I, Anne L Spieles-Engemann, 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:
The Neuroprotective Potential of Subthalamic Nucleus Deep Brain Stimulation in the 6-OHDA Rodent Model of Parkinson’s Disease

Student's name: Anne L Spieles-Engemann

This work and its defense approved by:

Committee chair: James Herman, PhD
Committee member: Michael Behbehani, PhD
Committee member: Timothy Collier, PhD
Committee member: Jack Lipton, PhD
Committee member: Fredy Revilla, MD
Committee member: Kim Seroogy, PhD
Committee member: Caryl Sortwell, PhD
The Neuroprotective Potential of Subthalamic Nucleus Deep Brain Stimulation in the 6-OHDA Rodent Model of Parkinson’s Disease

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience

by

Anne L. Spieles-Engemann

B.S. Western Michigan University 1998, M.A. Western Michigan University 2001

December 15, 2010

Committee Chair: James Herman, Ph.D.
Committee Members: Caryl Sortwell, Ph.D. (Advisor)
Tim Collier, Ph.D.
Jack Lipton, Ph.D.
Kim Seroogy, Ph.D.
Michael Behbehani, Ph.D.
Fredy Revilla, M.D.
Abstract

The studies in this dissertation are designed to examine the effects of subthalamic nucleus deep brain stimulation (STN DBS) in the 6-hydroxydopamine (6-OHDA) rodent model of Parkinson’s disease (PD). The first study examines whether STN DBS can provide neuroprotection to the dopaminergic neurons of the substantia nigra (SN) in the face of previous large-scale dopamine neuron loss, similar to what patients have upon initial diagnosis. The second study examines the effects of STN DBS on trophic factors within the STN itself and its target structures in both unlesioned and 6-OHDA lesioned animals. Findings from these studies demonstrate that STN DBS can provide neuroprotection to remaining dopaminergic neurons in the SN. Furthermore, the results show that STN DBS can upregulate brain-derived neurotrophic factor (BDNF) within the SN, striatum, globus pallidus interna, and M1 motor cortex. Taken together, these results demonstrate that STN DBS has the potential to be a disease-modifying therapy for PD, with important effects on cell survival and plasticity within the basal ganglia and motor cortex. They also suggest the need for further study of the use of STN DBS as a treatment for early stage PD as well as its use in other disorders in which downregulation of BDNF has been implicated. Finally, the more thorough understanding of STN DBS gained by these types of studies may aid in the risk-benefit analysis when deciding upon STN DBS as a treatment option.
Dedication

This manuscript is dedicated to my son, Eli Jacob Engemann, who has taught me more about life, love, and happiness than I could have ever hoped to learn.
Acknowledgments

First and foremost I would like to thank my husband Kevin Engemann. He was the person who first encouraged me to go back to school and the person who kept me going through his encouragement and belief that I could do this, even when I didn’t believe it myself. He has been an amazing source of support and encouragement through this process and I probably haven’t thanked him enough. So thank you Kevin, and I love you! Next I would like to thank my parents for their love and encouragement over the years. They gave me so many amazing experiences as I was growing up and let me do most of the crazy things I asked them to let me do, such as leaving home for a year when I was 16 to be an exchange student. After having my own child I now realize what an incredible act of courage and love that was. You two have made me into the person I am today and I hope that I have made you proud.

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3' diaminobenzidine</td>
</tr>
<tr>
<td>DAergic</td>
<td>Dopaminergic</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine membrane transporter</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsal ventral</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EP</td>
<td>Entopeduncular nucleus</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>F-DOPA</td>
<td>3,4-dihydroxy-6-¹⁸F-fluoro-L-phenylalanine</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFRα1</td>
<td>GDNF family receptor α1</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus externa</td>
</tr>
<tr>
<td>GPI</td>
<td>Globus pallidus interna</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanilic acid</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LBs</td>
<td>Lewy bodies</td>
</tr>
<tr>
<td>MANF</td>
<td>Mesencephalic-astrocyte-derived neurotrophic factor</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>MK-801</td>
<td>5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine</td>
</tr>
<tr>
<td>MPP+</td>
<td>N-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>NeuNir</td>
<td>Neuronal nuclei immunoreactive</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive-compulsive disorder</td>
</tr>
<tr>
<td>P75NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>phospholipase Cγ</td>
</tr>
<tr>
<td>PSTH</td>
<td>Peristimulus time histogram</td>
</tr>
<tr>
<td>RM-ANOVA</td>
<td>Repeated measures analysis of variance</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STN DBS</td>
<td>Subthalamic nucleus deep brain stimulation</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>THir</td>
<td>Tyrosine hydroxylase immunoreactive</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TrkB</td>
<td>Receptor tyrosine kinase B</td>
</tr>
<tr>
<td>UPDRS</td>
<td>United Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>VIM</td>
<td>Ventral intermediate nucleus of the thalamus</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventral posterior medial nucleus of the thalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>ZI</td>
<td>Zona incerta</td>
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</tbody>
</table>
Chapter 1: Introduction

History of Parkinson’s Disease

The Initial Description of Parkinson’s Disease

Parkinson’s disease (PD) was first described by James Parkinson in the seminal work “An Essay on the Shaking Palsy,” which was originally published as a monograph in 1817 (Parkinson, 2002). In this work, he described the similar symptoms affecting six cases he had observed as “[i]nvoluntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured” (Parkinson, 2002). Although several descriptions of the tremor associated with this disease had been described previously by Galen, Franciscus Sylvius, Juncker, and Cullen, Parkinson was the first to describe the multiple symptoms as belonging to one individual disease entity, which he termed paralysis agitans, rather than distinct diseases in and of themselves (Parkinson, 2002). Additionally, he was the first to describe the long progression of the disease from the first, barely perceptible tremor to the violent shaking, difficulty swallowing, loss of speech and delirium preceding death, as well as the non-motor symptoms such as constipation and sleep disturbances that accompany the disease (Parkinson, 2002). However, Parkinson did not have the benefit of any postmortem studies on the cases he observed and could therefore only hypothesize possible causes, which he suspected involved the medulla (Parkinson, 2002). It would take nearly 80 more years for the substantia nigra to be implicated in the pathogenesis of PD (Parent and Parent, 2010).
The Discovery of the Role of the Substantia Nigra in Parkinson’s Disease

The discovery of the substantia nigra (SN) is often mistakenly attributed to Samuel Thomas von Sœmmerring in 1778 (Parent and Parent, 2010). However, the SN was actually discovered by Félix Vicq d’ Azyr in 1786 (Faull and Carman, 1968; Parent and Parent, 2010). The first description of nigral neurons was provided by Jules-Bernard Luys in 1865 (Parent et al., 2002; Parent and Parent, 2010), and Domenico Mirto was the first to recognize them as projection neurons in 1896 (Parent and Parent, 2010). In 1910, Torata Sano described the pars compacta (SNc) and pars reticulata (SNr) subdivisions of the SN, explaining that the SNc contained densely packed pigmented neurons and the SNr contained fewer non-pigmented neurons within a dense fiber network (Parent and Parent, 2010).

The most important work involving the SN and its involvement in PD took place at the Salpêtrière hospital in Paris under the auspices of Jean-Martin Charcot and his students Georges Marinesco and Edourard Brissaud, as well as the pathologist Paul Oscar Blocq (Parent and Parent, 2010). It was in the 1860s when Charcot first realized that tremor was not the only hallmark of the paralysis agitans described by Parkinson. He recognized that the cardinal motor symptoms of this disease also included bradykinesia, rigidity, and balance impairment and suggested that the name be changed to Parkinson’s disease (Parent and Parent, 2010). Although Charcot himself was never able to elucidate the pathophysiology of PD, in 1893 Marinesco and Blocq hypothesized that lesions of the SN might cause parkinsonian tremor, based on their findings of a PD-like tremor in a patient with a SN tumor (Parent and Parent, 2010). This then led
Brissaud to conclude that the origin of PD likely lies in the SN (Parent and Parent, 2010). This conclusion was confirmed in 1919 by Constantin Trétiakoff who evaluated the brains of patients with PD and found a loss of the pigmented neurons of the SN, with inclusions in some of the remaining neurons that he termed Lewy bodies (corps de Lewy), as well as neurofibrillary alterations (Lees et al., 2008; Parent and Parent, 2010). These findings were later confirmed by Charles Foix; Jean Nicolesco, who added the observations of neuronal loss in the locus coeruleus, dorsal nucleus of the vagus nerve, and the substantia innominata; as well as Rolf Hassler, who also noted that the globus pallidus and striatum were mostly unaffected (it had been previously thought that degeneration in these two structures were the cause of PD) (Lees et al., 2008; Parent and Parent, 2010).

The Discovery of the Role of Dopamine in Parkinson's Disease

Although the importance of the SN in PD was established around the turn of the twentieth century, the role of dopamine in the disease would not be discovered until almost 60 years later. It was in the mid 1950s that Arvid Carlsson and his colleagues were investigating the effects of the vesicular monoamine transporter blocker reserpine, which produces motor symptoms of PD, on noradrenaline in mice and rabbits (Carlsson et al., 1957; Carlsson, 2002). While performing these experiments they discovered that administration of the catecholamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA), which was used because catecholamines do not cross the blood-brain barrier, would reverse reserpine-induced motor symptoms. They expected to find a concomitant restoration of noradrenaline levels but did not (Carlsson et al., 1957; Carlsson, 2002).
This forced them to look at L-DOPA’s effect on dopamine, since dopamine is the intermediate in the conversion of L-DOPA to noradrenaline (Carlsson, 2002). However, before they could do this, they first had to develop a method to detect dopamine. This was done using a fluroimetric method based on the hydroxyindole principle in which dopamine is oxidized and tautomerized to produce a stable fluorescence with a spectrum that was much different than that of noradrenaline (Carlsson and Waldeck, 1958; Atack, 1973; Roe, 1997). They were then able to show that dopamine was present in the brains of rabbits at much higher levels than would be expected if dopamine only functioned as a precursor to noradrenaline (Carlsson and Waldeck, 1958; Roe, 1997). This was followed by the discovery that administration of reserpine caused a depletion of dopamine that could be reversed by L-DOPA administration (Carlsson, 1959, 2002). At the same time, Carlsson’s students Åke Bertler and Evald Rosengren used the new fluorimetric method of dopamine detection to examine the distribution of dopamine in the brains of mammals. They discovered dopamine in the brains of cows, sheep, pigs, dogs, cats, rabbits, guinea pigs, and rats (Bertler and Rosengren, 1959) with the highest concentration of dopamine located in the basal ganglia, and in particular the caudate (Bertler and Rosengren, 1959; Roe, 1997). The depletion of dopamine in the brains of patients with PD was then confirmed by Oleh Hornykiewicz and Isamu Sano in 1960 (Ehringer and Hornykiewicz, 1960; Carlsson, 2002), followed by studies showing that intravenous L-DOPA could temporarily reverse akinesia and improve rigidity in patients with PD (Birkmayer and Hornykiewicz, 1961; Carlsson, 2002). However, L-DOPA did not become a routine treatment for PD until 1967, when George Cotzias and colleagues (Cotzias et al., 1967) developed an oral
dosing regimen that was finally able to adequately treat the motor symptoms (Carlsson, 2002). L-DOPA remains the standard treatment for the motor symptoms of PD today.

**Clinical Presentation of Parkinson’s Disease**

PD is a chronic, progressive disorder with an incidence of approximately 17 new cases per 100,000 people per year (Twelves *et al*., 2003). In the United States, it is estimated that there are 50,000 new cases of PD reported each year (NINDS, 2004). The cardinal motor symptoms of PD are tremor, rigidity, bradykinesia, and postural instability, although all four symptoms do not need to be present to attain a diagnosis of PD. Tremor is present in approximately 70% of patients and is typically present at rest with oscillations of 4 to 6 per second (Hoehn and Yahr, 1967; Rao *et al*., 2003). “Pill-rolling” is a common form of tremor in PD in which the index finger rubs against the thumb repeatedly (Ng, 1996; Rao *et al*., 2003). Rigidity occurs in approximately 90% of patients (Calne *et al*., 1992; Gelb *et al*., 1999; Frank *et al*., 2006) and is an involuntary stiffness of the muscles. When assessed for rigidity by the application of force across a joint, many patients will have “cog wheeling,” or a jerky motion of the joint (Colcher and Simuni, 1999; Rao *et al*., 2003). Bradykinesia is found in approximately 80% of patients (Calne *et al*., 1992; Gelb *et al*., 1999; Frank *et al*., 2006) and can refer either to an overall slowness of movement or slowness in initiating movement (Rao *et al*., 2003). Postural instability refers to the loss of postural reflexes leading to falls and does not usually appear until the later stages of the disease (Jankovic, 2008). Other common motor features of PD include gait changes (smaller stepping motions, forward flexion of the trunk, reduced arm swing), masked facies (hypomimia), micrography, and speech
and swallowing difficulties (Rao et al., 2003; Frank et al., 2006; Jankovic, 2008). Non-motor symptoms include autonomic dysfunction, cognitive and psychiatric problems, sleep disorders, and sensory disturbances (Jankovic, 2008), with the most common of these being orthostatic hypotension, constipation, depression, dementia, REM sleep disorder, insomnia, and hyposmia (Frank et al., 2006; Jankovic, 2008; Reichmann et al., 2009). The onset of PD symptoms is typically unilateral, and PD is diagnosed based on the presence of a combination of the cardinal motor features and the patients’ response to levodopa (Gelb et al., 1999; Jankovic, 2008).

Pathophysiology of PD

Neuronal Loss

That the loss of the dopamine in the brain is central to the pathophysiology of PD was established by the seminal work of Hornykiewicz and colleagues in 1960 (Ehringer and Hornykiewicz, 1960). However, it took a few more years and the development of new histochemical techniques before the role of the nigrostriatal system in the pathophysiology of PD would be elucidated. It was in 1964 that Annica Dahlström and Kjell Fuxe discovered that the nigrostriatal neurons in rats contain high levels of dopamine (Dahlstrom and Fuxe, 1964; Roe, 1997) and that Louis Poirier and Theodore Sourkes demonstrated that lesioning the SNc caused a decrease in catecholamines in the ipsilateral caudate and putamen (Poirier and Sourkes, 1964; Roe, 1997). Finally, in 1966, Poirer and Sourkes discovered that lesioning the midbrain of monkeys not only decreased catecholamines in the striatum, but also caused abnormal movement such as tremors and bradykinesia, indicating a possible role for the nigrostriatal dopamine
neurons in the pathophysiology of PD (Poirier et al., 1966; Roe, 1997). More recently it has been demonstrated that loss of nigrostriatal dopamine neurons is but one aspect in the pathophysiology of this disease and that even the nigral dopamine neurons are differentially affected.

The primary loss of SN dopaminergic neurons in PD occurs in those that project to the putamen, which is the motor portion of the striatum (Agid, 1991). The loss of projections in this area typically exceeds 95%, while the loss of the caudate projections is only about 80%, and this differential loss of striatal projections is a defining histological feature of PD (Bernheimer et al., 1973; Agid, 1991; Hornykiewicz, 2001). This is directly related to the fact that the ventral tier SNc neurons, which suffer greater loss in PD, innervate the putamen and the less affected dorsal tier SNc neurons innervate the caudate (Gibb and Lees, 1991; Hornykiewicz, 2001). The dopaminergic neurons projecting to the nucleus acumbens, hypothalamus, cortex, and limbic structures from the ventral tegmental area (VTA) are much less affected than the nigrostriatal projections (Agid, 1991) and dopaminergic neurons located outside of the SN, such as those in the periacqueductal grey matter, and the descending spinal neurons appear to be unaffected by the disease (Agid, 1991). The clinical symptoms of PD are not apparent until approximately 70–80% of the nerve terminals in the striatum and 50–60% of the cell bodies in the SN have been lost (Agid, 1991).

The loss of dopamine within the striatum also has a differential topographical distribution. Loss in the caudate is greatest in the rostral portion; in the putamen, it is
greatest in the caudal portion; and in both structures, it is greatest in the dorsal region (Nyberg et al., 1983; Kish et al., 1988; Hornykiewicz, 2001). Interestingly, although dopamine levels are decreased in the striatum of patients with PD, levels of its metabolite homovanillic acid (HVA) increase, indicating an increase in dopamine turnover that occurs as a compensatory mechanism in the remaining neurons (Bernheimer and Hornykiewicz, 1965; Hornykiewicz, 2001). D1 and D2 dopamine receptor levels also increase in the striatum (Hornykiewicz, 1998; Piggott et al., 1999) and become supersensitive (Buonamici et al., 1986; Rinne et al., 1990).

Although nigrostriatal dopamine loss is the hallmark of PD, loss of other types of neurons also occurs, including the noradrenergic neurons of the locus coeruleus, the serotonergic neurons of the raphe nucleus, the cholinergic neurons of the nucleus basalis of Meynert and the adrenergic neurons of the dorsal vagal nucleus and the sympathetic ganglia (Agid, 1991).

**Lewy Body Formation**

Another pathological hallmark of PD is the presence of Lewy bodies (LBs) within the remaining neurons of the SN. Lewy bodies in the dorsal vagal nucleus and the substantia innominatae of patients with PD were first described in 1912 by Friedrich Heinrich Lewy as “[i]nclusions, not identical with corpora amylacea, but with hyaline bodies” and “ball-like and serpiginous formations in cells where a nucleus could no longer be identified” (Holdorff, 2002). It was Constantin Trétiakoff who discovered these inclusions in the pigmented neurons of the SN of patients with PD in 1919 and coined the term *Lewy body* (Parent and Parent, 2010). LBs are abnormal aggregates of
proteins found in the perikarya of remaining SN neurons in patients with PD. Dauer and Przedborski (2003) describe them as “…spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins, including α-synuclein, parkin, ubiquitin and neurofilaments, and they are found in all affected brain regions (Forno, 1996; Spillantini et al., 1998). LBs are more than 15 μm in diameter and have an organized structure containing a dense hyaline core surrounded by a clear halo. Electron microscopy reveals a dense granulovesicular core surrounded by a ring of radiating 8–10 nm fibrils (Duffy and Tennyson, 1965; Pappolla, 1986).” The role of LBs in PD is currently unknown. It is possible that LBs themselves are toxic to neurons given that they contain aggregates of α-synuclein and ubiquitin, which have been shown to be associated with cell death in ventral mesencephalic cell cultures (McNaught et al., 2002). Alternatively, LB formation could be a neuroprotective mechanism whereby the cell is attempting to protect itself by removing toxic misfolded proteins (Auluck et al., 2002; Dauer and Przedborski, 2003).

The Progressive Nature of Parkinson’s Disease

An additional pathological hallmark of PD is that it is always progressive. In their seminal work in 1967, Margaret Hoehn and Melvin Yahr studied a group of 856 patients and invented a scale to classify their level of clinical disability. This scale is still used by neurologists today (see Table 1). However, this scale only classifies the clinical symptoms and not the underlying pathology. Braak and colleagues have more recently proposed a staging scale for the evolution of the histopathology of PD (Braak et al., 2003). Stage one consists solely of Lewy neurites and Lewy bodies within the dorsal
motor nucleus of the vagus nerve and/or the intermediate reticular zone. In stage 2, Lewy neurites and Lewy bodies begin to appear in the caudal raphe nuclei, the reticular formation and in the neuromelanin-containing cells of the coeruleus-subcoeruleus complex. In stage 3, the neuromelanin-containing cells of the SN begin to develop Lewy neurites and Lewy bodies, as do the magnocellular nuclei of the basal forebrain. In stage 4, loss of the neuromelanin-containing cells of the SN is seen, and pathology begins to appear in the forebrain. In stage 5, there is a loss of neuromelanin-containing neurons in the dorsal motor nucleus of the vagus nerve, intermediate reticular zone, reticular formation and coeruleus-subcoeruleus complex. Pathology also begins to extend to the sensory areas of the neocortex and the prefrontal neocortex. In stage 6, almost the entire neocortex is affected (Braak et al., 2003). Although this staging scheme was somewhat controversial when first proposed, it has largely been confirmed in a recent study by Kingsbury and colleagues (Kingsbury et al., 2010). Regardless, the progressive nature of PD is well known.

Table 1. Hoehn and Yahr Scale (Hoehn and Yahr, 1967)
The Hoehn and Yahr scale for the classification of the progression of Parkinson’s disease.

<table>
<thead>
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<th>Stage</th>
<th>Description</th>
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<td>I</td>
<td>Unilateral involvement only. Little functional impairment.</td>
</tr>
<tr>
<td>II</td>
<td>Bilateral or midline impairment. No balance impairment.</td>
</tr>
<tr>
<td>III</td>
<td>Impaired righting reflexes. Mild to moderate disability. Patient is still physically capable of being independent.</td>
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<tr>
<td>IV</td>
<td>Patient can still walk and stand unassisted but is severely disabled.</td>
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Progression of Nigrostriatal Degeneration

At the time of diagnosis, the typical Parkinson’s patient has already lost 70–80% of their striatal dopamine (DA) content and approximately 60% of SN DA neurons (Bernheimer et al., 1973). By 7 years after diagnosis, patients have lost 75–85% of their SN DA neurons, by 20 years they have lost approximately 90% and by 30 years they have lost approximately 95% (Damier et al., 1999). This correlation between cell loss and disease duration only occurs in the SNC, and not within the other cell populations affected by PD (Damier et al., 1999). Within the striatum, it has been demonstrated that dopaminergic function as measured by 3,4-dihydroxy-6-\textsuperscript{18}F-fluoro-L-phenylalanine (F-DOPA) uptake declines approximately 5–10% per year as compared to 1% in healthy controls (Leenders and Oertel, 2001, Dodiya et al., 2009).

Abnormal Neuronal Synchronization

Finally, more recent studies have identified a newer hallmark of PD, which is a synchronization of beta oscillations (15-30 Hz) within the basal ganglia-thalamocortical circuits (Bergman et al., 1998; Brown and Williams, 2005; Gatev et al., 2006; Hammond et al., 2007). These oscillations are reduced by both L-DOPA and STN DBS, with a concomitant improvement in motor functioning (Brown et al., 2004; Meissner et al., 2005; Silberstein et al., 2005; Kuhn et al., 2006, 2008; Wingeier et al., 2006; Bronte-Stewart et al., 2009; Giannicola et al., 2010). However, the origin of these oscillations remains unknown and research is ongoing (Holgado et al., 2010).
Etiology of Parkinson’s Disease

There are actually two distinct forms of PD: familial PD, in which genetic mutations are associated with the development of the disease, and sporadic PD, in which no known cause of PD can be identified (Shadrina et al., 2010). Familial PD accounts for approximately 10–15% of all PD cases, and to date, 7 genes have been implicated in PD pathogenesis (Shadrina et al., 2010).

Genetic Forms of Parkinson’s Disease

The first gene to be identified as associated with PD was the SNCA gene that codes for \( \alpha \)-synuclein (Polymeropoulos et al., 1997), which is a main component in Lewy bodies. Mutations in this gene might play a role in the pathophysiology of PD by causing an accumulation of \( \alpha \)-synuclein either through duplications and triplications of the gene or though mutations that cause a cell to be incapable of dealing with the protein through proteolytic degradation (Cookson, 2005; Shadrina et al., 2010). Another gene associated with PD is the PARK2 (also referred to as parkin) gene, which codes for the protein parkin (Kitada et al., 1998). Parkin is an E3 protein-ubiquitin ligase that can bind to and ubiquinate proteins for degradation (Zhang et al., 2000). Mutations in the parkin gene play a role in the pathogenesis of PD though the accumulation of its substrates resulting in cell death (Cookson, 2005). The third gene associated with familial PD is the PINK1 gene, which codes for the PINK1 protein, a serine/threonine-protein kinase (Valente et al., 2004). Currently, the role of PINK1 in the pathogenesis of PD is not clear. However, it has been shown to be present in mitochondria and can protect cells from proteasome inhibitor-induced cell death, so mutations may make cells vulnerable
to oxidative stress-induced cell death (Valente et al., 2004; Cookson, 2005; Shadrina et al., 2010). The fourth gene associated with familial PD is the PARK7 gene, which codes for the protein DJ-1 (Bonifati et al., 2003). Mutations in this gene may sensitize neurons to oxidative stress since DJ-1 may have antioxidant properties (Taira et al., 2004). The fifth gene associated with PD is the LRRK2 gene, which codes for the protein dardarin (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Currently, little is known regarding the function of dardarin and its role in the pathogenesis of PD (Shadrina et al., 2010). In addition to being associated with familial PD, mutations in this gene are present in approximately 3.6% of sporadic PD cases as well (Shadrina et al., 2010). Mutations in the UCH-L1 gene have also been linked to PD, although only in a single family (Leroy et al., 1998; Shadrina et al., 2010). This gene encodes residues at the C-end of ubiquitin hydrolase 1, which is found in Lewy bodies (Shadrina et al., 2010), and mutations may therefore play a role in Lewy body formation. The final gene associated with PD is the ATP13A2 gene, which encodes for a lysosomal ATPase (Di Fonzo et al., 2007; Shadrina et al., 2010). The function of this protein and its role in the pathogenesis of PD is unknown, although it is highly expressed in the dopaminergic neurons of the SN (Ramirez et al., 2006; Shadrina et al., 2010).

**Sporadic Parkinson's Disease**

Sporadic PD has no known cause and may involve several factors, including aging, environmental toxins and a genetic predisposition given that familial and sporadic PD are identical both neuropathologically and clinically and LRRK2 mutations are found in a small percentage of sporadic PD cases (Shadrina et al., 2010). Aging is the biggest risk
factor for developing PD (Hindle, 2010). Cell death in PD is thought to occur due to a variety of factors, including oxidative stress, mitochondrial dysfunction, glutamatergic excitotoxicity, ubiquitin-proteasomal dysfunction leading to the aggregation of misfolded proteins and impaired autophagy (Jenner and Olanow, 2006; Hindle, 2010), and many of these same pathologies are present in the normal aging process (Hindle, 2010).

Additionally, age-related loss of dopamine phenotype and increases in $\alpha$-synuclein follow the same regional patterns in the SN as those seen in PD, with greater loss occurring in the ventral tier of the SN (Chu and Kordower, 2007; Kanaan et al., 2010). Therefore, it has been hypothesized that PD is the normal aging process that has become intensified due to some unknown cause (Chu and Kordower, 2007). In addition to genetics and aging, environmental toxins have also been implicated in the etiology of sporadic PD. The toxin 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) was the first toxin to be implicated in PD when it was discovered that a group of heroin users with rapid onset of PD symptoms had accidentally injected themselves with MPTP contained in synthetic heroin (Langston et al., 1983). Additionally, the use of insecticides and herbicides has been associated with an increased risk of PD (Semchuk et al., 1992) and the insecticide rotenone has been shown to recapitulate the main features of PD in rats (Betarbet et al., 2000). An environmental role is also suggested by the low concordance rate in twin studies of PD (Schapira, 2009). Therefore, it appears that sporadic PD is likely to involve a combination of factors related to genetics, aging and environmental toxins.
Animal Models of Parkinson’s Disease

Genetic Models

Given that 10–15% of PD cases are due to known genetic mutations and that these mutations have the potential to help identify the key molecular players in sporadic PD, much time and energy has been devoted to developing genetic animal models of PD. Mutations in the SNCA gene encoding α-synuclein have been used to create genetic models of PD in Drosophila (Feany and Bender, 2000), C. elegans (Lakso et al., 2003), mice (Masliah et al., 2000), and rats (Lo Bianco et al., 2002). However, all of these are imperfect models as they either lack a progressive dopamine neuron loss, lack Lewy body formation, or fail to recapitulate the motor features of PD (Dawson et al., 2010). Genetic models of LRRK2 mutations also exist in Drosophila (Lee et al., 2007), C. elegans (Saha and Pahan, 2006) and mice (Li et al., 2009). Again, these are imperfect models. Drosophila and C. elegans don’t naturally express α-synuclein, and α-synuclein aggregation is a main pathological hallmark of LRRK2 mutations. Additionally, LRRK2 transgenic mice have little to no nigral dopamine neuron degeneration, one of the main pathological hallmarks of PD. Genetic models of parkin, PINK1 and DJ-1 mutations also exist in both Drosophila and mice; however, these knockout mice exhibit little to no dysfunction in the nigrostriatal dopamine system, and although loss of dopamine neurons does occur in Drosophila, it is the only PD trait seen, making the model of limited use (Dawson et al., 2010). Although these models are imperfect, they have provided useful insight into the role these gene mutations play in the pathogenesis of PD.
Neurotoxin Models

The other way to model PD in animals is via neurotoxin administration. The two most common neurotoxins used in PD research are MPTP (used in mice and non-human primates) and 6-OHDA (used in rats), as they are able to recapitulate the main pathological events that occur within the parkinsonian basal ganglia. For example, it has been demonstrated that STN activity becomes disorganized (Bergman et al., 1994; Hassani et al., 1996; Hutchison et al., 1998; Wichmann et al., 1999; Magnin et al., 2000) and the Globus pallidus interna (GPI) and SNr become hyperactive due to enhanced glutamatergic input from the STN, with subsequent compensatory downregulation of glutamate receptors in those structures (Difazio et al., 1992; Vila et al., 1997; Rodriguez et al., 1998) in patients with PD. It has similarly been demonstrated that 6-OHDA administration alters the firing properties of the STN (Hassani et al., 1996; Ni et al., 2001; Breit et al., 2005; Gajendiran et al., 2005) and both 6-OHDA and MPTP have been shown to increase neuronal activity and downregulate glutamate receptors in the SNr and GPI (Crossman et al., 1985; Schwartzman and Alexander, 1985; Porter et al., 1994; Wullner et al., 1994; Kaneda et al., 2005; Blandini et al., 2007). These models also reliably recapitulate parkinsonian motor impairments, although in the MPTP mouse model this can be dependent on strain and MPTP dosage (Colotla et al., 1990; Sedelis et al., 2000; Sedelis et al., 2001; Meredith and Kang, 2006). The 6-OHDA model has the added benefits of causing sensitization to L-DOPA that is similar to dyskinesia priming in PD patients, causing a shortening of the duration of L-DOPA effectiveness that is similar to the “wearing off” phenomena in PD patients (Papa et al., 1994; Lindgren et al., 2007; Jenner, 2008), and causing the rodent equivalent of dyskinesia in animals treated
with chronic L-DOPA (Cenci et al., 1998; Steece-Collier et al., 2003). However, L-DOPA induced dyskinesias are not unique to the 6-OHDA rodent model, as they can develop in the MPTP non-human primate model as well (Clarke et al., 1987). The two other neurotoxin models of PD involve the administration of rotenone and paraquat. However, these models have traditionally been limited by their high toxicity (Jenner, 2008), and lesioning with rotenone tended to cause neuronal death in other areas and neuronal populations of the brain besides the dopaminergic neurons of the SN, which is not typical of sporadic PD (Hoglinger et al., 2003). Although, recent advances in the dosing and vehicle used with rotenone administration appear to have eliminated the toxicity issues and the model now seems to more reliably recapitulate the major pathological hallmarks of PD (Cannon et al., 2009; Cannon and Greenamyre, 2010) One of the current studies characterizes a progressive 6-OHDA model of PD, which although it does not completely model PD etiology, recapitulates the major relevant characteristics of the disease for the purposes of our study.

