THE ROLE OF INTERHEMISPHERIC PATHWAYS
IN PARKINSON’S DISEASE AND DRUG-INDUCED DYSKINESIAS

A Dissertation in
Neuroscience

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2011
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ABSTRACT

Parkinson’s disease (PD) is the second most common neurodegenerative disorder that is characterized by cell death of the nigrostriatal pathway and loss of dopamine. Although current pharmacological therapies such as levodopa (LD) can significantly ameliorate symptoms in early stages of the disease, patients develop drug-induced motor complications in later stages. One such complication is the emergence of abnormal involuntary movements known as drug-induced dyskinesias (DID), which are disabling and limit effectiveness of anti-PD treatments. Despite significant advances in the field for understanding the mechanisms associated with DID, treatment for DID in PD is largely unsatisfactory for many patients. Advancing our understanding of the pathophysiological basis of DID will allow for better development of novel therapies that reduce or prevent DID.

The goals of this dissertation research were: 1) to examine novel experimental therapeutics that do not cause DID in animal models of PD; 2) to examine putative mechanisms through which such therapies exert their effects; 3) to examine the role of interhemispheric pathways in the genesis of DID.

In experiment 1, we evaluated if the Ayurvedic medication *Mucuna pruriens* would reduce DID in the rat and primate models of PD. Our results showed that a water formulation of *Mucuna pruriens* was highly effective in ameliorating parkinsonian deficits with reduced severity of DID. We also used a novel D₁ agonist EFF0311 in both the parkinsonian rat and monkey to show that EFF0311 can significantly decrease parkinsonism and reduce the risk of DID. These two experiments support the use of novel pharmacological agents to mitigate the problem of DID. In experiment 2, we showed that seventeen clinically hemiparkinsonian rhesus monkeys exposed to high doses of LD did not develop DID, and that such monkeys had profound unilateral loss of nigrostriatal neurons (90%). In experiment 3, we explored the putative mechanisms for the
resistance of hemiparkinsonian monkeys to DID using single-cell and local field potential electrophysiology in two hemiparkinsonian Rhesus monkeys. We show that chronic intermittent LD did not substantially alter firing rate or patterns in the subthalamic nucleus (a downstream nucleus critically implicated in parkinsonism and in genesis of DID). In experiment 4, we used a variety of tracing techniques (retrograde labeling and optogenetic viral vector systems) to label interhemispheric nigrostriatal connections in normal, partial and completely nigrostriatal lesioned hemiparkinsonian rats. We showed that LD administration to completely lesioned rats (the Ungerstedt model) and severely partial lesioned rats (the Winkler model) caused DID. Histological analysis showed loss of interhemispheric nigrostriatal fibers in these dyskinetic animals. Whereas, the partial striatal lesioned model (Sauer and Oertel model) did not develop DID and retained interhemispheric nigrostriatal fibers. In conclusion, these experiments provide novel insights into the pathophysiological mechanisms that underly DID. Furthermore, this series of experiments show that interhemispheric pathways may play a significant role in the genesis of DID. These findings could potentially lead to improved development of novel therapies that can reduce or prevent DID in patients suffering from PD.
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<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AIMS</td>
<td>Abnormal Involuntary Movements Scale</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-Posterior</td>
</tr>
<tr>
<td>BZ</td>
<td>Benserazide</td>
</tr>
<tr>
<td>CD</td>
<td>Carbidopa</td>
</tr>
<tr>
<td>CTX</td>
<td>Cortex</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>Dopamine- and cyclic AMP-regulated phosphoprotein of 32 kDa</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
</tr>
<tr>
<td>DDCI</td>
<td>Dopa-Decarboxylase Inhibitor</td>
</tr>
<tr>
<td>DID</td>
<td>Drug-Induced Dyskinesias</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsal-Ventral</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
</tr>
<tr>
<td>FMT</td>
<td>Fine Motor Task</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic Acid Decarboxylase</td>
</tr>
<tr>
<td>GID</td>
<td>Graft-Induced Dyskinesias</td>
</tr>
<tr>
<td>GPE</td>
<td>Globus Pallidus Externa</td>
</tr>
<tr>
<td>GPI</td>
<td>Globus Pallidus Interna</td>
</tr>
<tr>
<td>HP</td>
<td>Hemiparkinsonian</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-Radish Peroxidase</td>
</tr>
<tr>
<td>ICA</td>
<td>Intracarotid</td>
</tr>
<tr>
<td>LD</td>
<td>Levodopa</td>
</tr>
<tr>
<td>LFP</td>
<td>Local Field Potentials</td>
</tr>
<tr>
<td>LID</td>
<td>Levodopa-Induced Dyskinesias</td>
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<tr>
<td>M-STR</td>
<td>Multiple Striatal Injection of 6-OHDA Group</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial Forebrain Bundle</td>
</tr>
<tr>
<td>ML</td>
<td>Medial-Lateral</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium-Spiny Neurons</td>
</tr>
<tr>
<td>MPE</td>
<td>Water Extract of <em>Mucuna pruriens</em></td>
</tr>
<tr>
<td>MPEP</td>
<td><em>Mucuna pruriens</em> endocarp powder</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mUPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale Modified for Primates</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>OHP</td>
<td>Overlesioned Hemiparkinsonian</td>
</tr>
<tr>
<td>P-ERK</td>
<td>Phosphorylation of ERK</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PPN</td>
<td>Pedunculopontine Nucleus</td>
</tr>
<tr>
<td>PSD</td>
<td>Power Spectral Density</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelial</td>
</tr>
<tr>
<td>S-STR</td>
<td>Single Striatal Injection of 6-OHDA Group</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia Nigra</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia Nigra Pars Compacta</td>
</tr>
<tr>
<td>SNR</td>
<td>Substantia Nigra Pars Reticulata</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>STA</td>
<td>Spike-Triggered Average</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic Nucleus</td>
</tr>
<tr>
<td>STR</td>
<td>Striatum</td>
</tr>
<tr>
<td>TB</td>
<td>True Blue</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>THAL</td>
<td>Thalamus</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>VIM</td>
<td>Ventral Intermedius Nucleus of Thalamus</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would first like to thank Thyagarajan Subramanian, M.D. for his mentoring guidance and support during my doctoral dissertation. I would like to thank my doctoral committee, Kent Vrana, Ph.D., James Connor, Ph.D., Paul Eslinger, Ph.D. and Kathy Steece-Collier, Ph.D. for their valuable input and expertise. I would like to acknowledge the efforts of Thomas Wichmann, M.D., Thomas Pritchard, Ph.D., Milind Deogaonkar, M.D. and Erin Gilbert with primate experiments; Dhimant Desai, Ph.D. and Bala Manyam, M.D. for *Mucuna pruriens* experiments; Karl Deisseroth, M.D., Ph.D. and Charu Ramakrishnan, Ph.D. for optogenetic experiments; Richard Mailman, Ph.D and Vishu Murthy, Ph.D. for EFF0311 experiments. I would like to thank my fellow labmates, Kala Venkiteswaran, Ph.D., Tim Gilmour, M.S. and Donna Ford for their advice and support. I would like to thank Mark Nolt, Ph.D. for his expertise, advice and mentorship.

I would also like to thank my friends and family back home in California and in Hershey, Pennsylvania for their encouragement and love. Lastly, I would like to thank my family (Mom, Dad and Sister) and my girlfriend Cathy for all of their love and support. Without them, this would not have been possible.
Chapter 1

Pathophysiology of Parkinson’s Disease and Drug-Induced Dyskinesias

1 The following chapter represents a modified version of the chapter “Pathophysiology of Drug-Induced Dyskinesias” in the book Parkinson’s disease/Book 3 ISBN 978-953-307-464-1 edited by Abdul Qayyum Rana, MD,FRCPC. Authors: C Lieu, V Shivkumar, T Gilmour, K Venkiteswaran, M Nolt, M Deogaonkar, T Subramanian. CL and TS developed the concepts of the chapter. All authors contributed to writing the chapter and finalizing contents.
1.1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder that is characterized by cell death of the nigrostriatal pathway and loss of dopamine (DA). Although current pharmacological therapies such as levodopa (LD) can significantly ameliorate symptoms in early stages of the disease, patients develop drug-induced motor complications in later stages. One such complication is the emergence of abnormal involuntary movements known as drug-induced dyskinesias (DID), which are disabling and limit effectiveness of anti-PD treatments. The aim of this chapter is to introduce PD and the phenomenology of DID. This chapter will evaluate the current theories for DID, covering recent studies that identify the underlying pathophysiological changes on the biochemical/molecular (receptors, enzymes, and neurotransmitter systems), cellular (basal ganglia connections), electrophysiological (basal ganglia neuronal activity) and behavioral level that have been proposed to influence the development of DID. Since DID can be very disabling, understanding the factors that contribute to the onset of DID in PD will allow for the development of improved and novel treatment strategies that prevent or mitigate DID without diminution of anti-parkinsonian effects.

1.2. PD: Clinical Features And Pathophysiology

PD is an adult-onset neurological disease that causes disabling motor symptoms that include slowness of movement (bradykinesia), resting tremor, postural instability and increased muscle rigidity. It is the second most common neurodegenerative disease and affects > 1 million people in the United States. The mean age for onset is 55-60 years (Przedborski 2007) with most studies suggesting that a majority of patients have disease onset closer to age 60.

