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Improving Therapeutics for Parkinson Disease

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by

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Abstract

The following results are from studies designed with the overall goal of elucidating means for improving therapeutics for Parkinson’s disease. There is still much to be understood regarding the cellular and molecular mechanisms underlying the development of side effects to therapies for Parkinson’s disease such as levodopa-induced dyskinesias. Additionally, therapeutic intervention is further complicated when one considers the role of the functionally waning host environment, especially in the context of its responsivity to therapeutic agents. The work in this thesis was designed to focus on two aspects of Parkinson’s disease therapeutics, (1) improving graft efficacy by contributing additional information on how the host environment in regards to age impacts graft function in Parkinson’s disease, and (2) determining whether specific morphological changes of the nigral target neurons within the striatum impact development or severity of levodopa-induced dyskinesias. The first study demonstrates that while the aging striatum can, under specific conditions, support the survival of large numbers of grafted embryonic dopamine neurons, there is limited functional benefit even with robust cell survival. This realization is an important contribution to the field as it newly suggests the aged striatum is capable of supporting grafted dopamine neurons, but the limited efficacy of these grafts demonstrates the importance of the intricate balance between grafted cells and the aged host environment. The second study demonstrate that maintaining appropriate synaptic contacts that are presumably maintained when striatal medium spiny neuron cytoarchitecture is preserved, resulting in reduced drug-induced side-effects in parkinsonian rats. Future experiments are
needed to explore the specific requirements of the aging striatum for functional recovery and how to implement synaptic stability of medium spiny neurons in clinical practice to potentially decrease side-effects of dopamine replacement pharmacotherapy.
The scientist is not a person who gives the right answers, he’s one who asks the right questions.

Claude Lévi-Strauss, Le Cru et le cuit, 1964
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>A2A</td>
<td>adenosine-2A</td>
</tr>
<tr>
<td>AADC</td>
<td>aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>ad</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>ar</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>C</td>
<td>caudal</td>
</tr>
<tr>
<td>CamKII</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CB</td>
<td>carotid body</td>
</tr>
<tr>
<td>CeM</td>
<td>central medial thalamic nuclei</td>
</tr>
<tr>
<td>CL</td>
<td>central lateral thalamic nuclei</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-0-methyl transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>D1R</td>
<td>dopamine receptor 1</td>
</tr>
<tr>
<td>D2R</td>
<td>dopamine receptor 2</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>dopamine and cAMP-regulated phospho-protein</td>
</tr>
<tr>
<td>DBS</td>
<td>deep brain stimulation</td>
</tr>
<tr>
<td>dPL</td>
<td>dorsal prelimbic cortices</td>
</tr>
<tr>
<td>DR3</td>
<td>dopamine receptor 3</td>
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<tr>
<td>Erk1/Erk2</td>
<td>extracellular signal-related kinase 1 / 2</td>
</tr>
<tr>
<td>FFD</td>
<td>facial-forelimb dyskinesias</td>
</tr>
<tr>
<td>FosB</td>
<td>FBJ murine osteosarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GID(s)</td>
<td>graft-induced dyskinesias</td>
</tr>
<tr>
<td>Glut</td>
<td>glutamate</td>
</tr>
<tr>
<td>GP</td>
<td>globus pallidus</td>
</tr>
<tr>
<td>GPe</td>
<td>globus pallidus, externa</td>
</tr>
<tr>
<td>GPI</td>
<td>globus pallidus, interna</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
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</table>
hESC (human) embryonic stem cell

Hz Hertz

i.m. intramuscular

i.p. intraperitoneal

i.v. intravenous

IL infralimbic cortex

IMD intermediodorsal thalamic nuclei

LD levodopa

LID(s) levodopa-induced dyskinesias

LMN lower motor neuron

LTD long-term depression

LTP long-term potentiation

MAO-B monoamine oxidase-B

MAP mitogen-activated protein kinase

MD movement disorders

MEK mitogen-activated protein kinase kinase

Mm millimeter

mos months
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<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>MSN</td>
<td>medium spiny neuron</td>
</tr>
<tr>
<td>Nim</td>
<td>nimodipine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NR1</td>
<td>NMDA receptor subunit 1</td>
</tr>
<tr>
<td>NR2A</td>
<td>NMDA receptor subunit 2A</td>
</tr>
<tr>
<td>NR2B</td>
<td>NMDA receptor subunit 2B</td>
</tr>
<tr>
<td>PCN</td>
<td>paracentral thalamic nuclei</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPN</td>
<td>pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>PSTP</td>
<td>Physician Scientist Training Program</td>
</tr>
<tr>
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</tr>
<tr>
<td>PVN</td>
<td>paraventricular thalamic nuclei</td>
</tr>
<tr>
<td>R</td>
<td>rostral</td>
</tr>
<tr>
<td>RM-ANOVA</td>
<td>repeated measures - analysis of variance</td>
</tr>
<tr>
<td>RPEC</td>
<td>retinal pigmented epithelial cell</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra, pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>substantia nigra, pars reticulate</td>
</tr>
<tr>
<td>sq</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TH-ir</td>
<td>tyrosine hydroxylase immunoreactive</td>
</tr>
<tr>
<td>UC-COM</td>
<td>University of Cincinnati College of Medicine</td>
</tr>
<tr>
<td>um</td>
<td>micrometer</td>
</tr>
<tr>
<td>Veh</td>
<td>vehicle</td>
</tr>
<tr>
<td>VM</td>
<td>ventral mesencephalon</td>
</tr>
<tr>
<td>vPL</td>
<td>ventral prelimbic cortices</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<td>weeks</td>
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Dedication

to JMO and JJO for igniting my spark for discovery with my first book of science experiments. While I never could seem to just follow what was written on the page, you always encouraged my creative exploration of what was never written…

to JPO and JCO for being my faithful, albeit unknowing, subjects throughout volumes 1, 2, and 3 of said experiments. You taught me true unconditional love as only younger siblings can, and probably also the importance of IACUC approval…

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to JS and NL for keeping me going and always in check and whose friendship has meant the world…

to DOP and DPO for always reminding me that life never stops and there’s always at least part of it that is simply beautiful…

to EBD for making me see the true value of hard work, the precious gift of strong friendship, and the remarkable tenacity of life…

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Chapter 1: Introduction

Observe, record, tabulate, communicate. *Use your five senses. Learn to see, learn to hear, learn to feel, learn to smell, and know that by practice alone you can become expert.*

*Sir William Osler, MD*
1.1 History of Parkinson disease

Parkinson disease (PD), a devastating neurodegenerative condition, is characterized by loss of motor function, leaving its victims progressively less mobile, eventually frozen in their own bodies. PD was first described in 1817 by the English physician James Parkinson, who summarized his observations as paralysis agitans, or the “shaking palsy.” Parkinson’s initial description in his Essay on the Shaking Palsy (Figure 1.1.1) described six of his patients whom he observed as being affected with “involuntary tremulous motion, with lessened muscular power in parts not in action and even when supported; with a propensity to bend the trunk forward and to pass from a walking to a running pace; the sense and intellects being uninjured (Parkinson, 2002).” In the late 1800’s Charcot, who further characterized the disease in detail, suggested the name Parkinson disease (Goetz et al., 1987a; Goetz et al., 1987b). Nearly 200 years later, this first description of what we now know as Parkinson disease remains quite accurate. The symptoms of bradykinesia (slowed movement), rigidity, and resting tremor are the most classic characteristics of PD and are referred to as the “diagnostic triad.” Additional symptoms included masked fascies, stooped posture, shuffling gait, micrographia, microphonia (reduced speech volume) and a multitude of non-motor symptoms such as constipation, and dementia or depression (Olanow & Tatton, 1999; Fahn, 2003) (See section 1.7 for more detailed discussion of PD symptoms). With an incidence of 4.5-21 cases/100,000 people and a prevalence of 120/100,000 people, it is estimated that more than one million Americans are currently suffering from PD (Olanow & Tatton, 1999).
While the initial description of PD was purely based on clinical symptoms, it was not until the work of Meynert, when the underlying anatomical features of the disease became apparent as he suggested the involvement of the basal ganglia in movement disorders, such as PD (Meynert, 1871). Near the turn of the century, Brissaud (Brissaud, 1895) hypothesized that the substantia nigra (SN) or “black stuff” of the midbrain was the region affected by PD, which was subsequently confirmed by Tretiakoff who confirmed lesions of the SN in a small group of parkinsonian patients (Tretiakoff, 1919; Lees et al., 2008). Over the course of the next five decades, the work of Carlsson and colleagues revealed the ability of a compound called levodopa to reverse hypokinesia in reserpinized rabbits (Carlsson et al., 1957), confirmed dopamine (DA) expression in the brain (Carlsson et al., 1958; Carlsson & Waldeck, 1958; Carlsson, 1959b; a), and led to the hypothesis that PD was related primarily to reduced striatal DA (Bertler & Rosengren, 1959; Carlsson, 1959b; a; Sano et al., 1959). Significant loss of DA in the caudate and putamen specifically in parkinsonian patients (and not in patients with other movement disorders) was confirmed by Hornykiewicz and Ehringer in their seminal manuscript in 1960 (Ehringer & Hornykiewicz, 1960). Levodopa reached clinical trials in the late 1960's, which confirmed its efficacy in treating parkinsonian symptoms and further supported the current hypothesis that dopamine depletion was the factor underlying PD (Bernheimer et al., 1961; Barbeau, 1962; Cotzias et al., 1967; Yahr et al., 1969; Birkmayer & Hornykiewicz, 2001). Despite the evolution of newer DA agonists and DA replacement therapies, to this day, levodopa remains the mainstay pharmacological treatment for the hypokinesia experienced in PD.
It is now well established that loss of nigrostriatal DA due to death of dopaminergic neurons in the SN pars compacta (SNpc) is the underlying histopathology common to all cases of PD. Today's treatment options remain focused on restoring striatal DA, whether through pharmacology or surgical intervention. The struggle to maintain optimal efficacy while minimizing side effects, such as dyskinesias, remains, and while surgical intervention, such as deep brain stimulation or grafting, is also promising, usage is still often restricted to only the most severe cases. Much of the current research in the field of PD is focused on identifying specific risk factors for disease initiation/progression and side effect development, as well as tailoring therapeutic intervention designed to restore not only neurochemical deficiencies, but also aberrant morphology or cell signaling.
Figure 1.1.1: Image from the original manuscript by Dr. James Parkinson describing what is known today as Parkinson's disease. Reprinted: J Neuropsychiatry Clin Neurosci 14:2, Spring 2002; Image is reproduced under permission of public domain.
1.2 Histological changes in PD

In normal aging there is a gradual loss of midbrain dopaminergic neurons throughout life, however symptomatic PD develops when about 60% of these neurons have been depleted (60% of SN neurons lost, 80% striatal DA lost = symptomatic expression) (Birkmayer & Birkmayer, 1987). This cell loss is evident not only microscopically as a reduction in pigmented catecholamine-producing neurons from the SNpc, but is also strikingly apparent grossly due to the resulting loss of neuromelanin containing neurons as a consequence of diminishing DA neurons in the substantia nigra. Neuromelanin is lost as it is discharged into the neuropil where it is taken up by macrophages. This loss of dopaminergic cell bodies in the substantia nigra translates further to a reduction in DA levels in the target nucleus of the nigral neurons, the striatum.
Figure 1.2.1: The nigra of a patient with Parkinson's disease has markedly fewer neurons and reduced pigment (right), compared to that of a non-parkinsonian patient (left). Modified with permission from UC-COM Brain and Behavior Image Bank.

Figure 1.2.2: Gross depigmentation due to loss of neuromelanin in the substantia nigra in a parkinsonian patient (left), compared to normal (right). Modified with permission from UC-COM Brain and Behavior Image Bank.
The presence of Lewy bodies is a diagnostic histological hallmark of PD. Lewy bodies present as characteristic round lamellated eosinophilic cytoplasmic inclusions, composed of degenerated neurofilaments, alpha-synuclein, and ubiquitin. They are abnormal aggregates of protein that develop inside neurons and it is believed that they represent aberrant protein processing that may be related to disease etiology.

![Lewy body in nigral neuron](image)

*Figure 1.2.3: Lewy body in nigral neuron.* Modified with permission from UC-COM Brain and Behavior Image Bank.
Importantly, neuronal degeneration in PD is not restricted to the substantia nigra. Other affected areas include the locus coeruleus, the dorsal motor nucleus of the vagus nerve, other brain stem nuclei, the hypothalamus, basal forebrain, and sympathetic ganglia. Further, the nigra may not always be the first region with observable pathology. Studies have suggested initial lesions occurring in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and anterior olfactory nucleus, followed next by gradual involvement of the nuclear gray and cortical areas (Braak et al., 2003). Braak and colleagues also hypothesize that PD progresses in an ascending manner through the brain stem, followed by cortical involvement which starts with the anteromedial temporal mesocortex, followed by the neocortex. High order sensory association and prefrontal areas are then hypothesized to be followed by first order sensory association/premotor areas and primary sensory/motor fields (Braak et al., 2003). Thus, Braak and colleagues have proposed that Parkinson's disease (PD) begins as a synucleinopathy in nondopaminergic structures of the lower brainstem (and/or olfactory bulb), progressing rostrally to include the substantia nigra, leading to parkinsonism.

Current clinical criteria for diagnosis of PD do indeed support this pattern of lower brainstem involvement (Burke et al., 2008). However, such patterns of disease progression are often not observed, particularly in dementia with Lewy bodies and when synucleinopathy occurs without accompanying neurological symptoms (Burke et al., 2008).

Braak’s model suggests that the early involvement of lower brainstem pathology predicts later progression of SN involvement and development of PD, yet, in practice,
numerous postmortem observations made in asymptomatic aged individuals have shown varying ranges of pathology (Burke et al., 2008). Additionally, there is no relation between Braak stage and the clinical severity of PD, leaving the relationship between abnormal synuclein staining and PD progression unclear (Burke et al., 2008).

Further the lines between PD and other neurodegenerative diseases are sometimes blurred, even at the level of histology. For instance, dementia with senile plaques and neurofibrillary tangles, like those characteristic of Alzheimer’s disease, develops in about 20 to 30 percent of patients with PD (Farlow & Cummings, 2008). Of note, some patients with parkinsonism and dementia have Diffuse Lewy Body disease, demonstrating Lewy bodies in cortical neurons, in addition to the characteristic nigral pathology (Farlow & Cummings, 2008). Thus, it has become increasingly apparent that there are a number of diseases which, despite different symptoms and etiologies, appear to have in common particular characteristics, such as, for example, protein misfolding.
1.3 The basal ganglia

1.3.1 Neuroanatomy of the basal ganglia:

The basal ganglia are a bilateral group of nuclei located deep within the cerebral cortex, surrounding the thalamus. In primates, the basal ganglia are somewhat enveloped by the internal capsule, a major axonal tract through the brain. Their location deep inside the brain places these structures in intimate contact with some of the phylogenetically oldest parts of the brain such as the limbic system and midbrain. The basal ganglia function as a motor control system, carefully modulating cortical, thalamic,
and inter-structural signaling for movement control (Kandel et al., 2000). This critical role of the basal ganglia in movement control is grossly evidenced by the observation that lesions of structures within the basal ganglia almost always result in movement disorders. While their role in modulating movement is commonly considered the primary function of the basal ganglia, one cannot overlook the role of these structures in emotional and cognitive function, dysfunction of which frequently accompanies movement disorders of basal ganglia origin, and is sometimes one of the early, if not only symptoms heralding these diseases in their initial presentation.

The nuclei of the basal ganglia include the caudate and putamen (together comprising the striatum), globus pallidus (GP) pars interna (GPI) and pars externa (GPE), subthalamic nucleus (STN), nucleus basalis, nucleus accumbens, substantia nigra, ventral tegmental area (VTA), and pedunculopontine nucleus (Haines, 2008). Nomenclature for these structures can be somewhat confusing as different combinations of the primary nuclei are sometimes referred to collectively. For example, the caudate + putamen are referred to as the dorsal striatum, while the caudate + putamen + globus pallidus is generally referred to as the corpus striatum (Haines, 2008).

**Note:** Catecholaminergic neurons are categorized by the nomenclature A1-A17. Each region (e.g. A1) represents embryonic cells which are future catecholaminergic neurons in a specific region of the CNS, for example the diencephalon (A11, A12, A13, A14, A15), midbrain (A8, A9, A10), rostral rhombencephalon (A5, A6, A7), and medulla
(A1, A2, C1, C2). Relevant to PD, is the specific nomenclature for future dopaminergic neurons of the midbrain. A8 neurons will become residents of the retrorubral fields, A9 are the future dopaminergic neurons of the substantia nigra, and A10 neurons are the dopaminergic neurons of the ventral tegmental area (Manger et al., 2002b; a).

**Basal Ganglia Nomenclature**

**Striatum** = caudate + putamen  
**Lentiform nucleus** = putamen + globus pallidus  
**Corpus striatum** = caudate + putamen + globus pallidus  
**Ventral striatum** = nucleus accumbens + ventral parts of the caudate and putamen  
**Ventral pallidum** = basal nucleus of Meynert (nucleus basalis)

**Figure 1.3.2: Summary of Basal Ganglia Nomenclature**
1.3.1.1 The striatum

One primary area of the basal ganglia, which is critical to normal motor control, and largely implicated in the disintegration of this control in PD, is the striatum. The striatum includes two structures, the caudate nucleus and the putamen. These two structures are intimately located deep within the brain, and in primates, are somewhat delineated by the fibers of the internal capsule, which pass between the bodies of the caudate nucleus and putamen (Kandel et al., 2000; Haines, 2008). The striatum surrounds the thalamus, and the high degree of interconnective circuitry between the putamen, caudate, and thalamus reflect their intricate signaling pathways (Kandel et al., 2000; Haines, 2008).

Figure 1.3.3 emphasizes the continuity of the putamen with the head and body of the caudate nucleus. Rostrally, the head of the caudate nucleus and the putamen are continuous with one another through the nucleus accumbens. Further caudally, the caudate nucleus and putamen are partially separated by the fibers of the internal capsule; the thin webs of tissue that connect the putamen with the body of the caudate nucleus are called striatal bridges (Haines, 2008).

While the striatum comprises a critical target structure for the dopaminergic neurons of the substantia nigra, and thus plays a role in PD; other disorders can also arise from lesions to the striatum. For example, the most common result of a lesion to the caudate nucleus is abulia (apathy, loss of initiative, loss of spontaneous thoughts and emotions) (Haines, 1985), while lesion to the rostroventral caudate nucleus can result in the movement disorder choreoathetosis (Haines, 1985; Kandel et al., 2000).
Atrophy of the head of the caudate is associated with Huntington’s disease (Haines, 1985), and both motor disturbances and mood disorder seen in Huntington’s disease correlate with cell loss in the caudate nucleus (Haines, 1985; Nance, 1998; Vonsattel & DiFiglia, 1998). Obsessive-compulsive disorder is also associated with atrophy of the head of the caudate nucleus (Hamad, 1994), and neuroimaging studies provide evidence that the head of the caudate nucleus is involved in Tourette’s syndrome (Hamad, 1994). The vast majority of neurons (95%) in the striatum are GABAergic medium spiny neurons (Kandel et al., 2000).

Figure 1.3.3: The Striatum; lateral view of the caudate and putamen. Modified with permission from UC-COM Brain and Behavior 1 Syllabus, 2005
Anatomical organization of the striatum

Motor control of specific body regions has been well mapped over the motor cortex in humans, primates and rodents. The striatum shares the same pattern of regional localization of specific body parts, in other words, a “striatal” homunculus. The dorsal striatum consists of the caudate (dorsomedial striatum in rats) and the putamen (dorsolateral striatum in rats). The ventral striatum contains the nucleus accumbens, which is divided into a core and shell. Dopaminergic projections from the VTA target the ventral striatum while nigrostriatal dopamine arising from the SNpc targets the dorsal striatum (Amalric & Koob, 1993). Figure 1.3.4 summarizes the anatomical organization of the rat striatum. Excitatory input from the frontal cortex, midline and intralaminar thalamic nuclei, basal amygdaloid complex, and hippocampal formation project topographically to specific zones within the striatum. Frontal cortical areas and their corresponding striatal projection zones are shown in the same colors.
Figure 1.3.4: Schematic representation of somatotopic organization of the striatum. Abbreviations: ACC, anterior cingulate cortex; IL, infralimbic cortex; PFC, prefrontal cortex; dPL and vPL, dorsal and ventral prelimbic cortices; CeM, CL, IMD, MD, PC and PV, central medial, central lateral, intermediodorsal, mediodorsal, paracentral and paraventricular thalamic nuclei, respectively. Reproduced by permission from Elsevier Limited, Trends in Neuroscience, (Voorn et al., 2004); license 2252560274603.
1.3.1.2 The substantia nigra and ventral tegmental area

The substantia nigra and ventral tegmental area are groups of dopaminergic neurons located in the midbrain with projections to other regions of the basal ganglia, thalamus, limbic system, and cortical structures (Kandel et al., 2000; Haines, 2008). The substantia nigra is divided into three sections, the pars compacta (SNpc), the pars reticulata (SNpr) and the pars lateralis. As previously discussed, atrophy of neuronal cell bodies in the SNpc leads to DA loss and PD. The VTA lies just medial to the substantia nigra and supplies DA to the limbic system and cortex. Although largely intact in most parkinsonian patients, degeneration of the VTA in PD is thought to underlie symptoms of depression (Birkmayer & Birkmayer, 1987; Zesiewicz et al., 1999). Hyperactivity in the mesolimbic dopamine projection pathways from the VTA to the limbic system is thought to mediate the positive symptoms of psychosis (Birkmayer & Birkmayer, 1987; Girault & Greengard, 2004).

1.3.1.3 The sub-thalamic nucleus

The subthalamic nucleus (STN) lies below the thalamus and appears structurally contiguous with the substantia nigra at its caudal end (Haines, 2008). Cell bodies located in the globus pallidus, externa (GPe) segment project to the STN, providing inhibitory GABAergic input to this excitatory glutamatergic nucleus. Efferent glutamatergic fibers from the STN project to the globus pallidus, interna (GPI) segment and to a lesser degree the SNpr (Kandel et al., 2000; Haines, 2008). The subthalamic nucleus is a key component of a pathway known as the ‘indirect pathway’ (detailed
below) of the basal ganglia and is also a critical target of deep brain stimulation therapy for relief of parkinsonian motor symptoms (see discussion in treatment of PD).

1.3.1.4 The globus pallidus

The globus pallidus (GP) is divided into two smaller nuclei, the GPI, and GPe. The GP consists of predominately GABA producing neurons and thus provides inhibitory output to the thalamus both directly (from the striatum to the GPI; the *direct* pathway) and via the STN (from the striatum to the GPe, to the STN, then to the GPI; the *indirect* pathway) (Kandel et al., 2000; Haines, 2008). There is evidence that suggests that the combined damage to the frontal lobe, caudate nucleus, and the globus pallidus may account for the repetitive behavior seen in frontal lobe degeneration and possibly obsessive compulsive disorder (Alexander et al., 1990; Kandel et al., 2000).

1.3.1.5 The pedunculopontine tegmental nucleus

The pedunculopontine tegmental nucleus (PPN) is one of the main components of the reticular activating system and contains multiple neural cell types including cholinergic, glutamatergic and GABAergic cells (Kandel et al., 2000). The PPN has a wide range of projections sending axons to multiple targets in thalamus, basal ganglia, cerebral cortex, basal forebrain, and lower brainstem, and receives direct afferents from the medial pallidum. The PPN has many functions and is involved in arousal, attention,
Neuronal cell loss is seen in the pedunculopontine tegmental nucleus in Parkinson’s disease, Alzheimer’s disease, and progressive supranuclear palsy (PSP). There is evidence for this nucleus playing a role in the pill-rolling tremor of Parkinson’s disease. Additionally, the PPN has been implicated in the generation of PD motor symptoms such as akinesia, gait dysfunction, and postural instability (Munro-Davies et al., 1999; Pahapill & Lozano, 2000; Nandi et al., 2002). The PPN is a potential target for deep brain stimulation as several case reports have demonstrated the efficacy of low-frequency stimulation of the PPN in the reduction of PD motor symptoms (ie (Plaha & Gill, 2005). Although PPN stimulation appears inferior to STN-DBS (Stefani et al., 2007), there may be promise in combined PPN + STN-DBS (Stefani et al., 2007).

1.3.1.6 The nucleus accumbens

The nucleus accumbens is often referred to as the “pleasure center,” and is intricately involved in DA-mediated reward pathways. Abnormalities in the projection from the hippocampus to the nucleus accumbens can result in the thought disorganization and psychosis seen in schizophrenia (Kandel et al., 2000). The projections of the mesolimbic DA system to the nucleus accumbens serve as the final common pathway of reward. Importantly, numerous psychotropc drugs of abuse cause dopamine to be released into the nucleus accumbens yielding a much more intense reward than in drug-free reward circuitry (Kandel et al., 2000). As DA receptors on
neurons in the nucleus accumbens become sensitized to drugs of abuse the demand for receptor stimulation (and frequency/quantity of drug use) increases, leading to establishment of addiction circuitry (Kandel et al., 2000; Girault & Greengard, 2004). Anhedonia or depression experienced by parkinsonian patients may be due in part to depletion of DA signaling at the level of the nucleus accumbens.

1.3.1.7 The nucleus basalis (basal nucleus of Meynert)

The nucleus basalis is the major brain center for cholinergic neurons that project throughout the cortex. These neurons have the principal role in mediating memory formation (Garcia-Alloza et al., 2006). Specific degeneration of the nucleus basalis leads to cholinergic deficiency syndrome, which may be responsible for the more limited short-term memory problems associated with aging referred to as mild cognitive impairment (Chozick, 1987; Cummings & Benson, 1987; Jellinger, 1991; Donnet, 1993). Degeneration of cholinergic neurons in this nucleus is also associated with Alzheimer’s disease as well as PD (Chozick, 1987; Cummings & Benson, 1987; Jellinger, 1991). Generalized reduction of acetylcholine production underlies the cognitive dysfunction seen in Alzheimer’s, Lewy body dementia, and PD patients with cognitive impairment (Chozick, 1987; Cummings & Benson, 1987; Jellinger, 1991; Donnet, 1993).

1.3.2 General circuitry in the normal basal ganglia

The basal ganglia are a collection of nuclei at located in the ventral telecephalon. These structures receive signals from widespread areas of cerebral cortex
(sensorimotor, association, and limbic) and route integrated responses to these signals back to the frontal cortex. The primary role of the basal ganglia is to act as a processing station for cortical motor information. Cortical information is processed through a series of multiple parallel channels as afferent signals pass through the basal ganglia. The general motor circuitry involving the basal ganglia is: Cortex $\rightarrow$ Basal Ganglia $\rightarrow$ Thalamus $\rightarrow$ Cortex, and the basic mechanism of operation of the basal ganglia is through disinhibition (Kandel et al., 2000; Shepherd, 2004). Consequently, damage to the basal ganglia often results in the release of unwanted behaviors, such as those seen in Huntington’s disease, or with levodopa treatment in PD. In addition to being involved in strategic motor planning, the basal ganglia also operate in close harmony with the frontal lobes in the acquisition, retention, and expression of cognitive behavior.

There are five distinct circuits or loops through the basal ganglia: skeletomotor, oculomotor, limbic and two association circuits (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004). Each loop originates from multiple cortical regions that have similar general functions, and passes through different basal ganglia and thalamic nuclei, or separate portions of the same nucleus. These loops are somatotopically organized throughout, and the cortical targets of all loops are distinct regions of the frontal lobe (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004).

The primary function of the association circuits is their role in cognition. These circuits receive input from many areas of cortex and are responsible for relating motor activity to environmental targets. The association circuits project to the frontal association area of the cortex, including the dorsolateral prefrontal cortex, which are
important in organizing behavior (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004).

The limbic loop pathway is centered on the ventral striatum and is associated with emotional behavior and with the motivational aspects of motor behavior. Projections originate from many areas of the brain including the hippocampus, entorhinal cortex, medial and lateral temporal lobes, amygdala, VTA, and nucleus accumbens. The primary cortical target of the ventral pallidum is the anterior cingulate area and medial orbitofrontal cortex directly, and indirectly via the dorsomedial nucleus of the thalamus (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004).

The skeletomotor loop is centered on the putamen and its connections. The putamen receives somatotopically organized (arm, leg, face) inputs from the primary motor, primary sensory, somatosensory association, premotor, and supplementary motor cortices. The putamen projects to both internal and external segments of the globus pallidus and the substantia nigra pars reticulata (SNpr) (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004). Both the GPi and SNpr are the output nuclei of the basal ganglia and project to the thalamus. The motor loop is completed by thalamocortical projections to the supplementary motor, premotor, and primary motor cortices. There is a projection from the pallidothalamic component of the motor loop from the GPi to the pedunculopontine nucleus (PPN). This nucleus, located in the brainstem and part of the reticular formation, influences motor (locomotion) and vegetative (sleep-wake cycle) systems in the pons and medulla (Alexander et al., 1990; Kandel et al., 2000; Shepherd, 2004). A side loop in this motor pathway passes from
the putamen to the GPe/i and from there to the subthalamic nucleus and back to the GPi.

1.4 Basal ganglia circuitry in the parkinsonian brain

The motor symptoms of PD result from this preferential loss of dopaminergic neurons in the SNpc (Ehringer & Hornykiewicz, 1960; Agid, 1989). Dopaminergic output from the SNpc projects to the medium spiny neurons (MSNs) of the striatum where it acts as a modulator of cortical glutamatergic input. In PD, loss of nigral DA neurons leads to a reduction of dopaminergic input to the striatum, disrupting the critical balance between striatal DA and glutamate, and leading to alterations of both the direct and indirect pathway outputs to the thalamus (see below). Current treatment modalities for PD focus on restoration of DA levels in the striatum and include the use of DA agonists such as levodopa, the gold standard of treatment. Experimental therapies include targeted lesioning of basal ganglia structures, and embryonic dopaminergic tissue engraftment into the striatum.

Striatal MSNs are the main output neurons of this structure and synapse upon the main output nuclei of the basal ganglia; the GPi as well as the SNr via two distinct efferent pathways from the striatum, known as the direct and indirect pathways. The direct pathway involves inhibitory monosynaptic connections from GABAergic striatal MSNs expressing the D1 DA receptor (D1R) directly upon neurons of the GPi/SNr. In contrast, GABAergic striatal MSNs of the indirect pathway, which predominately express the D2 DA receptor (D2R), project first to the GPe and STN before reaching the
GPI and SNr (Crossman et al., 1987; Albin et al., 1989; DeLong, 1990). GABAergic neurons in the GPe form inhibitory synapses onto glutamatergic neurons in the STN, thus reducing excitatory output from the STN to the GPI and SNr. Endogenous DA excites D1R expressing cells and inhibits D2R expressing cells such that in the normal basal ganglia, DA from the SNc activates the direct pathway and inhibits the indirect pathway (Crossman et al., 1987; Albin et al., 1989; DeLong, 1990). In PD the loss of cells within the SNc results in hyperactivity of the STN and GPI leading to over-inhibition of excitatory glutamatergic thalamocortical pathways necessary in the initiation and execution of motor movements. Thus in PD, activity of the direct pathway is decreased, while activity of the indirect pathway is increased, effectively shutting down thalamic and brainstem motor circuits as summarized in Figure 1.4.1.

In PD, loss of DA output from the SN results in diminished dopaminergic stimulation of striatal DA receptors. Normally, DA-mediated activation of the direct pathway enables disinhibition of the thalamus, while activation of the indirect pathway maintains thalamic inhibition. Diminished nigral DA leads to dysregulation of both the direct and indirect pathways such that there is loss of stimulation of the direct pathway and loss of inhibition of the inhibitory indirect pathway. In the direct pathway, reduced nigral DA results in reduced activation of striatal D1 receptors, followed by subsequent reduction in GABA-mediated inhibition of the output nuclei of the basal ganglia, the GPI and SNpr. Disinhibition of the GPI and SNpr leads to GABA mediated over-inhibition of the thalamus, reducing excitatory glutamate signaling from the thalamus to the motor cortex, and thus inhibiting initiation of motor activities. In the indirect pathway, loss of nigral DA reduces the tonic inhibition of the GPe, which normally mediated by DA-
mediated stimulation of D2 receptors on striatal GABAergic, enkephalin expressing neurons. Over-inhibition of the GPe in the parkinsonian brain reduces inhibitory control of the glutamatergic STN. Hyperactivity of the glutamatergic STN results in hyperactivation of the major output nuclei of the basal ganglia, the GPi and SNpr. As in the direct pathway, where release of inhibition of GPi and SNpr results in thalamic overinhibition, glutamatergic hyperactivity from the STN also drives thalamic overinhibition, markedly reducing thalamic activation of motor cortex.

It is important to note that while classic dogma in the field has supported the concept of distinct indirect and direct pathways within the basal ganglia, there is increasing support of the hypothesis that signaling pathways of the basal ganglia involve a complex network that is not simply two distinct circuits (Nambu, 2004; Tachibana et al., 2004; Nambu, 2005; Leblois et al., 2006; Nambu, 2009). Further, it is postulated that neurons characteristically defined as belonging to either the direct or indirect pathway, likely participate in modulation of signaling in multiple pathways (Nambu, 2004; Tachibana et al., 2004; Nambu, 2005; Leblois et al., 2006; Nambu, 2009). Future studies will continue to reveal details outlining these intricate neural networks. With this caveat in mind, it remains clear that the basal ganglia supports integration and coordination of cortically-driven movements with both permissive and inhibitory signaling pathways.
Figure 1.4.1: Schematic of Normal and Parkinsonian basal ganglia circuitry.

Reproduced with permission from Dr. Christopher J. Brubaker, Ph.D.; 2005.
1.5 Risk factors for Parkinson disease

While there are some familial cases of PD associated with mutations in specific genes, the majority of cases appear sporadic in nature, and the factors that initiate and maintain the degradation of the SNpc underlying PD remain uncertain. The clearest and most decisive risk factor for PD is advancing age. Although the age of onset generally ranges from 35-85 years old, most patients develop symptoms after the age of 60 years old. In fact, 75% of all cases of PD begin after the age of 60, and the incidence of disease increases with each decade up to 80 years of age. Young onset PD (less than 40 years old) occurs, but is much rarer and is associated with a more rapid progression of disease course (Conn & Rakel, 2009).

Other risk factors for PD include gender, genetics, ethnicity, and environmental factors. Men are about 1.5 times more likely than women to develop Parkinson’s disease and there is some evidence that estrogen may be neuroprotective for dopamine neurons in women (Fauci, 2008; Conn & Rakel, 2009). Race also plays a role, as caucasians are at a greater risk for PD and research suggests that blacks and Asians have a slightly lower rate of Parkinson’s disease than whites (Fauci, 2008; Conn & Rakel, 2009).

More than half a dozen genes, including Parkin and alpha-synuclein, have been associated with Parkinson's disease (Figure 1.5.1), although the vast majority of PD cases (95%) are idiopathic, with no identifiable underlying genetic mutation. Identified genetic mutations and/or a family history of PD are both clear risk factors for development of PD. Those patients who do have genetic mutations are at greater risk for earlier onset PD, usually before the age of 50 (Fauci, 2008; Conn & Rakel, 2009).
Further, genetic forms of PD are generally associated with a longer disease course. One exception is the genetic form LRRK-2, which causes PD in the same age range as “sporadic” PD. Despite the observation that the great majority of PD cases have no apparent underlying genetic determinant, epidemiological studies support a complex interaction of genetic vulnerability and environmental factors. Further, although there is no clear unifying theme revealed by analysis of parkinsonism-associated genes, a potential site of action is the mitochondrion. Mitochondrial toxins have been implicated in the etiology of PD (Beal, 2003) and other studies have demonstrated that mitochondrial complex 1 activity is impaired in parkinsonian patients (Beal, 2003). Further, at least three genes (parkin, DJ-1, and PINK-1) associated with hereditary PD have been linked to mitochondria.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK 1</td>
<td>SNCA</td>
<td>alpha-synuclein</td>
<td>Uncertain: vesicle trafficking</td>
<td>ad</td>
</tr>
<tr>
<td>PARK 2</td>
<td>PRKN</td>
<td>Parkin</td>
<td>E3 ubiquitin ligase</td>
<td>ar</td>
</tr>
<tr>
<td>PARK 4</td>
<td>SNCA</td>
<td>alpha-synuclein (triplication or duplication)</td>
<td>Uncertain: vesicle trafficking</td>
<td>ad</td>
</tr>
<tr>
<td>PARK 5</td>
<td>UCH-L1</td>
<td>UCH-L1 (Ubiquitin carboxy-terminal hydroxylase L1)</td>
<td>Proteasomal processing</td>
<td>ad</td>
</tr>
<tr>
<td>PARK 6</td>
<td>PINK1</td>
<td>PINK1</td>
<td>Mitochondrial kinase</td>
<td>ar</td>
</tr>
<tr>
<td>PARK 7</td>
<td>DJ-1</td>
<td>DJ-1</td>
<td>Oxidative stress response</td>
<td>ar</td>
</tr>
<tr>
<td>PARK 8</td>
<td>LRRK2</td>
<td>Dardarin</td>
<td>Cytosolic kinase</td>
<td>ad</td>
</tr>
</tbody>
</table>

**Figure 1.5.1: Genetically Based Parkinson Disease** *(modified from Harrison’s Internal Medicine, 17th edition)*
Exposure to environmental toxins such as herbicides and pesticides likely increases one’s risk of PD. There is increased risk of PD associated with living in rural areas, drinking well water, or living on a farm; again most likely due to increased exposure to herbicides and pesticides (Fauci, 2008; Conn & Rakel, 2009). Rotenone is a well-known pesticide often used in organic farming that is established to cause parkinsonism in animal models (Hoglinger et al., 2006; Schmidt & Alam, 2006) and increase the incidence of PD in humans (T. Greenamyre, personal communication). Environmental toxicants such as rotenone act through multiple mechanisms including inhibition of mitochondrial complexes similar to that observed in PD (Schapira, 2008; Bueler, 2009) and increasing neuronal levels of alpha-synuclein, a protein strongly implicated in PD pathogenesis (Liu & Yang, 2005). Understanding mechanisms of environment toxicants and interactions with genetic susceptibility factors associated with PD will lead to elucidation of the etiology of PD.

Finally, there is some evidence that head trauma impacting dopaminergic neurons (Fauci, 2008; Conn & Rakel, 2009) or use of illicit drugs such as cocaine (Lloyd et al., 2003) may increase risk for PD due to deleterious effects on dopaminergic cell survival within the brain. In contrast, reduced incidence of PD is associated with coffee drinking, smoking, use of nonsteroidal anti-inflammatory drugs, and estrogen replacement in postmenopausal women (Fauci, 2008; Conn & Rakel, 2009).

In summary, the primary risk factor for PD is advanced age, however other factors including being male, Caucasian, a family history of PD, or exposure to particular environmental toxins can impact susceptibility to this neurodegenerative disease.
1.6 The role of age in Parkinson disease

As discussed in section 1.5, advanced age is the most significant risk factor for development of PD demonstrated by an increased incidence of PD with increasing age (Casetta et al., 1990; Mayeux et al., 1995; Morens et al., 1996; Tanner & Goldman, 1996; Marras et al., 2005; Totaro et al., 2005). Importantly, there are changes at both the cellular and behavioral levels in human and non-human primates that suggest a link between normal aging processes and pathogenesis of PD. For example, a significant decline in striatal dopamine levels and increase in the homovanillic acid/dopamine molar ratios in non-parkinsonian humans is reported to accompany advancing age (Kish et al., 1992). Further, the CNS demonstrates multiple global changes with age including but not limited to cell shrinkage, loss of synaptic connections, dysregulation of phenotype, and impaired neurotransmission (Dickstein et al., 2007).