The 6-hydroxydopamine Model of Parkinson’s Disease

Discovery

6-OHDA was first discovered in 1959 by Senoh and Witkop and was first used in experiments in 1963 to deplete peripheral neurons of noradrenaline in mice (Porter et al., 1963). Subsequently, Ungerstedt (Ungerstedt, 1968) injected 6-OHDA into the various areas known to contain monoaminergic neurons in rats, including the caudate nucleus, substantia nigra and the area dorsolateral to the nucleus interpeduncularis,
and found that it was able to deplete dopamine in these areas. He also observed that when injected into the SN, depletion of dopamine occurred in both the SN and the caudate, suggesting anterograde degeneration (Ungerstedt, 1968). Ungerstedt and Arbuthnott (Ungerstedt and Arbuthnott, 1970) were also the first to describe the use of amphetamine-induced rotations in unilaterally lesioned rats as a method for determining the functional state of the remaining nigrostriatal system. Animals used in this model are almost always unilaterally lesioned due to the high mortality rate associated with bilateral lesions that results from adipsia and aphagia (Ungerstedt, 1971; Blandini et al., 2008). Amphetamine-induced rotations are still in use today as a way of examining the effectiveness of a variety of PD treatments used in the 6-OHDA rodent model of PD.

**Methods of Use**

Initial studies utilizing 6-OHDA to create a rodent model of PD injected the toxin, which does not cross the blood-brain barrier, directly into the SN or into the medial forebrain bundle in order to create a fairly immediate and almost complete loss of dopamine within the nigrostriatal system that is most appropriate for studying late-stage PD (Deumens et al., 2002; Blandini et al., 2008). However, in 1990, Berger and colleagues discovered that 6-OHDA can also be transported in a retrograde fashion by cells and that when injected into the striatum it can cause destruction of the cell bodies in the SN (Berger et al., 1991). This method was thought to more accurately model the initial striatal dopamine terminal loss that is thought to occur before SN dopamine cell loss in PD (Berger et al., 1991), and is therefore a more clinically relevant model of the
disease. In 1994, Sauer and Ortel extended these findings to create a progressive intrastriatal lesion protocol in which the loss of the dopaminergic neurons in the substantia nigra occurs in a protracted manner over the course of eight weeks (Sauer and Oertel, 1994). The lesion paradigm that we characterized and used in the current studies was based upon this work, as it provides the most accurate representation of the clinical presentation of PD and allowed us to intervene with stimulation at a time when the animals had lost approximately 50% of the SN dopamine neurons. This is analogous to early PD and the time when patients typically first seek medical intervention (Agid, 1991; Deumens et al., 2002).

**Mechanism of Toxicity**

6-OHDA exerts its toxicity mainly though the oxidative stress it creates once it enters a cell (Sachs and Jonsson, 1975; Blandini et al., 2008). 6-OHDA is structurally similar to endogenous catacholamines and is therefore taken up into cells via the dopamine membrane transporter (DAT) (Simola et al., 2007). 6-OHDA then accumulates in the cytosol and is oxidized via monoamine oxidase (MAO), which generates toxic hydrogen peroxide (H$_2$O$_2$), which can then trigger the production of oxygen radicals, leading to cellular destruction (Simola et al., 2007). In addition to oxidation via MAO, 6-OHDA can auto-oxidate to create cytotoxic H$_2$O$_2$ and reactive oxygen species (Simola et al., 2007; Blandini et al., 2008). Inflammation may also play a role in the degenerative process, as activated microglia have been found in the nigrostriatal pathway of lesioned animals (Rodrigues et al., 2001). Additionally, 6-OHDA may also enter the mitochondria and
inhibit complex I (Glinka and Youdim, 1995), although this has only been demonstrated in isolated mitochondria and not in whole cells (Simola et al., 2007).

**Pathological Changes Associated with 6-OHDA**

In addition to the destruction of dopaminergic terminals in the striatum and neurons in the substantia nigra, the 6-OHDA model is also able to recapitulate other pathological changes that take place in PD. Specifically, 6-OHDA administration alters the firing properties of the STN (Hassani et al., 1996; Ni et al., 2001; Breit et al., 2005; Gajendiran et al., 2005); increases neuronal activity and downregulates glutamate receptors in the SNr and GPi (Porter et al., 1994; Wullner et al., 1994; Blandini et al., 2007); and causes sensitization to L-DOPA and a shortening of L-DOPA effectiveness (Papa et al., 1994; Lindgren et al., 2007; Jenner, 2008), similar to what takes place in patients with PD (Difazio et al., 1992; Vila et al., 1997; Hutchison et al., 1998; Magnin et al., 2000). The only aspects of PD that 6-OHDA lesioning fails to replicate are the degeneration of dopaminergic neurons outside of the nigrostriatal system (Betarbet et al., 2002; Del Tredici et al., 2002) and the formation of Lewy bodies (Schober, 2004).

**Advantages and Disadvantages of the 6-OHDA Model**

One of the main advantages of the 6-OHDA model is that it produces a stable, reliable lesion and motor deficits (Meredith and Kang, 2006; Blandini et al., 2008) that are easily assayed. For example, unilateral 6-OHDA lesioning produces deficits in motor functioning that can be tested via the examination of contralateral forepaw use in the cylinder task, deficits in proprioception that can be tested via forelimb placing in
response to vibrissae stimulation, and deficits in somatosensory functioning that can be tested via capacity for removing an adhesive placed on the forepaws (Schallert and Tillerson, 2000). In one of the current studies, the cylinder task is used to demonstrate deficits in motor functioning post lesion and improved motor functioning during STN DBS.

Although the 6-OHDA model of PD is not truly PD and does not fully recapitulate all of the pathological hallmarks of the disease, it is still the most widely used model of nigrostriatal dopamine loss for modeling PD that is in use today (Blandini et al., 2008). In addition to producing a stable, reliable lesion and concomitant motor deficits (Meredith and Kang, 2006; Blandini et al., 2008), it has the added benefit of being much less expensive and easier to perform than MPTP lesioning in non-human primates, which is the other reliable and well-known model of PD. Another benefit is that the site and amount of toxin can be adjusted to model early or late PD, so treatments can be tested at the various stages of nigrostriatal dopamine loss. The toxin can also be administered unilaterally, which leaves the contralateral nigrostriatal system intact allowing it to be used as a within subject control. Finally, this model can also be used to produce a progressive loss of nigrostriatal dopamine, making it highly amenable to studying neuroprotective strategies, as was done in one of the current studies.

Anatomy of the Basal Ganglia

The basal ganglia is a group of subcortical nuclei consisting of the striatum (made up of the caudate and putamen), the globus pallidus externa (GPe) (globus pallidus in rats
The main input to the basal ganglia derives from the cortex, and the main output structures are the GPi/EP and the SNr (Bolam et al., 2000). Although recent evidence has shown basal ganglia functioning to be a bit more complicated than previously thought (Obeso et al., 2008), basal ganglia output has traditionally been thought to be controlled via two parallel pathways, the direct and the indirect (Albin et al., 1989).

The Direct Pathway

In the direct pathway, the γ-aminobutyric acid (GABA)ergic neurons of the striatum that express D1 dopamine receptors project directly to the output nuclei of the basal ganglia; the GPi/EP and the SNr (Bolam et al., 2000). The output signal of the basal ganglia at rest is inhibitory since the striatum at rest does not discharge but the GABAergic neurons of the output nuclei are tonically active (Albin et al., 1989; Chevalier and Deniau, 1990; DeLong, 1990; Bolam et al., 2000). When the D1 dopamine receptors of the direct pathway are stimulated (or when the direct pathway is stimulated via the glutamatergic corticostriatal projections), the GABAergic projections to the GPi/EP and SNr are activated and these structures are thus inhibited (Bolam et al., 2000; Lewis et al., 2003). Since the projections of these nuclei are also GABAergic, inhibiting these nuclei causes a disinhibition of their target structure, which is the thalamus. The thalamus has a glutamatergic projection to the cortex; therefore activation of the direct pathway leads to facilitation of movement (see Figure 1).
The Indirect Pathway

In the indirect pathway, the GABAergic neurons of the striatum that express D2 dopamine receptors project to the output nuclei via the GPe and STN. The GABAergic fibers of the striatum project to the GPe, and the GABAergic fibers of the GPe then project to the STN. The glutamatergic fibers of the STN then project to the GPi/EP, the SNr and the SNc (Shink et al., 1996; Bolam et al., 2000). When the D2 dopamine receptors of the striatum are activated, the GABAergic projections to the GPe cause the GPe to be inhibited. This causes a disinhibition of the STN since the GPe projections to the STN are also GABAergic. The STN glutamatergic projections then activate the SNr, SNc and GPi/EP. The SNr and GPi/EP inhibit the thalamus and cortex via their GABAergic projections causing inhibition of movement (see Figure 2) (Lewis et al., 2003). In PD, it is believed that the loss of dopamine input to both the D1 and D2 receptors leads to overactivity of both the GPi/EP and the SNr, which in turn leads to
decreased thalamic and cortical output that is believed to underlie bradykinesia (Lewis et al., 2003).

**Subthalamic Nucleus Circuitry**

For the purpose of the current studies, it is also important to understand the anatomy of the STN projections. The STN is an important control structure within the basal ganglia that is believed to be responsible for the resting activity of the basal ganglia output nuclei (GPi/EP and SN) (Nakanishi et al., 1987; Bevan and Wilson, 1999; Bolam et al., 2000). In addition to the previously described glutamatergic projections to the GPi/EP, SNr and SNc, the STN also contains glutamatergic projections to the cortex, GPe and striatum (Jackson and Crossman, 1981; Kita et al., 1983; Kita and Kitai, 1987; Parent and Hazrati, 1995; Kitai and Kita, 2006; Degos et al., 2008) (see Figure 3). The STN also receives reciprocal GABAergic efferents from the GPe and glutamatergic efferents.
from the cortex. Orthodromic activation of these glutamatergic M1-STN efferents has been shown to reverse parkinsonian behaviors (Gradinaru et al., 2009). One of the current studies examines the possibility that glutamatergic activation of N-methyl-D-aspartic acid (NMDA) receptors resulting from STN DBS leads to the upregulation of trophic factors in STN target nuclei via either antidromic or orthodromic activation of glutamatergic pathways, thus an understanding of this anatomy is essential.

**Current Treatments for the Motor Symptoms of Parkinson’s Disease**

**Pharmacological Treatments**

L-DOPA is currently the standard pharmacological treatment for PD and has been for over 40 years due to its effectiveness in improving the motor symptoms of PD in the first few years of treatment (Hely et al., 2000). However, as the disease progress, L-DOPA becomes less effective. In advanced PD, cell loss begins to occur in non-dopaminergic pathways, leading to such issues as dysphagia, speech impairments, postural
instability, and a decrease in cognitive abilities (Bonnet et al., 1987; Hely et al., 2000). Since these symptoms are not related to dopamine loss, they are not responsive to L-DOPA (Hely et al., 2000). Additionally, with extended use L-DOPA becomes less effective to due to the motor fluctuations and dyskinesias that develop. This appears to be a universal phenomenon since several studies have shown that all patients within a study have these side effects after 10 years of treatment with L-DOPA (Schrag and Quinn, 2000; Hely et al., 1999). These side effects appear to be correlated with the severity of striatal dopaminergic denervation, as well as the nature of L-DOPA itself since higher doses are more likely to lead to these side effects (Agid et al., 1998). Therefore, the American Academy of Neurology now recommends the use of MAO-B inhibitors (which prevent the breakdown of monoamines thus increasing their availability) and dopamine agonists to treat the initial symptoms of PD in newly diagnosed patients, with the introduction of L-DOPA once the patient requires greater improvements in motor ability (Miyasaki et al., 2002). NMDA receptor antagonists are also sometimes used in the early stages to improve motor symptoms (Rao et al., 2006) by preventing the increase in SN and GPi inhibitory activity due to the overactive glutamatergic signaling from the STN. Anticholinergics may also be used, especially in tremor-predominant PD (Miyasaki et al., 2002) to counteract the activity of acetylcholine in the striatum that results from the loss of autoinhibition of acetylcholine release due to dopamine depletion and its synchronous oscillatory release due to the ongoing oscillatory activity in the cortico-basal ganglia-thalamocotical loop (Aosaki et al., 2010). In the later stages of the disease when motor fluctuations and dyskinesias have developed, the focus of treatment shifts to extending the benefit of L-DOPA, which
tends to have a shorter beneficial duration as the length of use increases (Hely et al., 2000; Rao et al., 2006). At this point, L-DOPA is often combined with dopamine agonists and MAO-B inhibitors to reduce “off” time (time during which L-DOPA is not effective) and Catechol-O-methyltransferase (COMT) inhibitors, which extend the half-life of L-DOPA to help eliminate the end of dose wearing-off effect that some patients experience (where effectiveness of L-DOPA wears off before they are ready to take the next dose) (Rao et al., 2006).

**Surgical Treatments**

Once motor fluctuations and dyskinesias arise that limit the benefit obtained from L-DOPA, patients may benefit from surgical treatments to help control motor symptoms (Pahwa et al., 2006). There are 2 types of surgical procedures that have been commonly used to treat PD: lesioning and electrical stimulation (Zesiewicz and Hauser, 2001). The main lesioning surgeries that have been performed are thalamotomy, in which the ventral intermediate nucleus (VIM) of the thalamus is lesioned, and pallidotomy, in which the GPi is lesioned (Zesiewicz and Hauser, 2001). Thalamotomy is useful in reducing contralateral tremor (Matsumoto et al., 1984), but bilateral thalamotomies have high complication rates resulting in swallowing and speech difficulties (Tasker, 1990), limiting the usefulness of this surgery. Pallidotomy is useful in reducing dyskinesias and can also improve tremor, rigidity and bradykinesia (Dogali et al., 1995; Lozano et al., 1995), which led to its surpassing thalamotomy in popularity in the mid 1990s (Lozano, 1996; Zesiewicz and Hauser, 2001). However, with the advent of Federal Drug Administration approval for deep brain stimulation of the VIM for tremor
in 1997, followed by approval of DBS of the GPi and STN as an adjunctive treatment for PD in 2002 (Collins et al., 2010), DBS quickly replaced lesioning procedures in popularity since it provided the same symptomatic benefit as pallidotomy with the added benefits of being adjustable and reversible (Zesiewicz and Hauser, 2001). DBS of the VIM is used to treat tremor whereas DBS of the STN and GPi are used to treat tremor as well as bradykinesia and rigidity (Zesiewicz and Hauser, 2001). In a nonrandomized trial comparing STN DBS with GPi DBS, patients with STN DBS tended to have better motor outcomes, although patients with GPi DBS tended to have fewer complications (Moro et al., 2010). In a randomized trial comparing the two, no differences were seen in motor outcomes. The only differences seen were that the GPi DBS group tended to do better on a measure of visuomotor processing speed than the STN DBS group, the GPi DBS group had slight improvement on the Beck Depression Inventory II scale whereas the STN DBS worsened slightly, and the STN DBS group had a greater reduction in medication use (Follett et al. 2010). The authors of this study concluded that one target did not appear to be superior to the other and that target selection should take into account the patient’s motor and nonmotor symptoms, the goal of stimulation, and the surgeon’s preference based on experience and any technical considerations (Follett et al. 2010).

Deep Brain Stimulation for the Treatment of Parkinson’s Disease

History

The history of DBS for the treatment of PD begins with the discovery by Benabid and colleagues (Benabid et al., 1987) that unilateral stimulation of the VIM at frequencies
over 100 Hz could mimic the effects of thalamotomy for the treatment of tremor when combined with contralateral thalamotomy (although it had been recognized that stimulation could mimic lesioning intraoperatively since 1968 (Toth and Tomka, 1968). While it is true that this study was the first to demonstrate that long-term high-frequency stimulation could improve tremor, the history of deep brain stimulation actually starts with the advent of stereotactic surgery pioneered by Ernest Spiegel and Henry Wycis in 1947 (Spiegel et al., 1947). The stereotactic device was developed as a tool to more accurately target regions of the brain for lesioning as a treatment for psychiatric disorders (Hariz et al., 2010), but by 1950, it was being used by these same surgeons to treat movement disorders via pallidotomies and thalamotomies (Spiegel and Wycis, 1950). During these surgeries, intraoperative deep brain stimulation was used as a means for determining the target of the lesion (Hariz et al., 2010). From the very beginning it was observed that low-frequency stimulation would worsen motor symptoms and high-frequency stimulation would improve them, but there lacked consensus on the definition of low and high frequencies (Hariz et al., 2010; Gildenberg, 2005) until Benabid published his study on VIM stimulation in 1991 (Benabid et al., 1991). After the introduction of L-DOPA in 1968, surgery to treat PD was stopped almost altogether (Gildenberg, 2006), although surgery continued for the treatment of medically intractable tremor (Schwalb and Hamani, 2008), thus leading to Benabid’s seminal discovery in 1987, followed by his publication in 1991, that chronic bilateral stimulation of the VIM could effectively treat tremor (Benabid et al., 1991), and to the eventual Food and Drug Administration (FDA) approval in 1997 of deep brain stimulation of the VIM for the treatment of tremor (Schwalb and Hamani, 2008).
Concurrently, in the 1990s, pallidotomy once again became a popular treatment for PD after its reintroduction by Laitinen in 1992 (Laitinen et al., 1992a, 1992b). By 1994, Siegfried and Lippitz were testing DBS of the GPi as an alternative to lesioning (Siegfried and Lippitz, 1994), eventually leading the way to FDA approval of DBS of the GPi as a treatment for PD in 2002 (Schwalb and Hamani, 2008). At the same time, several groups began experimenting with stimulating the STN as a way of improving PD motor symptoms based on the important role of this structure in PD symptomology as elucidated by DeLong in 1984 (DeLong et al., 1984; Schwalb and Hamani, 2008). The first human patient was implanted with an STN stimulator in 1994 by Benabid’s group (Benabid et al., 1994), and by 1998, groups were reporting that STN DBS might be superior to GPi DBS at relieving PD motor symptoms (Krack et al., 1998; Kumar et al., 1998a). STN DBS for the treatment of PD subsequently received FDA approval in 2002 (Schwalb and Hamani, 2008). Since the current studies utilize deep brain stimulation of the subthalamic nucleus, the remainder of this discussion will only address stimulation of this particular structure.

Candidacy for STN DBS Surgery

For a patient to be considered a candidate for DBS, the patient must have had the disease for a minimum of 5 years and be at least at stage 3 on the Hoehn-Yahr scale (Chang and Chou, 2006; Espay et al., 2006; Neimat et al., 2006; Limousin and Martinez-Torres, 2008), although the mean disease duration is 14 years before STN DBS is performed (Volkmann, 2004). Additionally, patients must have a diagnosis of idiopathic PD with the absence of atypical signs and must have a greater than 33%
reduction in their Unified Parkinson’s Disease Rating Scale (UPDRS) motor score after L-DOPA administration, as compared to their off-medication score, indicating that they are L-DOPA responsive (Espay et al., 2006; Limousin and Martinez-Torres, 2008). Patients are not considered candidates if they have cognitive, behavioral, or psychiatric problems (Limousin and Martinez-Torres, 2008). Patients are also not candidates if they are on anticoagulant or immunosuppressive therapies or if there are any neurosurgical or general surgical contraindications (Limousin and Martinez-Torres, 2008). Additionally, patients who have severe brain atrophy, extensive white-matter lesions, or focal lesions in the basal ganglia or in the trajectory of the electrode must be excluded from candidacy (Limousin and Martinez-Torres, 2008). Finally, although age itself is not exclusionary, it is recommended that extreme caution be utilized when recommending DBS for patients over the age of 70 (Limousin and Martinez-Torres, 2008).

**STN DBS Surgery**

STN DBS surgery is a stereotactic procedure in which the STN is first identified using either magnetic resonance or computed tomography imaging and then targeted using stereotactic coordinates. During the procedure the DBS lead containing 4 platinum-irridium electrode contacts is inserted into the brain such that the contacts are within the area of the STN. The lead is then connected to an extension at the skull, which is tunneled under the skin of the scalp and neck and connected to a pulse generator that is implanted subcutaneously in the infraclavicular area (Koller et al., 1999). Frequency, pulse width, amplitude, and number and location of active contacts are all adjustable parameters in the pulse generator, which can be changed using an external
programming device (Koller et al., 1999). Stimulation parameters may be changed many times over the course of treatment using STN DBS in order to achieve optimal therapeutic results.

**Efficacy of STN DBS**

The clinical efficacy of STN DBS is well established. Several studies have shown that STN DBS leads to a significant improvement of anywhere from 40–60% in UPDRS motor scores and an approximate 50% reduction in dyskinesia (Herzog et al., 2003; Krack et al., 2003; Rodriguez-Oroz et al., 2004; Schupbach et al., 2005; Ostergaard and Aa Sunde, 2006; Limousin and Martinez-Torres, 2008). STN DBS has also been shown to significantly improve patients' self-reported health-related quality of life (Martinez-Martin and Deuschl, 2007) and has been demonstrated to be superior to the best medical therapy at relieving the motor symptoms of PD (Deuschl et al., 2006b). STN DBS is also economically beneficial, as over the duration of the stimulator life, surgery is less expensive than medications (Charles et al., 2004; Meissner et al., 2005).

**Side Effects of STN DBS Surgery and Stimulation**

Although STN DBS is highly efficacious, it is associated with some stimulation-induced side effects, and the surgical procedure, as with any surgery, can lead to serious adverse events including death. In a meta-analysis of 29 studies, Kleiner-Fisman and colleagues (Kleiner-Fisman et al., 2006) reported that the most common stimulation-induced side effects are dysarthria (9.3% incidence), weight gain (8.4% incidence), depression (6.8% incidence), eyelid-opening apraxia (3.6% incidence), stimulation-
induced dyskinesias (2.6% incidence), and other motor problems such as muscle contractions, diplopia, or worsening of postural instability or gait (4% incidence when all are included). They also reported an incidence of 3.5% for psychiatric problems such as hypersexuality, mood disorders, psychosis, or hallucinations (Kleiner-Fisman et al., 2006). Many of these stimulation-induced side effects, especially the motor side effects, are transient or can be improved through adjustment of the stimulation parameters. The most common long-term problems associated with stimulation are a reduction in verbal fluency (Parsons et al., 2006; Smeding et al., 2006; Castelli et al., 2010; Fasano et al., 2010; Mikos et al., 2010) and accelerated cognitive decline in those with existing preoperative cognitive decline (Saint-Cyr et al., 2000; Krack et al., 2003). The most common surgical adverse events are transient confusion in the immediate postoperative period (15.6% incidence); intracranial hemorrhage (3.9% incidence); seizures (1.5% incidence); other surgical complications such as wound-healing issues, thrombophlebitis, hemiparesis and cerebrospinal fluid leak (3.3% incidence when included together); and pulmonary embolism (0.3% incidence) (Kleiner-Fisman et al., 2006). Since this surgery involves an implantable device, adverse events can occur related to the device itself. The most common device-related problems include broken electrodes or leads (wires connecting electrodes to the stimulus generator) (4.4% incidence), device malfunction (3% incidence), infection around the device (1.9% incidence) and migration of the device (1.5% incidence) (Kleiner-Fisman et al., 2006). Mortality rates associated with STN DBS appear to be low. In their meta-analysis of 29 studies, Kleiner-Fisman and colleagues (Kleiner-Fisman et al., 2006) only found 2 reports of death related to the surgery, and both were due to pulmonary embolism.
However, one study has reported a mortality rate as high as 1.8% (Umemura et al., 2003). In a review of the literature, Benabid and colleagues (Benabid et al., 2009) found that adverse event rates varied greatly between centers performing the procedure, which could be related to differences in surgical technique or expertise.

The Mechanism of Action of STN DBS

The mechanism of action of DBS has been hotly debated over the years. Originally, it was thought that STN DBS inhibited STN activity, mimicking the effects of STN ablation (Benazzouz et al., 1995, 2000; Dostrovsky and Lozano, 2002). However, clinically it was noted that STN DBS appeared to drive rather than inhibit the STN (Ceballos-Baumann et al., 1999; Hershey et al., 2003). Moreover, microdialysis measurements have demonstrated that glutamate levels in the SNr and GPi actually increase during STN DBS (Windels et al., 2000; Boulet et al., 2006). Importantly, one study has managed to unify these seemingly opposing studies by demonstrating dual inhibitory and excitatory effects of STN DBS at therapeutically relevant frequencies (Garcia et al., 2003). This study demonstrated that STN DBS can suppress spontaneous STN activity while at the same time inducing new activity that is time-locked to the stimulation in vitro. This gave rise to the “more” hypothesis of STN DBS, which predicts that STN DBS causes neurons to become stable oscillators that are completely driven by stimulation, which then overrides the spontaneous STN activity and produces high-frequency regular spiking (Garcia et al., 2003). In vivo studies have demonstrated that inhibitory responses occur at the STN stimulation site while equal numbers of inhibitory and excitatory responses were found in the GP and SNr (Shi et al., 2006), lending support to this “more” hypothesis. Studies demonstrating the STN DBS can attenuate the beta
oscillations within the basal ganglia-thalamocortical network (Brown et al., 2004; Meissner et al., 2005; Silberstein et al., 2005; Wingeier et al., 2006; Bronte-Stewart et al., 2009) also seem to support the idea that STN DBS overrides the pathological activity within the STN, and this could be an important mechanism by which DBS imparts its therapeutic effect.

The Neuroprotective Potential of STN DBS

As discussed previously, STN DBS has demonstrated convincing therapeutic efficacy in the treatment of the cardinal motor features of PD (Herzog et al., 2003; Krack et al., 2003; Rodriguez-Oroz et al., 2004; Schupbach et al., 2005; Ostergaard and Aa Sunde, 2006; Zhang et al., 2006; Limousin and Martinez-Torres, 2008). However, recent investigations have sought to determine whether STN DBS can also slow or halt the progression of PD. Preclinical studies have provided evidence to support this. In rats, it has been demonstrated that when STN DBS is administered immediately following intrastriatal 6-OHDA injection, the number of surviving tyrosine hydroxylase immunoreactive (THir) neurons in the SN is double compared to that of the inactive stimulator control animals (Maesawa et al., 2004). Subsequent studies have demonstrated that STN DBS administered one week after lesioning also results in protection of the SN THir neurons; however the magnitude of the neuroprotection is more modest than that associated with immediate STN DBS (Temel et al., 2006; Harnack et al., 2008). These rodent studies have been replicated in non-human primates, where it has been demonstrated that STN DBS given to non-human primates either before or 6 days after MPTP treatment also results in protection of SN DA
neurons (Wallace et al., 2007). The clinical literature also suggests that neuroprotection may be occurring, based on studies that have demonstrated that patients treated with STN DBS show maintenance of improvement in off-medication motor symptoms 3–5 years after surgery (Krack et al., 2003; Rodriguez-Oroz et al., 2004; Rodriguez-Oroz et al., 2005). However, in one prospective study of the effects of STN DBS in patients with advanced PD, patients receiving STN DBS displayed similar disease progression (utilizing striatal fluorodopa uptake) to PD patients that did not receive STN DBS, thus seeming to negate the concept of STN DBS-mediated neuroprotection (Hilker et al., 2005). However, these authors did acknowledge that had they examined patients in earlier stages of PD, they may have been able to observe a neuroprotective effect.