The main pathologic feature of PD is the chronic degeneration of dopaminergic neurons originating from the substantia nigra pars compacta (SNC) and terminating in the striatum, known as the nigrostriatal pathway, which leads to depletion of DA. Although the exact etiology of
neuronal cell death in PD is unclear, the putative cause of death of DA neurons is a combination of dysfunction in neuronal mitochondria, oxidative stress and inflammatory responses (Przedborski 2007). Motor symptoms of PD do not occur until there is significant loss of nigrostriatal neurons (60%) and DA (80%).

In addition to loss of nigrostriatal neurons, another pathological feature of PD is the presence of cytoplasmic, intraneuronal inclusions known as Lewy bodies. These inclusions are eosinophilic, spherical in shape and include aggregates of proteins (α-synuclein, parkin and ubiquitin) (Przedborski 2007).

The etiology of PD has been connected to both genetic susceptibility and environmental toxins. However, 90% of PD cases are idiopathic with no apparent genetic causation. There are well-known environmental neurotoxins, including rotenone, paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that can cause PD or increase the risk of developing PD.

Pharmacological treatment with LD (the precursor of DA) or other DA replacement therapies, such as synthetic DA agonists, are currently used to ameliorate parkinsonian symptoms. Among the currently available oral PD treatments, LD is still considered the gold-standard for PD treatment since no other treatment strategy is as effective at ameliorating PD symptoms. Although these treatments are successful at alleviating parkinsonism during the early stages of the disease (symptoms present unilaterally), most advanced PD patients (symptoms present bilaterally) develop drug-induced disabling, motor complications known as DID, requiring alterations to drug regimen or functional neurosurgical treatment. DID (or LD-induced dyskinesias – LID) are abnormal, excessive involuntary movements that occur with the chronic, daily administration of oral, pharmacological anti-parkinsonian medications. DID/LID are characterized by chorea, ballismus, flinging of arms, violent jerks or athetosis and dystonias (see descriptions in Table 1-1) (Fahn and Jankovic 2007). These disabling movements limit the effectiveness of pharmacological treatments, thus negating clinical benefits. Therefore, increased
understanding of the pathophysiological mechanisms of DID can greatly impact the way we treat dyskinesias in the clinic, resulting in better control of symptoms, increased quality of life and decreased financial burdens upon patients and their families.

<table>
<thead>
<tr>
<th>Movement</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Chorea</td>
<td>Nonrhythmic, rapid, purposeless, flow from one body part to another</td>
</tr>
<tr>
<td>Ballismus</td>
<td>Large-amplitude movements in limbs (flinging/flailing)</td>
</tr>
<tr>
<td>Athetosis</td>
<td>Writhing, continuous movements</td>
</tr>
<tr>
<td>Dystonia</td>
<td>Sustained, prolonged twisting/posturing</td>
</tr>
</tbody>
</table>

Table 1-1. Description of dyskinetic movements

1.3. Behavioral Characteristics of DID

DID in PD

Peak-dose dyskinesias are the most common form of DID and occur in 75% to 80% of the patients experiencing DID (Zesiewicz, Sullivan et al. 2007). The major risk factor has been considered to be severity of the disease. Other risk factors for DID include LD treatment duration, age of disease onset and initial LD dose (Grandas, Galiano et al. 1999). Peak-dose dyskinesias are due to a high dose of LD and represent an overdosed state. The plasma levels of LD are high and there is presumably excess striatal DA. Chorea is the most common form of involuntary movement in these cases. However, in later stages, dystonia can also occur. Chorea is more prominent in the head, trunk and upper limbs (Thanvi, Lo et al. 2007). Reducing the individual dose of anti-parkinsonian medication ameliorates the DID but can cause deterioration of parkinsonism. Hence these patients typically need more frequent dosing of anti-parkinsonian medications. Sustained-release LD formulations may decrease the duration of DID.
Diphasic DID develop when plasma levels are rising or falling but not with peak levels. These dyskinesias predominantly occur in the lower limbs and tend to be dystonic or choreiform. Treatment of diphasic DID is more difficult than peak-dose dyskinesias. The use of DA agonists with a longer duration of action and LD as supplementary drug is the most effective approach to treating diphasic DID (Bibbiani, Costantini et al. 2005; Zesiewicz, Sullivan et al. 2007).

Off-period dystonia occurs when the plasma levels of DA are low, particularly in the early morning. It can be precipitated by anxiety or attempts to walk. It is characterized by painful spasms of the foot on the more affected side. It is treated by preventing the “offs”. This can be achieved by use of DA agonists or sustained release LD formulations (Zesiewicz, Sullivan et al. 2007).

**Preclinical animal models of DID**

**Rodent models of DID**

Unilateral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) induces loss of nigrostriatal neurons (Ungerstedt and Arbuthnott 1970) in the rat. 6-OHDA also affects other neurotransmitter systems (e.g. noradrenergic and serotonergic systems). This causes extensive loss of nigrostriatal neurons unilaterally (>95% loss), and significant loss of DA in this pathway (known as the Ungerstedt model). This leads to a hemiparkinsonian (HP) rat model of PD that exhibits motor deficits contralateral to the lesion. Administration of pharmacological DA replacement therapies such as LD or DA agonists induces abnormal involuntary, dyskinetic movements and contraversive rotational behavior. These abnormal involuntary movements are separated into orolingual dyskinesias (rapid protrusion of the tongue and chewing movements), truncal and neck dystonic posturing contralateral to the lesion and hyperkinetic/dystonic posturing of the forelimb contralateral to the lesion (Cenci, Lee et al. 1998; Steece-Collier, Collier et al. 2003; Lieu, Kunselman et al. 2010) (Fig. 1-1). Similar to
the HP rat, 6-OHDA can be injected into the MFB or striatum to create HP dyskinetic mice (Lundblad, Picconi et al. 2004; Pavon, Martin et al. 2006). In mice, knockout of the PitX3 gene prevents the development of nigrostriatal neurons. LD exposure induces dyskinesias in these animals in the form of hyperkinetic movements of the forelimbs and hindlimbs (Ding, Won et al. 2011). These abnormal involuntary movements in the rodent exemplify more hyperkinetic, rhythmical movements and are less like choreiform-type movements seen in primate models or in PD patients. There continues to be controversy in the literature on the relevance of drug-induced hyperkinetic movements seen in the HP rat and mouse when compared to the drug-induced hyperkinetic movements seen in MPTP-treated monkey (see below) and PD patients with motor fluctuations. Regardless, many consider the HP rodent as a viable, clinically relevant preclinical model for the study of the behavioral aspects of DID.
Figure 1-1. Example of DID in the HP rat. Truncal (red arrows) and neck (yellow arrows) dystonic posturing, and hyperkinetic/dystonic posturing of the forelimb (blue arrow).

**Primate models of DID**

Primates have played an important role in understanding the pathophysiological basis of DID and in preclinical experimental therapeutics targeted at diminishing or preventing DID. Investigators have utilized various species to model DID that include squirrel monkeys, common marmosets, macaques and vervet nonhuman primates (Boyce, Rupniak et al. 1990; Pearce, Jackson et al. 1995; Heimer, Rivlin-Etzion et al. 2006; Liang, DeLong et al. 2008). Exposure to the neurotoxin MPTP leads to a relatively selective loss of dopaminergic nigrostriatal neurons, and is used in primates to induce parkinsonism. LD and other pharmacological DA replacement therapy induces hyperkinetic, abnormal involuntary movements (choreoathetosis, violent jerks, flailing of the limbs), dystonic, abnormal posturing in the extremities and trunk, and orolingual
dyskinesias (purposeless protrusion of the tongue). This animal model displays dyskinesias more clinically similar to DID in PD when compared to the parkinsonian rodent. Examples of DID in the parkinsonian primate are shown in Figure 1-2.

![Figure 1-2](image-url)

*Figure 1-2.* Example of DID in the parkinsonian primate. Dystonic posturing and choreiform movements in the feet/legs (red and blue arrows) and orolingual dyskinesias (yellow arrow).

Depending on route of MPTP-administration, investigators can create various primate models of PD (Emborg 2007). The HP model is induced by intracarotid MPTP injection unilaterally. This model displays parkinsonian symptoms on the side of the body contralateral to exposure. The systemic model is a bilateral parkinsonian model which is created by systemic injection (IM, SC, or IV) of MPTP. This model displays symptoms bilaterally which in some cases can lead to a severely parkinsonian animal. The overlesioned model is created by a combination of intracarotid and systemic injection. These animals display asymmetric
Parkinsonism. The bilateral intracarotid model is induced by MPTP injections both in the left and right carotid arteries. See Table 1-2.

Table 1-2. Primate models of PD summarized from (Emborg 2007).