The presence of PD motor symptoms in non-parkinsonian aged individuals increases with age such that over half of people over the age of 85 show at least two of the cardinal behavioral symptoms of PD, compared to 30% of individuals aged 75-84, and only 15% of individuals aged 65-74 (Bennett & Castiello, 1996). This observation is also true in aging non-human primates as studies have shown age-related motor deficits in nonhuman primates with accompanying nigrostriatal dysfunction (Emborg et al., 1998). Further, aged monkeys demonstrate generalized impairment in locomotion and skilled motor tasks accompanying spontaneous age-related loss of TH and DAT immunoreactivity in the SN (Emborg et al., 1998).

Normal aging is also associated with cellular and behavior changes in both the SN and striatum. The question of whether or not significant cell loss in the SN is
associated with normal aging has conflicting results, as there is considerable species-specific variability in the patterns of age-related changes in the DA system (Morgan et al., 1999). For example in nonhuman primates, while there is general agreement that striatal DA declines with advanced age, associated changes in the number and function of nigral DA neurons remains a point of debate (Goldman-Rakic & Brown, 1981; Irwin et al., 1994; Pakkenberg et al., 1995; Emborg et al., 1998; Gerhardt et al., 2002; McCormack et al., 2004). Nonetheless, the age-related reduction in expression of DA phenotype in the SN does appear to be accompanied by both a reduction in the number of neurons expressing DA markers as well as reduced immunoreactivity for these markers (Embrog et al., 1998).

Such observations of age-related changes in dopaminergic phenotype of the striatum and nigra have contributed to several theoretical models of PD as a model of pathologic aging. Initial interpretations portrayed PD as unsuccessful aging, followed by the Calne-Langston hypothesis, which proposed that PD is the result of an insult superimposed upon aging-related changes which subsequently leads to symptomatic DA-depletion (Calne & Langston, 1983). In the age of cancer research which introduced the two-hit hypothesis combining genetic and environmental events to contribute to the intiation of tumorigenesis, the Calne-Langston hypothesis further evolved to a multiple-hit hypothesis, where PD is a result of the combination of more than one environmental or genetic event with aging-related changes (Thiruchelvam et al., 2003; Thiruchelvam et al., 2004; Carvey et al., 2006). In both models the progressive depletion of striatal DA over the course of an individual’s lifetime brings them ever closer to the threshold for symptomatic PD.
Studies by Kanaan et. al. further extended the hypothesis regarding PD and aging by demonstrating significant striatal DA depletion accompanying advancing age in normal rhesus monkeys (Kanaan et al., 2008). Further, these studies demonstrate dramatic loss of nigrostriatal biochemical compensatory mechanisms in aged individuals (Kanaan et al., 2008). Prior studies in the same animals by Collier and colleagues demonstrated that while young animals respond to DA depletion by increasing striatal trophic factors, aged animals were incapable of increasing striatal trophic activity in response to DA depletion (Collier et al., 2005).

These studies led to conclusions by Kanaan et al. that the loss of compensatory mechanisms in aged subjects “may be more central to the role of aging in the development of PD,” and the hypothesis that loss of plasticity in advanced age leads to a reduced ability to recover from a lifetime of insults to the DA system that likely lead to parkinsonism. Kanaan and Collier expanded upon the Calne-Langston and multiple-hit hypotheses to develop the Cycle Acceleration Hypothesis of PD. In this newly proposed model, normal aging is associated with striatal DA loss and DA neuron dysfunction resulting from the interaction of numerous other factors that influence DA neuron dysfunction and susceptibility to degeneration. PD is modeled as a continuum of accelerated DA loss and accelerated aging. Factors such as prenatal infections, environmental toxins, genetic predispositions, and natural intracellular predisposing factors such as DA metabolism and oxidative stress are proposed to influence the susceptibility of DA neurons to degeneration (Kanaan et al., 2007; 2008).

In the striatum, both normal aging humans and aged non-human primates demonstrate decreased striatal DA and DA phenotypic markers (Kish et al., 1992;
Emborg et al., 1998; Collier et al., 2005). Aged monkeys with motor impairment have been shown to have concurrent DA loss in the putamen and such motor impairment is further associated with decreases in striatal DA and metabolites, decreased DA release, decreased number of TH neurons, decreased TH fiber density, and decreased soma size of TH+ neurons (Kanaan et al., 2008).

Additionally, aging is associated with reductions in dendritic spine density, loss or regression of some dendritic branches, and alterations in cytoarchitecture of neocortical neurons (Jacobs et al., 1997; Peters et al., 1998b; Duan et al., 2003). There also is a loss of general plasticity associated with aging, which extends to loss of synapses (Peters et al., 1998a; Peters et al., 1998b; Peters et al., 2008) and dendritic spine loss (Dickstein et al., 2007). Such age-related decline in plasticity is thought, at least in part, to be associated with the age-related loss of growth factors. The combined decline in plasticity and growth factors is regarded to be central to diminished efficacy of transplantation therapy in the aged parkinsonian striatum compared to younger graft recipients (Freed et al., 2001). Age related reduction of growth factors and plasticity likely impact DA graft efficacy in aged subjects due to resultant lack of trophic support for survival of young grafted cells (which are heavily dependent on such factors), and inability of surviving grafted cells to make appropriate synaptic contacts due to reduced plasticity in the aged striatum.

Since dendritic spines are the major recipients of cortical glutamtergic input and nigral dopaminergic input, this indicates that there is a loss of input from cortical neurons to striatal MSNs with age. Such loss of synapses has been demonstrated in rats (Chen et al., 1995; Wong et al., 1998), monkeys (Uemura, 1980), and humans
(Adams, 1987b; a; Masliah et al., 1993a; Masliah et al., 1993b). It is hypothesized that changes in striatal dendritic spine morphology and synaptic connectivity are implicated in the pathogenesis of LIDs in the parkinsonian brain. Further the loss of dendritic spine plasticity and the accompanying loss of physiological targets for DA reinnervation have significant ramifications for neural grafting or gene therapy aimed at restoring striatal DA terminal reinnervation.

1.7 Symptoms of Parkinson disease

The cardinal motor signs of PD are resting tremor, rigidity and bradykinesia. Diagnosis of PD requires presentation of at least two out of the three motor symptoms; reviewed in detail below. Early disease is also marked by masked facies, decreased eye blinking, stooped posture, and decreased arm swing. Patients may also report vague symptoms such as weakness, fatigue, aching, and discomfort (Conn & Rakel, 2009).

Resting Tremor: Resting tremor is present in 85% of patients with true PD, and is a particularly important factor in distinguishing true PD from other parkinsonian-like syndromes. Resting tremor occurs at 4-6 Hz in frequency and typically begins unilaterally and distally, principally involving the hand. The tremor is often described as “pill-rolling” due to the specific involvement of the digits and wrist early in presentation. Although initially unilateral, the tremor usually spreads from the arm first to the ipsilateral leg, and after a year or more to involve both sides of the body. Tremor can
eventually involve the lips, tongue and jaw (but usually spares the head and neck). The initially unilateral and gradual onset of symptoms, followed by increased severity and spread to both sides, is characteristic of PD (Conn & Rakel, 2009).

**Bradykinesia:** Bradykinesia is the phenomenon described as slowness or poverty of movements. A person with bradykinesia often also has incomplete movement, difficulty initiating movements and sudden stopping of ongoing movement. A person with bradykinesia tends to walk with short, shuffling steps referred to as a festinating gate. There is also marked loss of fine motor control, evidence by a decrease in dexterity, and by micrographia (shrinking handwriting). The voice can also be affected as a result of bulbar bradykinesia, presenting as soft speech (hypophonia). The characteristics of bradykinesia make it often described as the most disabling motor symptom of PD; one that greatly interferes with activities of daily living (Conn & Rakel, 2009).

**Rigidity:** Rigidity is defined as uniform resistance to passive movement about a joint throughout its full range of motion. “Cogwheel” rigidity is a diagnostic finding characterized by brief regular interruptions of passive resistance due to the presence of tremor. Rigidity can be quite troublesome and even dangerous for patients as they are unable to respond quickly to their environment or to impending threats (such as a sudden loss of balance, slipping on wet pavement, or an oncoming car).
Patients with PD also experience gait disturbance characterized by shuffling short steps and a tendency to turn “en bloc,” or in a squared off pattern, as opposed to a smooth arcing turn. Patients demonstrate a “festinating gait,” a result of the combination of flexed posture and loss of postural reflexes which results in patients accelerating during walking to “catch up” with their center of gravity. More advanced PD is characterized by freezing of gait upon onset of locomotion, attempts to change direction, or environmental challenges (such as entering a crowded room). Balance and postural problems increase with disease progression exhibited by flexion of the head, stooping posture, forward tilting of the upper trunk, and holding the arm in a flexed posture while walking. For patients with advanced PD, postural instability is one of most disabling motor symptoms of the disease, leading to falls and injuries, and associated with increased morbidity and mortality (Conn & Rakel, 2009).

**Non-motor:** While PD is classified as a movement disorder and clinically diagnosed based on presenting *motor* symptoms, it is also important to recognize that there are many *non-motor* symptoms that are also characteristic of the disease. Non-motor symptoms include autonomic dysfunction (i.e. orthostatic hypotension or urinary incontinence), cognitive impairment, and emotional or behavioral dysfunction (i.e. depression, anxiety, dementia, and psychosis) (Conn & Rakel, 2009). Additionally patients may experience sensory dysfunction including disturbances in sense of smell, taste, vision, and pain (Conn & Rakel, 2009). Many patients also suffer from sleep disorders, further aggravating concurrent cognitive or emotional disturbances. Figure 1.7.1 summarizes the motor and non-motor features of PD.
## Symptoms of Parkinson’s Disease

### Cardinal Motor Symptoms
- Bradykinesia
- Postural instability
- Rigidity
- Tremor

### Autonomic Dysfunction
- Cardiovascular (orthostatic hypotension)
- Gastrointestinal (dysphagia, drooling, constipation, delayed gastric emptying)
- Sexual (erectile dysfunction, decreased desire and arousal)
- Thermoregulatory (hyperhydrosis)
- Urologic (increased frequency and urgency, incontinence, nocturia)

### Neuropsychiatric Dysfunction
- Anxiety
- Depression
- Dementia
- Psychosis

### Sensory Dysfunction
- Olfactory disturbance (anosmia)
- Pain
- Paresthesia
- Visual disturbances (abnormal eye movements, blurred or double vision)

### Sleep Dysfunction
- Excessive daytime sleepiness
- Insomnia or fractionated sleep
- Sleep disorders (REM behavior disorder, sleep apnea, restless legs syndrome)

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**Figure 1.7.1:** Summary of symptoms of PD *(Modified from Conn’s Current Therapy 2009)*
1.8 Symptomatic treatment of Parkinson disease

A) Levodopa

B) Dopamine

Figure 1.8.1: The chemical structures of levodopa (A) and dopamine (B). In the brain, decarboxylation of the COOH from levodopa by AADC yields dopamine. Images originated in ChemDraw, 2006 and reproduced by permission of GNU Free Documentation License.

1.8.1 Pharmacotherapy:

Symptomatic treatment of PD is aimed at restoration of striatal DA and reversal of the motor deficits described previously. Several pharmacological options are available to patients, however, the “gold standard” and most commonly used agent is the dopamine precursor levodopa (Figure 1.8.1). Levodopa is administered orally in combination with a peripheral dopamine decarboxylase inhibitor such as benserazide or carbidopa (Carbidopa + Levodopa = Sinemet). Coadministration of levodopa with
benserazide or carbidopa prevents peripheral degradation of levodopa, reducing the
dose of levodopa needed for symptomatic relief, and maximizing CNS concentration of
the drug. Peripheral decarboxylase inhibition is also of great importance in preventing
or reducing peripheral side-effects of levodopa such as nausea, vomiting, and
cardiocascular effects. Levodopa is absorbed from the gut and crosses the blood-brain
barrier via the large neutral amino acid transporter. In the brain, levodopa is converted
to DA via the aromatic amino acid decarboxylase (AADC) enzyme and packaged into
synaptic vesicles for neuronal-activation mediated release. Figure 1.8.2 summarizes
the peripheral and central metabolic pathways of levodopa.
Other direct acting DA receptor agonists are also used clinically, but none are as efficacious in relieving parkinsonian motor symptoms as levodopa. Most DA agonists act at specific DA receptors, but none are entirely selective, thus generally acting on combinations of DA receptors. While it is known that changes in DA receptor activity are involved in parkinsonian behaviors, the exact pattern, timing, and degree of receptor stimulation necessary in order to relieve motor symptoms is yet unknown. In contrast to specific receptor agonists, levodopa is converted into DA in the brain, and can therefore
act as endogenous DA in the parkinsonian brain, acting at whichever combination of DA receptors necessary for maximal therapeutic benefit.

Additional drug classes such as catechol-0-methyl transferase (COMT) and monoamine oxidase-B (MAO-B) inhibitors are employed to reduce the peripheral and central degradation of DA. The rationale behind the use of these drugs is aimed at 1) increasing availability of the deficit neurotransmitter and 2) reducing the amplitude of fluctuations in striatal DA and prolonging its availability in the synapse.

Non-DA drugs are also used as adjunct therapeutics based on the neurochemistry of the striatum. For example, the large interneurons of the striatum contain the neurotransmitter acetylcholine (ACh). There is an important balance between DA and ACh in striatum for normal motor function. Motor symptoms in Parkinson’s disease are secondary to an altered dopamine-acetylcholine balance: specifically reduced striatal dopaminergic tone and subsequent cholinergic overactivity. Thus, anticholingeric drugs are an important symptomatic adjunct therapy for PD, and were the primary therapeutic option for PD prior to the discovery of levodopa. Adenosine receptor antagonists (A2A), and NMDA antagonists have also been shown to have anti-parkinsonian actions, although these drugs are not as effective in relieving motor symptoms as levodopa.

Ongoing clinical trials are focused on improved pharmacotherapy options for patients via multiple approaches such as (1) dopaminergic modulators (such as safinamide or pardoprunox), which would have multiple mechanisms of action (i.e. pharmacological targets) and allow for elimination or reduction of levodopa dosage, (2) new formulations of levodopa (long-acting formulations, pump administration) to help
prevent side effects related to pulsatile delivery, (3) enhanced endogenous dopamine
release (nitisinone) to maximize endogenous function and reduce levodopa usage, (4)
non-dopaminergic transmitters involved with basal ganglia function (A2A antagonists,
serotonin agonists and antagonist, anti-glutamatergics, anti-adrenergics) to increase
the arsenal of therapeutic choices available, (5) non-motor targets (such as sleep
disorders, orthostatic hypotension, urinary dysfunction, constipation) to expand
symptomatic relief beyond motor symptoms, (6) relief of neuropsychiatric symptoms
(including depression, PD psychosis), and (7) disease modification or neuroprotection
strategies (e.g. isradipine, uric acid, coQ10) to slow progression of the disease. While
research continues for more efficacious pharmacotherapy, symptomatic PD treatment
remains limited by waning pharmaceutical efficacy with progressing disease and
development of side effects such as LIDs.
Limitations of PD Therapy

- Symptomatic therapy works well in most PD patients for only 3-5 years
  - Side effects: Dyskinesias
  - Symptoms become resistant to therapy

- Patients treated with LDOPA for 4-6 years have a 40% risk of developing LIDs
  - Uncontrollable hyperkinetic movement and dystonia
  - Onset of LID is unpredictable and occurrence is highly variable
  - Likely due to unregulated levels of DA and glutamate within the basal ganglia

- Understanding cellular mechanisms related to side-effect development and waning therapeutic efficacy will allow for the development of improved treatment strategies.

**Figure 1.8.3:** Summary of the limitations of PD pharmacotherapy. Photos of parkinsonian patient experiencing LIDs. Photos courtesy of Dr. Kathleen Shannon, Department of Neurology, Rush University, Chicago IL
1.8.2 Therapeutic surgical options

Surgical options for late stage disease or PD refractory to pharmacotherapy include pallidotomy (rarely today), and deep brain stimulation (DBS). Experimental options include engraftment of fetal dopaminergic cells into the substantia nigra, caudate, and/or putamen. Further, many researchers and patients are interested in the potential therapeutic and disease modifying effects of gene therapy options for PD. Historically, surgical interventions for PD are considered invasive and are reserved for only those patients whose disease is refractory to pharmacotherapy, or those patients with end stage disease. Current research however has suggested that earlier interventions such as DBS or grafting may offer superior treatment compared to delayed intervention (Volkmann, 2007; Volkmann & Deuschl, 2007; Laguna Goya et al., 2008), and may also slow the course of disease progression via neuroprotective mechanisms (Breit et al., 2004). Each of these surgical interventions are discussed in detail in the following sections.

1.8.2.1 Gene therapy

Multiple clinical trials are examining the efficacy of gene therapy for PD. Gene therapy employs viral vectors to introduce a gene of interest to a brain region of interest. Examples of gene products of interest in PD therapy include glia cell-line derived neurotrophic factor (GDNF) and neurturin delivered to the striatum to enhance sprouting of DA terminals in this region, and the enzyme glutamic acid decarboxylase (GAD) induced in the STN to dampen glutamatergic overactivity in this region.
Most gene therapy trials remain in early stages of development, such as the recent phase one trial of glutamic acid decarboxylase (GAD) gene transfer into neurons of the STN. As previously discussed, loss of inhibitory GABAergic input results in overactivity of the STN in the parkinsonian brain. As a result the uninhibited STN drives other basal ganglia regions such as the GPi and SNpr into hyperactive states. Introduction of GAD into the STN via an adeno-associated viral (AAV) vector is hypothesized to allow the STN to produce GABA as well as release GABA onto its target structures, ideally restoring normal patterns of cell signaling within the basal ganglia; further resulting in reduced motor symptoms, and potentially avoiding more invasive interventions such as DBS. Animal studies (Bankiewicz et al., 1998; Emborg et al., 1998; Luo et al., 2002; Lee et al., 2005; Emborg et al., 2007) followed by initial clinical trials, determined the safety of GAD gene therapy (During et al., 2001; Kaplitt et al., 2007; Kaplitt, 2008). Recruitment for phase 2 trial of GAD gene therapy is ongoing and follow-up trials are beginning in effort to determine whether this treatment is in fact clinically efficacious in parkinsonian humans (During et al., 2001; Kaplitt et al., 2007; Kaplitt, 2008).

Other gene therapy trials are now further in development, such as the double blind placebo controlled study which examined the safety and efficacy of CERE-120 (adeno-associated virus serotype 2 [AAV2]-neurturin [NTN]) in the treatment of patients with idiopathic PD. Neurturin is a neurotrophic factor shown to support the survivability of midbrain DA neurons (Rosenblad et al., 1999). Striatal delivery of human NTN via the AAV2 gene vector has been shown to enhance activity of the dopaminergic nigrostriatal system in aged monkeys (Herzog et al., 2007), suggesting its utility as a
clinical therapeutic intervention. Initial clinical data supports that administration of CERE-120 is a safe, well-tolerated, and potentially efficacious treatment for PD (Marks et al., 2008), however these results remain preliminary until publication of results from a recently completed double-blinded placebo control trial in which CERE-120 was administered via bilateral stereotactic injections into the parkinsonian putamen of 34 patients, with 17 patients receiving sham stereotactic surgery (no CERE-120 administered) (ClinicalTrials.gov identifier: NCT00400634).

Multiple other targets are being investigated as potential gene therapy targets in animal models. For instance, the trophic factors pleiotrophin and glial derived neurotrophic factor (GDNF) are of particular interest for their potentially neuroprotective properties. As we begin to understand more about the etiology of PD and treatment related side effect such as LIDs, additional targets of interest will be explored with gene therapy in the hopes of developing a minimally invasive, long term treatment option that may offer neurorestorative or neuroprotective advantages over traditional pharmacotherapy.

**1.8.2.2 Pallidotomy**

Pallidotomy involves targeted lesioning of the globus pallidus in PD patients. Stereotaxtic pallidotomy was developed by Dr. Hirotaro Narabayashi (Narabayashi, 1962b; a; c; Shimazu et al., 1962), however it is rarely performed anymore as it has become replaced by DBS, which is associated with fewer risks and is considered less invasive as it is a non-destructive process. The most efficacious use of pallidotomy is
the reduction of LIDs resulting from long-term levodopa therapy. Patients with limited response to levodopa are not considered good candidates for pallidotomy. Complications of pallidotomy can include stroke and the risk of infection or seizures.

1.8.2.3 Deep brain stimulation

Deep brain stimulation (DBS) is a promising avenue for treatment of PD, especially in severe cases of PD refractory to other pharmacological treatments or those with severe side effects to other treatment options. For example, surgical candidates for DBS are those individuals with intractable tremor, or severe dyskinesias (Volkmann, 2004). DBS can be very efficacious in relieving parkinsonian motor symptoms and improving a patient’s quality of life. The most common and effective sites for implantation of the stimulator are the STN and GPi, but the STN is the target of choice as it is considered superior in relieving akinesia, allows greater reductions in medication dosages, and is more energy efficient (Volkmann, 2004). In fact, STN-DBS relieves each of the cardinal motor symptoms in PD (Volkmann, 2007; Volkmann & Deuschl, 2007). The utility of other sites including the caudal zona incerta and the pallidofugal fibers located medial to the STN are also being considered as potential stimulation targets.

High frequency DBS was developed in the late 1980s as an alternative to destructive lesioning, such as pallidotomy (Benabid, 1993; Benabid et al., 1993a; Benabid et al., 1993b). Delivery of high frequency stimulation (HFS) to the STN via DBS is thought to override aberrant circuitry associated with PD, thus reducing the
inhibition of thalamic output to the motor cortex, enabling reduced bradykinesia and improved motor control. Delivery of HFS to the STN mimics the effects of lesion surgery, but does not cause permanent destruction of tissue as would pallidotomy or other lesioning procedures. Chronic stimulation is achieved by implanting a stimulating electrode within the target region (e.g. the STN). The electrode is connected to a pacemaker and battery pack implanted under the skin of the chest (Figure 1.8.4). This design enables one of the major advantages of DBS over lesion surgeries in that the stimulator can be turned on and off, and adjusted remotely. Stimulator settings can be adjusted for amplitude, pulse width and pulse frequency in order to achieve optimal therapeutic efficacy throughout different stages of disease. Additional advantages of DBS over ablative (lesioning) surgeries include avoidance of tissue destruction, making DBS a reversible procedure. Further, DBS is relatively safe, and can be performed bilaterally (Volkmann, 2007; Volkmann & Deuschl, 2007).

A retrospective review of 921 patients from 37 cohorts receiving DBS revealed dramatic improvement in multiple aspects of disease including: improved UPDRS activities of daily living and motor scores (50% in on-medication state, and 52% in the off-medication state), reduction of dopaminergic drug dosage (55.9%), reduction of LIDs (69.1%), and reduction in daily 'off' periods (68.2%) (Kleiner-Fisman et al., 2006). Adverse side effects of DBS include intracranial hemorrhage (3.9%) and psychiatric problems such as depression and suicide (Kleiner-Fisman et al., 2006). This study concluded that DBS appears effective, but further assessment of safety needs to be conducted (Volkmann, 2007; Volkmann & Deuschl, 2007).
A large multicenter randomized controlled study compared STN-DBS with best medical management over 6 months (Deuschl et al., 2006). This study involved a total of 156 parkinsonian patients with severe motor impairment. This study demonstrated that neurostimulation resulted in a 25% improvement in health-related quality of life for patients receiving STN-DBS, versus effectively no change in the group receiving best medical care (Deuschl et al., 2006). Serious adverse events in the surgical group included fatal intracerebral hematoma and a suicide. However, this study found no evidence of increased risk of cognitive or neuropsychiatric complication with STN-DBS (Deuschl et al., 2006).

Nonetheless, the psychosocial effect of DBS remain an issue of concern as many patients experience some degree of social maladjustment or struggle with reintegration into society post-surgery (Schupbach et al., 2006). Thus, despite excellent motor recovery, patients describe problems with reintegration into their social lives, including problems with work, family, and interpersonal relationships. Some hypothesize that this social maladjustment may be due to the fact that most surgical patients are in advanced stages of PD and have been living with disease for many years (Schupbach et al., 2007; Volkmann, 2007; Volkmann & Deuschl, 2007). This is supported by data that suggests advanced age is associated with reduced post-surgical benefit as patients over the age of 70 may have increased risk of post-surgical cognitive decline, and do not experience as great a degree of benefit with surgery as younger patients (Russmann et al., 2004). Further, early data from studies of DBS in younger patients at less severe stages of disease show marked post-surgical motor improvements with minimal, mild, and transient adverse effects (Schupbach et al.,
2007). These data suggest that in young patients, DBS may be better tolerated and more effective in slowing disease progression and improving quality of life than medication alone.

While STN-DBS is clearly effective in relieving motor dysfunction of PD, and these benefits appear to be long-lasting (Krack et al., 2000; Schupbach et al., 2005), it is clear that further studies examining the long-term benefits, risk factors, and optimal point(s) of intervention are warranted.

Figure 1.8.4: Deep brain stimulation involves implantation of a stimulating electrode in the STN. The electrode is connected by wires under the skin to its power source; a pacemaker placed in the chest wall. Image modified from Wired Magazine, original source unknown per author Mr. Brandon Keim.
1.8.2.4 Transplantation

1.8.2.4.1 Preclinical animal studies

The rationale for neural transplantation is simple: replace cells lost to damage or disease. There has long been interest in whether cells of the nervous system which die from trauma or disease could be replaced by new ones. While very early attempts at brain cell grafting were unsuccessful, Dr. Elizabeth Dunn (Dunn, 1917) at the turn of the twentieth century was first to show that brain tissue grafted from one newborn rat to another newborn rat could survive transplantation. Despite this landmark discovery, little progress was made in the area of neural transplantation over the next fifty years. However, resurgent interest in the 1970’s, particularly in the field of Parkinson’s disease, led to an explosion of research, which continues through today.

The promise of neural transplantation as a true possibility gained validity throughout the 1970s as multiple neuronal tissues were transplanted in the anterior chamber of the eye including catecholamine and serotonin neurons (Olson & Seiger, 1972), cerebellar cortex (Hoffer et al., 1974), locus coeruleus (Olson & Seiger, 1976), and hippocampal formations (Hoffer et al., 1977a; Hoffer et al., 1977b; Olson et al., 1977; Woodward et al., 1977). Transplantation as a therapeutic possibility came to the forefront of PD research in the late 1970s when Bjorklund and colleagues transplanted monoaminergic neurons into adult hippocampus (Bjorklund et al., 1976), and Stenevi and colleagues transplanted central and peripheral monoamine neurons into adult rat brain (Stenevi et al., 1976). In 1979, the potential promise of neural transplantation for patients with PD began to emerge as studies in the DA-depleted rat revealed transplant-mediated recovery via intracerebral nigral transplants (Bjorklund & Stenevi, 1979) and
transplant-mediated reduction of motor abnormalities in the DA-depleted rat (Perlow et al., 1979).

Difficulties surrounding the use of fetal ventral mesencephalic tissue for transplantation including ethical issues, scarcity of tissue, and source heterogeneity led to the search for alternative tissue sources. Possibilities have included autologous grafts using mesenchymal stem cells (MSCs) and DA-secreting cells from carotid bodies (CB), as well as allografts of human embryonic stem cells (ESCs) and retinal pigment epithelial cells (RPECs). Early preclinical and clinical studies used adrenal autografts and other adult DA-secreting cells such as those found in the carotid body of the rodent (Espejo et al., 1998) and primate (Luquin et al., 1999), or the retinal pigment epithelium of the rat (Subramanian et al., 2002) and monkey (Doudet et al., 2004), as alternative sources of DA in response to public criticism of the use of human fetal and embryonic tissue.

Despite some promise in the lab where transplanted adrenal chromaffin cells were shown to reduce rotational behavior in the parkinsonian rat (Freed et al., 1981), and to survive and express catecholamines in the primate brain (Freed et al., 1981; Morihisa et al., 1984), subsequent clinical trials of transplantation of adrenal medullary tissue into the parkinsonian striatum were largely unsuccessful and subsequently abandoned (Backlund et al., 1985; Goetz et al., 1989; Rehncrona, 1997; Hallett & Litvan, 2000). Nonetheless, such studies provided important information about the role of the host environment in successful clinical grafting. Specifically, adrenal medullary grafting studies in MPTP treated mice result in robust TH-ir fiber staining in the parkinsonian striatum. Bohn and colleagues demonstrated that such adrenal medullary
grafts exert a neurotrophic action in the host brain to enhance recovery of endogenous DA neurons, such that the observed increase in TH-ir fibers in response to grafting, are fibers from the host striatum rather than from the grafted tissue (Bohn et al., 1987). Thus, these studies highlight the potential of the host striatum in transplant-mediated recovery of parkinsonism.

Although adrenal medulary grafts have been abandoned clinically, the potential of MSCs, CBs, RPECs and ESCs as alternative transplant tissues continues to be studied.

MSCs can be obtained from adult bone marrow throughout life, and thus could be a potential autologous source of graft tissue. While MSCs in vivo support hematopoietic stem cells, they do retain pluripotency, and are able to differentiate into osteocytes, chondrocytes, and adipocytes in vitro. Recently, MSCs have been successfully experimentally manipulated to differentiate into neurons (Kim et al., 2006; Rivera et al., 2006). Especially important for their consideration as a viable source for transplantation therapy, efficient and highly specific induction of DA neurons from rat and human MSCs has also been demonstrated (Dezawa et al., 2004). Further, transplantation of hMSC-derived DA neurons into the parkinsonian rat yields substantial motor recovery and transplanted cells exhibit extensive migration, produce DA, and produce no evidence of abnormal cell division (Dezawa et al., 2004). Further characterization of the mechanisms underlying hMSC-derived DA neuron mediated effects from transplantation is necessary before clinical implementation of this tissue source.
Carotid bodies are chemosensory glomus cells that secrete DA in response to hypoxia (McGregor et al., 1984). Transplantation of carotid body cells into the striatum of parkinsonian rodents (Espejo et al., 1998; Toledo-Aral et al., 2003) and monkeys (Luquin et al., 1999) have been effective in reducing parkinsonism. A recent clinical trial (Minguez-Castellanos et al., 2007) transplanting CB cells demonstrated motor improvement in some patients equivalent to that seen in the double blind fetal VM transplant trial (Laguna Goya et al., 2008). Patients had significantly better outcome (measured as reduction in UPDRS motor score) associated with both absence of fibrotic atrophy of CB and milder disease severity at time of grafting. Despite motor improvements, no increase in striatal fluoro-dopa with CB transplants was observed. The investigators hypothesize that functional improvements related to CB transplantation may be due to release of trophic factors from the cells since survival of DA neurons post-transplantation was only approximately 10% (Minguez-Castellanos et al., 2007).

Retinal pigmented epithelial cells (RPECs) are retinal support cells that produce levodopa as part of the melanin synthesis pathway. These cells have demonstrated neurotrophic effects in cultures of rat striatal and mesencephalic neurons (McKay et al., 2006) and improve motor symptoms in parkinsonian rats when co-transplanted while attached to cross-linked porcine gelatin microcarriers into striatum (Subramanian et al., 2002). In the clinics, hRPECs attached to microcarriers are referred to as Spheramine. Open-label clinical studies with Spheramine demonstrated a 48% improvement in UPDRS scores of patients one year post-transplantation. Of note, there is no report of graft-induced dyskinesias in transplanted patients at this early time point (Stover et al.,
Currently, a phase II double-blind, randomized, multicenter, placebo-controlled (sham surgery) study is underway to evaluate safety, tolerability, and efficacy of Spheramine implanted bilaterally into the postcommissural putamen of patients with advanced PD (Stover & Watts, 2008). Spheramine is administered by stereotactic implantation into the striatum of PD patients and the use of immunosuppression is not required (Stover & Watts, 2008). Spheramine may represent a treatment approach with the potential of supplying a more continuous delivery of levodopa to the striatum in advanced PD than can be achieved with oral therapy alone (Stover & Watts, 2008).

Human ESCs can be differentiated into neurons via supplement with bFGF and EGF (Reubinoff et al., 2001), or with FGF-2 (Zhang et al., 2001), and DA neurons have been derived from mouse ESCs via a combination of neurotrophic factors (Perrier et al., 2004). Further, A9 DA neurons from hESCs co-cultured with immortalized human fetal midbrain astrocytes demonstrate symptomatic benefit upon transplantation into parkinsonian rats. However, undifferentiated neuroepithelium was found in the core of such grafts, and had tumorigenic potential (Perrier et al., 2004). Studies directly comparing ESC-derived neurons to VM tissue engrafted into the striatum of the parkinsonian rat revealed similarities and distinct differences in the manner in which ESC and VM grafts interact with the denervated striatum (Yurek & Fletcher-Turner, 2004). TH-ir fiber outgrowth and cell numbers were similar in both ESC and VM grafted rats, but only VM grafted rats demonstrated significant reduction of amphetamine induced rotational asymmetry with grafting (Yurek & Fletcher-Turner, 2004). Further studies to optimize potential of ESCs as a transplantation therapy must be completed before relevant clinical studies can be designed. Specifically, the major drawback of
using stem cells converted to dopaminergic neurons for grafting in PD is that these cells may retain the potential to revert from their induced DA phenotype. This particular issue must be resolved to make grafting of ESCs a truly viable transplantation therapy.

Transplantation of fetal neural cells into the primate brain was intensely studied in the 1980s, and it was established that DA grafts into the MPTP primate striatum were viable, integrated with the host striatum, and were able to reverse parkinsonian behaviors (Bakay & King, 1986; Redmond et al., 1986b; a; Bakay et al., 1987; Brundin et al., 1987; Collier et al., 1987; Sladek et al., 1987; Sladek et al., 1988a; Sladek et al., 1988b). Additional studies confirmed utility of aborted human fetal tissue as viable transplantation tissue into rats (Brundin et al., 1988b; Clarke et al., 1988; Nilsson et al., 1988) and monkeys (Redmond et al., 1988).

Numerous transplantation studies in the laboratory continued around the globe throughout the 1980s and 1990s. Implantation of DA neurons into the striatum of rodents, marmosets, and monkeys was performed in hundreds of animal studies examining multiple factors influencing transplanted cell survival. The optimal embryonic ages for cell survival after transplantation were determined for the rodent (Strecker et al., 1989; Yurek et al., 1990), primate (Collier et al., 1987; Sladek et al., 1993a; Sladek et al., 1993b; Elsworth et al., 1996; Annett et al., 1997), and human (1988; Brundin et al., 1988a; Brundin et al., 1988b; Hitchcock et al., 1988; Lindvall et al., 1988; Lindvall et al., 1989; Freeman & Olanow, 1991; Freeman et al., 1995a). Grafted cell survival ranged from 5% to 45%, depending on the study (Rosenstein, 1995). Parameters of grafting were refined to yield cells expressing TH, with increased concentrations of DA throughout the graft location, neurite outgrowth, and integration with host environment.
Electrophysiology studies confirmed that grafted cells exhibit the same profile as DA neurons, and respond in a manner characteristic of dopamine neurons (Fisher et al., 1991). Further, grafted neurons have been shown to respond to afferent stimulation (Fisher et al., 1991), and form synaptic connections, based on electron microscopy studies (Freund et al., 1985).

Initially, graft functional efficacy in the 6-OHDA rat model of PD was primarily measured by reversal of amphetamine-induced rotational asymmetry (Dunnett et al., 1983). While intrastriatal grafts were capable of reversal of amphetamine-induced rotational asymmetry, there was no evidence of graft-mediated improvement in deficits in feeding and drinking, sensorimotor orientation disengage behavior, or skilled forepaw reaching (Dunnett et al., 1987; Mandel et al., 1990; Montoya et al., 1990; Nikkhah et al., 1993a). Later studies have shown transplant-mediated improvement in more complex task and parkinsonian motor behaviors by optimizing the graft size, location, or distribution (Steece-Collier et al., 1995; Lee et al., 2000; Steece-Collier et al., 2003; Maries et al., 2006; Winkler et al., 2006; Steece-Collier et al., 2009).

From preclinical experiments, it was established that optimal cells for grafting should; (1) have completed migration to their target location in the developing brain, (2) no longer undergo cell division, (3) express the desired phenotype according to their future location, and (4) have yet to extend neuronal processes, which can easily be shorn in transplantation (Brundin et al., 2000a). In general, tissue for grafting for PD is collected via microdissection of the embryonic brain in the region of the ventral floor of the mesencephalic flexure, an area containing developing A8, A9 and A10 dopamine cell groups (Figure 1.8.5). The extracted tissue is either implanted as solid pieces or
dissociated into a cell suspension. The process of grafting is traumatic to the young neurons, disrupting normal cell-cell contacts and posing numerous challenges such as ischemia, oxidative stress, and attack from the host immune system. As a result, only 5-20% of grafted neurons survive in the host environment, regardless of species (Rosenstein, 1995; Brundin et al., 2000a).

Figure 1.8.5: Example of an embryonic brain taken at gestational day 14 from a rat. This example shows the mesencephalic flexure (circled area) and the floor of this structure (arrows) that contains the developing A8-10 dopamine cell groups.
**1.8.2.4.2 Transplantation: clinical trials**

Engraftment of fetal dopaminergic cells into the parkinsonian brain is complicated not only by ethical issues and tissue availability, but also by recent clinical trials which have shown inconsistent efficacy of surgical engraftment. Initial clinical trials for fetal transplantation in parkinsonian patients were small safety and efficacy studies such as those in Sweden (Lindvall et al., 1988), Mexico (Madrazo et al., 1988), and England (Hitchcock et al., 1988). Initial attempts to graft into the brain were widely variable in both methods and outcome. These initial clinical trials were open-label, and involved small groups of patients, but nonetheless provided the observations that engrafted embryonic DA neurons can survive in the parkinsonian brain and provide some clinical benefit. These studies also demonstrated that while grafted DA neurons could be efficacious in relieving PD symptoms, they also resulted in the development of graft-induced dyskinesias (GIDs), an observation further supported by later clinical trials (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003) and in animal models (Steece-Collier et al., 2003; Maries et al., 2006; Soderstrom et al., 2008; Steece-Collier et al., 2009). These abnormal motor movements have some similarities to LIDs, however GIDs are persistent, even in the absence of levodopa. Pallidotomy has been used to alleviate severe cases of GIDs (Vergani et al., 2006). GIDs are discussed in more detail in the following section.

The first double-blind placebo-controlled grafting trials for PD was performed by Freed and colleagues, and reported in 2001 (Freed et al., 2001). This study involved 40 patients with severe PD who ranged in age from 35-75 years old. Patients were randomized into either DA neuron-graft or sham-graft treatment groups. DA neuron-
grafted patients received human embryonic DA neurons cultured up to 4 weeks before being implanted in the putamen. While the primary outcome measure of global change in disease severity one year post-surgery revealed no statistically significant difference between sham-graft and DA neuron-grafted treatment groups, it was found that those patients who were under 60 years of age who received DA neuron-grafts had reduced disease severity as measured by both the UPDRS, and the Schwab and England scales. In this study, GIDs were observed in 15% of patients, even in the absence of levodopa (Freed et al., 2001).

Several years later in 2003, results of a second major double-blind placebo-controlled grafting trial were published by Olanow and colleagues. Similar to the Freed study, this trial involved 34 patients with severe PD between the ages of 30-75 years old. These patients received tissue from 1-4 donors grafted bilaterally into the post-comissural putamen. In this study, the grafted cells were prepared as a cell suspension and stored for only 2 days before implantation. Survival of grafted neurons was confirmed by increased striatal fluorodopa (F-dopa) uptake on PET imaging. The primary endpoint of this trial was a change in the UPDRS scores during off phases. Similar to the trial lead by Freed (Freed et al., 2001), those DA neuron-grafted patients with mild disease performed better than sham subjects in this measure, however, there was overall no difference in post-surgical UPDRS scores for DA neuron-graft versus sham-graft patients. Further, over half (56%) of patients developed GIDs, in the absence of levodopa in this study. Of note, it was observed that there was a trend for increased functional improvement with increased number of donors; however this did not reach statistical significance.
Failure to meet the primary endpoint for both the Freed et al. (2001) and Olanow et. al. (2003) studies, combined with evolution of GIDs in 15-56% of grafted patients after surgery, led to a halt of clinical grafting trials world-wide. Although paused at the bedside, exploration of embryonic grafts as therapy for PD continues at the bench as researchers continue to refine and further define the characteristics needed for optimal graft performance. In particular, the observations suggesting greater efficacy of grafts in younger patients may be especially important in the clinics as clinical grafting trials in PD patients are poised to begin again in Europe in 2009-2010. Indeed, the impact of host age on graft efficacy is part of the rationale underlying the design of Aim 1: The Age Study, in this dissertation.

