There were no differences in cell numbers among the vehicle-treated of the four weeks of stress exposure, the stressed animals still weighed significantly less than non-stressed animals, but in the last week of the experiment, this effect had disappeared. C: High power photomicrographs of TH immunostaining in the SNpc of animals receiving CVS and RU486 flanking the lesion. Note that whereas the four 6-OHDA lesioned groups exhibited substantial TH+ cell loss, no differences in cell numbers are apparent among the groups. $n = 8$ for Control/Vehicle/Vehicle, Control/Vehicle/6-OHDA, Control/RU486/Vehicle, Control/RU486/6-OHDA, CVS/Vehicle/Vehicle, CVS/RU486/Vehicle; $n = 10$ for CVS/Vehicle/6-OHDA, CVS/RU486/6-OHDA.
lesion surgeries, non-stressed animals receiving RU486 weighed less than the non-stressed vehicle-injected animals ($P < 0.05$), which continued throughout the experiment. After the end of the four weeks of stress exposure, the stressed animals still weighed significantly less than non-stressed animals, but in the last week of the experiment, this effect had disappeared.

**Adrenal glands and thymus weight**

In this RU486/CVS experiment, there were no significant differences in the weights of either the adrenal glands or thymus among any of the experimental groups (Figs. 7).

**Behavior**

In this experiment, animals received RU486 prior to each CVS stressor in an attempt to prevent the worsening of motor deficits in the combined CVS/PD model. Baseline behavior was taken before administration of 6-OHDA and no differences were observed between any of the eight experimental groups (Fig. 8). In all four lesioned groups, there was a significant reduction in motor behavior compared to the four unlesioned groups at both two and four weeks after the lesion ($F_{7,52} = 8.385$ (2 weeks post-lesion); $F_{7,47} = 7.131$ (4 weeks post-lesion); Fig. 8; $P < 0.01$); however there were no differences among any of the lesioned groups themselves. There were also no behavioral differences among any of the unlesioned groups.

**Stereological Cell Counts**

In this RU486 experiment, the lesioned animals all displayed loss of TH+ cells compared to unlesioned controls ($F_{7,44} = 37.16$; Fig. 9A; $P < 0.001$), but there were no differences between the lesioned groups or between the unlesioned groups. Quantification of NeuN+ cells revealed a similar pattern of degeneration as the TH+ neurons, indicating frank cell death rather than phenotypic downregulation of the TH enzyme.
Discussion

In this study, administration of CORT twice daily flanking the lesion did not worsen motor dysfunction at either time point or exacerbate neurodegeneration compared to animals receiving only 6-OHDA. Administration of the GR antagonist RU486 prior to each stressor during the CVS flanking regimen also did not alter the pattern of motor deficits seen in the cylinder test or the amount of TH+ neurodegeneration in the SNpc. These data suggest CORT is not sufficient by itself to cause the worsened behavioral dysfunction and nigral dopaminergic cell degeneration observed in the combined CVS/6-OHDA model (see Chapter 2). In addition, preventing CORT signaling in the SNpc by blocking the GR with RU486 did not protect the dopaminergic neurons from degeneration.

As mentioned earlier, glucocorticoids can affect the plasticity of neurons in many regions, particularly those of the limbic system (e.g. Magariños and McEwen, 1995; Wellman, 2001). Chronic stress and glucocorticoid exposure decrease hippocampal volume in animals (Duman, 2002) and the hippocampus and PFC exhibit decreases in volume in depressed patients (Sheline et al., 1996; Drevets, 2000; Salvadore et al., 2001). Glucocorticoids can also prime neurons for subsequent or concomitant injury (Sapolsky, 1994; Stein-Behrens et al., 1994). Parkinson’s disease is a disease of aging and there are elevated levels of cortisol in the aged brain and PD patients (Charlett et al., 1998; Gould and Tanapat, 1999). Age-related dysfunction of the HPA axis can cause memory impairment, demonstrating the ability of glucocorticoid hyperactivity to result in behavioral deficits (Issa et al., 1990). These data support the idea that elevated glucocorticoids could worsen the neurodegeneration in the impaired SNpc. However, in this study, it appears that high levels of glucocorticoids alone are not able to foster increased injury upon the dopaminergic neurons. The dosage of CORT used has previously been able to
mirror effects of CVS, including weight loss and thymic atrophy (Zhang et al., 2010). Although we did, in fact, observe a reduction in weight gain in CORT-injected animals, indicating effectiveness of the CORT regimen, we did not see any effects on nigral neurodegeneration.

The GR antagonist RU486 demonstrates antidepressant efficacy in the forced swim test (Wulsin, et al., 2010; Iijima et al., 2010) and acts on par with the commonly used antidepressant imipramine (Cryan et al., 2005). RU486 can improve psychotic symptoms in depression (Belanoff et al., 2001; DeBattista et al., 2006; Flores et al., 2006), but does not appear to improve depression symptomatology (DeBattista et al., 2006; Flores et al., 2006). Importantly for the purposes of our study, the blockade of GR by RU486 is able to reverse chronic stress effects in the hippocampus (Oomen et al., 2006; Krugers et al., 2006), indicating the potential to do so in the SNpc as well. Yet the administration of RU486 prior to each stressor was unable to improve dysfunction in lesioned animals, either stressed or unstressed. Given in the inability of the glucocorticoid CORT to elicit increased neurodegeneration in the 6-OHDA model in the first part of this study, this is perhaps not unexpected. It is, therefore, not unlikely that the stress-induced increase in behavioral dysfunction and neuronal loss previously observed (Chapter 2) occurs via some other pathway or mechanism.

Surprisingly, in the experiment to block the action of glucocorticoids, RU486 not only made no difference in the amount of neurodegeneration, but our positive control group, Vehicle/CVS/6-OHDA was no different than Vehicle/Control/6-OHDA. This means we were not able to precisely duplicate our data from Chapter 2 where CVS before and after the lesion worsened motor deficits and neurodegeneration compared to lesioned only animals. However, the experiment was not an exact replication, and it may be that the numerous s.c. injections the animals received throughout the course of the experiment (56 total) caused habituation to the
stress response so that CVS was no longer effective in creating a more neurotoxic environment. Additionally, during the second half of the RU486 experiment, the animal vivarium underwent treatment for a pinworm outbreak, which resulted in changes in the animal housing conditions and diet. While all conditions were equally affected, it is unknown if the midstream changes detrimentally affected the experiment and/or differentially distressed separate groups within the experiment. There is also the finding that the RU486 treatment reduced the weight of non-stressed rats. Thus, it is possible that chronic injections of RU486 have a negative effect on the physiology of otherwise healthy rats, though it did not affect behavior or cell loss.

Depression is a complex disorder, and while there is evidence of HPA axis dysfunction in numerous patients, in many patients there is not (Kanner, 2004). It is, therefore, unlikely that glucocorticoid hyperactivity is able to account for all the changes observed. Other theories for the causes of depression include the neurotrophic factor hypothesis and the neuroinflammatory and neurodegenerative hypothesis (Nestler, 2002b; Maes et al., 2009; Zunszain et al., 2009). Many of these theories also have links to PD etiology. Alteration of neurotrophic factor expression is observed in regions involved in depression (Duman and Monteggia, 2006) and changes in neurotrophic levels affect the survival of neurons in the SNpc (Chiocco et al., 2007). High levels of cytokines are present in depressed patients (Howren et al., 2009), and there are increased levels in the hippocampus after CVS (Tagliari et al., 2011) that may affect cellular plasticity. Neuroinflammation is also believed to play a key role in PD development (Whitton, 2007). Additionally, cellular apoptosis may contribute to the pathogenesis observed in depression and chronic stress, and is present in SNpc of PD models as well (Marti et al., 2002; Lucassen et al., 2006; Kosten et al., 2007). Glucocorticoid actions may link many of these other hypotheses together, but it is also possible for the above mentioned scenarios to influence the
dysfunction and neurodegeneration present in the combined CVS/6-OHDA model independently.