Despite these findings, several questions remain unanswered in the literature. First, all of these preclinical studies exclusively examined the DA cell bodies in the SN. No morphological or neurochemical examination of the DA terminals in the striatum was performed. Without demonstrated preservation of striatal dopaminergic transmission, the relevancy of nigral cell body protection is unclear. Second, all of these studies examined the impact of STN DBS that occurred either prior to or within a few days of neurotoxic insult, when the nigrostriatal system was relatively intact. No preclinical study to date has examined the impact of STN DBS when the nigrostriatal system has been significantly denervated, as is the case when PD patients are initially diagnosed. Finally, although all of these studies were able to demonstrate neuroprotection as a result of STN DBS, none of them examined the mechanism behind that neuroprotection. The current studies seek to investigate these issues, including the effects of STN DBS on
the trophic factors glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) due to their potential as neuroprotective mechanisms.

**Trophic Factors**

**The Discovery of Nerve Growth Factor**

Trophic factors are proteins that have a multitude of important functions in both development and adulthood, as well as in response to injury. These functions include neuron survival, cell proliferation and differentiation, control of target innervation via the promotion of neurite outgrowth, synapse formation and the regulation of synaptic strength and plasticity (Huang and Reichardt, 2001). Nerve growth factor (NGF) was the first trophic factor to be isolated and identified by Stanley Cohen in 1954 (Cohen *et al.*, 1954). However, the story of the discovery of NGF actually begins in the mid 1930s with the experiments of Viktor Hamburger in which he discovered that removal of the limb bud of chick embryos led to the reduction of sensory and motor neurons in the spinal cord on the operated side (Hamburger, 1934) and that when a limb was transplanted onto the chick embryo, it led to increased numbers of sensory and motor neurons on the operated side (Hamburger, 1939), which he hypothesized was due to the target providing a signal that recruited undifferentiated cells to develop into sensory and motor neurons. In the early 1940s, Rita Levi-Montalcini began repeating these experiments while in Italy, but added the use of silver staining, which allowed her to distinguish between neurons and undifferentiated cells. She obtained the same results as Hamburger, but was able to determine that the mechanism was cell death/survival and not cell differentiation (Cowan, 2001). After reading her paper, Hamburger invited Levi-
Montalcini to work with him in his lab at Washington University, and she arrived in 1947 (Cowan, 2001). Then, in 1948, one of Hamburger’s former students, Elmer Bueker, published a paper demonstrating that when mouse sarcoma 180 was transplanted into a chick embryo, the dorsal root ganglia innervating the transplant became enlarged (Bueker, 1948). Levi-Montalcini and Hamburger decided to further investigate this phenomenon and found that the sensory and sympathetic ganglia of chick embryos bearing mouse sarcoma 180 or 37 transplants were enlarged compared to the contralateral side, with the conclusion that the tumor cells must produce growth factors (Levi-Montalcini and Hamburger, 1951). Additional studies demonstrated that sympathetic ganglia that were not in contact with the tumor also were enlarged and that when the sarcoma was transplanted on the chorio-allantoic membrane, which prevented direct contact between the embryonic and transplanted tissue, the sympathetic ganglia still became enlarged (Levi-Montalcini, 1952). This led to the hypothesis that the tumor was releasing a diffusible growth factor. Levi-Montalcini then decided to investigate this further using tissue culture and tumor extract instead of the whole tumor. The results of these experiments demonstrated that when sensory and sympathetic ganglia from chick embryos were cultured with the tumor extracts, they developed a dense halo of nerve fibers within 12 to 24 hours (Levi-Montalcini et al., 1954). The tissue culture assay then allowed for the isolation and identification of NGF by Cohen, who had joined Hamburger and Levi-Montalcini at Washington University in 1953 (Levi-Montalcini, 1982). Cohen and Levi-Montalcini later discovered that the male mouse salivary glands are a rich source of NGF (Levi-Montalcini and Cohen, 1960), and Cohen was then able to produce an antiserum to NGF (Cohen, 1960). These two discoveries paved the way for
subsequent experiments examining structure, mechanism of action and functions of NGF, and in 1986 the Nobel Prize in medicine was awarded to Levi-Montalcini and Cohen for their discovery of NGF.

The Discovery of Brain-Derived Neurotrophic Factor

Another important discovery occurred when Yves-Alain Barde and colleagues (Barde et al., 1980) found that rat brain extract had an effect similar to, yet slightly different than NGF on cultured neurons (i.e., it promoted sensory but not sympathetic neuron survival). They also found that when both these extracts and NGF were added to the culture medium, there was a greater than additive effect. Taken together, these results indicated that the brain extracts must contain other trophic factors similar to NGF. This was followed by the isolation and purification of BDNF in 1982 (Barde et al., 1982). Once it was discovered that BDNF and NGF had 50% homology, investigators were then able to rapidly identify other members of the neurotrophin family of trophic factors, including neurotrophin 3, neurotrophin 4/5 and neurotrophin 6 (Oppenheim and Johnson, 2003).

The Discovery of Glial Cell Line-Derived Neurotrophic Factor and Other Trophic Factor Families

One of the most important findings with respect to dopaminergic neurons was the finding by Engele and colleagues in 1991 (Engele et al., 1991) that glia-conditioned media promoted the survival of dopaminergic neurons. The factor promoting
dopaminergic neuron survival in these cultures was later isolated and identified by Lin and colleagues in 1993 (Lin et al., 1993) as GDNF.

Since the original discovery of NGF more than 50 years ago and the subsequent identification of the other members of the neurotrophin family of trophic factors, many more trophic factors and trophic factor families have been discovered. Other major families besides the neurotrophins include the transforming growth factor β superfamily, the cytokines, the epidermal growth factors, the fibroblast growth factors, the interleukins, the tumor necrosis factors, the chemokines, the colony-stimulating factors, the interferons, the heparin-binding growth factors, and the more recently discovered mesencephalic-astrocyte-derived neurotrophic factor (MANF) family, among others (Lindholm et al., 2007; Oppenheim and Johnson, 2003; Landreth, 1999). The most potent dopaminotrophic factors belong to the neurotrophin, transforming growth factor β and MANF families with conserved dopamine neurotrophic factor, MANF, neurturin, GDNF and BDNF having the most potent effects on dopamine neurons (Voutilainen et al., 2009; Lindholm et al., 2007; Kordower et al., 2006; Collier and Sortwell, 1999). One of the current studies examines the effects of STN DBS on GDNF and BDNF in particular.

**Glial Cell Line-Derived Neurotrophic Factor**

**GDNF Expression**

As stated previously, GDNF was first isolated and identified by Lin and colleagues in 1993 (Lin et al., 1993) after Engele and colleagues demonstrated that glia-conditioned
media promoted the survival of dopaminergic neurons in culture (Engele et al., 1991). Several studies have since demonstrated that GDNF is widely expressed both within the central nervous system and in the periphery during development and into adulthood (Schaar et al., 1993; Choi-Lundberg and Bohn, 1995; Nosrat et al., 1996). However, with regard to the dopaminergic nigrostriatal system, the highest levels of GDNF gene expression in rats occur in the striatum between postnatal day 0 and postnatal day 10, with lower expression levels seen in the adult striatum (Choi-Lundberg and Bohn, 1995). In the substantia nigra, GDNF gene expression levels are highest at postnatal day 0 and are seen at very low levels in the adult as detected via PCR (Choi-Lundberg and Bohn, 1995). This expression pattern is consistent with the role of GDNF in increasing the survival, dopamine uptake and neurite outgrowth of dopamine neurons (Lin et al., 1993). Although the expression level of GDNF in the substantia nigra is very low, it has been demonstrated that GDNF can be retrogradely transported from the striatum to the substantia nigra (Tomac et al., 1995a), which may be a method for dopaminergic neuron survival in the adult.

**GDNF Structure, Signaling Mechanisms and Function**

GDNF was classified as a member of the transforming growth factor β superfamily of trophic factors based on the fact that it contains seven cystine residues with the same spacing as the other members of the transforming growth factor β superfamily, although it shares less than 20% amino acid homology with any of the other members of this family (Lin et al., 1993). GDNF also appears to be distantly related to the neurotrophin family of trophic factors based on its cystine knot structure consisting of “…an eight-
member ring held together by two disulfide bonds, with a third disulfide bond threaded though the middle of the ring…” (Kingsley, 1994), which is also seen in the neurotrophins (Saarma and Sariola, 1999). Interestingly, GDNF is N-glycosylated at two amino acid residues, which is rare for mature growth factors (Saarma and Sariola, 1999), although this glycosylation does not appear to affect its trophic effects on dopaminergic neurons (Garbayo et al., 2007).

GDNF production is regulated in an activity-dependent manner (Saavedra et al., 2008), and there are several different neurotransmitters that can affect its expression. First, dopamine appears to regulate GDNF expression, as it has been demonstrated that activation of both D1 and D2 receptors increases GDNF mRNA expression in both dopaminergic neuron cultures and astroglial cultures (Ohta et al., 2000, 2003, 2004; Guo et al., 2002). Second, serotonin has also been shown to increase GDNF mRNA expression via activation of the 5-hydroxytryptamine (5-HT)2 receptor in astroglial cultures (Hisaoka et al., 2004). Third, adenosine increases both GDNF mRNA and protein in astroglial cultures via activation of the A2B receptor (Yamagata et al., 2007). This mechanism may be of particular importance for the role of GDNF in responding to neuronal injury, since adenosine levels have been shown to increase in an ischemic model of neuronal injury (Parkinson et al., 2002). Fourth, glutamate has been shown to increase GDNF mRNA both in vivo in the striatum and in astroglial cultures (Ho et al., 1995; Battaglia et al., 2009). It appears that this effect can be mediated both via NMDA receptor activation (Ho et al., 1995) and via metabotropic glutamate receptor activation (Battaglia et al., 2009). Finally, it appears that cytokines such as interleukin (IL)-1β, IL-6,
tumor necrosis factor (TNF)-α and TNF-β can also increase GDNF mRNA and protein in astroglial cultures (Appel et al., 1997; Verity et al., 1998). GDNF is initially synthesized in its precursor form pro-GDNF, and then is cleaved to form mature GDNF (Carnicella and Ron, 2009). In culture, cleavage has been demonstrated to be mediated by the endoproteases furin, PACE4, PC5A, PC5B and PC7, and cleavage may occur both intercellularly and in the extracellular matrix (Carnicella and Ron, 2009).

GDNF signaling actually involves two receptors. GDNF must first bind to the GDNF family receptor α1 (GFRα1), which is a glycosylphosphatidylinositol-linked cell surface receptor (Jing et al., 1996). This GDNF-GFRα1 complex then binds to the tyrosine kinase receptor Ret, which induces its tyrosine phosphorylation (Jing et al., 1996; Treanor et al., 1996). Activation of the Ret receptor then leads to the activation of several cell signaling cascades, including the mitogen-activated protein kinase (MAPK) cascade, the extracellular signal-regulated kinase 1/2 (ERK 1/2) cascade, the phosphatidylinositol 3 kinase (PI3K) cascade and the phospholipase Cγ (PLC-γ) cascade (Airaksinen and Saarma, 2002). These signaling cascades are involved in cell survival, neurite outgrowth, neurotransmission and cell proliferation (Cobb, 1999; Airaksinen and Saarma, 2002).

Given the widespread expression of GDNF, it is not surprising that it has many more functions than promoting dopaminergic neuron survival. In the periphery, GDNF is necessary for normal kidney and enteric nervous system development (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Additionally, GDNF promotes the
survival of developing motor neurons (Oppenheim et al., 1995) and can rescue adult motor neurons from injury-induced cell death (Li et al., 1995). In the central nervous system, GDNF can help maintain the cholinergic phenotype in injured basal forebrain cholinergic neurons (Williams et al., 1996) and can protect CA1 hippocampal cells from excitotoxic injury (Martin et al., 1995) and the noradrenergic cells of the locus coeruleus from 6-OHDA-induced injury (Arenas et al., 1995).

GDNF as a Treatment for Parkinson's Disease

After it was established by Lin and colleagues (Lin et al., 1993) that GDNF promoted the survival of dopaminergic neurons in vitro, intense interest was generated in its potential use as a treatment for PD and several investigators began to examine its effects in animal models of the disease. Hoffer and colleagues (Hoffer et al., 1994) demonstrated that intranigral injection of GDNF could increase dopamine and reduce apomorphine-induced rotational behavior in 6-OHDA lesioned animals. Subsequently, Beck and colleagues (Beck et al., 1995) demonstrated that GDNF protected SN dopamine neurons from degeneration following transection of the medial forebrain bundle, Kearns and Gash (1995) demonstrated that GDNF could protect SN dopamine neurons from both intranigral and intrastratal 6-OHDA, and Tomac and colleagues (Tomac et al., 1995b) demonstrated that GDNF could protect SN dopamine neurons and striatal dopaminergic terminals as well as preserve dopamine levels in the MPTP mouse model of PD. In 1996, Gash and colleagues (Gash et al., 1996) extended these studies to the MPTP monkey model and found that intracerebral GDNF could improve bradykinesia, rigidity and postural instability as well as increase dopamine and dopaminergic neuron
size and fiber density. After such promising results, the intracerebroventricular application of GDNF was extended to PD patients in several clinical trials. Kordower and colleagues (Kordower et al., 1999) were the first to report no improvement in PD symptoms in a single patient who had received direct GDNF protein infusions to the putamen, followed by a similar report from a multicenter, randomized, double-blind, placebo-controlled trial (Nutt et al., 2003). Although these results were disappointing, many people still hold out hope that neurotrophic factors may prove to be an efficacious treatment for PD and a new clinical trial using magnetic resonance imaging-guided targeting of GDNF to the putamen along with a new delivery system is currently being planned (Richardson et al., 2011).

**Brain-Derived Neurotrophic Factor**

**BDNF Structure, Function and Expression Patterns**

As stated previously, BDNF was first isolated and identified by Barde and colleagues in 1982 (Barde et al., 1982). Their investigation into the existence of BDNF began in 1978 when they noticed that culture media obtained from glial cells as well as extracts from the rat central and peripheral nervous system promoted the survival of sensory but not sympathetic neurons *in vitro*. This effect on sensory neurons was not affected by the application of NGF antibodies, suggesting the existence of another trophic factor similar to NGF (Barde et al., 1978). After its identification in 1982, BDNF was classified as a member of the neurotrophin family of trophic factors based on the fact that it was found to be 50% homologous with NGF, contained the same six cystine residues, and had a similar arrangement of disulfide bridges (Leibrock et al., 1989).
After the demonstration that BDNF could promote the survival of sensory neurons \textit{in vitro} (Barde \textit{et al.}, 1982), Johnson and colleagues (Johnson \textit{et al.}, 1986) set out to determine if any populations of central nervous system neurons were BDNF responsive and they decided to use fetal retinal ganglion cells since they are easy to identify and large numbers of them die during development. They found that BDNF does promote their survival in culture. Then, in 1990, Wetmore and colleagues (Wetmore \textit{et al.}, 1990) set out to determine where BDNF is expressed via in situ hybridization. They found that BDNF is expressed in the hippocampus, cortex, claustrum and cerebellum, with little expression in the basal ganglia and no expression in the periphery. Hofer and colleagues (Hofer \textit{et al.}, 1990) also examined BDNF mRNA expression and, in addition to the areas seen by Wetmore, also found BDNF in the olfactory bulb, thalamus, preoptic area, hypothalamus and pontine nuclei. In addition, they found BDNF expression in the heart and lungs. Other studies have also found BDNF expression outside the central nervous system in platelets and Schwann cells (Yamamoto and Gurney, 1990; Acheson \textit{et al.}, 1991). The breakthrough in BDNF in relation to PD came in 1991 when Hyman and colleagues (Hyman \textit{et al.}, 1991) found that BDNF promotes the survival of dopaminergic neurons in culture. Then, in 1993, Seroogy and Gall (Seroogy and Gall, 1993) demonstrated the existence of BDNF mRNA in the dopaminergic neurons of the SN, as well as the GPI/EP and ventral tegmental area, giving further support to the hypothesis that BDNF is a trophic factor for dopaminergic neurons \textit{in vivo}. 

BDNF Synthesis, Release and Signaling

As with GDNF, BDNF is synthesized and released in an activity-dependent manner (Gall et al., 1992; Griesbeck et al., 1999), and BDNF mRNA has specifically been shown to be upregulated by glutamatergic and acetylcholinergic activity (Lindholm et al., 1994; Bustos et al., 2004). Specifically, stimulation of muscarinic, but not nicotinic, acetylcholine receptors and stimulation of both NMDA and non-NMDA glutamate receptors regulates BDNF mRNA expression (Zafra et al., 1990; Lindefors et al., 1992; Favaron et al., 1993; Gwag and Springer, 1993; Lapchak et al., 1993; Hartmann et al., 2001; Matsuda et al., 2009). BDNF is initially synthesized in its precursor form proBDNF, which is converted to mature BDNF intracellularly by either endoproteases or proprotein convertases (Mowla et al., 1999). However, proBDNF can also be released from a cell without undergoing proteolytic conversion (Mowla et al., 2001), where it can either act on receptors or be cleaved to form mature BDNF via plasmin (Lee et al., 2001). There are two receptors that exist for BDNF: receptor tyrosine kinase B (TrkB) and p75 neurotrophin receptor (p75NTR). The mature form of BDNF binds to and activates the TrkB receptor, which in turn activates the ERK, PI3K and PLC-γ signaling pathways that promote cell survival, neurite outgrowth, neurotransmission and cell proliferation (Cobb, 1999; Airaksinen and Saarma, 2002). The pro form of BDNF binds to the p75NTR receptor, which when in complex with the receptor sortilin, initiates apoptosis (Teng et al., 2005; Domeniconi et al., 2007; Jansen et al., 2007). However, p75 can also form a complex with the TrkB receptor, which increases the affinity and specificity of the TrkB receptor for the mature form of BDNF and the promotion of cell survival (Chao, 2003; Bibel et al., 1999). With regard to the current studies, it is
interesting to note that high-frequency STN DBS for PD improves symptoms, whereas low frequency worsens symptoms (Rizzone et al., 2001; Moro et al., 2002; Garcia et al., 2005), and high-frequency stimulation has been shown to induce the secretion of mature BDNF while low-frequency stimulation induced the secretion of proBDNF in hippocampal neuronal cultures (Nagappan et al., 2009). Additionally, high-frequency stimulation also increased tissue plasminogen activator (tPA), which converts plasminogen to plasmin, which is responsible for cleaving pro-BDNF to form mature BDNF (Nagappan et al., 2009).

As originally discovered by Barde and colleagues in 1982, BDNF is well known for promoting neuron survival (Barde et al., 1982; Johnson et al., 1986; Kalcheim et al., 1987; Hofer and Barde, 1988). However, BDNF has many other roles. BDNF has been shown to promote neuronal differentiation (Kalcheim and Gendreau, 1988; Knusel et al., 1991), regulate axonal and dendritic growth and guidance (Beck et al., 1993; Paves and Saarma, 1997; Li et al., 2005), potentiate neurotransmitter release (including dopamine and glutamate) (Blochl and Sirrenberg, 1996; Takei et al., 1997; Li et al., 1998; Sala et al., 1998), modulate synaptic plasticity and long-term potentiation (Kang and Schuman, 1995; Figurov et al., 1996; Patterson et al., 1996) and can increase dendritic spine density and modulate spine morphology (Shimada et al., 1998; Tyler and Pozzo-Miller, 2003; Matsutani and Yamamoto, 2004).
**BDNF as a Treatment for Parkinson’s Disease**

After Hyman and colleagues (Hyman et al., 1991) discovered that BDNF could promote the survival of the dopaminergic neurons in the SN, interest was generated in using BDNF as a possible treatment for PD, especially since BDNF expression is reduced in the SN of patients with PD (Parain et al., 1999; Howells et al., 2000). Before BDNF was used in any animal models of PD, it was first demonstrated by Spina and colleagues (Spina et al., 1992) that BDNF could protect mesencephalic dopaminergic neurons from N-methyl-4-phenylpyridinium (MPP+) and 6-OHDA-induced cell death *in vitro*. The next step in investigating its potential use as a PD treatment came when Altar and colleagues demonstrated that intrastriatal or supranigral application of BDNF can increase dopamine turnover and/or release *in vivo* (Altar et al., 1992). Then, in 1994, Frim and colleagues (Frim et al., 1994) demonstrated that BDNF could protect the SN dopamine neurons from MPP+ *in vivo* in rodents. In 1995, Yoshimoto and colleagues (Yoshimoto et al., 1995) examined the effects of BDNF on amphetamine-induced rotations in the 6-OHDA rodent model of PD and found that BDNF decreased these rotations. Tsukahara and colleagues (Tsukahara et al., 1995) then extended these studies to monkeys and found that BDNF treatment prior to MPTP administration prevented the development of severe parkinsonian motor signs and SN cell loss as seen in the non-BDNF treated group. In 1996, BDNF was explored as a way to improve the efficacy of transplanted fetal dopaminergic neurons when Yurek and colleagues (Yurek et al., 1996) demonstrated that BDNF application enhanced the innervation of striatum by the grafted dopamine neurons in the 6-OHDA rodent model of PD. However,
after the failure of fetal cell transplants and intracerebral GDNF application to improve PD symptoms in clinical trials, BDNF was never tested clinically as a treatment for PD.

**Trophic Factors in the Current Studies**

In order to elucidate the mechanism of STN DBS induced neuroprotection seen in the current studies, we also examined the effects of high-frequency STN DBS on GDNF and BDNF within the STN and its target structures. The STN serves as an important modulating influence within the basal ganglia circuitry, providing glutamatergic projections to the cortex, GPe, GPi, SN and striatum (see Figure 3) (Jackson and Crossman, 1981; Kita et al., 1983; Kita and Kitai, 1987; Parent and Hazrati, 1995; Kitai and Kita, 2006; Degos et al., 2008). It is known from studies of other systems that high-frequency stimulation of hippocampal glutamatergic synapses can induce the presynaptic and postsynaptic release of BDNF (Hartmann et al., 2001; Matsuda et al., 2009; Nagappan et al., 2009) and pharmacological activation of hippocampal GABA(B) receptors also induces BDNF release (Fiorentino et al., 2009). Preclinical and clinical studies have demonstrated that STN DBS can increase glutamate and GABA levels in numerous basal ganglia structures (Windels et al., 2000, 2003; Bruet et al., 2003; Boulet et al., 2006; Stefani et al., 2006) and increased glutamatergic transmission can increase BDNF mRNA in the SN (Bustos et al., 2004). Therefore, it is possible that STN DBS-mediated increases in glutamate and GABA triggers the upregulation and release of BDNF and/or GDNF, since both of these trophic factors are present in the nigrostriatal system (Schaar et al., 1993; Seroogy and Gall, 1993; Seroogy et al., 1994; Choi-Lundberg and Bohn, 1995; Kawamoto et al., 1996; Conner et al., 1997). In
addition, HF STN DBS upregulates immediate early gene (IEG)-encoded proteins in a number of structures, including the SN (Salin et al., 2002; Bacci et al., 2004; Schulte et al., 2006). Prolonged IEG expression precedes long-term adaptive processes that can affect cellular metabolism, excitability, transmitter synthesis, synaptic plasticity and trophic factor synthesis (Nedivi et al., 1993; Porter et al., 1994; Herdegen and Leah, 1998), indicating that the IEG proteins induced by STN DBS could lay the groundwork for a subsequent increase in BDNF and/or GDNF.

As neuroprotective agents, GDNF and BDNF have been shown to be highly effective in the preclinical literature. Numerous studies have demonstrated that infusion of BDNF or GDNF to the SN of rats with 6-OHDA lesions of the nigrostriatal pathway provides significant neuroprotection (Collier and Sortwell, 1999) and gene transfer of GDNF can provide significant neuroprotection from parkinsonian insult in both rats and monkeys (Sortwell and Kowdower, 2006). Additionally, the preclinical findings that STN DBS is protective against two different neurotoxins, 6-OHDA and MPTP (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008), suggest a common neuroprotective mechanism not related to the toxicity of the agents themselves. Collectively, these findings suggest that STN DBS has the potential to increase BDNF and/or GDNF mRNA and/or protein levels in STN target structures.
Chapter 2: A Functionally Relevant and Long-Term Model of Deep Brain Stimulation of the Rat Subthalamic Nucleus: Advantages and Considerations

Abstract

In this review we outline some relevant considerations with regards to the rat model of deep brain stimulation of the subthalamic nucleus (STN DBS). In order to optimize the rat STN DBS model in terms of predictive validity for the clinical situation we propose that the STN stimulation experimental design parameters in rodents should incorporate the following features: (i) stimulation parameters that demonstrate functional alleviation of symptoms induced by nigrostriatal dopamine (DA) denervation; (ii) stimulation duration that is relatively long-term and continuous; (iii) stimulation that is initiated at a time when the denervation status of the nigrostriatal system is known to be partial and progressing; (iv) stimulation current spread that is minimized and optimized to closely approximate the clinical situation; (v) the appropriate control conditions are included; and (vi) implantation to the STN target is verified post-mortem. Further research that examines the effect of long-term STN DBS on the neurophysiology and neurochemistry of STN circuitry is warranted. The rat model of functionally relevant long-term STN DBS provides a most favorable preclinical experimental platform in which to conduct these studies.
Introduction

Deep brain stimulation (DBS) of the subthalamic nucleus (STN) has become the most often practiced neurosurgery for treatment of the cardinal motor features of Parkinson’s disease (PD), specifically bradykinesia, rigidity and resting tremor. More than 40,000 patients have received this treatment as of 2007 (Pereira et al., 2007). High-frequency DBS was successfully developed to replace ablative procedures due to the advantage of it being reversible and adjustable. Initially, bilateral stimulation of the STN or the pars interna of the globus pallidus (Gpi) were both utilized with similar frequency to treat PD. However, in recent years STN DBS has gained greater popularity and is now practiced more often by most centers performing DBS due to greater improvements in motor performance, which allows for a greater reduction in dopaminergic medication (Vitek, 2002; Benabid et al., 2005; Moro et al., 2010). Bilateral high-frequency STN DBS is a safe, stable and reliable therapy that leads to dramatic long-term improvements in the quality of life for PD patients (Benabid et al., 2005; Hamani et al., 2005; Lyons and Pahwa, 2005). Recent clinical studies indicate that bilateral STN DBS may be more effective in treating PD than medication alone (Deuschl et al., 2006). Additionally, STN DBS is economically beneficial as it is less expensive than medications for PD, with the advantage in cost realized within the second year (Charles et al., 2004; Meissner et al., 2005). More recently, STN DBS has also been investigated as a treatment strategy for other disorders such as obsessive-compulsive disorder (OCD) and seizures (Vercueil et al., 1998; Lado et al., 2003; Usui et al., 2005; Winter et al., 2008; Klavir et al., 2009).

The clinical use of STN DBS has proceeded without a complete understanding of its mechanism of action. This circumstance has spurred intense investigation into the
impact of STN DBS in preclinical animal models, with the overwhelming majority of preclinical research utilizing rats as experimental subjects. We, and others, have used the 6-hydroxydopamine (6-OHDA)-lesioned rat as a model of PD to study the effects of STN DBS. This model offers many advantages, not the least of which are the lack of financial and experimental design constraints that are associated with basic research in non-human primates. Further, rats are better suited for STN DBS studies than their mouse counterparts on a number of levels. However, the rat model of STN DBS is not without its limitations. In this review we outline some relevant considerations with regards to the rat model of STN DBS.

Rat STN Neuroanatomy

Rats represent good candidates for preclinical investigation as the organization of structures and circuitry adjacent to the STN compares favorably between rats and humans. The STN is a small, densely populated structure located between the zona incerta and the cerebral peduncle. It is primarily comprised of projection neurons with axons that extend for > 750 μm. The GPi (in rodents, entopeduncular nucleus) and the substantia nigra pars reticulata (SNr) are the principal target structures of the glutamatergic neurons of the STN. These same structures also receive inhibitory input from the striatal GABAergic direct pathway, making the GPi and the SNr the major output structures of the basal ganglia (Whittier and Mettler, 1949; Carpenter and Strominger, 1967). The STN exerts powerful glutamate-mediated excitatory effects on the GPi and the SNr as well as the frontal cortex (FC), the pars externa of the globus pallidus (GPe) and the striatum (Jackson and Crossman, 1981; Kita et al., 1983; Kita
and Kitai, 1987; Parent and Hazrati, 1995; Kitai and Kita, 2006; Degos et al., 2008) whereas it receives inhibitory input from GABAergic neurons of the GPe via the indirect pathway. As a result, the STN is in the unique position of being the main relay nucleus of the indirect pathway that can act in concert with the direct pathway to modulate the activity of the GPi and SNr. Therefore, the STN is in a strategic position to influence the output of motor information from the basal ganglia.

Targeting the small STN deep within the brain can be difficult. From a neuroanatomical standpoint, rats represent much more favorable candidates than mice to serve as a preclinical model of STN DBS as the size of the rat STN is approximately twice that of the mouse STN (1.2 x 1.2 mm in rats [Hardman et al., 2002] vs. 0.6 x 0.65 mm in mice [Franklin and Paxinos, 2001]) making targeting of the STN easier in rats. That being said, the STN of rats is approximately 300 x smaller than the human STN (rat STN ≈ 0.8 mm$^3$; human STN ≈ 240 mm$^3$ [Hardman et al., 2002]) whereas the size of the stimulating electrode used in rodent studies is only 6 x smaller than electrodes used clinically. The large size of the stimulating electrode relative to the size of the rat brain increases the likelihood that current aimed at the rat STN will spread to structures outside of the STN. Previous studies in our laboratory have directly examined this issue (Figure 4A [Spieles-Engemann et al., 2010]). We estimated the distance of current spread associated with STN stimulation by utilizing simultaneous STN stimulation and extracellular recordings. Concentric bipolar stimulating electrodes were placed within the STN of anesthetized rats using extracellular guided recording techniques. A tungsten recording electrode was then used to record from sites at varying distances
from the STN. We estimated that under our experimental stimulation parameters current spread impacts not only the STN but also a sphere immediately surrounding the STN with an approximate radius of at least 250 μM. These findings indicate that the interpretation of results generated in rat STN DBS studies should always consider that observed effects may be attributable to stimulation of fibers and structures outside of the STN.