Figure 1.8.6 summarizes the major details of four open label and two double-blind placebo-controlled clinical trials in transplantation for PD. Each study varies according to the average years of disease, position of graft placement, number of embryos transplanted, age of donor tissue, graft preparation and use of immunosuppression. However, in general, most studies involved patients with greater than a decade of disease, bilateral transplantation (usually putaminal), with multiple embryos between 5-9 weeks of age, in either solid or dissociated state. With the exception of the Freed 2001 study, all studies involve immunosuppression after grafting.

In regards to measures of graft efficacy, changes in the UPDRS motor scores in ‘on’ and ‘off’ phases, as well as the percent of time in ‘off’ phase were evaluated. The mean reduction in levodopa dosage and mean reduction in LIDs were also assessed. Finally, fluorodopa uptake was, in general, increased with transplantation.
Although there is no standard grafting protocol, and, as a result, significant variability in the design and results of the major clinical trials (both open-label, and double-blind), several factors determining successful grafts have been realized. The low efficiency of DA cell survival (4-5%) in transplantation (Mendez et al., 2005) means about three fetuses are needed per side such that at least 100,000 neurons survive and graft-mediated reinnervation of at least 1/3 putaminal volume results from the graft. Further, those patients with less severe disease, patients with DA loss restricted to the dorsolateral striatum (Piccini et al., 2005), patients less than 60 years of age at time of grafting, and patients receiving more grafted tissue (higher number of cells and/or fetuses) demonstrate the greatest graft efficacy. Overall, the best effect is achieved by maximizing the number of surviving transplanted cells. However, the concern remains that once the obstacle of poor graft survival is overcome, whether DA neuron grafting can provide optimal benefit in all PD patients. Factors such as advanced age, loss of plasticity and ability to respond or incorporate new circuitry, and altered target neuron morphology remain obstacles that may impede graft success even if optimal survival of grafted tissue is achieved.

The most significant roadblock remaining in advancement of the therapeutic potential of transplantation of fetal VM DA cells into the parkinsonian brain are the ethical issues surrounding this therapy. While the ethics concerning sources of transplantation tissue certainly merit careful attention, the risk of development of GIDs with grafting is the primary ethical factor restricting use of grafting as a PD therapy. Subjecting a patient to GIDs is counter to the basic Hippocratic tenet of clinical
medicine, “First, do no harm.” Eliminating the risk of GIDs is a critical step towards restoring the utility of transplantation in the clinics.
<table>
<thead>
<tr>
<th>Study</th>
<th>(Freed et al., 1992)</th>
<th>(Hauser et al., 1999)</th>
<th>(Hagell et al., 1999)</th>
<th>(Brundin et al., 2000b)</th>
<th>(Freed et al., 2001)</th>
<th>(Olanow et al., 2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of study</td>
<td>Open-label</td>
<td>Open-label</td>
<td>Open-label</td>
<td>Open-label</td>
<td>Double-blind</td>
<td>Double-blind</td>
</tr>
<tr>
<td>Number of patients</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>19‡</td>
<td>11/12</td>
</tr>
<tr>
<td>Average years of disease</td>
<td>13.4</td>
<td>18.2</td>
<td>11.6</td>
<td>12.6</td>
<td>14</td>
<td>10.6/8.2</td>
</tr>
<tr>
<td>Position of graft placement</td>
<td>2 unilateral C + Pu</td>
<td>Bilateral Pu</td>
<td>4 bilateral Pu</td>
<td>Bilateral C + Pu</td>
<td>Bilateral Pu</td>
<td>Bilateral Pu</td>
</tr>
<tr>
<td>Amount of graft transplanted</td>
<td>1 embryo</td>
<td>3 – 4 embryos (per side)</td>
<td>4 – 8 embryos (per side)</td>
<td>3 – 5 embryos (per side)</td>
<td>2 embryos (per side)</td>
<td>1 or 4 embryos (per side)</td>
</tr>
<tr>
<td>Age of donor tissue</td>
<td>6–8 wks</td>
<td>6.5–9 wks</td>
<td>6–8 wks</td>
<td>5–7 wks</td>
<td>7–8 wks</td>
<td>6–9 wks</td>
</tr>
<tr>
<td>Graft preparation</td>
<td>Solid</td>
<td>Solid</td>
<td>Dissociated</td>
<td>Dissociated</td>
<td>Solid</td>
<td>Solid</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>Cs + Pred in 4 patients</td>
<td>Cs (6 mo)</td>
<td>Cs + Az + Pred (constant)</td>
<td>Cs + Az + Pred (12 – 24 mo)</td>
<td>None</td>
<td>Cs (6 mo)</td>
</tr>
</tbody>
</table>

**Results**

<table>
<thead>
<tr>
<th></th>
<th>∆% in UPDRS motor in ‘on’ phase</th>
<th>n.s.</th>
<th>N/A</th>
<th>N/A</th>
<th>N/A</th>
<th>72%/73%</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆% in UPDRS motor in ‘off’ phase</td>
<td>N/A</td>
<td>-30%</td>
<td>-30%</td>
<td>-40%</td>
<td>-18%</td>
<td>9/-1%</td>
</tr>
<tr>
<td>∆% of time in ‘off’ phase</td>
<td>N/A</td>
<td>-43%</td>
<td>-59%</td>
<td>-43%</td>
<td>N/A</td>
<td>21/-2%</td>
</tr>
<tr>
<td>∆% in total UPDRS in ‘off’ phase</td>
<td>N/A</td>
<td>-32%</td>
<td>N/A</td>
<td>N/A</td>
<td>-15%</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean reduction in levodopa dosage</td>
<td>39%</td>
<td>16% at12 – 24 mo</td>
<td>37%</td>
<td>54% at 18 – 24mo</td>
<td>N/A</td>
<td>20/11% at 20 mo</td>
</tr>
<tr>
<td>Mean reduction (%) in ‘on’ time with LID</td>
<td>N/A</td>
<td>53%</td>
<td>69%</td>
<td>25%</td>
<td>N/A</td>
<td>14/12%</td>
</tr>
<tr>
<td>Fluorodopa uptake</td>
<td>Only studied in 2 patients</td>
<td>61% increase at 12 mo</td>
<td>85% at 12 – 18 mo</td>
<td>61% increase at 10 – 23 mo</td>
<td>40% at 12 mo</td>
<td>Increase*</td>
</tr>
</tbody>
</table>

Heterogeneities in the preparation and therapeutic outcomes are compared between selected open-label and double-blind clinical trials. Major differences between the preparation of these studies included the severity of disease in recruited patients, the age, amount, preparation, and position of the grafted tissue, as well as the use of immunosuppression. Such disparities in transplantation protocol might be the underlying causes for the various level of clinical benefits, including the mean changes in UPDRS score, time spent in ‘off’ phase, levodopa dosage, severity of LID, and putaminal fluorodopa uptake.

*% can not be calculated as the fluorodopa uptake at baseline is not given in the original manuscript.

‡ Subsequently, 14 of the 20 sham-operated patients received a VM transplant (i.e., 33 patients in total were ultimately grafted in this study).

Az: Azathioprine; C: Caudate; Cs: Ciclosporin A; LID: Levodopa-induced dyskinesias; mo: Month; N/A: Not assessed; ns: Nonsignificant; Pred: Prednisolone; Pu: Putamen; UPDRS: Unified Parkinson’s Disease Rating Scale.

**Figure 1.8.6:** Summary of major clinical trials for neurotransplantation in PD.

*Modified from Goya and Barker, 2007.*
1.9 *Levodopa- and graft-induced dyskinesias*

Although highly efficacious in relieving the motor deficits associated with PD, LD treatment carries with it the risk of the development of LIDs in almost half of patients after 4-6 years of treatment (Ahlskog & Muenter, 2001). LIDs are involuntary, abnormal movements that can significantly interfere with normal motor movements and compromise quality of life of persons with PD. Although younger patients and those with more severe DA depletion are at higher risk for LID development, the onset of LIDs is unpredictable (Shaw et al., 1980). Significantly, even in patients with highly comparable clinical characteristics of PD the occurrence of LIDs remains highly variable (Mones *et al.*, 1970; Mones *et al.*, 1971; Marconi *et al.*, 1994; Blanchet *et al.*, 1996; Schrag *et al.*, 1998; Olanow & Tatton, 1999; Baas, 2000; Fahn, 2000; Ahlskog & Muenter, 2001; Blanchet, 2003; Kostrzewa *et al.*, 2005). LIDs are primarily choreic (rhythmic, sometimes repetitive, “dance-like” motions) with secondary characteristics of dystonia (sustained muscle contractions causing twisting or abnormal posturing) (Blanchet *et al.*, 1996; Fahn, 2000). LIDs commonly affect the upper limbs, usually on the side contralateral to that of the highest degradation of the SNpc DA neurons (Marconi *et al.*, 1994; Fahn *et al.*, 2000). The most common time for LIDs to occur is at peak plasma and brain levels of levodopa, where the therapeutic benefit is also maximized (Figure 1.9.1). Peak dose dyskinesias are generally referred to as “on-dyskinesias.” However LIDs have also been observed both early and late in the dosing schedule as plasma levels are increasing or decreasing. These are referred to as bi- or diphasic dyskinesias. Finally, dyskinetic behaviors can also be noted when levodopa
levels are low (generally 12 hours after the last dose of levodopa), in a time referred to as the ‘off’ period. Thus, these are referred to as ‘off dyskinesias’ (Figure 1.9.1).

Figure 1.9.1: LIDs correspond to plasma levels of levodopa.

In contrast to LID, GID are noted only in PD patients that have been grafted with DA neurons. The development of GIDs has been observed in multiple patients after extended postgraft intervals as discussed in the previous section. These novel (not seen prior to grafting), post-graft dyskinesias persist despite improvement in pre-graft LIDs. GIDs tend to be more restricted or focused in their localization and varied in presentation compared to their pre-operative levodopa-induced counterparts (Freed et al., 2001; Hagell et al., 2002, Olanow et al., 2003). Additionally, in the 6-OHDA rat
model of PD, GIDs have been observed as focal dyskinesia generally expressed as in the form of facial-forelimb stereotypy (FFS) and correlate with upregulation of FosB/deltaFosB localized specifically to the ventrolateral striatum (Maries et al., 2006). In rat similar to human, these GID behaviors develop as the graft matures and are noted in conjunction with decreased LIDs (Maries et al., 2006; Soderstrom et al., 2008).

Graft-induced dyskinesias are distinct from LIDs as they present when patients are off dopaminergic medication, have been reported in between 15-55% of transplant recipients (Freed et al., 2001; Olanow et al., 2003). According to the retrospective analysis by Hagell and colleagues in which they further examined transplant recipients and dyskinesia development, the severity and development of GIDs are not correlated with UPDRS motor scores, putaminal F-dopa uptake on PET scanning, amount of daily dopaminergic medication, or pre-transplant LID severity (Hagell et al., 2002).

Multiple mechanisms have been proposed to explain the emergence of GIDs including patchy inhomogeneous graft-mediated dopaminergic reinnervation of the host putamen defined by PET study of dyskinetic transplant recipients (Ma et al., 2002) and by other rodent studies of graft placement, distribution, and size (Winkler et al., 2002; Lane et al., 2006; Maries et al., 2006; Winkler et al., 2006; Lane et al., 2009a; Lane et al., 2009b). Specifically, incomplete patchy reinnervation of the ventral striatum resulting from grafts placed in the dorsal striatum is hypothesized to be one factor contributing to the development of GIDs (Lee et al., 2000; Hagell & Cenci, 2005; Piccini et al., 2005). Further, the uneven distribution of DA released from transplanted cells may be further exacerbated by D1R supersensitivity in the regions of the striatum not innervated by the graft (Lee et al., 2000; Hagell & Cenci, 2005; Piccini et al., 2005).
Other hypotheses regarding GID development include that GIDs may result from the presence or “contamination” of the transplant by ectopic cells other than A9 DA neurons. Preclinical studies designed to isolate A9 DA neurons upon dissection are complicated by the lack of reliable techniques to sort developing neurons while maintaining viability for transplantation. Studies have demonstrated the presence of non-dopaminergic neurons and astrocytes in embryonic striatal grafts and at the graft-host border (Barker et al., 1996). Reactive astrocytes lead to gliosis and release of cytokines, both of which may present physical or neurochemical barriers to graft efficacy (Bona et al., 1999).

Finally, GIDs may also be associated with both withdrawal of immunosuppression and inappropriate synaptic connectivity. This is supported by studies in our laboratory which have demonstrated increasing development of specific graft-mediated dyskinetic behaviors associated with escalating immune activation in parkinsonian grafted rats (Soderstrom et al., 2008). In the same study, ultrastructural analyses showed increases in TH-ir axo-dendritic synapses, TH-ir asymmetric specializations, and non-TH-ir perforated synapses in grafted, compared to intact, striata (Soderstrom et al., 2008). These features were exacerbated in rats with the highest immune activation and correlated statistically with GID-like behaviors, thus suggesting that immune-mediated aberrant synaptology may contribute to aberrant behaviors mediated by grafting (Soderstrom et al., 2008).
1.10 Potential mechanisms underlying dyskinesia in the parkinsonian striatum

Despite intensive research involved in the behavioral and neurochemical characterization of LIDs and GIDs, the specific molecular mechanisms and synaptic adaptations underlying the manifestation of these behaviors remain unclear. In fact, multiple mechanisms underlying the development of both graft- and levodopa-induced dyskinesias have been proposed, including, but not limited to, excessive, low, or intermediate levels of dopamine release, patchy innervation and dopamine receptor supersensitivity, and abnormal graft-to-host connections (Hagell & Cenci, 2005). In regards to GIDs, studies in both humans (Ma et al., 2002) and rodents (Maries et al., 2006) suggest that discrete and unbalanced increases in dopaminergic function within the striatum likely contribute to GID presentation. Recent compelling evidence from Soderstrom and colleagues suggests that specific abnormal synaptic profiles are correlated with the occurrence of GIDs in the parkinsonian rat model (Soderstrom et al., 2008). Further, these aberrant synapse profiles and their associated behaviors are enhanced in the presence of immune plasticity factors associated with allograft protocols (Soderstrom et al., 2008).

Briefly, with regard to LIDs, it is likely that genetic differences between individual patients may influence their general susceptibility to these behaviors. Observations in both the clinic (Calon et al., 2003) and at the bench (Konradi et al., 2004) support this hypothesis. For example, in the outbred Sprague-Dawley rat strain approximately 50% of parkinsonian rats develop LIDs, despite being age-matched, equally lesioned, and undergoing identical levodopa treatment schedules (Konradi et al., 2004). Additionally, data from our lab has shown that in highly inbred Fischer 344 rats LID development is
robust and uniform (Steece-Collier et al., 2003), whereas the highly inbred Lewis RT.1 rats do not develop LIDs even when subjected to exceptionally high and prolonged levodopa treatment (Steece-Collier, unpublished results). The paucity in our knowledge of mechanisms underlying dyskinesias caused by current treatments of PD is a significant barrier to improving therapeutic intervention for this devastating disease.

1.10.1 Factors underlying induction of LIDs

Multiple factors are thought to underlie the induction and maintenance of dyskinesias. The presentation of dyskinesias can be highly variable and dopaminergic drugs differ widely in their ability to induce dyskinesias. The gradual sensitization phenomenon associated with the induction of levodopa-induced dyskinesias is referred to as priming, in which the presentation of dyskinetic behaviors results due to repeated administration of particular dopaminergic agents. The phenomenon of priming has been compared to that of sensitization, or “reverse tolerance,” as observed with stimulants such as cocaine or amphetamine. Sensitization occurs when repeated administration of a drug leads to an elevated response to the same dose of the drug when it is given subsequently. Sensitization has been artfully described as a “model of neural plasticity within which drug-induced changes in complex behavior can be linked to drug induced changes in molecular processes (Carlezon & Nestler, 2002).” Additionally, levodopa priming has also been compared to development of epileptic foci in response to electrical stimulation, where repetitive electrical stimulation of a particular area leads to enhanced sensitivity and aberrant responsivity of the affected region.
(Kalichman, 1982b; a; Kalichman et al., 1982). Whichever the comparison, levodopa priming in the parkinsonian brain is associated with changes in striatal plasticity (Calabresi et al., 1992c; Calabresi et al., 2000a; Calabresi et al., 2000b; Picconi et al., 2003; Picconi et al., 2005; Pisani et al., 2005; Calabresi et al., 2008) and molecular processes (e.g. (Calabresi et al., 2008), and is a major factor underlying the emergence of behavior alterations, such as dyskinesias.

The key features underlying the induction of dyskinesias are (1) the extent of nigral dopamine cell loss, (2) the type of drug, and (3) the mechanism of action of the drug. With regard to extent of nigral DA loss, it is clear that the degree of striatal DA denervation determines the level and duration of drug exposure required to induce dyskinesias. For instance, in normal humans, monkeys, and rats, dyskinesias largely do not develop in response to levodopa, even when administered repeatedly and at high doses (Cotzias et al., 1967; Mena et al., 1970; Mones et al., 1971; Klawans et al., 1972a; Klawans et al., 1972b; Chase et al., 1973; Paulson, 1973; Sassin, 1975; Schneider, 1989; Boyce et al., 1990b; a; Rajput, 1996). There are some reports of dyskinesias developing in normal animals after very high levels of levodopa administration, but these are exceptions to the rule, and suggest that very high levels of levodopa may be associated with some toxicity (Pearce et al., 2001). However, in parkinsonian humans, monkeys, and rats, LIDs develop in response to levodopa, but generally in relative degree dependent on the extent of striatal DA-depletion. Systematic study of this phenomenon in MPTP monkeys shows that the onset of dyskinesia development is directly linked to the extent of the lesion (Jenner, 2003).
In addition to the degree of DA-depletion, the pulsatile nature of oral levodopa therapy is another major factor contributing to LID induction. In the striatum under normal conditions, DA is released tonically. Pharmacological drug delivery of levodopa generally does not mimic this physiological tonic DA tone found in the normal striatum. Indeed, levodopa has a relatively short half-life and is absorbed from the gut somewhat erratically in PD patients, leading to fluctuating plasma levels of the drug. Further, striatal DA tone is dependent on the integrity of DA terminals. As fewer DA terminals exist with disease progression to regulate release of DA in the striatum, the pulsatility of DA produced from exogenous levodopa is effectively exacerbated. The combination of DA denervation, with exposure to intermittment levodopa intake leads to the presentation of LIDs. The pulsatile (and non-physiological) nature of oral LD therapy is essential to the development of LIDs, as continuous infusion of LD even at very high dosages does not result in LIDs (reviewed by (Calon et al., 2000). Indeed, continuous infusion of levodopa, which eliminates the pulsatile nature accompanied by oral therapy, prevents the emergence of LIDs, even at high doses of levodopa (Stocchi et al., 2005; Olanow et al., 2006). Further, long-acting formulations of levodopa, and repeated subcutaneous administration of levodopa tend to be associated with decreased severity of LIDs. However, although use of continuous drug delivery can delay onset of LIDs, or switching from pulsatile to continuous delivery can even temporarily reverse LID expression (Hadj Tahar et al., 2003; Smith et al., 2006; Jackson et al., 2007), as DA denervation progresses, LIDs eventually emerge despite efforts to maintain steady levodopa concentrations in the brain.
Glutamatergic hyperactivity in the basal ganglia is also implicated in LIDs (Blanchet et al., 1998; Verhagen Metman et al., 1998a; Blanchet et al., 1999; Chase et al., 2000; Blanchet, 2003). This contention is supported by the fact that as previously discussed, NMDA receptor antagonists (such as amantadine and MK-801) have been shown to reduce LID severity in both the lab and the clinic. Deep brain stimulation of the STN may also function to reduce the glutamatergic hyperactivity of the parkinsonian basal ganglia which may directly lead to decreased LIDs, or allow a decrease in dopaminergic pharmacotherapy, also reducing LIDs (Benabid et al., 2005a; Benabid et al., 2005b; Benabid et al., 2006a; Benabid et al., 2006b; Gubellini et al., 2006; Hurley & Jenner, 2006).

The glutamatergic system is intimately linked to synaptic plasticity within the CNS and there is increasing evidence that LIDs are related to aberrant changes in synaptic plasticity in the parkinsonian striatum (Calabresi et al., 1992a; Calabresi et al., 1992b; Calabresi et al., 1992c; Calabresi et al., 1992d; Mercuri et al., 1992a; Mercuri et al., 1992b; Calabresi et al., 1993a; Calabresi et al., 1993b; Calabresi et al., 1993c; Calabresi et al., 2000a; Calabresi et al., 2000b; Picconi et al., 2003; Picconi et al., 2005; Pisani et al., 2005; Santini et al., 2007; Calabresi et al., 2008; Santini et al., 2008). Specifically, dendritic spines of striatal MSNs serve as morphological “gates,” that are critical to the regulation and balance of cortical glutamatergic and nigral dopaminergic input to the striatum. As such, when dendritic spines are lost with DA depletion (Robinson & Kolb, 1997), there is a resulting loss of physiological control over glutamatergic signaling in the parkinsonian striatum. Diminished dopamine and unregulated glutamate in the parkinsonian striatum contribute to excess LTP (Calabresi
et al., 1992d), which can be translated as excessive, unnecessary motor memories. Dyskinesias have been associated with an inability to “erase” these excessive motor memories (Picconi et al., 2003) and it is hypothesized that such changes in electrochemical and morphological plasticity factors underly the emergence and maintenance of LIDs in the parkinsonian brain. The role of aberrant plasticity in the evolution of LIDs is discussed in further detail in section 1.10.3.

### 1.10.2 The aging striatum and dyskinesias

While advanced age is the greatest risk factor for development of PD, there appears, however, to be an inverse relationship between age and dyskinesia development. In particular, young patients (< 60 years of age) (Kostic et al., 1991; Blanchet et al., 1996; Schrag et al., 1998) and those with high levels of DA depletion (Blanchet et al., 1996; Di Monte et al., 2000) are more likely to develop LIDs. However, it is not just the degree of DA depletion that determines a patient’s susceptibility to LIDs, since studies of parkinsonian brains found a similar extent of degeneration of the SN in both dyskinetic and non-dyskinetic parkinsonian patients (Calon et al., 2003). More recent studies have begun to outline critical changes in synaptic and morphological plasticity as contributing factors in dyskinesia development. Further, it is currently hypothesized that age-related changes in synaptic plasticity also contribute to dyskinesia development and influence pharmacological and surgical efficacy. It is well established that plasticity declines with age (Adams, 1987b; a; Collier et al., 2007), and that aberrant mechanisms of plasticity have been shown to underlie LID development.
(Calabresi et al., 2000a; Picconi et al., 2003; Picconi et al., 2005; Pisani et al., 2005; Calabresi et al., 2008; Picconi et al., 2008). Specifically, excessive motor memories are hypothesized to be one of the factors underlying dyskinesia development (Picconi et al., 2003). Thus, it follows, that in the younger, more plastic, adaptive brain, such individuals may be at higher risk of developing LIDs compared to older subjects, with reduced plasticity.

1.10.3 Striatal plasticity and dyskinesias

1.10.3.1 Synaptic plasticity in the striatum

The concept of synaptic plasticity was defined in 1949 with the work of Hebb (Hebb & Konzett, 1949; Hebb, 1949), who observed that a synapse connecting two cells strengthened only if both pre- and postsynaptic sites are active simultaneously. Synaptic plasticity is also defined by changes in synaptic strength that occur quickly, and are long-lasting. Long term potentiation (LTP), long-term depression (LTD) and synaptic depotentiation are all activity-dependent synaptic changes considered hallmarks of synaptic plasticity. Long term potentiation, or the long-lasting increase in the efficacy of communication between two neurons, is inducible when the presynaptic neuron is exposed to persistent high-frequency stimulation (HFS). Depotentiation involves the administration of low-frequency stimulation (LFS) over the course of several minutes to reverse the LTP. Both phenomena are highly dependent on glutamate release and stimulation of NMDA receptors which leads to downstream signal cascades involved in regulation of specific gene expression and protein synthesis.
The cAMP response element-binding protein (CREB), FOS, and JUN families of transcription factors are examples of those that are consistently increased in animal models of LIDs (Calabresi et al., 1996; Gerfen, 2000b; a; Gerfen et al., 2002).

Once assumed to be exclusive to classic learning and memory within the hippocampus, synaptic plasticity is now known to be associated with many brain areas, including the striatum (Graybiel, 1995b; a), where motor memories are processed, stored, reinforced, or deleted. The striatum processes cortical motor information, acting as a filter for motor circuitry. Synaptic plasticity or “cellular memory” within the striatum is unique in that it involves both nigrostriatal DA and corticostriatal glutamate and the reciprocal interaction of these two neurotransmitters (Calabresi et al., 1994). Striatal synaptic plasticity also depends on both the level of membrane depolarization as well as the specific glutamate receptor subtype involved (Calabresi et al., 1992a; Calabresi et al., 1992b; Calabresi et al., 1992c; Calabresi et al., 1992d). Further supporting the role of synaptic plasticity in the striatum, it is established that repetitive activation of corticostriatal pathways has the potential to induce both LTD (likened to erasure of motor memories) and LTP (strengthening of motor memories) of synaptic transmission in the striatum (Calabresi et al., 1992c; Calabresi et al., 1994; 1996; Picconi et al., 2003; Picconi et al., 2005; Pisani et al., 2005). Thus, the striatum exhibits motor memory, and is able to both block the execution of unwanted movements (which is reinforced by development of LTD) and allow execution of appropriate motor programs (reinforced by development of LTP).
1.10.3.2 Changes in striatal plasticity with DA-depletion; potential role in LIDs

The role of aberrant striatal plasticity in LIDs and PD is becoming increasingly clearer. Both LTD and LTP are lost in the DA-denervated striatum and chronic levodopa treatment restores LTP, but not LTD (Picconi et al., 2003). LTD has been likened to the brain’s process of memory erasure or “forgetting”, and is a process considered necessary for the management of both emotional and motor memories (Picconi et al., 2003). It has been hypothesized that this loss of the ability to erase unnecessary motor memories within the striatum is a key factor underlying the development of LIDs. Normally, induction of LTP with high frequency stimulation can be reversed with low frequency stimulation (synaptic depotentiation). This bidirectional plasticity is lost specifically in dyskinetic rats treated with levodopa, such that LTP is restored, but synaptic depotentiation remains diminished. In contrast, non-dyskinetic rats undergoing the same protocol are said to maintain bidirectional plasticity, in that both LTP and synaptic depotentiation are preserved (Picconi et al., 2003). Additionally, altered phosphorylation of striatal DA and cAMP-regulated phosphoprotein of 32 Da (DARPP-32) is observed in rats with LIDs (Picconi et al., 2003). Cumulatively, these data supports the hypothesis that a loss of the capacity for depotentiation within striatal synapses of dyskinetic rats, accompanied by molecular changes downstream of cAMP regulated pathways within the striatum, are associated with dyskinesia development.

While changes in LTP, LTD, and depotentiation are clear indicators of synaptic plasticity, morphological changes such as axonal regeneration, sprouting, synaptogenesis, and neurogenesis are also important factors (Brown et al., 2004). LTP is associated with synaptic remodeling, and changes in the length and density of
dendritic spines are examples of morphological changes critically involved in establishing and maintaining synaptic strength. Dendritic spine atrophy is observed with DA-depletion in humans and 6-OHDA lesioned rats (McNeill et al., 1988; Zaja-Milatovic et al., 2005; Stephens et al., 2005; Day et al., 2006). Synaptic remodeling in the striatum is a key feature underlying the development and persistence of neuroleptic-induced dyskinesias (Meredeth et al., 2000). Transcriptome analysis of striata of dyskinetic lesioned rats revealed upregulation of genes involved in structural and synaptic plasticity as well as those related to calcium homeostasis and signaling (Konradi et al., 2004), further supporting the hypothesis that aberrant striatal plasticity changes at both the biochemical and morphological levels are integral to abnormal motor learning and are key factors underlying the development of LIDs.

1.10.4 Dendrite morphology and LIDs

1.10.4.1 Dendritic spines

Dendritic spines are small protrusions of dendritic membrane ranging in volume from less than 0.01 μm$^3$ to 0.8 μm$^3$ (Alvarez & Sabatini, 2007). These small structures play a big role in synaptic transmission as they are the site of nearly 90% of the excitatory synapses in mature brain (Harris, 1999). Observation of dendritic spines was first reported over a century ago by Ramon y Cajal (Garcia-Lopez et al., 2007) using Camillo Golgi’s “reazione nera” (black reaction) protocol (Sala et al., 2008). Today the reazione nera is known as Golgi staining, and remains a standard method used in laboratories to visualize and study multiple elements of neuronal cytoarchitecture,
including dendritic spines. While spines are rarely found in organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans*, studies have shown spines are present at synapses in the giant squid (Young et al., 1973) supporting the conclusion that spines may have evolved to accommodate the demands of more complex nervous systems. Although many dendritic spines have a characteristic “bowling pin” appearance, with an head (~0.004-2um3) connected to the dendritic shaft by a thin neck (diameter ~0.04-1um and length ~0.1-2um), spines can differ widely in their morphological characteristics across different cell types (Alvarez & Sabatini, 2007). Most spines have constricted necks and are either mushroom shaped with heads exceeding 0.6 microns in diameter or thin shaped with smaller heads (Harris et al., 1992). Extensive electron microscopy studies of brain tissue have shown that spines can be also stubby, cup-shaped or branched protrusions with two or more heads, or single protrusions with multiple synapses along the head and neck (Harris & Kater, 1994; Hering & Sheng, 2001) and that these different shapes can be found at the same time on the same dendrites (Spacek & Harris, 1998). Thus, based on their size and shape, spines can be classified as “mushroom,” “thin,” or “stubby.” Further, it is hypothesized that such variability in spine morphology correlates with the differences in synaptic strength established at these spines (Yao et al., 2008). It is important to recognize the highly plastic nature of spines, which exist as very dynamic structures, intimated involved in synaptic plasticity (Alvarez & Sabatini, 2007). In this nature, several studies suggest that dendritic spines function are possible repositories of long-term memory in the brain (Segal, 2005). Further, classification of spines must be recognized as a snapshot of that spine at a particular moment in time, as *in vivo*, at
least in developing neurons, the majority of the spines change their shape over periods of minutes or hours (Parnass et al., 2000). Spine motility is developmentally regulated and, in mature neurons, there are fewer transitions between morphological categories (Dunaevsky et al., 1999; Parnass et al., 2000). Spines are a site of interaction not only between neurons, but also with glia, as more than half of synapses on dendritic spines have been shown to be partially covered by astrocyte processes (Ventura & Harris, 1999). Typical mature spines have a single excitatory synapse located at the head, but the same spines can also have an inhibitory input (Knott et al., 2002), such as in the striatum, where nigral DA gates glutamatergic signaling by synapsing on the neck of MSN spine. Despite coordination of inputs from both glia and other neurons, spines generally represent the main postsynaptic compartment for excitatory input.

The intracellular scaffold and machinery of post-synaptic dendritic spines is unique and highly specialized. First, the highly dynamic cytoskeleton of the dendritic spine is comprised almost entirely and exclusively of F-actin. Further, the head of the spine contains the postsynaptic density (PSD), an electron-dense thickening of the membrane where the synaptic junction is located (Sheng & Sala, 2001). PSDs vary in size and structure, ranging from small discs to large irregular shapes that can be either continuous or perforated, but is generally a relatively large structure within the spine, occupying around 10% of the surface area exactly opposite the presynaptic input (Sheng & Sala, 2001). The PSD is highly complex organelle in which hundreds of components are associated each other via protein-protein interactions (Sheng & Sala, 2001). Further, the PSD contains hundreds of proteins including NMDA, AMPA, and metabotropic glutamate receptors; scaffolding proteins such as PSD-95; and signaling
proteins such as CamKII (Kennedy, 2000). There is also a relationship between PSD and spine structure, for example, mushroom spines have larger and more complex PSDs with a higher density of glutamate receptors (Matsuzaki et al., 2001; Nicholson et al., 2006; Matsuzaki, 2007).

In addition to the PSD, other organelles are localized at spines such as smooth endoplasmic reticulum (SER) (Spacek & Harris, 1997), and sometimes the spine apparatus, an organelle formed by two or more disks of SER separated by an electron-dense material (Westrum et al., 1980). Larger spines typically have larger synapses and contain varied types of organelles and it has been observed that both SER and spine apparatus are usually associated with larger spines and are absent in small spines (Spacek & Harris, 1997). Further, larger spines are more likely to contain polyribosomes (Ostroff et al., 2002) and endosomal compartments (Cooney et al., 2002; Park et al., 2006), and more often associated with perisynaptic astroglia (Witcher et al., 2007). It is further hypothesized that since SER is known to play a role in calcium handling (Burgoyne, 1983; Andrews et al., 1988), calcium homeostasis may be controlled differently depending on the size of the spine. Thus, larger spines are considered to provide a stronger response to glutamate, local regulation of intracellular calcium, endosomal recycling, protein translation and degradation, and interaction with astroglia. Conversely, smaller spines may be more plastic, with rapid changes in size in response to subsequent activation (Segal, 2005).

Studies in hippocampus (Nusser et al., 1998; Takumi et al., 1999a; Takumi et al., 1999b; Racca et al., 2000) have shown that number of glutamate receptors is proportional to both spine volume and the area of PSD, as receptor density is uniform...
within the PSD. Further, the PSD and spine volume are proportional to the area of the active zone, which is in turn proportional to the number of docked vesicles (Schikorski & Stevens, 1997), correlating with the amount of neurotransmitter release per action potential (Murthy et al., 1997). These data support the claim that large spines represent stronger synapses, and that increasing size of the head of the spine likely correlates with increasing synaptic strength.

Finally, localization of translation of mRNA into protein has been demonstrated in the dendritic spine suggesting complex mechanisms of local translation in response to synaptic inputs. Localization of proteins at the dendritic spine is important for both synaptic transmission and synaptic plasticity, and involves not only protein transport, but also local protein synthesis (Steward & Levy, 1982; Steward & Schuman, 2001; Jiang & Schuman, 2002; Bassell & Kelic, 2004; Kelleher et al., 2004; Martin, 2004; Takei et al., 2004). Some synaptic proteins are translated in the neuronal cell body and then transported to the postsynaptic site, including AMPA (Setou et al., 2002; Setou et al., 2004) and NMDA (Setou et al., 2000) glutamate receptors. Transportation of AMPA and NMDA receptors to spines depends on the kinesin family of microtubule-associated motor proteins. In contrast, the mRNAs of other proteins, such as type 1 inositol triphosphate receptor (IP3R1) (Furuichi et al., 1993), the alpha subunit of calcium/calmodulin-dependent protein kinase II (aCaMKII) (Steward & Schuman, 2001), and synArfGEF(Inaba et al., 2004), are transported through dendritic shafts and translated locally, at the postsynaptic sites. Such localization of mRNA translation at subcellular compartments, like the dendritic spine, is an efficient way to make the proteins needed at a particular location. There is increasing evidence supporting the
importance of the dendritic mRNA transport and translation for synaptic function (Steward & Levy, 1982; Steward & Schuman, 2001; Jiang & Schuman, 2002; Bassell & Kelic, 2004; Kelleher et al., 2004; Martin, 2004; Takei et al., 2004). For example, blocking the dendritic translocation of mRNA for aCaMKII suppresses the late phase of long-term potentiation in the hippocampus (Miller et al., 2002). The potential role of localized translation in plasticity changes underlying the development of LIDs remains to be elucidated, but may offer important insight regarding the role of dendritic spine morphology and such behaviors.

1.10.4.2 Role of dendritic morphology in neuronal communication in the striatum

Striatal MSNs comprise approximately 90% of neurons in the intact striatum and as their name implies, these neurons contain numerous dendritic spines, or stalk-like extensions on surfaces of the dendrites. Dendritic spines of MSNs are the primary site of afferent input for nigral DA neurons ascending from substantia nigra, and glutamate input from the cortex, and thalamus (Smith et al., 1994; Ingham et al., 1998). Nigral DA neurons almost exclusively synapse onto the shaft of dendritic spines of MSNs, while glutamate afferents synapse specifically onto the heads of the same spines (Smith et al., 1994; Ingham et al., 1998).

Again, there is accumulating evidence to suggest that aberrant striatal plasticity is important in the development of LIDs in parkinsonian subjects (Centonze et al., 1999; Calabresi et al., 2000b; Picconi et al., 2003; Picconi et al., 2005), and this aberrant plasticity may be linked to dendrite pathology associated with DA-depletion. As
previously mentioned, it is well documented in postmortem parkinsonian brains that
there is a significant atrophy of dendritic spines with advanced disease (McNeill et al.,
1988; Stephens et al., 2005; Zaja-Milatovic et al., 2005). Specifically, MSNs from PD
brains showed not only a significant reduction in the length of the dendrites compared to
age-matched controls, but the remaining dendrites often show few-to-no spines (McNeill
et al., 1988). It is important to remember that the dendrites (and especially the
associated dendritic spines) are critical structural input sites for incoming afferent fibers.
Alterations in MSNs, particularly loss of dendritic spines, would be anticipated to result
in aberrant postsynaptic interactions between DA and glutamate in the striatum. Indeed
postsynaptic interactions between DA and glutamate, which are critical for normal motor
behavior, are known to be disrupted in the parkinsonian brain (Calabresi et al., 1993b).
While it is difficult to test the contribution of spine loss to development of motor deficits
and/or LIDs in humans, the same loss of MSN dendritic spines is observed in mice and
rats with severe DA depletion (Day et al., 2006).

Indeed, it has been in parkinsonian rodents that the underlying dendritic spine
loss following DA-depletion has been recently identified to involve intraspine Cav1.3 L-
type Ca²⁺ channels (Day et al., 2006). Administration of the Cav1.2/1.3 calcium channel
antagonist nimodipine to rats, or the absence of these calcium channels in transgenic
mice prevents spine loss despite severe DA depletion (Day et al., 2006). The precise
mechanism by which DA depletion influences synaptic plasticity, and the degree to
which alterations in spine density and/or morphology contribute to parkinsonian motor
deficits and/or LID development remains unclear. However, identification of this Cav1.3
dysregulation mechanism has allowed testing the hypothesis that maintaining spine
integrity will contribute to a reduction of LID behaviors which is the basis of Aim 2 of this thesis.