In conclusion, glucocorticoids do not appear to play a primary role in the increased behavioral deficits and cellular neurodegeneration seen within our comorbid chronic stress/PD model. It remains to be determined what mechanisms exacerbate the neuronal loss, but could include excitotoxicity, neuroinflammation, loss of neurotrophic support, or a combination of several factors. To explore other potential mechanisms for the increased dysfunction and neurodegeneration, in the next chapter we will examine the effects of CVS and antidepressant treatment on neurotrophic factor expression in regions involved in depression and PD.
References


Chapter 4

Modulation of neurotrophic factors by chronic variable stress and antidepressant treatment

Abstract

There is speculation that Parkinson’s disease (PD) and affective disorders such as depression share common pathologies either directly or indirectly. However, current models of PD focus on the nigrostriatal pathway and the resulting motor dysfunctions associated with the disease. In order to investigate specifically depression in rats, we utilized the chronic variable stress (CVS) model to induce depressive-like symptoms. We hypothesized that CVS would modulate neurotrophic factor expression in areas implicated in both PD and depression. We also hypothesized that chronic antidepressant treatment would alter neurotrophic factor expression in an opposite manner to stress-modulation. In the first experiment, rats were exposed to a two-week CVS regimen. In the second experiment, rats were administered the tricyclic antidepressant desipramine (10 mg/kg) once daily for three weeks. After completion of each experiment, the animals were sacrificed and processed for in situ hybridization. We examined the mRNA levels of several neurotrophic factors known to be neuroprotective for midbrain dopaminergic neurons: transforming growth factor alpha (TGFα), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). The high affinity receptors for BDNF and NT-3, trkB and trkC, respectively, were also studied. Finally, the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, was analyzed as well. In animals exposed to chronic stress, we found that levels of TGFα and NT-3 mRNA were increased in the hippocampal formation. Expression of TGFα mRNA was also increased in the nigrostriatal system. In select cortical regions, BDNF mRNA levels were decreased and trkC mRNA levels
were increased. The locus coeruleus (LC) appeared to be a plastic region, with TH expression increased and BDNF mRNA decreased. No changes in levels of trkB were found in stressed animals in the regions examined. In the antidepressant-treated animals, elevated levels of BDNF mRNA were found in the anterior cingulate, frontal and piriform cortices. Expression of trkB was increased in the striatum and piriform cortex. Levels of NT-3 mRNA were increased in the piriform cortex, but decreased in the anterior cingulate cortex. No alterations were found in trkC or TH mRNA levels in the desipramine-treated animals. These findings indicate that CVS perturbs neurotrophic factor levels in specific areas involved in PD, as well as depression, and raises the possibility that stress-induced depression could be harmful to dopaminergic neurons. Additionally, we found that in some regions, such as BDNF mRNA in the piriform cortex, antidepressant modulation of neurotrophic factors occurred in a reciprocal fashion to animals exposed to CVS. However, in most cases, there was no apparent relationship in trophic factor plasticity in antidepressant-treated animals versus chronically stressed animals. Nevertheless, our findings of changes in dopamine-associated neurotrophic factors in specific areas involved in both PD and depression lead to the notion that antidepressants could be beneficial for injured dopaminergic neurons.
Introduction

Being diagnosed with an affective disorder increases the risk of being diagnosed with Parkinson’s disease (PD) (Leentjens et al., 2003; Alonso et al., 2009), and studies indicate that 40-45% of PD patients develop depression (Cummings, 1992). However, given the focus on treating the motor symptoms in PD, there is little understanding of the underlying pathology of comorbid depression. One possible mechanism may be the alteration of neurotrophic factor expression in areas involved in depression and PD. In the neurotrophic hypothesis of depression, the neuropathology observed may, in part, be the result of a deficiency in neurotrophic support in key brain regions, whereas antidepressant actions may be effective by reversing this trophic loss (Nestler et al., 2002). Thus, maladaptive alterations of neurotrophic factors in depression may increase the vulnerability of dopaminergic neurons to neurodegeneration and development of PD.

Dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis also plays a role in depression pathology. In humans with depression, levels of corticotrophin-releasing hormone (CRH) are elevated in the cerebral spinal fluid (Nemeroff et al., 1984) and many depressed patients exhibit a lack of feedback regulation of cortisol, indicating impairment of the HPA axis (Kanner, 2004). Chronic activation of the stress response is believed to be maladaptive and can lead to neuronal defects (Herman and Cullinan, 1997). The hippocampus and the other parts of the limbic system are implicated in neuropsychiatric diseases, as well as regulation of stress levels (Herman et al., 1989). Reduction in the volume of the hippocampus and frontal cortex is observed in humans (Sheline et al., 1996; Salvadore et al., 2001) and chronic stress in animals can lead to dendritic hypertrophy and a decrease in neurogenesis in the hippocampus (Magariños and McEwen, 1995a,b; Wellman, 2001; Duman et al., 2002). Glucocorticoids and chronic stress
models also decrease the expression of brain-derived neurotrophic factor (BDNF), a member of
the neurotrophin family, within the hippocampus and frontal cortex (Smith et al., 1995a; Schaaf
et al., 1997, 1998; Mao et al., 2010). Other regions of the brain and additional trophic factors are
less well studied in chronic stress paradigms.

Neurotrophic factors, including BDNF, neurotrophin-3 (NT-3) and transforming growth
factor alpha (TGFα), are known to have neurotrophic and neuroprotective effects on various
neuronal populations, including catecholaminergic neurons (Thoenen, 1995; Collier and
Sortwell, 1999; Yurek and Seroogy, 2001; Aron and Klein, 2011). Aberrant chronic stress-
induced modulation of these factors may increase the vulnerability of affected neuronal
populations and systems to maladaptive neuroplasticity and, perhaps, neurodegeneration. In this
study, we will utilize chronic variable stress (CVS), a well-accepted model of depressive-like
symptoms (Wilner et al., 2005), which mimics behavioral, hormonal and physiological changes
seen in humans. Chronic variable stress produces anhedonic behavior, learned helplessness,
adrenal hypertrophy and elevated CRH levels (Herman et al., 1995; Wilner et al., 2005; Cryan
and Mombereau, 2004; Ulrich-Lai and Herman, 2009), all of which have correlates in human
depression. We examined regions throughout the brain, including regions of the limbic system,
the nigrostriatal pathway, and brainstem nuclei locus coeruleus (LC) and dorsal raphe nucleus
(DRN), hypothesizing that alterations of expression will occur in the regions involved in both PD
and depression.

Antidepressant treatment has the potential to reverse the structural and behavioral
aberrations and cell loss caused by stress and depression (Nibuya et al., 1995; Nestler et al.,
2002). In human imaging studies, chronic antidepressant treatment is protective against volume
loss in the frontal cortex and hippocampus (Lavretsky et al., 2005; Sheline, 2003).
Administration of antidepressants can both reverse the loss of cell number and reduction of BDNF levels by increasing hippocampal neurogenesis as well as BDNF expression, and this may contribute to therapeutic action (Tardito et al., 2006). Expression the truncated (inactive) form of trkB, the high-affinity receptor of BDNF, prevents antidepressant actions, emphasizing the importance of BDNF/trkB signaling in treating depression (Saarelainen et al., 2003). As with chronic stress paradigms, regions of the brain outside the hippocampus and additional trophic factors have been less well examined in antidepressant studies. Little is known of the impact antidepressants have on the mesostriatal dopaminergic system. The responses of trophic factors, as well as of TH, to antidepressants in the nigrostriatal system and other regions involved in PD have received little attention. Thus, in the second part of this study, we chronically treated animals with desipramine (DES), a tricyclic antidepressant (TCA) that inhibits the reuptake of norepinephrine. Desipramine can elevate BDNF in the frontal cortex and hippocampus of normal rats and reverse corticosterone-induced decreases in BDNF (Dwivedi et al., 2006; Balu et al., 2008). We examined the expression of trophic factors, their receptors and TH within several regions of the adult rat brain after chronic antidepressant treatment to test the hypothesis that alterations will occur in directions contrary to those of CVS-treated animals.

Materials and Methods

Animals

Adult male Sprague Dawley rats (320-340 g) were acquired from Harlan Laboratories, Indianapolis, IN (n=6 per group) for the CVS study and adult male Wistar rats (280-330 g) were used for the antidepressant study (n=6 per group). Animals were housed two per cage under normal conditions (12 hours on/off light cycle) and food and water were available ad libitum. All
procedures were in compliance with University of Cincinnati Institutional Animal Care and Use Committee.

**Chronic variable stress**

Rats were randomly divided into two weight-matched groups, one group exposed to CVS and the other serving as the control group. The CVS protocol utilized has been characterized extensively previously (Herman et al., 1999). The CVS regimen used the following seven stressors: cold exposure (1h in a 4°C room), vibration (1h on shaker), hypoxia (9% oxygen, 30 min), cold swim (10 min in 16-18°C water), warm swim (20 min in 31-33° water), crowding (6/cage, overnight), isolation (1/cage, overnight). Animals in the CVS group were exposed to two stressors each day during the light cycle, one in the morning and one in the afternoon, irregularly timed for 14 days, with overnight stressors given six times intermittently. Stressors were administered in a random fashion to prevent habituation. Weights of the animals were collected on day seven and day fourteen of the regimen.

**Antidepressant administration**

Rats were administered the tricyclic antidepressant DES (10 mg/kg) (Bravo et al., 2009) or vehicle (saline) once daily (i.p.) for a three-week period.