The question in terms of clinical relevancy is: does the mismatch in the scale of the rat brain relative to the size of the stimulating electrode, as compared to stimulators placed in the STN of PD patients, introduce the probability of current spread outside the STN that may not normally occur in the clinical situation? In PD patients stimulation parameters are meticulously refined over multiple post-surgical sessions in order to optimize functional improvements (Deuschl et al., 2006a). The area of current spread, which has been termed the volume of tissue activation (Butson and McIntyre, 2005), is not a factor in this programming. Functional efficacy of the stimulation is the ultimate goal. The stimulation parameters routinely used in PD patients have been estimated to result in stimulation of a large volume of neural tissue that is not limited to the STN proper, resulting in activation of neighboring large-diameter myelinated axons (McIntyre et al., 2004b; Butson et al., 2007). A schematic representation of structures and fiber pathways that can potentially be impacted by STN stimulation is illustrated in Figure 4B. Nonetheless, it is reasonable to assume that the relative stimulation area in the rodent model of STN DBS will be larger. Under these conditions careful positioning of the stimulating electrode confirmed by postmortem placement analysis becomes critical.
Further, the stimulation parameters, particularly stimulation amplitude, must be carefully considered, as large-amplitude current will impact a larger volume of tissue. Lastly, and in our opinion most importantly, the STN stimulation parameters utilized in rat studies must demonstrate functional alleviation of symptoms induced by nigrostriatal dopamine (DA) denervation.

**STN DBS in the Rat**

*Stimulation Parameters*

In PD patients with STN stimulation, the precise stimulation parameters are determined after implantation surgery for each patient in extensive consultation with their neurologist. Nonetheless, there are ranges within which most stimulus parameters fall: stimulus frequency between 130 and 185 Hz, pulse duration between 60 and 210 μs, and stimulus amplitude between 1 and 3.5 V (Kuncel and Grill, 2004). With the exception of stimulus amplitude (intensity), preclinical studies in the rodent model of STN DBS are likely to be most informative when STN stimulus parameters are set within these same ranges. Further, while therapeutic STN stimulation frequencies have been reported to be > 100 Hz, frequencies < 50 Hz are non-therapeutic (Garcia *et al.*, 2005) and stimulation at 5–10 Hz has been demonstrated to worsen parkinsonism (Rizzone *et al.*, 2001; Moro *et al.*, 2002). With regards to stimulus amplitude, due to the large differences in STN volume between rodents and humans, the intensity used in rats cannot replicate clinical parameters and every attempt should be made to minimize current spread to neuronal structures not normally impacted in the clinical setting. Estimates of the volume of tissue activated in the human brain range from 30 to 114...
mm³ with approximately 70 mm³ generating the most therapeutic benefit (Maks et al., 2009). This volume of activation would likely include the lenticular fasciculus and ansa lenticularis (see Figure 4B) but not the substantia nigra (SN). Based on our current-spread studies in anesthetized rats (Figure 4A), stimulation of the STN at amplitudes > 100 μA should therefore be interpreted with caution as stimulation of the SN cannot be excluded. Further, stimulus amplitudes utilized in anesthetized rats may not directly translate to awake rats as anesthesia can impact the firing properties of neurons (Chen et al., 2001; Erchova et al., 2002). When possible, refinements in stimulus parameters should be made in awake rats in order to minimize the effects of STN stimulation on SN and the medial forebrain bundle. For example, the behavioral responses of differing stimulus intensities can be immediately observed after stimulation is turned on in awake rats (Maesawa et al., 2004; Spieles-Engemann et al., 2010). These responses range from minor to major involuntary movements. For example, at 30–50 μA contralateral dyskinetic orofacial movements are typically apparent, increasing the stimulation intensity to 120 μA leads to dyskinetic movements of the contralateral forepaw, and stimulation > 200 μA results in severe tonic twisting or rotational behavior. These rotational responses are indicative of direct SN stimulation (Arbuthnott and Ungerstedt, 1975; Watanabe et al., 1983). Therefore, we contend that stimulation intensities set to just below the level of apparent contralateral orofacial dyskinetic movements (approximately 30–50 μA) may be optimal in terms of restricting current spread.
Figure 4. What exactly is stimulated by STN DBS? A. Schematic of area of current spread in rat STN DBS experiments. Short-term dual stimulation-recording experiments were conducted using concentric bipolar stimulating electrodes (black line) identical to the electrodes we use for chronic STN stimulation. A tungsten recording electrode was then placed at varying distances from the stimulating electrode; at each site we recorded from individual cells before and after STN stimulation (AP -3.8 mm, L +2.5 mm, DV -7.6–8.0 mm, frequency 130 Hz; pulse duration 60 μs, amplitude 100 μA). Locations of the recording sites were verified histologically at the conclusion of the experiment utilizing Kluever Barrera staining. At each site a single neuron was isolated and the response of the cell to STN stimulation (frequency 130 Hz; pulse duration 60 μs, amplitude 100 μA) was recorded by constructing a peristimulus time histogram (PSTH) using 100 sweeps and bin width of 1 ms, prestimulation of 40 ms and post stimulation of 200 ms. At this current intensity the stimulus artifact was between 2 and 3 ms. During the recording, the occurrence of antidromic activation of the cell was tested by measuring the response jitter. If the PSTH showed a distinct peak with latency between 5 and 8 ms, it was concluded that the cell was activated orthodromically. It was assumed that the cell was activated by spread of current if (i) no distinct peak in PSTH was observed during the first 10 ms after the stimulus artifact, (ii) the cell was not antidromically activated, and (iii) the number of spikes in bins within the 10 ms following the onset of the stimulation current was > 3 x the number of spikes during the prestimulation period. No response was detected when the cells recorded from were located approximately 300 μM or further rostral to the STN (green lines). However, when recording electrodes were placed in the rostral SN, approximately 250 μM from the stimulating electrode, we did detect a current spread-induced response (red line). Based on these experiments we estimate that under our stimulation parameters, in anesthetized rats, current spread impacts not only the STN but also a sphere immediately surrounding the STN with an approximate radius of at least 250 μM (red sphere). Therefore, in order to avoid direct stimulation of the SN we position our stimulating electrode in the anterior portion of the STN (Bregma – 3.5), a full 900 μM rostral to the rostral border of the SN. Sagittal section of the rat brain reprinted with permission from Paxinos & Watson (2005). B. Schematic of the human STN and surrounding structures. The STN is a small, densely populated structure located between the zona incerta and the cerebral peduncle, and is primarily comprised of glutamatergic projection neurons. Its main targets are the substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPI); however it also has projections to the SN pars compacta (SNC), external GP (GPe), striatum and cortex (red arrows). The STN receives glutamatergic input from the cortex and GABAergic input from the GPe (blue dashed arrows). Immediately dorsal and ventral to the STN are the lenticular fasciculus and ansa lenticularis (green arrows), which are comprised of GABAergic projections from the GPe to the thalamus. Also dorsal to the STN is the zona incerta, which contains both glutamatergic and gamma-aminobutyric acid (GABA)ergic projections to areas of the cortex, diencephalon, basal ganglia, brainstem, and spinal cord. During STN DBS, STN efferents, afferent inputs to the STN and fibers of passage all have the potential to be stimulated as a result of current spread. Further, STN DBS can also increase the activity of target structures of the STN efferents.
**Chronic vs. Acute Stimulation**

An additional consideration to keep in mind when establishing a clinically relevant model of rat STN DBS is an appreciation for the duration of stimulation. The overwhelming majority of studies published to date examine the acute effects of STN DBS lasting from minutes to hours (Benazzouz *et al.*, 2000; Windels *et al.*, 2000, 2003, 2005; Salin *et al.*, 2002; Bruet *et al.*, 2003; Boulet *et al.*, 2006; Schulte *et al.*, 2006; Shi *et al.*, 2006). However, patients that receive STN DBS experience stimulation 24 hours a day, 7 days a week, often for many years. The brain is a remarkably plastic environment and the biology associated with acute effects of DBS may not be representative of the biology generated by chronic stimulation. Therefore, an important part of studying the impact of STN DBS in the clinical situation is examining in the rat model the long-term effects of STN DBS, which has been the subject of only a very limited number of reports (Maesawa *et al.*, 2004; Temel *et al.*, 2006; Harnack *et al.*, 2008; Spieles-Engemann *et al.*, 2010).

**Verification of Stimulator Placement and Damage to the STN**

The rat STN is a relatively small structure, which spans only 200 μM in the dorsal-ventral axis. Targeting the STN can therefore be extremely difficult. While extracellular-guided recordings can lower STN placement failure rates they cannot ensure correct placement. Correct placement must be verified post-mortem and data analyzed accordingly. Such a postmortem analysis has often yielded important findings, indicating that placement of STN stimulating electrodes outside of the STN can actually have detrimental effects on the nigrostriatal system (Chang *et al.*, 2003; Shi *et al.*, 2004;
Spieles-Engemann et al., 2010). Additionally, examination of the STN itself is critical as lesions of the STN can directly counteract the effects of parkinsonian insults (Delfs et al., 1995; Guridi et al., 1996; Piallat et al., 1996, 1999; Blandini et al., 1997; Carvalho and Nikkhah, 2001). Many previous studies have carefully determined the status of the STN following stimulator implantation to eliminate the confounding effects of STN lesions on the interpretation of results (Aziz et al., 1991; Dostrovsky and Lozano, 2002; Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Spieles-Engemann et al., 2010). Finally, implantation of the stimulator can have unknown consequences on brain neurochemistry and excitability. Rats in which stimulators are implanted but in which stimulation is never initiated serve as the best control condition to determine the specific effects of stimulation. Our laboratory and others consistently incorporate these critical quality control measures into our experimental procedures (Chang et al., 2003; Maesawa et al., 2004; Shi et al., 2004; Spieles-Engemann et al., 2010).

**STN DBS in the Parkinsonian Rat**

*Neurotoxin Models of PD*

Established neurotoxin models of PD (6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]) offer the distinct advantage of allowing investigators to study the effects of STN DBS in a severely DA-depleted environment (Langston et al., 1984; Sauer and Oertel, 1994; Lee et al., 1996; Meredith et al., 2008). The nigrostriatal degeneration induced by the toxins produces measurable motor impairments and triggers a succession of events within the basal ganglia that parallels many of those characteristic of PD. Following dopamine depletion in PD, STN activity becomes
extremely disorganized (Bergman et al., 1994; Hassani et al., 1996; Hutchison et al., 1998; Wichmann et al., 1999; Magnin et al., 2000). Additionally, significant alterations occur within the GPi and the SNr, which become hyperactive due to enhanced glutamatergic inputs from the STN. Similarly, experiments utilizing the 6-OHDA lesion model of PD in rodents reveal that after DA depletion the firing properties of the STN are altered, specifically leading to an increase in both the rate and the percentage of STN neuron burst firing (Hassani et al., 1996; Ni et al., 2001; Breit et al., 2005; Gajendiran et al., 2005). Additionally, 6-OHDA and MPTP lesions of the nigrostriatal tract display increases in markers of neuronal activity (Crossman et al., 1985; Schwartzman and Alexander, 1985; Blandini et al., 2007) and a downregulation of glutamate receptors in the SNr and GPi (Porter et al., 1994; Wullner et al., 1994; Kaneda et al., 2005). These findings are consistent with the findings in humans that DA depletion leads to hyperactivity in SNr and GPi and a compensatory downregulation of receptors in those structures due to enhanced glutamatergic input from the STN (Difazio et al., 1992; Vila et al., 1997; Rodriguez et al., 1998).

Another feature of the neurotoxin models is the ability to perform precise analysis of motor performance, which can detect impairments due to DA depletion and improvements in response to STN DBS. In this regard the 6-OHDA rat model appears to hold a slight advantage over the MPTP-lesioned mouse as the lesion and detection of motor deficits is more stable and reliable in 6-OHDA-treated rats as compared to MPTP-treated mice (Meredith and Kang, 2006). Specifically, MPTP treatment in mice may have no effect on motor performance, or it may cause a transient deficit followed by full
recovery, or it can even produce hyperactivity (Colotla et al., 1990; Sedelis et al., 2000, 2001; Meredith and Kang, 2006) whereas 6-OHDA treatment produces a reliable lesion and motor deficits (Meredith and Kang, 2006; Spieles-Engemann et al., 2010).

Previously in our laboratory we characterized an intrastriatal 6-OHDA lesion protocol in rats that results in relatively protracted nigral DA neuron degeneration that is first expressed as a loss of DA phenotype and ultimately leads to overt cell loss of nigral DA neurons. In contrast, the loss of striatal DA innervation and the behavioral manifestations of this loss occur over a much more condensed time frame, specifically within 2 weeks after 6-OHDA lesioning (Spieles-Engemann et al., 2010) (Figure 5).

Figure 5. Summary of the time course, magnitude, and behavioral impact of nigrostriatal degeneration following intrastriatal 6-OHDA. Schematic illustrating the time course of degeneration of nigral THir neurons (red squares), nigral NeuNir neurons (red triangles), striatal THir neurites (white circles) and impairments in contralateral forepaw use (black circles) following intrastriatal 6-OHDA. In contrast to the progressive loss of nigral neurons that we observe over the course of 4 weeks after 6-OHDA, the loss of striatal THir and the behavioral manifestations of this loss occurs over a much more condensed time frame and is essentially complete within 2 weeks post 6-OHDA.
The time course of degeneration produced by our lesion parameters in the rat allows for STN DBS to be initiated at specific intervals that can model either early or late PD. For example, the changes in motor function symptomatic of PD are not usually apparent until DA levels in the striatum have dropped to < 20% of normal accompanied by a 60% loss of nigral DA neurons (Bernheimer et al., 1973). Further, more recent evidence indicates that loss of terminals in the putamen of early PD patients precedes nigral neuron loss (Dodiya et al., 2009). This early-stage pattern of nigrostriatal degeneration is most accurately modeled at the 2-week post-lesion time point in our rat paradigm, with later-stage PD more accurately modeled at 4 or 6 weeks following 6-OHDA. Initiation of STN DBS at various points after 6-OHDA may reveal differences in the impact of STN DBS during early vs. late stages of PD. Up to recently, the typical PD patient receiving STN DBS is in late-stage PD, having a mean disease duration of 14 years (Volkmann, 2004). However, an ongoing clinical trial seeks to determine the safety and tolerability of STN DBS in early PD (Charles, 2010). Our intrastriatal lesion protocol allows us to evaluate the effects of STN DBS within a partially DA-depleted or a near-complete DA-depleted environment. In conclusion, although the causes of PD are unknown and the neurotoxin models of PD (6-OHDA, MPTP) are unlikely to completely model PD etiology, these models recapitulate the major relevant characteristics of the disease within the structures of the basal ganglia and provide an appropriate background on which to test the impact of STN DBS.

**Functionally Relevant STN DBS in the Rat Model**

The nigrostriatal DA denervation in the rat that results from intrastriatal 6-OHDA yields
numerous motor impairments that can be objectively quantified. As stated previously, one of the considerations of the rat model of STN DBS is the fact that the structures and pathways within the area of current spread may not accurately model the clinical situation. However, current spread can be minimized by reducing stimulus amplitude and observing stimulus-induced behaviors in awake rats. Further, as stimulation parameters in PD patients are programmed to optimize symptomatic relief a case can be made for similarly “programming” rat stimulation parameters at current intensities that alleviate 6-OHDA-induced motor impairments. In this regard the ability to demonstrate functional efficacy of stimulation in the rat model becomes essential and, ideally, examinations of the impact of STN DBS on neurochemical and morphological outcome measures should utilize functionally effective stimulation parameters.

Previous work has demonstrated that short-term STN DBS can positively impact deficits in motor performance induced by 6-OHDA by its ability to reverse or attenuate reaction time deficits and amphetamine-induced contralateral rotations, and improve treadmill locomotion, walking speed, forelimb akinesia and rearing activity (Meissner et al., 2002; Chang et al., 2003; Darbaky et al., 2003; Shi et al., 2004, 2006; Fang et al., 2006; Vlamings et al., 2007). The main focus of these studies was to determine the impact of acute STN DBS on these behavioral outcomes with the duration of stimulation lasting from minutes to hours. Only 2 studies have evaluated the ability of long-term STN DBS to alleviate 6-OHDA-induced deficits in motor performance. The first study demonstrated that 2 weeks of continuous STN DBS can ameliorate amphetamine-induced contralateral rotations (Maesawa et al., 2004). In our laboratory we recently
evaluated the impact of long-term STN DBS on 6-OHDA-induced deficits in rat rearing activity and forepaw akinesia in the cylinder task (Spieles-Engemann et al., 2009). Rats that received 2 weeks of continuous active STN stimulation exhibited significant increases in both rearing activity and contralateral forepaw use, demonstrating that long-term STN DBS is functionally effective in alleviating parkinsonian symptomology. Using identical stimulation parameters we have previously demonstrated that long-term STN DBS can provide neuroprotection in the face of previous significant nigral DA neuron loss (Spieles-Engemann et al., 2010).

**Optogenetics Provide Insight into Circuitry Involved in Functionally Relevant STN DBS**

In a recent study by Gradinaru and colleagues (Gradinaru et al., 2009) the power of optogenetics was harnessed to specifically uncover which distinct component of STN circuitry may be involved in alleviating 6-OHDA-induced motor deficits in rats and mice. Unlike electrical DBS, in which the volume of tissue activated can include local STN circuitry, afferents, efferents and neighboring fibers of passage (See Figure 4B), the elegant technique of optogenetic stimulation can be tailored to drive or inhibit specific cell populations or circuits. Using this approach it was revealed that antidromic activation of primary motor cortex (M1)-STN afferents was sufficient to impact 6-OHDA-induced deficits whereas inhibition of local STN neurons or astroglia was not. In this study motor performance was assessed by measuring rotational asymmetry in response to amphetamine, correction of head position bias and distance and speed of locomotion. Further, only high-frequency stimulation (> 100 Hz), not stimulation at lower frequencies
(20 Hz), elicited the therapeutic effects. These optogenetic results functionally confirm the findings from previous studies in which antidromic M1 activation was revealed to be a consequence of STN DBS in anesthetized rats (Li et al., 2007). Collectively these results highlight the potential importance of activation of M1-STN circuitry in the therapeutic efficacy of STN DBS in rodent models and may aid in optimizing clinical stimulation parameters in the patient population.

**Predictive Ability of the 6-OHDA Rat Model of STN DBS**

Beyond the advantages and limitations of the rat model of STN DBS that have been raised, a final advantage of the 6-OHDA rodent model of STN DBS for preclinical research is that the cost of utilizing rodents compared to non-human primates allows for greater flexibility in experimental design, including treatment and control groups and types of *in vivo* and postmortem analyses. Clearly the ultimate utility of the rat model of STN DBS can be determined by evaluating its predictive ability for results in non-human primate studies and clinical trials. Although fewer studies have been conducted in this regard, evidence exists which demonstrates that results in monkey and human studies are consistent with previous findings in rat studies. Specifically, studies in rats, primates and humans have all shown that STN DBS inhibits neural activity in the STN itself (Hashimoto *et al.*, 2003; Tai *et al.*, 2003; Meissner *et al.*, 2005; Shi *et al.*, 2006) while exciting STN output structures (Windels *et al.*, 2000, 2003; Hashimoto *et al.*, 2003; Stefani *et al.*, 2005, 2006). Additionally, examinations of the impact of STN DBS on levels of striatal DA suggest that the level of nigrostriatal degeneration will predict whether STN DBS will increase (Bruet *et al.*, 2001; Zhao *et al.*, 2009) or not affect
(Meissner et al., 2001, 2002; Hilker et al., 2003; Strafella et al., 2003) striatal DA. Lastly, STN DBS has been shown to be neuroprotective for the DA neurons of the SN in both rats and monkeys (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Spieles-Engemann et al., 2010). These findings illustrating the predictive validity of STN DBS are detailed in Table 2. In conclusion, while the 6-OHDA rat model of STN DBS is not without limitations, it is an important experimental platform in which findings can aid

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<td>STN DBS inhibits the STN</td>
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<td>STN DBS increases striatal DA (partial lesions)</td>
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Table 2. Predictive Validity of the Rat Model of STN DBS

in understanding the mechanism of STN DBS. This understanding can inform the experimental design of necessary follow-up studies in non-human primates and PD patients and ultimately provide insights into the mechanisms of STN DBS.

Conclusions

Numerous in vitro and in vivo studies have been conducted to determine the effect of stimulation on the rat STN and its circuitry (for review see Chang et al., 2008; Gubellini et al., 2009; McIntyre et al., 2004a). To date there have been more than 45 manuscripts
published that utilize the *in vivo* rat model of STN DBS. These published reports vary in experimental design features including stimulus intensity, frequency and duration as well as in quality control measures including verification of stimulator placement within the STN and lack of STN damage. The features and outcome measures utilized in any particular study provide criteria with which to evaluate how findings in rodent studies can be extrapolated to the clinical situation. Most notably, only five studies have evaluated the impact of continuous STN stimulation for a period of > 4 days (Bacci *et al.*, 2004; Maesawa *et al.*, 2004; Oueslati *et al.*, 2007; Harnack *et al.*, 2008; Spieles-Engemann *et al.*, 2010) with the majority of these examining the issue of whether STN DBS can provide neuroprotection from 6-OHDA (Maesawa *et al.*, 2004; Harnack *et al.*, 2008; Spieles-Engemann *et al.*, 2010). Further research that examines the effect of long-term STN DBS on the neurophysiology and neurochemistry of STN circuitry is warranted. The rat model of functionally relevant long-term STN DBS provides an optimal preclinical experimental platform in which to conduct these studies.
Chapter 3: Stimulation of the Rat Subthalamic Nucleus Is Neuroprotective Following Significant Nigral Dopamine Neuron Loss

Abstract

Deep brain stimulation of the subthalamic nucleus (STN DBS) is efficacious in treating the motor symptoms of Parkinson’s disease (PD). However, the impact of STN DBS on the progression of PD is unknown. Previous preclinical studies have demonstrated that STN DBS can attenuate the degeneration of a relatively intact nigrostriatal system from dopamine (DA)-depleting neurotoxins. The present study examined whether STN DBS can provide neuroprotection in the face of prior significant nigral DA neuron loss similar to PD patients at the time of diagnosis. STN DBS between 2 and 4 weeks after intrastriatal 6-hydroxydopamine (6-OHDA) provided significant sparing of DA neurons in the SN of rats. This effect was not due to inadvertent lesioning of the STN and was dependent upon proper electrode placement. Since STN DBS appears to have significant neuroprotective properties, initiation of STN DBS earlier in the course of PD may provide added neuroprotective benefits in addition to its ability to provide symptomatic relief.

Key Words: Deep brain stimulation, Subthalamic nucleus, Parkinson’s disease, Neuroprotection, 6-hydroxydopamine, Stereology
Introduction

Deep brain stimulation of the subthalamic nucleus (STN DBS) is now the most frequently practiced surgical therapy for the treatment of Parkinson’s disease (PD). The efficacy of STN DBS in the relief of the cardinal motor symptoms of PD is well documented with recent reports indicating that symptomatic relief is both long-lasting and comparable to the best medical therapy (Krack et al., 2003; Schupbach et al., 2005; Weaver et al., 2009). However, the clinical use of this treatment has proceeded without systematic study of the impact of STN DBS on the progression of the disease itself. Currently, PD patients are not considered candidates for STN DBS unless symptoms have been present for a minimum of 5 years (Chang and Chou, 2006; Neimat et al., 2006). The average PD patient has a mean disease duration of 14 years before STN DBS is performed (Volkmann, 2004) during which time it is likely that the disease has progressed significantly (Goetz et al., 2000). Therefore, by the time patients initiate DBS therapy they are well into the later stages of PD, which makes it difficult to determine whether STN DBS can preserve remaining DA neurons. Positive indications of STN DBS’ neuroprotective efficacy in parkinsonian animal models would suggest that expansion of this treatment to PD patients in early and moderate stages of the disease should be seriously considered.

Preclinical studies in both rats and monkeys have demonstrated that STN DBS can prevent the degeneration of nigral dopamine (DA) neurons from the insult produced by DA-depleting neurotoxins (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008). While these studies are a promising preliminary indication
of STN DBS’ neuroprotective potential, the overwhelming majority of nigral DA neurons were present when STN stimulation was initiated either immediately prior to or soon after toxin administration. While this experimental paradigm provides the best opportunity to observe neuroprotection, it is confounded by the possibility that the reduction in nigral DA neuron degeneration associated with STN DBS is due to prevention of toxin uptake/metabolism and does not accurately model the DA neuron loss that already exists in PD patients who undergo the procedure.

In the present study, we utilized unbiased stereology and analysis of striatal DA to characterize the time course and magnitude of nigral DA neuron and dopaminergic terminal loss following unilateral intrastriatal 6-OHDA injection to rats. Our goal was to precisely define the rate of progression of the relatively protracted nigrostriatal degeneration induced by intrastriatal 6-OHDA and then utilize these same lesion parameters to establish partial nigrostriatal degeneration prior to initiating long-term high-frequency STN DBS. The impact of STN stimulation on SN DA neuron number was evaluated. Additionally, we examined effects on the degree of striatal dopaminergic innervation and levels of striatal DA and DA metabolites, parameters that prior studies did not examine. Our results demonstrate that STN DBS initiated after significant nigral DA neuron loss can prevent further DA neuron degeneration, but did not preserve striatal DA innervation and levels of striatal DA, effects attributed to limitations of the 6-OHDA model employed here.
Materials and Methods

Animals

Male, Sprague Dawley rats (Harlan, 200-250 g) were used in these studies. For Experiment 1: Time Course of Nigrostriatal Degeneration Following Intrastriatal 6-OHDA, a total of 24 rats were divided amongst 4 groups (2 weeks post 6-OHDA, 4 weeks post 6-OHDA, 6 weeks post 6-OHDA and 6 weeks post intrastriatal vehicle injection). In the final analysis there were a total of 5 rats in the 2-week group, 4 rats in the 4-week group, 6 rats in the 6-week group and 6 rats in the vehicle injected group. For Experiment 2: Impact of STN DBS after 6-OHDA Lesion, a total of 30 rats were divided into either the ACTIVE stimulator group ($n = 19$) or the INACTIVE stimulator control group ($n = 11$). Animals were only included in the final analyses if they completed the full two-week stimulation interval. All animals were given food and water ad libitum, and housed in reversed light-dark cycle conditions in the University of Cincinnati Vontz Center vivarium, which is fully AAALAC approved.

Experimental Overview

Time Course of Nigrostriatal Degeneration Following Intrastriatal 6-OHDA. Rats were either unilaterally lesioned via intrastriatal injections of 6-OHDA or injected with an equal volume of vehicle into the striatum. Rats were culled into three separate groups for sacrifice at either 2 weeks, 4 weeks, or 6 weeks after surgery. Forelimb akinesia was assessed via the cylinder task (details on the procedure are presented in the following text) prior to surgery and at each of these time points in the 6-week post surgery 6-OHDA and vehicle groups. Amphetamine-induced rotational asymmetry was quantified
for the 6-OHDA rats at these same time points. After sacrifice stereological cell counts of the tyrosine hydroxylase immunoreactive (THir) and the neuronal nuclei immunoreactive (NeuN) neurons were performed in both the ipsilateral lesioned and contralateral unlesioned substantia nigra of the 6-OHDA injected rats. Stereological counts of THir neurites within the striatum also were made in the ipsilateral lesioned striatum and contralateral unlesioned striatum.

Stimulation of the STN after Intrastriatal 6-OHDA. Prior to 6-OHDA injection and stimulating electrode implantation surgery, all rats were assessed for forelimb akinesia utilizing the cylinder task. The following day all rats were unilaterally lesioned via injections of 6-OHDA into the striatum and implanted unilaterally (ipsilateral to 6-OHDA) with stimulating electrodes into the STN during the same surgical session. All rats were allowed to recover from surgery for exactly 2 weeks. At 2 weeks all rats were reassessed for the degree of contralateral forelimb akinesia via the cylinder task. Rats that displayed contralateral forelimb akinesia were divided into 2 separate groups: ACTIVE (n = 19) or INACTIVE (n = 11) stimulation. Rats in the ACTIVE group had their stimulators connected to an external stimulation source and received STN stimulation continuously for 2 weeks. Rats in the INACTIVE group received no stimulation during the 2-week interval. At the end of the 2 weeks all rats were sacrificed and their brains processed for stereological cell counts of THir neurons in the SN, THir neurites in the striatum or levels of DA and DA metabolites in the striatum. Placement of the stimulating electrode was analyzed histologically utilizing Kluver-Barrera staining. This experimental design is illustrated in Figure 6.
Figure 6. Experimental design for STN DBS after intrastriatal 6-OHDA. On Day 0 forelimb akinesia was assessed in all rats via the cylinder task. On Day 1 all rats were unilaterally lesioned via injections of 6-OHDA into the striatum and implanted unilaterally (ipsilateral to 6-OHDA) with stimulating electrodes into the STN during the same surgical session. During Days 2–13 all rats were allowed to recover from surgery. On Day 14 all rats were reassessed for degree of contralateral forelimb akinesia via the cylinder task. Rats that displayed a 20% reduction in contralateral forelimb use were divided into two separate groups: ACTIVE (n = 19) or INACTIVE (n = 11) stimulation. Rats in the ACTIVE stimulation group had their stimulators connected to an external stimulation source and received STN stimulation during Days 15–27 24 h a day. Rats in the INACTIVE group received no stimulation during Days 15–27. On Day 28 all rats were sacrificed and their brains processed for stereological cell counts of THir neurons in the SN, THir neurites in the striatum or levels of DA and DA metabolites in the striatum and frontal cortex. Appropriate placement of the stimulating electrode was confirmed histologically utilizing Kluver–Barrera staining.