**Morphological striatal plasticity**

- Striatal input pathways involve two NTs
  - Nigrostriatal DA
  - Corticostriatal Glutamate

- Reciprocal interaction of glutamate and dopamine receptors is unique to striatal plasticity
  - Calabresi, 1994

- Nigrostriatal DA and cortical glutamate inputs converge on dendritic spines of MSNs in the striatum in an ordered and specific manner

*Figure 1.10.4.1: Morphological striatal plasticity.* This overly simplified diagram represents the structural organization of dopaminergic and glutamatergic afferent input at the dendritic spine of striatal MSNs. It is important to note that this figure does not portray involvement of multiple additional neurotransmitters and signaling molecules involved in striatal neurotransmission and plasticity.
1.10.4.3 Role of dendrite and synapse pathology in LIDs

Changes in behavior of an organism are thought to involve structural modifications in the nervous system, particularly alterations in patterns of synaptic connectivity (Robinson & Kolb, 1997). Indeed there is profound reorganization of striatum at the cellular level that has been well documented in animal models of PD (Ingham et al., 1989) and in human PD (McNeill et al., 1988; Anglade et al., 1996). Further, chronic levodopa administration to parkinsonian subjects results in numerous additional neurochemical alterations of the nigrostriatal DA system (Calabresi et al., 2000a; Calabresi et al., 2000b; Picconi et al., 2003; Picconi et al., 2005; Pisani et al., 2005). While the precise mechanism by which DA depletion contributes to development of LIDs remains unclear, it is known is that: 1) chronic treatment of parkinsonian animals or humans with levodopa can lead to abnormal, dyskinetic movements that persist long after the drug is withdrawn (Nutt et al., 1994; Canales & Graybiel, 2000; Westin et al., 2001); and 2) increasing severity of DA denervation appears to increase the likelihood of development of LIDs (Mena et al., 1970; Mones, 1971; Mones et al., 1971; Chase et al., 1973; Langston & Ballard, 1984; Caligiuri & Peterson, 1993; Fahn, 2000). Interestingly, Meredith and colleagues (De Souza et al., 2003) found that chronic treatment of rats with the D2 receptor antagonist, haloperidol, resulted in persistent orolingual dyskinesias, which correlates with long-term increases in spine density, dendritic branching, and numbers of terminal segments in MSNs of the nucleus accumbens. Similarly, persistent structural modifications of MSNs in the nucleus accumbens and in neurons of the prefrontal cortex are noted following sensitization to chronic amphetamine (Robinson & Kolb, 1997). Accordingly, it is logical to examine
whether similar changes in striatal MSNs occurs with chronic levodopa in parkinsonian rats that develop dyskinesias. Further, since severe dopamine depletion is noted to lower the threshold for development of LIDs, it is further pertinent to assess the development of LIDs in an environment of DA-depletion but maintained spine status, as with the nimodipine treated animals in Aim 2 of this dissertation.

1.11 Cellular targets involved in development and maintenance of dyskinesias

As discussed in early sections, multiple factors are beginning to emerge as important contributors to LID development including electrophysiological and morphological changes in the parkinsonian striatum. It is clear that the nature of levodopa administration combined with the degree of dopamine denervation play a role in determining initiation of LIDs. The question of molecular signaling accompanying morphological and electrophysiological changes in the dyskinetic parkinsonian brain is a critical component of both understanding the presentation of dyskinesias and designing therapeutic intervention to reduce or prevent LIDs. While there are endless potential targets when discussing cell signaling pathways, the clear place to focus when trying to understand LIDs are the targets of nigral dopamine and cortical glutamate in the striatum. We now know that both the indirect and direct pathways are altered in the expression of dyskinesias; however some of these changes are transient, raising the question as to whether signaling changes observed in the dyskinetic basal ganglia are a cause of aberrant motor behaviors, or a result of aberrant motor behaviors. It is important to keep this caveat in mind as we consider potential molecular mechanisms underlying dyskinesias. Further, while some cell signaling alterations are expressed as
simply an increase or decrease in protein expression, one must not overlook the importance of protein \textit{activation} or conformational changes. For example, while total protein levels may remain unchanged, specific phosphorylation patterns may be critical players in the development of LIDs.

\section{Parallels between dyskinesia induction and cocaine sensitization}

Both levodopa and dopaminergic stimulants such as cocaine and amphetamine alter intracellular signaling via dopaminergic pathways. For this reason, cocaine sensitization can be considered analogous to levodopa sensitization or priming, and chronic ‘addictive’ cocaine intake akin to the levodopa maintenance dosing. Accordingly, it follows that better understanding of the cell signal pathways shared by levodopa and other dopaminergic stimulants will yield better understanding of the intricate balance of protein activation and expression underlying adaptations of the parkinsonian brain to levodopa administration and LID development.

Both cocaine (also, amphetamine) and levodopa increase striatal DA, and subsequent stimulation of dopamine receptors, albeit via slightly different mechanisms. Conversion of levodopa to DA in the dopaminergic terminals results in the increased vesicular release of DA. Acute cocaine administration blocks the presynaptic reuptake of DA, mobilizing the release of DA from synaptic storage pools of DA (Venton et al., 2006), leading to increased DA receptor signaling. Released DA can act at one of many DA receptor subtypes. There are five subtypes of metabotropic G-protein linked dopamine receptors, D\textsubscript{1}, D\textsubscript{2}, D\textsubscript{3}, D\textsubscript{4}, and D\textsubscript{5}. The D\textsubscript{1} and D\textsubscript{5} receptors are members of
the D₁-like family of dopamine receptors, whereas the D₂, D₃ and D₄ receptors are members of the D₂-like family (Girault & Greengard, 2004). The striatum is specifically enriched in D₁, D₂, and D₃ receptors (Meador-Woodruff et al., 1996).

Within the striatum, activation of the D₁ receptor, whether by cocaine, levodopa, or other dopaminomimetic drugs, increases cAMP levels, leading to activation of protein kinase A (PKA). PKA mediates phosphorylation of target molecules including voltage-gated Na⁺ and Ca²⁺ channels, ligand-gated GABA-A channels, and glutamate receptors (AMPA and NMDA). Thus, PKA is one substrate which mediates communication between DA and glutamatergic signaling in the striatum.

Both chronic cocaine and levodopa administration upregulates D₁/PKA signaling, and also leads to the activation of the transcription factor deltaFosB, which in turn induces the cyclin-dependent protein kinase Cdk5 allowing phosphorylation of DARPP-32 by Cdk5 at the Thr-75 residue. When DARPP-32 is phosphorylated in this manner, it functions as an inhibitor of PKA, whereas phosphorylation of DARPP-32 at amino acid Thr-34 leads to inhibition of protein phosphatase-1 (PP-1), allowing for PKA activation. In both cocaine- and levdopa-mediated stimulation of this signaling pathway, site specific phosphorylation of DARPP-32 amino acids allows delicate autoregulation of signaling cascades downstream of both dopaminergic and glutamatergic signaling pathways (Carta et al., 2008a). Further aberrant phosphorylation of DARPP-32 has been correlated with altered plasticity specifically in dyskinetic rats (Picconi et al., 2003; Santini et al., 2007). This signaling cascade is outlined in Figure 1.11.1.
Figure 1.11.1: Signaling pathways involved in acute vs chronic DA receptor stimulation with levodopa or cocaine. Image modified from (Santini et al., 2007)
1.11.2 Striatal dopamine receptors and associated pathways

Changes in dopaminergic systems including not only the striatum, but also limbic regions, motor cortex, and other basal ganglia nuclei, occur in response to levodopa (Braak et al., 2004). Basic pharmacology principles of receptor sensitization would suggest that in response to DA-denervation, there would be an upregulation of striatal DA receptors, and subsequent down-regulation in response to levodopa replacement of lost DA. However, the expression of DA receptors and associated changes with LIDs is not quite this simple. For example, changes in D2 receptor expression are not consistently seen in dyskinetic striata, suggesting a more specific role for D1 receptors in dyskinesias expression (Calabresi et al., 2008; Berthet et al., 2009; Darmopil et al., 2009). Indeed, MPTP primates with LIDs do demonstrate increased density of striatal D1 receptors, which is thought to lead to changes in receptor trafficking (Aubert et al., 2005; Guigoni et al., 2005b; Guigoni et al., 2007). Dyskinetic 6-OHDA rats also demonstrate changes in mRNAs specific to striatal DA receptor trafficking (Lee et al., 2008). Altered D1 receptor signaling leading to dyskinesias has also been associated with changes in multiple intracellular signaling cascades including (1) G-proteins and proteins that are concerned with receptor desensitization, (2) extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), (3) G-protein coupled receptor kinases, and (4) arrestin (Corvol et al., 2004; Bezard et al., 2005). Despite these findings, it would be remiss to assume that LIDs are mediated exclusively via D1R signaling as both D1 and D2 agonists can induce LIDs after priming (Blanchet et al., 1993), and antagonists of both D1 and D2 receptors are efficacious in relieving LIDs (Taylor et al., 2005). The D3 receptor also appears to play a role in LIDs as an increase in striatal D3
receptor expression as a result of levodopa treatment following nigrostriatal loss has also been reported (Bordet et al., 1997). However, the role of this receptor is more controversial as D3 antagonists may or may not be able to suppress dyskinesias (Hurley et al., 1996a; Hurley et al., 1996b; Bordet et al., 2000; Bezard et al., 2003; Guigoni et al., 2005a).

While changes in DA receptors with LIDs are not always predictable or clear, a systematic investigation of intracellular signaling pathways downstream of DA receptors are ongoing in several laboratories in attempt to more clearly identify the mechanisms that result in induction of dyskinesia. As mentioned above, increased DA D1 receptor sensitivity and downstream signaling have been reported in primates with LIDs (Aubert et al., 2005). This D1 receptor sensitization results in enhanced activation of cAMP-dependent protein kinase A (PKA), which phosphorylates various downstream proteins such as DARPP-32.

In 6-OHDA-lesioned rats, DARPP-32 phosphorylation at Thr-34 is significantly increased. This increase of DARPP-32 phosphorylation at Thr-34 is reversed with levodopa specifically in non-dyskinetic animals (Santini et al., 2007). Significantly, this specific change in DARPP-32 phosphorylation persists uniquely in dyskinetic animals suggesting that DARPP-32 may allow induction of dyskinesia via sensitization of D1 receptors to levodopa.

As previously discussed, the role of DARPP-32 was further confirmed by the observation that abnormally high levels of phospho[Thr-34]DARPP-32 accompanied loss of synaptic depotentiation specifically in dyskinetic animals. Phospho[Thr-
DARPP-32 inhibits protein phosphatase-1 (PP1), activation of which is required for depotentiation (Picconi et al., 2003). This was an important link between observed alterations of synaptic plasticity and accompanying cell signaling changes, both associated with LID development (Picconi et al., 2003). Thus, irreversibility of DARPP-32 phosphorylation at Thr-34 and loss of depotentiation are unique to the dyskinetic animal.

Further support for the role of DARPP-32 in LIDs was garnered by the observation that genetic inactivation of DARPP-32 reduced the occurrence of LIDs (Santini et al., 2007). Additionally, it was shown that via the MAP-kinases, ERK1 and ERK2, DARPP-32 plays a role in regulation of gene expression observed in the striatum of dyskinetic animals. Through different and complex mechanisms, activation of ERK leads to the activation of the transcription factors cAMP-responsive element-binding (CREB) protein (Gerfen et al., 2002; Santini et al., 2007).

In summary, both D1 and D2 receptor pathways are involved with LID expression, with specific involvement of targets within the cAMP/PKA and MAPK intracellular signalling pathways, such as DARPP-32. Further understanding of how changes in DARPP-32 phosphorylation states mediate cell signaling at the receptor and intracellular levels, and how such changes contribute to LID development are critical steps towards preventing these unwanted treatment side effects in patients.
1.11.3 *Striatal glutamatergic receptors and associated pathways*

In addition to ascending nigral dopaminergic signaling pathways, changes in corticostriatal glutamatergic signaling also contribute to LIDs. Loss of striatal DA leads to dysregulation of corticostriatal transmission (Picconi *et al.*, 2005; Pisani *et al.*, 2005; Calabresi *et al.*, 2007; Surmeier, 2007; Calabresi *et al.*, 2008), and underlies the emergence of aberrant striatal LTP and LTD in the parkinsonian brain, as discussed in previous sections. While much of the work in the PD field has classically been “dopamine-centric,” the role of the striatal glutamatergic system in PD and therapeutic motor complications is becoming increasingly more apparent and must not be overlooked.

A critical component of midbrain DA is modulation of glutamate transmission. While glutamate-dopamine interactions in the striatum are complex, exogenously applied glutamate evokes DA release (Cheramy *et al.*, 1986a; Cheramy *et al.*, 1986b; Girault *et al.*, 1986), and DA acting via D2 receptors on glutamate terminals in the striatum inhibits glutamate release (Yamamoto & Davy, 1992; Peris *et al.*, 1998; Tseng *et al.*, 2004). In the parkinsonian striatum, glutamate modulation by DA is diminished and hyperactivity of glutamatergic synapses is observed (Lindfors & Ungerstedt, 1990; Yamamoto & Davy, 1992; Ingham *et al.*, 1998; Meshul *et al.*, 1999).

Additionally, DA D1 receptors closely interact with glutamate NMDA receptors in striatal neurons. D1 receptor activation has been shown to enhance striatal trafficking of NMDA receptor subunits, promoting clustering of NR1 and NR2 subunits with the postsynaptic density scaffolding protein PSD-95 and enhancing receptor surface
expression (Hallett et al., 2006). Further, chronic levodopa treatment causes significant changes in striatal glutamatergic signaling, including adaptive modification of NMDA receptor subunits (Hurley et al., 2005), with an abnormal redistribution of the NR2B subunit between synaptic and extrasynaptic membranes (Gardoni et al., 2006).

The role of the NMDA receptor and glutamate in LIDs is further supported by clinical data that shows the glutamate antagonist amantadine can suppress dyskinesias in humans and MPTP primates (Papa & Chase, 1996; Blanchet et al., 1998; Verhagen Metman et al., 1998a; Verhagen Metman et al., 1998b; Blanchet et al., 1999; Nash et al., 2000; Blanchet, 2003; Blanchet et al., 2003; Nash et al., 2004; Morissette et al., 2006a; b). In fact, both NMDA and AMPA receptor antagonists have been shown to be effective in suppressing dyskinesias (Konitsiotis et al., 2000; Steece-Collier et al., 2000; Bibbiani et al., 2005). Additionally, such studies provide evidence of altered striatal glutamate signaling, including changes in glutamate receptor expression, phosphorylation, and synaptic organization in LIDs (Chase et al., 2000). The role of NMDA receptors in LIDs has been closely examined by the use of drugs that act selectively on the NR2B subunit of NMDA receptors, which is highly expressed in the striatum. It is becoming increasingly more evident that LIDs likely involve both NMDA and AMPA receptor mediated glutamatergic pathways.

The expression of glutamatergic receptors on dendritic spines of striatal medium spiny neurons in parkinsonian brains has been studied and although there is evidence for altered NMDA receptor density with development of LIDs (Chase et al., 1998; Oh et al., 1998; 1999a; Oh et al., 1999b; Dunah et al., 2000; Calon et al., 2003; Hurley et al., 2005), the specific impact on NR1, NR2A and NR2B subunits are unclear. There is also
evidence for altered phosphorylation of NMDA and AMPA receptors in levodopa treated, 6-OHDA-lesioned rats (Chase & Oh, 2000; Chase et al., 2000; Wessell et al., 2004; Bibbiani et al., 2005), but results are somewhat inconsistent between studies, and changes observed in 6-OHDA rats do not hold true for MPTP primates (Hallett et al., 2005). Both NMDA and AMPA receptors undergo serine phosphorylation via the actions of protein kinase A (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CamKII). Inhibition of these targets results in motor changes, such that inhibition of PKA and CamKII suppresses levodopa-induced motor behaviors in 6-OHDA-lesioned rats, whereas inhibition of PKC increases the rate of appearance of altered motor-responses post-levodopa administration (Chase et al., 2000). These findings support the roles of molecules such as PKA, PKC, and CamKII as intermediaries between glutamate signaling and motor response to levodopa, supporting the hypothesis that changes in intracellular signaling cascades that lead to the sensitization of glutamate underlie the induction of motor complications, such as LIDs.

The specific role of such intercellular signaling pathways in the initial priming or sensitization phase versus maintenance of LIDs remains unclear. It is yet to be determined if changes for these two phenomenon are the same, or whether such signaling changes are a cause, or result of LID development.
1.11.4 The role of serotonin in LIDs

Recently, the potential role of striatal serotonergic innervation in development of LIDs has been a controversial issue of much debate in PD research. Some researchers strongly defend the idea that serotonergic neuron release of vesicular dopamine is the underlying (pre-synaptic) cause of LIDs. In early PD, remaining striatal DA terminals are capable of producing the therapeutic effect of levodopa by increasing physiological DA tone within the striatum. Levodopa is taken up into existing DA terminals, stored in vesicles, and released into the synaptic cleft in an activity-dependent manner, thus approximating physiological release of endogenous DA. At early stages, the parkinsonian striatum is still able to maintain some degree of 'control' over DA levels, preventing excessive variability in extracellular DA levels via auto-regulatory feedback mediated by the DA transporter and the D$_2$/D$_3$ autoreceptors present on remaining DA terminals.

With disease progression and progressive DA terminal loss, fewer DA terminals are present to modulate conversion of levodopa and extracellular DA tone. It is proposed that as DA terminals diminish serotonin terminals become involved in regulating levodopa. Serotonin terminal arise from the raphe nucleus and normally supply a sparse innervation of the striatum, particularly dorsal striatum. With disease progression in PD, there is a sprouting of serontonergic terminals in striatum (Gaspar et al., 1993). Serotonin neurons have the capacity to uptake levodopa, convert it to DA through the same aromatic amino acid decarboxylase enzyme found in DA neurons, and store it in vesicles since serotonergic neurons have the same vesicular transporter as DA neurons (Carta et al., 2008b).
As serotonin neurons lack the DA auto-receptors for feedback control found on DA neurons, stimulation of serotonergic neurons causes release not only of endogenous serotonin, but also the levodopa-derived DA that accumulated in the serotonin storage vesicles. Thus, DA release from serotonergic terminals is poorly regulated and results in uncontrolled, excessive release of DA. In this model, it is proposed that the inability of serotonergic neurons to control release of levodopa-derived DA, coupled with the lack of DA-specific autoregulatory feedback in serotonergic neurons results in aberrant DA signaling and induction of LIDs. Further, engraftment of serotonergic neurons into the parkinsonian striatum has been shown to exacerbate LIDs (Carlsson et al., 2007; Carlsson et al., 2009), while lesion of serotonergic projections in parkinsonian rats has been shown to abolish LIDs (Carlsson et al., 2009). The efficacy of serotonin receptor agonists in suppression of LIDs in 6-OHDA rats further supports this model, suggesting that regulation of striatal serotonergic activity also regulates striatal DA release.

While this mechanism may play a role in LID development from the presynaptic standpoint, similar to the understanding that pulsatile DA leads to LIDs, post-synaptic, downstream factors determining development of dyskinesias remain unclear.
1.12 Modeling Parkinson’s disease: The rat model of PD and LIDs

For many diseases, basic science turns to animal models to enable the study of detailed behaviors or mechanisms not easily assessed in human patients or mathematical models. Animal models are invaluable to the progress of basic science and medical research. Animals models of PD have been employed in basic science research since the 1960’s, beginning with the development of the 6-OHDA rat model of PD by Ungerstedt and colleagues (Ungerstedt, 1968). The neurotoxin 6-OHDA was also used in primates, and was followed by the development of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) primate model of PD. Serendipity plays a role in many great scientific discoveries, and the MPTP model of PD is no exception as part of the development of the MPTP model was dependent upon the mistaken calculations of an unfortunate graduate student. MPTP is a byproduct of the synthesis of MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), an opioid analgesic drug, a painkiller analog of pethidine (Demerol), with approximately 70% the potency of morphine (Langston et al., 1983; Langston & Ballard, 1983; Johannessen & Markey, 1984). MPPP is not used clinically and is listed as a Schedule 1 drug in the USA, but has been illegally manufactured for recreational drug use. The first documented case of MPTP synthesis was by a 23 year old graduate student named Barry Kidston (Langston & Ballard, 1983). It is reported that Kidston had studied a paper by Albert Ziering (Ziering & Lee, 1947) that described the potential to produce a drug with potency near that of morphine by reverse esterification of meperidine (Langston & Ballard, 1983; Langston & Palfreman, 1995). What Kidston did not know is that the intermediate in this step is temperature and pH sensitive, and under the right (or wrong, depending on one’s point
of view) conditions will lead to the formation of the neurotoxin MPTP as a major impurity (Johannessen & Markey, 1984). In 1976, Kidston successfully synthesized MPTP in his attempt to produce MPPP, and injected himself with his preparation. Within three days Kidston began showing symptoms of PD (Langston & Ballard, 1984; Langston & Palfreman, 1995; Fahn, 1996). Interestingly, the NIH found MPTP traces in Kidston’s lab and tested the substance on rats, but due to the tolerance of rats to MPTP toxicity, the animals remained asymptomatic (Langston & Palfreman, 1995). Kidston was successfully treated with levodopa, but died less than 2 years later from a cocaine overdose (Fahn, 1996). Autopsy verified nigral DA denervation in Kidston’s brain (Langston & Palfreman, 1995; Fahn, 1996).

It was not until the 1980’s when a second incident of recreational drug abuse involving MPTP opened the avenue of primate PD research. In 1982 seven young drug abusers in northern California developed signs and symptoms of PD including rigidity, akinesia, bradykinesia, tremor, and bent posture after using intravenous MPPP contaminated with MPTP (Langston et al., 1983; Langston & Ballard, 1984; Langston & Palfreman, 1995). Their drug-induced parkinsonism combined with that of Kidston’s lead to the discovery that MPTP can cause parkinsonism in humans (Langston et al., 1983). These events, serendipitous for science although not so much for those unfortunate patients, lead to the development of the MPTP primate model of PD.

MPTP, a so-called ‘magic bullet’ can be administered systemically to primates (i.v., i.p., s.q., i.m.) to yield bilateral parkinsonism, or via intracarotid injection for a unilateral model. The unilateral model enables the subject to maintain the ability to feed and care for itself, and provides an inherent internal control via the unaffected, intact
side. Administration of MPTP leads to a relatively selective DA neuron loss in the SN and some loss of cells in the adjacent VTA, and DA depletion in the striatum (Hantraye et al., 1993).

The cytotoxic effects of MPTP are related to its reactive metabolite, MPP+ (1-methyl-4-phenylpyridinium ion) (Langston et al., 1983; Johannessen & Markey, 1984). MPTP is enzymatically converted to MPP+ via monoaminooxidase B (MAOB) in glia. MPP+ then enters DA neurons via the dopamine transporter (DAT), which subsequently leads to inhibition of mitochondrial complex I, resulting in an abundant increase in reactive oxidative species and neuronal death (Soderstrom et al., 2006). Not only does this profile of elevated reactive oxygen species and reduced complex one activity mirror findings in post-mortem parkinsonian human brains (Langston et al., 1983), the MPTP primate also demonstrates the classic parkinsonian motor symptoms of rigidity, tremor and bradykinesia (Elsworth et al., 1987b; a). Further, MPTP primates are responsive to levodopa therapy, and are also subject to the development of levodopa-induced dyskinesias (Calon et al., 2000).

The MPTP primate model of PD is undoubtedly a robust and valuable model; however it is not without limitations. Non-human primates can be challenging to maintain, especially those with bilateral disease, and are also very expensive. Further the ethical considerations regarding use of non-human primates in medical research are not insignificant.

Fortunately, there is a more widely accessible and less expensive model of PD that is available: the 6-OHDA treated rat. First developed by Ungerstedt and
colleagues as a bilateral model (Ungerstedt, 1968), rats with unilateral depletion of midbrain DA neurons following stereotaxic injection of the neurotoxin 6-hydroxydopamine (6-OHDA) are a well established and frequently used model of PD and LIDs (Ungerstedt, 1970; Ungerstedt & Arbuthnott, 1970). Precise stereotaxic injection of 6-OHDA enables accurate delivery directly to the target of choice; commonly the substantia nigra, and/or medial forebrain bundle, or more recently, directly into the striatum. Administration of 6-OHDA leads to degeneration of monoaminergic neurons as it is transported into cell bodies and neuronal processes of dopaminergic and noradrenergic neurons, where it inhibits mitochondrial complexes I and IV (Ungerstedt, 1968; Dabbeni-Sala et al., 2001). The neurotoxin 6-OHDA also produces oxidative stress through additional mechanisms that lead to death of these neurons (Glinka et al., 1997; Hanrott et al., 2006).

The original bilateral 6-OHDA model presented with the same drawbacks as systemic administration of MPTP to non-human primates; high mortality and morbidity. The unilateral 6-OHDA rat model is much easier to use, and again, as in the MPTP primate model, provides the experimenter with an internal control, the intact side. The neurotoxic effects of 6-OHDA injected into the midbrain result in both (1) marked reduction in the number of neurons immunoreactive for tyrosine hydroxylase in the substantia nigra, and (2) reduction of TH immunoreactivity in the striatum, thus indicating severe depletion of DA in both regions. Lesion severity can be assessed behaviorally by a number of tests including amphetamine-induced rotational asymmetry, cylinder task, forepaw step-adjustment task, or the vibrissae task (Schallert, 2006; Anstrom et al., 2007). Each of these tests is based on either (1) the imbalance of
striatal DA between the lesioned and intact hemispheres (rotational asymmetry) or (2) overall diminished levels of DA. Overall reductions in DA are reflected by either reduced locomotor activity on the side of the body corresponding to the DA-depleted hemisphere or comparatively enhanced locomotor activity on the intact side of the animal.

Our laboratory (Steece-Collier et al., 2003; Maries et al., 2006; Soderstrom et al., 2008; Steece-Collier et al., 2009) and others (Cenci et al., 1998; Andersson et al., 1999; Westin et al., 2001; Cenci et al., 2002; Lundblad et al., 2002; Winkler et al., 2002; Andersson et al., 2003; Steece-Collier et al., 2003) have demonstrated that unilaterally DA-denervated rats administered repeated doses of levodopa develop significant abnormal involuntary movements, or dyskinesias in response to repeated levodopa. The word ‘dyskinesia,’ derived from two Greek roots: dys- (trouble), and kinesis (movement), is used throughout this dissertation to describe the abnormal involuntary movements including dystonia, hyperkinesia, and/or stereotypies in rats following levodopa administration. The general characteristics of dyskinesias in parkinsonian rats are similar to those observed in both PD patients as well as non-human primate models of PD. Rats administered levodopa demonstrate dystonia in the form of sustained twisting, hyperextension, and/or rigidity in the trunk, neck, and limbs. Dyskinetic rats also exhibit hyperkinetic behaviors such as forelimb, facial, or orolingual movements with repetitive stereotypic or choreic patterns. It is important to note that these movements are involuntary, and may only be briefly interrupted, if at all. Further, Lundblad and colleagues have documented that dyskinesias in rats have pharmacological profiles that are strikingly similar to those of LIDs in parkinsonian non-
human primates and human patients (Lundblad et al., 2002). Additionally, recent data has shown that specific cytoarchitectural changes that occur in humans with PD also accompany 6-OHDA mediated DA depletion (Day et al., 2006). Specifically, as previously discussed in Section 1.12, in the PD striatum, there is a marked decrease in the density of dendritic spines on striatal MSNs. This spine loss has also been demonstrated in the 6-OHDA rat, further supporting the utility of this model in studying detailed morphological and cell-cell signaling pathways underlying PD.

Observations in our lab have shown that there is some strain-dependent variability (as seen also in primates) in the susceptibility and expression of LIDs between different strains of rats. For instance, the inbred Fischer 344 rat responds quickly, robustly and uniformly to levodopa with a variety of hyperkinetic and dystonic movements, while in the inbred Lewis rat, rats are uniquely resistant to LID development even in the face of high doses of levodopa. Further, outbred strains, such as Sprague-Dawley rats demonstrate a dose-dependent, variably penetrant presentation of LIDs. This is reflective of the same

Figure 1.13.1: The 6-OHDA rat model of PD;

Example of trunk, neck, and limb dystonia in the levodopa treated parkinsonian rat.
variability we see in human patients with PD, where some patients are uniquely resistant to LIDs, and others remain largely susceptible, for reasons yet unknown. Closer study of these strain-related differences in LID presentation is warranted to further define risk factors for LIDs and better tailor pharmacotherapy to individual patients.

In summary, the 6-OHDA rat model of PD is reflective of human disease on multiple levels including, but not limited to; (1) depletion of nigrostriatal DA similar to that observed in human PD, (2) motor behaviors similar in both presentation and response to pharmacotherapy as in human PD, and (3) cytoarchitectural changes which mirror those in human PD. Additionally, the 6-OHDA model is reproducible and cost-effective. Thus, the 6-OHDA lesioned rat provides an established, reliable, and clinically relevant animal model of PD for preclinical investigation.
Chapter 2; Introduction to the Specific Aims

Every honest researcher I know admits he's just a professional amateur. He's doing whatever he's doing for the first time. That makes him an amateur. He has sense enough to know that he's going to have a lot of trouble, so that makes him a professional.

Charles Franklin Kettering (1876-1958) U. S. Engineer and Inventor.
2.1 Aim 1: Improving efficacy of DA terminal replacement strategies: Impact of age on graft function

Over the past decade, multiple studies have focused on the therapeutic efficacy of transplanted dopaminergic cells into the parkinsonian striatum (Olanow et al., 1996; Olanow et al., 1997; Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). While there has been painstaking effort to characterize the optimal donor cells used for such transplantation (ie (Annett et al., 1997), comparatively little has been done to determine the optimal host environment conditions for successful transplantation. In this regard, it has been shown that dopaminergic grafts are more efficacious in relieving parkinsonian symptoms in younger recipients (Freed et al., 2001), however, the adaptability to therapeutics of the aging parkinsonian striatum and resulting consequences in regard to disease progression remain unknown. Age is a risk factor not only for the development of PD (Allam et al., 2003; Driver et al., 2009) but also for development of levodopa-induced dyskinesias in the parkinsonian patient. While it is clear that increasing age is a risk factor for PD, conversely, LIDs are found to occur more readily in younger patients (e.g.: (Wagner et al., 1996)).

In Aim 1, we employed unilaterally parkinsonian Fischer F344 rats to investigate the role of age in the ability of the parkinsonian brain to respond to pharmacological and cell replacement therapies. Young (3 m.o.), middle aged (12 m.o.) and aged (22 m.o.) parkinsonian rats received daily levodopa treatment followed by a dopamine neuron or sham graft placed into the dopamine-depleted striatum. Young, middle aged and aged subjects were grafted with DA-grafts of 200,000, 500,000, or 900,000 cells, respectively, according to data from Collier et al., which demonstrates reduced DA-graft survivability.
in the aged striatum (Collier et al., 1999). Subsets of parkinsonian rats from each age and treatment group were treated with levodopa and resulting LIDs were measured. Parkinsonian rats treated with levodopa developed LIDs regardless of age at time of lesion. DA-grafted animals demonstrated recovery of amphetamine-induced rotational asymmetry compared to sham-grafted rats, regardless of age. Further, grafting reduced LIDs to the same level by the final post-graft evaluation timepoint across all age groups. This post-graft reduction in LIDs was initially delayed in aged DA-grafted rats, compared to young DA-grafted rats. Graft volume, grafted cell survival, neurite outgrowth, striatal integrity, and FosB expression were assessed in each grafted animal and compared across age groups. A significantly higher number of grafted dopaminergic cells survived in the aged striatum (22m.o.), which translated to a significantly greater graft volume, compared to middle aged and young DA-grafted rats. Although DA-grafted rats demonstrate greater striatal disruption than sham-grafted rats, and despite significantly larger grafts in the aged cohort, there was minimal disruption of normal striatal cytoarchitecture across all ages (as assessed with DARPP-32 staining). Further, neurite outgrowth from DA grafted neurons was extensive regardless of host age, although there were differences in direction of neurite extension between ages. Finally, dorsolateral expression of FosB, which is elevated by DA-depletion, and by levodopa administration and correlated with LID expression, was normalized with grafting in all age groups. Together, these findings suggest a preservation of adaptability of the aged host striatum to support transplanted DA cells, which is counter to the current notion that the older host environment offers poor survivability to grafted cells (Collier et al., 1999; Sortwell et al., 2001).
2.2 Aim 2: Understanding mechanisms of levodopa-induced dyskinesias: The role of striatal pathology on levodopa-induced dyskinesias

Although the primary neurochemical deficiency in Parkinson’s disease is related to death of nigral dopamine neurons that innervate the striatum, it is not clear precisely how this alteration leads to symptoms of the disease (Day et al., 2006). It is known that DA denervation results in numerous changes in transmitter function and altered neuron morphology. Specifically, the primary site for afferent input from nigral DA neurons and cortical glutamate neurons is the striatal medium spiny neuron (MSN). As indicated by its name, the MSN contains numerous dendritic spines, small stalk-like protrusions covering the surfaces of its dendrites. The numerous dendritic spines found on normal MSNs are critical sites for synaptic integration of DA and glutamate signaling, which is essential for normal motor behavior. In advanced PD there is a marked atrophy of dendrites and spines on these neurons (McNeill et al., 1988; Stephens et al., 2005; Zaja-Milatovic et al., 2005). Similar pathology is observed in mice and rats with severe DA depletion (e.g.: (Day et al., 2006)). Importantly, a mechanism involving intraspine Cav1.3 L-type Ca\(^{2+}\) channels has been found to account for spine loss following striatal DA depletion. Administration of the calcium channel antagonist nimodipine to rats, or the absence of Cav1.3 calcium channels in transgenic mice prevents dendritic spine loss despite severe DA depletion (Day et al., 2006). The impact of altered dendritic morphology of MSNs in the parkinsonian striatum on efficacy and/or development of side-effects of standard symptomatic therapy remains unclear. However, it is anticipated that an absence of these critical input sites in the parkinsonian striatum make it difficult for standard levodopa therapy to recapitulate normal physiological
responses in the face of altered synaptic connectivity, and may lead to aberrant plasticity resulting in development of LIDs.

Aim 2 of this dissertation tests the primary hypothesis that loss of dendritic spines on striatal MSNs is a key step in the emergence of pathological activity in the basal ganglia that is intimately linked to the development of LIDs, and preventing such spine loss will eliminate, or reduce development of these aberrant behaviors. The design of these studies also allowed us to test two other hypotheses not previously addressed in the scientific literature: 1) that aberrant dendritic spine remodeling in the striatum occurs following chronic levodopa and is a key feature underlying the development of LIDs; and 2) that reduction of spine density on MSNs plays a key role in classical basal ganglia motor deficits and decreased anti-parkinsonian drug responsiveness in severely parkinsonian subjects.

To test the proposed hypotheses I examined the role that dendritic spine loss plays in development of abnormal dyskinetic movements and/or generalized motor deficits by comparing a battery of specific behavioral tests (see methods) in severely DA depleted rats treated either with: 1) nimodipine to prevent spine loss, or 2) control vehicle which allows for typical dendritic spine loss. The findings of my studies in Aim 2 demonstrate that 1) spine loss that accompanies severe DA depletion contributes to development of aberrant levodopa-induced behaviors, and 2) alterations in spine morphology occur in the striatum following levodopa, which may occur regardless of spine density alterations related to DA depletion.
Chapter 3: Materials and Methods

*If your experiment needs statistics, then you ought to have done a better experiment.*

3.1 Aim 1- Impact of age on graft function

Care and use of animals were in compliance with all applicable state laws and regulations as well as principles expressed in the National Institutes of Health and the United States Public Health Service Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at University of Cincinnati approved this study prior to initiation.

3.1.1 Brief summary of the experimental design

Details for methods in Aim 1 are described in the following sections and summarized in Figure 3.1.1 and 3.1.2. Briefly, male Fischer 344 rats were rendered parkinsonian via unilateral stereotaxic injection of 6-OHDA into the substantia nigra and medial forebrain bundle. Three age groups of rats were used such that young rats were 3 months old at time of lesion, middle aged rats were 15 months old at time of lesion, and aged rats were 22 months old at time of lesion. Parkinsonian motor behaviors were evaluated using amphetamine-induced rotations and the lateral forepaw displacement test as further described below. Three weeks after lesioning, animals received daily levodopa injections (M-F) for the remainder of the study (15 weeks). Eight weeks after lesioning, subsets of lesioned rats from each age group received either fetal ventral mesencephalic DA grafts or acellular sham grafts. The number of grafted cells was varied between aged groups as previously explained, such that young rats received grafts of 200,000 cells, middle aged rats received grafts of 500,000 cells, and aged rats received grafts of 900,000 cells. Levodopa therapy was continued one week post-
grafting, and animals were assessed 30 minutes post-levodopa for LIDs. Eleven weeks after grafting, rats were sacrificed via saline/paraformaldehyde perfusion as described below.

**Figure 3.1.1** Timeline of all surgical procedures and experimental treatments in Aim 1

<table>
<thead>
<tr>
<th>Age Group</th>
<th>N</th>
<th>Successfully Lesioned (excluded)</th>
<th>Successfully Grafted (excluded)</th>
<th>Died during study</th>
<th>Survived and included in final study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (3 mos) Sham</td>
<td>8</td>
<td>4 (4)</td>
<td>n/a</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Young (3 mos) Graft</td>
<td>8</td>
<td>4 (4)</td>
<td>4 (1)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Middle (15 mos) Sham</td>
<td>15</td>
<td>11(4)</td>
<td>n/a</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Middle (15 mos) Graft</td>
<td>15</td>
<td>10(5)</td>
<td>8(2)</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Aged (22 mos) Sham</td>
<td>15</td>
<td>13(2)</td>
<td>n/a</td>
<td>8</td>
<td>11; 5(final timepoint)</td>
</tr>
<tr>
<td>Aged (22 mos) Graft</td>
<td>15</td>
<td>11(4)</td>
<td>10(1)</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

**Figure 3.1.2** Summary of animals used in Aim 1. Number of rats excluded from study are shown in parenthesis. n/a: not applicable
3.1.2 Nigrostriatal dopamine lesion

Adult male Fischer 344 (F344) rats aged 3 months, 15 months, or 22 months at time of lesion received unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA) into both the left medial forebrain bundle (MFB) and left substantia nigra (SN). Nigral coordinates were 4.8 mm posterior to bregma, 1.7 mm lateral to midsaggital suture, and 8.0 mm deep from skull; MFB coordinates were 4.3 mm posterior to bregma, 1.2 mm lateral to midsaggital suture, and 8.0 mm below skull. Animals were anesthetized prior to surgery with a chloropent solution (3.0 ml/kg; chloral hydrate 42.5 mg/ml + sodium pentobarbital, 8.9 mg/ml) and placed in a stereotaxic frame. The neurotoxin 6-OHDA (5ug 6-OHDA HBr/μl 0.9% saline with 0.2 mg/ml ascorbate) was injected at a rate of 0.5 ul/min (2 ul total at each site) using a Hamilton 5 ul syringe with a 26-gauge needle.

3.1.3 Amphetamine-induced rotational asymmetry

Amphetamine rotations at 2 weeks post-lesion and 10 weeks post-graft were used to confirm the presence of an adequate lesion and to assess behavioral efficacy of neural grafts, respectively. Lesioned rats were injected with amphetamine sulfate (5 mg/kg, i.p.; 0.1 cc/100 g body weight). Rotational behavior was monitored for 90 min using automated rotometers (TSE Systems, Germany). Rats rotating prior to transplantation at a rate of ≥7 turns per minute over 90 min were included in this study. It has been our experience that rats with this rotational rate have >90% nigral DA cell depletion and develop levodopa-induced dyskinesias (LIDs) (Steece-Collier 2003, Maries 2006).
3.1.4 Lateral forepaw step adjustment testing

Forepaw step adjustment was tested at 4, 6, and 10 weeks post-grafting surgery by gently restraining each animal and allowing only one forepaw to make contact with an automated conveyer moving at 7cm/second according to the protocols established by Schallert and colleagues (Schallert, 2006). DA-depleted rats exhibit decreased ability to adjust the forepaw corresponding to the lesioned side as it is laterally displaced by the moving conveyor. Data are expressed as average steps/minute for the forepaw corresponding to the DA-depleted side.

3.1.5 Levodopa priming

Three weeks after a 6-OHDA lesion, but prior to grafting, animals in the levodopa treatment groups (defined below) were injected daily to induce dyskinesias prior to grafting. The schedule for levodopa priming included 1 week of once daily levodopa at a dose of 25 mg/kg levodopa plus 25 mg/kg benserazide (a peripheral decarboxylase inhibitor) followed subsequently by daily 12.5 mg/kg levodopa plus 12.5 mg/kg benserazide for an additional 3 weeks. A separate control cohort of animals (defined below) received the vehicle saline instead of levodopa. Levodopa or saline was injected intraperitoneally at the same time daily, Monday through Friday.