**Tissue collection**

In the CVS study, the morning after the last stressor, the rats were sacrificed via rapid decapitation and the brains were frozen in isopentane. The thymus and adrenal glands were collected for analysis. Both the thymus and adrenal glands were cleaned and weighed to determine efficacy of the CVS regimen. Animals receiving antidepressant treatment were anesthesized and decapitated the day after the last injections. Their brains were collected and frozen in dry ice.
**In situ hybridization**

The fresh-frozen brains were serially sectioned at 10 µm coronally throughout the PFC, striatum, dorsal hippocampus, ventral midbrain, DRN and LC using a Microm cryostat, thaw-mounted onto Superfrost plus microslides (VWR, Batavia, IL), and subsequently stored at -20°C until hybridization. Semi-adjacent sections were processed for the *in situ* hybridization localization of BDNF, trkB, NT-3, trkC, TGFα, and TH mRNAs using linealized cDNA plasmids and were labeled using the proper polymerase (T3 for BDNF and NT-3; T7 for TH, trkB, trkC, and TGFα) and 35S-UTP (PerkinElmer, Boston, MA), as previously described (Seroogy et al., 1991, 1994; Seroogy and Herman, 1997; Numan et al., 2005; Dickerson et al., 2009).

The BDNF and NT-3 cDNA plasmids were gifts from Christine Gall and Julie Lauterborn, University of California at Irvine. The BDNF cRNA included 384 bases complementary to the rat BDNF mRNA coding region (Isackson et al., 1991; Gall et al., 1992), whereas the NT-3 cRNA was complementary to 392 bases of the rat NT-3 mRNA coding region (Ernfors et al., 1990; Maisonpierre et al., 1990; Gall et al., 1992). The trkB and trkC plasmids resulted in antisense RNA transcripts of 200 and 577 bases, respectively (Lamballe et al., 1991; Jelsma et al., 1993; Dixon and McKinnon, 1994). The trkB riboprobe detects only the full-length form of the rat trkB receptor (Middlemas et al., 1991; Goodness et al., 1997), whereas the trkC riboprobe recognizes both the full-length and truncated forms of the rat trkC receptor (Valenzuela et al., 1993; Dixon and McKinnon, 1994). The TGFα plasmid was a gift from H. Kornblum, UCLA.

For pretreatment, slides were brought to room temperature and fixed with 4% paraformaldehyde (pH 7.4) for 10 minutes. After fixing, the slides were put in a series of five
minute washes made with diethyl procarbonate (DEPC)-treated water. The washes were 0.1 M phosphate buffered saline (PBS) (twice), followed by 0.1 M PBS/0.2% glycine (twice), and again 0.1 M PBS (twice). The slides were then treated with triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 minutes. Lastly, the slides were dehydrated via a series of ethanol washes of increasing concentration, delipidated using chloroform, and air-dried.

After the pretreatment, slides were hybridized. The hybridization solution for each probe included the following: deionized formamide, 50% dextran sulfate, 10 mg/ml denatured salmon sperm DNA, 15 mg/ml tRNA, 5 M dithiothreitol, DEPC H2O, and the appropriate 35S-labeled cRNA probe. The sections were hybridized with 50 μl of the solution and subsequently coverslipped. The resulting concentration of the hybridization solution was 1 x 10^6 cpm/slide. The slides were incubated at 60°C for 18-24 hours inside a sealed, humidified chamber. After hybridization, the coverslips were removed and the slides were washed in RNase buffer and a series of standard sodium citrate washes of decreasing concentration. The hybridized slides were exposed to BioMax MR film (Kodak, Rochester, NY) for appropriate periods for each probe (BDNF: 12-19 days; trkB: 7-10 days; NT-3: 20-38 days; trkC: 6-8 days; TGFα: 6 days; TH: 1 day). The films were then developed using GPX (Kodak) developer and fixer.

**Analysis**

Film autoradiograms were analyzed by densitometry using Scion Image software (NIH) to compare the optic densities (OD) of hybridization in various brain regions of the CVS-treated versus control animals and the antidepressant-treated versus control animals. The following regions were examined: PFC, anterior, frontal, parietal, piriform and entorhinal cortices, striatum, hippocampus, substantia nigra pars compacta (SNpc), ventral tagmental area (VTA), dorsal raphe nucleus (DRN), and LC. At least six measurements were taken for each probe from
each animal and averaged together. Background values were taken from an unlabeled region of each section measured and subtracted from the OD obtained in order to determine the mean corrected grey level. The CVS and antidepressant data are shown as a percentage of the control data. Group differences of hybridization OD, animal weights, and weights of the thymus and adrenal glands were determined by t-test (GraphPad Prism). Differences were considered significant when $P < 0.05$.

**Results**

*Animal weights*

At the beginning of the experiment, there were no significant differences in body weight between the stressed and non-stressed groups. The weights taken of both groups at seven and fourteen days into the experiment verified the efficacy of the chronic stress regimen. At both time points, chronic-stressed animals gained significantly less weight compared to the control non-stressed animals ($t(30) = 5.367, P < 0.001$ at seven days; $t(30) = 4.524, P < 0.001$ at fourteen days) (Fig. 1).

The effectiveness of the CVS regimen was also verified using the weights of the adrenal glands and the thymus. The thymus of stressed animals weighed significantly less than non-stressed animals ($t(30) = 4.617, P < 0.001$), indicating atrophy (Fig. 2A). The adrenal glands of stressed animals weighed significantly more compared to the control animals ($t(30) = 4.184, P < 0.001$), suggesting hypertrophy the adrenals (Fig. 2B).

**Experiment 1: Neurotrophic factor expression after CVS exposure**

*NT-3 mRNA*

Two of the regions examined demonstrated altered levels of NT-3 mRNA expression in
Figure 1. Weights of Control and CVS animal throughout the course of the experiment.

Animals were divided into weight-matched groups at the beginning of the study. During the two weeks of CVS, stressed-exposed animals gained weight at a lesser rate compared to control animals. Data are expressed as mean ± SEM. *$P < 0.001$ compared to controls.
Figure 2

**Figure 2.** A: The thymus weighed significantly less in rats that were exposed to the CVS regimen. In addition, the thymus made up a smaller percentage of total body weight in CVS rats compared to the controls (data not shown). B: Both adrenal glands from the rats were removed at the time of sacrifice and weighed. Quantification of the average weight for each condition showed that the adrenals weighed more in the CVS-conditioned animals compared to the controls. Data are expressed as mean ± SEM. *$P < 0.05$ compared to control values.
Figure 3. Densitometric analysis of NT-3 mRNA expression of Control versus CVS rats. A: Expression of NT-3 mRNA was decreased in the anterior cingulate cortex after two weeks of CVS. B: In the granule cell layer of the dentate gyrus an increase in mRNA expression was detected. Data are expressed as mean ± SEM. *P < 0.05 compared to control values.
Figure 4. Film autoradiograms showing modulation of NT-3 mRNA expression in limbic cortical regions and hippocampus.  

A: Note the decrease in labeling for NT-3 mRNA in CVS versus control (Con) animals in the anterior cingulate cortex (AC Cortex).  

B: In the dentate gyrus stratum granulosum (DG), note the increase in NT-3 cRNA hybridization in the CVS exposed animals.
CVS-treated animals. In the anterior cingulated cortex (AC Ctx), stressed animals exhibited decreased NT-3 mRNA levels compared to the non-stressed controls ($t(10) = 3.165, P < 0.01$) (Figs. 3A, 4A). In contrast, NT-3 levels increased within the dentate gyrus in the chronic stressed animals versus the controls ($t(14) = 4.201, P < 0.001$) (Figs. 3B, 4B). There were no changes found in the PFC, frontal, parietal, piriform, and entorhinal cortices, striatum, SNpc, VTA, DRN, or LC (data not shown).

**TH mRNA**

Expression of TH mRNA within the LC increased slightly in CVS animals compared to controls ($t(11) = 2.423, P < 0.05$) (Figs. 5A, 6A). No alterations were observed in either the SNpc or VTA (data not shown).

**trkC mRNA**

The entorhinal cortex demonstrated elevated levels of trkC mRNA in the stressed animals compared to the unstressed controls ($t(13) = 2.860, P < 0.05$) (Figs. 5B, 6B). There were no changes found in the PFC, anterior, frontal, parietal, and piriform cortices, striatum, hippocampus, SNpc, VTA, DRN, or LC (data not shown).