<table>
<thead>
<tr>
<th>Primate Models of PD</th>
<th>Hemiparkinsonian</th>
<th>Overlesioned</th>
<th>Bilateral</th>
<th>Bilateral Intracarotid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route</td>
<td>Intracarotid</td>
<td>Intracarotid + Systemic</td>
<td>Systemic (IM, SC, IV)</td>
<td>Intracarotid</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>0.4 - 0.5</td>
<td>0.4-0.5 - 0.2-0.6</td>
<td>0.2 - 0.6</td>
<td>0.4 - 0.5</td>
</tr>
<tr>
<td>Advantages</td>
<td>Minimal care, reproducible, rapid symptom onset</td>
<td>Asymmetric parkinsonism, Slower progression, mimics advanced disease</td>
<td>Critical case needed</td>
<td>Critical case needed</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Extensive surgical procedure</td>
<td>Predictability of symptoms</td>
<td>Critical case needed</td>
<td>Critical case needed</td>
</tr>
<tr>
<td>Dyskinesias?</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The extent of nigrostriatal damage necessary to induce DID in animal models has demonstrated varying results. In rats and mice, development of DID is typically after extensive loss of nigrostriatal neurons via MFB lesion, typically upward to 95% loss of dopaminergic neurons when compared to the unlesioned side. Although, as we discuss later, the critical threshold of nigrostriatal DA loss that is needed to cause DID in the rodent is yet to be fully established. Single striatal injection of 6-OHDA as described previously (Sauer and Oertel 1994) induces a partial lesion with approximately 50% degeneration. These rats do not develop DID with LD exposure. However, multiple striatal injection sites can lead to extensive degeneration where such animals develop DID similar to the MFB-lesioned rat (Winkler, Kirik et al. 2002). In the primate, some have demonstrated that normal monkeys (i.e. squirrel monkeys) without MPTP exposure or that monkeys with minor nigrostriatal degeneration develop DID, whereas other investigators argue the necessity that extensive nigrostriatal damage bilaterally is essential for the development of DID (Kurlan, Kim et al. 1991; Pearce, Heikkila et al. 2001; Togasaki, Tan et al. 2001; Heimer, Rivlin-Etzion et al. 2006; Liang, DeLong et al. 2008). We have recently shown that macaque Rhesus monkeys that are clinically HP by intracarotid MPTP do not develop DID.
Despite high dose chronic intermittent LD treatment (described in detail in chapter 3 and published in (Lieu, Deogaonkar et al. 2011)), indicating that bilateral parkinsonian Rhesus monkey is a more suitable model for DID in this species.

1.4. Differential Diagnosis of Dyskinesias

Accurate identification of DID and its phenomenology is critical. There are many movements that occur in PD patients and in various animal models of PD that can be easily mistaken for DID. In this section, we describe the commonly mis-identified conditions that mimic DID in PD patients and how they can be differentiated clinically. Along the same lines, we also describe frequently misdiagnosed abnormal involuntary movements in the various animal models of PD. Accurate identification of DID is a critical and an essential step to understanding the pathophysiology of this disorder and to develop improved treatment strategies. Since by definition DID are caused by pharmacological treatments, it is also important to understand the pharmacology of individual drugs and how they interact with other drugs in PD patients and in various animal models of the disease.

Tremor

In contrast to DID which are involuntary, continual, abrupt, brief and irregular, tremors are oscillatory, rhythmic and regular and tend to affect the more distal parts of the upper extremities. Peak-dose dyskinesias, by definition, will appear only after administration of anti-PD medications (typically 60-90 min) while tremor in PD will frequently mitigate upon administration of anti-PD medication. Rarely, diphasic dyskinesias may have to be distinguished from lower extremity tremor. Diphasic dyskinesias and tremor are both seen when anti-PD medication levels are low. However, on administration of anti-PD medications, diphasic
dyskinesias tend to disappear sooner and abruptly as compared to parkinsonian tremor, which will mitigate slowly.

**Huntington’s disease**

Chorea is a frequent manifestation of Huntington’s disease. However, Huntington’s chorea is easily distinguished by the family history, absence of temporal relation to dosing of anti-PD medications and by presence of several other typical findings in Huntington’s disease that separates this entity from PD. This issue is more complicated in juvenile Huntington’s disease. In this case, typically the patient is parkinsonian (Roos 2010), does not exhibit chorea and is often treated with anti-PD medications. In this scenario, if the patient develops choreiform movements, they need to be distinguished from DID as opposed to natural occurrence due to progression of Huntington’s disease. Following points may be used to make this distinction:

- a) DID have a temporal course to timing of anti-PD medications while chorea occurring in Huntington’s disease is random and has no temporal course.

- b) Juvenile Huntington’s disease is a more severe form and invariably the patient will have more symptoms in other neurological domains beyond simple parkinsonism (dementia, ataxia, etc.)

A third scenario is when an adult Huntington’s disease patient is treated with antiparkinsonian medications (e.g. Haloperidol). This drug can produce tardive dyskinesias which need to be distinguished from DID.

**Tics and Stereotypies**

Tics are abrupt, brief, repetitive and stereotyped movements which vary in intensity and are repeated at irregular intervals (Jankovic 2009). Patients usually have a generalized urge preceding the actual movement or local discomfort in the region of the body where the tic
appears. Tics can be voluntarily suppressed but these result in mounting inner tension leading to a rebound of tics. Tics can also persist during sleep. Stereotypies are involuntary, patterned, repetitive, continuous, coordinated, ritualistic movements or utterances. Unlike tics, stereotypies are not preceded by an urge and usually occur during periods of stress, excitement or when engrossed. They can be ceased by distraction or initiation of a new activity.

Tics and stereotypies can be differentiated from dystonia by the absence of worsening on attempted movements. Tics and stereotypies can be differentiated from DID which are choreiform. Also, DID cannot be voluntarily suppressed.

Stereotypies can be displayed by non-human primates as a typical response to stress. They are worsened by isolation, lack of human interaction, loss of peer or other stressors. Tics are also quite common, learned from parent-sibling or peers in laboratory housed non-human primates. They are repetitive movements and can include grimacing, rearing behavior, scratching and chewing movements when there is no food. Distinguishing these from DID in monkeys require careful videotaping, knowing pre-existing tics and stereotypies, and relationship to LD dosing.

**Tardive dyskinesias**

These are involuntary movements that are seen as a complication of long-term DA receptor antagonist therapy and present with rapid, repetitive, stereotypic movements involving oral, buccal and lingual areas. In cases where the patient is currently on DA receptor antagonists and exhibits signs of parkinsonism and oro-bucco-lingual involuntary movements, it is easy to make the distinction between tardive dyskinesias and DID. However, in very rare cases where the patient has been previously treated with one or more DA receptor antagonists for a short period but such information is not available at the time of clinical presentation, it becomes essential to differentiate between DID and tardive dyskinesias. In such cases, the predominant oro-bucco-
lingual involvement, lack of limb and trunk involvement and the absence of improvement on withdrawal of drugs help to differentiate tardive dyskinesias from DID.

**Myoclonus**

Myoclonus is a sequence of repeated, often nonrhythmic, brief shock-like jerks due to sudden involuntary contraction or relaxation of one or more muscles. Myoclonus can be differentiated from dystonia by the lack of distinctive postures. Rhythmic myoclonus can be distinguished from chorea occurring in DID by the predictable timing of movements. Asynchronous multifocal myoclonus is more difficult to distinguish but can be done so due to the simpler, shock-like movements of myoclonus compared to the more complex, randomly distributed movements in chorea.

1.5. **Physiology Of Drug-Induced Dyskinesias**

**Functional models of the basal ganglia**

The basal ganglia are a set of subcortical nuclei that play an important role in the motor system. One of the main functions of the basal ganglia motor loop is to select and maintain specific motor behaviors. The nuclei of the basal ganglia include the substantia nigra pars compacta (SNC), striatum (STR) (caudate and putamen), globus pallidus interna and externa (GPI and GPE, respectively), subthalamic nucleus (STN) and substantia nigra pars reticulata (SNR).

One of the prevalent models for the functional connectivity for PD is based on the classic rate model of the “direct” and “indirect” pathways of the basal ganglia motor loop (Albin, Young et al. 1989; Alexander, Crutcher et al. 1990; DeLong 1990). In this model, the dopaminergic nigrostriatal pathway modulates the activity of two separate pathways in the STR (DA D1-receptor mediated “direct” and DA D2-receptor mediated “indirect” pathways) (Figure 1-3).
Figure 1-3. Medium-spiny neuron in the striatum and its connections to downstream basal ganglia nuclei (globus pallidus externa (indirect) and interna, and substantia nigra reticulata (direct) (A). Convergent afferent glutamate cortical and nigral dopaminergic connections to the medium spiny neuron (B). Reproduced with permission, (Purves, Augustine et al. 2001).

With subsequent loss of DA due to nigrostriatal degeneration (Fig. 1-4A), the activity of the motor loops is altered, leading to parkinsonism. In the parkinsonian “direct” pathway, γ-Aminobutyric acid (GABA)-ergic striatal input to the GPI/SNR is reduced, leading to a disinhibition and overactivity of the GABAergic GPI/SNR. In the parkinsonian “indirect” pathway, there is an increase in GABAergic striatal neuron activity to the GPE leading to inhibition of the GABAergic GPE. This leads to disinhibition of the glutamatergic STN and overactivity of this nucleus. This pathway also causes increased activity of the GPI/SNR. Taken together, in the parkinsonian state, both the “direct” and “indirect” pathways lead to an excessive inhibition of the motor thalamus (THAL) and subsequently the motor cortex (CTX).

Based on this model, DA replacement therapy should result in balanced activity of the “direct” and “indirect” pathways (Fig. 1-4B). However, our group as well as other investigators
has demonstrated that with oral pharmacological DA replacement treatment, various basal ganglia nuclei activity do not become “balanced” as hypothesized by this classic rate model (Heimer, Bar-Gad et al. 2002; Heimer, Rivlin-Etzion et al. 2006; Gilmour, Lieu et al. 2011).