Intrastriatal 6-OHDA Injections. Rats were anesthetized prior to surgery with Equithesin (0.3 ml/100 g body weight i.p.; chloral hydrate 42.5 mg/ml + sodium pentobarbital 9.72 mg/ml) and injected in two sites in the striatum with either 6-OHDA (MP Biomedicals, Solon, OH; 5 μg/μl 6-OHDA in 0.02% ascorbic acid, 0.9% saline solution) or vehicle. The coordinates for these injections were AP +1.6 mm, ML +2.4 mm, DV -4.2 mm and AP -0.2 mm, ML + 2.6 mm, DV -7.0 mm. A total of 2 μl 6-OHDA (total dose of 6-OHDA = 20 μg over two sites) or vehicle was injected at 0.5 μl/min. at each site.
**Behavioral Testing.** Non-drugged, spontaneous use of the forepaws was measured in rats as described by Schallert (2006). A 20% reduction in contralateral forelimb use was utilized to confirm 6-OHDA lesion prior to enrollment in either the ACTIVE or INACTIVE groups. Amphetamine-induced rotational asymmetry was assessed as described previously (Terpstra et al., 2007). Forelimb akinesia and rotations were not assessed in rats connected to stimulator cables as our external hardware connections interfered with rat mobility and paw placement and are incompatible with the rapid rotations induced by amphetamine.

**Extracellular microelectrode recordings.** Extracellular microelectrode recording was used to provide STN targeting guidance as described previously (Maesawa et al., 2004). Potentials of the electrode were amplified 10,000x, conducted through a band pass filter, and monitored with the aid of an oscilloscope display, audio monitor and an online computer. Bregma was carefully delineated and marked. Initial coordinates for electrode implantation were at AP -3.6 mm and L +2.5 mm according to the atlas of Paxinos and Watson (2005). The electrode was then lowered to 5.0 mm ventral (using a micromanipulator) where the approximate dorsal border of the ventral posterior medial nucleus of the thalamus (VPM) is located. Upon entering the vicinity of the VPM and proceeding ventrally, sensory responses to stimulation of the contralateral vibrissae were confirmed (Friedberg et al., 2004; Maesawa et al., 2004). As the electrode was advanced ventrally a relatively silent area representing the zona incerta (ZI) was encountered which spans approximately 0.5–1.0 mm. Immediately ventral to the ZI, the
STN was readily distinguishable by a sudden increase of irregular spikes firing at a high rate. Located immediately ventral to the STN is the cerebral peduncle, which also is a strikingly silent structure. The STN spans approximately 100–200 μm along its dorsal ventral (DV) axis (Paxinos and Watson, 2005). In sessions where the zone of sudden irregular spikes was found to be wider in DV span (> 300 μm) the electrode position was assumed to be within the SN and therefore too posterior. If this happened, the electrode was withdrawn and repositioned 200 μm in the rostral direction and recording was resumed. The precise coordinates of the dorsal border of the STN were then carefully recorded to guide subsequent electrode placement. This procedure is illustrated in Figure 7.

Figure 7. Extracellular recording-guided electrode placement in STN and long-term STN DBS platform. A. Response of a neuron within the ventral posterior medial nucleus of the thalamus (VPM) to stimulation of the contralateral vibrissa, stimulation period indicated by horizontal lines beneath x-axis. This VPM landmark is used to determine appropriate AP and ML electrode placement; the STN is located 0.5–1.0 mm ventral to this site and is readily distinguishable by a sudden increase of irregular spikes firing at a high rate. B. **Dye infusion within STN recording site** (arrow). After the coordinates of the STN were identified a bipolar concentric microelectrode was implanted with the electrode fixed in place using dental acrylic and bone screws. Scale bar = 1000 μM. C. **Schematic of an individual stimulation setup**, including an Accupulser Signal Generator (World Precision Instruments, WPI) connected to a Constant Current Bipolar Stimulus Isolator (WPI) that is connected to the concentric bipolar stimulating electrode implanted into the rat STN and secured using dental cement and bone screws. The rat is housed in a Ratum System Bowl (BioAnalytical Systems, Inc., BASI). The stimulator cable is routed through a commutator to allow the rat to move freely during the two-week stimulation interval. D. **Photograph of an individual rat undergoing STN DBS during the two-week stimulation interval.**
Estimation of Current Spread. In order to ensure we were not stimulating the SN during STN stimulation, we estimated the distance of current spread associated with STN stimulation by utilizing simultaneous STN stimulation and extracellular recordings. Concentric bipolar stimulating electrodes identical to the electrodes we ultimately used for chronic STN stimulation (inner electrode projection 1 mm, inner insulated electrode diameter 0.15 mm, outer electrode gauge 26, Plastics One, Roanoke, VA) were placed within the STN (AP -3.8 mm, L +2.5 mm, approximately DV -7.6 from dura) using the extracellular guided recording techniques described previously. A tungsten-recording electrode was then used to record from varying sites between STN and SN (Site 1: AP -6.12, ML +2.0, DV -4.5 from dura; Site 2: AP -5.88, ML +2.4, DV -8.0 from dura; Site 3: AP -5.2, ML +2.4, DV -8.0 from dura; Site 4: AP -4.00, ML +2.5, DV -8.0 from dura). Locations of the recording sites were verified histologically at the conclusion of the experiment utilizing Kluver-Barrera staining. At each site a single neuron was isolated and the response of the cell to STN stimulation (frequency 130 Hz; pulse duration 60 μs, amplitude 100 μA) was recorded by constructing a peristimulus time histogram (PSTH) using 100 sweeps and bin width of 1 ms, prestimulation of 40 ms and post stimulation of 200 ms. At this current intensity the stimulus artifact was between 2 and 3 ms. During the recording, the occurrence of antidromic activation of the cell was tested by measuring the response jitter. If the PSTH showed a distinct peak with latency between 5 ms and 8 ms, it was concluded that the cell was activated orthodromically. It was assumed that the cell was activated by spread of current if 1) no distinct peak in PSTH was observed during the first 10 ms after the stimulus artifact, 2) the cell was not antidromically activated, and 3) the number of spikes in bins within the 10 ms following
the onset of the stimulation current was more than 3 times the number of spikes during
the prestimulation period. Using this technique current spread was not detected when
the cell recorded from was located in the caudal mesencephalon, either outside the SN
(Site 1) or within the SN (Sites 2 and 3); all of these sites were at minimum 1.4 mm
caudal to the stimulating electrode. In contrast, current spread was detected when the
recording electrode was placed in the rostral SN (Site 4), approximately 0.25 mm caudal
to the stimulating electrode. Based on these experiments we estimate that under our
experimental stimulation parameters current spread impacts not only the STN but also a
sphere immediately surrounding the STN with an approximate radius of at least 250 μM.
In order to minimize the possibility that our STN stimulation generated current that
spread to any portion of the SN all stimulating electrodes were placed in the anterior
portion of the STN in subsequent experiments (Bregma – 3.6 mm), a full 900 μM rostral
to the rostral border of the SN.

Electrode Implantation. Stimulating electrode implantation took place immediately
following STN recording and intrastriatal 6-OHDA injection. A bipolar concentric
microelectrode (as described in previous section) was lowered to the dorsal border of
the STN with coordinates for each rat predetermined by extracellular guided recordings.
The electrode was then fixed in place using dental acrylic and bone screws. The dorsal
border of the STN was chosen as the implantation location in order to minimize any
damage resulting from electrode implantation. At the conclusion of all experiments
stimulating electrode placement was verified morphologically utilizing Kluver Barrerra
staining.
**Long-Term Stimulation.** Rats assigned to the ACTIVE group were individually housed in Plexiglas Raturn bowls (BASi, Inc., West Lafayette, IN) and connected to a commutator (Plastics One, Roanoke, VA) via a stimulating cable connected to the external plug of the STN microelectrode. The commutators were connected to an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments). This is illustrated in Figure 7. Rats were stimulated at frequency of 130 Hz, 60 μs pulse width and an intensity of 30–50 μA, which was below the threshold of contralateral forepaw and orofacial dyskinesia, thereby preventing problems with feeding or locomotion during the stimulus interval. In subsequent studies we have confirmed that these identical STN stimulation parameters can provide functional improvements in contralateral forelimb akinesia (Spieles-Engemann et al., 2009). Stimulation commenced 2 weeks following 6-OHDA/electrode implantation surgery and was active 24 h/day for a two week period. INACTIVE animals were housed individually in standard shoebox cages for the identical two-week interval. Our long-term STN DBS stimulation platform is depicted in Figure 7.

**Sacrifice.** At the conclusion of the two-week stimulation interval rats for morphological analysis were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with saline. Brains were removed, post fixed for 24 h in 4% paraformaldehyde and transferred to 30% sucrose in 0.1M PO₄ buffer. Rats utilized for HPLC analysis of striatal and frontal cortex DA and metabolites were perfused intracardially with heparinized saline at 37 °C followed by ice-cold saline in order to remove endogenous circulating catecholamines from blood vessels. The brains were
immediately removed and flash frozen in 3-methyl butane. Brains were stored at -80 °C until analysis.

Tyrosine Hydroxylase and Neuronal Nuclei Immunohistochemistry for SN Neurons and Striatal Neurites. Paraformaldehyde perfused brains were frozen on dry ice and sectioned at a 35 μm thickness using a sliding microtome. Every sixth section was processed for labeling with antisera against TH or NeuN using the free-floating method. Following blocking in serum, tissue was incubated in primary antisera directed against TH (SN: Chemicon MAB318, mouse anti-TH 1:4000) or NeuN (Chemicon, mouse anti NeuN Clone A60 1:1000), overnight at room temperature. Triton-X (0.3 %) was added to the 0.1M Tris buffer during incubations and rinsed to permeabilize cell membranes. Following primary incubation, sections were incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). Antibody labeling was visualized by exposure to 0.5 mg/ml 3,3’ diaminobenzidine (DAB) and 0.03% H₂O₂ in Tris buffer. Sections were mounted on subbed slides, dehydrated to xylene and coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Kluver-Barrera Histology. Kluver-Barrera staining was used to visualize electrode placement and quantification of the STN cell number. Staining was performed in every sixth section throughout the STN according to previously described methods (Kluver and Barrera, 1953). Stimulating electrode placement was considered to be appropriately
targeted to the STN if the tip of the stimulating electrode was observed to be within or at the border of the STN within any of the sections examined.

Stereology. Stereology was performed using a BX52 Olympus microscope (Olympus America Inc.) equipped with Stereo Investigator stereological software (Microbrightfield Bioscience, Williston, VT) and a Microfire CCD camera (Optronics, Goleta, CA) as described previously (Kordower et al., 2006). Using the optical fractionator principle, the SN or the STN was individually outlined on each section under a low magnification (1.25X) and cell counts of THir (SN) or Kluver-Barrera stained (STN) neurons were made according to stereological principles while focusing down through the z-axis at 60x. In order to assure that only the SN was included in the NeuNir analysis, the Virtual Slices Module for Stereo Investigator (Microbrightfield Bioscience) was used to superimpose the midbrain sections immunolabeled for TH over adjacent midbrain sections immunostained for NeuN to delineate the region of interest. Analysis of THir neurite density was performed utilizing the Space Balls probe in Stereo Investigator. This hemispheric probe was used to obtain an unbiased estimate of THir neurite length in the striatum using previously reported methods (Koprich et al., 2003a,b). Every sixth coronal section through the entire rostral-caudal span of the striatum was analyzed. This lateral area of the striatum was chosen for analysis based on its involvement in paw reaching and other sensorimotor tasks (Kelley et al., 1988; Pisa, 1988; Brown et al., 2002). CEs for all analyses were < 0.10.
Dissection of Striatum and Frontal Cortex for HPLC Analysis. Frozen brains were equilibrated at a temperature of -18 °C prior to dissection. 1–2 mm coronal slabs were blocked from each brain utilizing a brain blocker (Zivic, Pittsburgh, PA) and striatal and frontal cortex tissues from both hemispheres were microdissected while being held at a constant -12 °C on a cold plate (Teca, Chicago, IL). Frozen dissected structures were placed individually in vials and stored at – 80 °C until analysis.

High Protein Liquid Chromatography (HPLC). Homogenized samples were analyzed as described previously (Koprich et al., 2003a,b). Samples were separated on a Microsorb MV C-18 column (5 Am, 4.6_250 mm, Varian, Palo Alto, CA) and simultaneously examined for DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Compounds were detected using a 12-channel coulometric array detector (CoulArray 5200, ESA, Chelmsford, MA) attached to a Waters 2695 Solvent Delivery System (Waters, Milford, MA) under the following conditions: flow rate of 1 ml/min; detection potentials of 50, 175, 350, 400 and 525 mV; and scrubbing potential of 650 mV. The mobile phase consisted of a 10% methanol solution in distilled H₂O containing 21 g/L (0.1 M) citric acid, 10.65 g/L (0.075 M) Na₂HPO₄, 176 mg/L (0.8 M) heptanesulfonic acid and 36 mg/L (0.097 mM) EDTA at a pH of 4.1. Data are expressed as ng/mg protein.

Statistical Analysis. For the experiment Time Course of Nigrostriatal Degeneration, a one-way analysis of variance (ANOVA) was performed to look at between group differences in THir and NeuNir neuron numbers, and THir terminal density. Significant
differences in main effects were determined by Fisher LSD post-hoc analysis. To determine significant differences between the lesioned and non-lesioned side within each group, a one-way repeated measures ANOVA (RM-ANOVA) was performed, followed by a Holm-Sidak (THir neurons and terminal density) or Dunn’s (NeuN) post-hoc test. A one-way RM-ANOVA followed by a Holm-Sidak post-hoc test was also used to look at group differences in behavioral tasks.

For the experiment *Effects of Long-Term STN Stimulation on Nigrostriatal Neurons and Biochemistry*, two-way RM-ANOVAS followed by a Tukey’s post-hoc test were used to determine significant differences in THir neuron number and terminal density as well as DA and DA metabolite levels in the striatum and cortex. A one-way ANOVA followed by a Fisher LSD post-hoc test was used to determine differences between groups with good stimulator placement and those with poor stimulator placement. SigmaStat 3.0 software (Systat Software, San Jose, CA) was used for all statistical analyses and the level of statistical significance was set at 0.05.

**Results**

*Time Course of Nigrostriatal Degeneration After Intrastriatal 6-OHDA*

*Reductions in THir Neurons in the SN.* At the two-week, four-week and six-week post 6-OHDA intervals there were significantly fewer THir neurons in the SN ipsilateral to intrastriatal 6-OHDA injection as compared to the contralateral SN (2 week: \( F_{(1, 5)} = 34.641, p = 0.004 \); 4 week: \( F_{(1, 4)} = 42.486, p = 0.007 \); 6 week: \( F_{(1, 6)} = 329.345, p \leq 0.001 \)). No significant differences were observed between the number of THir neurons
in the SN contralateral to 6-OHDA injection at any of the time points examined (p ≥ 0.05). Significant loss of THir neurons in the ipsilateral SN occurred between 2 and 4 weeks and 2 and 6 weeks post intrastriatal 6-OHDA ($F_{(2, 15)} = 13.292$, $p ≤ 0.001$). There were no significant differences between the number of THir neurons in the SN ipsilateral to 6-OHDA between 4 and 6 weeks after lesion (p ≥ 0.05). Therefore, our intrastriatal 6-OHDA lesion parameters caused a 46 ± 6% decrease in THir neurons in the SN at 2 weeks, progressing significantly further to a 75 ± 4% reduction at 4 weeks, and a 76 ± 3% decrease at 6 weeks. These results are depicted in Figures 8A-D, I.

Loss of NeuNir Neurons in the SN. In order to distinguish between loss of TH phenotype and actual nigral neuronal death, we quantified NeuNir neurons within the SN of these same rats. There were significantly fewer NeuN positive cells in the SN ipsilateral to intrastriatal 6-OHDA injection as compared to the contralateral SN at all time points examined (2 week: $F_{(1, 4)} = 22.319$, $p = 0.018$; 4 week: $F_{(1, 3)} = 20.697$, $p = 0.045$; 6 week: $F_{(1,6)} = 105.906$, $p ≤ 0.001$). No significant differences were observed between the numbers of NeuNir neurons in the SN contralateral to 6-OHDA injection at any of the time points examined (p ≥ 0.05). Significant loss of NeuNir neurons in the ipsilateral SN occurred between 2 and 4 weeks and 2 and 6 weeks post intrastriatal 6-OHDA ($F_{(2, 13)} = 11.579$, $p = 0.002$). There were no significant differences between the number of NeuNir neurons in the SN ipsilateral to 6-OHDA between 4 and 6 weeks after lesion (p ≥ 0.05). Therefore, our intrastriatal 6-OHDA lesion parameters caused a 13 ± 1.9% decrease in NeuNir neurons in the SN at 2 weeks, progressing significantly further to a 31 ± 9.1% reduction at 4 weeks, with no further significant neuronal loss observed at 6 weeks (37
Figure 8. Time course, magnitude and behavioral impact of nigrostriatal degeneration following intrastriatal 6-OHDA. Micrographs of THir neurons in the SN and THir terminals in the striatum at 2 (A), 4 (B) and 6 (C) weeks after unilateral intrastriatal 6-OHDA (left). Progressive degeneration of nigral THir neurons is illustrated by comparing the number of THir neurons evident in the left hemisphere with the normal complement of THir nigral neurons in the contralateral mesencephalon. Note the almost complete absence of THir neurites at the level of the striatum starting at 2 weeks (A) after 6-OHDA. Scale Bar = 1000 µM. D. Stereological counts of THir neurons in the SN reveal significantly fewer THir neurons in the lesioned SN (grey bars) relative to the intact SN (black bars) at all time points examined (*p < 0.001). In addition, significantly fewer THir neurons were present in the SN ipsilateral to 6-OHDA at 4 weeks compared to the ipsilateral SN at 2 weeks (p < 0.001). There were no significant differences between the number of THir neurons in the SN ipsilateral to 6-OHDA between 4 and 6 weeks after lesion (p ≥ 0.05). E. Stereological counts of NeuNir neurons in the SN reveal significantly fewer NeuNir neurons in the lesioned SN relative to the intact SN at all time points examined (*p < 0.001). Further, significantly fewer NeuNir neurons are present in the SN ipsilateral to 6-OHDA at 4 weeks compared to the ipsilateral SN at 2 weeks (*p < 0.005). There were no significant differences between the number of NeuNir neurons in the SN ipsilateral to 6-OHDA between 4 and 6 weeks after lesion (p ≥ 0.05). F. THir neurite density in the striatum was significantly reduced at 2, 4 and 6 weeks following lesion (*p < 0.0001) with no progression observed after 2 weeks. G. Contralateral forelimb akinesia in the cylinder task at 2, 4 and 6 weeks after intrastriatal vehicle injection (black solid triangles) or 6-OHDA injection (white open circles). All rats receiving 6-OHDA exhibited a significant reduction in contralateral forepaw use at each time point compared to both pre-6-OHDA baseline contralateral forepaw use and vehicle injected controls (*p < 0.002). No significant differences in contralateral forepaw use were observed between 2, 4 and 6 weeks after 6-OHDA (p ≥ 0.05). H. Amphetamine-induced rotational asymmetry after intrastriatal 6-OHDA. All rats receiving 6-OHDA exhibited ipsilateral rotations at all post-6-OHDA time points examined. No significant differences in rotational asymmetry were observed between 2, 4 and 6 weeks after 6-OHDA (p ≥ 0.05). I. Schematic illustrating the time course of degeneration of nigral THir neurons (black squares), nigral NeuNir neurons (white triangles) and striatal THir neurites (white circles) in the ipsilateral hemisphere following intrastriatal 6-OHDA. Specifically, our lesion parameters lead to loss of 46% THir and 13% NeuNir nigral neurons in the SN at 2 weeks, progressing further to a loss of 75% THir and 31% NeuNir nigral neurons at 4 weeks. Nigral DA neuron loss is essentially completed by 4 weeks under these lesion parameters as analysis of SN neurons at 6 weeks post-6-OHDA is essentially identical to the numbers of THir and NeuNir nigral neurons at 4 weeks. In contrast to the progressive loss of nigral neurons that we observe over the course of 4 weeks after 6-OHDA, the loss of striatal THir and the behavioral manifestations of this loss occurs over a much more condensed time frame and is essentially complete within 2 weeks post-6-OHDA. The interval of STN DBS used in subsequent experiments is indicated by the grey box.
Importantly, when we compared the numbers of total neurons lost between the THir counts and the NeuNir counts at the three time points after intrastriatal 6-OHDA it was evident that at 2 weeks post 6-OHDA a loss of TH phenotype occurred prior to overt neuronal loss in the SN (5854 THir neurons lost compared to 2748 NeuNir neurons lost). However, at 4 and 6 weeks post 6-OHDA reductions in both THir and NeuNir neurons ipsilateral to lesion appeared to be relatively identical (4 weeks: 8292 THir neurons, 9848 NeuNir neurons. 6 weeks: 8776 THir neurons, 8433 NeuNir neurons). These numbers of nigral THir and NeuNir neurons, arrived at using unbiased stereology, indicate that during the first 2 weeks after 6-OHDA a mixture of TH phenotype loss and cell death of SN neurons occurred, whereas between 2 and 4 weeks after 6-OHDA further overt neuronal loss took place that remained stable at 6 weeks post 6-OHDA. These results are illustrated in Figures 8E and I.

Reductions in THir Neurites in the Striatum. We also determined the impact of intrastriatal 6-OHDA injection on THir neurite density in the striatum. Again, there were significantly fewer THir positive neurites in the striatum ipsilateral to 6-OHDA compared to the contralateral striatum at all time points examined (2 week: $F_{(1, 6)} = 272.84, p \leq 0.001$; 4 week: $F_{(1, 5)} = 267.159, p \leq 0.001$; 6 week: $F_{(1, 5)} = 122.675, p \leq 0.001$). However, in contrast to the gradual reduction in THir and NeuNir nigral neurons that we observed at 2 and 4 weeks post lesion, marked reductions in striatal THir neurite density were observed in the ipsilateral striatum at the 2-week post 6-OHDA time point that were maintained over the course of 4 and 6 weeks. No significant differences were observed between the number of THir neurites in the striatum contralateral to 6-OHDA.
injection at any of the time points examined ($p \geq 0.05$). There were no significant differences in striatal THir neurites in the ipsilateral striatum between the three different post 6-OHDA time points ($p \geq 0.05$). Therefore, our intrastriatal 6-OHDA lesion parameters caused a $93 \pm 4.2\%$ decrease in striatal THir neurite density at 2 weeks, with no significant further neurite loss observed at 4 ($93 \pm 3.6\%$) and 6 weeks ($97 \pm 1.4\%$). These results are depicted in Figures 8F and I.

**Induction of Contralateral Forelimb Akinesia and Rotational Asymmetry.** In order to determine the impact of our lesioning protocol on motor performance we analyzed both forelimb akinesia via the cylinder task and amphetamine-induced rotational asymmetry. In the cylinder task the affected paw is contralateral to the side of the 6-OHDA injection. For the cylinder task, all rats receiving 6-OHDA exhibited a significant reduction in contralateral forepaw use at all time points examined compared to both pre-6-OHDA baseline contralateral forepaw use ($F_{(3, 6)} = 9.726, p \leq 0.001$), as well as to vehicle injected control rats ($F_{(1,12)} = 18.652, p = 0.002$). However, no significant differences in contralateral forepaw use were observed between 2, 4 and 6 weeks after 6-OHDA ($p \geq 0.05$). These results are illustrated in Figure 8G.

Similarly, all rats receiving 6-OHDA exhibited ipsilateral amphetamine-induced rotations at all post-6-OHDA time points examined. No significant differences in ipsilateral rotations were observed between 2, 4 and 6 weeks after 6-OHDA ($p \geq 0.05$). These results are illustrated in Figure 8H.
Effects of Long-Term STN Stimulation on Nigrostriatal Neurons and Striatal DA

**Stimulator Placement.** Examination of coronal sections through the level of the STN revealed postmortem tissue damage that occurred during the detachment of the electrode from the dental acrylic and the removal of the electrode from the brain (Figure 9A). Approximately 6 out of the 19 rats in the ACTIVE group had stimulating electrode tips that were determined to be located outside of the STN during postmortem histological analysis. In general, the majority of poorly placed stimulating electrodes were observed to be located caudal to the STN, in between the STN and the SN. Rats in the ACTIVE group with misplaced STN stimulating electrodes were subsequently separated into a separate group and henceforth referred to as ACTIVE MISPLACED.

**STN Cell Number.** In order to determine whether either electrode implantation or stimulation resulted in significant cell loss in the STN, we utilized unbiased stereology of Kluver-Barrera stained sections to quantify the number of cresyl violet stained cells in the STN on the side ipsilateral to the electrode, as well as the contralateral STN in both the ACTIVE and INACTIVE groups. Damage that occurred during postmortem electrode removal did not prevent the quantification of cells within the STN and neighboring sections (Figures 9B, C). There were no significant differences observed in the number of cellular profiles in the STN between the ACTIVE and INACTIVE groups nor the ipsilateral implanted side versus the contralateral intact side ($p \geq 0.05$). These results are illustrated in Figure 9D. Therefore, neither implantation nor electrical stimulation impacted total cell number in the STN.
Survival of THir Neurons in the SN following STN Stimulation. After 2 weeks of stimulation there were significantly more THir neurons remaining in the ipsilateral SN of ACTIVE rats compared to the number of THir neurons in the ipsilateral SN of both the INACTIVE and the ACTIVE MISPLACED rats ($F_{(2, 19)} = 32.261$, $p \leq 0.001$). Further, rats in the ACTIVE MISPLACED group possessed significantly fewer THir neurons in the contralateral SN compared to the number of THir neurons in the contralateral SN of both the ACTIVE and INACTIVE groups ($F_{(2, 19)} = 5.586$, $p = 0.014$). In the most rostral sections through the SN of rats in the ACTIVE MISPLACED group, THir neurons appeared smaller with shorter neurites, often with lighter staining intensity. Therefore, it appears that 2 weeks of stimulation of the STN can halt the nigral THir neuron degeneration that normally would be expected to occur between 2 and 4 weeks after intrastriatal 6-OHDA using our lesion parameters. Further, improper placement of active...
stimulating electrodes outside of the STN not only will not halt the nigral degeneration, but it also can cause nigral neuron degeneration by itself. These results are illustrated in Figures 10A–I.

**Impact of STN DBS on Striatal THir Neurites.** We also examined the impact of long-term STN DBS on THir neurite survival in the striatum. Rats in both the ACTIVE and the INACTIVE groups exhibited significant loss of ipsilateral striatal THir neurite density as a result of intrastriatal 6-OHDA ($F_{(1, 9)} = 88.175, p \leq 0.001$). Two weeks after stimulation, there were no significant differences in the density of THir neurites in the striatum ipsilateral to STN stimulation in rats in the ACTIVE group as compared to the density of THir neurites in the ipsilateral striatum of rats in the INACTIVE control group ($p \geq 0.05$). These results are depicted in Figure 10J.

**DA and DA Metabolite Levels.** We examined the impact of long-term STN DBS on levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum and the frontal cortex. These results are listed in Table 3. Rats in both the ACTIVE stimulation group and the INACTIVE control group exhibited a significant loss of striatal DA within the ipsilateral striatum as a result of intrastriatal 6-OHDA ($F_{(1, 9)} = 111.239, p \leq 0.001$). Two weeks after stimulation, there were no significant differences in the levels of striatal DA in the ipsilateral striatum of rats in the ACTIVE stimulation group as compared to the levels of DA in the ipsilateral striatum of rats in the INACTIVE control group ($p \geq 0.05$). This is depicted in Figure 10K. Similar results were observed
for levels of DOPAC and HVA in the ipsilateral striatum of both the ACTIVE and INACTIVE groups with both groups displaying significant loss of both DA metabolites.