**BDNF mRNA**

Two regions exhibited changes in BDNF expression in CVS animals compared to the unstressed control animals. Levels of BDNF mRNA were diminished in the piriform cortex of stressed animals ($t(10) = , P < 0.05$) (Figs. 7A, 8A). In the LC, BDNF expression in chronic stressed animals also decreased compared to the control group ($t(10) = 2.802, P < 0.05$) (Figs. 7B, 8B). There were no changes found in the PFC, anterior, frontal, parietal, and entorhinal cortices, hippocampus, SNpc, VTA, or DRN (data not shown).
Figure 5. Densitometric analysis of TH and trkC mRNA expression in cortical regions and locus coeruleus (LC). **A**: Expression of TH mRNA in the LC is increased slightly, but significantly, after CVS exposure. **B**: In the entorhinal cortex, expression of trkC mRNA is increased after two weeks of CVS. Data are expressed as mean ± SEM. *P* < 0.05 compared to respective control values.
Figure 6. Film autoradiograms showing modulation of trkC mRNA expression in cortical regions and TH mRNA in locus coeruleus. A: A slight increase in TH mRNA labeling is evident in the LC following CVS exposure. B: Elevated trkC mRNA hybridization is detected in the entorhinal cortex (Ent Ctx) of CVS-treated versus control (Con) animals.
Figure 7. Densitometric analysis of BDNF mRNA expression in the piriform cortex and locus coeruleus (LC). Levels of BDNF mRNA in both piriform cortex (A) and LC (B) were significantly decreased after CVS exposure. Data are expressed as mean ± SEM. *$P < 0.05$ compared to respective control values.
Figure 8. Film autoradiograms showing BDNF mRNA expression in CVS versus Control (Con) animals. A: In the piriform cortex (Pir Ctx), hybridization for BDNF mRNA is decreased in CVS-treated versus control rats. B: Expression of BDNF mRNA in the locus coeruleus (LC) is decreased in animals after two weeks of CVS exposure.
Figure 9. Densitometric analysis of TGFα mRNA expression in hippocampus and striatum. **A:** Hybridization signal for TGFα mRNA was increased in the hilar region of CA3 stratum pyramidale in the hippocampus post-CVS. **B:** Elevated levels of TGFα mRNA were detected in the striatum after CVS exposure. Data are expressed as mean ± SEM. *P < 0.05 compared to respective control values.
Figure 10. Film autoradiograms showing elevated TGFα mRNA expression in CVS versus control (Con) animals in the region CA3c of hippocampal region (A) and striatum (B). Levels of TGFα mRNA expression were increased in both regions two weeks after CVS exposure.
TGFα mRNA

Two regions demonstrated altered TGFα mRNA expression in chronically stressed animals. In the striatum, there was an elevation of TGFα mRNA in the stressed animals ($t(10) = , P < 0.05$) (Figs. 9A, 10A). The hilar region of CA3 stratum pyramidale of the hippocampal formation also showed enhanced TGFα expression in the CVS animals compared to the controls ($t(13) = 3.197, P < 0.01$) (Figs. 9B, 10B). No changes were found in the PFC, frontal, parietal, piriform, and entorhinal cortices, SNpc, VTA, DRN, or LC (data not shown).

trkB

No alterations were observed in this experiment for trkB mRNA expression in any of the regions examined in stressed animals versus controls (data not shown).

Experiment 2: Neurotrophic factor expression after antidepressant treatment

BDNF

Expression of BDNF was altered in three brain regions in animals receiving chronic antidepressant treatment. Compared to controls, hybridization for BDNF mRNA was elevated in DES-treated animals in the AC Ctx ($t(10) = 6.159; P < 0.001$; Figs. 11A, 12), the frontal cortex ($t(10) = 2.447, P < 0.05$; Figs. 11B, 12), and the piriform cortex ($t(10) = 2.310, P < 0.05$; Figs. 11C, 12). There were no other alterations in the other regions examined (data not shown).

NT-3

Hybridization for NT-3 mRNA was decreased in the AC Ctx in animals receiving chronic DES treatment compared to the controls ($t(8) = 25.93, P < 0.05$; Figs. 13A, 14). In contrast, NT-3 expression was elevated in the piriform cortex within DES-treated animals ($t(9) = 4.990, P < 0.001$; Figs. 13B, 14). No other regions examined were found to exhibit significant changes.
Figure 11. Densitometric analysis of BDNF mRNA expression in anterior cingulate (A), frontal (B) and piriform (C) cortices. Hybridization signal was elevated in all three regions in the desipramine (DES)-treated animals after three weeks of antidepressant administration. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.001 compared to respective control values.
**Figure 12** BDNF mRNA after DES

**Cortical Regions**

*Figure. 12.* Densitometric analysis of BDNF mRNA hybridization in several cortical regions.

In the desipramine (DES)-treated animals, expression was elevated in the anterior cingulate (AC), frontal (Fr), and piriform (Pir) cortices compared to the saline-treated control (Con) animals.
Figure 13. Densitometric analysis of NT-3 hybridization in cortical regions.  

**A:** Expression of NT-3 mRNA in the anterior cingulate cortex was decreased after desipramine (DES) treatment.  

**B:** In the piriform cortex, NT-3 mRNA levels were enhanced after three weeks of antidepressant treatment.  

Data are expressed as mean ± SEM.  *$P < 0.05$, **$P < 0.001$* compared to respective control values.
Figure 14. Film autoradiograms showing modulation of NT-3 mRNA expression in anterior cingulate (AC) cortex and piriform cortex. **A:** A decrease in NT-3 mRNA labeling was evident in the AC cortex following desipramine (DES) administration. **B:** Elevated NT-3 mRNA hybridization was detected in the piriform (Pir) cortex of antidepressant-treated versus control animals.
trkB

In the striatum, hybridization for trkB mRNA increased significantly in animals that received chronic DES administration (t(6) = 2.905, \( P < 0.05 \); Figs. 15A, 16). Additionally, DES-treated animals exhibited enhanced trkB expression in the piriform cortex (t(10) = 2.477, \( P < 0.05 \); Figs. 15B, 16). Changes were not observed in any of the other regions examined (data not shown).

trkC and TH

In this experiment, expression of trkC and TH mRNAs was not altered in the regions examined in antidepressant-treated versus control animals (data not shown).

Discussion

In the first experiment in this study, chronic unpredictable stress was found to elicit changes in expression of neurotrophic factors, neurotrophic factor receptors, and TH in select cortical, striatal and brainstem regions. For example, in several limbic cortical regions expression of BDNF and NT-3 mRNAs was reduced, whereas that of trkC mRNA was elevated after chronic stress. We also observed that TH mRNA expression, similar to other models of chronic stress, was elevated in the LC after exposure to CVS; however, BDNF levels were reduced in the chronically stressed LC. Additionally, in CVS treated rats, TGF\(\alpha\) mRNA was upregulated in the striatum, and in the hippocampus, levels of both TGF\(\alpha\) and NT-3 were increased in a region-specific manner. These results suggest aberrant regulation of neurotrophic factors and/or their receptors and of TH by CVS in limbic and catecholaminergic systems. These chronic stress-induced alterations raise the possibility of increased vulnerability of affected
Figure 15. Densitometric analysis of trkB mRNA expression in striatum and cortex. **A:**

Hybridization for trkB mRNA was increased in the striatum after three weeks of DES administration. **B:** In the piriform cortex a small, but significant increase in hybridization was detected in the antidepressant-treated animals. Data are expressed as mean ± SEM. *P < 0.05 compared to control values.
Figure 16. Film autoradiograms showing modulation of trkB mRNA expression in the striatum and piriform cortex.  

A: Note the increase in labeling for trkB mRNA in desipramine (DES) versus control (Con) animals in the striatum (mainly dorsal aspects).  

B: In the piriform (Pir) cortex, a slight but significant increase in trkB cRNA hybridization was observed in antidepressant-treated animals.
neuronal populations to maladaptive neuroplasticity.

In the second experiment, chronic antidepressant (desipramine) treatment induced an upregulation of BDNF expression in several mesocorticolimbic regions that receive dopaminergic innervation, including the anterior cingulate and piriform cortices. The trkB receptor was also upregulated in the piriform cortex and striatum after chronic antidepressant treatment. The neurotrophin NT-3 was increased in the piriform cortex as well, but mRNA levels decreased in the anterior cingulate cortex. These results raise the possibility that enhanced neurotrophin expression afforded by antidepressant administration may counter the decreased neurotrophin expression induced by CVS in some of the same brain structures. However, relevant mRNA expression in other mesotelencephalic components of central stress and PD circuitry, although altered, did not reveal complementary regulation with CVS compared to antidepressant treatment. Overall, these findings of antidepressant-induced modulation of pertinent trophic factors, their receptors and synthesizing-enzymes in the mesotelencephalic system and relevant stress circuitry may reveal involvement of such factors in the comorbidity of PD and depression.

Chronic variable stress is a well-characterized model of depression symptomatology and closely mimics the behavioral and hormonal changes observed in depression. It has advantages over other chronic stress models in that it prevents habituation to the stress response (Magariños and McEwan, 1995). Chronic, but not acute antidepressant treatment reverses the anhedonic behavioral effects seen in the model (Roth and Katz, 1981). During CVS, there is a hypersecretion of corticosterone and adrenocorticotropic hormone, an upregulation in CRH mRNA levels in the paraventricular nucleus and adrenal hypertrophy (Herman et al., 1995). The expression of both mineralocorticoid and glucocorticoid receptors are decreased in hippocampal
subfields (Herman et al., 1995). Chronic stress and corticosterone administration can also modulate neurotrophic factor expression (Smith et al., 1995a; Schaaf et al., 1997, 1998).