The classic model described above would also predict that DID would be accompanied by increased thalamocortical activity, reduced inhibitory output from GPI/SNR to the THAL, and therefore reduced STN glutamatergic output in the “indirect” pathway and increased striatal output in the “direct” pathway (Fig. 1-4C). While some studies have supported this hypothesis, others have argued against it utilizing lesioning and electrophysiological studies of various basal ganglia nuclei (Bergman, Wichmann et al. 1990; Hamada and DeLong 1992; Papa, Desimone et al. 1999; Baron, Vitek et al. 2000). Therefore, this model does not adequately explain the pathophysiology of DID.

**Figure 1-4.** Classic rate model of the basal ganglia. Red – GABA (Inhibitory); Green – Glutamate (Excitatory); Blue – Degenerated Dopaminergic Nigrostriatal Pathway. Size of arrows indicate extent of activity. In the normal state, the “direct” and “indirect” pathways would appear the same as Fig. 1-4B, except that SNC is intact.
Another model, the pattern model of the basal ganglia, takes into account that neuronal firing pattern in the basal ganglia is altered in PD. Most studies have demonstrated that there is both an increase in firing rate and increasing bursting firing pattern in the STN and GPI in PD (Fig. 1-5). This is discussed in detail in Chapter 5. To note, in the figure below, PD is accompanied with increases in STN and GPI/SNR firing rate but also an increase in altered firing pattern in these two nuclei.

**Figure 1-5.** Pattern model of the basal ganglia in the normal, PD and DID state. Black arrows – GABA/inhibitory; Gray arrows – glutamate/excitatory. Size of arrow indicates firing rate (i.e. wider lines = increase in firing rate). Hashed lines indicate increase in altered firing patterns. Reproduced and adapted with permission, (Vitek, Chockkan et al. 1999).
Another model was introduced by Nambu and colleagues which modified the classic rate model above to include an additional pathway known as the “hyperdirect” pathway (Nambu, Tokuno et al. 2002; Nambu 2005). This pathway originates in the cortex and directly communicates with the STN. This pathway conveys powerful effects from the motor cortex directly to the GPI/SNR via the STN, bypassing information processing through the striatum. It is suggested that during movement initiation, the hyperdirect pathway first modulates thalamocortical activity. Then subsequent processing of movement information via the “direct” and “indirect” pathway are executed. It is suggested that this processing sequence occurs when certain movements are selected and others inhibited (Fig. 1-6).

![Diagram of the basal ganglia pathways](image)

**Figure 1-6.** Hyperdirect pathway of the basal ganglia. CX – cortex, Th – thalamus. Reproduced with permission, (Nambu 2005).
Another model was also proposed by Obeso and colleagues (Obeso, Rodriguez-Oroz et al. 2000) as shown in Figure 1-7. In this model, there are cortico-basal ganglia-cortical somatotopically organized parallel motor loops (black) which act as positive signaling for processing and execution of movement. Also, horizontal loops (internal circuits) provide “internal stabilization” of the basal ganglia. These include positive (blue) and negative (red) feedback loops, and excitatory-inhibitory loop (green). This model suggests that the processing of motor information in the basal ganglia is not a linear system as suggested by the rate model. Rather, it suggests that the basal ganglia is a multifaceted neuronal network that consists of multiple, parallel and feedback loops that are involved in the processing, selection/inhibition and proper execution of complex motor tasks.

**Figure 1-7.** Modern view of basal ganglia motor circuitry. Abbreviations: CM/Pf, centromedian-parafascicular complex; GPe, globus pallidus externa; GPi, globus pallidus interna; SNC, substantia nigra pars compacta; SMA, supplementary motor area; STN, subthalamic nucleus; VL, ventralis lateralis. Reproduced with permission, (Obeso, Rodriguez-Oroz et al. 2000).
Continuous versus pulsatile dopaminergic stimulation

DA is constitutively available to the striatum by the nigrostriatal pathway in the normal state. With degeneration of the nigrostriatal pathway and subsequent loss of DA in PD, the striatum has less constitutive availability of DA, and thus DA receptors are no longer tonically stimulated to a physiological level. After oral administration of pharmacological anti-PD medications, plasma levels of these therapies will fluctuate throughout the day based on pharmacokinetics and half-life of the drug in PD patients, thus causing pulsatile, intermittent activation of striatal DA receptors. Since LD is a prodrug and has to be converted to DA, remaining intact nigrostriatal neurons are able to buffer, store, release on demand, and reuptake DA in early stages of PD, providing constitutive DA to the striatum. It is hypothesized that this provides continuous stimulation of striatal dopaminergic receptors, similar to the physiological state in the normal condition.

In more advanced stages of PD, it has been proposed that chronic intermittent, pulsatile treatment and stimulation of striatal dopaminergic receptors with pharmacological DA replacement therapies such as LD or DA agonists leads to supersensitivity (an enhanced biochemical, physiological, and behavioral response to dopaminergic drugs such as LD or DA agonists) of striatal dopaminergic receptors (Chase, Baronti et al. 1989; Kostrzewa 1995). In this context, as the disease progresses to more advanced stages, there is extensive loss of nigrostriatal neurons and the neurotransmitter mechanisms fail, leading to a more pulsatile, intermittent activation of dopaminergic receptors than early stages of PD. Drugs with longer half-lives, combinational pharmacological therapies and cell transplantation studies have provided evidence that continuous dopaminergic stimulation can provide better symptomatic relief and mitigate/prevent DID in more advanced stages of PD (Blanchet, Konitsiotis et al. 1998; Pearce, Jackson et al. 1999; Subramanian, Marchionini et al. 2002; Bibbiani, Costantini et al. 2005; Soderstrom, O'Malley et al. 2010; Murthy, Gowdahalli et al. 2011).
1.6. Biochemical and Molecular Mechanisms of DID

DA receptors

DA receptors are metabotropic G-protein-coupled receptors widely expressed throughout the basal ganglia but mainly in the striatum. The two main family subtypes of DA receptors are D₁-like and D₂-like receptors. The prevailing hypothesis for DID have been attributed to striatal DA receptor supersensitivity. Earlier studies have shown that the presence of mRNA encoding for D₁ receptors decreases and D₂ receptors increases in response to dopaminergic denervation in rodent studies (Gerfen, Engber et al. 1990), which is further confirmed in PD patients and MPTP-treated monkeys (Lee, Seeman et al. 1978; Alexander, Schwartzman et al. 1993; Morissette, Goulet et al. 1996). More recently, Aubert et al. found differential changes of D₁ and D₂ receptor expression in dyskinetic monkeys, showing an increase in D₂ mRNA and D₂ ligand-binding compared to controls. D₁ mRNA is also downregulated in MPTP monkeys but comparable to normal in LD treated parkinsonian monkeys. Further, they demonstrate that increased D₁ receptor signaling (second messenger signaling – see section below) is linearly related to DID (Aubert, Guigoni et al. 2005). It had also been reported that there is an increase in both membrane and cytoplasmic striatal D₁ receptor expression in MPTP-treated dyskinetic monkeys compared to normal monkeys, with only moderate changes in D₂ receptor expression (Guigoni, Doudnikoff et al. 2007).

Therefore, previous studies demonstrate that plastic changes of DA receptors occur in response to DA denervation and pharmacological dopaminergic treatments. The notion of DA receptor supersensitivity is a combination of alterations to striatal DA receptor expression and subsequent G-protein second messenger signaling (see below). Although our understanding of DA receptor plasticity and supersensitivity has been studied mainly in the striatum for the underlying molecular changes associated with DID, future studies that examine alterations to the
extrastriatal DA and receptor expression in the GPE, GPI, STN and SNR are warranted (Rommelfanger and Wichmann 2010).

Second messenger signals

Neuronal second messenger signaling cascades are important for synaptic plasticity, modulation of downstream proteins and control of gene transcription factors (immediate early gene expression) and are modulated by DA receptors (Fig. 1-8). It has been reported that there is a significant increase of phosphorylation of ERK (P-ERK) in the DA denervated striatum in dyskinetic animals, and that blockade of ERK phosphorylation with the MEK1/2 inhibitor SL-327 or Ras inhibitor lovastatin significantly decreases dyskinesias (Pavon, Martin et al. 2006; Schuster, Nadjar et al. 2008; Ding, Won et al. 2011). It has further been shown that phosphorylation of ERK increases in striatal medium-spiny neurons (MSN) after acute LD exposure but chronic LD exposure translates into a decrease in MSN ERK phosphorylation and reciprocal increases in cholinergic striatal interneurons. Similarly, another related second messenger signal, the dopamine- and cAMP-regulated 32 kDa phosphoprotein (DARPP-32) has also been implicated in the onset of DID (Santini, Sgambato-Faure et al. 2010). More specifically, increased phosphorylation on the Thr34 (P-Thr34) site of the protein seems to be associated with onset of DID (Guan, Zhan et al. 2007). We have shown that striatal DARPP-32 expression is selectively decreased in dyskinetic HP rats compared to normal rats (Lieu, Stull et al. 2008). In the normal rat, we found relative uniformity of DARPP-32 immunohistochemical staining throughout the striatum bilaterally. However, in the LD-treated dyskinetic rat, we found decreased staining in dorsolateral areas of the striatum in the unlesioned hemisphere and an overall decrease in the density of staining in various regions of the lesioned hemisphere (Fig. 1-9). Studies in dyskinetic rats and monkeys evaluating the transcription factors ΔFosB and ΔJunD within the STR have also been implicated in the onset and maintenance of dyskinesias (Pavon,
Martin et al. 2006; Berton, Guigoni et al. 2009; Cao, Yasuda et al. 2010). As mentioned previously, these maladaptive changes of second messenger signals are likely to be the direct result of DA receptor supersensitivity.