3.1.6 Preparation of tissue for transplantation

Ventral mesencephalic tissue containing developing A8-A10 DA cell groups was dissected from E14 (CRL 10.0–11.5 mm) F344 rats. Ventral mesencephalic tissue was
collected and pooled in 4°C calcium–magnesium free buffer (CMF: 137 mM NaCl/ 2.7 mM KCl/ 8 mM Na2HPO4/ 1 mM KH2PO4/ 5.5 mM glucose). Tissue was then transferred to CMF containing 0.1% trypsin, warmed to 37°C in a water bath for 10 min, rinsed in CMF again, and triturbated in 0.004% DNase using Pasteur pipettes of 1.0 mm and 0.5 mm tip diameter. The resulting suspension was pelleted by centrifugation at 500 xg for 10 min. The pellet was suspended in 1.0 ml of Neurobasal Media (Gibco). Estimation of cell number and viability (via trypan blue exclusion) was determined. Final suspensions were prepared at a density of 33,333 cells/ul for young rats; 66,666 cells/ul for middle aged rats, and 150,000 cells/ul for aged rats according to the design detailed below. Cells were kept on ice during transplantation surgery and used within 3–4 hours of preparation.

### 3.1.7 Dopamine neuron transplantation

Rats were randomized into sham graft and DA graft groups. Pre-graft LID scores were equal for sham graft and DA graft groups. Dopamine grafted animals received fetal ventral mesencephalic cells at a single rostral/caudal site (AP +0.2, ML +2.8), with tissue dispersed along three dorsal/ventral locations at this site; −4.4, 5.1 and 5.8 ventral to dura. Rationale for the graduated increase in the numbers of grafted VM cells for middle and advanced aged rats is based on our previous experience (Collier et al., 1999) showing a progressive decline in DA graft survival with advancing age in rats. The youngest rats, at 3 mos of age received 200,000 cells/animal. Due to a predicted 50% reduction in cell survival in middle aged rats (Collier et al., 1999), 15 mos rats received 400,000 cells/animal. Similarly, due to a predicted five-fold reduction in cell
survival in aged rats (Collier et al., 1999), 22 mos rats received a total of 900,000 cells/animal (Collier et al., 1999). Injections were delivered using a 10ul Hamilton syringe with a 26 gauge needle. Each dorsal-ventral injection site received 2ul of VM cells for a total volume of 6ul for each rat. Sham-graft subjects received injections of 6ul of cell-free vehicle media using the same stereotaxic coordinates employed for cell deposits in the DA grafted animals. Injection of identical volumes of fluid in sham-grafted animals was included to control for impact of potential tissue damage on experimental outcomes of each treatment. The needle was left in place for three minutes after the last injection. All levodopa treatment groups were given a 7-day levodopa drug-free period following grafting (Lee et al. 2000; Steece-Collier 1999, Maries 2006). One week after transplantation, rats resumed their daily levodopa (12.5 mg/kg levodopa: 12.5 mg/kg benserazide, i.p.). The vehicle groups were given the same 7-day injection-free period.

Ten weeks after grafting, animals were tested for their rotational response to amphetamine (5 mg/kg, i.p.) for additional assessment of graft efficacy. Prior to the amphetamine test, animals were withdrawn from levodopa for 48 hours to avoid direct interaction of levodopa with amphetamine.

3.1.8 Dyskinesia rating

The term “dyskinesia” is used throughout the text to refer to abnormal involuntary movements including dystonia, hyperkinesia, and/or stereotypies noted in rats in response to levodopa. Details of these behaviors and the rating scale used for
rating are reported elsewhere (Steece-Collier et al., 2003). Dyskinetic behaviors were rated for 2 min, 30 minutes after the levodopa injection, a time that corresponds to peak dose dyskinesias. Ratings were performed daily by the same individual throughout the study. This investigator was blinded to the treatment conditions and checked weekly with inter-rater reliability by a second blinded investigator. During the rating session for each animal, both severity and the frequency of individual dyskinetic behaviors were evaluated and recorded. A total daily dyskinesia severity score was obtained by multiplying frequency and intensity. All experimental animals were randomized at the beginning of the experiment and rated in the same order throughout the experiment. Injections were spaced such that each animal was rated precisely 30 minutes after levodopa injection. Total LID scores were computed by adding the severity scores of individual dyskinetic behaviors.

### 3.1.9 Necropsy and preparation of tissue

Eleven weeks after grafting, animals were perfused transcardially with room temperature heparinized 0.9% saline (150 ml) followed by 100 ml buffered fresh 4.0% paraformaldehyde. Brains were postfixed for 24 hours in 4.0% paraformaldehyde followed by transfer to 30% sucrose solution for 48–72 hours. Brain sections were cut on a sliding microtome at 40 μm, and all sections were stored at 0°C in a cryoprotectant solution before processing.
3.1.10 *Immunohistochemistry*

Paraformaldehyde-fixed sections through the striatum were used for immunohistochemical staining of tyrosine hydroxylase (Kordower *et al.*, 1995; Steece-Collier *et al.*, 1995), FosB/deltaFosB, and DARPP-32. A separate series of every sixth section through the striatum were employed for either TH or DARPP-32 immunochemistry, and animals from all groups were assayed at the same time. For TH immunohistochemistry, sections were incubated with TH primary antibody (1:8000; Immunostar, Hudson, Wisconsin) for 36 hours at room temperature followed by 90 minutes in horse anti-mouse biotinylated IgG (1:200; Vector Laboratories, Burlingame, CA) and developed using diaminobenzadine (DAB). For DARPP-32 immunohistochemistry tissue was incubated overnight at room temperature in monoclonal Darpp-32 primary antibody (1:1000; Cell Signaling Technology) followed by incubation for one hour in goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA) and developed using DAB. Every sixth section through the striatum was incubated overnight at room temperature in an antibody against FosB/deltaFosB isoforms (1:2000, Santa Cruz Biotech, CA), followed by incubation in biotinylated goat–anti rabbit IgG (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in TBS containing 1% goat serum for 90 min at room temperature. Sections were developed using the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) and DAB. Controls consisted of processing tissue in an identical manner except for omitting the primary antibody. Sections were mounted on gelatin-coated slides, dehydrated, and cover slipped. The slides were visualized using light microscopy.
3.1.11 Stereological quantification of graft cell number

The number of grafted TH-ir cells located within the striatum was estimated using an optical dissector sampling design (Gundersen et al., 1988; West et al., 1991; Kordower et al., 2001) by an investigator blinded to the grafting conditions. Approximately three to five equi-spaced sections were sampled along the entire length of the graft within the striatum of each brain. The region of the graft was outlined using a 4x objective. A systematic sample of the area occupied by the graft was made from a random starting point determined by the software (Stereoinvestigator 2000 software; MicroBrightField, Colchester, VT). The counting frame was established at 50μm x 50μm such that the number of sampling sites covered the entire area of the graft within each section, without overlap, or gaps, thus enabling complete enumeration of the analyzed tissue. These parameters resulted in roughly 700-1400 sample sites per section. Counts of TH-ir cells were made at regular predetermined intervals (x = 50μm, y = 50μm), and a counting frame (50 x 50 μm = 2500 μm²) was superimposed on the image of the tissue sections. These sections were then analyzed using a 60x planapo oil immersion objective. Section thickness averaged approximately 24 μm. The optical dissector height was 20 μm. This method allowed for guard zones of 2μm on either surface of the tissue sample, such that cell counting was only conducted within the optical dissector. TH-ir cells were only counted if the first recognizable labeled profiles of the cell came into focus within the counting frame (West et al., 1991).
3.1.12 Graft volume quantification

Striatal and graft volumes were calculated using the Neurolucida stereology program. Briefly, contours were defined for both striatum and graft and the outlines of each structure were traced in TH-ir sections throughout the entire length of the striatum. The same tissue used for graft cell counts was used for graft volume analysis. The Neurolucida Explorer program was used to calculate total graft and striatal volume for each animal from the tracings obtained through Neurolucida.

3.1.13 Striatal integrity

Volume of striatal displacement under all grafting conditions (e.g. VM or sham) was assessed via DARPP-32-ir staining of striatum in each experimental subject. As with graft volume measurement, striatal outlines were traced throughout the striatum in its entirety. The area devoid of DARPP-32 staining was clearly demarcated in 3-5 sections of the striatum for all subjects, corresponding to the VM graft or sham-graft area. This area was traced with a contour specific for striatal area devoid of DARPP-32 immunoreactivity. The Neurolucida Explorer program was used for volume calculations. Data is expressed by percent of total striatum with positive DARPP-32 staining.
3.1.14 Densitometry analysis of FosB/deltaFosB-ir cells

Three sections of dorsolateral precommissural striatum were analyzed for each animal by an investigator blinded to the experimental treatment. Tissue section selected for analysis corresponded to (1) pre-graft striatum, (2) the first section of grafted striatum, and (3) the second section of grafted striatum. Slides were visualized at 40x using light microscopy (Olympus BX60, Olympus, USA) and were digitized using a Nikon DM1200 camera and ACT software (Nikon Microscopy, USA). At each of the three striatal levels, two sampling images were obtained for both the DA-depleted and intact striatum. The first image of striatal FosB/deltaFosB positive cells was obtained adjacent to the lateral border of the striatum at a mid-dorsoventral level. The second imaged area was of the same size, adjacent and just medial to the first area. Densitometry of FosB/deltaFosB-ir cells within the digitized images was performed using ImageJ software. The investigator blinded to the experimental conditions outlined each of the FosB/deltaFosB-positive cells in the digitized images, with the optical density of each cell then being measured by the software. A mean optical density reading was generated for each FosB-ir cell. An average of 50 cells was sampled per field of view (giving a total of optical density samples from 300 cells per animal per side). An average optical density was calculated for all FosB/deltaFosB-ir cells present in the two adjacent field of view images. The background level of optical density was determined by tracing small non-cellular areas within the striatum located between the FosB/deltaFosB-ir cells. An average background optical density value was obtained from a total of 60 regions per animal per side. The corrected FosB/deltaFosB optical density was obtained by subtracting the average background optical density from the
average optical density obtained for the FosB/deltaFosB-ir cells. The optical density of
the FosB/deltaFosB-positive cells present in the dorsolateral lesioned striatum at each
level was expressed as a percent increase from the value found in the unlesioned
(intact) striatum at the same level.

3.1.15 Quantification of neurite outgrowth via densitometry

Densitometry. Densitometry of neurite outgrowth was quantified using the
Nikon NIS-Elements software to assess TH immunoreactivity in multiple fields of view
extending from the dorsal, ventral, lateral, and medial borders of the grafts. Measurements are expressed as the “Area Fraction,” which represents the percentage
of the field of view occupied by TH-ir fibers on the microscope. All fields of view for all
animals were set to identical light settings on the microscope and identical threshold
levels (0; 215) to subtract background staining. Background readings were taken from
multiple fields of view with the fiber tracts of the corpus callosum for each animal to
establish this threshold before data collection began. The investigator was blinded to
the age and treatment conditions of each animal before data collection. For each
animal, the dorsal, medial, ventral, and lateral borders of the graft were identified under
the microscope. The section with the largest portion of graft was used for analysis for
each animal. Beginning at the dorsal border of the graft, images were acquired at 40X
as consecutive adjacent fields of view, with no overlap between regions. This results in
each field of view representing a total area of 281.6um X 225.28um. Images were
collected from the border of the graft to the corresponding striatal border (i.e., dorsal
border of graft to dorsal border of striatum), resulting in data from 3-12 fields of view for any given direction. The first six fields of view extending from the graft border in each direction were used for analysis as reliable detection of staining was reduced beyond this distance. A screen shot from the Nikon software used to collect densitometry data is shown in Figure 3.1.3. The portion of the image highlighted in red designates the area counted as immunoreactive for TH. In this particular example, this yields an area fraction of 51.16%. Data was analyzed for neurite extension proximal to the graft (1-3 fields of view from graft border) and distal to the graft (4-6 fields of view from graft border) in each measured axis (dorsal, medial, ventral, lateral), for young, middle aged, and aged DA-grafted rats (Figure 3.1.4).
Figure 3.1.3: Screen shot from Nikon NIS-Elements software used for neurite outgrowth densitometry. Arrow indicates Threshold setting window; Arrowhead indicates Area Fraction. Area highlighted in red represents staining within threshold limits, corresponding to an Area Fraction of 51.16% in this particular sample.
Figure 3.1.4: Neurity density analysis was performed in consecutive fields of view (boxes) from the dorsal (D), medial (M) ventral (V) and lateral (L) borders of the graft. Blue arrow denotes proximal fields of view and red arrow denotes distal fields of view.

3.1.16 Quantification of THir fiber density in the striatum using Spaceballs

Fiber density analyses were performed utilizing the Space Balls hemispheric probe to obtain an unbiased estimate of TH-ir neurite densities in the striatum based on previously reported methods. Contours were drawn for three fields of view matching the dimensions of the fields of view used for the densitometry analysis at the lateral border of the graft. Contours of the same side were drawn on the intact side to collect fiber
density for the intact side of each rat. The same section of grafted tissue analyzed in the densitometry analysis was used for analysis via the Spaceballs software. Neurites were counted using a BX52 Olympus microscope (Olympus America Inc.) equipped with a Microfire CCD camera (Optronics). The camera settings were maintained throughout the entirety of each experiment and the investigator was blinded to the treatment conditions during analysis.

3.1.17 **Statistical analysis**

Weekly dyskinesia scores were obtained as an average of the three daily assessments from each week (i.e.: Wednesday, Thursday, Friday). Dyskinetic behavior was analyzed using three-way analysis of variance employing Sigma Stat 3.0 software (Aspire Software International, Leesburg, VA) to examine significant differences of (1) severity of LIDs between ages, (2) behavioral efficacy of grafting between age groups. Student-Neuman-Keuls (SNK) test was used to control for multiple comparisons when there was a significant ANOVA. An ANOVA followed by Student-Neuman-Keuls (SNK) test was also used to examine the presence of significant differences between sham and DA grafts, as well as the impact of host age on survival of grafted TH-ir cells, DA graft volume, and ability of TH-ir cells to normalize optical density of the FosB/deltaFosB marker. Two-way ANOVAs were used to determine statistical differences in neurite outgrowth, followed by the SNK post-hoc test. The level of statistical significance was set at 0.05 for all statistical analyses.
3.2 **Aim 2- Understanding mechanisms of levodopa-induced dyskinesias: The role of striatal pathology on levodopa-induced dyskinesias**

The experiments for Aim 2 summarize data from two series of experiments, my first “pilot studies” and the “follow-up studies.”

### 3.2.1 Pilot study experimental design

Animals for these pilot studies underwent experimental procedures to be rendered parkinsonian as outlined in Figure 3.2.1. Experimental groups for pilot data are outlined in Figure 3.2.2. The details of each protocol employed in these experiments follow in subsequent sections of this methods chapter.

Briefly, rats either DA-depleted with 6-OHDA, or used as intact controls. DA-depleted rats received with vehicle or nimodipine pellets, and subsets of each treatment group received levodopa or saline challenge, such that there was a final n=10 rats for each group (Figure 3.2.2). Rats were trained and pre-tested for parkinsonian motor behaviors before DA-depletion. Two days after lesion, 21-day continuous release nimodipine pellets (0.8mg/kg/day) were implanted into the subscapular space as described in the methods section. Nimodipine pellets were replaced every 20 days to ensure continuous availability of nimodipine throughout the experiments. One to two weeks after nimodipine administration, post-lesion parkinsonian motor behaviors were evaluated (Figure 3.2.1). Levodopa treatment was initiated two weeks after initial nimodipine pellet implantation/ DA-depletion surgery. In pilot studies, escalating
dosages of levodopa were tested for behavioral response in the ranges of 6-25mg/kg (1:1; levodopa:benserazide). Final parkinsonian motor behaviors were evaluated before sacrifice of rats at the study endpoint.

Figure 3.2.1: Experimental timeline for pilot nimodipine data. Animals are implanted with 21-day continuous release nimodipine pellets two days after being rendered Parkinsonian via 6-OHDA. Pellets are replaced every 20 days. Post-lesion parkinsonian motor behaviors are evaluate periodically throughout the study. Levodopa administration begins three weeks after DA-depletion, and resulting LIDs are evaluated throughout the week.
<table>
<thead>
<tr>
<th>Group</th>
<th>DA Status</th>
<th>Treatment</th>
<th>Challenge</th>
<th>Spine Status</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Depleted</td>
<td>Vehicle pellets</td>
<td>Levodopa</td>
<td>atrophy</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Depleted</td>
<td>Vehicle pellets</td>
<td>Saline</td>
<td>atrophy</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Depleted</td>
<td>Nimodipine pellets</td>
<td>Levodopa</td>
<td>normal</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Depleted</td>
<td>Nimodipine pellets</td>
<td>Saline</td>
<td>normal</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Intact</td>
<td>--</td>
<td>Levodopa</td>
<td>normal</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>Intact</td>
<td>--</td>
<td>Saline</td>
<td>normal</td>
<td>10</td>
</tr>
</tbody>
</table>

*Figure 3.2.2: Experimental groups for data from pilot nimodipine study*
3.2.2 Follow-up study data: Experimental design and timeline

The following figures summarize the results for the experiments designed to follow-up the pilot nimodipine study, just described. The experimental design and timeline are summarized in Figure 3.2.3, and Figure 3.2.4, respectively. Briefly, cohorts of rats were rendered parkinsonian and treated with either 21 day continuous release nimodipine or vehicle pellets. Nimodipine and vehicle treated rats were then divided into two subsets to receive either levodopa or saline challenge. Each treatment group consisted of 8 rats (Figure 3.2.3). Levodopa administration began three weeks after grafting and continued to the end of the study (approximately 5 weeks). In this experiment, rats received levodopa at 12.5-25 mg/kg per dose. Rats were sacrificed 60-80 minutes post-levodopa injection and saline perfused, followed by paraformaldehyde (Figure 3.2.4).
**Figure 3.2.3: Experimental Design for Aim 2; Follow-up Study.** Groups of male Sprague-Dawley rats were rendered parkinsonian and administer nimodipine or vehicle +/- levodopa as indicated.
Figure 3.2.4: Timeline for Aim 2 experiments for follow-up study. Animals underwent the same course of interventions as in preliminary data, except levodopa doses were administered at 12.5mg/kg or 25mg/kg throughout the study. At the end of the study, rats were anesthetized and sacrificed via perfusion with saline and paraformaldehyde at 60-80 minutes post-levodopa administration.
3.2.3 Experimental subjects and dopamine denervating lesion

Adult male Sprague-Dawley (200-225g) rats were rendered parkinsonian via unilateral striatal DA depletion through stereotaxic injection of the neurotoxin 6-hydroxy-dopamine into the substantia nigra and medial forebrain bundle. Using this lesion paradigm our lab routinely achieves ~99% nigral DA depletion. Successful nigrostriatal DA lesion was confirmed by rotational asymmetry in response to levodopa. Total clockwise and counter-clockwise rotations were counted by an investigator blinded to treatment conditions for one minute, 30 minutes post levodopa injection. Rotational scores were calculated as described in section 3.1.3. Success of nigrostriatal DA lesion was also confirmed using the vibrissae task as described below.

3.2.4 Nimodipine administration

Twenty four hours post 6-OHDA DA-depletion, rats were implanted with 21 day continuous-release nimodipine pellets (0.8 mg/kg per day nimodipine) or inert control pellets (all pellets from Innovative Research of America) into the interscapular space (Day et al., 2006). Pellets were replenished every 20 days throughout the duration of the study to ensure no lapse in drug-release. Two weeks after DA-depletion daily levodopa or sterile saline vehicle injections were begun. Dyskinetic behaviors were rated as detailed below, and after these behaviors stabilized animals were sacrificed as described below.
3.2.5 Levodopa treatment and LID rating

Two weeks after DA-depletion, levodopa was introduced to a subset of vehicle and nimodipine treated rats. All rats were evaluated for both parkinsonian motor behavior as well as LIDs throughout the study. Two weeks after DA-depletion, both nimodipine and levodopa treated rats were randomized to saline or levodopa treatment groups. Those rats designated for treatment with levodopa were started at 12.5mg/kg of levodopa + 12.5mg/kg the dopa decarboxylase inhibitor benserazide. Levodopa injections varied between the pilot study and the follow-up study for rationale discussed below. Rats received levodopa 5 days a week (M-F) and were evaluated blindly for LIDs either three times a week (W-F) in the pilot study, or twice weekly (Monday and Thursday) in the follow-up study. In the follow-up study, I chose to focus on LID behavior early in the week as observations from the pilot studies demonstrated that the differences in dyskinesia development between nimodipine and vehicle treated rats are largest early in the week and tended to diminish later in the week after repeated doses of levodopa. That is, the neuroprotective effect of spine preservation waned with repeated dosing over the week of treatment.

In the pilot study, after approximately 4 weeks of high dose levodopa, rats were switched to a lower dose of the drug. Rationale for this switch was that preliminary data from parallel studies in our lab suggested that reducing the dose of levodopa from 12.5 to 6.25 mg/kg appeared to allow for an additional perspective on the differential effect of spine preservation on LIDs development. Specifically, once levodopa priming occurs, rats are well-known to maintain the peak high level of aberrant LIDs behavior even when the drug dose is decreased. Thus, I was interested in determining whether
lowering the dose of levodopa might “re-instate” the buffering capacity of spine preservation against LID severity in parkinsonian rats with normal spine density, while maintaining the same high level of LID in the parkinsonian rats with severe spine loss.

In the follow-up study, rats were treated at 12.5mg/kg levodopa for approximately 4 weeks, followed by challenge doses of 25mg/kg for two weeks in order to evaluate differences in severity of LIDs between parkinsonian rats with normalized spine density versus those without at both doses of levodopa. Challenge doses at the high dose of 25mg/kg were employed maximize the differences in LID severity between both treatment groups. During challenge doses, rats were rated for LIDs every 20 minutes post injection for 2 minutes at a time until they were no longer exhibiting dyskinesias (180 minutes at 12.5mg/kg; 240 minutes for 25 mg/kg dose).

3.2.6 Sensori-motor integration (vibrissae) test

For this test, animals are gently suspended by the tester so that all limbs hang freely except the forelimb not being tested, which is restrained. The animal is then moved toward the corner of a platform (e.g.: laboratory bench top) so that the vibrissae/whiskers on only one side of the face make contact with the platform. The animal responds by reaching for the platform edge with the corresponding paw. Striatal DA depletion results in a dose-dependent decrease in the number of successful forepaw responses with the limb contralateral to the lesion. For example, before lesioning, rats will perform in the vibrissae task with a 100% success rate (i.e. 10/10 forepaw taps in response to ipsilateral whisker stimulation). Successfully lesioned rats
maintain a 100% successful tapping rate on the “intact” side of the body, but exhibit less than 10% successful tapping on the “lesioned” side of the body (0-1/10 successful taps).

### 3.2.7 Dendritic spine density quantification using a modified Golgi-Cox impregnation method

Rats from pilot study experiments were sacrificed one hour after levodopa, a time when peak dose LIDs are expressed (peak dose LIDs are noted from 30-80 minutes post levodopa, e.g.: Maries et al., 2006). Prior to sacrifice, rats were evaluated for LID severity 30 minutes post levodopa. At time of sacrifice, rats were deeply anesthetized with sodium pentobarbitol and perfused with 0.9% saline, followed by 200ml 4% paraformaldehyde. Rats were decapitated, and the brains were quickly removed and sectioned for Golgi staining and immunohistochemistry. Brains were post-fixed in 4% paraformaldehyde for an additional 60 minutes and then transferred to 0.1M phosphate buffer prior to sectioning. Sections through the striatum were collected in series of six sections at 50µm, which were placed in cryoprotectant and retained for immunohistochemistry. Each seventh section was cut at 100 µm and retained for Golgi-impregnation. Sections retained for Golgi were placed on the end of a non-subbed glass slide. Two such samples were placed on each slide, and then a second slide was gently placed on top without compressing the samples. The end of the slides opposite the samples was gently wrapped with standard electrical tape and the whole construct was submerged in Golgi fixative and placed in a dark storage area, completely devoid of light (Golgi fixative: 10g mercuric chloride, 10g potassium dichromate, 10g potassium chromate in 1L water). The fixative was exchanged on the second day of impregnation
and the samples were allowed to develop for two weeks. The fixative was replaced by a 1% solution of potassium dichromate, and the following day the slices were teased off the slides directly into a Petri dish containing 1% potassium dichromate by gently running a single-edge razor blade along the slide underneath the slice. The slices were then transferred to 4% gel-coated slides. After drying in a humidity chamber for 20 minutes, the slides were placed in 28% ammonia hydroxide for 30 minutes, followed by a 5-minute rinse in tap water, a 15-minute rinse in 15% Kodak developer and an additional rinse in tap water. Finally, the slides were put through a dehydration series of alcohol (50%, 70%, 95% and 100%), cleared in xylene and coverslipped using DPX (distyrene, plasticizer, xylene) mounting medium.

**Criteria for spine counts:**

Dendritic spine density was quantified via light microscopy using the Neurolucida stereological neuronal reconstruction software suite. Dendrites were screened for selection for spine analysis at 20x, and confirmed and traced at 60x. Individual dendrites for analysis were selected on the basis of the Golgi-stain quality of the whole neuron. A neuron of adequate stain appears dark translucent brown with an intact cell body, and complete staining of the majority of associated dendrites. Dendritic spine density is evaluated for both proximal and distal segments of selected full-length dendrites (140-160 µm in total length from soma to dendritic end). A proximal segment starts no closer than 50 µm to the soma and consists of a segment 30-50 µm in length. Distal segments encompass the final 30-50 µm of the same dendrite. Importantly, there is no overlap between proximal and distal segments, with a minimal distance of 20
µm between segments (shown in Figure 3.2.5: Parameters for dendrite selection and quantification of dendritic spine density using Golgi stained tissue. Dendrites and spines were traced at 100x. In quantification of spine density, careful attention was paid to total spine morphology such that spines included in counts were at least 1.3 µm in length, with no breaks or ‘floating heads’. Generally, spines have a slight ‘bowling pin’ appearance, though they may be more extreme in the swelling at their terminal ends (i.e. mushroom spine) or bifurcated.
**Figure 3.2.5:** Parameters for dendrite selection and quantification of dendritic spine density using Golgi stained tissue. A proximal segment starts no closer than 50 µm to the soma and consists of a segment 30-50 µm in length. Distal segments encompass the final 30-50 µm of the same dendrite. Importantly, there is no overlap between proximal and distal segments, with a minimal distance of 20 µm between segments. Dendrites and spines were traced at 100x.
3.2.8 Immunohistochemistry

**Tyrosine hydroxylase:** One millimeter forebrain slabs designated for immunohistochemistry analysis from the pilot studies, or whole brains from the follow-up studies were immediately post-fixed in 4% paraformaldehyde, transferred to 30% sucrose, and cut at 40um on a freezing microtome. These sections were then processed for tyrosine hydroxylase (TH) immunohistochemistry to confirm successful nigral DA cell depletion for each rat. Sections were incubated with a monoclonal TH (1:4000; Chemicon Inc., CA) primary antibody for 24 hours at 4°C. Sections were then incubated for 2.5 hours in goat anti-mouse biotinylated secondary antisera (1:400; Vector Laboratories, Burlingame, CA) and developed using 3,3-diaminobenzidine (DAB). Sections were mounted on gelatin-coated slides, dehydrated, and cover slipped. The slides were visualized using light microscopy. Only those rats with greater than 95% nigral DA neuron loss are included in the behavioral analysis.

**FosB/deltaFosB:** In the follow-up study, every sixth section of brain (including the striatum) was incubated overnight at room temperature in an antibody against FosB/deltaFosB isoforms (1:2000, Santa Cruz Biotech, CA), followed by incubation in biotinylated goat–anti rabbit IgG (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in TBS containing 1% goat serum for 90 min at room temperature. Sections were developed using the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) and DAB. Controls consisted of processing tissue in an identical manner except for omitting the primary antibody. Sections were mounted on gelatin-coated slides, dehydrated, and cover slipped. Optical density of FosB/deltaFosB was quantified using light microscopy.
3.2.9 Densitometry analysis of FosB/deltaFosB-ir cells

Three sections of dorsolateral precommissural striatum were analyzed for each animal by an investigator blinded to the experimental treatment. This specific location of dorsolateral precommissural striatum was chosen for analysis as elevation of FosB/deltaFosB immunoreactivity in this region is well established to be associated with LID development. Slides were visualized at 40X using an Olympus BX60 microscope (Olympus, USA) and were digitized using a Nikon DM1200 camera and ACT software (Nikon Microscopy, USA). At each of the three striatal levels, two sampling images were obtained for both the DA-depleted and intact striatum. The first image of striatal FosB/deltaFosB positive cells was obtained adjacent to the lateral border of the striatum, adjacent to the corpus callosum and at a mid-dorsoventral level. The second imaged area was of the same size, adjacent and just medial to the first area. Densitometry of FosB/deltaFosB-ir cells within the digitized images was performed using ImageJ software. The investigator (JAO) blinded to the experimental conditions outlined each of the FosB/deltaFosB-positive cells in the digitized images, with the optical density of each cell then being measured by the software. A mean optical density reading was generated for each FosB-ir cell. An average of 50 cells was sampled per field of view (giving a total of optical density samples from 300 cells per animal per side). An average optical density was calculated for all FosB/deltaFosB-ir cells present in the two adjacent field of view images. The background level of optical density was determined by tracing small non-cellular areas within the striatum located between the FosB/deltaFosB-ir cells. An average background optical density value was obtained from a total of 60 regions per animal per side. The corrected FosB/deltaFosB
optical density was obtained by subtracting the average background optical density from the average optical density obtained for the FosB/deltaFosB-ir cells. The optical density of the FosB/deltaFosB-positive cells present in the dorsolateral lesioned striatum at each level was expressed as a percent increase from the value found in the unlesioned (intact) striatum at the same level.

3.2.10 Area under the curve calculations

In the follow-up study, LID severity was compared between parkinsonain rats with intact dendritic spine density versus parkinsonian rats with depletion of dendritic spine density using the area under the curve. The area under the curve (AUC) of LID development for each rating session was calculated using the trapezoidal method, which approximates the definite integral, or the area bounded by the X and Y axes and the data points. While most commonly used to compare pharmacological properties of drugs, AUC measurements in this context enable comparison of total LIDs between treatment groups.

Values were assessed over the total timecourse of levodopa-induced dyskinesias (from 0-180 minutes for 12.5mg/kg levodopa, or 0-240 minutes for 25mg/kg levodopa), as well as for subsets of peak dyskinesias development such as 20-80 minutes. Data is represented as the average AUC for a given treatment group at a given dosage of levodopa. Error is expressed as the standard error of the mean for the average of AUCs for a given treatment group at a given dosage of levodopa.
3.2.11 Medial Terminal Nucleus (MTN) guided confirmation of DA-depletion

DA-depletion of nigral neurons in lesioned rats was confirmed using the MTN guided counting method established by Sauer and Oertel (Sauer & Oertel, 1994). Briefly, TH+ neurons were counted on the lesioned side at 20x via light microscopy throughout the nigra, using the medial terminal nucleus as an anatomical marker of the border of the substantia nigra. Only those neurons with robustly stained cell bodies, visible within the plane of focus, were counted. Three to five sections of nigra were used for each animal. Both the lesioned and intact sides were counted using this method and data is analyzed as (# cells on lesioned side) / (# neurons intact side)x 100 = percent lesioned. The investigator was blinded to all treatment conditions during quantification.

3.2.12 Statistical analyses

Dyskinesia scores were obtained from pilot studies as an average of the three daily assessments from each week (i.e.: Wednesday, Thursday, Friday). Dyskinesia scores from follow-up studies are assessed on either Monday or Thursday of each week. Dyskinetic behavior was analyzed using two-way analysis of variance employing Sigma Stat 3.5 software (Aspire Software International, Leesburg, VA) to determine significant differences. Difference between nimodipine and vehicle treated groups for LID development, rotational response to levodopa, FosB/deltaFosB expression, and parkinsonian motor behaviors were also analyzed with ANOVA. Student-Neuman-Keuls (SNK) test was used to control for multiple comparisons when there was a
significant ANOVA. The level of statistical significance was set at 0.05 for all statistical analyses.
Chapter 4; Experimental Results and Discussion for Specific Aims

*Research is to see what everybody else has seen, and to think what nobody else has thought.*

4.1 Aim 1: Impact of age on graft function

4.1.1 Results

Grafting reduces amphetamine-induced rotational asymmetry across all ages:

In all age groups, post-lesion amphetamine induced rotations were at least 7rpm ipsilateral rotations or greater (rats for grafting; young: 8.607 ± 1.511 rpm; middle: 8.206 ± 0.925 rpm; aged: 7.093 ± 0.827 rpm; rats for sham-grafting: young: 8.544 ± 1.5111 rpm; middle: 7.69 ± 0.925 rpm; aged: 7.606 ± 0.872 rpm). In sham-grafted rats, there was no reduction in the number of rotations per minute in any age group (young: 7.944 ± 1.511 rpm; middle: 6.154 ± 0.925 rpm; aged: 6.683 ± 1.85 rpm). There was significant reduction in the number of amphetamine-induced rotations in DA-grafted animals regardless of age (young: 4.052 ± 1.511 rpm *post-graft sham vs. graft for young p=0.014; middle: 2.1 ± 1.068 rpm *post-graft sham vs. graft for middle, p<0.001; aged: 1.959 ± 1.068 rpm *post-graft sham vs. graft for aged, p<0.001) (Figure 4.1.1).
Figure 4.1.1: Assessing lesion and graft efficacy via amphetamine-induced rotational behavior: Light gray: Post-lesion/Pre-graft rotations; Mid-tone gray: Post-sham-graft rotations; Dark gray: Post-DA-graft rotations. Asterisk denotes p<0.05 for Post-DA-graft animals versus Post-lesion and Post-sham-graft scores within each age group.
4.1.2 Grafted cell survival and total graft volume across ages

Aged rats demonstrate grafts 5-fold greater in DA-neuron number and total volume:

Stereological quantification of TH+ cell number showed that regardless of age, rats exhibited greater than 99% depletion of TH+ dopamine neurons in the substantia nigra on the lesioned side (Figure 4.2.1, A-C). The number of grafted TH positive neurons in the striatum of aged graft recipients was significantly greater than in corresponding grafted middle or young rats (total number TH+ cells: young: 1505.667 ± 590.564; middle aged: 3250.857 ± 616.967; aged: 5261.500 ± 989.093; ANOVA, P = 0.040) (Figure 4.1.2). The overall percentage of grafted DA neuron survival did not vary with age (young: 4.015% ± 1.575; middle aged: 3.468% ± 0.658; 3.118% ± 0.586; ANOVA, P = 0.788).

Stereological quantification of graft volume showed that the aged graft recipients had a significant increase in graft volume compared to their younger counterparts (young: 0.157 ± 0.015 mm$^3$; middle aged: 0.462 ± 0.088 mm$^3$; aged: 0.828 ± 0.313 mm$^3$; ANOVA: P = 0.001) (Figure 4.1.3).

Thus, in the aged grafted rat, as a result of receiving a graft of nearly one million cells, these animals have significantly larger grafts compared to younger cohorts, both in TH+ cell number, and in total graft volume.
Figure 4.1.2: TH immunocytochemistry in DA-grafted young (A,D,G), middle aged (B,E,H), and aged (C,F,I) rats. 6-OHDA-mediated DA-depletion is 99% or greater across all ages (A-C). High magnification view at medial border of graft in young (D), middle (E), and aged (F) DA-grafted rat. Low magnification view of DA-graft in the striatum of young (G), middle (H), and aged (I) DA-grafted rats. Aged rats have significantly greater numbers of surviving TH+ neurons in the grafted striatum compared to middle and young DA-grafted rats. Asterisk denotes p<0.05.
Figure 4.1.3: Graft volume analysis: (A) Graft volume in aged DA-grafted rats is significantly greater than in middle aged or young DA-grafted rats. Asterisk denotes $p<0.05$. (B) Reconstruction of a typical representative example of graft reconstruction from a middle aged rat. R: rostral; C: caudal. Volume occupied by graft is shown in red, outline of striatum is shown in white.
4.1.3 Confirmation of neuronal integrity in the grafted striatum

Neuronal integrity is maintained in the grafted striatum across all ages:

The findings discussed in Figure 4.1.2 and Figure 4.1.3 demonstrate that in the aged grafted rat, as a result of receiving a graft of nearly one million cells, these animals have significantly larger grafts compared to younger cohorts, both in TH+ cell number, and in total graft volume. To determine the degree of striatal damage with grafting, the total volume of striatal DARPP-32 immunoreactivity for grafted and sham-grafted rats was quantified using striatal reconstruction via the stereological software Neurolucida as described in the methods. Robust total DARPP-32 immunoreactivity was detected throughout the striatum, and a distinct area devoid of such staining was clearly visible in each grafted animal (Figure 4.1.4 A-B).

The total volume of striatum expressing DARPP-32 was quantified as the difference between total striatal volume and volume of the area devoid of DARPP-32 immunoreactivity. Despite the observed difference in graft volume and grafted cell numbers, there is no age-dependent difference in the degree of striatal displacement as quantified as the volume of striatum devoid of DARPP-32 immunoreactivity, indicating equivalent disruption of striatal cytoarchitecture across ages. There is, however a small, but significant decrease in the percent of intact striatum with DA-grafting in middle and aged rats as shown in Figure 4.1.4C (in DA-grafted rats: young: 99.85% ± 0.86; middle aged: 98.30% ± 0.57; aged: 96.93% ± 0.473; in sham-grafted rats: young: 99.97% ± 0.75; middle aged: 99.95% ± 0.53; aged: 99.97% ± 0.669; 2-way ANOVA of grafted vs. shams across ages: P = 0.001). Despite this age-related reduction in the percent of intact striatum, in both the sham-grafted and DA-grafted rats, greater than
96% of the striatum remains intact, indicating minimal disruption of striatal cytoarchitecture, despite the presence of varying sizes of DA-grafts. Further, the greater degree of striatal displacement observed in middle and aged DA-grafted rats compared to sham-grafted rats confirms the larger graft volumes seen in middle aged and aged DA-grafted rats compared to young DA-grafted rats.

**Figure 4.1.4: Dopamine and cAMP-regulated phospho-protein (DARPP-32):**  
(A) Representative photomicrograph of striatal DARPP-32 staining at 1.25x.  
(B) Representative photomicrograph at border of region devoid of DARPP-32 staining, 10x.  
(C) Quantification of the percent of striatal volume with DARPP-32+ immunoreactivity.  
Light gray: DA-grafted rats; Dark gray: Sham-grafted rats. Asterisk indicates p<0.05 for sham vs. DA-grafted rats at that age.
4.1.4 Neurite outgrowth of grafts in young, middle, and aged rats

As the aged rats demonstrated significantly higher numbers of grafted cell survival and graft volume, it follows to ask whether this translates into greater neurite outgrowth. Neurite outgrowth was quantified by two methods: densitometry and stereology. The first method used to quantify neurite outgrowth in the DA-grafted rats involved densitometric quantification of TH expression in fields of view adjacent to the graft. This method is advantageous over the stereology program called “Spaceballs”® in that analysis was performed along four axis of neurite outgrowth, instead of just in the lateral direction from the graft border.

Neurite outgrowth via densitometry: Sphere of influence

Neurite outgrowth is extensive in young, middle, and aged grafted rats:

Using densitometric quantification of total TH staining, the degree of neurite outgrowth from the graft along four axes (medial, lateral, dorsal, and ventral) in each age group was determined. This data is expressed as the percent field of view with TH+ neurite staining. Although semi-quantitative in nature, utilizing this method of neurite outgrowth densitometry data, the collective extent of neurite extension in dorsal, medial, ventral, and lateral directions was assessed to illustrate total neurite outgrowth in multiple directions from the graft in each age category. For each age group, the average neurite density measure obtained from the Spaceballs analysis was normalized to the corresponding average number of surviving grafted TH+ neurons. Quantification of the average grafted neurite density per surviving grafted TH+ neuron reveals that the
density per surviving TH+ neuron is significantly greater in young rats versus other age groups (Figure 4.1.5 A-C) (young: 0.0000353 ± 1.15E-05 µm/mm³; middle aged: 0.0000107 ± 1.01E-06 µm/mm³; aged: 0.0000105 ± 1.16E-06 µm/mm³; ANOVA p < 0.05).