The lack of trophic support in regions of the limbic system is thought to play a part in neuropathology in depression, and this loss may be reversed by antidepressant actions (Nestler et al., 2002). The downstream alterations in plasticity caused by neurotrophic factors may explain the delay in antidepressant behavioral effects despite the ability of antidepressants to acutely affect the levels of monoamines (Nestler et al., 2002). Neurotrophic factors promote neuronal survival and protection of injured systems (Skaper et al., 1993; Altar et al., 1994; Martin-Iversen et al., 1994; Zhang et al., 1997; Farkas and Krieglstein, 2002; Zhang et al., 2004). Neurotrophic factors, and neurotrophins in particular, exert positive and sometimes reciprocal actions with serotonin and norepinephrine, the two neurotransmitters commonly associated with depression. After injury, both BDNF and NT-3 are able to promote sprouting of serotonergic neurons (Mamounas et al., 1995; Grider et al., 2005). Conversely, alterations in serotonin affect BDNF levels in both the hippocampus and frontal cortex (Zetterström et al., 1999). Norepinephrine and BDNF display a similar mutual relationship, with BDNF able to increase the number of noradrenergic neurons (Holm et al., 2003) and norepinephrine able to increase levels of BDNF in hippocampal cell cultures (Patel et al., 2010). The ability of antidepressants to acutely increase serotonin and norepinephrine neurotransmitter levels may be the reason for the increase in neurotrophic factors (Nestler et al., 2002; Mattson et al., 2004). It is likely that the neurotrophic factors themselves are not antidepressive, but instead activate signaling genes such as cAMP-response-element-binding protein (CREB) to bring about many of the plasticity changes observed and help reorganize neuronal networks (Nestler et al., 2002; Castrén and Rantamäki, 2009).
Clinicians use many different classes of antidepressants to treat depression in PD, including selective serotonin reuptake inhibitors (SSRI), selective norepinephrine reuptake inhibitors, TCAs and, more recently dopamine agonists (Schrag, 2006; Barone et al., 2010). There are, however, very few well-controlled studies evaluating antidepressant effectiveness in PD (Weintraub, 2005). In this study, we chose to examine the effects of the TCA DES on neurotrophic factor administration. Desipramine is a potent inhibitor of norepinephrine reuptake transporters and at very high concentrations inhibits serotonin and dopamine reuptake (Tremblay and Blier, 2006). Administration of DES increases levels of norepinephrine and augments adrenoreceptor transmission (Tremblay and Blier, 2006). Desipramine is able to inhibit oxidative stress and apoptosis in many neural systems and disease models including 6-OHDA (Lauterbach et al., 2010), suggesting potential neuroprotective actions. The antidepressant DES also increases the expression of glucocorticoid receptor in hippocampal neurons (Okugawa et al., 1999), which are decreased in chronic stress. In treating depression symptoms in PD, DES is more effective than the SSRI citalopram, but also has more side effects (Devos et al., 2008). In a genetic depression model, DES decreased α-synuclein in the striatum, indicating potential therapeutic benefits even in PD without depression (Jeannotte et al., 2009). Treatment with DES can also increase BDNF levels in select brain regions (Nibuya et al., 1995; Balu et al., 2008). Thus, accumulating evidence suggests that desipramine exerts varied, often neuroprotective, effects on various transmitter and trophic factor systems. Moreover, these neurochemical actions of DES point towards its potential as being effective in treating depression in PD.

Many depression studies predominately focus on neurotrophic factor expression in the hippocampus. We were unable to detect changes in BDNF or trkB expression in the hippocampus either after chronic stress or chronic antidepressant treatment. This is intriguing, as
BDNF modulation in the hippocampus is one of the most commonly reported alterations after stress exposure (Smith et al., 1995a; Schaaf et al., 1997; Zhang et al., 2010) and antidepressant treatment (Duman and Monteggia, 2006; Zhang et al., 2010). The receptor trkB is often altered as well (Schaff et al., 1997; Nibuya et al., 1999). However, BDNF changes, particularly after stress, may not be as straightforward as first suspected. Similar to our results, other studies have found no alterations of BDNF in the hippocampus at all after chronic stress exposure (Lee et al., 2006; Allaman et al., 2008; Hanson et al., 2011). These findings indicate that stress-induced BDNF changes in the hippocampus may not be as general as thought and might be sensitive to the specific stressors used and the length of exposure. It also emphasizes the need to use appropriate depression models, as some commonly used (such as learned-helplessness) may not be actually modeling depressive symptoms (Nestler and Hyman, 2010). Alterations after antidepressant treatments may also be sensitive to the class of antidepressant, route of administration and the dosage used. In contrast to the lack BDNF alterations in the hippocampus, we found an increase of TGF\(\alpha\) in the hippocampal CA3c region and of NT-3 in the DG after CVS exposure. Other stress studies have also noted NT-3 expression increases in the hippocampus (Smith et al., 1995a; Song et al., 2006). It is likely this is a response to the increase in glucocorticoid levels caused by the CVS regimen, as NT-3 increases in the hippocampus are corticosterone-dependent (Smith et al., 1995a) and dexamethasone increases NT-3 expression in the hippocampus after ischemia and traumatic brain injury (Yang et al., 2002, 2005). There are few findings on the modulation of TGF\(\alpha\) in the hippocampus, but the increases may protect against stress-induced increases in dendritic atrophy. However, despite these changes and numerous relevant publications, the direct involvement of the hippocampus in depression is not yet proven (Nestler et al., 2002).
In the present study, we observed several trophic factor alterations in cortical regions involved in the limbic system and learning and memory. Although we did not detect modulation of any neurotrophic factors examined in the hippocampus after chronic antidepressant treatment, we did observe increases in BDNF in the anterior cingulate and frontal cortices. Both the frontal and anterior cingulate cortices are part of the mesocorticolimbic system of mood and stress control and receive dopaminergic projections from the midbrain. Our results are similar to a previous study, in which DES did not alter BDNF protein expression in the hippocampus, amygdala, olfactory bulb, and brainstem, but did increase expression in the frontal cortex (Balu et al., 2008). Also in the anterior cingulate cortex, NT-3 mRNA levels were downregulated after both CVS and DES treatment. The loss of NT-3 expression in the anterior cingulate cortex after stress might increase the susceptibility neurons to plasticity changes such as dendritic atrophy.

The piriform cortex is a particularly plastic region in our experiments. After treatment with DES, levels of BDNF, NT-3 and trkB were all increased and chronic stress downregulated BDNF expression. The increase of BDNF in the piriform after DES treatment may counteract the decrease brought about by stress. The trophic factor alterations in piriform cortex are not treatment-specific. Other antidepressants and other classes of drugs are able to increase BDNF expression in the piriform cortex (Nibuya et al., 1995; Meredith et al, 2002; Hemmerle et al., 2006) and in several brain injury models both BDNF (Tsukahara et al., 1998; Hicks et al., 1999) and trkB (Mudó et al. 1993) are increased in this region. Importantly, the piriform cortex receives dopaminergic projections from the SNpc and BDNF and NT-3 can be retrogradely transported (DiStefano et al., 1992; Sobreviela et al., 1996). One could speculate that as the trophic environment increases in the piriform cortex after DES treatment, the piriform cortex may be a source of retrograde neuroprotection for the dopaminergic neurons within the SNpc.
Conversely, the loss of BDNF levels in the piriform region after CVS may negatively influence the dopaminergic afferent neurons.

The NT-3 high-affinity receptor, trkC was upregulated in the entorhinal cortex following CVS. The entorhinal cortex both receives and sends inputs to the hippocampus (Naber et al., 2001). Injection of glutamate receptor agonists into the entorhinal cortex increases both BDNF and trkB mRNA in the hippocampus, indicating that changes in the entorhinal cortex can influence gene expression in the hippocampus (Flakenberg et al., 1996). Whether the trkC increase observed in the entorhinal cortex could influence NT-3 levels in the hippocampus or vice versa remains unknown.

The LC contains most of the noradrenergic cell bodies in the brain. The noradrenergic system is affected by gene expression changes during depression and loss of noradrenergic axonal projections and cell bodies are observed in chronic stress depression models (Kitayama et al., 1997, 2008; Krishnan, and Nestler, 2010). A loss of LC neurons occurs within PD patients as well (Rommelfanger and Weinshenker, 2007). The LC is involved in a myriad of functions including sleep, mood and stress regulation. We observed a slight increase in LC TH mRNA after CVS treatment, but no changes after treatment with DES. Previous studies of TH expression in the LC have found an increase after acute, but not chronic stress (Smith et al., 1991) and a decrease after treatment with multiple classes of antidepressants (Nestler et al., 1990). This discordance in findings may be explained by the difference in experimental design. Smith et al. (1991) used chronic restraint, which causes habituation of the stress response (Magariños and McEwan, 1995), and Nestler et al. (1990) examined protein, not mRNA, and did not specifically examine DES. Modulation of NT-3 mRNA in the LC has also been reported, with an increase after chronic restraint and a decrease after treatment with several
antidepressants, including DES (Smith et al., 1995b). We did not observe any such alterations of NT-3 expression in our experiments, but did find a decrease in BDNF in LC after chronic stress. Interestingly, this latter data also stands in contrast to a previous report (Smith et al., 1995b).