Figure 1-8. Alterations to signal transduction in DID in PD.
**Figure 1-9.** DARPP-32 in the striatum. (Above) Normal rat - DARPP-32 staining shows relative uniformity throughout the striatum. (Below) Dyskinetic HP rat - DARPP-32 not only showed less uniformity in the dorsolateral areas of the striatum in the unlesioned side (*) but also an overall decrease in density of staining in various regions in the lesioned hemisphere (#).

**GABA**

GABA, being one of the major inhibitory neurotransmitters in the basal ganglia, is present in striatonigral and striatopallidal neurons. These 2 subsets of neurons express 2 isoforms of the GABA synthesizing enzymes (glutamic acid decarboxylase – GAD), GAD65 and GAD67 (Mercugliano, Soghomonian et al. 1992). Systematic administration of LD induces significant
increases in GAD gene expression in striatonigral neurons (Soghomonian, Pedneault et al. 1996; Cenci, Lee et al. 1998; Carta, Fenu et al. 2003; Nielsen and Soghomonian 2004; Katz, Nielsen et al. 2005; Yamamoto and Soghomonian 2009) and a small increase in GAD gene expression of striatopallidal neurons (Carta, Fenu et al. 2003; Nielsen and Soghomonian 2004; Carta, Tronci et al. 2005). However, more recently, a study found a decrease in GAD65/67 mRNA in GPI neurons in dyskinetic monkeys. Although GABAergic striatal interneurons are not primarily affected in PD, as a result of progressive DA depletion, expression levels of GABA receptors change in the striatum. Subchronic administration of LD to 6-OHDA-lesioned rats induces marked increases in GABA release in the SNR (Yamamoto, Pierce et al. 2006), and this increase was blocked by subchronic administration of an mGlutR5 agonist (Mela, Marti et al. 2007). This suggests that an mGlutR5 agonist can efficiently decrease the severity of DID in animal models of PD. Pulsatile dopaminergic stimulation can cause up-regulation of GABA receptors in GPI. GABA receptors were reported to be up-regulated in GPI of primates with DID (Calon, Morissette et al. 1999) and dyskinetic PD patients (Calon and Di Paolo 2002). There have been reports on Modafinil preventing the upregulation of GABA-A receptor binding in the GPI of MPTP-treated marmosets (Zeng, Smith et al. 2004). Modafinil is currently used to treat narcolepsy and excessive daytime sleepiness. Modafinil’s exact mechanism of action still remains unclear but is thought to increase levels of catecholamine release including DA release from its synaptic terminals.

**Glutamate**

Glutamate is the main excitatory neurotransmitter in the basal ganglia. PD and DID are associated with basal ganglia glutamatergic hyperactivity. As a result of DA depletion in the basal ganglia and treatment with LD or DA agonists, glutamate levels and glutamate receptor expression is increased, resulting in dyskinetic behavior (Calon, Morissette et al. 2002; Ouattara, Hoyer et al. 2010). Earlier studies by Calon and colleagues (Calon, Morissette et al. 2002; Calon,
Rajput et al. 2003) have shown an elevation of NMDA receptor binding in the putamen (53%) during motor fluctuation when compared to patients without motor fluctuation. A recent study used a PET marker for activated NMDA receptor ion channels in PD patients (dyskinetic and non-dyskinetic). They demonstrated that dyskinetic patients showed a higher uptake of the NMDA receptor PET marker in the striatum and precentral gyrus (motor cortex) compared to non-dyskinetic patients. (Ahmed, Bose et al. 2011). In a separate study, Conte-Perales and colleagues found no significant changes in the expression of glutamatergic mRNA markers for the vesicular glutamate transporter vGlut1 or vGlut2 between dyskinetic and non-dyskinetic monkeys (Conte-Perales, Rico et al. 2011).

A number of glutamate receptor antagonists have been shown to reduce DID. Amantadine is a glutamate NMDA receptor antagonist that has been utilized in PD patients as an antidyskinetic treatment. In clinical practice, Amantadine is used to give temporary relief of DID, however, this effect is short lived. The reason why this effect occurs is currently unknown (Berg, Godau et al. 2011).

In a study using 6-OHDA lesioned rats (Marin, Bonastre et al. 2011), metabotropic glutamate receptor type 5 (mGluR5) antagonist + LD co-administration had a significant attenuation of DID. In these animals, expression of vGlut2 significantly decreased, but vGlut1 expression remained unchanged. These vGlut changes were not seen in dyskinetic rats exposed to LD alone, demonstrating that an mGluR5 antagonist + LD can alter vGlut2 expression.

A recent study showed a beneficial motor effect with the novel drug AFQ056 (an mGluR5 antagonist) with LD in dyskinetic MPTP-treated monkeys, supporting the therapeutic use of an mGluR5 antagonist to decrease dyskinesias (Gregoire, Morin et al. 2011). Similarly, Berg and colleagues also evaluated AFQ056 in PD patients with moderate to severe dyskinesias in a placebo-controlled double-blinded study. Patients received chronic treatment of either AFQ056 or placebo BID (2x/day). The study showed that there was a significant reduction in
dyskinesias in the AFQ056-treated group with no significant changes in the motor portion of the Unified Parkinson’s Disease Rating Scale (UPDRS) (Appendix A) (Berg, Godau et al. 2011).

Serotonin (5-HT)

Dorsal raphe serotonergic neurons project to many regions of the brain, including the STR, SNR and GPI. Furthermore, there are multiple subtypes of serotonin receptors, and each regulates the actions of DA neurotransmission differently (Alex and Pehek 2007). Some studies in human PD patients and MPTP-treated monkeys suggest that parkinsonism is associated with a loss of striatal serotonin and serotonergic neuronal innervation as shown by pathological and serotonin transporter binding imaging studies (Perez-Otano, Herrero et al. 1991; Guttman, Boileau et al. 2007; Kish, Tong et al. 2008). On the other hand, recently Zeng et al. observed that striatal serotonin hyperinnervation follows nigrostriatal pathway loss (Zeng, Iravani et al. 2010). Their study also suggests that chronic LD treatment and the onset of DID are associated with a marked hypertrophy of striatal serotonin axonal varicosities.

The main hypothesized pathophysiological role of the serotonergic system in DID is non-regulated DA synthesis of LD by serotonergic neurons (Carta, Carlsson et al. 2007). Therefore, investigators have evaluated both serotonin pharmacological agents targeted to specific serotonin receptor subtypes and serotonergic cell transplantation to identify if certain serotonergic treatments can reduce dyskinesia severity.

In the dyskinetic state, Riahi and colleagues evaluated changes to serotonin receptor binding (5-HT(2A)) expression in dyskinetic parkinsonian monkeys (Riahi, Morissette et al. 2011). They found a decrease in striatal serotonin binding after MPTP-treatment. Animals with DID had increases in serotonin receptor binding in both the striatum and cortex. Treatment with a glutamate antagonist or D2 DA receptor agonist led to a normalization of striatal and cortical serotonin receptor binding.
Earlier studies that have targeted the 5-HT1A receptors or the 5-HT2 receptors have shown that they can decrease of dyskinesia severity. These include buspirone, mirtazapine, and quetiapine (Kleedorfer, Lees et al. 1991; Bonifati, Fabrizio et al. 1994; Meco, Fabrizio et al. 2003; Eskow, Gupta et al. 2007). For example, Eskow and colleagues reported that the partial 5-HT1A agonist buspirone reduced development and expression of DID (Eskow, Gupta et al. 2007). Another study by Oh and colleagues demonstrate that quetiapine, a 5HT2A/C and D2/3 antagonist, decreases DID without decreasing the therapeutic effects of LD in parkinsonian monkeys (Oh, Bibbiani et al. 2002). ACP-103, a 5-HT2A receptor inverse agonist and antagonist, can decrease DID in parkinsonian monkeys without affecting the anti-parkinsonian effects of LD (Vanover, Betz et al. 2008). In a more recent open-label, pilot clinical study, aripiprazole (D2 and 5-HT1A partial agonist and 5-HT2A antagonist) was shown to effectively decrease severity of dyskinesias in PD patients at low doses (Meco, Stirpe et al. 2009). Aripiprazole did not significantly worsen parkinsonian symptoms in these patients.

Investigators using cell transplantation studies (serotonergic and dopaminergic cell transplantation) in preclinical models have demonstrated that serotonin neurons play a critical role in the development of DID (Carlsson, Carta et al. 2009). Recently Politis and colleagues (Politis, Wu et al. 2010) showed that dyskinesias were markedly attenuated by systemic administration of a serotonin receptor agonist (5HT1AR agonist) which dampened the transmitter release from serotonergic neurons, indicating that DID were caused by the serotonergic hyperinnervation (i.e serotonergic neurons mediate dyskinetic side effects in PD patients with normal transplants).