Figure 10. STN DBS initiated 2 weeks after intrastriatal 6-OHDA halts ongoing nigral DA neuron degeneration. Rats received unilateral intrastriatal injections of 6-OHDA and stimulation was initiated 2 weeks later (corresponding to 50% nigral DA neuron loss) and continued for a period of 2 weeks (associated with significant nigral degeneration to 75% loss). A–D. No stimulation (INACTIVE). Representative micrographs of THIR neurons 4 weeks after 6-OHDA in the contralateral (A) and ipsilateral (B) SN. Note higher magnification (D) of nigral THIR neurons from the area delineated by the black box in C demonstrating striking degeneration. Scale bar in A = 500 μM, D = 325 μM. E–H. STN stimulation (ACTIVE). THIR nigral neurons 4 weeks after 6-OHDA in the contralateral (E) and the ipsilateral (F) SN. Note the presence of more THIR nigral neurons (G, H) in rats that received 2 weeks of STN DBS. I. Stereological counts of THIR neurons in the SN of rats implanted with inactive electrodes in the STN (INACTIVE, black bars), rats receiving 2 weeks of STN stimulation (ACTIVE, grey bars) and rats receiving 2 weeks of stimulation outside of and caudal to the STN (ACTIVE MISPLACED, white bars). THIR SN neurons in the mesencephalon contralateral to 6-OHDA/stimulating electrode (Contra) and ipsilateral to 6-OHDA/stimulating electrode (Electrode) were quantified. Unilateral stimulation of the STN (ACTIVE) completely halted the progression of nigral DA neuron degeneration normally observed between 2 and 4 weeks after 6-OHDA (*p < 0.05). In contrast, lesion progression continued to expected levels of approximately 75% loss in rats implanted with INACTIVE STN stimulators. ACTIVE MISPLACED stimulation did not confer neuroprotection exhibiting similar numbers of THIR neurons in the SN ipsilateral to 6-OHDA/electrode as rats in the INACTIVE group and significantly fewer THIR neurons in the contralateral SN compared to the number of THIR neurons in the contralateral SN of both the ACTIVE and INACTIVE groups (*p < 0.02). J. and K. Two weeks of ACTIVE STN stimulation did not restore striatal THIR neurite density (J) or levels of striatal DA (K). Rats in both the ACTIVE stimulation group and the INACTIVE control group exhibited a significant loss of striatal THIR neurite density and DA levels within the ipsilateral striatum as a result of intrastriatal 6-OHDA (*p < 0.001).
due to 6-OHDA compared to the contralateral striatum (DOPAC: $F_{(1, 9)} = 57.801$, $p \leq 0.001$; HVA: $F_{(1, 9)} = 52.796$, $p \leq 0.001$) with no significant differences observed in ipsilateral DOPAC or HVA in the ipsilateral striatum of ACTIVE and INACTIVE rats ($p > 0.05$) as a result of 2 weeks of stimulation. There was also no significant increase in DA turnover rates in the striatum as a result of ACTIVE stimulation. Rats in both the ACTIVE and INACTIVE groups also exhibited a significant loss of DA and HVA in the cortex ipsilateral to 6-OHDA injection (DA: $F_{(1, 9)} = 8.331$, $p = 0.014$; HVA: $F_{(1, 9)} = 23.872$, $p \leq 0.001$), but not DOPAC ($p \geq 0.05$). There were no significant differences observed in DA, DOPAC, or HVA levels no DA turnover rates in either the ipsilateral or contralateral frontal cortex as a result of 2 weeks of stimulation ($p > 0.05$).

Table 3. Levels of DA, DOPAC and HVA in the striatum and frontal cortex. Rats in both the ACTIVE stimulation group and the INACTIVE control group exhibited a significant loss of DA, DOPAC and HVA within the ipsilateral striatum and a significant loss of DA and HVA in the frontal cortex as a result of intrastriatal 6-OHDA ($^*p < 0.001$). No significant differences were observed in the levels of any of the catecholamines examined in either structure as a result of ACTIVE stimulation of the STN ($p > 0.05$). Values are expressed in units of ng/mg protein ± S.E.M. Below detectable limits = b.d.l.
Discussion

The present experiments characterize an intrastriatal 6-OHDA lesioning paradigm resulting in relatively protracted nigral DA neuron degeneration that is expressed first as a loss of TH phenotype and ultimately leads to frank cell loss of nigral DA neurons. Specifically, our lesion parameters lead to loss of 46% of THir neurons in the SN at 2 weeks, progressing further to a loss of 75% of THir neurons at 4 weeks. In contrast, the loss of striatal dopaminergic (DAergic) innervation and the behavioral manifestations of this loss occur over a much more condensed time frame; specifically within 2 weeks after 6-OHDA lesioning. Subsequent studies utilizing these lesion parameters indicate that striatal DAergic denervation may be complete even sooner than 2 weeks after lesion (within days, unpublished observations). Interestingly, this initial loss of terminals followed by nigral neuronal loss may recapitulate an important feature of the sequence of events in PD, albeit over a shorter period of time. The changes in motor function symptomatic of PD are not usually apparent until DA levels in the striatum have dropped to less than 20% of normal (Hornykiewicz, 1988). In the postmortem putamen of PD patients with disease duration ≤ 5 years a substantial loss of both TH and dopamine transporter immunoreactivity was observed while numerous melanin-containing neurons in the SN were still present (Dodiya et al., 2009).

A thorough examination of the time course and magnitude of both nigral DA neuron and striatal terminal loss was an essential first experiment in order to understand the impact of STN DBS initiated at 2 weeks following toxin injection. Our intrastriatal 6-OHDA lesion strategy was based on the work of Sauer and Oertel (1994) who were the first to
inject 6-OHDA into the striatum (instead of nigral cell bodies or proximal axons). Since that study intrastriatal 6-OHDA has been utilized extensively to evaluate the therapeutic potential of numerous agents (Sortwell and Kowdower, 2006; Garcia-Arencibia et al., 2007; Xue et al., 2007; Madhavan et al., 2009). Sauer and Oertel reported an initial downregulation of DA phenotype followed by nigral neuronal loss that continued to significantly impact the nigral DA neuron population for up to 4 weeks. No observations regarding the time course of striatal terminal loss were reported. In the present study, with the benefit of modern stereological quantitation methods, we have been able to confirm and refine the original findings of Sauer and Oertel as well as to demonstrate the time course of striatal terminal loss in this model. Specifically: (i) DA phenotype loss precedes frank neuronal loss of nigral DA neurons, (ii) significant nigral neuron loss occurs over 4 weeks, and (iii) striatal terminal loss is not protracted, occurring within days of intrastriatal 6-OHDA injection. While other laboratories have characterized the extent of nigrostriatal DA neuron loss after intrastriatal 6-OHDA (Lee et al., 1996; Kirik et al., 1998; Blandini et al., 2007; Aponso et al., 2008) this is the first published report to utilize stereological methods to document the magnitude and time course of loss of nigral DA neuron phenotype, nigral neurons and striatal terminals.

The critical finding of the present study is that STN DBS initiated 2 weeks post intrastriatal 6-OHDA, at a time when approximately 50% SN DA cell loss has already occurred, halts continued DA neuron death. This neuroprotective effect of STN DBS was not due to a lesion of the STN and was dependent upon proper electrode placement. Previous studies have examined whether STN DBS can slow or halt the
progression of PD in preclinical animal models. However, significant nigral neuron loss was not confirmed prior to the initiation of stimulation, nor could it be expected based on the experimental paradigm. In rats STN DBS administered within a few hours to 7 days after intrastriatal 6-OHDA injection significantly increases the number of surviving THir nigral neurons (Maesawa et al., 2004; Temel et al., 2006; Harnack et al., 2008). STN DBS given to non-human primates either before or 6 days after MPTP treatment also results in protection of SN DA neurons (Wallace et al., 2007).

Despite these encouraging findings, the question of whether STN DBS can provide neuroprotection when applied following considerable nigrostriatal degeneration was unknown. Further, no previous study reported on the impact of STN DBS at the level of the striatum. The present study examined the effects of STN DBS at a critical time point when approximately 50% of DA neuron loss in the SN had occurred. Here we have shown that although STN DBS can provide neuroprotection for the DA neurons in the SN, this protection does not extend to the THir neurites or DA levels in the striatum. Given that 93% of striatal DAergic terminals had already succumbed to the 6-OHDA at the time that stimulation was initiated, this is not surprising. Experimental treatment strategies that seek to evaluate neuroprotection at the level of the striatum should appreciate the limitations of the intrastratal 6-OHDA model in this regard. While it is possible that lower concentrations of 6-OHDA may lead to protracted loss of striatal DA terminals, this would need to be specifically determined. While we observed no increase in THir striatal neurites following 2 weeks of STN stimulation it is possible that longer periods of stimulation may induce significant compensatory sprouting of remaining
DAergic neurites. Previous work in which viral vectors have provided continuous delivery of trophic factors (Eslamboli et al., 2005; Sortwell et al., 2008) indicates that this compensatory process can take many weeks.

The timing of STN DBS in this study relative to 6-OHDA intrastriatal injection rules out for the first time the possibility that STN DBS provides its neuroprotective benefits by preventing 6-OHDA uptake/metabolism. Previously it has been hypothesized that STN DBS may provide neuroprotection via inhibition of the overactive STN resulting from striatal DA denervation, thus preventing excitotoxic cell death in the SN. However, growing evidence suggests that STN DBS drives and synchronizes the STN instead of inhibiting it (Ceballos-Baumann et al., 1999; Windels et al., 2000, 2003; Hershey et al., 2003; Hilker et al., 2005; Boulet et al., 2006; Zhang et al., 2008). Therefore, it is unlikely that decreased excitotoxicity is involved in the STN DBS-mediated neuroprotection that we observe. While we did not directly investigate the mechanism of STN DBS-mediated neuroprotection in this study additional experiments in our laboratory have documented that under these same lesion and stimulation parameters a significant increase in nigrostriatal brain-derived neurotrophic factor (BDNF) is associated with STN DBS (Spieles-Engemann et al., 2009). It is possible that increased BDNF induced by the STN stimulation may be the mechanism responsible for this neuroprotection. Augmentation of BDNF in the nigrostriatal system by either exogenous protein infusion or vector-mediated delivery can similarly protect from 6-OHDA (Altar et al., 1994; Shults et al., 1995; Klein et al., 1999; Sun et al., 2005; Singh et al., 2006). Future investigations will directly examine this issue.
Investigating the neuroprotective effects of STN DBS in a clinical population is difficult. However, Hilker and colleagues (2005) conducted a prospective study in advanced PD patients using $^{18}$F-fluorodopa PET to measure disease progression. The authors concluded that their findings did not support a neuroprotective effect of clinically effective STN DBS. However, they also acknowledged that neuroprotective effects may be observable in patients in earlier stages of PD. Our present results illustrate the limitations of intervening too late in the disease process: it is by definition impossible to protect what has already been lost. We appreciate that the intrastriatal 6-OHDA model is not PD however, the exact cause of DA neuron degeneration in sporadic PD is currently unknown. Decades of research have indicated that the disease is likely due to cumulative effects of genetic and environmental factors (Poirier et al., 1991; Mouradian, 2002; Elbaz et al., 2007; Migliore and Coppede, 2009). Oxidative stress has been consistently implicated in contributing to the pathology of PD (Przedborski et al., 2001; Jenner, 2007; Henchcliffe and Beal, 2008). Similarly, oxidative stress has been implicated as the mechanism of cell death following intrastriatal 6-OHDA (Rodriguez-Pallares et al., 2007, 2009; Sanchez-Iglesias et al., 2007; Smith and Cass, 2007; Joglar et al., 2009). Until the exact causes of PD are elucidated the intrastriatal 6-OHDA model provides a useful tool to screen potential neuroprotective therapies.

Recent reports conclude that DBS is as effective or more effective than the best medical therapy in alleviating disability in moderate to severe PD patients (Krack et al., 2003; Schupbach et al., 2005; Weaver et al., 2009). The conclusion that STN DBS is neuroprotective in parkinsonian animal models would suggest that this treatment
strategy holds great potential to delay the progression of PD and that this therapy should be expanded and offered to PD patients in early stages of the disease, a time when a greater complement of DA neurons and terminals remain to be influenced. As illustrated by our inability to protect striatal THir neurites, the neuroprotective potential of STN DBS appears unavoidably linked to the magnitude of nigrostriatal degeneration that has already occurred. Under present treatment protocols PD patients are not considered for STN DBS until symptoms have been present for a minimum of 5 years (Chang and Chou, 2006; Neimat et al., 2006) and the typical PD patient has a mean disease duration of 14 years before STN DBS is performed (Volkmann, 2004) during which time the disease has progressed significantly. Therefore, by the time patients initiate DBS they are well into the later stages of PD with STN DBS often considered as a treatment option of last resort. However, the possibility of STN DBS-mediated neuroprotection must be carefully weighed against the risks associated with the procedure as adverse events are significantly higher in DBS compared to best medical therapy in patients with advanced PD (Weaver et al., 2009). An ongoing clinical trial seeks to determine the safety and tolerability of STN DBS in early PD and to compare these results to optimal drug therapy (Vanderbilt University). Careful consideration of the results from this trial, and previous trials, will allow patients to determine what treatment option represents their best course of action.
Chapter 4: Subthalamic Nucleus Stimulation Increases Brain-Derived Neurotrophic Factor in the Nigrostriatal System and Primary Motor Cortex

Abstract

The mechanisms underlying the effects of long-term deep brain stimulation of the subthalamic nucleus (STN DBS) as a therapy for Parkinson’s disease (PD) remain poorly understood. The present study examined whether functionally effective, long-term STN DBS modulates brain-derived neurotrophic factor (BDNF). Rats that received two weeks of continuous unilateral STN DBS exhibited significant improvements in parkinsonian motor behaviors in tests of forelimb akinesia and rearing activity. Unilateral STN DBS increased BDNF protein 2-3 fold bilaterally in the nigrostriatal system with the location (substantia nigra vs. striatum) dependent upon lesion status. Further, BDNF protein was bilaterally increased in primary motor cortex (M1) by as much as 2 fold regardless of lesion status. STN DBS did not impact cortical regions that receive less input from the STN. STN DBS also was associated with bilateral increases in BDNF mRNA in the substantia nigra (SN) and internal globus pallidus (Gpi). The increase observed in Gpi was completely blocked by pretreatment with 5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), suggesting that the activation of N-methyl-D-aspartate (NMDA) receptors was involved in this phenomenon. The upregulation of BDNF associated with long term STN DBS may exert pronounced and underappreciated effects on plasticity in the basal ganglia circuitry that may play a role in the symptomatic effects of this therapy as well as support the neuroprotective effect of stimulation documented in this rat model.
**Key Words:** Deep brain stimulation, Parkinson's disease, Basal ganglia, Trophic factors
Introduction

Over 40,000 patients have received high-frequency electrical stimulation of the subthalamic nucleus (STN DBS) to treat the motor symptoms of Parkinson’s disease (PD) since 2007 (Pereira et al., 2007). Previous studies have demonstrated that STN DBS can provide neuroprotection for the dopaminergic neurons of the substantia nigra (SN) in both the 6-hydroxydopamine (6-OHDA) rodent model and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) non-human primate model of PD (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008; Spieles-Engemann et al., 2010). It has also been demonstrated that electrical stimulation of the dopaminergic neurons of the (VTA) increases brain-derived neurotrophic factor (BDNF) mRNA and protein in its primary target area, the prefrontal cortex (Friedman et al., 2009). BDNF is known to be a potent trophic factor that is important for the survival of the dopaminergic neurons in the SN (Collier and Sortwell, 1999). However, no investigation of the effect of STN DBS on trophic factors in the SN or other STN target structures has been conducted.

The STN serves as an important modulating influence within the basal ganglia circuitry, providing glutamatergic projections to the cortex, globus pallidus externa (GPe)(globus pallidus [GP] in rats), globus pallidus interna (GPi) (entopeduncular nucleus [EP] in rats), SN and striatum (Jackson and Crossman, 1981; Kita et al., 1983; Kita and Kitai, 1987; Parent and Hazrati, 1995; Kitai and Kita, 2006; Degos et al., 2008). High-frequency stimulation of hippocampal glutamatergic synapses can induce the presynaptic and postsynaptic release of BDNF (Hartmann et al., 2001; Matsuda et al.,
2009; Nagappan et al., 2009), and in basal ganglia, increased glutamatergic transmission can increase BDNF mRNA in the SN (Bustos et al., 2004). Additionally, STN DBS has been shown to increase γ-aminobutyric acid (GABA) levels in STN target structures (Windels et al., 2000, 2003, 2005; Bruet et al., 2003; Boulet et al., 2006), and pharmacological activation of hippocampal GABA(B) receptors induces BDNF release (Fiorentino et al., 2009). Finally, direct current stimulation has been reported to induce BDNF secretion in the M1 motor cortex when applied to M1 slices (Fritsch et al., 2010). Collectively, these findings suggest that STN DBS has the potential to similarly alter BDNF mRNA and/or protein levels in STN target structures.

We hypothesized that STN DBS would increase neurotrophic factor levels in STN target structures. We examined this issue in our recently described rat model of STN DBS (Spieles-Engemann et al., 2010). In the current study we initially investigated the functional impact of long-term STN DBS on parkinsonian motor behaviors in the cylinder task. Next, we examined the effects of STN DBS on protein levels within the SN and striatum of two neurotrophic factors known to be important for dopamine neuron viability: glial cell line-derived neurotrophic factor (GDNF) and BDNF. We subsequently measured BDNF mRNA and protein levels within specific regions of the cortex, GP, EP, hippocampus and the STN itself. Finally, to assess whether STN DBS induced increases in BDNF gene expression may be linked to glutamate transmission we examined the effects of NMDA receptor blockade with MK-801.
Materials and Methods

Animals. A total of 76 male, Sprague Dawley rats (Harlan, 200-250 g) were used in these studies. Thirty-one of these animals were given unilateral intrastriatal 6-hydroxydopamine (6-OHDA) injections in addition to STN stimulator implantation, 41 of these animals received unilateral STN stimulator implantation with no 6-OHDA, and 4 additional animals served as naïve controls. All animals were given food and water ad libitum, and housed in reverse light-dark cycle conditions in the University of Cincinnati Vontz Center vivarium, which is fully AAALAC approved. Animals were only included in the final analyses if 1) their SN dopamine neurons were appropriately lesioned after 6-OHDA injection, 2) they completed the full two-week stimulation interval and 3) they were verified to have correct STN stimulator placement following postmortem analysis.

Experimental Overview

STN DBS in Unlesioned Rats: All rats (n=41) were implanted unilaterally with stimulating electrodes into the STN. Rats were allowed to recover from surgery for 2 weeks. One subgroup of animals (n = 16) was implanted with osmotic pumps (Model 2002, Azlet Osmotic Pumps, Cupertino, CA) 10 days post-electrode implantation to allow for the continuous delivery of MK-801 or saline. These rats were then allowed to recover for 4 more days before beginning stimulation (2 weeks post electrode implantation). Rats were then randomly divided into ACTIVE (n =17) or INACTIVE (n =24) stimulation groups. Rats in the ACTIVE group had their stimulators connected to an external stimulation source and received STN stimulation continuously for two weeks. Rats in the INACTIVE group received no stimulation during the identical two-
week interval. At the end of the two weeks all rats were sacrificed within 24 hours of the cessation of stimulation and brains processed for ELISA and/or qPCR analysis.

STN DBS in 6-OHDA Lesioned Rats: All rats (n=31) were assessed for forelimb akinesia and rearing activity utilizing the cylinder task prior to 6-OHDA lesion surgery. The following day rats were unilaterally lesioned via injections of 6-OHDA into the striatum and implanted unilaterally (ipsilateral to 6-OHDA) with stimulating electrodes into the STN during the same surgical session. Following a two-week recovery period, all rats were reassessed for the degree of contralateral forelimb akinesia and rearing via the cylinder task and were then divided into ACTIVE (n =20) or INACTIVE (n =11) stimulation groups. Rats in the ACTIVE group had their stimulators connected to an external stimulation source and received STN stimulation continuously for two weeks. Rats in the INACTIVE group received no stimulation during the two-week interval. At the end of the two weeks a subset of rats were reassessed for forelimb akinesia (ACTIVE n= 8, INACTIVE n=7) and rearing activity (ACTIVE n=12, INACTIVE n=5) via the cylinder task both on and after 24 hours off of stimulation. All rats were then sacrificed and their brains processed for ELISA analysis.

Intrastriatal 6-OHDA Injections. Rats were unilaterally injected with 6-OHDA as described previously (Spieles-Engemann et al., 2010). Briefly, rats were injected in two sites in the striatum (AP +1.6 mm, ML +2.4 mm, DV -4.2 mm and AP -0.2 mm, ML + 2.6 mm, DV -7.0 mm relative to bregma) with 6-OHDA (MP Biomedicals, Solon, OH; 5 µg/µl 6-OHDA in 0.02% ascorbic acid, 0.9% saline solution, injection rate 0.5 µl/minute, 2 µl per site).
Electrode Implantation. All rats were implanted unilaterally with stimulating electrodes. Electrode implantation occurred immediately following intrastriatal 6-OHDA injection in rats in the STN DBS in DA Lesioned Rats experiment. A bipolar concentric microelectrode (Plastics One, Roanoke VA) was lowered to the dorsal border of the STN (AP -3.4, L +2.5, and DV -7.7 from bregma) as identified by previous experiments in which extracellular-guided recording was utilized (Spieles-Engemann et al., 2010). The electrode was then fixed in place using dental acrylic and bone screws. The dorsal border of the STN was chosen as the implantation location in order to minimize any damage to the STN resulting from electrode implantation. Post-operative pain relief was provided via subcutaneous injections of buprenorphine (0.05 ml/100 g BW).

Osmotic Pump Implantation and MK-801 Delivery. Osmotic pumps (Model 2002, Alzet Osmotic Pumps, Cupertino, CA) filled with either 7.5 mg/ml (+)-MK-801 hydrogen maleate (MK-801) (Sigma, St. Louis, MO) in 0.9% saline or 0.9% saline were implanted subcutaneously to the interscapular space in a subset of anesthetized, unlesioned animals. This concentration and osmotic pump configuration allowed for the continuous delivery of a total of 0.3 mg/kg/day MK-801 for the entire 2-week stimulation interval. This osmotic pump concentration was chosen based on previous reports (Armentero et al., 2006). Pumps were examined at the time of sacrifice to confirm that the contents had been delivered.
Cylinder Testing. Non-drugged, spontaneous use of the forepaws and rearing activity was measured in rats as originally described by Schallert (2006) and using standard procedures in our laboratory (Spieles-Engemann et al., 2010). Briefly, during the dark cycle rats were placed in a clear Plexiglas cylinder and videotaped for 5 minutes or 20 weight bearing paw placements on the side of the cylinder, whichever came first. A rater blinded to treatment analyzed videotapes. The number of times the rat reared (both forepaws raised off the platform) and used its left, right, or both paws for weight bearing on the side of the cylinder was determined and recorded. Data for forepaw use is reported as the percentage of contralateral (to 6-OHDA injection) forelimb use:

\[
\frac{\text{contralateral} + \frac{1}{2} \text{both}}{\text{ipsilateral} + \text{contralateral} + \text{both}} \times 100
\]

as described previously (Spieles-Engemann et al., 2010; Schallert, 2006). Data for rearing activity is reported as the total number of rears/minute. A 20% reduction in contralateral forelimb use was utilized to confirm an adequate 6-OHDA striatal lesion prior to enrollment in either the ACTIVE stimulation or INACTIVE stimulation groups.

Long-Term Stimulation. Rats assigned to the ACTIVE group were stimulated in a freely moving setup as described previously (Spieles-Engemann et al., 2010). The stimulus signal was supplied by an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments, Sarasota, FL). Rats were stimulated at frequency of 130 Hz, 60 μs pulse width, and an intensity of 30-50 μA, which was set below the threshold of contralateral forepaw and orofacial dyskinesia, thereby preventing problems with feeding or locomotion during the stimulus interval. Stimulation commenced 2 weeks
following 6-OHDA/electrode implantation surgery and was active 24 hours/day for a 2-week period. Animals with INACTIVE electrodes were housed individually in standard shoebox cages for the identical 2-week interval.

Sacrifice. Twenty-four hours after the cessation of stimulation rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized saline at 37°C followed by ice-cold saline in order to remove endogenous circulating growth factors from blood vessels. The brains were immediately removed, flash frozen in 3-methyl butane, and stored at -80°C. This same perfusion and storage procedure was also used for the naïve rats.

Microdissections. Brains were placed in a -20°C cryostat for approximately one hour. The brains were then sliced into 1–2 mm slabs using chilled (-20°C) single edge razor blades and an aluminum brain blocker. The brain slabs were dissected on a cold plate set at -12°C (ThermoElectric Cooling America Corp, Chicago, IL). All microdissection instruments and the cold plate were wiped down with RNase Away (Invitrogen, Carlsbad, CA) to prevent contamination from RNase and DNA. The EP, GP, STN, SN, hippocampus, M1 cortex, M2 cortex, and parietal cortex (cortical regions delineated as per Degos et al., 2008) were dissected out of the frozen brain slabs using either iridectomy scissors or a small tissue punch. Slabs containing the STN were examined for visual verification of electrode placement prior to dissection of this nucleus. For animals in which the STN was not used for ELISA or PCR, this STN slab was then immediately immersed in 10% phosphate buffered formalin for subsequent electrode
placement analysis. Each dissected brain structure was placed in a separate pre-frozen RNase free microcentrifuge tube and immediately placed back on dry ice. All samples were stored at -80 °C until time of assay.

**STN brain slab processing.** STN slabs were stored in 10% phosphate buffered formalin for a minimum of 1 week and then transferred to 30% sucrose in 0.1M PO₄ buffer. For sectioning, slabs were frozen on dry ice and sectioned at 80 μm thickness using a sliding microtome. Every section throughout the STN was stained using the Kluver-Barrera technique according to previously described methods (Kluver and Barrera, 1953). Briefly, mounted sections were washed in xylene then ETOH and then stained with Luxol Fast Blue (0.1 g Solvent Blue 38, Sigma, St. Louis, MO, 95% ETOH, and 10% Glacial Acetic Acid) for 15 minutes. The sections were then washed again with ETOH and distilled water and then differentiated in 0.05% Lithium Carbonate, ethanol, and water. Sections were then stained with Cresyl Violet (0.1%, Harleco, Lawrence, KS) for 5 minutes. Sections were then dehydrated to xylene and coverslipped with Cytoseal. Stimulating electrode placement was considered appropriately targeted to the STN if the tip of the stimulating electrode was observed to be within or at the dorsal border of the STN within any of the sections examined (see Figure 11). Our laboratory has previously documented that neither electrode implantation nor stimulation under identical conditions impacts STN cell survival (Spieles-Engemann et al., 2010).
Protein Assay. In order to determine total protein levels, tissue was homogenized in T-PER (Pierce, Rockford, IL) using a 300V/T Ultrasonic Homogenizer (BioLogics, Manassas, VA) and a portion of each sample for ELISA was added to 2% SDS solution and then added to a BD Falcon 96-well Microtest plate (Fisher, Morris Plains, NJ) along with a BSA standard curve (Pierce, Rockford, IL). CuSO$_4$ (4%) was then added to the samples and the plate was incubated at 37°C for 20 minutes and then read at 590 nm on a spectrophotometer.

ELISA. The ELISA reaction was completed (in triplicate) using Immunolon 4 HBX or Costar 96 well plates (Fisher, Morris Plains, NJ) according to the ELISA manufacturer’s instructions (GDNF or BDNF Emax ImmunoAssay Systems Kit, Promega, Madison, WI). The samples were then read at 450 nm within 30 minutes using a spectrophotometer with unknown values determined through interpolation against the

**Figure 11. STN stimulator placement.** A representative micrograph showing stimulating electrode placement in a coronal section through the rat STN stained via the Kluver-Barrera method. The luxol fast blue component of Kluver-Barrera stains the fiber tracts and the cresyl violet component stains the cell bodies. Therefore, this staining makes the dense cellular swath of the STN immediately dorsal to the internal capsule easily identifiable. Electrode placement was considered to be appropriately targeted to the STN if the tip was within or at the border of the STN within any of the sections examined. STIM=stimulator, IC=internal capsule, OPT=optic tract, STN=subthalamic nucleus. Scale bar=500µm.
GDNF or BDNF standard curve. Each structure was run individually with both ACTIVE and INACTIVE animals on the same plate. Results are expressed as GDNF or BDNF pg/mg of protein. Data was normalized to the percent of INACTIVE rats control. The control for the ACTIVE left hemisphere (stimulator and 6-OHDA hemisphere) was the mean BDNF pg/mg protein value of the INACTIVE left hemisphere and the control for the ACTIVE right hemisphere was the mean BDNF pg/mg protein value of the INACTIVE right hemisphere.

*RNA isolation.* RNA was isolated using the QIAshredder (Qiagen, Valencia, CA) and RNeasy Plus Mini kit (Qiagen, Valencia, CA). The Qiagen protocol for purification of total RNA from animal tissue was used with the addition of an extra wash with 500 μl buffer RPE to remove residual salts.

*BDNF qPCR.* RNA from the tissue was then converted to cDNA via a 2-step process using random hexamers and MultiScribe Reverse Transcriptase (ABI, Carlsbad, CA). The RNA was assumed to be converted 100% to cDNA. The PCR reactions were carried out with 1X TaqMan Universal MasterMix (ABI, Carlsbad, CA), and the TaqMan gene expression assay kit for BDNF (Rn02531967_s1, ABI, Carlsbad, CA). This kit includes 900 nM of each manufactured primer and 900 nM of a BDNF MGB-florescent probe. A total of 20 ng of cDNA was added to each 50 μl reaction mixture. Additional reactions containing RNA only (no cDNA) were amplified to ensure no genomic contamination. qPCR reactions to control for cDNA quantities were run using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control.
(Rn99999916_s1, ABI, Carlsbad, CA). The qPCR reactions were run on an ABI 7500 real-time thermocycler using the following method: Step 1: incubation at 50°C for 2 min. Step 2: incubation at 95°C for 10 min. Step 3: denaturation at 95°C for 15 s followed by annealing-elongation at 60°C for 1 min followed by data collection. Step 3 was cycled 40 times for the qPCR run. Cycle thresholds were chosen during the linear phase of amplification using the AutoCT function. Structures were run individually, and all plates contained structures from both ACTIVE and INACTIVE animals as well as at least 1 naïve animal, which were used as the controls. Analysis was first carried out using the 2^ΔCT method (Schmittgen and Livak, 2008). The 2^ΔCT values for the naïve animals were then averaged and used as the control for the percent control conversion.