Many neurotrophic factors are neuroprotective for dopamine neurons, particularly in in vitro studies, but also in vivo (Collier and Sortwell, 1999; Yurek and Seroogy, 2001; Aron and Klein, 2011). In both human PD patients and aged animals, there is a decrease in trophic support in the nigrostriatal system (Howells et al., 2000; Ling et al., 2000; Dickerson et al., 2009). After lesioning, the compensatory neurotrophic response in the nigrostriatal system is also attenuated in older animals (Yurek and Fletcher-Turner, 2001, 2002). The altered, perhaps insufficient, trophic environment may explain the particular vulnerability of the dopaminergic neurons to neurodegeneration in the aged. Importantly, there is a greater decrease in BDNF serum levels in PD patients with depression than non-depressed PD patients, perhaps indicating a greater general loss of BDNF in dressed PD patients (Ricci et al., 2010). However, serum levels do not necessarily reflect brain BDNF levels.

Both NT-3 and BDNF, as well as their receptors, are co-localized within dopaminergic neurons in the SNpc (Seroogy et al., 1994; Numan et al., 1999) and are able to protect against neurotoxins in vivo (Hagg, 1998). However, while TGFα is also present in the nigrostriatal system (Seroogy et al., 1991; Yurek and Seroogy, 2001), it does not have as robust a neuroprotective effect (Alexi et al., 1996). On the other hand, in our CVS model TGFα is upregulated in the striatum, indicating dysregulation of the neurotrophic environment in response to the stress-induced effects. The BDNF receptor trkB was elevated after DES administration in the striatum. While BDNF content in the nigrostriatal pathway was not changed, an elevation in trkB could increase BDNF signaling in the striatum. We found no TH mRNA alterations in the
SNpc after chronic stress or antidepressant treatment, indicating neither CVS nor DES directly alters TH mRNA content in the nigrostriatal pathway in non-lesioned animals within the timeline of our studies.

In conclusion, these data suggest that chronic unpredictable stress alters the neurotrophic environment in the mesotelencephalic system and other regions involved in both depression and PD. This aberrant trophic condition could be postulated to increase the vulnerability of dopaminergic neurons to injury or degeneration. Administration of antidepressants may well help counteract these changes and provide neuroprotection or neurorestoration, emphasizing the important of detecting and treating depression in PD. Finally, our results expand the known regions in limbic and other systems demonstrating neurotrophic factor alterations in the present two paradigms. Further examination of other antidepressants as well as assessing trophic factor modulation in the combined chronic stress/PD animal model (see Chapter 2) is warranted to help clarify the potential roles of neurotrophic factors in both depression and PD.
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Chapter 5
General Discussion

In Parkinson’s disease (PD), the presence of motor symptoms is essential for clinical diagnosis and this component of the disorder is the focus of most clinical and basic research. However, the prevalence of non-motor symptoms presents a more complicated picture, which detrimentally affects patient quality of life. The lack of definitive therapies for these symptoms demonstrates the great need for the development of better animal models. The goal of this dissertation was to explore the effects of chronic variable stress (CVS) on the progressive loss of dopaminergic neurons after lesioning with 6-hydroxydopamine (6-OHDA) and the possible mechanisms that may be behind these outcomes. In Chapter 2, we described the generation of the combined model as well as several different temporal iterations of the effects of chronic stress on motor dysfunction and nigral dopaminergic cell degeneration. In Chapters 3 and 4, several different mechanistic aspects of CVS were explored to ascertain what underlies the exacerbated behavioral impairment and neuronal loss. Thus, in Chapter 3, the potential critical role of glucocorticoids alone, without CVS, in the increased neurodegeneration was evaluated. Finally, in Chapter 4, alterations of the neurotrophic environment after chronic stress and antidepressant treatment were examined to determine if there were changes in regions of the brain involved in depression and PD, which may underlie an increased susceptibility to neurodegeneration.
Chronic variable stress and neurodegeneration

The principle finding of this dissertation is that animals exposed to chronic unpredictable stress flanking a nigrostriatal lesion display a worsening of PD symptoms, both behaviorally and pathologically (Chapter 2). Studies focusing on the role of stress and depression alone have noted alterations in cell number and volume in several other brain regions. Chronic stress decreases the volume of the hippocampus, likely a result of dendritic atrophy and decrease in neurogenesis (Kanner, 2004). Many other studies have also observed structural alterations in the prefrontal cortex (PFC), an essential region in the formation of depression (Wellman, 2001; Radley et al., 2004; Cerqueira et al., 2005; Dias-Ferreira et al., 2009). There are also important human correlates, with a decrease in hippocampal and PFC volume observed in depressed human patients (Sheline et al., 1996; Bremner et al., 2000; Sheline, 2003; Salvadore et al., 2001). The changes in brain structure in depressive patients mean that depression is likely not simply caused by changes in neurotransmitter levels, but is a result of physical alterations in brain circuitry (Kanner, 2004). However, no definitive link between depression, stress dysfunction, and volume loss in humans has been discovered as of yet (Kanner, 2004; Tata and Anderson, 2010).

Interestingly, in regard to PD, the striatum also exhibits some alterations after chronic stress (Dias-Ferreira et al., 2009). In contrast to other areas, stress causes the amygdala to increase in dendritic complexity (Vyas et al., 2002; Mitra and Sapolsky, 2008), indicating that stress can have regionally specific effects. Importantly, impairment of learning occurs in animals with a hyperactive hypothalamic-pituitary-adrenal (HPA) axis, indicating elevated glucocorticoids result in hippocampal dysfunction and produce behavioral alterations (Issa et al., 1990). Whether hypersecretion of glucocorticoids can affect the nigrostriatal pathway and result in worsened motor outcomes, too, was one of the focuses of this dissertation.
Research on the effects of stress on the dopaminergic system demonstrates physiological alterations, but mainly in the mesolimbic and mesocortical pathways rather than the mesostriatal pathway (e.g. Pani et al., 2000). While this has important relevance for the formation of depression, it does not indicate whether stress-induced depression could possibly affect the motor symptoms of PD. Most stress research does not usually focus on the nigrostriatal system, and those few studies that do have found some, but not many, changes (e.g. Kim et al., 2005). It is likely that stress does not have as readily apparent affects on the uninjured substantia nigra pars compacta (SNpc), at least not in the common timeframe of most experiments. However, these studies are also often carried out in otherwise healthy animals. Research has demonstrated that stress can increase subsequent injury to neurons compared to non-stressed animals (Sapolsky, 1994; Chen et al., 2009). While the increased loss of dopaminergic neurons in our PD model when animals are exposed to stress-induced depressive symptoms is intriguing, it is not completely surprising. A few other studies demonstrate stress elevating the levels of neurodegeneration in the injured SNpc. Animals exposed to acute restraint prior to lesioning had a worsened outcome (Smith et al., 2002). Chronic restraint also appears to induce behavioral alterations and increased neurodegeneration in the SNpc, although specific decreases in tyrosine hydroxylase (TH) loss were not observed. However, these paradigms do not accurately reflect many of the physiological alterations likely occurring in depression, as our model does.

This does raise the question of whether our paradigm elicits the increased dopaminergic neuronal deficits due to the presence of depressive effects or simply via chronic stress, which are not one and the same. To address this, future experiments must compare lesioned animals exposed to CVS to lesioned animals given chronic restraint stress. Chronic restraint creates habituation to the stress response and does not result in depressive symptoms such as adrenal
hyperplasia or learned helplessness (Magariños and McEwan, 1995; Gregus et al., 2005). If restrained rats do not present with worsened dysfunction, this likely means that the increased neurodegeneration requires the presence of the strongly activated HPA axis and formation of depressive symptoms brought about by the chronic unpredictable stress paradigm. If the restrained rats do display increased dopaminergic loss in the SNpc as well, there are still clinical implications for our model, as alterations in the stress response occur in PD patients and would emphasize the need for controlling stress levels in PD (Charlett et al., 1998; Müller and Muhlack, 2007; Macht et al., 2007).

There are many behavioral correlates to human depression in the CVS model. Most importantly is the presence of anhedonia, whether demonstrated by decrease in sucrose water intake or brain reward stimulation (Zacharko et al. 1983; Willner, 1997). Other behavioral symptoms include increased immobility in the forced swim test, potentiated learned helplessness, decreased male sexual behavior and aggression, sleep dysfunction and decreased grooming, though not every study has found such results (Willner, 2005). Some studies have noted the presence of anxiogenic behavior in CVS animals (Zurita et al., 2000), though others have found an increase in anxiolytic-like behavior (D’Aquila et al., 1994). This has relevance to PD patients, as many with depression also have anxiety (Cummings and Masterman, 1999), and both anxiety and depression are often present before the clinical diagnosis (Shiba et al., 2000; Jacob et al., 2010).