**Acetylcholine**

Acetylcholine has been another neurotransmitter implicated in the modulation of DID. A number of studies have demonstrated that long term exposure to nicotine, which acts on nicotinic
acetylcholine receptors, can reduce DID in 6-OHDA lesioned rats and MPTP dyskinetic monkeys. Interestingly, the nicotinic receptor antagonist mecamylamine can also provide similar effects (Quik, Cox et al. 2007; Bordia, Campos et al. 2008; Bordia, Campos et al. 2010). It has been recently demonstrated that DID can be decreased with selective nicotinic receptor agonists in dyskinetic rats (Huang, Campos et al. 2011), suggesting that nicotinic receptors on dopaminergic terminals may play a role in modulating DID. As mentioned previously, studies have also demonstrated an increase in striatal cholinergic interneuron activity in dyskinetic mice (Ding, Won et al. 2011). Thus, although acetylcholine is not as widely expressed as other neurotransmitters (glutamate, GABA, and dopamine) within the basal ganglia, its role in DID warrants further research.

Adenosine

Adenosine A2A receptors are G-protein-coupled receptors that have been implicated in the pathophysiology of DID. Adenosone A2A receptors are mainly present in GABAergic striatopallidal neurons, colocalized with enkephalin and D₂ receptors. Adenosine A2A receptors mRNA levels in the putamen are known to be increased in PD patients with DID, when compared to normals or PD patients without DID. Furthermore, specific-binding to adenosine A2A receptors is elevated in dyskinetic PD patients compared to controls in the putamen, and elevated in PD patients when compared to normals in the GPE (Calon, Dridi et al. 2004). These significant increases in A2A receptor mRNA have been further confirmed in the 6-OHDA lesioned dyskinetic rat when compared to sham-treated animals with LD treatment or 6-OHDA lesioned rats without LD (Tomiyama, Kimura et al. 2004). Interestingly, increases in A2A receptor mRNA has been found in normal cynomolgus monkeys displaying DID with chronic LD treatment (Zeng, Pearce et al. 2000). Recently, it had been shown that genetic knock-out of forebrain A2A receptors in mice can attenuate DID after LD treatment (Xiao, Bastia et al. 2006).
Adenosine A2A receptor antagonist administration in parkinsonism has been extensively examined, demonstrating that antagonism can increase anti-parkinsonian effects of pharmacological DA replacement therapies without exacerbating DID in preclinical models and in PD patients (Kanda, Jackson et al. 1998; Lundblad, Vaudano et al. 2003; Xiao, Bastia et al. 2006; LeWitt, Guttman et al. 2008). Taken together, adenosine A2A receptor antagonists and pharmacological DA replacement treatment can be a useful combinational therapy to target dyskinesias.

Other chemical systems

Other chemical systems are present throughout the basal ganglia and play an important role in DID. It has been shown that neuropeptide mRNA levels of striatal preproenkephalin and preprodynorphin are increased in dyskinetic MPTP-lesioned monkeys compared to control MPTP-lesioned monkeys, similar to previous reports in the 6-OHDA lesioned parkinsonian rat (Gerfen, Engber et al. 1990; Zeng, Pearce et al. 2000; Morissette, Dridi et al. 2006; Guan, Zhan et al. 2007; Tamim, Samadi et al. 2010).

The opioid system has also been implicated in DID. Using opioid receptor-stimulated G-protein activation techniques, Chen and colleagues demonstrated that µ-opioid receptor-mediated G-protein activation is increased in the brain, specifically the cortex and basal ganglia in dyskinetic monkeys treated with LD. They also found binding changes in δ- and κ-opioid receptor in these animals (Chen, Togasaki et al. 2005). Furthermore, µ-opioid and δ-opioid stimulated binding in the striatum was positively correlated to DID severity in their study. In a later study, it was shown that pre-treatment with the κ-opioid agonist U50,488 can reduce DID in the parkinsonian rat and primate (Cox, Togasaki et al. 2007). However, U50,488 had the deleterious effects of reducing the anti-PD effects of LD.
Another system is the cannabinoid system which is also widely expressed in the basal ganglia, and modulates the activity of other neurotransmitter systems such as glutamate and DA. Cao and colleagues demonstrate the selective antagonism of cannabinoid type 1 receptor can increase the efficacy of LD without affecting the severity of dyskinesias in parkinsonian rhesus monkeys (Cao, Liang et al. 2007).

These biochemical studies demonstrate that there are a wide-range of distinct pathophysiological changes that occur in response to DA denervation to the striatum and with DID in the basal ganglia. It is also evident that multiple neurotransmitter systems influence the severity of DID and its pathophysiology at multiple levels, both within the basal ganglia and outside of the basal ganglia. At the level of receptors and their expression patterns, there are alterations, and the most obvious and well studied example is the notion of receptor supersensitivity. In addition, the attached second messenger systems show alterations that modulate medium spiny neuronal function. Although a unifying hypothesis that stitches together the various abnormalities in neurotransmitter systems remains elusive, the notion that numerous neurotransmitter systems are simultaneously influenced by the pathophysiology of DID is now established. The other unifying concept appears to be the notion that normal animals and animals with minor lesions in the nigrostriatal pathways do not appear to be vulnerable to the classic DID phenotype exhibited by PD patients. Also, it is clear that PD patients with early, mild disease remain resistant to DID. These findings and the neurochemical changes that are largely described in animals and in PD patients with advanced disease strongly support the notion that DID is a disease that requires profound loss of the nigrostriatal dopaminergic systems and, as we discuss later, this loss has to be bilateral to trigger the downstream changes described in this section with neurochemistry. Future studies which examine the complex interaction between multiple basal ganglia neurotransmitter systems are warranted to advance the development of novel anti-
dyskinetic therapies and for us to come away with a unifying single hypothesis that involves all the neurotransmitter systems.

1.7. Electrophysiological Changes In DID

**Globus pallidus externa (GPE) and interna (GPI)**

Most of the present electrophysiological studies of DID are linked to an excessive decrease in activity in the GPI. In a study by Filion et al. (Filion, Tremblay et al. 1991), apomorphine was injected in MPTP monkeys. They showed that all GPI neurons decreased their firing rate following apomorphine administration. The reverse was true of the predominant neuronal population in the GPE. A similar study by Boraud et al. (Boraud, Bezard et al. 1998) supports the correlation between dyskinesias and an excessive decrease in the firing frequency of GPI neurons. A similar excessive decrease was reported by Papa and colleagues (Papa, Desimone et al. 1999) in MPTP-treated monkeys treated with LD. This study showed that during DID, the firing rates declined profoundly in almost all cells of GPI, with a decrease as low as 97% in individual cells. From recordings in parkinsonian patients treated with apomorphine, Lozano and colleagues suggested that dopaminergic agents act by decreasing GPI and STN activity, and increasing GPE activity (Lozano, Lang et al. 2000). They went on to suggest that DID resulted from a large reduction in GPI firing.

The predominant electrophysiological signature of DID at present is the excessive decrease in GPI activity, which is in support of the classic rate model of the basal ganglia (see Fig 1-4C). However, the idea that the hypoactivity in GPI is the primary mechanism by which DID occurs is challenged by the fact that a pallidotomy, which abolishes activity in GPI, eliminates dyskinesias (Baron, Vitek et al. 2000; Lozano, Lang et al. 2000). As a result, some have suggested that the pathophysiology of dyskinesias is not simply the result of the hypoactivity observed in GPI, but rather the result of a change in the firing pattern output of GPI. This would
explain why a pallidotomy eliminates DID, as the abnormal firing pattern is removed (Obeso, Rodriguez-Oroz et al. 2002). However this theory remains unproven since the discharge rates are so low that firing patterns cannot be discerned in this condition during electrophysiological recordings. The notion that abnormal pattern is the reason for dyskinesias in the GPI, and removal of it as relief of DID, is not completely proven. The more viable hypothesis at the present time is the notion that discharge rates are suppressed in the act of DID.

Other studies have examined the role of oscillatory activity in the basal ganglia with respect to DID. In MPTP monkeys, Heimer and colleagues (Heimer, Rivlin-Etzion et al. 2006) found increases in oscillatory activity and synchronization in GPI and GPE after induction of parkinsonism, and decreases in both following DA replacement therapy. Although DA replacement therapy had a reversal effect on the changes resulting from MPTP, they noted an imbalance in the oscillatory power and synchronization between GPI and GPE. Further studies on the electrophysiology of DID must take into account the present results and explore the finer aspects of neuronal discharge characteristics like the firing patterns, multiple rhythms, oscillations, and synchronization in various regions of the basal ganglia circuitry.

Subthalamic nucleus (STN) and substantia nigra reticulata (SNR)

Since a change in the firing pattern of neurons from bursting to random pattern has also been implicated in the genesis of DID, recent work from our laboratory examined the effects of chronic LD treatments on the firing rate and firing pattern of STN and SNR neurons in the stable HP monkey model of PD without DID (Gilmour, Lieu et al. 2011). We also evaluated local field potentials (LFP) of both nuclei before and after LD treatments. We found that LD treatments did not significantly change the mean firing rate of STN neurons or bursting neuronal firing patterns. However, LD treatments induced a significant reduction of the mean firing rates of SNR neurons and a trend toward increased burstiness. The entropy of the spike sequences from STN and SNR
was unchanged by LD treatment, but there was a shift of spectral power into higher frequency bands in LFP. The results of our study act as a prelude to what may occur prior to the development of dyskinesias. This work is described in detail in Chapter 5.