From this data, altered directionality of neurite outgrowth across ages becomes apparent. In general, compared to older rats, young DA-grafted rats demonstrate greater neurite extension from the dorsal border of the graft. Conversely, compared to young DA-grafted rats, middle and aged DA-grafted rats display increased neurite extension in the lateral and medial directions from the graft borders. Specifically, compared to young DA-grafted rats, middle and aged DA-grafted rats display increased neurite extension in the region proximal to the graft (0-850 um) in both lateral (young: 30.93 ± 3.0%; middle: 55.05 ± 3.0%;* aged: 63.46 ± 3.0%; *p<0.05) and medial (young: 41.75 ± 2.3%; middle: 54.50 ± 2.3%;* aged: 54.73 ± 2.3%; *p<0.05) directions from the graft borders (Figures 4.1.5 H and 4.1.5 I). Thus, neurite outgrowth from the lateral and medial borders of the graft was increased in both middle aged and aged DA-grafted rats versus young DA-grafted rats (2-way ANOVA, p<0.05).

Further, young rats demonstrate a dominance of dorsal neurite extension compared to middle and aged grafted rats such there is significantly greater neurite outgrowth at the distal range from the dorsal border of the graft in young DA-grafted rats compared to both middle and aged DA-grafted rats (Figure 4.1.5 I) (2-way ANOVA, p<0.05). Of note, aged rats demonstrate markedly reduced ventral outgrowth compared to other age groups at the distal region from the ventral border. This finding is primarily due to the ventral location of the DA-grafts in aged rats, some of which were close to
the ventral border of the striatum, thus limiting the number of fields of view sampled from these animals.

**Density of neurite outgrowth via stereology program Spaceballs**

The stereological program, Space Balls®, was used on a subset of data to quantify density of TH+ positive neurites in three consecutive fields of view beginning at the lateral border of graft and extending laterally towards the lateral border of the striatum. As a control measure, neurites were counted on the intact side of the striatum at the same level and location as on the grafted side. While the average neurite density is greater on the intact side versus the DA-grafted side for all age groups (p < 0.001), there is no age-dependent difference in neurite density between the intact and lesioned sides (Figure 4.1.5 A) (DA-grafted side: young: 0.0428 ± 0.0119 µm/mm³; middle: 0.0532 ± 0.0103 µm/mm³; aged: 0.0615 ± 0.0119 µm/mm³; sham-grafted side: middle: 0.149 ± 0.0119 µm/mm³; young: 0.172 ± 0.0119 µm/mm³; aged: 0.149 ± 0.0119 µm/mm³; p > 0.05). This analysis did not demonstrate any significant age-dependent difference in neurite outgrowth from the lateral border of the graft. However, this analysis was underpowered (Figure 4.1.5 A) (Power for age: 0.0500), and limited by insufficient sample sizes.

For each age group, the average neurite density measure obtained from the Spaceballs analysis was normalized to the corresponding average number of surviving grafted TH+ neurons. When the average grafted neurite density per surviving grafted TH+ neuron is quantified, the density per surviving TH+ neuron is significantly greater in
young rats versus other age groups (Figure 4.1.5 B) (young: $0.0000353 \pm 1.15E-05 \mu m/mm^3/cell$; middle aged: $0.0000107 \pm 1.01E-06 \mu m/mm^3/cell$; aged: $0.0000105 \pm 1.16E-06 \mu m/mm^3/cell$; ANOVA $p < 0.05$).

Figure 4.1.5 A: Quantification of lateral striatal neurite density in DA-grafted rats using the stereological program Spaceballs. Neurite density is significantly greater in the intact striatum (light gray) compared to grafted striatum (dark gray) at all ages. Asterisk denotes $p<0.05$. There is no age related difference in neurite density on either the intact or DA-grafted striatum.
Figure 4.1.5 B: Quantification of lateral neurite density via Spaceballs in young, middle aged, and aged DA-grafted rats adjusted to number of surviving TH+ grafted cells. Young rats have greater neurite outgrowth on a per-grafted cell basis when compared to both middle aged and aged grafted rats. Asterisk denotes $p<0.05$. 
Figure 4.1.5 C: Sphere of influence, Young DA-grafted rats. Extent of neurite outgrowth from the center of the graft in medial, lateral, dorsal, and ventral directions is depicted along each arm of the graph as shown. Each circle represents the percent of that field of view occupied by TH-ir fibers. The location of each circle along its axis corresponds to the from the center of the graft in the specified direction.
Figure 4.1.5 D: Sphere of influence, Middle aged DA-grafted rats. Extent of neurite outgrowth from the center of the graft in medial, lateral, dorsal, and ventral directions is depicted along each arm of the graph as shown. Each circle represents the percent of that field of view occupied by TH-ir fibers. The location of each circle along its axis corresponds to the from the center of the graft in the specified direction.
Figure 4.1.5 E: Sphere of influence, Aged DA-grafted rats. Extent of neurite outgrowth from the center of the graft in medial, lateral, dorsal, and ventral directions is depicted along each arm of the graph as shown. Each circle represents the percent of that field of view occupied by TH-ir fibers. The location of each circle along its axis corresponds to the from the center of the graft in the specified direction.
Figure 4.1.5 F: Sphere of influence, Overlay of all ages, DA-grafted rats. Extent of neurite outgrowth from the center of the graft in medial, lateral, dorsal, and ventral directions is depicted along each arm of the graph as shown. Each circle represents the percent of that field of view occupied by TH-ir fibers. The location of each circle along its axis corresponds to the from the center of the graft in the specified direction.
**Figure 4.1.5 G: Sphere of influence, DA-grafted rats.** Alternative depiction of neurite outgrowth sphere of influence for young (green), middle aged (blue), and aged (red) grafted rats. This data reflects the average percentage of the field of view occupied by TH-ir fibers over the first six fields of view adjacent to the border of the graft along the axis indicated. In young rats, there is a significantly greater extent of neurite outgrowth from the dorsal border of the graft.
**Figure 4.1.5 H: Neurite outgrowth dorsal/ventral:**  (i) Densitometric quantification of neurite outgrowth from the dorsal border of the graft in young, middle aged, and aged DA-grafted rats.  (ii) Densitometric quantification of neurite outgrowth from the ventral border of the graft in young, middle aged, and aged DA-grafted rats. Asterisk denotes p<0.05. Young rats demonstrate elevated neurite outgrowth at distal regions from dorsal graft border (i), while little difference is observed at the ventral border between ages. **Proximal:** 0-850um from graft border; **Distal:** 850-1700um from graft border.
Figure 4.1.5 I: Neurite outgrowth medial/lateral: At proximal, but not distal ranges from graft border, both middle and aged DA-grafted rats demonstrate increased neurite outgrowth in directions medial (i) and lateral (ii) from the center of the graft compared to young DA-grafted rats. Asterisk denotes p<0.05. Proximal: 0-850um from graft border; Distal: 850-1700um from graft border.
4.1.5 Pre- and post-graft levodopa-induced dyskinesias across ages

Graft-mediated improvement in levodopa-induced dyskinesias is inferior in the aged parkinsonian rat:

Total LIDs: All levodopa treated rats developed LIDs to equal levels across ages at all pre-graft timepoints. The severity of LIDs was significantly reduced with grafting across all ages at each of the post-graft timepoints in comparison to sham-grafted rats (Figure 4.1.6). At the early post-graft timepoint, young grafted rats showed significantly greater reduction in LID severity compared to aged grafted rats (Figure 4.1.7 D-E) (Early post-graft timepoint Dyskinesia Severity Score: young: 6.458 ± 1.997; aged: 12.019 ± 1.153; sham: 16.075 ± 1.094; Mid post-graft timepoint: young: 7.375 ± 1.997; aged: 10.032 ± 1.153; sham: 17.595 ± 1.307; Late post-graft timepoint: young: 6.333 ± 1.997; aged: 7.911 ± 1.307; sham: 15.633 ± 1.547; 2-way ANOVA, p<0.05 for sham vs. graft at all timepoints, and young vs. aged at early timepoint). Although the aged rats show a protracted recovery in LID severity compared to their younger counterparts, grafting yields equivalent reduction of LID severity across ages at the final LID evaluation timepoint (Late post-graft time point Dyskinesia Severity Score: young: 6.333 ± 1.997; aged: 7.911 ± 1.307; sham: 15.633 ± 1.547; 2-way ANOVA, p<0.05 for sham vs. graft at all timepoints, and young vs. aged at early time point). When the degree of improvement in LID severity post-grafting is adjusted to the number of surviving grafted dopaminergic cells, young rats demonstrate a significantly greater degree of improvement per cell compared to middle and aged grafted rats (Figure 4.1.7 F) (% reduction in LID severity post grafting per cell: young: 0.0469 ± 0.0131%; middle: 0.0232 ± 0.00428%; aged: 0.0105 ± 0.00446%; ANOVA, p = 0.017).
**Individual LIDs:** Individual dyskinetic behaviors were analyzed for sham-graft and DA-grafted animals at all time points (Figure 4.1.8 A-O). In general and as seen with the total LID data in Figures 4.1.6 and 4.1.7 individual dyskinetic behaviors show the same pattern of reduction in dyskinesias severity with DA-grafting and delayed or diminished graft efficacy with age.

**Figure 4.1.6:** Pre- and post-graft total levodopa-induced dyskinesias, all ages. Asterisk denotes $p<0.05$ for sham-graft versus DA-graft at the time point for all ages.
Figure 4.1.7: Total levodopa induced dyskinesias: (A) young rats, (B) middle aged rats, (C) aged rats. Sham rats: dashed line; Grafted rats: solid line. (D) Total levodopa induced dyskinesias in young and aged DA-grafted and sham-grafted rats across all timepoints. Asterisk denotes p<0.05 for DA-graft vs sham-graft. (E) Total levodopa-induced dyskinesias in sham grafted, young DA-grafted, and aged DA-grafted animals at early post graft time point. Asterisk denotes p<0.05 for indicated comparison. (F) Percent improvement in dyskinesias severity per grafted TH+ cell. Asterisk denotes p<0.05 for indicated comparison.
**Figure 4.1.8:** *Individual levodopa-induced dyskinesias severity scores* for young (A,D,G,J,M), middle aged (B,E,H,K,N), and aged (C,F,I,L,O) rats at pre-graft (weeks -4, -3, -2, -1) and post-graft (early, middle, late) evaluation intervals. Trunk (A-C), RFPD (D-F), Hindpaw (G-I), Forepaw (J-L), Orolingual (M-O). Dashed line represents sham-grafted rats. Solid line represents DA-grafted rats.
4.1.6 Evaluation of parkinsonian motor behaviors; pre- and post- graft

Motor behavior was assessed in all rats using the forepaw lateral step adjusting task as described in the methods. Data is expressed as percent right paw use: Percent right paw use = (number of right paw adjustments in trial/ trial time)/((number of right paw adjustments in trial/ trial time) + (number of left paw adjustments in trial/ time))*100. Before DA-depletion all rats demonstrated successful completion of the forepaw lateral step adjusting task with each forepaw such that each paw was used approximately 50% of the time, regardless of age (pre-lesion, Figure 4.1.9) (percent left paw use: rats for grafting; young: 38.347 ± 5.515%; middle: 54.478 ± 3.377%; aged: 48.576 ± 3.021%; rats for sham-grafting: young: 41.066 ± 4.776%; middle: 48.183 ± 3.377%; aged: 47.895 ± 3.184%). After lesioning with 6-OHDA, all rats demonstrated marked loss of function of the forepaw contralateral to the lesioned side, regardless of age (post-lesion, Figure 4.1.9)(percent left paw use: rats for DA-grafting: young: 2.381 ± 5.515%; middle aged: 11.426 ± 3.611%; aged: 8.111 ± 3.021%; rats for sham grafting: young: 8.054 ± 4.776%; middle 8.452 ± 3.377%; aged: 6.51 ± 3.184%). This loss of function was not restored with grafting (post-graft, Figure 4.1.9) (percent left paw use: rats for DA-grafting: young: 0.762 ± 5.515%; middle aged: 0.00 ± 3.611%; aged: 4.774 ± 3.9%; rats for sham-graft: young: 9.134 ± 4.776%; middle aged: 6.638 ± 3.184%; aged: 6.897 ± 6.755%).
Figure 4.1.9: Forepaw step adjustment task. Before DA-depletion, rats use each paw equally, approximately 50% left, and 50% right. After DA-depletion, there is dramatic reduction of use of the paw corresponding to the lesioned side. This loss of function does not recover in these animals with grafting (post-graft), and there is no difference in performance with age.
4.1.7 FosB/deltaFosB immunoreactivity is reduced with grafting across all ages

FosB/deltaFosB transcription factors are known to be elevated with DA-depletion, and further deregulated with levodopa treatment. Further, FosB expression is definitively linked to LIDs. We quantified expression of FosB/deltaFosB in sham and grafted rats across all ages. FosB expression was increased with levodopa treatment and remained elevated in sham-grafted rats. This elevation of FosB was greater in young and aged sham-grafted rats than in middle aged sham-grafted rats. FosB expression in the DA-grafted striatum on the lesioned (DA-grafted) side was reduced and restored toward normal values across all ages compared to sham-grafted rats (Figure 4.1.10) (2-way ANOVA, p<0.05: young sham-grafted: 116.773 ± 19.331%; middle aged sham-grafted: 69.623 ± 7.892%; aged sham-grafted: 120.733 ± 8.645%). This reduction in FosB/deltaFosB expression with grafting was greatest in aged rats (Figure 4.1.10) (2-way ANOVA, p<0.05: young DA-grafted: 51.161 ± 11.161%; middle aged DA-grafted: 41.521 ± 7.892%; aged DA-grafted: 15.145 ± 9.665%).
**Figure 4.1.10: FosB densitometry in DA-grafted and sham-grafted rats.** (A) FosB expression is elevated in sham-grafted rats treated with levodopa across all ages. DA-grafting significantly reduces this elevation in FosB across all ages. This reduction of FosB expression with DA-grafts is greatest in the aged rats. Light gray: DA-grafted rats; Dark gray: sham-grafted rats. Single asterisk denotes p<0.05 for sham vs. DA-graft; double asterisk denotes p<0.05 for comparison between sham-graft groups; triple asterisk indicates p<0.05 for aged DA-grafted rats compared to both young and middle aged DA-grafted rats. (B-E) Photomicrographs depicting neuronal cell bodies with FosB immunoreactivity from a sham-grafted young rat in the lesioned (B) and intact (C) striatum, and from a DA-grafted rat in the lesioned (D) and intact (E) striatum. Outlines of cell body and background tracings used for densitometry quantification shown in panels (B-E).
4.1.8 Discussion of results for Aim 1

The therapeutic potential of dopaminergic tissue engraftment into patients with Parkinson’s disease remains controversial and incompletely understood. While clinical and preclinical data support the idea that dopamine neuron engraftment into parkinsonian individuals is most effective in younger individuals (Collier et al., 1999; Freed et al., 2001) with less severe dopamine-depletion (Hagell et al., 2002; Picconi et al., 2005; Breysse et al., 2007), most clinical grafting trials to date have chosen only patients with advanced PD. Rationale for choosing this population of patients is that they represent those most in need of improved therapeutics, and because there are ethical issues associated with grafting into less severely debilitated individuals that continue to respond to standard pharmacotherapy. Although results in this patient population have been largely disappointing, younger patients even with advanced disease have been observed to have significantly better post-surgical outcome than their aged counterparts (Freed et al., 2001).

While there has been painstaking effort both in the clinic and the laboratory to characterize the optimal conditions for donor cells used for such transplantation; addressing issues such as donor cell age, density of grafted cells, origin of grafted cells, immune factors, and growth factors, (e.g.: (Goetz et al., 1989; Cahill & Olanow, 1990; Olanow et al., 1990; Freeman & Olanow, 1991; Freeman et al., 1995a; Freeman et al., 1995b; Olanow et al., 1996; Annett et al., 1997; Kordower et al., 1997a; Kordower et al., 1997b; Sable et al., 1997; Fitoussi et al., 1998; Kordower et al., 1998; Collier et al., 1999; Borlongan et al., 2001; Freed et al., 2001; Olanow et al., 2001; Hagell et al., 2002; Arjona et al., 2003; Freed et al., 2003; Matarredona et al., 2003; Toledo-Aral et
al., 2003; Yoshizaki et al., 2004; Hagell & Cenci, 2005; Ferrari et al., 2006; Maries et al., 2006; Winkler et al., 2006; Breysse et al., 2007; Torres et al., 2007), comparatively little has been done to determine the optimal host environment for successful transplantation, and has mostly been limited to optimal transplant location within the host brain (Stromberg et al., 1986; Goren et al., 2005; Breysse et al., 2007). Again, in humans (Freed et al., 2001) and in rats (Collier et al., 1999; Breysse et al., 2007) grafting into aged parkinsonian subjects is significantly less efficacious than in younger counterparts. It has long been known that the aged brain lacks many trophic and support factors found in younger brains (Collier et al., 1999; Ling et al., 2000; Collier et al., 2005), and is generally considered a hostile environment for grafted embryonic neurons. Many researchers are exploring methods of increasing survival of grafted DA neurons for PD, regardless of host age. However, the purpose of the current study was to examine whether, even when the challenge of limited cell numbers is overcome, will cell replacement therapy in the aged parkinsonian subject be as effective as that found in their young counterpart?

Previous data from our laboratories (Collier et al., 1999) has provided information indicating that when the same numbers of embryonic ventral mesencephalic (VM) DA neurons are grafted into striatum of parkinsonian rats of varying ages, there is a proportional decrease in survival of the engrafted neurons with increasing age. Based on specifications from this previous data (Collier et al., 1999), a five-fold or two-fold increase in the number of VM neurons was placed into 22 or 15 month old parkinsonian rats compared to their 3 month old counterpart. The behavioral efficacy of embryonic VM grafts with equal or greater numbers of surviving DA neurons in rats of advanced
(22 mo) and middle (15 mo) age compared to young (3 mo) rats was evaluated. The current study suggests that even when approaches to achieve optimal survival of grafted neurons can be achieved for all parkinsonian subjects, additional factors found in the aged brain may continue to limit the rate of symptomatic relief from grafted neurons.

Contrary to previous literature which suggests the aging parkinsonian striatum may be refractory to transplantation therapy due to markedly decreased survivability of DA neurons in the aged host (Collier et al., 1999), this study demonstrates one specific set of conditions in which the normally hostile environment of the aged striatum is overcome by transplantation of five times more DA neurons than in the young host. This is an important and timely finding as the efficacy of fetal transplantation for Parkinsonian patients remains controversial due to disappointing results of the major clinical trials in the field (Freed et al., 2001; Freed et al., 2003; Olanow et al., 2003; Hagell & Cenci, 2005). Nonetheless, the promise of fetal DA transplantation as a therapy for PD has not been dismissed and research continues in an effort to both optimize efficacy and minimize side effects of grafting therapies.

One of the major limitations of grafting as a therapeutic option for PD has been the emergence of GIDs in a significant portion of patients post-graft (Freed et al., 2001; Olanow et al., 2003). In fact, GID development is the primary reason such clinical trials have been halted in the United States. In this regard, it is of particular interest that the animals in this study developed little to no GIDs, regardless of age. Previous studies from our lab have demonstrated the robust development of GIDs in parkinsonian rats with fetal transplants in the dorsal striatum (Maries et al., 2006; Soderstrom et al.,
2008). Unlike previous studies, the majority of DA-grafted animals in this study have large grafts in the ventral striatum. The specific somatotopic organization of the striatum has been well described (West et al., 1990; Brown, 1992; Ebrahimi et al., 1992; Mittler et al., 1994; Brown & Sharp, 1995; Fricker et al., 1996; West, 1998; Alloway et al., 1999; Leergaard et al., 2000; Hoover et al., 2003; Shih et al., 2006), ascribing functionality of specific limbs to distinct regions of the striatum. Further, patchy or inadequate reinnervation of the ventral striatum due to dorsal graft placement, or grafts resulting in little reinnervation of the ventral host striatum, is hypothesized to be one of the factors underlying the development of GIDs in both parkinsonian animals and humans (Hagell et al., 2002; Maries et al., 2006). Thus, the ventral location of the grafts in this study provides the most logical explanation as to why the classic forelimb-facial stereotypies typical of GIDs resulting from dorsal grafting were not observed. Importantly, this observation may suggest that alternative placement of transplanted tissue in the parkinsonian striatum may be one practical method of preventing unwanted GIDs.

In these experiments, the number of grafted DA neurons was adjusted to account for diminished survival with increasing age (Collier et al., 1999). Dopamine neuron grafts were capable of decreasing pre-graft levodopa-induced dyskinesias across the three age groups tested at all post-graft evaluation time points, regardless of host age. Further, at the final post-graft time point, there were no differences in the degree of LID expression between young (3m.o.), middle aged (12 m.o.) and aged (22m.o.) DA-grafted rats. However, although robust cell survival was achieved in the aged striatum in this study, there remained a delayed and diminished behavioral efficacy of DA-
grafting in the aged striatum when compared to the young DA-grafted rats in the study. This delayed efficacy, specifically in the aged DA-grafted rats, may be due to altered neurochemical signaling related to LID development, such as expression of the immediate early gene, FosB.

The expression of the transcription factor, FosB has been intimately linked with LID development such that in the parkinsonian rat, immunoreactivity of FosB serves as an indicator of the activation of neurocircuitry associated with LIDs (Andersson et al., 1999; Andersson et al., 2003). For example prodynorphin mRNA was shown by Andersson and colleagues to be co-induced with FosB-like proteins in dynorphin-containing neurons (presumably direct pathway neurons), and intrastriatal infusion of FosB antisense mRNA inhibited not only prodynorphin mRNA upregulation, but also dyskinesia development (Andersson et al., 1999; Andersson et al., 2003). Thus, analysis of FosB expression DA-grafted rats of varying ages was warranted in this study. Analysis of FosB expression demonstrated a significant reduction of FosB elevation with DA-grafting in young, middle aged, and aged DA-grafted parkinsonian rats. Further, this change was greatest in aged rats, thus restoring FosB levels less than 20% greater than on the intact side. Combined with the robust cell survivability and large graft volume, the recovery in FosB expression in aged rats is an additional indicator of graft efficacy in the aged striatum. Further, DA-grafted aged rats demonstrate extensive neurite outgrowth, comparable to that of younger DA-grafted rats. Interestingly, despite these indicators of robust DA-graft survival in the aged host (cell survivability, graft volume, neurite outgrowth, FosB restoration), there is still an initial delay in reduction of LIDs with grafting, specifically in the aged DA-grafted rats.
Further, when graft efficacy in terms of neurite outgrowth or reduction in LIDs is evaluated on per grafted DA-neuron basis, the younger DA-grafted rats far outperform their aged counterparts.

Why aged rats are unable to respond as quickly to DA-grafts, or why DA-grafts in the aged striatum are less efficacious on a \textit{per cell} basis remains unknown, but the answer is likely explained by a combination of factors, including (1) additional downstream signaling pathways associated with LID development (such as glutamate signaling via NMDA/AMPA receptors as discussed in the introduction), (2) more complex aspects of intracellular signaling, such as phosphorylation status changes in target molecules (i.e. DARRP-32, Erk) (Santini et al., 2007) or age-related expansion of DNA trinucleotide repeats (Hands et al., 2008), and (3) generalized alterations or reductions in synaptic plasticity associated with aging and/or dyskinesia development (Picconi \textit{et al.}, 2003; Picconi \textit{et al.}, 2005; Pisani \textit{et al.}, 2005; Collier \textit{et al.}, 2007; Hands \textit{et al.}, 2008).

One issue that also remains unresolved in this study is the long-term behavioral impact beyond the timeframe of this study. At eleven weeks post-grafting, all DA-grafted rats demonstrated significantly reduced dyskinesia severity and reduced amphetamine-induced rotational asymmetry to the same degree. However, it is possible that continued behavioral improvement may have developed with extended observation as the levels of post-graft dyskinesias had not yet plateaued in grafted rats. One may speculate that over a greater post-graft evaluation time period, an age-dependent difference in LID reduction with DA-grafting might emerge such that aged
rats would exhibit significantly less (or even greater) recovery than middle or young DA-grafted rats.

In this study, we examine the degree of neurite outgrowth in four directions from the grafts. While all DA-grafted rats demonstrate extensive neurite outgrowth, there are some age-dependent differences in the directionality of this outgrowth. While some of these differences may be due to variation in graft size or specific placement within the striatum, there is a clear increase in dorsal innervations associated with grafting in the young host. Further, while TH+ fiber in the grafted striatum indicate the presence of viable grafted dopaminergic cells, this analysis does not demonstrate that the graft-host connections are indeed restored, normal connectivity. Previous data from our lab has shown a shift in distribution of the type of synaptic connections made by grafted tissue within the parkinsonian striatum of the host environment (Soderstrom et al., 2008). Ultrastructural studies examining the graft-host connectivity within the striatum between ages would yield invaluable information as to the degree of successful restoration of intracellular synaptic connectivity within the grafted striatum. While the DARPP-32 data from this study suggests minimal striatal damage with grafting across all ages, there is a small by significant reduction in the percent of intact striatum associated with larger grafts such as those in the middle and aged rats. The combination of reduced graft-host interactions and elevated striatal damage could likely contribute to reduced graft efficacy in the aged host, but further studier are necessary to confirm such hypothesis.

Regardless of factors such as graft-host connectivity or specific activation of intracellular pathways, the observation remains that the aged striatum requires a far greater number cells to reach therapeutic efficacy (as measured by restoration of
amphetamine-induced rotational asymmetry, and reduction of levodopa-induced
dyskinesias) than in the young DA-grafted rat. This requirement of the aged striatum for
more cells is likely explained by the general decrease in trophic factor support in the
aged striatum. Increasing the number of grafted cells is one manner in which to
overcome the relatively hostile environment of the aged striatum. The combination of
optimal grafted cell density and trophic factors coupled with appropriate synaptic
connections is necessary to achieve therapeutic efficacy in the aged parkinsonian
striatum.
4.2 Aim 2: Understanding mechanisms of levodopa-induced dyskinesias: The role of striatal pathology on levodopa-induced dyskinesias

4.2.1 Results

The results for Aim 2 summarize data from two series of experiments, my first “pilot studies” and the “follow-up studies” as outlined in the methods. The pilot nimodipine studies were designed to generate preliminary “proof of principle” data, (1) demonstrating that I could demonstrate the occurrence of spine loss on dendrites of striatal MSNs in response to DA-depletion, (2) demonstrating that I could model preventing spine loss with continuous release nimodipine pellets, (3) examining whether there was any behavioral effect of acute nimodipine and levodopa drug-drug interaction, and (4) examining whether there was evidence of a dose or time dependent effect of spine loss on reduction of LIDs. Following discussion of data from this pilot study, the confirmatory follow-up experiment is reported. This follow-up experiment based on preliminary findings from the pilot study was designed to further characterize the effect of maintaining spine integrity on LID development, and also to go one step further, and attempt to begin to elucidate potential downstream effects of maintaining intact spine density, focusing specifically on quantification of the transcription factor FosB expression in rats with intact dendritic spines versus those with denuded dendrites.
4.2.2 Confirmation of dendritic spine loss with DA-depletion, and spine preservation with nimodipine

Throughout the pilot experiments of Aim 2, a modified Golgi-Cox protocol was utilized to quantify dendritic spine density on striatal MSNs as described in the methods section. Figure 4.2.1 is an image of a histological section of rat brain stained with this method showing neuronal staining through the entire brain.

![Image](image.png)

Figure 4.2.1: Representative photomicrograph of modified Golgi-Cox staining of rat brain section at the level of the rostral striatum at 1.25X. Even at low magnification robust staining of cortical and striatal neurons can be appreciated.
Analysis of Golgi-stained neurons with the neuronal reconstruction software, Neurolucida and Neurolucida Explorer provided 3-D reconstruction of a neuron and its entire dendritic arborization, including dendritic spines, as shown in Figure 4.2.2.

**Figure 4.2.2:** 3-D reconstruction of striatal MSN and dendritic spines using *Neurolucida* software. Central cell body is surrounded by primary dendrites and their branches in blue, yellow, and white.
We found that severe DA-depletion resulted in marked reduction of dendritic spine density as can be seen in Figure 4.2.3A, an example of a dendrite of a MSN in the intact striatum demonstrates the normal appearance of striatal dendrites, covered in numerous spines (proximal density: 10.5±0.53 spines/10um, distal density: 8.50±0.60 spines/10um). Panel B of Figure 4.2.3 demonstrates the marked loss of such spines as a result of DA-depletion (DA-depleted: proximal 6.29±0.47* spines/10um, distal 5.84±0.46* spines/10um; asterisk denotes p<0.05 compared to normal). Panel C of Figure 4.2.3 further illustrates restoration of spines in the levodopa-treated parkinsonian striatum, however with an apparent increase in spine morphology that is atypical for striatal MSNs, ie increases in bifurcated and mushroom spines (data not quantified).

In this study, we demonstrate significant spine loss in response to DA-depletion and spine preservation observed with nimodipine treatment post DA-depletion in the parkinsonian rat (Figure 4.2.4 and Figure 4.2.5). Quantification of dendritic spine numbers using Neurolucida® software shows a significant reduction in the number of mature dendritic spines on both proximal (segment approx 50 to 100 um from cell body) and distal (segment approx 150 to 200 um from cell body) dendritic segments in rats with 99.4% ± 0.22% depletion of nigral TH-ir cells compared to controls (6-OHDA+VEH) (*p<0.001). This is in contrast to DA-depleted rats treated with chronic nimodipine showing a significant sparing of dendritic spines despite severe DA depletion (6-OHDA+NIM). Intact: proximal: 10.5±0.53 spines/10um, distal: 8.50±0.60 spines/10um; DA-depleted: proximal 6.29±0.47* spines/10um, distal 5.84±0.46* spines/10um; DA-depleted + Nimodipine: proximal 10.44±0.58 spines/10um, distal 8.74±0.41 spines/10um (asterisk denotes p<0.05 compared to normal).
**Figure 4.2.3: Golgi-Cox staining of striatal MSNs.** A) Abundant dendritic spines are noted in the striatum of a control rat brain. B) In the DA-depleted striatum there are markedly fewer dendritic spines and increased tortuosity of dendrites noted here by increased variation in focal planes of the dendrites. C) Levodopa treatment appears to restore spine number with an appearance of abnormal spine morphology. a’ denotes higher magnification of region defined by a. b’: refocused image of portion of dendrite which is out of the plane of focus in b. Arrow: bifurcated spine.
Figure 4.2.4: Continuous, low-dose nimodipine prevents spine loss in parkinsonian rats with severe DA depletion. Rats with severe dopamine depletion in this study showed a significant decrease in number of dendritic spines on striatal MSN. Chronic release nimodipine pellets (0.8mg/kg/day) completely protected MSN against spine loss despite a mean nigral dopamine cell loss of 99.65 ± 0.13%. Micrographs of Golgi stained MSNs from: A) normal (non-6-OHDA, vehicle control) rat striatum, B) DA-depleted rat striatum, and C) DA-depleted striatum of a rat treated with chronic nimodipine pellets. D) Quantification of dendritic spine density.
Figure 4.2.5: Continuous, low-dose nimodipine prevents spine loss in parkinsonian rats with severe DA depletion. Quantification of dendritic spines using Neurolucida® software shows a significant reduction in the number of mature dendritic spines on both proximal (segment approx 50 to 100 um from cell body) and distal (segment approx 150 to 200 um from cell body) dendritic segments in rats with 99.4% ± 0.22% depletion of nigral TH+ cells compared to controls (6-OHDA+VEH) (*p<0.001). This is in contrast to DA-depleted rats treated with chronic nimodipine showing a significant sparing of dendritic spines despite severe DA depletion (6-OHDA + NIM). N =10 rats per group; 10 distal and 10 proximal dendrites per rat quantified for spine density.
4.2.3 Impact of nimodipine-mediated spine preservation in DA-depleted rats on parkinsonian motor behaviors

Parkinsonian motor behaviors were evaluated in nimodipine and vehicle treated rats before and after DA-depletion to examine whether maintaining striatal dendritic spine density impacted the animal’s performance in sensori-motor integration task (vibrissae test), or postural adjustment (forepaw step adjustment test). We found that behaviors were markedly impaired with DA-depletion in both vibrissae (pre-lesion: 100% accuracy; post-lesion: <10% accuracy) and forepaw step adjustment tasks (Levodopa treated: vehicle pre-lesion: 17.00±1.34 steps/minute; vehicle post-lesion: 7.70±1.11* steps/minute; nimodipine pre-lesion: 17.60±2.08 steps/minute; nimodipine post-lesion: 8.40±1.63* steps/minute // saline treated: vehicle pre-lesion: 24.30±2.77 steps/minute; vehicle post-lesion: 8.60±1.07* steps/minute; nimodipine pre-lesion: 24.00±3.22 steps/minute; nimodipine post-lesion: 6.25±0.96* steps/minute; asterisk denotes p<0.05 for pre- versus post-lesion).

There is no difference in the degree of improvement in performance of either vibrissae (Figure 4.2.6 B) or lateral forepaw adjustment (Figure 4.2.7 B) tests post DA-depletion in either nimodipine or vehicle treated rats treated with saline, indicating no influence of dendritic spine integrity (e.g.: with or without nimodipine) on sensorimotor integration or lateral forepaw adjustment in parkinsonian rats (saline treated: vehicle pre-lesion: 24.30±2.77 steps/minute; vehicle post-lesion: 8.60±1.07* steps/minute; vehicle post-saline: 3.45±0.76* steps/minute; nimodipine pre-lesion: 24.00±3.22 steps/minute; nimodipine post-lesion: 6.25±0.96* steps/minute; nimodipine post-saline: 3.65±0.65 steps/minute; asterisk denotes p<0.05 for pre- versus post-lesion values).
Further, we found no observable influence of dendritic spine integrity (e.g.: with or without nimodipine) on step adjusting test performance in parkinsonian rats exposed to daily levodopa injections. Specifically when rats were given daily (M-F) DA replacement through an intraperitoneal injection of levodopa (12.5mg/kg), they exhibit the same degree of behavioral deficit as those injected with the vehicle (Dyskinesia severity score: Levodopa treated: vehicle pre-lesion: 17.00 ± 1.34; vehicle post-lesion: 7.70 ± 1.11*; vehicle post-levodopa: 3.45 ± 0.76*; nimodipine pre-lesion: 17.60 ± 2.08; nimodipine post-lesion: 8.40 ± 1.63*; nimodipine post-levodopa: 4.90 ± 1.26*; asterisk denotes p<0.05 for pre- versus post-lesion values).

In contrast, when rats with intact spine morphology were treated with levodopa (12.5mg/kg) and challenged with a different motor task, the vibrissae motor task, 12 hours after levodopa there was a significant improvement compared to vehicle treated controls (Percent intact: Levodopa treated: vehicle post-lesion 1.00 ± 1%; vehicle post-levodopa: 12.83 ± 6.18%; nimodipine post-lesion: 3.00 ± 1.53%; nimodipine post-levodopa: 38.83 ± 9.89*; Saline: vehicle post-lesion: 2.00 ± 1.33%; vehicle post-saline: 20 ± 6.37%; nimodipine post-lesion: 3.00 ± 1.53%; nimodipine post-saline: 21.83 ± 7.03%; asterisk denotes p<0.05) (Figure 4.2.6 A).
A  
Vibrissae Test: Levodopa

![Graph showing percentage of successful trials (n/10) over time.](Image)

- **Pre-lesion**
- **2 wks Post-lesion**
- **4 wks Post-lesion**

B  
Vibrissae Test: Saline

![Graph showing percentage of successful trials (n/10) over time.](Image)

- **Pre-lesion**
- **2 wks Post-lesion**
- **4 wks Post-lesion**
**Figure 4.2.6: Vibrissae Sensorimotor Function: Impact of Spine Integrity.**  

(A) Daily levodopa does provide for significantly improved behavior during ‘OFF’ (no levodopa for 12 hours) specifically in rats with preserved spine integrity (nimodipine treated). Asterisk denotes p<0.05.  

(B) Preservation of dendritic spine density on MSNs in the presence of severe DA-depletion (99.65 + 0.13 % nigral TH-ir cell loss) does not improve motor disability in this model in the absence of levodopa. Black: nimodipine-treated; Gray: vehicle-treated
A  
Lateral Forepaw Step Adjustment: DA-depleted side + Levodopa

Introduce levodopa

Average steps/trial with parkinsonian paw

<table>
<thead>
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B  
Lateral Forepaw Step Adjustment: DA-depleted Side + Saline

Introduce saline

Average steps/trial with parkinsonian paw

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Figure 4.2.7: Forepaw Step Adjustment to Postural Change during “OFF” (no levodopa for 12 hours): Impact of Spine Integrity. Unlike the vibrissae motor test (Figure 4.2.8), there is no influence of dendritic spine integrity on motor function measured with the step adjusting test, either with (A) or without (B) chronic daily levodopa. Black: nimodipine-treated; Gray: vehicle-treated
4.2.4 *Acutely administered nimodipine does not alter the expression of LIDs*

To test whether the low dose of nimodipine (0.8 mg/kg/day) I planned to use in the chronic release pellets to prevent dendritic spine loss would itself impact LIDs, dyskinesia behavior was examined in a group of parkinsonian rats, distinct from rats used for the chronic nimodipine pellet studies. In these parkinsonian rats, an *acute* injection of nimodipine was administered in conjunction with levodopa to determine whether nimodipine had either negative or positive influences on LIDs in our model. Rats were rendered severely parkinsonian, again *without* any pellet implants. All drugs were administered on the test day by intraperitoneal injection. Levodopa was administered at one of three doses: 6.0, 8.0, or 12.5 mg/kg. Doses of levodopa were varied to ensure that we were not overwhelming any potential *nimodipine effect* with our usual “high” dose of 12.5 mg/kg levodopa. Dyskinesia severity was analyzed 30 minutes post-levodopa (Figure 4.2.8; *pre-nimodipine; gray bars*), which was followed by an injection of one of four test doses* of nimodipine (0.08, 0.8, 8.0, or 20 mg/kg). Thirty minutes following the nimodipine injection, dyskinesias were rated a second time (Figure 4.2.8; *post-nimodipine; black bars*). A 48-hour washout was given between drug tests. *Test doses of nimodipine were chosen to be 10-fold higher and lower than that used in the chronic release pellets we will use in the proposed studies (i.e.: 0.8 mg/kg). We also examined the same nimodipine dose as the pellets (0.8 mg/kg), plus a dose of 20 mg/kg, which is a higher dose commonly employed in the literature (Opacka-Juffry et al., 1998). This highest dose was included as a precaution (prior to our testing for levels of plasma nimodipine in pelleted animals) in case this lipophilic drug might have accumulated in fatty tissue and raised plasma levels of the drug. None of the
nimodipine doses tested directly interfered with or potentiated LIDs. Thus, we are able to rule out any behavioral impact of nimodipine-levodopa drug-drug interactions and focus our analysis on the contribution of MSN spine density and morphology. (Figure 4.2.8).
Figure 4.2.8: Acutely Administered Nimodipine Does Not Alter the Expression of Levodopa-induced Dyskinesias. A group of parkinsonian rats, distinct from rats used for the chronic nimodipine pellet studies, were employed to determine whether co-administration of (acute) nimodipine (i.p.) with levodopa negatively or positively influenced LIDs. Rats were rendered parkinsonian without any pellet implants. All drugs were administered on the test day by i.p. injection. LID severity was analyzed 30 minutes post-levodopa (light gray bars), which was followed by an injection of one of four doses of nimodipine (0.08, 0.8, 8.0, 20 mg/kg). Thirty minutes following the nimodipine injection, dyskinesias were rated again (black bars). Dashed arrows on x-axis indicate escalating doses of nimodipine tested in combination with the various doses of levodopa (6.0, 8.0, or 12.5 mg/kg). The horizontal dashed lines indicate the statistical average severity of dyskinesia for each dose of three doses of levodopa used.
4.2.5 Pilot Study Data: Impact of nimodipine-mediated spine preservation in DA-depleted rats on LIDs:

Rats in this pilot study underwent three weeks of levodopa treatment on an escalating dose schedule. Thus, for the first week (M-F) rats were administered 5mg/kg levodopa, i.p; followed by 8mg/kg levodopa, i.p for week 2, and 12.5 mg/kg levodopa, i.p. for week three. Resulting levodopa-induced dyskinesias were evaluated and are summarized in Figure 4.2.9. Regardless of the dose of levodopa, there was no difference in severity of LIDs between rats with maintained dendritic spine density and those with dendritic spine loss (Dyskinesia severity score: Nimodipine treated: 6mg/kg levodopa: 2.88 ± 1.11; 8mg/kg levodopa: 5.21 ± 0.87; 12 mg/kg levodopa: 7.92 ± 1.27; Vehicle treated: 6mg/kg levodopa: 3.53 ± 1.09; 8mg/kg levodopa: 6.53 ± 1.33; 12 mg/kg levodopa: 7.6 ± 1.28; p >0.05 for nimodipine vs. vehicle at each dosage).
Figure 4.2.9: Preservation of striatal MSN spine density did not have a statistically significant effect on LIDs with acute (one week) levodopa at the doses tested here. While total severity levels were similar between these acutely treated groups (nimodipine versus vehicle), there were qualitative differences in behaviors that are being further investigated. Chronic nimodipine was administered via subcutaneous pellet at a dose of 0.8 mg/kg per day beginning two days post-lesion. Two weeks after the lesion, animals were begun on a daily (M-F) levodopa injection paradigm with ascending doses of levodopa, with each dose given for one week. The doses of levodopa employed allowed examination of low, moderate and high doses of this drug on dyskinesia in animals with intact or depleted dendritic spine morphology.
Shortly after completion of the pilot nimodipine study, another cohort of rats treated with either nimodipine or vehicle, in the same manner as in the pilot study was carried out by Dr. Steece-Collier. These rats however, underwent more extensive, chronic levodopa treatment, beginning treatment with high dose levodopa (12.5mg/kg) for several weeks, followed by additional weeks of treatment at low dose levodopa (6.25mg/kg) levels. The results from this dosing paradigm are shown in Figure 4.2.10. In this paradigm, rats with intact spines are initially less dyskinetic than their spine depleted counterparts. However, with continued levodopa administration, rats with preserved spine density become increasingly more dyskinetic such that after 15 days of treatment, there is no difference in dyskinesia severity compared to spine-depleted rats.