Whereas we did find increased parkinsonian deficits in animals exposed to the depression-relevant CVS model, we did not fully examine how the 6-OHDA lesion possibly affected depressive symptoms in the chronically stressed animals. Stress alone did not cause motor behavior alterations or neuronal loss. One of the major markers we used for CVS
effectiveness was the decrease in weight gain, commonly observed in CVS animals (Willner 1997, 2005). Both the lesioned and unlesioned stressed animals did not have differences in the decreased rate of weight gain in any of the experimental iterations. Still, there are many other depressive symptoms present in the CVS model that might be affected by the 6-OHDA lesion, including decreases in the sucrose water intake as a sign of anhedonia, potentiation of learned helplessness, and increases in immobility time in the forced swim test (Nestler et al., 2002; Willner, 2005). Because the striatal lesion results in mobility deficits, the examination of learned helplessness and the forced swim test is difficult to assess in the PD model. However, evaluation of changes in sucrose water consumption, believed to be independent of the weight loss, is possible (Willner 1997, 2005). Although we have preliminary data indicating that sucrose water consumption is not varied between lesioned and unlesioned animals (Knecht et al., 2010; data not shown), others have in a “premotor” stage bilateral lesion model of PD showed a decrease in sucrose consumption (Tadaiesky et al., 2008). Future studies should determine whether unilateral dopaminergic loss in the SNpc alters the presentation of depressive symptoms compared to the use of chronic stress alone.

The presence of depression in PD could worsen the severity of both motor and non-motor functions. In depressed patients, the incidence of sleep disorders is elevated, possible indicating a more severe disease process (Tandberg et al., 1998; Happe et al., 2002; van Rooden et al., 2009). Some studies indicate that PD patients with depression have more severe motor symptoms (Pålhagen et al., 2008). The increased dopamine loss caused by depression may in turn worsen the depressive symptoms as the dorsomedial and ventral thalamic nuclei of the basal ganglia have dopaminergic projections into the prefrontal and frontal cortices (Malhi and Berk, 2007). Therefore, as stated earlier, it is important to recognize and treat depression in PD.
In the present experiments, it was necessary to have animals exposed to the effects of stress both prior to and after the lesion (flanking of lesion) in order to obtain exacerbated motor dysfunction. Some PD patients may have depression symptomatology both before and after the onset of motor symptoms, and perhaps it is these patients with longer exposure to depression pathophysiology that have a worsened disease outcome. It is unknown whether patients diagnosed with depression only preclinically or only after onset of motor symptoms have such detrimental effects, and our model did not reveal alterations in animals only exposed before or after the lesion. Worsening of motor behavior in the concomitant model did not occur until four weeks post-lesion, which is also the timepoint that TH+ cell numbers were evaluated. It would be interesting to determine whether the two week mark exhibited a difference in neuronal loss in stressed versus non-stressed animals that had not yet resulted in behavioral changes, particularly given the alterations in neurotrophic factor expression observed after two weeks of CVS (Chapter 4). A large caveat to our findings is that the pre- and post-CVS models encompass a shorter total time exposed to the stressors compared to the flanking regimen, and it may be this shorter total exposure period, not the precise chronological placement of the lesion, that precludes observation of motor deficiencies.

In preclinical stages of PD, a small loss of dopaminergic neurons in the SNpc is apparent, but many other biochemical changes are occurring, such as a decrease in TH expression and increase in oxidative stress in the SNpc (Ferrer et al., 2011). Oxidative stress is also elevated in the PFC in preclinical PD (Ferrer et al., 2011). Interestingly, it is not until the later stages of PD that α-synuclein inclusions are present in brain regions associated with depression (Braak et al., 2004). However, neuropathology is present in the brainstem in preclinical stages in the locus coeruleus (LC) and dorsal raphe nucleus (DRN) (Braak et al., 2004), the primary nuclei of the
noradrenergic and serotonergic systems, respectively. Degeneration of the LC possibly precedes the loss of neurons in the SNpc (Rommelfanger and Weinshenker, 2007). Whether this contributes to depression in early PD is unknown, though the LC and DRN are involved in stress regulation (Chaouloff, 2000; Ressler and Nemeroff, 2000). Still, the presence of the α-synuclein inclusions does not necessarily correlate with actual cell loss, so it is too early to tell the implications. It may also be that mechanisms for neurodegeneration independent from α-synuclein formation occur in the PD brain (Ferrer et al., 2011).

There are several potential concerns for our combined model that should be noted. While many patients with depression display hyperactivation of the HPA axis, a large percentage with depression does not (Kanner, 2004). This means that dysfunction of the HPA axis does not equal depression. Therefore, it is important to have other behavioral makers to help validate the model. As mentioned above, CVS shares several behavioral correlates with depression, including anhedonia, emphasizing the validity of CVS as a model of the disorder. The question also arises whether we are modeling the appropriate type of depression observed in PD. There is the contention that there are several different subtypes of depression in PD, such depressive symptoms with or without anxiety (Brown et al., 2011). Still, given the role of stress in PD (Snyder et al., 1985), the combined CVS/6-OHDA model should have clinical significance, allowing the study of the role of stress dysfunction on PD symptomatology.

The present results have important implications for the progression of PD symptoms in patients with depression comorbidity and emphasize the need for clinicians to be aware of the potential of non-motor complications in PD. It may also be advisable for psychiatrists dealing with depressed patients, particularly in the older age set, to scrutinize for the onset of PD motor symptoms and other neurodegenerative diseases that have a high rate of depression. The
increased recognition of preclinical PD presents the opportunity to catch the initial formation of the disorder and may permit early intervention.

**Potential mechanisms for increased neurodegeneration in the SNpc**

While glucocorticoids are directly implicated in increasing neuronal vulnerability in many instances (Sapolsky, 1994; Chen et al., 2009), we did not find this to be the case for our experiments (Chapter 3). Neither glucocorticoid injections nor the administration of the glucocorticoid antagonist mifepristone (RU486) altered the levels of neurodegeneration in the 6-OHDA model. The role of glucocorticoids and neurodegeneration is complex. In many cases, increased glucocorticoid levels decrease neurogenesis in the hippocampus, but adrenalectomy and subsequent loss of glucocorticoids can result in a decrease in neurogenesis as well (Reagan and McEwen, 1997). While in many cases, administration of dexamethasone diminishes dopamine content in MPTP-treated animals at a high dose, it is able to protect against dopamine loss at a lower dose, demonstrating that steroids may also have potential neuroprotective properties at the right concentration (Kurkowska-Jastrzębska et al., 2004). Dysfunction of the glucocorticoid receptor in the SNpc can also increase the vulnerability of neurons to neurotoxic loss (Morale et al., 2003). Nevertheless, we did not observe any neuroprotective or neurodegenerative effects of corticosterone (CORT) in the lesioned rats. It is possible that the dosage levels of CORT were not sufficient despite the effects on weight. We did not determine the levels of CORT or other stress hormones throughout the experiment and therefore must presume that the regimen mimicked the intermittent increase of glucocorticoids sufficiently. However, we did use a CORT dose that previously duplicated the effects of CVS (Zhang et al. 2010). Another potential confounding factor in the CORT experiment is the extent of the 6-
OHDA lesion. Though the previous CVS/6-OHDA experiments in Chapter 2 cannot be directly compared, it appears that the lesions are more extensive in the CORT experiment animals. Thus, the lack of worsened behavior and neurodegeneration in the CORT/6-OHDA animals may result from the lesion being too extensive for CORT administration to have an effect.

In Chapter 4, the function of neurotrophic factors in both the neuropathology and treatment of depression was explored. We found alterations of several neurotrophic factors after both CVS and chronic administration of the tricyclic antidepressant desipramine. However, with the exception of brain-derived neurotrophic factor (BDNF) in the piriform cortex, there was no reciprocity in the modulation of neurotrophic factor expression between the chronic stress and chronic antidepressant experiments. Neurotrophic factors were altered in the nigrostriatal pathway after both CVS and antidepressant treatment, as well as in relevant regions for depression. Loss of neurotrophic support in PD is hypothesized to increase the susceptibility of neurons to injury (Ling et al., 2000; Dickerson et al., 2009). This is also thought to be the case in depression. The neurotrophic hypothesis of depression asserts that loss of neurotrophic factors in limbic regions leads to neuronal vulnerability that ultimately results in depressive symptoms and that antidepressant treatment reverses this loss (Nestler et al., 2002). The stress-induced changes in trophic availability may suggest a mechanism for the increased neuronal loss observed in the CVS/6-OHDA model (Chapter 2).

The neurotrophins BDNF and neurotrophin-3 (NT-3) often are regulated in contrasting fashion. For example, loss of glucocorticoid levels decreases NT-3, while stimulation of mineralocorticoid receptors can reverse this loss (Chao et al., 1998). In contrast, BDNF mRNA levels are increased in adrenalectomized rats and reversed by glucocorticoid receptor activation (Chao et al., 1998). In our studies, BDNF mRNA levels in the hippocampus were unaltered in
either the CVS or antidepressant experiment, but the CVS regimen in our study did cause an increase in NT-3 expression. Particular emphasis has been placed on the modulation of BDNF in the hippocampus. Alterations of BDNF in the hippocampus after stress and depression often form the core basis for the neurotrophic factor hypothesis (Smith et al., 1995; Schaaf et al., 1997, 1988; Zhang et al., 2010). Loss of BDNF is linked to the decrease in neurogenesis observed in the hippocampus after chronic stress (Duman, 2002). However, some researchers have found that certain stressors, such as tail shock, do not cause a reduction in neurogenesis in the hippocampus (Hanson et al., 2011). Many other studies have also observed that stress does not always alter hippocampal BDNF expression (Lee et al., 2006; Allaman et al., 2008; Hanson et al., 2011). Accordingly, modulation of neurotrophic factors and changes in neurogenesis are likely not as clear-cut as first reported. It is likely that alterations in the hippocampus and possibly other regions are sensitive to the strength of the stressors. Still, we were able to find alterations in other regions of the rat brain, including areas not previously reported such as the piriform cortex. The variability in the stress-induced alterations of neurotrophic factors emphasizes the importance of using chronic stress paradigms such as CVS over other chronic homogenous stress regimens in depression studies, as such models are more relevant to human depression.