In a recent study that recorded LFP from STN using an externalized deep brain stimulator electrode, a desynchronizing effect of LD was noticed on two separate rhythms of STN (Priori, Foffani et al. 2004). The oscillatory activity increased at low frequency (2–7 Hz range), while the beta oscillations significantly decreased in the low-beta range. Similar effects were observed with apomorphine. Others have shown that an increase in the theta/alpha band of the oscillatory activity can lead to DID in the presence of excess DA in the SNR of the 6-OHDA-lesioned rat (Meissner, Ravenscroft et al. 2006). These studies add to the evidence that the imbalance of multiple rhythm systems may lead to DID.

Another recent study examined the activity changes in the SNR of non-parkinsonian monkeys with apomorphine induced orofacial dyskinesias (Nevet, Morris et al. 2004). Recordings were performed before (no dyskinesias) and after (with dyskinesias) administration of apomorphine. They found that 96% of the cells recorded exhibited a change in firing rate after the dose of apomorphine, with 70% showing a reduction. As in our study with LD (Gilmour, Lieu et al. 2011), they did not observe significant changes in firing pattern. As a result, the authors suggest that DID are more related to a decrease in neuronal firing rate in the SNR, rather than a change in the firing pattern. More work is needed to understand the role of basal ganglia neuronal firing patterns and its relationship to the long-term symptomatic effects of LD treatment and DID.

**Striatal medium-spiny neurons (MSN)**

Striatal MSN respond to DA input to the striatum, mediated by D1 (excitatory) and D2 (inhibitory) DA receptors. The “direct” striatal output pathway largely consists of the D1 receptor type, whereas the “indirect” pathway consists of the D2 type as described above. In a recent study,
Liang and colleagues (Liang, DeLong et al. 2008) recorded from MSN of severely parkinsonian monkeys during three periods: 1.) “OFF” states in which the monkeys exhibited parkinsonian disability; 2.) “ON” states in which the monkeys were treated with LD and regained motor control; and 3.) during high doses of LD which induced DID. During the OFF state, the authors found a significant increase in neuronal firing rate (2.7 – 52Hz), which is in contrast to what has been classically observed in the normal animal (0.5-2Hz). This increase in firing rate was observed in MSN from both the D₁ and D₂ pathway. In the ON state, an overall increase in activity was observed, although some neurons exhibited an increase in firing rate (63.6%) and some a decrease (33.6%). It is assumed that the increases and decreases corresponded to the excitatory and inhibitory pathways, respectively. In the dyskinetic state, the overall firing rate was similar to that observed in the ON state. However, some neurons showed an increase in firing rate from OFF to ON to ON with DID, and some neurons showed an increase from OFF to ON and then a decrease during ON with DID. The authors suggest that this combination of uni- and bidirectional changes with increases in DA leads to an imbalance of MSN activity. Interestingly, this result correlates with the suggestion by others that although enabling movement, this imbalance of striatal activity may result in DID (Wichmann and DeLong 1996; Mink 2003).

1.8. Graft-Induced Dyskinesias

Cell grafts have been increasingly researched over the past three decades as a method of endogenously resupplying DA to the depleted basal ganglia in a continuous fashion. The primary type of cells used in the early studies was fetal ventral mesencephalic cells, progenitors to the nigral cells which degenerate in PD. In the 1990s and early 2000s, after animal studies and open-label clinical trials had shown therapeutic benefits of cell transplants, two double-blind placebo-controlled multicenter studies were funded by the National Institutes of Health (NIH).
The results were disappointing; some patients received symptomatic benefits, but many patients did not (Freed, Greene et al. 2001; Olanow, Goetz et al. 2003). Additionally, up to half of the patients developed dyskinetic movements that persisted even after multi-day withdrawal of dopaminergic medications. These symptoms have since been labeled graft-induced dyskinesias (GID) and look similar to diphasic dyskinesias. Several hypotheses have been proposed for the cause of these GID.

One of the first proposed causes of GID was that the grafts were producing "hotspots" of excessive DA in small, localized areas of the striatum. A related factor of dorsal versus ventral striatal placement was suspected. It was suggested that small dopamine-producing grafts might be reducing the striatal DA supersensitivity only in a small area around each graft, and this imbalanced and patchy reinnervation produced GID. In support of this hypothesis, Ma and colleagues (Ma, Feigin et al. 2002) saw significantly increased uptake of \(^{18}\)F-dopa in five patients with GID, with the increase localized to small focal areas in the grafted putamen. This hypothesis was also supported by two separate studies. In a study by Maries and colleagues, they show differential effects of single, focal transplantation striatal sites versus wide-spread grafts at multiple striatal sites on dyskinesias in parkinsonian rats (Maries, Kordower et al. 2006). Similarly, Carlsson and colleagues in parkinsonian rats show differential effects on DID comparing rostral versus caudal striatal grafts (Carlsson, Winkler et al. 2006). However, Piccini and colleagues showed using another \(^{18}\)F-dopa experiment that patients with GID did not show abnormal DA release from graft areas (Piccini, Pavese et al. 2005), and subsequent post-hoc analysis of larger numbers of grafted patients showed no correlation between striatal reinnervation and GID (Hagell, Piccini et al. 2002; Olanow, Goetz et al. 2003).

Another hypothesis was that GID were caused by immune system rejection of the grafts. Early analyses showed that some patients with GID showed low-level inflammation around their grafts (Hagell, Piccini et al. 2002), and GID worsened in some patients after immunosuppression
was stopped (Piccini, Pavese et al. 2005). An experiment which induced graft rejection in a rat model did not show an increase in abnormal involuntary movements. (Lane, Soulet et al. 2008). This experiment showed that a complete graft rejection is not associated with amphetamine-induced dyskinesias. This suggested that inflammation alone is insufficient to cause DID. A slow, chronic inflammation experiment by the same authors also failed to alter amphetamine-induced dyskinesias. These authors did not test LD-induced DID. However, a separate set of experiments by Soderstrom et al. using striatal FVM transplantation showed that synaptic connectivity between the graft and the host is significantly modulated by the host immune responses, and this indeed influences DID (Soderstrom, Meredith et al. 2008).

Because the risk of developing GID has been shown to vary depending on the patient, another hypothesis for the origin of GID is that it stems from the same pathophysiology as DID. All of the original patients in the Freed and Olanow NIH studies who developed GID had previously been exposed to many years of intermittent LD therapy, suggesting possible priming and hypersensitization of the striatum. Pre-transplant DID has been implicated in the development of GID and is increasingly seen as a counterindication for transplant (Lane, Bjorklund et al. 2010). Experiments in rodent PD models have similarly shown correlations between pre-transplant DID and post-transplant amphetamine-induced dyskinesias (Lane, Winkler et al. 2006; Lane, Brundin et al. 2009; Lane, Vercammen et al. 2009). However, Steece-Collier and colleagues have shown that GID develops regardless of LD priming status (Steece-Collier, Soderstrom et al. 2009). It remains unclear how the interaction of DA replacement pharmacological therapy history and cell transplants influence GID, and future studies that address this complexity are necessary.

Another hypothesis is that GID is the result of aberrant synaptic graft-host connectivity. Studies have previously shown degeneration of dendrites and dendritic spines on the MSNs in advanced PD (Stephens, Mueller et al. 2005). Soderstrom and colleagues recently looked at
synaptic connections to dendrites near graft sites, showing that decreases in tyrosine hydroxylase-positive synapses onto striatal dendritic spines and increases in asymmetric excitatory synapses correlate with GID in rat models (Soderstrom, Meredith et al. 2008). Further studies showed that chronic administration of nimodipine, a calcium channel blocker which had been shown to preserve striatal dendritic spines, reduced GID (Soderstrom, O'Malley et al. 2010). Another approach to avoiding aberrant graft synaptic connectivity and prevention of GID has been the use of retinal pigment epithelial (RPE) cells, which produce LD and possibly DA but do not form axons or synaptic connections (Subramanian, Marchionini et al. 2002; Bakay, Raiser et al. 2004). These RPE cells have been shown to provide symptomatic benefit in both animal trials and open-label clinical studies. However a recent placebo-controlled clinical trial with post-natal RPE failed to meet primary endpoint. It is thought that the switch from embryonic to post-natal source of RPE was the cause for this failure (Gross, Watts et al. 2011). Promising recent research with new inducible pluripotent stem cells and inducible neuron-like cells shows progress toward dopaminergic therapeutic grafts developed from patients’ own donor cells (Wernig, Zhao et al. 2008; Vierbuchen, Ostermeier et al. 2010).