When levodopa dosages are decreased by half (to 6.25 mg/kg), those animals with intact spines show a reversal in dyskinesia severity such that by 20 days post-levodopa, there is again, a reduced level of LIDs in rats with intact spines versus those with denuded spines (Figure 4.2.10). This ability of rats with intact spines to effectively “buffer” their response to levodopa is of particular interest considering that neuronal alterations secondary to levodopa priming are generally thought to be long-lasting if not irreversible (Obeso et al., 2000a; Obeso et al., 2000b; Obeso et al., 2000c; Olanow & Obeso, 2000b; a; Rascol, 2000; Westin et al., 2001).
Dendritic Spine Preservation through Chronic Release Nimodipine Pellets Buffers Severity of Levodopa-induced Dyskinesias

- High Dose Levodopa: 12.5 mg/kg
- Low Dose Levodopa: 6.25 mg/kg
- Vehicle Pellets
- Nimodipine Pellets

Y-axis: Dyskinesia Severity
X-axis: Treatment Timing (Days: 1, 6, 12, 15, 18, 20)
Figure 4.2.10: Long-term levodopa administration: This figure refers to an additional study in our lab conducted by Dr. Steece-Collier. (A) In severely parkinsonian rats maintaining normal dendritic spine integrity dampens severity of LIDs, even at high dose levodopa. However, with repeated daily dosing of this high dose of levodopa, the buffering capacity is lost (i.e. Day 15). Of particular interest, subsequent lowering of daily dose of levodopa allows nimodipine treated PD rats to show reversal of LID severity. (B) Of note, in this particular cohort of rats there was an observed improvement in vibrissae test scores for nimodipine treated rats such that nimodipine treated rats demonstrated improvement in sensori-motor integration on the lesioned side from 4-10 weeks post-lesion, while vehicle treated rats showed no recovery in this behavior. P= 0.004.
Follow-up study data

4.2.6 Confirmation of DA-denervation and impact of nimodipine-mediated spine preservation in DA-depleted rats on parkinsonian motor behaviors

Successful DA-denervation was confirmed in all lesioned rats using the medial terminal nucleus (MTN) counting method (Sauer & Oertel, 1994). All animals included in this study were greater than 85% lesioned (average percent lesioned nimodipine: 0.65 ± 2.03%; vehicle: 2.01 ± 1.38%). Amphetamine-induced rotational asymmetry is a classical measure of severity of DA-denervation in the parkinsonian rat (Ungerstedt & Arbuthnott, 1970), however amphetamine alone induces changes in spine density and morphology (Robinson & Kolb, 1997). Thus, as in all our nimodipine studies, the use of amphetamine-induced rotational asymmetry was avoided to eliminate the drug as a potential confounding factor. Instead, levodopa-induced rotational asymmetry was quantified during peak dosage of levodopa at both 12.5mg/kg and 25mg/kg levodopa. Levodopa-induced rotational asymmetry data is summarized in Figure 4.2.11. All rats exhibited some degree of rotational asymmetry in response to levodopa. At 12.5 mg/kg levodopa, there was no difference in degree of rotational asymmetry between nimodipine and vehicle-treated rats (Nimodipine: 3.52 ± 1.18 rpm; Vehicle: 6.14 ± 1.18 rpm; nimodipine vs. vehicle at 12.5mg/kg levodopa, p > 0.05).

However, at 25mg/kg levodopa, the degree of levodopa-induced rotational asymmetry was markedly reduced in parkinsonian rats with preserved spine density compared to those without spine preservation (Nimodipine: 3.34 ± 1.03 rpm; Vehicle: 9.57 ±1.03 rpm; Figure 4.2.11, nimodipine vs. vehicle at 25mg/kg levodopa, p<0.05).
Rats were also assessed for degree of parkinsonism via the vibrissae test. All rats were pre-tested before DA-depletion and performed the vibrissae task with 100% accuracy with both the left and right forepaws. After DA-depletion, only those rats with 20% or lower performance with the right forepaw (the “lesioned” side) were included in the study. Contrary to our findings in the pilot study, where levodopa-treated rats with intact dendritic spine density (nimodipine-treated rats) showed some degree of improvement in vibrissae task performance compared to rats with reduced dendritic spine density (vehicle-treated rats) (Figure 4.2.6 and Figure 4.2.10), there was no improvement in vibrissae test scores in levodopa or saline treated parkinsonian rats regardless of spine status in this study (Pre-lesion Nimodipine: 100%; Pre-lesion Vehicle: 100%; Post-lesion Nimodipine: 0% at all timepoints; Post-lesion Vehicle; 0% at all timepoints). Finally, any rat whose peak dyskinesia scores were two or more standard deviations outside of the mean for their treatment group for two or more consecutive rating periods were eliminated from data analysis. The combination of these criteria (>85% lesioned, <20% success rate on post-lesion vibrissae, and within 2 standard deviations of the mean for LID behavior) led to the exclusion of two nimodipine and two vehicle-treated rats from final analysis.
Figure 4.2.11: Preserving dendritic spines dampens levodopa-induced rotational asymmetry. Levodopa-induced rotational asymmetry was quantified during peak (20-60 minutes) dosage of levodopa at both 12.5mg/kg and 25mg/kg levodopa. All DA-depleted rats exhibited some degree of rotational asymmetry in response to levodopa. At 12.5 mg/kg levodopa, there was no difference in degree of rotational asymmetry between nimodipine and vehicle-treated rats. However, at 25mg/kg levodopa, the degree of levodopa-induced rotational asymmetry was markedly reduced in parkinsonian rats with preserved spine density (nimodipine) compared to those without spine preservation (vehicle). Dark gray: Vehicle; Light gray: Nimodipine. Asterisk denotes p<0.05.
4.2.7 Follow-up study data: impact of nimodipine-mediated spine preservation in DA-depleted rats on LIDs

Parkinsonian rats were divided into levodopa or saline treatment cohorts. Those animals receiving saline injections did not demonstrate any dyskinetic behavior at any timepoint, regardless of spine status. Total LIDs are displayed graphically in Figures 4.2.12-4.2.13 as development of LIDs over the complete timecourse of levodopa-induced behaviors, instead of simply peak dose LIDs (i.e., total LID at 40 minutes post-injection). There is a significant difference in the degree of LID severity between parkinsonian rats with preserved spine density and those without such that rats with preserved spine density are markedly less dyskinetic at both 12.5mg/kg (AUC Nimodipine: 445 ± 50.27*; AUC Vehicle: 589.05 ± 28.58; *p<0.05) (Figure 4.2.12) and 25mg/kg (AUC Nimodipine: 670.95 ± 99.76*; AUC Vehicle: 1037.14 ± 80.44; * p<0.05) (Figure 4.2.13) doses of levodopa. The magnitude of reduction in dyskinesia severity in parkinsonian rats with preserved spine density (nimodipine-treated) is more pronounced (reduction in LIDs in nimodipine-treated rats compared to vehicle-treated rats over total timecourse (54.6% at 25mg/kg levodopa and 32.4% at 12.5mg/kg levodopa) and maintained over a longer timecourse (LIDs are reduced in nimodipine treated rats from 40-80 minutes at 12.5mg/kg levodopa versus 40-180 minutes at 25mg/kg levodopa; 2-way ANOVA; p<0.05) at 25mg/kg compared to 12.5mg/kg.

The expression of anatomically specific dystonias and hyperkinesias in response to levodopa was also assessed. Parkinsonian rats with preserved dendritic spine density developed significantly reduced dystonias, specifically in the neck, trunk, and right forepaw compared to parkinsonian rats with spine depletion (Figure 4.2.14 A, C, D, ...
E). Reduction in severity of each dystonic behavior was seen only at the higher dose of 25mg/kg levodopa (Nimodipine: 3.00 ± 1.35*; Vehicle: 5.52 ± 0.98; *p<0.05) and not at the lower dose of 12.5mg/kg (Nimodipine: 2.88 ± 1.50; Vehicle: 3.25 ± 0.98; p>0.05). In contrast to dystonic behaviors, there is no difference in the severity of total hyperkinesias, regardless of levodopa dosage, between rats with or without preserved spine density (Dyskinesia severity score: 25mg/kg levodopa (Nimodipine: 2.11 ± 0.34; Vehicle: 2.50 ± 0.17); 12.5 mg/kg levodopa (Nimodpine: 2.11 ± 0.37; Vehicle: 2.54 ± 0.20); p > 0.05; Figure 4.2.14 B). However, there is a significant reduction specifically in right forepaw hyperkinesia in parkinsonian rats with preserved spine density compared to their denuded counterparts.

In contrast to all other behaviors evaluated, there were two behaviors in which severity was worsened in parkinsonian rats with preserved dendritic spine density. Regarding dystonia of the hindpaw, none of the vehicle treated rats displayed hindpaw dystonia at any dosage, however two nimodipine-treated rats developed mild hindpaw dystonia at 12.5mg/kg levodopa leading to a significant increase of hindpaw dystonia in nimodipine treated rats at 12.5 mg/kg (Dyskinesia severity scores: Nimodipine: 0.20 ± 0.16*; Vehicle: 0 ± 0; *p = 0.04) (Figure 4.2.14 G). Additionally, there was a small but significant increase in the level of stereotypic forelimb-facial hyperkinesias in rats with preserved spine density at 25mg/kg levodopa (Dyskinesia severity score: Nimodipine: 1.09 ± 0.41; Vehicle: 0.48 ± 0.35; *p<0.05; Figure 4.2.14 H). Despite the increased severity of these behaviors, there remains a significantly reduced level of total dyskinesias development in rats with preserved spine density compared to rats with depleted spines.
Levodopa-induced Dyskinesias; 12.5mg/kg

Average AUC: Trapezoid Method

Minutes post levodopa (ip)
B

Total levodopa-induced dyskinesias; 12.5mg/kg

Area Under Curve: Trapezoid Method

- Vehicle
- Nimodipine

20-60 minutes | 20-100 minutes | Total (0-180 minutes)

* Indicates significant difference

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Figure 4.2.12: Nimodipine-mediated spine preservation reduces LID severity at 12.5mg/kg dose levodopa. (A) Total LIDs are reduced in nimodipine-treated rats compared to vehicle-treated rats treated with 12.5mg/kg levodopa. This reduction in dyskinesias in animals with preserved spine density is present during peak-dose levodopa, from 40-80 minutes post-levodopa. (B) Total LIDs are reduced for the duration of levodopa-induced activity (0-180 minutes) in nimodipine-treated rats by 32.4% compared to vehicle-treated rats. Reduced LIDs in nimodopine rats is also apparent from 20-100 minutes, but not during early time period of 20-60 minutes. Light gray: Nimodipine-treated; Dark-gray: Vehicle-treated. Asterisk denotes $p<0.05$. 
Total levodopa-induced dyskinesias; 25mg/kg

Area Under Curve: Trapezoid Method

- Vehicle
- Nimodipine

20-60 minutes
20-100 minutes
Total (0-240 minutes)
Figure 4.2.13: Nimodipine-mediated spine preservation reduces LID severity at 25 mg/kg dose levodopa. (A) Total LIDs are reduced in nimodipine-treated rats compared to vehicle-treated rats treated with 25 mg/kg levodopa. This reduction in dyskinesias in animals with preserved spine density is present during the majority of levodopa-induced activity, from 40-180 minutes post-levodopa. (B) Total LIDs are reduced for the duration of levodopa-induced activity (0-240 minutes) in nimodipine-treated rats by 54.6% compared to vehicle-treated rats. Reduced LIDs in nimodipine-treated rats is also apparent from 20-100 minutes and during early time period of 20-60 minutes. Light gray: Nimodipine-treated; Dark-gray: Vehicle-treated. Asterisk denotes $p<0.05$. 

Figure 4.2.14: Breakdown of individual dystonic and hyperkinetic behaviors in nimodipine and vehicle treated rats. (A) Dystonias of the neck, trunk and forepaw were evaluated in nimodipine and vehicle treated rats at 12.5 and 25 mg/kg ip levodopa. Dystonias are reduced in parkinsonian rats with preserved spine density compared to rats with spine loss at 25mg/kg levodopa, but not at 12.5 mg/kg levodopa. (B) There was no difference in development of hyperkinesias in parkinsonian rats at either 12.5 or 25 mg/kg levodopa, regardless of spine status. (C) Dystonia of the neck is reduced with spine preservation specifically at 25mg/kg levodopa. (D) Dystonia of the trunk is reduced with spine preservation specifically at 25mg/kg levodopa. (E) Dystonia of the right forepaw is reduced with spine preservation specifically at 25 mg/kg levodopa. (F) Hyperkinesia of the right forepaw is reduced with spine preservation specifically at 25 mg/kg levodopa. In contrast to other dyskinetic behaviors, hindpaw dystonia (G) and stereotypic forelimb-facial hyperkinesia (i.e. CCL (H) are elevated in rats with preservation of spine density specifically at 25 mg/kg levodopa.
4.2.8 Follow-up study data: impact of nimodipine-mediated spine preservation in DA-depleted rats on FosB expression

FosB is a rapid/transient transcription factor related to neuronal plasticity and elevation of FosB expression has been well correlated with the development of LIDs. FosB is upregulated with DA depletion and can be re-regulated by successful DA grafts, as shown in Aim 1. Further, FosB is significantly upregulated in the lateral striatum of PD rats following chronic levodopa (i.e. Andersson et al 1999), and expression in the lateral striatum is definitively linked to our expression of LIDs. In support of this relationship, intrastriatal infusion of antisense FosB mRNA prevents LID development (Andersson 1999, 2003). The following data demonstrate that in the parkinsonian rat with reduced spine density (vehicle treated) FosB expression is elevated with DA-depletion (184.5 ± 30.1%), and significantly further elevated with levodopa treatment (264 ± 25.4%) (Figure 4.2.15; 2-way ANOVA, p<0.05). This classic pattern of FosB regulation in response to DA-denervation and subsequent levodopa therapy is altered in rats with preserved spine density (nimodipine treated) (Figure 4.2.15). As in vehicle-treated rats, striatal FosB is elevated with both DA-depletion (300.8 ± 33.6%) and levodopa (224.8 ± 25.4%) in nimodipine-treated parkinsonian rats, however in the nimodipine rats, there is no significant difference in the level of FosB elevation with levodopa compared to FosB elevation in the absence of levodopa (saline-treated). Further, there is no significant difference between nimodipine and vehicle-treated rats in degree of FosB elevation with levodopa, however, in the absence of levodopa (saline-treated), FosB expression is significantly greater in nimodipine-treated rats compared to vehicle-treated rats (Figure 4.2.15, 2-way ANOVA; p<0.05).
Figure 4.2.15: FosB expression: In the parkinsonian rat with reduced spine density (vehicle treated) FosB expression is elevated with DA-depletion, and significantly further elevated with levodopa treatment. This classic pattern of FosB regulation in response to DA-denervation and subsequent levodopa therapy is altered in rats with preserved spine density (nimodipine treated). FosB is also elevated with both DA-depletion and levodopa in nimodipine-treated parkinsonian rats, however there is no significant difference in the level of FosB elevation between saline and levodopa treated animals in this group. Further, there is no difference between nimodipine and vehicle-treated rats in degree of FosB elevation with levodopa, in the absence of levodopa (saline-treated) FosB expression is significantly greater in nimodipine-treated rats compared to vehicle-treated rats. Blue: Saline-treated; Red: Levodopa-treated. Asterisk denotes p<0.05.
4.2.9 Discussion for Aim 2

Dendritic spines are important biochemical compartments essential to normal neuronal signaling. Loss of these morphological specializations on striatal output neurons following DA depletion doubtless impacts synaptic transmission, the exact consequence of which is just beginning to be explored. Loss of spines and accompanying synaptic connectivity also appears be important in the development of adverse motor side-effects of levodopa therapy. Maintenance of spines may provide normalization of information flow through the striatum and rest of the basal ganglia nuclei, thereby reducing aberrant plasticity associated with expression of LIDs.

In the pilot study data, I have demonstrated that in our lab, as demonstrated by others (Ingham et al., 1989; Stephens et al., 2005; Day et al., 2006), we find marked reduction in dendritic spine density of striatal MSNs with 6-OHDA mediated DA-depletion (Figure 4.2.5). Further, this spine loss is prevented with administration of continuous release nimodipine pellets, 1-2 days post DA-depletion ((Day et al., 2006) (Figure 4.2.4 and Figure 4.2.5). Although there is some evidence that maintaining spine integrity can relieve LIDs (Rylander et al., 2009; Schuster et al., 2009) the role of maintaining intact dendritic spines in the development of LIDs remains largely undetermined.

The central hypothesis for the experiments outlined in Aim 2 is that preventing loss of dendritic spines will allow for maintenance of corticostriatal synaptic integrity and will prevent or reduce the incidence of levodopa-induced dyskinesias. Prior to testing this hypothesis, we examined whether there was any direct drug-drug interaction between levodopa and nimodipine that would complicate data interpretation. Employing
multiple doses of both levodopa (6, 8, 12.5 mg/kg) and nimodipine (0.08, 0.8, 8, 20 mg/kg) we found NO interaction between these two drugs on dyskinetic behaviors, allowing confidence that in this model, the impact of spine integrity on LID development, not simply calcium channel blockade, is being investigated (Figure 4.2.8).

Analysis of parkinsonian motor function in nimodipine and vehicle treated rats suggest that maintaining integrity of normal spine density on MSNs, even in an environment of DA-depletion, can result in improved motor function for particular behaviors in the parkinsonian rat. It is not surprising that an animal with a severe absence of striatal DA despite a normal compliment of dendritic spines would show severe motor deficits. However, it would be anticipated that maintaining normal spine density and the accompanying synaptic organization/machinery would result in superior “symptomatic” relief with DA replacement therapy. Our initial data suggest that maintaining spine integrity in an environment of severe DA-depletion does little to improve general motor dysfunction (Figure 4.2.7), but does appear to provide for improved sensorimotor function in those rats receiving levodopa (Figure 4.2.6). In the follow-up study, there was no observable improvement in sensori-motor integration in the absence of levodopa. The specific improvement in vibrissae scores observed only in rats with intact spine density and receiving levodopa suggests that perhaps repeated stimulation of spinous DA receptors with daily DA-replacement therapy (i.e.: levodopa) in parkinsonian subjects with normal dendritic spine numbers could result in preservation of the integrity and/or functionality of these receptors and related behavioral impact.
The animals in the follow-up study do however, demonstrate reduced rotational asymmetry in response to levodopa in those animals with preserved dendritic spine density (Figure 4.2.11), suggesting that preservation of spine density does contribute to improved parkinsonian motor functions in some manner, especially in the presence of DA restoration via levodopa. Further, maintaining architectural integrity of dendritic spines likely contributes to normalized physiological gating of glutamatergic input onto MSNs, which would be expected to contribute to normalized motor behavior. It remains unknown why some behaviors (vibrissae task) improve in the presence of maintained spine integrity, while others (lateral forepaw step adjustment) do not (Figures 4.2.6, 4.2.7, 4.2.10B), but it is likely due to a combination of the degree of complexity of a behavior, the required balance of DA and glutamate for initiation and maintenance of that behavior, and the degree and pattern of spine morphology throughout the parkinsonian striatum. Understanding the impact of degeneration of structures such as MSNs in the parkinsonian brain and their role in response to standard and/or experimental therapies may create opportunities for novel interventions for PD.

In these experiments, I have demonstrated that in the parkinsonian rat with reduced spine density (vehicle treated) FosB expression is elevated with DA-depletion (184.5 ± 30.1%), and significantly further elevated with levodopa treatment (264 ± 25.4%) (Figure 4.2.15; 2-way ANOVA, p<0.05). This classic pattern of FosB regulation in response to DA-denervation and subsequent levodopa therapy is altered in rats with preserved spine density (nimodipine treated) (Figure 4.2.15). As in vehicle-treated rats, striatal FosB is elevated with both DA-depletion (300.8 ± 33.6%) and levodopa (224.8 ± 25.4%) in nimodipine-treated parkinsonian rats, however in the nimodipine rats, there is
no significant difference in the level of FosB elevation with levodopa compared to FosB elevation in the absence of levodopa (saline-treated). Further, there is no significant difference between nimodipine and vehicle-treated rats in degree of FosB elevation with levodopa, however, in the absence of levodopa (saline-treated), FosB expression is significantly greater in nimodipine-treated rats compared to vehicle-treated rats (Figure 4.2.15, 2-way ANOVA; p<0.05). These data suggest preservation of dendritic spine density may prevent further upregulation of FosB normally accompanying levodopa administration. Thus, the preservation of dendritic spine density may buffer aberrant signaling patterns leading to LIDs, perhaps partly explaining the reduced severity of LIDs observed in those rats with normalized spine density. This finding reinforces the importance of not simply intact morphology, but also intact synaptic connectivity in the quest for improving striatal signaling in the parkinsonian striatum.

While it appears that spine loss is not a causative factor for expression of parkinsonian-like motor deficits seen in this rat model, the current data do suggest that a decrease in dendritic spine density following severe DA-depletion influences motor behaviors in response to DA therapy, such as levodopa-induced rotational asymmetry and levodopa-induced dyskinesias. Preliminary data also suggest that dendritic spines are highly plastic and that repeated levodopa exposure is capable of increasing the number of dendritic spines in the DA-depleted striatum, and that levodopa may further cause abnormal structural adaptations (Figure 4.2.3). Thus, one can conclude that intact spine morphology is necessary, but not sufficient for effective recovery of the parkinsonian striatum. It is clear that maintaining spine density is not sufficient to correct dysregulation of intracellular signaling molecules such as FosB in response to DA-
depletion, however it does appear sufficient to dampen FosB dysregulation in response to levodopa. In combination with the observation of reduced LIDs in rats with preserved spine density these data support the conclusion that additional plasticity factors such as electrical, chemical, or downstream signaling pathways likely contribute to the presentation of dyskinesias and correction of aberrant signaling on multiple levels is necessary for adequate control of PD symptoms and avoidance of treatment-related dyskinesias (modeled in Figure 4.2.16). Nonetheless, the use of drugs like nimodipine provide a novel model to explore drug-related plasticity specifically in the parkinsonian striatum. Future studies in the lab will investigate the possibility that maintenance of spine compartments might provide for improved DA replacement therapy in severely parkinsonian subjects through preservation of DA receptors on these morphological entities.
**Figure 4.2.16: Summary of several of the factors underlying development of LIDs.**

It is clear that a combination of factors included dysregulation of synaptic communication, aberrant morphology, and aberrant changes in plasticity factors underlie LIDs. It follows the prevention of LIDs will require intervention on multiple levels to address each underlying factor.
Chapter 5; Discussion

But are we sure of our observational facts? Scientific men are rather fond of saying pontifically that one ought to be quite sure of one's observational facts before embarking on theory. Fortunately those who give this advice do not practice what they preach. Observation and theory get on best when they are mixed together, both helping one another in the pursuit of truth. It is a good rule not to put overmuch confidence in a theory until it has been confirmed by observation. I hope I shall not shock the experimental physicists too much if I add that it is also a good rule not to put overmuch confidence in the observational results that are put forward until they have been confirmed by theory.

Chapter 5: Discussion

5.1 Summary of the results of the Specific Aims

The experiments described in this dissertation were designed to address means of improving current therapeutics for patients with Parkinson's disease. Currently, optimal therapeutic benefit from available treatments is often limited by disabling side effects. For instance, the presentation of levodopa-induced dyskinesias in response to long-term levodopa treatment plagues over half of PD patients. Further, limited and/or variable efficacy and the emergence of graft-induced dyskinesias, have significantly derailed development of fetal DA neuron transplantation as a potential PD therapy.

Mechanisms underlying variations in efficacy and development of side effects related to pharmacological and surgical intervention in PD patients remain uncertain. Identifying factors impeding the success of DA replacement in the parkinsonian striatum, whether through grafting or other means, will allow for maximized therapeutic outcome.

The studies in this dissertation explore improving therapeutics for PD from two specific angles; (1) optimizing graft efficacy in the aged host, and (2) understanding the role of morphological alterations on striatal cytoarchitecture in presentation of LIDs. The issue of optimizing graft efficacy in the aged host is addressed in Aim 1 of this dissertation, in which I used a rat model of PD to examine if equivalent behavioral efficacy between young, middle, and aged graft recipients can be achieved by compensating for decreased cell survival known to occur in the aged brain. Understanding the role of morphological alterations of striatal cytoarchitecture in
presentation of LIDs is addressed in Aim 2, in which I explored the effects of preserved dendritic spine density on parkinsonian motor behaviors, levodopa-induced dyskinesias, and induction of FosB expression in the parkinsonian rats.

**Aging and PD (Specific Aim 1)**

Advanced age is well-established as the greatest risk factor for the development of PD (Driver et al., 2009). Further, the overall efficacy of fetal transplantation for parkinsonian patients remains controversial due to the lack of statistical significance in the primary behavioral endpoints in the major clinical trials in the field (Freed et al., 2001; Freed et al., 2003; Olanow et al., 2003; Hagell & Cenci, 2005). Nonetheless, the promise of fetal DA transplantation as a therapy for PD has not been dismissed and research continues in an effort to both optimize efficacy and minimize side effects of grafting therapies.

In Aim 1, I examined whether compensating for reduced survival of grafted DA neurons, known to occur in the aging host, would allow for enhanced functional recovery. In this Aim, I tested the hypothesis that when the obstacle of poor graft cell survival in the aged parkinsonian brain can be overcome, equivalent survival of grafted DA neurons across host ages will result in equal behavioral efficacy. Previous data from our group (Collier & Sortwell, 1999) indicated that when the same numbers of embryonic ventral mesencephalic DA neurons (i.e.: 200,000) were transplanted into the striatum of parkinsonian rats of varying ages, there was a proportional decrease in survival of engrafted neurons with increasing age. Based on specifications from this
previous data, we grafted either a two-fold or five-fold increase in the number of mesencephalic neurons into 15 or 22 month old parkinsonian rats compared to their 3 month old counterparts. This approach was taken in an attempt to obtain an equal final number of grafted DA neurons in each age group.

Indeed, this approach did allow for aged rats to have enhanced numbers of grafted DA neurons, and I found approximately 5-fold more TH-ir neurons in 22 mo rats compared to the 3 mo rats. Similarly, 15 mo grafted rats also showed enhance DA neuron survival and a 2-fold increase in TH-ir neurons in their grafts. Thus, this finding challenges the long-held premise that aged brain does not allow for robust survival of grafted embryonic neurons.

In contrast to my hypothesis, I found that even when DA grafts in aged (22 mo) rats contained 5-fold more TH-ir neurons compared to young (3 mo) rats, the degree of TH-ir neurite outgrowth was equal between aged and young graft recipients (although direction of distribution varied between ages). Further, graft-mediated behavioral improvement in LIDs was significantly delayed, requiring 10 weeks to achieve a comparable level seen in younger rats with significantly fewer neurons. As would be expected based on robust survival of grafted DA neurons and significant decrease in LIDs across all age groups, I also found that aberrant FosB signaling was corrected in all age groups. These data demonstrate that under particular conditions, robust, functional survival of engrafted embryonic DA neurons can be obtained in the aged parkinsonian brain. Reduction of compensatory mechanisms (Collier et al., 2005) and growth and plasticity factors (Collier et al., 1999; Sortwell et al., 2001; Yurek & Fletcher-Turner, 2001) normally make the aged striatum less hospitable to transplanted neurons.
In this particular instance, the large number of cells transplanted at a high density into the aged striatum likely enabled secretion of autotrophic support factors from engrafted cells, supporting their own survival. With greater numbers of surviving cells, improved graft efficacy in the aged striatum would be expected. However, even when a large number of grafted DA neurons survive in the aged parkinsonian rat brain, the behavioral impact is overall inferior to that seen with fewer neurons in younger subjects.

Given that grafting paradigms in PD patients are carried out in individuals with advanced disease, severe DA depletion, and often in patients of advanced years, improving graft efficacy in the aged host is of significant clinical value and interest. Previous data has suggested that repairing the severely diseased brain might present challenges that are insurmountable, particularly in individuals of advanced age, again due to decreased trophic support, decreased compensatory mechanisms in response to DA-depletion, and decreased plasticity factors. The results of Aim 1 experiments demonstrate that even when a large number of DA neurons are shown to survive in the aged parkinsonian rat brain, the behavioral impact is delayed, and inferior on a per cell basis to that seen with significantly fewer neurons in younger subjects. As clinical trials re-emerge, consideration of grafting into individuals of younger age may be warranted.

**Alterations of striatal morphology: therapeutic efficacy**

In addition to consideration of grafting into younger patients, data also indicate that grafting into patients with less severe disease may also be advisable. Dendritic spines are important biochemical compartments essential to normal neuronal signaling.
Loss of these morphological specializations on striatal output neurons following DA depletion doubtless impacts synaptic transmission, the exact consequences of which are just beginning to be explored. Loss of spines and accompanying synaptic connectivity may be an important factor that contributes to lack of full behavioral efficacy in grafting. Indeed, while dendritic spines are highly plastic structures, appearing and disappearing quite readily, there is a decrease in this plasticity with aging. If the presence of dendritic spines is required for physiological re-innervation following grafting, the absence of these structures in the PD brain would be deleterious and could account for suboptimal efficacy. Our laboratory, in studies outside of my thesis, has data demonstrating that in young subjects, DA-depletion mediated reduction in dendritic spine density is reversible following DA grafting (unpublished observation, KSC). However, it is possible that there is lack of “re-growth” of spines following DA graft placement in aged subjects. It is feasible to consider that this may be one factor contributing to a less efficacious outcome despite large numbers of grafted neurons.

Loss of dendritic spines also appears be important in the development of adverse motor side-effects of levodopa therapy as shown by the studies in Aim 2, and by Schuster and colleagues (Schuster et al., 2009). Maintenance of spines may provide normalization of information flow through the striatum and rest of the basal ganglia nuclei, thereby reducing aberrant plasticity associated with expression of LIDs (Day et al., 2006).
Alterations of striatal morphology and LIDs (Specific Aim 2)

In Aim 2, I tested the hypothesis that striatal neuron pathology, specifically the loss of dendritic spines on MSNs that accompanies severe DA depletion, results in suboptimal therapeutic benefit of levodopa in parkinsonian subjects. Behavioral tests confirmed no acute impact of nimodipine on test behaviors, allowing confidence that in this model, the impact of spine integrity on LID development, not simply calcium channel blockade, is being investigated (Figure 4.2.8).

Our initial data show that maintaining spine integrity in an environment of severe DA-depletion does little to improve general motor dysfunction (Figure 4.2.7), but does appear to provide for improved sensorimotor dysfunction in those rats receiving levodopa (Figure 4.2.6). In previous sections of this dissertation I have discussed the importance of the intricate and highly specific arrangement of nigral dopaminergic and cortical glutamatergic input on spines of striatal MSNs (Figure 1.10.4.1). Preserving this cytoarchitecture in turn likely preserves some degree of normal, physiological regulation of glutamate by DA in the parkinsonian striatum. Hyperactivity of the glutamatergic corticostriatal inputs is well-established as a contributing factor to dyskinesias, and is discussed in the introduction. For example, increases in perforated synapses (indicating increased corticostriatal activity, i.e. increased glutamate) has been associated with DA-depletion in the parkinsonian striatum (Muriel et al., 2001). Maintaining control of unregulated glutamatergic signaling in the parkinsonian striatum is a critical step in reducing or preventing unwanted motor behaviors, such as LIDs.
The specific integration of sensory and motor interaction in the striatum is only improved in those rats with a supply of DA (from levodopa) and with intact spine morphology (nimodipine-treated). Since rats used in these studies are more than 98% DA-depleted, the majority of striatal DA is from levodopa administration. Thus, it is not surprising that improvement would only be observed in those rats receiving levodopa treatment. The lateral forepaw adjustment task is a test of postural instability, a parkinsonian symptom often difficult to restore with DA replacement therapy (Jankovic, 2008). In contrast, the vibrissae test evaluates a completely distinct behavior, sensorimotor integration. It is well known that different motor behaviors involve distinct motor circuitry. Thus, it is not surprising that maintaining spine density may improve circuitry involved in some tasks, especially those responsive to treatment with DA agonists (i.e. vibrissae), but not others.

Rats in the follow-up study demonstrate reduced rotational asymmetry in response to levodopa in those animals with preserved dendritic spine density (Figure 4.2.11). The combination of improvement in sensorimotor integration and reduced rotational asymmetry specifically in those rats with preserved spine density receiving levodopa suggests that preservation of spine density does contribute to improved parkinsonian motor functions in some manner, especially in the presence of DA restoration via levodopa. One explanation for these observations may be that the presence of DA agonists, such as levodopa, may maintain the integrity of striatal DA receptors, especially when spine morphology remains intact. Aberrant D1 receptor expression and localization has been correlated with LIDs in primates (Guigoni et al., 2007), and translocation of DA receptors from dendritic spines to shafts has also been
hypothesized to occur in the dyskinetic striatum. Thus, in addition to regulation of hyperglutamatergic input from the cortex, maintenance of dendritic spines in the parkinsonian striatum may support normalized behaviors not only through preservation of normal cytoarchitecture, but also through maintenance of DA receptor expression and localization.

In Aim 2, studies revealed a significant and dose-dependent reduction in LID severity in an environment of preserved dendritic spine density (Figures 4.2.12-4.2.14). Thus, one can conclude that intact spine morphology is necessary, but not sufficient for effective recovery of the parkinsonian striatum. Current data suggest that spine preservation may also impact regulation of downstream signaling factors related to DA-depletion and LIDs, such as FosB (Andersson et al., 1999; Westin et al., 2001). In Figure 4.1.15, it appears that while FosB is upregulated with DA-depletion regardless of spine status, further increases in FosB expression (as classically observed with development of LIDs) are absent in those rats with preserved spine density. The FosB data reinforce the importance of not simply intact morphology, but also intact synaptic connectivity in the quest for improving striatal signaling in the parkinsonian striatum. While it is clear that maintaining spine density is not sufficient to prevent LIDs, it does appear that maintaining intact striatal morphology is one critical factor in preventing the presentation of dyskinesias and correcting aberrant signaling associated with PD symptoms and avoidance of treatment-related dyskinesias (modeled in Figure 4.2.16).
5.2 Optimizing graft efficacy

While clinical trials have revealed that two of the most significant factors linked to graft efficacy are the survival of DA neurons in the transplant and the extent of graft-mediated reinnervation in the host striatum (Laguna Goya et al., 2008), there are multiple additional technical considerations for successful grafting, including the mode of graft delivery, the age of grafted tissue, the source of grafted tissue, the type of graft (dissociated cells versus whole tissue), storage conditions of grafted tissue before implantation, and graft “dosage” or the number of cells grafted.

Studies in graft efficacy have revealed multiple causes of graft failure (Figure 5.2.1), including the following; (1) excessive cell death (apoptotic or necrotic) due to trauma (shearing of neurites or loss of cell-cell contacts due to preparation techniques), storage or culture techniques, oxidative stress, excitotoxicity, hypoxia or ischemia, loss of trophic factors, and loss of estrogen; (2) surgical factors including hemorrhage, hypothermia, or host trauma (excessive gliosis); (3) immune factors resulting in graft rejection; (4) increased host age resulting in diminished graft efficacy; (5) poor distribution of cells within the target tissue; (6) untreated regions of deficit (i.e. beyond the sphere of influence of the graft, or extra-striatal locations); and (7) aberrant plasticity in the form of circuitry or graft-host connectivity (Laguna Goya et al., 2008).

Countermeasures to these causes of graft failure have included refined microdissection techniques; providing key growth and trophic factors such as GDNF, bFGF, NGF, BDNF, and others; reducing oxidative stress through the use of free radical scavengers; limiting hypoxia/ischemia via use of VEGF to promote vascularization of newly grafted tissue; counteracting effects of increased age of recipient on cell survival;
and attempting to preserve or restore neural circuitry through techniques such as spine preservation (Figure 5.2.1).

While the search for the optimal transplant conditions continues, investigators do agree on several pieces of the puzzle. (1) Fetal cells can survive transplantation in the human brain for long periods of time. (2) Transplants are capable of demonstrating functional effects (behavioral improvements). (3) Transplants are reasonably safe. (4) Transplant outcome is variable. (5) Clinical transplants have been less successful than animals models may have predicted, most likely due to the high degree of variability in clinical methods and human patients. (6) Transplants can reduce a patient’s need for medication. (7) Imaging studies suggest that grafts can increase DA uptake over long periods of time.

The areas that still remain undecided include: (1) the need for immunosuppression, (2) the best target and distribution for graft placement, (3) optimal tissue preparation methods, dissociation, and holding media, (4) the amount of tissue needed (current studies have varied from 1-8 fetuses), and (5) how many DA cells are needed for graft survival.

As discussed in earlier sections, while there has been painstaking effort to characterize the optimal donor cells used for transplantation (Annett et al., 1997), comparatively little has been done to determine the optimal host environment conditions for successful transplantation. The results from Aim 1 contribute to the question of optimal grafting conditions by addressing issues of (1) optimizing the host environment, and (2) optimizing transplantation conditions despite a suboptimal (in this case aged)
host. Specifically, in Aim 1, I have shown that despite the disadvantages of reduced adaptability, reduced plasticity and reduced trophic support associated with the aged striatum, these factors can be compensated by increasing the number of grafted cells. Although graft efficacy in the aged host remains delayed and diminished on per cell basis, when compared to young graft recipients, it is important to note that at the endpoint of the study, there is no difference age-dependent difference in reduction of LIDs with grafting. Thus, although the grafts are inferior to those in young patients, they do offer greater symptomatic improvement than smaller grafts in the aged striatum.