Although these results provide a promising start for determining the role neurotrophic factors have in chronic stress-induced degeneration, there are many additional experiments needed. First, we would like to examine neurotrophic factor protein expression after CVS and chronic antidepressant treatment, as mRNA alterations do not always correlate to translational changes. Additionally, we need to examine these alterations in the more relevant animal models. The study of the effects of CVS on neurotrophic factor expression in a PD model is very
important, as this model will likely have more relevance to changes that occur with depression in PD patients. Also, examining trophic factor alterations in the CVS model concomitant with antidepressant treatment will allow for a more accurate look at the effects of antidepressants in depression. Exploration of other classes of antidepressants may be performed as well, as there are several classes used to treat depression in PD. In particular, examining selective serotonin reuptake inhibitors is important, as this class is the most common first choice among clinicians (Richard and Kurlan, 1997; Yamamoto, 2001).

There are several caveats to the experiments in this study. First, we used normal, healthy animals to examine neurotrophic alterations after antidepressant treatment. While this is often the case when screening antidepressants effectiveness, it does not accurately model the situation encountered in depression, nor does it not allow us to examine directly how desipramine may counteract neurotrophic factor alterations brought about by chronic stress. Additionally, given the collaborative nature of the antidepressant project, a different strain of rats (Wistar) was used instead of Sprague Dawley as in the CVS experiment, and the brains collected from each study were preserved in slightly different ways. This again makes absolute comparisons between the two projects difficult. Finally, some of the mRNA expression alterations we observed, while significant, were small. Whether these minor changes have actual physiological relevance remains to be determined. Nevertheless, many of the alterations were quite robust and likely indicate a shift in the neurotrophic environment and possibly in neuronal vulnerability.

**Future Directions/Conclusions**

The findings from this dissertation give a promising start to understanding the consequences and deciphering the underlying neuropathology in comorbid depression and PD.
However, there are many issues left to be address. The most important risk factor for PD is aging, and depression is elevated in the elderly (Krishnan et al., 2002). Cortisol levels are elevated in aged brains, as well as in PD patients compared to age-match controls (Charlett et al., 1998; Gould and Tanapat, 1999), possibly signaling increased stress reactivity. Many studies have also found a loss of volume in the SNpc of aged humans, indicating the increased vulnerability of SNpc neurons during the normal aging process (Stark and Pakkenberg, 2004). In animals, the increased susceptibility of neurons in the SNpc of aged brains is linked to a multitude of changes, including decreases in neurotrophic support, decreases in dopaminergic cell number, and increases in cortisol levels (Fearnley and Lees, 1991; Gould and Tanapat, 1999; Yurek and Fletcher-Turner, 2000). Compensatory reactions to neuronal injury are attenuated in the striatum of older animal brains (Yurek and Fletcher-Turner, 2001). However, in most studies of PD (and depression), including ours, younger animals are used. While this is done primarily because of logistical concerns, it is important to evaluate our combined model in aging rats to more accurately understand depression in PD. Studies have found differences in aged animals compared to young animals that could have implications for our model. Aged animal brains still have upregulation of BDNF in response to antidepressants, but the hippocampal subregions where the mRNA elevation occurs are different than those in younger animals, indicating a possible change of the functional physiology in hippocampus of older animals (Garza et al., 2004). HPA axis dysfunction also causes memory impairment in aged rats (Issa et al., 1990). In older rats, there is a loss of neurotrophic support in the nigrostriatal system including decreases of BDNF, trkB, glial cell line-derived neurotrophic factor, and the neuregulin receptor ErbB4 (Croll et al., 1998; Katoh-Semba et al., 1998; Yurek and Fletcher-Turner 2001; Dickerson et al., 2009). Aging may cause a more severe neuronal loss in our standard 6-OHDA model and others
have observed worsened motor behavior in older rats compared to young (Tamas et al., 2005). These data raise the possibility that the combination of both CVS and the 6-OHDA lesion in aged rats will cause even greater dysfunction.

Rat strains also display different sensitivity to the CVS regimen, with the Sprague Dawley strain, which we used almost exclusively in this dissertation, not displaying as robust a response as others (Wu and Wang, 2010). However, the out-bred Sprague Dawley line may more accurately reflect human populations. Using a rat breed with a more hyperactive HPA axis, such as the Fischer line, could perhaps demonstrate a stronger modification of motor symptoms.

It is likely that the increased neurodegeneration and resulting motor deficits are not the result of a single mechanism. As mentioned, further exploring of the role of neurotrophic factors in CVS alteration as well as in antidepressant treatment should be conducted in the combined model. Additionally, given the role that oxidative stress and neuroinflammation have in the deleterious effects of both depression and PD (Whitton, 2007; Frank-Cannon et al., 2009; Howren et al., 2009; Zunszain et al., 2011), examination of cytokine levels and oxidative stress alterations in the CVS/6-OHDA animals should be investigated. Apoptotic mechanisms are also involved in the pathogenesis of both depression and PD. Apoptosis is observed in both animal and human PD brains (Hartmann et al., 2001; Marti et al., 2002). In depression, apoptosis likely has a role in stress-induced structural alterations (Lucassen et al., 2001, 2006; Duman, 2004) and antidepressant treatment can reverse apoptotic gene alterations caused by chronic stress, indicating both stress and antidepressants can affect apoptotic gene expression (D’Sa and Duman, 2002; Kosten et al., 2007). To determine whether apoptosis is involved in the increased cell loss in our model, both pro- and anti-apoptotic gene expression should be examined.
An important step remaining in evaluating this model is the further examination of antidepressant treatment. For example, it will be important to test antidepressant therapies aged animals as the brain environment is closer to what is observed in the clinical setting. At this time, no standard antidepressant regimen is known to be most effective for treatment of depression in PD, and there are few well-controlled clinical studies (Weintraub, 2005; Wood et al., 2010). There is also some controversy over whether some classes of antidepressants increase symptoms (Richard and Kurlan, 1997; Arbouw et al., 2007). The combined model gives us the opportunity to evaluate the effectiveness of different categories of antidepressants in reversing the physiological and behavioral consequences of CVS and uncover the neurotransmitters affected. For example, comparing the selective-serotonin reuptake inhibitors, the norepinephrine reuptake inhibitions and tricyclic antidepressants will allow us to determine whether serotonergic or noradrenergic systems or both are involved in the motor and non-motor dysfunction.

The role of the dopaminergic system in depressive symptoms can also be studied. Dopamine agonists are often used as an alternative to or in combination with L-DOPA in treating early motor symptoms in PD (Piercey et al., 1998; Rektorova et al., 2003). There is also the intriguing possibility of using dopamine agonists like pramipexole to treat depressive symptoms. In clinical trials, pramipexole can reduce depression scores in patients with (Rektorova et al., 2003; Lemke et al., 2005; Kano et al., 2008) and without (Corrigan et al., 2000; Gupta et al., 2006) PD. Some studies suggest pramipexole has neuroprotective properties and may slow the progression of dopaminergic cell loss (Yamamoto and Schapira, 2008). However, some argue that the antidepressant effects are not clinically relevant (Leentjens, 2011). The combined model
presents the opportunity for further assessment of dopamine agonists treating motor and/or non-motor symptoms.

This dissertation sought to examine the relationship between depression and PD by developing a new animal model merging the pathologies of both disorders. The combined animal model produced worsened motor deficits and elevated levels of dopaminergic cell loss in the SNpc when the animals were exposed to the CVS regimen flanking the 6-OHDA lesion. In an effort to determine potential mechanisms behind the increase cell loss, the role of glucocorticoids was investigated. Glucocorticoids do not appear to have a large role in the enhanced neurodegeneration as they were neither necessary nor sufficient. Preliminary data, however, propose that alterations of the neurotrophic environment in the mesotelencephalic regions involved in depression and PD may increase the susceptibility of neurons to degeneration via loss of trophic support. In conclusion, the data from this dissertation suggest that the presence of depression within PD patients may have detrimental effects on the progression of the disease. However, there is still much research needed with this new model to determine the exact neurobiological underpinnings behind the increased motor dysfunction and neurodegeneration. The overall goal, of course, is to develop better therapies to alleviate both motor and depressive symptoms in PD.
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