Statistical Analysis. One-way, two-way, or two-way repeated measures analysis of variance (RM-ANOVA) followed by a Holm-Sidak or Tukey’s post-hoc test were used to determine significant differences in behavior, GDNF and BDNF protein levels, and BDNF gene expression levels. SigmaStat 3.0 software (Systat Software, San Jose, CA) and SPSS (Chicago, IL) was used for all statistical analyses and the level of statistical significance was set at 0.05. All results are expressed as the mean ± the standard error of the mean (SEM).

Results

Impact of STN DBS on Parkinsonian Motor Behaviors

In 6-OHDA lesioned animals, all rats exhibited reductions in both rearing activity and contralateral forepaw use compared to prelesion behaviors during spontaneous
exploration in a Plexiglas cylinder (cylinder task). Overall there was a significant difference between the ACTIVE and INACTIVE groups across time in both contralateral forepaw use and rearing activity (forepaw $F_{(3, 13)} = 20.915, p \leq 0.001$; rearing $F_{(3, 15)} = 17.348, p \leq 0.001$). Rats that received 2 weeks of continuous active STN stimulation (ACTIVE) exhibited significant increases in both rearing activity and contralateral forepaw use compared to the INACTIVE control group ($p < 0.05$). This difference was not apparent 24 hours after the cessation of stimulation ($p > 0.05$). These results indicate that our long-term STN stimulation parameters provide functional improvement in the parkinsonian motor behaviors induced by intrastriatal 6-OHDA. These results are depicted in Figure 12.

*Impact of STN DBS on GDNF Protein Levels in the Nigrostriatal System*

There were no significant differences in GDNF protein levels between ACTIVE and INACTIVE stimulation groups in either the SN ($p>0.05$) or the striatum ($p>0.05$) in unlesioned rats. ACTIVE rats ($n=4$) possessed $189.37 \pm 47.72$ pg/mg GDNF in the striatum compared to $122.22 \pm 21.49$ pg/mg GDNF in INACTIVE rats ($n=4$). Nigral levels of GDNF protein were $118.65 \pm 23.39$ pg/mg in ACTIVE rats and $110.67 \pm 25.26$ pg/mg in INACTIVE rats. Since no significant differences in GDNF protein were observed in the nigrostriatal system due to STN stimulation we focused our ensuing efforts on the evaluation of BDNF levels.
Figure 12. Long-term STN DBS reverses 6-OHDA-induced deficits in forepaw akinesia and rearing activity. A-H. Spontaneous rearing and forepaw use in the cylinder test. Rats received either no stimulation (A-D, n=7) or 2 weeks of STN DBS (E-H, n=12). Rats were videotaped and analyzed for the number of spontaneous hind leg rears and number of weight bearing forepaw placements (red plusses) on the cylinder wall before 6-OHDA (A, E, Pre), 2 weeks following intrastriatal 6-OHDA (B, F, Post-lesion), during stimulation on day 14 (C, G, On Stim), and 24 hours after the cessation of stimulation (D, H, Off Stim). I-J. Quantification of rearing activity (I) and forepaw placement (J). All rats exhibited a significant decrease in rears/minute and contralateral forepaw use as a result of 6-OHDA lesioning. Rats stimulated continuously for a two-week period (red triangles) exhibited significantly more rears/minute and significantly increased use of their contralateral forepaw (*, p<0.05) compared to rats that received no stimulation. Twenty-four hours after cessation of stimulation, stimulation-associated improvement was no longer apparent. Values represent the mean for each group at each time point ± SEM.
Impact of STN DBS on BDNF Protein Levels in the Nigrostriatal System

BDNF protein levels were significantly increased in both striatal hemispheres of unlesioned ACTIVE rats as compared to the striatum of unlesioned INACTIVE rats ($F_{(1, 8)}=11.674$, $p=.005$). ACTIVE rats possessed $115.80 \pm 20.69$ pg/mg BDNF in the unlesioned striatum compared to $35.93 \pm 6.60$ pg/mg BDNF in unlesioned INACTIVE rats. There was no significant difference ($p>0.05$) in BDNF protein levels between the ACTIVE and INACTIVE groups in the SN of unlesioned animals. Nigral levels of BDNF protein were $114.19 \pm 21.97$ pg/mg in ACTIVE rats and $82.89 \pm 18.61$ pg/mg in INACTIVE rats. These results indicate that STN DBS induces a significant upregulation of striatal BDNF protein in rats with an intact nigrostriatal system.

STN DBS also was associated with significant increases in BDNF protein in the nigrostriatal system of rats lesioned with intrastriatal 6-OHDA, however the location of this BDNF protein upregulation differed from the location in unlesioned rats. Specifically, there was a significant bilateral increase in BDNF protein in the substantia nigra of the ACTIVE group in lesioned rats as compared to the INACTIVE group of lesioned rats ($F_{(1,17)}=6.733$, $p=.019$). ACTIVE rats possessed $29.85 \pm 3.24$ pg/mg BDNF protein in the lesioned nigra compared to $12.87 \pm 1.66$ pg/mg BDNF protein in lesioned INACTIVE rats. No significant difference in striatal BDNF protein levels was observed between INACTIVE and ACTIVE lesioned rats ($p>0.05$). Striatal levels of BDNF protein were $8.15 \pm 1.93$ pg/mg in ACTIVE rats and $3.81 \pm 1.69$ pg/mg in INACTIVE rats and many of the samples were at or slightly below the minimum sensitivity of the assay. Collectively, these results indicate that STN DBS induces a significant bilateral increase in BDNF
protein levels in the nigrostriatal system and that the location of this upregulation is dependent upon lesion status. The effects of STN DBS on BDNF protein levels in the nigrostriatal system are illustrated in Figure 13.

Figure 13. Long-term STN DBS increases BDNF protein in the nigrostriatal system. No increase in nigral BDNF protein was seen with ACTIVE stimulation in intact rats (n=4) (A). In 6-OHDA lesioned animals BDNF protein is bilaterally increased in the substantia nigra of rats receiving ACTIVE stimulation (n=12, white bars) as compared to INACTIVE stimulator controls (n=6, black bars) (B, * p<0.05). BDNF protein was bilaterally increased in the striatum of rats receiving ACTIVE stimulation (n=4, white bars) as compared to INACTIVE stimulator controls (n=4, black bars) in intact rats (C, * p<0.05), but was not increased in the striatum of 6-OHDA lesioned rats (ACTIVE n=12, INACTIVE n=6) (D). Values expressed as the mean percent of control ± SEM for each group. The control for the ACTIVE left hemisphere (stimulator and 6-OHDA hemisphere) was the mean BDNF pg/mg protein value of the INACTIVE left hemisphere and the control for the ACTIVE right hemisphere was the mean BDNF pg/mg protein value of the INACTIVE right hemisphere.
Impact of STN DBS on BDNF protein levels in the cortex

We also examined BDNF protein levels in cortical areas that receive varying innervation from the STN, including the M1, M2 and parietal cortices (Kita and Kitai, 1987; Degos et al., 2008). In the M2 and parietal cortices that receive sparse STN innervation there were no significant differences in BDNF protein levels between the ACTIVE and INACTIVE groups in lesioned animals (p>0.05). BDNF protein levels in the M2 and parietal cortex of ACTIVE rats were 5.78 ± 0.92 and 3.10 ± 0.15 pg/mg, respectively. BDNF protein levels in the M2 and parietal cortex of INACTIVE rats were 5.95 ± 0.78 and 3.83 ± 0.44 pg/mg, respectively. However, in the M1 cortex, which receives the preponderance of innervation from the STN (Degos et al., 2008), we found a significant bilateral increase in BDNF protein in the ACTIVE as compared to the INACTIVE group in both unlesioned (F(1, 26)=5.935, p=.019) and lesioned (F(1,18)=5.611, p=.031) rats. BDNF protein levels in the M1 cortex of ACTIVE unlesioned and lesioned rats were 11.25 ± 1.47 and 17.0 ± 3.15 pg/mg, respectively whereas BDNF protein levels in the M1 cortex of INACTIVE unlesioned and lesioned rats were 7.15 ± 0.62 and 7.48 ± 1.37, respectively. These results indicate that STN DBS is associated with a significant bilateral increase in BDNF protein in the M1 cortex in both unlesioned and 6-OHDA lesioned rats. These results are illustrated in Figure 14.

Impact of STN DBS on BDNF protein levels in the STN, GP, EP and hippocampus.

In addition, we measured BDNF protein levels in the hippocampi of unlesioned animals in order to determine whether integration into STN circuitry was required for STN DBS to impact BDNF levels. Indeed, we observed no significant difference (p>0.05) in BDNF
protein levels between the ACTIVE and INACTIVE groups in these animals.

Hippocampal levels of BDNF protein were 19.83 ± 1.63 pg/mg in ACTIVE unlesioned rats and 21.82 ± 1.42 pg/mg in INACTIVE unlesioned rats. Lastly, we attempted to measure BDNF protein levels in the GP and EP, as well as in the STN itself. However, the BDNF protein levels in these structures were below the detectable limits of the ELISA assay for both the ACTIVE and INACTIVE groups in both unlesioned and lesioned animals.

Figure 14. Long-term STN DBS increases BDNF protein in the M1 cortex. Long term STN DBS does not increase BDNF protein in the parietal or M2 cortex of 6-OHDA lesioned rats (ACTIVE n=5, INACTIVE n=4) (A-B). Long term STN DBS bilaterally increases BDNF protein (* p<0.05) in the M1 cortex of rats receiving ACTIVE stimulation (white bars) as compared to INACTIVE stimulator controls (black bars) in both 6-OHDA lesioned (ACTIVE n=12, INACTIVE n=7) and intact rats (ACTIVE n=8, INACTIVE n=10) (C-D). Values expressed as the mean percent of control ± SEM for each group. The control for the ACTIVE left hemisphere (stimulator and 6-OHDA hemisphere) was the mean BDNF pg/mg protein value of the INACTIVE left hemisphere and the control for the ACTIVE right hemisphere was the mean BDNF pg/mg protein value of the INACTIVE right hemisphere.
Impact of STN DBS and MK-801 on BDNF gene expression in STN target structures

STN DBS significantly increased BDNF mRNA in both the SN and EP in unlesioned rats (SN: $F_{(2,36)}= 2.955$, $p = 0.046$; EP: $F_{(4,14)}=5.941$, $p=0.005$). Rats in the ACTIVE treatment group displayed an approximate 30% increase in BDNF mRNA in the SN whereas a doubling in EP BDNF mRNA was observed compared to both INACTIVE and naïve rats. Further, while MK-801 administration had no impact on STN DBS associated increases in nigral BDNF mRNA ($p>0.05$), in the EP MK-801 completely prevented the STN DBS induced increase in BDNF mRNA. These results are illustrated in Figure 15A and B.

When additional structures including GP, striatum, M1 cortex, or the STN itself were examined, there were no significant differences in BDNF mRNA levels associated with either STN DBS or MK-801 administration ($p>0.05$).

Figure 15. The effects of long-term STN DBS on BDNF mRNA expression in the EP and SN. A. Long term STN DBS bilaterally increased BDNF gene expression in the SN of rats receiving ACTIVE (n=12) stimulation regardless of MK-801 status (black bars, *p<0.05) as compared to INACTIVE (n=18) stimulator control animals (white bars) or naïve rats with no stimulators (n=4, grey bars). B. Long-term STN DBS bilaterally increased BDNF gene expression in the GPi of saline treated rats receiving ACTIVE stimulation (n=2, black bars, *p<0.05) as compared to INACTIVE stimulator control rats (saline n=4, MK801 n=6, white bars) or naïve rats with no stimulators (n=4, grey bars). Further, ACTIVE stimulation rats treated with MK-801 (n=3) also expressed significantly lower levels of BDNF mRNA in the GPi compared to ACTIVE saline treated rats (n=4, black bars, *p<0.05). Values are expressed as the mean percent of control where the control is the mean $2^{-\Delta Ct}$ of the naïve rats.

Values are expressed as the mean percent of control where the control is the mean $2^{-\Delta Ct}$ of the naïve rats.
Discussion

The present experiments characterize the effects of STN DBS on parkinsonian motor behaviors and the expression of GDNF and BDNF within the STN and its target structures in a rat model of PD. Specifically, we found that our STN DBS parameters reversed the 6-OHDA lesion induced decrease in contralateral forepaw use and rearing activity as measured in the cylinder task. This functional recovery was dependent on active STN DBS as it was not observed 24 hours after stimulation was turned off. In addition, we found that STN DBS does not affect the level of GDNF protein within the intact nigrostriatal system. In contrast, we did find a STN DBS associated increase in BDNF mRNA and protein levels in several of the STN target structures examined. Specifically, STN DBS bilaterally upregulated BDNF protein expression in the nigrostriatal system and the M1 cortex. Further, STN DBS increased BDNF mRNA in both the EP and the SN. In the EP, the STN DBS associated increase in BDNF mRNA expression was blocked by the administration of the NMDA receptor antagonist MK-801.

Previous work has demonstrated that acute STN DBS can reverse 6-OHDA lesion-induced impairments in motor behaviors, including contralateral forepaw akinesia and reduced rearing activity (Shi et al., 2004; Gubellini et al., 2006; Akita et al., 2010) and other motor deficits induced by striatal DA denervation (Temel et al., 2005; Li et al., 2010). Here we have demonstrated that 24 hours after the cessation of stimulation both contralateral forepaw use and rearing activity returned to impaired prestimulation performance levels. In contrast, increased STN DBS associated BDNF protein levels
persisted. Therefore, whether any relationship exists between improved performance in these motor tasks and increases in BDNF is unclear. However, several studies have shown that STN DBS can improve motor deficits other than forepaw akinesia and rearing that are associated with 6-OHDA lesioning (Temel et al., 2005; Li et al., 2010). Therefore, the possibility remains that increased BDNF could have an effect on motor tasks not measured in these experiments. Additionally, previous work has shown that immediately following the cessation of STN DBS total forepaw use in the cylinder task is enhanced and can be reversed by MK-801 (Akita et al., 2010). In the present study we did not examine the impact of MK-801 on 6-OHDA-induced motor impairments. It is tempting to speculate that had we measured BDNF levels during or immediately following stimulation that more marked increases in BDNF would have been detected. However, in the absence of this data it is unclear what relationship STN DBS induced increases in BDNF protein have on behavioral improvement. Regardless, the finding of improved motor function resulting from STN DBS remains important as it indicates that our long-term STN DBS model is functionally effective, and thus clinically relevant for continued translational research to enhance our understanding of the biological underpinnings of this therapy.

The mechanism whereby long term SNT DBS increases BDNF remains to be determined. However, previous in vitro studies provide possible answers. High frequency stimulation of hippocampal glutamatergic synapses can induce the dendritic release of BDNF protein (Hartmann et al., 2001) with prolonged stimulation inducing axonal release (Matsuda et al., 2009). Further, direct current stimulation combined with
low-frequency stimulation induces BDNF secretion and TrkB activation in M1 cortex slices (Fritsch et al., 2010). The results of the present study provide in vivo evidence of stimulation-induced increases in BDNF release, as has previously been observed following stimulation of the VTA (Friedman et al., 2009), perhaps via similar mechanisms as reported in vitro. Alternatively, STN DBS-mediated increases in BDNF may be due to an increased activity level resulting from improved motor functioning in the ACTIVE group. Exercise has been shown to increase BDNF levels in the rat brain (Neeper et al., 1996; Marais et al., 2009; Wu et al., 2011). However, several differences between our study and the exercise studies decrease the likelihood that exercise is involved in the elevated BDNF observed in the present experiments. Exercise-induced increases in BDNF result from rigorous wheel or treadmill running (Wu et al., 2011; Marais et al., 2009; Neeper et al., 1996). Although ACTIVE rats in our study were more physically active than INACTIVE rats, their activity was considerably less than treadmill or wheel running and stimulation did not return their activity to even baseline levels. Further, exercise-induced increases in BDNF consistently demonstrate upregulation in the hippocampus (Carro et al., 2000; Neeper et al., 1996). We observed no impact of STN DBS on hippocampal BDNF protein levels. Therefore, it is unlikely that the slight increase in activity associated with long term STN DBS is a key contributor to the increases in BDNF observed in ACTIVE rats.

Unilateral STN DBS produced a significant bilateral increase in BDNF mRNA in the SN. In intact rats the increase in BDNF protein was localized to the striatum whereas in lesioned rats with depleted striatal DA terminals (Spieles-Engemann et al., 2010) the
STN DBS-associated increase in BDNF protein was instead located in the SN. STN DBS did not impact BDNF mRNA levels in either the M1 or the STN. Collectively, our results suggest that STN DBS-associated increases in the nigrostriatal system result from increased SN BDNF mRNA that ultimately enhances BDNF protein levels in either the SN or striatum, depending upon the integrity of the nigrostriatal connections. Increases in BDNF within the nigrostriatal system have the potential to exert a multitude of effects. First, several preclinical studies have demonstrated STN DBS to be neuroprotective to the dopaminergic neurons in the SN (Spieles-Engemann et al., 2010; Harnack et al., 2008; Wallace et al., 2007; Temel et al., 2006; Maesawa et al., 2004). STN DBS-induced increases in BDNF may participate in this neuroprotection. Second, BDNF plays an essential role in the maintenance of postsynaptic spine density of the striatal medium spiny neurons (MSNs) (Rauskolb et al., 2010; Saylor et al., 2006). Loss of MSN spines has been demonstrated in both preclinical models and postmortem PD patients (Zaja-Milatovic et al., 2005; Stephens et al., 2005; McNeil et al., 1988; Ingham et al., 1993). Striatal MSN spines are the site of interaction for nigral dopamine neurons and glutamatergic cortical and thalamic neurons (Smith et al., 1994) and this interaction is necessary for normal basal ganglia functioning and thus normal motor function (Obeso et al., 2008). It is possible that STN DBS induced BDNF may impact MSN spine density and morphology, thus leading to the improved motor functioning seen with STN DBS (Liang et al., 2006; Rodriguez-Oroz et al., 2005; Vesper et al., 2002; Kumar et al., 1998). Finally, it has been previously demonstrated that BDNF can increase dopamine turnover and/or release in the nigrostriatal system (Altar et al., 1994; Martin-Iverson et al., 1994; Shults et al., 1994; Altar et al., 1992). Upregulation of BDNF within the
nigrostriatal system may also impact striatal dopaminergic transmission and thus improve parkinsonian motor symptoms.

STN DBS induced increases in BDNF within the nigrostriatal system have the potential to exert a multitude of effects. First, several preclinical studies have demonstrated STN DBS to be neuroprotective to the dopaminergic neurons in the SN (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008; Spieles-Engemann et al., 2010). STN DBS derived increases in BDNF may play a role in this neuroprotection. Second, BDNF plays an essential role in the maintenance of postsynaptic spine density of the striatal medium spiny neurons (MSNs) (Saylor et al., 2006; Rauskolb et al., 2010). Loss of spines on the MSNs has been demonstrated in both preclinical models and postmortem analysis of patients with PD (McNeill et al., 1988; Ingham et al., 1993; Stephens et al., 2005; Zaja-Milatovic et al., 2005). The spines of striatal MSNs are the site of interaction for nigral dopamine neurons and glutamatergic cortical and thalamic neurons (Smith et al., 1994) and this interaction is necessary for normal basal ganglia functioning and thus normal motor function (Obeso et al., 2008). It is possible that STN DBS induced BDNF may impact MSN spine density and morphology, thus leading to the improved motor functioning seen with STN DBS (Kumar et al., 1998b; Vesper et al., 2002; Rodriguez-Oroz et al., 2005; Liang et al., 2006). Finally, it has been previously demonstrated that BDNF can increase dopamine turnover and/or release in the nigrostriatal system (Altar et al., 1992, 1994; Martin-Iverson et al., 1994; Shults et al., 1994). Upregulation of BDNF within the nigrostriatal system may also impact striatal dopaminergic transmission and thus improve
parkinsonian motor symptoms.

The primary motor cortex (M1) is central to the execution and planning of movement. Reductions in motor cortex excitability and output have been shown in PD (Ridgel et al., 2009; DeLong, 1990; Albin et al., 1989), which is thought to underlie bradykinesia (Jenkins et al., 1992). STN DBS associated increases in BDNF in the M1 cortex may impact excitability (Kafitz et al., 1999) and thereby increase cortical output. Further, BDNF may participate in the decreases in cortico-subcortical oscillatory activity believed to underlie STN DBS associated improvements in tremor (Kuhn et al., 2008; Timmermann et al., 2003). Increases in BDNF have been directly linked to complex motor learning tasks (Kleim et al., 2006; Klintsova et al., 2004), which at the cellular level may reflect the effects of BDNF on fiber sprouting and the induction of action potentials (Hiebert et al., 2002; Kafitz et al., 1999). Further, Vall66Met mice (with impaired activity-dependent BDNF release) exhibit deficits in motor skill acquisition (Fritsch et al., 2010). Ultimately, the significance of STN DBS induced increases in M1 BDNF remains to be elucidated.

Our finding that STN DBS upregulates BDNF protein in the M1 cortex but not the M2 or parietal cortex is interesting when viewed in an anatomical context. In the rat the M1 cortex receives the preponderance of STN glutamatergic projections as compared to the M2 and parietal cortices. The M1 also provides a dense reciprocal projection to the STN (Degos et al., 2008). Discrete antidromic activation of M1-STN circuitry improves 6-OHDA induced motor deficits (Gradinaru et al., 2009) suggesting that activation of this
same pathway may play a role in the motor improvements associated with STN DBS in patients. Combined with findings that high frequency stimulation of neurons can cause dendritic release of BDNF (Hartmann et al., 2001), this raises the possibility that the mechanism of BDNF upregulation in the M1 is antidromic activation of M1-STN circuitry. However, since high frequency activation of neurons can also induce axonal release of BDNF (Matsuda et al., 2009), orthodromic activation of the STN-M1 cannot be ruled out. Further investigation will focus on the source of increased BDNF in the M1 associated with STN DBS.

STN DBS increased BDNF mRNA expression in both the SN and the EP. The EP and SN are the major output structures of the basal ganglia (Parent & Hazrati, 1995; Carpenter & Strominger, 1967; Whittier & Mettler, 1949), receiving both glutamatergic efferents from the STN (Degos et al., 2008; Kitai & Kita, 2006; Parent & Hazrati, 1995; Jackson & Crossman, 1981) and GABAergic inhibitory input from the striatal direct pathway (Parent & Hazrati, 1995; Carpenter & Strominger, 1967). STN DBS increases glutamate in the STN, SN, GP, and striatum; increases cGMP (as an indirect measure of glutamate concentration) in the GPi; and increases GABA in the SN and striatum (Stefani et al., 2006; Boulet et al., 2006; Windels et al., 2005; Windels et al., 2003; Windels et al., 2000). Glutamatergic stimulation of NMDA and GABA(B) receptors can lead to BDNF release (Matsuda et al., 2009; Fiorentino et al., 2009; Hartmann et al., 2001). Our findings could therefore be interpreted to support the concept that STN DBS leads to enhanced neurotransmitter release in the SN and the EP, which in turn leads to activation of NMDA and/or GABA(B) receptors, which culminates in increased BDNF
mRNA synthesis and release. However, whereas MK-801 completely blocked the stimulation-associated increase in BDNF in the EP, the increase in BDNF mRNA in the SN was unaffected. It is possible that the BDNF mRNA increase in the EP is mediated solely by NMDA receptors, which are blocked by MK-801, whereas the BDNF increase in the SN may be mediated by both NMDA and GABA(B) receptors and would therefore be less susceptible to MK-801 blockade of BDNF mRNA upregulation. Ultimately, the mechanism of STN DBS-mediated BDNF upregulation remains to be elucidated.

In summary, the finding that long term STN DBS increases BDNF indicates that this therapy may exert pronounced and underappreciated effects on plasticity in the basal ganglia circuitry, beyond basic inhibition and excitation of neuronal activity. The present study focused on the impact of STN DBS on the trophic factor BDNF. It remains to be determined whether long term STN DBS impacts the expression of additional trophic factors. A complete understanding of the impact of STN DBS on the trophic environment and the consequences of this for efficacy and side effects has the potential to influence patient selection and refinement of the therapy itself. It may also lead to the development of less invasive and more efficacious treatments for PD. The ability of stimulation to modulate BDNF expression also opens up the possibility for this therapy to be used to treat other disorders which may involve BDNF, such as Alzheimer’s, Huntington’s, traumatic brain injury, post traumatic stress disorder, or mood disorders. Ultimately, a deeper understanding of this ever-expanding therapeutic approach is essential to optimizing its safety and efficacy.
Chapter 5: Summary and Conclusions

The experiments described in this dissertation were designed to examine the neuroprotective effects of high-frequency deep brain stimulation in rats with large-scale dopamine neuron loss in the SN. Preclinical studies in both rodents and non-human primates have demonstrated that HF STN DBS administered either before or shortly after either 6-OHDA or MPTP lesioning can protect the dopamine neurons in the SN (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008). Additionally, clinical evidence is beginning to accumulate indicating a lack of progression of motor symptoms in patients receiving HF STN DBS up to 5 years after surgery as measured by “off medication” UPDRS scores (Krack et al., 2003; Rodriguez-Oroz et al., 2004; Rodriguez-Oroz et al., 2005; Tagliati et al., 2010). Together these findings indicate that HF STN DBS may prevent further degeneration of the SN dopamine neurons in PD.

The experiments in this dissertation explore STN DBS-mediated neuroprotection by first examining whether STN DBS is neuroprotective in the face of large-scale dopamine neuron loss in the SN. At the time of initial diagnosis, patients have already lost about 60% of their SN dopamine neurons (Bernheimer et al., 1973) and will continue to lose them as time progresses (Hoehn and Yahr, 1967; Braak et al., 2003; Kingsbury et al., 2010). Therefore, we sought to extend the findings of previous groups and determine if STN DBS would have neuroprotective effects even when initiated in a progressive lesion model after 50% of SN dopamine neurons had already been lost, similar to the
clinical situation when patients are first diagnosed. We then sought to elucidate a possible mechanism of neuroprotection by examining the effects of STN DBS on BDNF and GDNF, two potent dopaminotropic factors that are present in the nigrostriatal system (Schaar et al., 1993; Seroogy and Gall, 1993; Seroogy et al., 1994; Choi-Lundberg and Bohn, 1995; Kawamoto et al., 1996; Conner et al., 1997) and have previously been demonstrated to have neuroprotective effects in animal models of PD (Frim et al., 1994; Beck et al., 1995; Kearns and Gash, 1995; Tomac et al., 1995b; Tsukahara et al., 1995; Gash et al., 1996).

**Stimulation of the Rat Subthalamic Nucleus is Neuroprotective Following Significant Nigral Dopamine Neuron Loss**

In the first set of experiments for this study, we used unbiased stereological cell counts of THir neurons in the SN, NeuNir neurons in the SN, and THir neurite density in the striatum to characterize several different lesioning paradigms in order to find one that would result in protracted loss of the SN dopamine neurons. The paradigm that was ultimately used to test the neuroprotective effects of STN DBS led to a loss of 46% of THir neurons in the SN at 2 weeks (similar to the loss PD patients have at initial diagnosis [Bernheimer et al., 1973]), progressing further to a loss of 75% of THir neurons at 4 weeks (similar to late-stage PD). We also characterized motor behaviors via amphetamine-induced rotations and the cylinder task, as these have been shown to correlate with dopamine loss (Ungerstedt and Arbuthnott, 1970; Schallert, 2006) and allowed for a functional assessment of our lesioning paradigm. We wanted to find a progressive lesion paradigm in order to make our study clinically relevant. Additionally,
the characterization of this lesion paradigm will allow for the testing of this and other therapies at time points that accurately model early or late-stage PD, which could reveal differences in effectiveness based on how many SN dopaminergic neurons remain. For example, until recently, the typical PD patient receiving STN DBS is in late-stage PD, having a mean disease duration of 14 years (Volkmann, 2004), and no clinically neuroprotective effect as measured by $^{18}$F-fluorodopa uptake has been seen in such patients (Hilker et al., 2005). However, there is interest in utilizing STN DBS as a treatment in early PD based on the preclinical and clinical evidence suggesting the possibility of a neuroprotective effect (Krack et al., 2003; Maesawa et al., 2004; Rodriguez-Oroz et al., 2004, 2005; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008; Tagliati et al., 2010). Currently there is an ongoing Phase I clinical trial to determine the safety and tolerability of STN DBS in early PD (Charles, June 22, 2009). By utilizing the lesioning paradigm characterized in these studies, we can evaluate the effects of STN DBS within a partially DA-depleted environment similar to early PD or a near complete DA-depleted environment similar to late-stage PD, which may help to inform its use clinically.