While Aim 2 is not a grafting study, the results from these studies are also valuable in regard to considering optimizing the host environment in grafting. Specifically, the data from Aim 2 supports the importance of intact striatal morphology in optimizing benefit from pharmacological treatment. Other studies in our lab have revealed that maintained spine morphology contributes to enhanced graft efficacy (unpublished results, KSC).

Thus, as the field of transplantation in PD continues to move forward, a critical component in optimizing grafting parameters is the role of the host striatum. The results from this dissertation support the conclusion that intervention at both early age (Aim 1) and early disease (Aim 2) will yield optimal therapeutic efficacy.
<table>
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<tr>
<th>Causes of Graft Failure</th>
<th>Countermeasure/Strategy</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>1) EXCESSIVE CELL DEATH</td>
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<tr>
<td>necrosis and apoptosis...</td>
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<tr>
<td>➢ Anoikis/apoptosis/dissection</td>
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<tr>
<td>trauma</td>
<td></td>
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<tr>
<td>Loss/shearing of neurites</td>
<td>Dissect at optimal age</td>
<td>Bjorklund et al 1983; Sladek, Elsworth, et al 1993; Freeman et al 1995</td>
</tr>
<tr>
<td>Preparation, suspension</td>
<td>Small pieces versus suspensions</td>
<td>Watts et al 2000</td>
</tr>
<tr>
<td>Loss of cell-cell contact</td>
<td>Tenascin</td>
<td>Gates et al 1996; Marchionini et al 2001</td>
</tr>
<tr>
<td>➢ Holding, storage media</td>
<td></td>
<td></td>
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<tr>
<td>Limit to 12 days</td>
<td></td>
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<tr>
<td>Improved holding media</td>
<td></td>
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<tr>
<td>Cryopreservation</td>
<td>Vitrification</td>
<td>Rall and Wood 1994; Van Wagendonk- De Leeuw et al 1995; Foellmer et al 1998</td>
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<tr>
<td>Culture</td>
<td></td>
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<tr>
<td>Prevent selective death, proper</td>
<td></td>
<td>Stromberg et al 2001; Sanchez-Pernaute et al 2001</td>
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<td>factors</td>
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<td>➢ Oxidant stress</td>
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<tr>
<td>Melatonin</td>
<td></td>
<td>Mayo et al 1999; Reiter et al 1999; Gultekin et al 2001</td>
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<tr>
<td>➢ Excitotoxicity</td>
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<tr>
<td>Flunarizine</td>
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<td>Kaminski Schierle et al 1999</td>
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<td>➢ Hypoxia, ischemia</td>
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<tr>
<td>VEGF</td>
<td></td>
<td>Rosenstein et al 1998; Silverman et al 1998; Pitzer et al 2001</td>
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<tr>
<td>➢ Loss of trophic factors</td>
<td></td>
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<tr>
<td>GDNF, Neurturin</td>
<td></td>
<td>Sautter et al 1998; Yurek 1998; Helt et al 2001; Rosenblad et al 1999</td>
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<tr>
<td>bFGF</td>
<td></td>
<td>Takayama et al 1995</td>
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<tr>
<td>NGF, BDNF, NT3, NT4/5- no increases</td>
<td></td>
<td>Brundin, Karlsson, et al 2000</td>
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<tr>
<td>Fetal kidney factors</td>
<td></td>
<td>Chiang et al 2001</td>
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<tr>
<td>➢ Loss of estrogen</td>
<td>Replace estrogen</td>
<td>Leranth et al 2000</td>
</tr>
</tbody>
</table>

2) SURGICAL FACTORS

➢ Hemorrhage                        | Use smaller cannula      | Brundin et al 1990 |
➢ Hypothermia                        | Lower host temperature   | Karlsson et al 2000 |
➢ Trauma to host                     | Avoid host trauma        | Sinclair et al 1999 |
|                                   | Imunosuppression         | Widner and Brundin 1988; Borlongan et al 1996 |

3) IMMUNE REJECTION

Block immune recognition sites, as Brevig et al 2001
<table>
<thead>
<tr>
<th>Causes of Graft Failure</th>
<th>Countermeasure/Strategy</th>
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<tr>
<td></td>
<td>for porcine tissue</td>
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<tr>
<td></td>
<td>Autologous or immune-modified stem cells</td>
<td></td>
</tr>
<tr>
<td>➢ More cells die</td>
<td>Identify and supplement factors</td>
<td>Ling et al 2000; Sortwell et al 2001</td>
</tr>
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</table>

4) INCREASED HOST AGE

➢ Postsynaptic receptors lost Recovery may be slower/no known strategy Coller and Sortwell, 1999; Aim 1 of this dissertation

➢ Reduced survivability Increase number of grafted cells in aged host | Coller and Sortwell, 1999; Aim 1 of this dissertation |

5) POOR DISTRIBUTION OF CELLS Smaller, better distributed grafts Use trophic factors—many trophic factors also have trophic effects

➢ Hotspot vs. widespread grafts Widespread distribution of grafted cells reduces GIDs | Maries et. al, 2006 |

6) UNTREATED DEFICIT REGIONS Include putamen, caudate, GP, others?


8) ABBERRANT GRAFT-HOST NEURONAL CONNECTIVITY Graft-host cell-cell contacts are altered and correlated with dyskinesias development Soderstrom 2008

Maintain dendritic spine density in parkinsonian striatum Ongoing studies in Steece-Collier laboratory

Table 5.2.1: Summary of the typical causes of graft failure and current countermeasures,strategies to improve graft efficacy. *Table modified from Redmond, 2002.*
5.3 Efficacy of grafting in the aging parkinsonian patient

One significant drawback to dopaminergic grafting strategies are the multiple reports of GIDs in parkinsonian patients, which persist even at low plasma levels of levodopa (Freed et al., 2001; Hagell & Cenci, 2005). Both LIDs and GIDs present significant quality of life issues for PD patients and can obstruct adequate disease treatment. Multiple mechanisms underlying the development of LIDs and GIDs have been proposed (reviewed by (Hagell & Cenci, 2005)), yet the exact details behind development of these dyskinesias remain obscure.

The studies in Aim 1 were designed to address one potential factor that recent clinical data suggests may contribute to both LID and GID development; the age of the graft recipient. The age of the graft recipient may be an especially important factor in the development of GIDs as recent reports of aggravated post-graft dyskinesias occurred only in patients 60 years of age or younger (Freed et al., 2001).

It is well known that plasticity in the nervous system peaks during development and wanes over the course of one’s lifetime. It has been hypothesized that LIDs as well as other drug-induced sensitization paradigms arise from maladaptive plasticity responses (Calabresi et al., 2000a). Indeed, the sensitization seen in PD patients with LIDs appears to have similar underlying mechanisms to DA sensitization seen during repeated administration of psychomotor stimulants such as cocaine and amphetamine (Canales & Graybiel, 2000). Cocaine, like levodopa results in an increase in striatal DA. In support of the hypothesis that younger graft recipients may be more susceptible to dyskinesias, Brandon and colleagues (Brandon et al., 2001) recently demonstrated that sensitization of midbrain DA systems following repeated cocaine administration is more
pronounced and more prolonged in younger rats compared to older ones. Given the multiple parallels between the emergence of LIDs in experimental animals given levodopa and the progressive induction of behavioral sensitization in experimental animals given psychomotor stimulant drugs (Canales & Graybiel, 2000), it is possible that younger subjects may be more prone to altered circuitry underlying graft-mediated worsening of dyskinesias. However, in my thesis study, there was no significant development of GID behaviors, likely due to the ventral location of graft placement as discussed earlier in the discussion for Aim 1. This conclusion is supported by previous data from our lab, which has demonstrated development of these behaviors when grafts are located in the dorsal striatum (Maries et al., 2006). Further, it is hypothesized that GIDs result in part from incomplete, patchy reinnervation of the ventral striatum (Hagell et al., 2002).

Importantly, in the experiments of Aim 1, regardless of age, there was no difference in severity of LIDs in parkinsonian grafted and sham-grafted rats. This is counter to clinical observations that suggest young patients have a greater risk for the development of dyskinesias. These data should be interpreted somewhat cautiously however, as it is important to remember that increased dyskinesia susceptibility is not only associated with young age, but also with increased disease severity. In the controlled laboratory environment, despite differences in ages between treatment groups, severity of the lesion is controlled, and is a nearly complete ablation of endogenous dopamine. Thus, lack of differential severity in LID development may be explained by the fact that regardless of age, rats are rendered equally parkinsonian. Despite this potential confounder (or explanation), an age-dependence in graft efficacy
remains. Thus, despite equal DA-depletion, and equal dyskinesia severity, young rats are much more amenable to therapeutic intervention with transplantation. The question of age versus disease severity could be addressed in a future experiment comparing graft efficacy between age groups of animals with partial nigrostriatal lesion, and is suggested in the Future Directions.

**Factors affecting graft survivability in the aged host**

Two additional issues regarding diminishing graft efficacy with age are addressed by the experiments in Aim 1. First, previous data suggest reduced survivability of DA grafts in aged hosts (Collier et al., 1999). Secondly, grafting into the aged striatum is associated with reduced functional improvement in the clinics (Freed et al., 2001). While reduced functional improvement may indeed accompany diminished transplant survival, it is imperative to evaluate both issues independently since transplant survivability is not by any means in a direct linear relationship with therapeutic efficacy. This is evidenced by abundant cell survival accompanied by lesser functional efficacy in the aged subjects of Aim 1.

Clinical trials have revealed that two of the most significant factors linked to graft efficacy are the survival of DA neurons in the transplant and the extent of graft-mediated reinnervation in the host striatum (reviewed by Laguna Goya, 2007). Experimental data further support these findings as the level of DA cell survival has been positively correlated with their ability to reduce motor abnormalities in the grafted parkinsonian rat (Brecknell et al., 1996). The role of the host environment in supporting
transplanted cell survival is clear from in vitro studies in which striatal tissue extracts significantly enhance ventral mesencephalic DA neuron survival and neurite extension (Nakajima et al., 2001). Survival factors supplied by the striatum likely involve both BDNF (Zhou et al., 2000) and GDNF, which is elevated in the 6-OHDA lesioned striatum (Nakajima et al., 2001). However, expression of BDNF and its receptor, trkB, are reduced with age throughout the brain (Croll et al., 1998). The aged brain presents multiple additional challenges to graft survival including decreased compensatory mechanisms, decreased trophic factor support, and decreased cell-cell contacts, each of which are addressed in the following paragraphs.

**Decreased compensatory mechanisms in the aged brain**

With 6-OHDA depletion, no significant age effects were detected for BDNF or NT-3 protein expression in the striatum (Yurek & Fletcher-Turner, 2001). However, compared to old rats, young rats showed higher GDNF protein levels in both the striatum and ventral midbrain (Yurek & Fletcher-Turner, 2001). These data show that two endogenous neurotrophic factors, BDNF and GDNF, are differentially affected by a 6-OHDA lesion in the aging nigrostriatal system with young brain showing a significant compensatory increase of these two factors in the denervated striatum while no compensatory increase is observed in aged brain (Yurek & Fletcher-Turner, 2001). As discussed in the introduction, studies in young and aged monkeys by Kanaan et al. also demonstrate dramatic loss of nigrostriatal biochemical compensatory mechanisms in aged individuals (Kanaan et al., 2008). Prior studies in the same animals by Collier et
(Collier et al., 2005) demonstrated that while young animals respond to DA depletion by increasing striatal trophic factors, aged animals were incapable of increasing striatal trophic activity in response to DA depletion, further supporting earlier studies by Yurek and colleagues (Yurek et al., 1996; Yurek & Fletcher-Turner, 2001). The observations that compensatory mechanisms were lost in aged subjects led to the hypothesis that loss of plasticity in advanced age leads to a reduced ability to recover from a lifetime of insults to the DA system that likely lead to parkinsonism (Kanaan et al., 2008).

**Decreased trophic support in the aged brain**

There is evidence of change in trophic factor expression with age. With 6-OHDA depletion, no significant age effects were detected for BDNF or NT-3 protein expression in the striatum (Yurek & Fletcher-Turner, 2001). However, compared to old rats, young rats showed higher GDNF protein levels in both the striatum and ventral midbrain (Yurek & Fletcher-Turner, 2001). These data show that two endogenous neurotrophic factors, BDNF and GDNF, are differentially affected by a 6-OHDA lesion in the aging nigrostriatal system with young brain showing a significant compensatory increase of these two factors in the denervated striatum while no compensatory increase is observed in aged brain (Yurek & Fletcher-Turner, 2001).

In addition to such changes in trophic factor expression with age and DA-depletion, the withdrawal of trophic factors has been identified as one factor underlying the decreased survival of grafted cells (Sortwell, 2003). In fact, trophic factor withdrawal has been shown to be involved in the initial phase of cell death post-grafting (Collier,
An increase in both number and functional capacity of TH grafted neurons up to 30-35% (Sortwell, 2003) has been shown in response to trophic factor supplementation with various trophic factors, including BDNF, NT3, GDNF, aFGF, and bFGF (Giacobini et al., 1993; Rosenblad et al., 1996; Yurek et al., 1996; Granholm et al., 1997; Timmer et al., 2004). In addition to the presence of trophic factor support, the density of grafted cell also impacts grafted neuron survival. This was first demonstrated by Nikkah and colleagues who showed that decreasing graft volume, while maintaining constant concentration of grafted cells resulted in increased survival rates of TH-ir grafted neurons (Nikkah et al., 1994a; Nikkhah et al., 1994b), likely related to a decrease in necrosis of grafted tissue, which is not vascularized until several weeks post-transplantation. In further support of the role of grafted neuron density influencing grafted neuron survival, recent studies by Terpstra et al. demonstrated that increasing the concentration of embryonic VM grafted neuron numbers led to both increased grafted neuron survival rate and soma size (Terpstra et al., 2007). Based on previous data from cell culture studies that suggested that increasing cell density results in the release of survival factors into the media (Fujita et al., 2001), Terpstra and colleagues hypothesize that increased survival associated with increased concentration of grafted neurons is likely due to increased trophic factors such as GDNF, GDF5, PDGF, and BDNF (Terpstra et al., 2007), all of which have been associated individually with increased cell survival in grafting paradigms as previously discussed (Nikkah et al., 1993b; Smits et al., 1993; Rosenblad et al., 1996; Yurek et al., 1996; Granholm et al., 1997; Sautter et al., 1998a; Sautter et al., 1998b; Sautter et al., 1998c; Sortwell et al., 1998; Collier et al., 1999; Rosenblad et al., 1999). In light of
this data, successful grafting into the aged striatum requires compensation for reduced trophic factor support. In Aim 1, the grafted neuron density was highest in aged rat, which received the largest number of cells compared to younger rats. This increased density may in part explain the increased neuron survival observed in the aged grafted rats. Further, it is likely that increased trophic factors accompany such dense grafts of high neuronal number, which may also contribute to the reduction in LID severity observed in the aged grafted rats.

**Alteration in cell-cell and extracellular matrix contacts in the aged brain**

In addition to altered compensatory mechanisms and trophic factor expression, the aging brain also experiences alterations in cell-cell contacts and extracellular matrix (ECM) contacts, critical for the maintenance of cell signaling and interaction. Such changes are most likely reflected by age-related gliosis which often accompanies neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases. In fact, age-related gliosis is thought to be in part due to age-related changes in cell-cell and ECM proteins and contacts between glia and neurons (Labourdette, 2002). In addition to trophic factors, such cell-cell and ECM contacts also impact grafted neuron survival. Specifically, the loss of cell-cell contacts and ECM contacts has been associated with low survival of transplanted cells (Raff et al., 1992; Meredith & Wouterlood, 1993). Further, supplementation of grafted neurons with the ECM glycoprotein tenascin-C, which is endogenously secreted by astrocytes (Meiners et al., 1995; Mahler et al., 1996), increases TH neuron survival in both cell culture (Marchionini et al., 2003) and in
low-density grafts. Such data, combined with their observation that higher neuron density leads to increased grafted neuron survival, led Terpstra and colleagues to hypothesize that increases in grafted cell survival resulting from increased density of grafted cells may also be explained by accompanied increased in cell-cell and ECM contacts (Terpstra et al., 2007). Thus, one may further extrapolate this hypothesis in application to the data presented in Aim 1, suggesting that in addition to potential changes in trophic factor concentration and restoration of compensatory mechanisms, implantation of dense TH-ir neuron grafts in the aged striatum may also involve increases in cell-cell contact molecules and ECM factors, thus contributing to the enhanced survivability of grafted neurons in the aged host observed in these studies.

The results from Aim 1 addressed both reduced survivability of DA grafts in aged hosts and reduced functionality of DA grafts in aged hosts, suggesting that reduced survivability in the aged host can indeed be overcome by introduction of greater numbers of embryonic cells, resulting in larger grafts in aged hosts compared to younger counterparts receiving smaller grafts. Despite this marked degree of transplanted cell survival in the aged host, improvement in LIDs is delayed and overall inferior on a per cell basis when compared to younger grafted animals. The key to the ability of the aged host to support such a large number of grafted cells was, I hypothesize, due to the fact that the grafts were embryonic, thus endowing the cells with a microenvironment rich in gestational growth factors (Sauer et al., 1993; Haque et al., 1994; Haque et al., 1995; Wang et al., 1996; Sable et al., 1997; Sautter et al., 1998b; Collier et al., 1999; Chaturvedi et al., 2006; Jensen et al., 2008), and potentially
“exogenously” providing the aged host restored compensatory mechanisms in place of those lost with age and disease.

The results from Aim 1 are in line with the Kanaan-Collier Cycle Acceleration Hypothesis of PD. According to this model, the aged rat has accumulated/experienced a greater degree of insults than his younger counterpart, and thus is closer to reaching the threshold for degree of DA-depletion that would render it parkinsonian. It follows, when comparing young to aged subjects, that in the aged rat, when presented with the additional challenge of DA-depletion, the animal exhibits reduced compensatory mechanisms, and is less able to adapt to therapeutic intervention with grafting and levodopa. This would be reflected, as in the results of Aim 1, by inferior graft efficacy and therapeutic response.

Of interest, recent data in our lab have confirmed that maintenance of spine integrity in the grafted striatum leads to lesser severity of dyskinesias than in those grafted parkinsonian rats with depleted spine density (Soderstrom et al 2009, in press). As spine density is reduced with aging, it follows that preservation of 6-OHDA mediated DA-depletion in the aged rat, may slow progression towards the PD threshold, and provide a more effective host environment in which to introduce grafted cells.
5.4 *Thoughts on how spine preservation might reduce levodopa-induced dyskinesias*

The majority of striatal neurons are GABAergic medium spiny neurons (MSNs) whose numerous dendritic spines serve as the primary postsynaptic targets of both ascending afferent input from nigral dopaminergic neurons and descending excitatory cortical glutamatergic inputs in the adult brain (McKinney, 2005). Such striatal postsynaptic interactions between dopamine and glutamate are critical for normal motor behavior and are disrupted in the parkinsonian brain. Further, dendritic spines are known to change shape, even appearing and disappearing entirely, and are classically associated with neuroplasticity, such as long-term potentiation and accompanying growth of new spines (e.g. (Carlisle & Kennedy, 2005). In the striatum, synaptic plasticity is thought to involve the “writing” and “rewriting” of motor memories, a process that in this region involves the specific interactions of cortical glutamate and nigral dopamine on dendritic spines of MSNs. Accumulating evidence suggests that aberrant striatal plasticity is important in the development of levodopa-induced dyskinesias in parkinsonian subjects (Centonze *et al.*, 1999; Calabresi *et al.*, 2000b; Picconi *et al.*, 2003; Picconi *et al.*, 2005). While it is clear that severity of dopamine denervation plays an important role in the development of LIDs (Mones *et al.*, 1971; Langston & Ballard, 1984; Caligiuri & Peterson, 1993; Fahn, 2000), it remains unknown how loss of dendritic spines secondary to loss of dopamine impacts glutamate-dependent synaptic plasticity, or whether this contributes to LID development. From the studies in Aim 2, it appears that preservation of spine density does reduce dyskinesia severity, however the mechanism by which this occurs remains unclear.
Altered morphology of dendritic spines on MSNs in the parkinsonian striatum is one manner in which synaptic plasticity may be involved in LIDs. Indeed, it is well documented in postmortem parkinsonian brains that there is significant atrophy of dendritic spines with advanced disease (McNeill et al., 1988; Stephens et al., 2005; Zaja-Milatovic et al., 2005). Specifically, MSNs from parkinsonian brains showed a significant reduction in the length of the dendrites compared to age-matched controls, and the remaining dendrites often show few-to-no spines (McNeill et al., 1988). Alterations in MSNs, particularly loss of dendritic spines, would be anticipated to result in aberrant postsynaptic interactions between striatal dopamine and glutamate (Calabresi et al., 1993b). Loss of MSN dendritic spines is observed in rodents with severe dopamine depletion (Day et al., 2006) and is now known to occur via dysregulation of intraspine Cav1.3 L-type Ca\textsuperscript{2+} channels, which leads to striatopallidal dendritic spine loss following striatal dopamine depletion (Day et al., 2006). Administration of the calcium channel antagonist nimodipine to rats, or the absence of Cav1.3 calcium channels in transgenic mice prevents spine loss on striatopallidal neurons despite severe dopamine depletion (Day et al., 2006). This model enabled us to test the hypothesis that aberrant spine morphology associated with the parkinsonian striatum contributes to LID development as in Aim 2 of this dissertation.

Despite our understanding that spine loss involves dysregulation of intraspine Cav1.3 L-type Ca\textsuperscript{2+} channels, the precise mechanism by which DA depletion influences synaptic plasticity, and the degree to which alterations in spine density and morphology contribute to parkinsonian motor deficits and LIDs remains unclear (Day et al., 2006). Answering such questions will likely significantly impact a variety of therapeutic
approaches for PD. Severely altered neuronal morphology, such as that observed in striatal MSNs in advanced PD could translate into: 1) motor abnormalities associated with parkinsonian subjects, and 2) aberrant plasticity manifest as dyskinetic behavior and/or additional alteration of spine morphology. Aberrant spine morphology could also contribute to suboptimal efficacy of DA-replacement therapies. Thus, understanding the role of dendritic spine morphology in the development of parkinsonian motor behaviors is a critical step towards improved patient care.

While it appears that spine loss is not the only factor underlying expression of parkinsonian-like motor deficits seen in this rat model, the data from Aim 2 do suggest that maintaining dendritic spine density following severe DA-depletion improves motor behaviors in response to DA therapy, such as levodopa-induced rotational asymmetry and LIDs. Thus, one can hypothesize that intact spine morphology is necessary, but not sufficient for effective recovery of the parkinsonian striatum. Current data suggests that spine preservation may also impact regulation of downstream signaling factors related to DA-depletion and LIDs, such as FosB (Andersson et al., 1999; Westin et al., 2001). In Figure 4.1.15, it appears that while FosB is upregulated with DA-depletion regardless of spine status, further increases in FosB expression (as typically observed with development of LIDs) are absent in those rats with preserved spine density. The FosB data reinforces the importance of not simply intact morphology, but also intact synaptic connectivity in the quest for improving striatal signaling in the parkinsonian striatum. While it is clear that maintaining spine density is not sufficient to prevent LIDs, it does appear that maintaining intact striatal morphology is one critical factor in preventing the presentation of dyskinesias and correcting aberrant signaling associated
with PD symptoms and avoidance of treatment-related dyskinesias (modeled in Figure 4.2.16). Additional plasticity factors such as alterations in electrical, chemical, or downstream signaling pathways likely contribute to the presentation of dyskinesias and correction of aberrant signaling on multiple levels is necessary for adequate control of PD symptoms and avoidance of treatment-related dyskinesias (modeled in Figure 4.2.16).

**A model to explain the role of dendritic spines in dyskinesia development**

To summarize the influence of dendritic spine loss on parkinsonian behavior, I am proposing four hypothetical scenarios modeling the morphological changes occurring on the dendrites of MSNs in the parkinsonian striatum in the presence or absence of spine preservation (Figure 5.4.1). I hypothesize that the progressive alteration of inputs accompanying both age-related and disease-mediated spine loss is further exacerbated by associated disorganization of synaptic inputs at these critical modulatory sites. As described in the introduction, dendritic spines of MSNs are the primary site of afferent input for nigral DA neurons ascending from substantia nigra, and glutamate input from the cortex, and thalamus (Smith et al., 1994; Ingham et al., 1998). Nigral DA neurons almost exclusively synapse onto the shaft of dendritic spines of MSNs, while glutamate afferents synapse specifically onto the heads of the same spines (Smith et al., 1994; Ingham et al., 1998) (depicted in Scenario #1 of Figure 5.4.1). Alterations in MSNs, particularly loss of dendritic spines, would be anticipated to result in aberrant postsynaptic interactions between DA and glutamate in the striatum.
Indeed postsynaptic interactions between DA and glutamate, which are critical for normal motor behavior, are known to be disrupted in the parkinsonian brain (Calabresi et al., 1993b). Further, in the parkinsonian striatum, glutamate modulation by DA is diminished and hyperactivity of glutamatergic synapses is observed (Lindefors and Ungerstedt 1990; Yamamoto and Davy 1992; Ingham et al., 1993; Meshul et al., 1999). Loss of striatal DA leads to dysregulation of corticostriatal transmission (Picconi 2005, Calabresi 2007, Surmeier 2007), and underlies the emergence of aberrant striatal LTP and LTD in the parkinsonian brain. Thus, in addition to ascending nigral dopaminergic signaling pathways, changes in corticostriatal glutamatergic signaling also likely contribute to LIDs. The role of the glutamate, and glutamatergic receptors such as the NMDA receptor in LIDs is further supported by clinical data that shows the glutamate antagonist amantadine can suppress dyskinesias in humans and MPTP primates (Blanchet 1998, Blanchet 2003, Del et al 2001, Verhagen et al 1998, Blanchet et al 1999, Hadj 2004, Morissette 2006, Morissette 2006, Nash et al 2000, Nash et al 2004, Papa and Chase 1996, Verhagen and Chase 1998). Such alterations in cortical glutamatergic striatal inputs combined with progressive loss of striatal DA from the SN supports my hypothesis that in early PD, in the presence of spine preservation, dopaminergic input at the neck of the spine is diminished, however the presence of intact spines maintains intact glutamatergic synapses at the heads of spines (depicted in Scenario #2 of Figure 5.4.1). Further, in late-stage PD when spine density is maintained, there is further reduction of DA input and potentially aberrant glutamatergic input at the spine neck in addition to its normal location at the spine head, however normal cytoarchitecture of glutamatergic input remains largely intact (depicted in
Scenario #3 of Figure 5.4.1). In the parkinsonian striatum where spine integrity is not maintained, marked loss of spines results in aberrant glutamatergic connectivity, with glutamatergic synapse formation along the dendritic shaft, likely leading to unopposed hyperactivity of glutamatergic synapses along striatal MSNs (depicted in Scenario #4 of Figure 5.4.1). Such dysregulation of glutamatergic input combined with marked reduction or absence of endogenous dopamine is one potential explanation for the elevated levels of dyskinesias observed in parkinsonian rats with diminished dendritic spine density compared to parkinsonian rats with preserved spine density as demonstrated in Aim 2 of this dissertation.
**Figure 5.4.1:** Dopamine and glutamate interaction at the dendritic spines of MSNs. Normal arrangement of dopaminergic and glutamatergic inputs at the dendritic spine in the intact striatum (Scenario #1). Potential scenarios for synaptic architecture in the parkinsonian striatum during early (Scenario #2), or late (Scenario #3) post-lesion status in the presence of nimodipine-mediated spine preservation, or with spine depletion (Scenario #4). Checkered arrowheads depict DA input at the DA receptor (red), small black dots represent glutamatergic inputs at glutamate receptors (white). Black polygons in scenario #3 depict aberrant sites of glutamatergic input in the presence of spine-preservation despite DA-depletion.
5.5 The role of calcium signaling in the parkinsonian striatum; modeling an explanation for LIDs and reduced graft efficacy

It is now understood that multiple factors underly the etiology of both PD and LIDs, and that alterations in neuronal plasticity are clear contributors to both processes. The studies in this dissertation have examined how two such factors, the age of a subject at therapeutic intervention (Aim 1), and striatal morphological integrity (Aim 2) influence the efficacy of two important forms of PD therapy, transplantation, and levodopa.

Nigral neurons are unique pacemakers

The specific loss of nigral DA neurons inherent to PD, may actually be due to unique features of the cells themselves, leading to their position as the primary target of neuronal degeneration in this disease. Nigral neurons are unique in that, unlike many other neurons, they are autonomously active. Thus, these neurons have been compared to cardiac pacemaker cells, as they generate regular, rhythmic action potentials in the absence of synaptic input (Tepper et al., 1998). Further, the autonomously activated action potentials of nigral DA neurons are calcium based, and dependent upon L-type Ca^{2+} channels, whereas classical action potentials in other neurons are Na^{+} based (Ping & Shepard, 1996; Grobaski et al., 1997; Bonci et al., 1998; Ping & Shepard, 1999; Puopolo et al., 2007).
Ca^{2+} homeostasis is metabolically expensive for the autonomous pacemaker

The Ca^{2+} channels involved in the pacemaking activity of nigral neurons are comprised of the Cav1.3 subunit, which is different from the Cav1.2 subunit found in cardiac pacemakers (Chan et al., 2007). A steep concentration gradient of Ca^{2+} is maintained by neurons such that Ca^{2+} flows easily into neurons, but requires active ATP-dependent export out of the cell. Usually, as in neurons with sodium based action potentials, the calcium flux across the cell membrane is comparatively minimal, and the gradient is easily maintained in this manner. However, in neurons with calcium based action potential, such as the nigral DA neurons, the burden of Ca^{2+} flux is much greater (Wilson & Callaway, 2000). Thus, this energy expensive process constitutes a significant metabolic burden for these cells (Surmeier, 2007). To accommodate such large influxes of calcium, calcium entering neurons is sequestered by the mitochondria or endoplasmic reticulum. The process of capturing, storing, and exporting calcium from the cell requires consumption of ATP in both organelles, and places high demands on oxidative phosphorylation by mitochondria (Berridge, 1998; Rizzuto & Pozzan, 2006). The constant calcium buffering and transport out of nigral neurons is thus a highly energy consuming process, thought by some to explain the acceperate aging of SN dopamine neurons (Surmeier, 2007).

Calcium, mitochondria, and aging

The mitochondria have been described as the cellular “biologic clock,” as the production of free radicals and reactive oxygen species from oxidative phosphorylation...
in the mitochondria leads to accumulated mitochondrial DNA damage, and it is this damage that is hypothesized to underlie aging (Harman, 1972; Wallace, 2005). Thus, the rate of mitochondrial damage parallels the rate of cellular metabolism, and that particular cell’s rate of aging (Surmeier, 2007). It follows that cells with a higher metabolic rate would age faster, and indeed this is found to be the case in the substantia nigra, where loss of highly metabolically active dopamine neurons occurs at a faster rate than in other neurons in the brain (Stark & Pakkenberg, 2004). Further, nigral neurons have elevated levels of mitochondrial DNA mutations and reduced complex I function (Swerdlow et al., 1996; Bender et al., 2006; Kraytsberg et al., 2006).

*The potential role of nigral Ca\(^{2+}\) in PD and the concept of ‘Rejuvenation’*

High metabolic demands experienced by the nigral cell by nature of its calcium-dependent autonomous pace-making may explain the increased rate of cellular dysfunction with age. Even greater metabolic activity in PD supports the hypothesis that PD is an example of accelerated aging as proposed by Kanaan and colleagues (Kanaan et al., 2008). *It follows then that in the aged transplant recipients of Aim 1, the reduced efficacy of grafted cells may be explained, at least in part, by an extended duration of Ca\(^{2+}\)-based metabolic activity in the aged nigra.*

If PD is in fact a direct consequence of accelerated aging of nigral DA neurons, due to Ca\(^{2+}\)-mediated mechanisms, inhibition or reduction of Ca\(^{2+}\) dependence may delay disease onset or progression (Surmeier, 2007). There is evidence of altered Ca\(^{2+}\) dependence of nigral neurons in developing mice. In young neurons, autonomous
activity is regulated by $\text{Na}^+$ dependent channels, rather than $\text{Ca}^{2+}$ dependent channels. In nigral neurons this mechanism switches to $\text{Ca}^{2+}$ dependence in adulthood, but the mechanism of $\text{Na}^+$ dependent autonomy is also retained. Sustained block of the L-type Cav1.3 $\text{Ca}^{2+}$ channels in adult mice re-activates the latent $\text{Na}^+$ dependent autonomous pacemaking activity, a phenomenon referred to in the literature as “rejuvenation” (Paladini et al., 2003).

The concept of “rejuvenation” provides further support for the potential beneficial effect of calcium channel blockers in PD. While there are multiple classes of calcium channels used in pharmacotherapy as antihypertensives (Eisenberg et al., 2004), only the dihydropyridines are effective antagonists at the Cav1.3 channels, which are specifically involved in the $\text{Ca}^{2+}$ mediated autonomous pacemaking of nigral DA neurons. It is this class of drugs that were used to “rejuvenate” nigral pacemaking from a $\text{Ca}^{2+}$ dependent to $\text{Na}^{2+}$ dependent process in mice (Paladini et al., 2003). Retrospective studies of calcium channel blocker use and incidence of PD found no correlation with drugs such as verapamil, or diltiazem, which are block calcium at other channels (Ton et al., 2007). However, retrospective study of the use of dihydropyridines (such as nimodipine and isradipine) did report reduced incidence of PD in patients treated with these drugs for hypertension (Rodnitzky, 1999).

In Aim 2, we have demonstrated that use of the dihydropyridine Cav1.3 antagonist nimodipine results in preservation of spine loss in response to DA-denervation of the SN. Further, preventing such spine loss reduces severity of LIDs and prevents upregulation of FosB in response to levodopa treatment. Thus, it is plausible that in addition to morphological protection (i.e. preservation of spine density),
this regimen involving nimodipine may also result in altered autonomous activity of remaining nigral DA neurons. Further, it follows that as DA neurons are lost with age, (and from the observations in this dissertation that suggest anti-parkinsonian therapeutic benefit may be minimized with age and/or disease progression), such intervention may pose greater benefit at younger rather than older ages.

The aged striatum demonstrates reduced plasticity and responsivity as well as reduced density of dendritic spines. Further, transplantation into the aged brain is less efficacious than in younger brains. The question of the capacity to “rejuvenate” the aging parkinsonian brain, whether through alterations of cell autonomy, restoration of striatal DA through transplantation, maintenance of spine density and morphology, or changes in other intracellular pathways is critical to parkinsonian patients.

Figure 5.5.1 proposes a model of transplantation efficacy in an environment of striatal spine preservation in the aged parkinsonian subject based on my hypothesis that preservation of spine density in the aged parkinsonian rat will contribute to greater graft efficacy, requiring fewer grafted cells and yielding more rapid reduction in LID severity in the aged host. In this model, the most effective disease modulation strategy is the combination of both early intervention and spine preservation (Figure 5.5.1 A and 5.5.1 C). Further, preservation of spine loss in aged grafted rats is hypothesized to both (1) reduce the number of grafted cells necessary for reduction of LID severity in the aged parkinsonian rat, and (2) improve the rate of LID reduction in response to transplantation, such that it more closely approximates that seen in younger grafted rats (depicted in 5.5.1A by the hypothetical green line and in 5.5.1.C by blue arrow showing
shift of curve depicting LID development in aged subjects towards timecourse of LID development in young subjects when grafted in the presence of spine preservation).

As PD diagnostic techniques become further refined and therapeutic options are optimized, the legitimacy of early disease intervention and modulation is becoming increasingly more realistic. Age remains the greatest risk factor for PD, and slowing the accelerated aging associated with the disease whether through pharmacological, surgical, or combined techniques, will likely offer the most promising therapeutic option for patients.
The influence of age and calcium channel blockade on disease progression
B

Improvement of post-graft LIDs is delayed in the aged host

C

Improvement of post-graft LIDs is delayed in the aged host
**Figure 5.5.1: The role of age and calcium channel modulation in progression of PD in the 6-OHDA rat model.** (A) Preservation of dendritic spine density in the parkinsonian striatum may delay the course of disease progression as indicated by the rightward shifted green line. Notice the difference in hypothetical degree of disease severity between subjects with intact spine density (green line) versus those without (red line) at both early (corresponding to younger age or earlier in disease progression) and late (corresponding to older age or later in disease progression). In this model, the most effective disease modulation strategy is the combination of both early intervention and spine preservation.

(B) The role of age and calcium channel modulation in development of LIDs in the 6-OHDA rat model. In this figure, the X-axis denotes Time, the Y-axis denotes severity of post-graft LIDs, and the Z-axis denotes differences in number of surviving grafted cells. Reduction in LIDs with grafting is delayed in aged hosts versus young or middle aged grafted rats, model based on results from Aim 1. At the early post-graft evaluation timepoint, younger graft recipients showed greater improvement in LID severity than did aged graft recipients, despite greater numbers of grafted DA neurons in the aged cohort. By the late post-graft evaluation timepoint, grafting reduces LID severity (y-axis) to the same level across ages. (C) Preservation of spine loss in aged grafted rats is hypothesized to both reduce the number of grafted cells necessary for reduction in LID severity, and improve the rate of LID reduction, such that it more closely approximates that seen in younger grafted rats. This is reflected by the green dotted line, which shifted towards the curve for young grafted rats, requires fewer grafted cells, and less time to reach the same degree of graft efficacy.
5.6 Future directions

The model proposed in Figure 5.5.1 suggests the next logical step in further understanding the roles of both spine preservation and subject age is to combine the use of nimodipine to preserve spine density in young and aged grafted rats. Characterizing the outcome of this combined intervention may reveal further steps in optimizing transplant efficacy and reducing LID severity.

There are a number of additional questions that remain unanswered and are relevant to the studies reported in this dissertation. For instance, changes in both DA and glutamate receptor localization in the environment of spine loss and spine preservation will provide valuable insight regarding the endogenous compensatory activities of striatal MSNs in response to morphological changes at the ultrastructural level.

Our lab has observed levodopa-induced alterations in spine number and morphology, although these changes remain to be quantified. Such morphological alterations results from levodopa pharmacotherapy are hypothesized to be a contributory factor in the development of dyskinesias, akin to changes in spine density observed with amphetamine treatment.

Assessment of dendritic spine loss resulting from DA-depletion, and nimodipine-induced prevention of spine density remains to be verified in inbred strains of rats, such as the Fischer 344 rat. Confirmation of this model will enable use of Fischer rats, which eliminate the potential confounding variable of genetic variability inherent to outbred strains such as Sprague-Dawley rats, as used in the experiments in Aim 2. Additionally,
quantification of spine loss in Lewis rats, which are resistant to development of LIDs, will answer whether there are strain-dependent differences in 6-OHDA mediated spine loss that correlate with strain-dependent differences in LID susceptibility.

Detailed characterization of intracellular signaling pathways underlying dyskinesia development, such as quantification of shifts in phosphorylation states of DARPP-32, and correlation of these changes with spine status is another important step in understanding associated downstream changes in neuronal communication.

Finally, assessing the effects of spine preservation in a partial lesion model of PD is necessary to begin detailed assessment the therapeutic capacity of spine preservation in a clinical setting.
In response to the question, “Which single medical advance would benefit most people?”

The discovery of a pill that would stop people talking nonsense, without necessarily killing them.

Dr. Oleh Hornykiewicz; one of the “fathers of dopamine research” who discovered underlying DA-deficiency in parkinsonian patients, and restoration of motor behaviors with levodopa.


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