As part of this study, we also utilized dual stimulation and recording electrodes in order to determine the amount of current spread we would obtain utilizing stimulation parameters of 130 Hz, 60 μsec, and 100 μA. These parameters were chosen to replicate the parameters used clinically, with the exception of stimulus intensity. Although in the clinical situation the stimulation parameters are determined post-surgery for each patient in extensive consultation with their neurologist in order to obtain
the best functional improvement (Deuschl et al., 2006a), most stimulus parameters are between 130–185 Hz, 60–210 μs, and 1–3.5 V (Kuncel and Grill, 2004), and 130 Hz and 60 μs are usually utilized as the initial starting point (Volkmann et al., 2002). Further, while therapeutic STN stimulation frequencies have been reported to be greater than 100 Hz, frequencies less than 50 Hz are nontherapeutic (Garcia et al., 2005) and stimulation at 5–10 Hz has been demonstrated to worsen parkinsonism (Rizzone et al., 2001; Moro et al., 2002). Therefore, we chose a frequency of 130 Hz and a pulse width of 60 μs for these experiments. With regard to intensity, the STN of rats is approximately 300 times smaller than the human STN (rat STN ≈ 0.8 mm³; human STN ≈ 240 mm³, (Hardman et al., 2002) whereas the size of the stimulating electrode used in rodent studies is only 6 times smaller than electrodes used clinically. The large size of the stimulating electrode relative to the size of the rat brain increases the likelihood that current aimed at the rat STN will spread to structures outside of the STN and therefore necessitates that a lower intensity be used to limit current spread. Estimates of the volume of tissue activated in the human brain range from 30–114 mm³ with approximately 70 mm³ generating the most therapeutic benefit (Maks et al., 2009). This volume of activation would likely include the lenticular fasciculus and ansa lenticularis but not the SN. In our current spread experiment we found that with our electrodes at an intensity of 100 μA (130 Hz, 60 μs) current spread in a sphere with a radius of 250 μm from the electrode tip. Therefore, we decided to implant electrodes for all subsequent experiments in the anterior portion of the STN and utilize intensities less than 100 μA in order to prevent current from spreading to the SN. We felt that individualizing the intensity by setting it just below each rat's threshold for contralateral orofacial dyskinesia
was the best way to determine intensity for these experiments. First, since our studies were long-term studies in which the animals received stimulation continuously for 2 weeks, this ensured that our animals would not have any feeding or grooming issues, which could have resulted in early termination of the study. Second, setting intensity in this manner seemed to best replicate the clinical situation in which the intensity is increased until side effects are seen and then decreased to the point where patients have symptomatic benefit but no side effects (Volkmann et al., 2002). Third, limiting the intensity limits current spread since it has been demonstrated that as intensity increases, the distance of current spread increases (Ranck, 1975) and we wanted to ensure we did not stimulate the SN directly.

In the next set of experiments we examined the effects of long-term STN DBS on STN cell number, SN THir cell number, striatal THir neurite density and striatal and cortical dopamine levels in our progressive lesion model. We felt that examination of the STN itself to ensure that neither the physical implantation of the stimulator nor the electrical stimulation caused a lesion was critical since lesions of the STN have also been shown to improve parkinsonian motor symptoms and be neuroprotective for SN dopamine neurons (Delfs et al., 1995; Guridi et al., 1996; Piallat et al., 1996, 1999; Blandini et al., 1997; Carvalho and Nikkhah, 2001). We wanted to ensure any effects we saw were due to STN stimulation and not STN lesioning, and utilizing unbiased stereology, we found no differences in STN cell number due to either stimulator insertion or stimulation.
Interestingly, no research has been done to explain why lesioning and stimulation have similar effects on cell survival in the basal ganglia. This is perhaps because the notion that stimulation inhibited STN activity and mimicked the effects of STN ablation (Benazzouz et al., 1995, 2000; Dostrovsky and Lozano, 2002) persisted for so long. However, it should be noted that the bulk of the studies demonstrating neuroprotection as a result of STN lesioning performed the STN lesion prior to 6-OHDA lesion (Piallat et al., 1996, 1999; Carvalho and Nikkhah, 2001) and a more recent study has demonstrated that STN lesioning 15 days after 6-OHDA injection does not provide neuroprotection for SN dopamine neurons, although motor deficits were improved (Rizelio et al., 2010). Therefore, it is unlikely that lesioning and stimulation function through similar mechanisms since stimulation administered 2 weeks post 6-OHDA lesion provides neuroprotection for SN dopamine neurons and STN lesioning does not. Regardless, it was important for us to eliminate any doubt surrounding the possibility of neuroprotection resulting from STN lesion due to stimulator insertion or stimulation.

Perhaps the most important finding from this set of experiments relates to the effect of STN DBS on SN THir cell number. We found that STN DBS initiated 2 weeks post intrastriatal 6-OHDA, at a time when approximately 50% of SN DA cell loss has already occurred, halts continued DA neuron death. Previous studies have examined whether STN DBS can slow or halt the progression of PD in preclinical animal models. However, significant nigral neuron loss was not confirmed prior to the initiation of stimulation, nor could it be expected based on the experimental paradigm. (Maesawa et al., 2004; Temel et al., 2006; Wallace et al., 2007; Harnack et al., 2008). Prior to the current study,
the question of whether STN DBS could provide neuroprotection when applied following considerable nigrostriatal degeneration was unknown. The timing of STN DBS in this study relative to 6-OHDA intrastriatal injection rules out for the first time the possibility that STN DBS provides its neuroprotective benefits by preventing 6-OHDA uptake/metabolism and demonstrates the need to investigate other possible mechanisms of neuroprotection.

Additionally, we also felt it important to examine the impact of STN DBS at the level of the striatum since no other study had ever examined this question. Although we did see neuroprotection in the SN, that protection did not extend to the THir neurites in the striatum. This is likely due to the fact that the majority of the THir neurites in the striatum have already succumbed to 6-OHDA within a few days of the toxin administration, leaving only about 7% remaining at the time we initiated stimulation. It is not possible to protect what isn’t there, and this is a major limitation of the 6-OHDA model used for this particular experiment. Determining the impact of STN DBS at the level of the striatum will require a 6-OHDA model that results in protracted loss of THir neurites and such a model remains to be discovered. It is interesting to note, however, that our model in which striatal THir terminals are lost prior to THir neurons actually mimics the clinical situation in which it has been demonstrated that PD patients with a disease duration ≤5 years had a substantial loss of both TH and dopamine transporter immunoreactivity in the putamen while numerous melanin-containing neurons in the SN were still present during postmortem examination (Dodiya et al., 2009). While we observed no increase in THir striatal neurites following 2 weeks of STN stimulation, it is
possible that longer periods of stimulation may induce significant compensatory sprouting of remaining DAergic neurites. Previous work in which viral vectors have provided continuous delivery of trophic factors (Eslamboli et al., 2005; Sortwell et al., 2008) indicates that this compensatory process can take many weeks.

We also felt that it was important to investigate the effects of STN DBS on the levels of dopamine and its metabolites DOPAC and HVA, as well as the rate of DA turnover in the striatum within our lesion model as there have been conflicting reports in the literature as to whether STN DBS increases striatal dopamine (Bruet et al., 2001) or whether it increases its metabolism without an increase in dopamine itself (Paul et al., 2000; Meissner et al., 2001, 2002). Interestingly, in our model of long-term STN DBS we found no differences in striatal DA, DOPAC, or HVA nor DA turnover rates between the active and inactive stimulation groups. It is likely that we did not see the changes in DA or DOPAC and HVA seen by the other groups due to the fact that they measured DA and its metabolites via microdialysis during short-term STN DBS while we measured them via HPLC 24 hours after the cessation of stimulation. Had we measured DA, DOPAC and HVA levels during stimulation it is possible we might have seen increases as well. We also chose to examine levels of DA and its metabolites in the frontal cortex since a previous study utilizing STN DBS at an intensity of 300 μA found an increase in DA in the nucleus accumbens that was mediated by the ventral tegmental area (VTA). Since the VTA also has a dopaminergic projection to the frontal cortex (mesocortical pathway) (Oades and Halliday, 1987) we felt that measuring DA in this area was another way to ensure we were not invoking excessive current spread with our
stimulation parameters. We saw no increase in DA, its metabolites, nor DA turnover in this area. However, had we seen an increase, we would not have been able to rule out activation of the VTA due to antidromic activation of its projections to the STN (Hassani et al., 1997) rather than current spread.

Although this study confirmed our hypothesis that STN DBS would provide neuroprotection to the dopaminergic neurons in the SN even in the face of large-scale dopamine loss, several questions remain. First, as previously mentioned, in the future it would be interesting to carry out stimulation for longer periods of time in order to see if longer periods of stimulation affect compensatory sprouting of striatal THir neurites. Second, the major question that remains to be answered by this study is what are the functional effects of protecting SN DA neurons when few if any striatal THir neurites remain and there is no increase in striatal DA or DA metabolism? Therefore, a range of functional motor tests both during and after stimulation should be carried out within our model to address this question. Third, STN DBS should be initiated at a later time point within our progressive 6-OHDA model, which would be similar to late-stage PD, in order to compare its effects with the current study in which we intervened at a time point akin to early PD. Finally, it would be interesting to test the neuroprotective potential of STN DBS in other progressive models of PD, such as the new viral vector-mediated overexpression of α-synuclein model of PD (Ulusoy et al., 2010), which appears to more closely mimic PD than the 6-OHDA model.
Subthalamic Nucleus Stimulation Increases Brain-Derived Neurotrophic Factor in the Nigrostriatal System and Primary Motor Cortex

The purpose of the first set of experiments in this study was to elucidate the possible mechanism responsible for the nigral neuroprotective effect of STN DBS demonstrated in Aim 1 of this project. Therefore, we chose to examine GDNF and BDNF protein levels in the nigrostriatal system of unlesioned rats after receiving 2 weeks of STN DBS. We chose to examine the effects of STN DBS on these particular trophic factors for several reasons. First, GDNF and BDNF are 2 potent dopaminotropic factors that are present in the nigrostriatal system (Schaar et al., 1993; Seroogy and Gall, 1993; Seroogy et al., 1994; Choi-Lundberg and Bohn, 1995; Kawamoto et al., 1996; Conner et al., 1997) and have previously been demonstrated to have neuroprotective effects in animal models of PD (Frim et al., 1994; Beck et al., 1995; Kearns and Gash, 1995; Tomac et al., 1995b; Tsukahara et al., 1995; Gash et al., 1996). Second, STN DBS increases glutamate in the SN and striatum (Windels et al., 2000, 2003; Bruet et al., 2003; Boulet et al., 2006) and stimulation of hippocampal glutamatergic synapses in vitro leads to both presynaptic and postsynaptic BDNF release (Hartmann et al., 2001; Matsuda et al., 2009; Nagappan et al., 2009). Additionally, increased glutamatergic activity to the SN as a result of STN overactivity has been shown to increase BDNF mRNA in the SN (Bustos et al., 2004). Third, STN DBS has been shown to increase GABA levels in the SN and striatum (Windels et al., 2000, 2003, 2005; Bruet et al., 2003; Boulet et al., 2006) and pharmacological activation of hippocampal GABA(B) receptors in vitro induces BDNF release (Fiorentino et al., 2009). Finally, there is precedent in other systems, as
electrical stimulation of the VTA has been shown to increase BDNF protein and mRNA in its primary target structure, the prefrontal cortex in vivo (Friedman et al., 2009). Collectively, we felt that these previous studies suggested that STN DBS could potentially alter GDNF and/or BDNF protein levels within the nigrostriatal system. Performing these initial experiments in unlesioned animals eliminated any confounding issues we would have had due to the nearly immediate loss of striatal DA terminals that occurs in our lesion model. Our results showed that STN DBS had no effect on GDNF protein within the nigrostriatal system or BDNF protein in the SN, but it did increase BDNF protein bilaterally within the striatum (see Figure 16).

Given that we saw no neuroprotective impact of STN DBS on striatal DA terminals but did see an effect on SN DA neurons, we decided to see what happened to BDNF protein levels within the nigrostriatal system when we used the same lesion parameters and intervened with STN DBS at the same time point we had in our neuroprotective experiment in Aim 1. The results showed no increase in BDNF protein in the striatum. Again, this is likely due to the fact that at the time we intervene with STN DBS in our lesion model there are virtually no DAminergic terminals left in the striatum and therefore there is no place for BDNF to be stored or released in this location. However, we did see a bilateral increase in BDNF protein in the SN (see Figure 16).

It could be argued that the increase in BDNF that we saw in the SN in lesioned animals was due to lesioning and not related to STN DBS since several studies have shown BDNF protein in the SN to increase as a result of 6-OHDA lesioning and to remain
elevated for as long as 4 weeks after lesioning (Yurek and Fletcher-Turner, 2000, 2001). However, these studies utilized lesions in which the toxin was administered near the medial forebrain bundle (MFB) and SNc. Other studies utilizing partial striatal 6-OHDA lesions have shown a transient increase in BDNF mRNA at 1 week post lesion, which by 2 weeks post lesion had decreased and was not significantly different from controls (Aliaga et al., 2000; Bustos et al., 2009). It is possible that the differences observed in these studies could be due to the differences in lesion paradigms, or differences in the amount of time protein remains elevated as compared to mRNA. It could also be argued that the BDNF upregulation was a result of stimulator insertion.
and the resulting glial reaction (Elkabes et al., 1996; Sun et al., 2008). Therefore, we addressed these issues in our study design by utilizing animals that were lesioned and had stimulators implanted but never turned on as controls to which we compared our stimulated animals. Even if BDNF levels were elevated due to lesioning and/or stimulator implantation, any increase in BDNF we saw in stimulated animals as compared to the non-stimulated lesioned controls should be due to stimulation effects. However, to be thorough, in the future we should examine BDNF levels in lesioned implanted non-stimulated animals at the time point we intervene with STN DBS (2 weeks post lesion) to ensure STN DBS is actually driving the increase in BDNF and not merely maintaining an endogenous increase due to 6-OHDA lesioning and/or stimulator insertion.

It could also be argued that the increase in BDNF we saw in ACTIVE animals was the result of increased activity levels as compared to INACTIVE animals since several studies have demonstrated that exercise can increase BDNF levels in the rat brain (Neeper et al., 1996; Marais et al., 2009; Wu et al., 2011). However, the studies demonstrating exercise-induced increases in BDNF demonstrated that BDNF increased specifically as a result of either wheel (Neeper et al., 1996; Marais et al., 2009) or treadmill running (Wu et al., 2011) and that exercise increases BDNF in the hippocampus (Neeper et al., 1996) in addition to the nigrostriatal system. Since animals in our experiments were not given access to a running wheel or treadmill and we detected BDNF increases in specific target structures of the STN and not in the hippocampus, it is unlikely that the increases in BDNF were the sole result of increased
activity among the animals receiving STN DBS. However, repeating these experiments with 4 experimental groups; ACTIVE sedentary, ACTIVE exercised (given access to wheel or treadmill), INACTIVE sedentary, and INACTIVE exercised, would help to deliniate the role increased activity might play in the previously demonstrated increases in BDNF.

It should be noted that BDNF protein and mRNA were increased bilaterally in all structures in which we reported an increase (SN, striatum and M1 for protein, SN and EP for mRNA). This is consistent with the clinical literature in which unilateral STN DBS has been demonstrated to have bilateral effects on the motor symptoms of PD (Chung et al., 2006; Alberts et al., 2008; Arai et al., 2008). Additionally, this also makes sense anatomically given that the nigrostriatal neurons have contralateral projections (Gerfen et al., 1982; Royce and Laine, 1984; Morgan and Huston, 1990), as do the cortico-striatal projections (Parent and Hazrati, 1995).

Additionally, we examined the impact of STN DBS on motor functioning in a subset of lesioned animals. A previous study demonstrated the potential for DBS to reverse 6-OHDA lesion induced contralateral forepaw akinesia and reduced rearing activity (Shi et al., 2004). However, in this study, STN DBS was performed acutely and the intensity was increased during the cylinder task until forepaw asymmetry disappeared. Our study is unique in that we demonstrated the reversal of forepaw asymmetry in a long-term model of STN DBS and without expressly looking for that reversal in order to set the stimulus intensity. Although, it is unlikely that the alterations detected in BDNF levels in
our study are the sole factor in the behavioral improvement seen given that forepaw asymmetry returned once stimulation was stopped while the alterations in BDNF persisted for at least 24 hours after the end of stimulation. Regardless, the finding of improved motor function resulting from STN DBS remains important as it indicates that our long-term model is functionally effective, and thus clinically relevant. However, it is possible that other motor skills that were not assessed in the present study could be impacted by augmented BDNF levels, as several studies have shown that STN DBS can improve other motor deficits associated with 6-OHDA lesioning (Temel et al., 2005; Li et al., 2010). Future studies should examine this issue.

After seeing increases in BDNF protein after STN DBS in the nigrostriatal system, which receives glutamatergic projections from the STN, we decided to look at BDNF protein levels after STN DBS in the other structures that receive glutamatergic innervation from the STN, namely the GP, EP and several areas of the frontal cortex including the M1, M2 and parietal cortex (Jackson and Crossman, 1981; Kita et al., 1983; Kita and Kitai, 1987; Parent and Hazrati, 1995; Kitai and Kita, 2006; Degos et al., 2008). We also wanted to see what was happening to BDNF levels within the STN itself. Protein levels in the GP, EP and STN were below the detectable limits of our ELISA assay (15.6 pg/ml). However, this does not necessarily mean that there were no STN DBS-mediated increases in BDNF protein in these structures. It simply means that the ELISA assay we used was not sensitive enough to pick up any BDNF protein in these very small structures. In the future, if we want to be able to detect any possible changes in BDNF protein in these structures we will need to use another assay such as a protein...
microarray that has been demonstrated to be much more sensitive than ELISA (Lebrun and VanRenterghem, 2006; Zhong et al., 2010). However, we did detect an STN DBS-mediated increase in BDNF in the M1 cortex but not in the M2 or parietal cortex (see Figure 17). Anatomically this is consistent with the fact that the M1 cortex receives the preponderance of glutamatergic innervation from the STN as compared to the M2 or parietal cortex (Degos et al., 2008). The M1 also provides a dense reciprocal glutamatergic projection back to the STN (Degos et al., 2008), and antidromic activation of this projection has been shown to improve 6-OHDA-induced motor deficits (Gradinaru et al., 2009). If glutamatergic stimulation is indeed responsible for increased BDNF upregulation and release, it makes sense that we see that increase in the area of the cortex that receives the most glutamatergic projections from the STN. It is interesting to note that the source of BDNF protein in the cortex could either be the neurons themselves or cortical oligodendrocytes, both of which have been shown to release BDNF in response to glutamatergic stimulation (Bagayogo and Dreyfus, 2009; Fritsch et al., 2010).

The mechanism of BDNF upregulation in the M1 cortex could potentially be antidromic activation of M1-STN circuitry. First, discrete antidromic activation of the M1-STN projection has been shown to improve 6-OHDA-induced motor deficits (Gradinaru et al., 2009). Second, several studies have shown STN DBS antidromically activates fiber tracts in vivo (Walker et al., 2010; Deniau et al., 2010) and in particular can antidromically activate the motor cortex causing increased cortical excitability (Kuriakose et al., 2010). Finally, high frequency stimulation of neurons can cause
dendritic release of BDNF (Hartmann et al., 2001), and antidromic activation has been shown to play a role in long term potentiation and synaptic plasticity (Colbert, 2001). However, since high frequency activation of neurons can also induce axonal release of BDNF (Matsuda et al., 2009) and the design of this study did not address this question, orthodromic activation of the STN-M1 cannot be ruled out.

After finding increases in BDNF protein in several target structures of the STN, we decided to investigate changes in BDNF mRNA in the STN target structures in an effort to identify where BDNF upregulation could be occurring and to where it might be transported. We also decided to administer the NMDA antagonist MK-801 to a subset of these animals in order to elucidate the role of glutamate in BDNF upregulation. The only structures in which we found BDNF mRNA to be upregulated were the SN and the EP.
Interestingly, MK-801 blocked the mRNA increase in the GPi, but not in the SN. The most likely explanation for this lies in the types of neurotransmitters that have been shown to be increased in each structure as a result of STN DBS. In the GPi only glutamate (as measured indirectly via cGMP levels) has been shown to be increased (Stefani et al., 2006) whereas both glutamate and GABA have been shown to be increased in the SN (Windels et al., 2000, 2003, 2005; Bruet et al., 2003; Boulet et al., 2006; Lee et al., 2007). Both glutamate and GABA(B) receptor activation have been linked to BDNF secretion in vitro (Aliaga et al., 1998; Marini et al., 1998; Chun et al., 2000; Fiorentino et al., 2009). Our findings of increased BDNF in vivo as a result of STN DBS are consistent with these previous studies. However, based on...
our findings it is likely that only glutamatergic stimulation is responsible for BDNF synthesis in the EP whereas both glutamatergic and GABAergic stimulation may play a role in BDNF synthesis in the SN. To test this hypothesis in the future, we will need to administer a GABA(B) antagonist during STN DBS, and if glutamatergic stimulation is solely responsible for the increase in BDNF mRNA in the EP, we should not see any blockade of that upregulation. The major weakness of these MK-801 experiments was that we did not measure the levels of MK-801 present in the animals at the time of sacrifice. Therefore, it is possible (although unlikely given that we did ensure the contents of the pumps had been delivered at the time of sacrifice) that we included some animals that had very low levels of MK-801 in their system and thereby diluted the effects seen. Had we measured MK-801 levels and excluded animals that had received very low levels, we might have seen a more robust effect. Based on our results, there is a need to examine BDNF changes in the thalamus given the recent findings of a direct glutamatergic subthalamo-thalamic projection (Rico et al., 2010) as well as the fact that the thalamus is a major target of the SN and EP (see Figure 18) (Ilinsky et al., 1997; Kultas-Ilinsky et al., 1997; Sidibe et al., 1997).

To summarize our data, we saw BDNF protein increases in the striatum and M1 cortex of unlesioned animals and in the SN and M1 cortex of lesioned animals (see Figures 16 and 17). Additionally, the only increases in BDNF mRNA we observed were in the EP and SN (see Figures 16 and 18). The origin of BDNF protein in the striatum has three possible sources: the STN, the SN, or the cortex, all of which have projections to the striatum (Kita and Kitai, 1987; Smith and Parent, 1988; Parent and Hazrati, 1995; Kitai
and Kita, 2006) and all of which contain BDNF mRNA that has been demonstrated to be functionally coupled to the striatum (Rite et al., 2005). Previous studies have indicated that approximately 66% of striatal BDNF protein comes from the M1 while only about 14% comes from the SN (Altar et al., 1997) leading one to believe that any increase in BDNF seen in the striatum likely had its origin in the M1 cortex. However, at least for the STN DBS-mediated increase in striatal BDNF protein, this appears not to be the case. The source of the STN DBS-mediated increase in striatal BDNF protein appears to be anterograde transport from the SN. This is based on our observations that there was no upregulation of BDNF protein seen in the SN in unlesioned animals yet upregulation did occur in the SN in lesioned animals when there were very few striatal terminals remaining, that this did not occur in the M1 cortex where both unlesioned and lesioned animals had a significant increase in BDNF protein, and that BDNF mRNA increased in the SN but not in the M1 cortex. The major weakness of our attempt at mapping out where BDNF upregulation is occurring and where it is being transported and released is related to the fact that we did not examine changes in BDNF protein or mRNA in the thalamus. Given the recent findings of a direct glutamatergic subthalamo-thalamic projection (Rico et al., 2010) as well as the fact that the thalamus is the main target of the SN and EP (Ilinsky et al., 1997; Kultas-Illinsky et al., 1997; Sidibe et al., 1997) and the importance of the thalamus in motor functioning and its degeneration in PD (Halliday, 2009), it is possible that changes in BDNF that could impact the motor symptoms of PD may also be occurring in this structure. The fact that we saw protein increase in the M1 but not mRNA could be explained by the 2 different ways BDNF is affected by glutamatergic stimulation. First, high-frequency activation of glutamatergic
synapses has been shown to induce BDNF release from dendritic secretory granules (Hartmann et al., 2001) and evidence points to the existence of a vesicle pool of BDNF, which during repeated depolarization results in prolonged and/or repetitive BDNF release (Kolarow et al., 2007). Pharmacological activation of glutamate receptors has also been shown to increase BDNF mRNA expression within cells, including those of the mesencephalon (Bessho et al., 1993; Aliaga et al., 1998). In our model it appears likely that the increase in BDNF protein we measured in the M1 cortex is due to stimulation-induced vesicular release of intracellular stores. This is because we saw no increase in BDNF mRNA in the M1 cortex itself, or the STN, from which BDNF could be transported. However, it is possible that BDNF mRNA could be increasing in the thalamus and is then transported to the M1 cortex via the thalmo-cortical projections. Since we did not measure BDNF protein or mRNA in the thalamus this cannot be ruled out. However, our evidence does point to receptor-mediated increases in mRNA within the SN and EP.

Although this study at least partially confirmed our hypothesis that STN DBS would increase neurotrophic factors in STN target structures, several questions remain. First, would we see an increase in GDNF if we repeated these experiments in lesioned animals? Although we saw no changes in GDNF as a result of STN DBS in normal animals, it is possible that we could see an increase within the pathological context of a lesioned brain. Second, is BDNF the only trophic factor that is increased by STN DBS? This question may be answered in the future by ultilizing a low-density PCR array such as the Neurotrophins and Receptors Array available from Qiagen. Utilizing such an
array would also help to address another weakness of this study, which is that we did not look at how STN DBS altered levels of the BDNF receptor TrkB. An increase in BDNF without a concomitant increase in TrkB may not have much of an effect since it is via receptor-mediated signaling that BDNF exerts its effects. Third, is BDNF actually responsible for the neuroprotective effects seen in Aim 1? This question could be addressed in several different ways. First, there exist transgenic mice with the Val66Met polymorphism that impedes activity-dependent BDNF release. STN DBS could be performed in these mice to see if similar neuroprotective effects for SN DA neurons occur as a result of stimulation. However, targeting the STN in mice could prove to be difficult as it is roughly half the size of that of a rat (Hardman et al., 2002). Another possible way would be to use short hairpin RNA (shRNA) to knock down BDNF expression in rats and then perform STN DBS. However, shRNA is not ideal since it can also knock down genes other than the target gene (Jackson et al., 2003) and can induce an interferon response (Bridge et al., 2003). Therefore, this method is less than ideal. Perhaps the best way to examine the role of BDNF in neuroprotection would be to use a viral vector to introduce the Val66Met polymorphism in rats. Then STN DBS could be performed in these animals and if BDNF is truly responsible for the neuroprotective effects, we would expect to see no neuroprotection of SN DA neurons after 2 weeks of stimulation. Utilizing a Tet regulatable vector, you could turn on expression of this polymorphism at a time point during stimulation and expect to see less of a neuroprotective effect than animals without the polymorphism (again assuming that BDNF is responsible for the neuroprotective effect). Fourth, does BDNF have any effects on the motor symptoms seen in the 6-OHDA model of PD? This question could
be addressed by performing STN DBS in rats that have been transfected with the Val66Met polymorphism and measuring various motor tasks before, during and after stimulation. Fifth, would we see greater increases in BDNF or increases in additional structures if we examined BDNF protein and mRNA levels shortly after initiating STN DBS, or conversely how long is this increase maintained? Would we still see increases after 3 or 4 weeks of stimulation? Sixth, if STN DBS was performed in a model with protracted effects on the striatal terminals, it would be interesting to measure spine density and examine morphology as well as measure dopamine levels in order to elucidate possible effects of BDNF within the striatum. It would also be interesting to measure tPA levels in the various nuclei in which we saw increases in BDNF since high-frequency stimulation has been shown to increase levels of tPA in vitro, leading to the cleavage of pro-BDNF to create the mature form (Nagappan et al., 2009). Finally, STN DBS has been shown to decrease GABA in the VTA (Winters et al., 2008) and decreased BDNF in the prefrontal cortex, which is a target of the VTA, has been shown to play a role depressive symptoms (Friedman et al., 2009). Since depression can be a side effect of STN DBS (Rodriguez-Oroz et al., 2005; Zhang et al., 2006; Kleiner-Fisman et al., 2006), it would be interesting to examine BDNF levels in the prefrontal cortex after long-term stimulation to determine if they decrease in a certain percentage of animals.

PD is a progressive disease with no known cure. As such, the search continues for a treatment that can halt, or at least slow, the progression of this disease. Based on the evidence presented in these studies, STN DBS has the potential to be that treatment. In
fact, the potential for this treatment is so promising that a Phase I clinical trial is underway to investigate the efficacy and safety of STN DBS in early PD with the hope that the data will provide the basis for a full-scale multicenter trial that will investigate the hypothesis that STN DBS can slow or halt the progression of PD (Clinical trials.gov NCT00282152). With the additional revelation that STN DBS can increase BDNF expression, it gives hope that this therapy could be used as a treatment in other disorders in which BDNF expression is reduced, such as Alzheimer’s, Huntington’s, traumatic brain injury, post-traumatic stress disorder, or mood disorders. Understanding the effects of stimulation on the trophic environment within the basal ganglia could also help lead to less invasive and more efficacious therapies for PD. At the very least, the more thorough understanding of STN DBS gained by these types of studies could aid patients and physicians in weighing the risks versus benefits when making the decision of whether or not to opt for STN DBS surgery.
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