A final hypothesis for the cause of GID is the accidental inclusion of serotonergic cells in the dopaminergic graft. Serotonergic neurons are known to be able to convert, store, and release DA under certain conditions (Tanaka, Kannari et al. 1999; Carlsson, Carta et al. 2009). The patients in the Freed NIH study who developed GID had been transplanted with neurons which had been cultured for several days, a technique which is known to increase the proportion of serotonergic neurons compared to dopaminergic neurons. It was recently shown in a rat PD model that serotonergic striatal grafts increased drug-induced dyskinesia activity (Carta, Carlsson et al. 2007; Carlsson, Carta et al. 2009). DID is not identical to GID, as DID is seen during and after the surge in DA which follows LD ingestion (de la Fuente-Fernandez, Sossi et al. 2004), and some studies show no relationship between GID and serotonergic innervation in the rat model.
(Lane, Brundin et al. 2009). However, recent studies in human patients continue to support the serotonergic cograft hypothesis for GID. Politis and colleagues used $^{11}$C-DASB PET to show that two grafted patients with GID showed much higher levels of striatal serotonin receptor expression than other grafted patients without GID (Politis, Wu et al. 2010). Furthermore, GID was significantly reduced by systemic administration of buspirone, a 5-HT agonist which reduces serotonin (and possibly DA) release from serotonin terminals.

In summary, the exact cause of GID still remains to be clarified, and several factors may well be involved. Promising hypotheses for reducing GID are garnering increasing experimental support, including selection of patients without severe DID, optimization of novel cell sources and transplant techniques to reduce immune reaction and decrease serotonergic progenitor cells, and pharmacological methods of preserving striatal spines and increasing striatal dopaminergic reinnervation.

1.9. Conclusion

In this chapter, we describe the pathophysiological mechanisms associated with DID in PD. Despite significant advances in the field for understanding the mechanisms associated with DID, treatment for DID in PD is largely unsatisfactory for many patients. There are numerous treatments including adjunct drugs for DID and functional neurosurgical treatments (described in Chapter 2) that are currently available to treat DID. However, these treatments are still limited in their efficacy to mitigate/prevent dyskinesias and can be out of reach for many PD patients. In Chapter 2, we also describe treatment strategies for DID in preclinical studies from our laboratory that could act as novel alternatives to currently available anti-PD and anti-dyskinetic therapies.

The prevailing hypothesis of DA receptor supersensitivity due to loss of continuous dopaminergic stimulation is still the unifying conceptual idea for DID. However, this idea still does not entirely explain the phenomenology of DID. Furthermore, based on various
electrophysiological reports in preclinical and clinical studies, the classic rate model of the basal ganglia does not completely explain DID. There are a number of alternate theories based on work from our laboratory for DID which include: interhemispheric inhibition (Chapters 3, 4 and 6) and basal ganglia neuronal firing pattern abnormalities (Chapter 5) as complementary pathophysiological mechanisms for DID. Advancing our understanding of the pathophysiological basis of DID will allow for better development of novel therapies that reduce or prevent DID.
Chapter 2

Current and emerging treatments for dyskinesias: adjunct treatments, functional neurosurgery, and novel therapies

2 The following chapter represents a modified version of the chapter “Pathophysiology of Drug-Induced Dyskinesias” in the book Parkinson’s disease/Book 3 ISBN 978-953-307-464-1 edited by Abdul Qayyum Rana, MD, FRCP. Authors: C Lieu, V Shivkumar, T Gilmour, K Venkiteswaran, M Nolt, M Deogaonkar, T Subramanian. CL and TS developed the concepts of the chapter. All authors contributed to writing the chapter and finalizing contents.

This chapter also contains a modified version of the journal article “A water extract of Mucuna pruriens provides long-term amelioration of parkinsonism with reduced risk for dyskinesias” published by Lieu et al., 2010 in Parkinsonism and Related Disorders, Volume 16, pp. 458-465. Authors: C Lieu, A Kunselman, B Manyam, K Venkiteswaran, T Subramanian. CL, AK, BV and TS developed the contents of the study. CL and KV conducted experiments. CL and AK did the statistics. CL and TS wrote the manuscript. All authors finalized the manuscript.

This chapter also contains a modified version of the manuscript “The anti-parkinsonian and anti-dyskinetic mechanisms of Mucuna pruriens in the non-human primate and rat: A novel treatment for Parkinson's disease” in revision in Parkinsonism and Related Disorders. Authors: C Lieu, K Venkiteswaran, T Gilmour, A Rao, A Petticoffer, E Gilbert, M Deogaonkar, D Desai, B Manyam, T. Subramanian. CL, KV, TG, AR, AP, EG, MD, and TS conducted animal experiments. DD evaluated extract content. BM aided in experimental design and methods. CL, TG and TS wrote initial drafts and finalized contents.

This chapter also contains a modified version of the manuscript “The novel D1 full agonist EFF0311 ameliorates parkinsonism in the rat model of Parkinson’s disease” in preparation. Authors: C Lieu, V Murthy, R Mailman, T Subramanian. All authors contributed in experimental design. CL and VM conducted the experiments. CL wrote the initial draft. All authors finalized contents.
2.1. Introduction

There have been significant advances in understanding the pathophysiological mechanisms associated with DID as described in Chapter 1. This had led to the development of various treatment strategies that target DID. We first describe adjunct drug treatments that reduce the severity of dyskinesias. These treatments are utilized in conjunction with DA replacement therapies, typically LD. We next describe functional neurosurgical options when pharmacological therapies provide minimal anti-PD effects or when patients experience severe DID. Unfortunately, most of these treatments are currently limited in effectively diminishing DID or unattainable for many patients. Finally, we describe two preclinical studies we have conducted that examine potential, novel alternative treatments to currently available anti-PD and antidykinetic therapies. Results from our studies suggest that these novel therapies could be alternative treatments that both ameliorate parkinsonian motor disability with reduced risk for the development of DID.

These various treatment strategies provide insights into pathophysiological mechanisms that underlie DID. The scientific knowledge gained through the use of various pharmacological agents that target neurotransmitter systems provide underpinnings for putative pathophysiological mechanisms. Similarly, functional neurosurgical therapies such as ablative treatments and deep brain stimulation (DBS) performed at various targets of the basal ganglia provide putative clues for the pathophysiology of DID. In this chapter, we summarize the literature to date that either support existing pathophysiological theories or contradict them, setting the stage for the development of novel hypotheses for DID.

2.2. Adjunct Drugs To Reduce DID

Amantadine
Amantadine is a NMDA receptor antagonist and has been found to be efficacious in the treatment of DID without reducing antiparkinsonian benefits. Its antidyskinetic effect gives support to the glutamatergic theory as a pathophysiological mechanism. Double-blind placebo-controlled studies have demonstrated 45% to 60% reductions in dyskinesias. Benefits are typically seen in 3 weeks following initiation of treatment. Although the benefits of Amantadine have been shown to last for only 8 months to 1 year in some studies (Sawada, Oeda et al. 2010), a more recent study has shown that the antidyskinetic effects last longer than 1 year and has advocated the continued use of Amantadine for the treatment of dyskinesias (Wolf, Seppi et al. 2010). Also, discontinuation of Amantadine has been shown to worsen dyskinesias. It is likely that there are plasticity effects that underlie the decrease in efficacy of Amantadine.

Sarizotan

Sarizotan is a 5-HT1A receptor agonist and is a high affinity antagonist for D3 and D4 receptors. It is possible that its beneficial effects come from both 5-HT receptor agonism and DA receptor antagonism. It has been found to reduce dyskinesias in 6-OHDA lesioned rats and in MPTP-lesioned monkeys (Bibbiani, Oh et al. 2001). In open label studies, Sarizotan showed promising results in decreasing DID (Olanow, Damier et al. 2004). A double-blinded placebo-controlled study demonstrated significant decrease in the duration and severity of DID on the UPDRS (Appendix A) with 2mg/day Sarizotan compared to placebo. UPDRS is a standard rating tool used in clinical research (see Appendix A) (Goetz, Damier et al. 2007).

Levetiracetam

Levetiracetam, a GABA analog, is an anti-epileptic drug that appears to have some anti-DID effects. The mechanisms of the antidyskinetic effects of Levetiracetam are unknown. It has been found to reduce dyskinesias in MPTP lesioned primates. Tousi and Subramanian were the
first to report improvement in DID upon treatment with low doses of Levetiracetam in an open
label study (Tousi and Subramanian 2005). Other open label studies provided mixed efficacy
results and poor tolerability due to somnolence. More recently, two double-blinded placebo-
controlled studies have evaluated the efficacy and safety of Levetiracetam in the treatment of
dyskinesias (Stathis, Konitsiotis et al. 2010; Wolz, Lohle et al. 2010). Both studies found a
significant decrease in DID. Furthermore, both studies did not demonstrate worsening of
parkinsonian symptoms. The most common side effects are somnolence and dizziness. However,
in contrast to the open label studies that reported intolerable side effects leading to high dropout
rates, the double-blinded placebo-controlled studies did not report severe adverse effects. It has
been hypothesized that the beneficial effects of Levetiracetam could be due to modulation of the
pathological synchronization and desynchronization of neuronal circuits of the basal ganglia,
specifically of GABAergic nuclei, and correction of maladaptive DA release and reuptake.

DA agonists

DA agonists are often used as adjuncts to LD in advanced PD. DA agonists exert their
pharmacological effect by directly activating the DA receptors bypassing the presynaptic
synthesis of dopamine. These include non-ergot compounds, such as Ropinirole and Pramipexole,
and apomorphine. DA agonists when used as the initial form of therapy can help to delay onset of
LD-induced complications. In patients who have already developed DID, addition of a dopamine
agonist may permit a reduction in LD dose without worsening of parkinsonism. The addition of a
DA agonist might result in worsening of DID in some patients already taking significant
quantities of LD, but this can be corrected by lowering the dose of LD. If lowering the dose of
LD results in increased “off” states, then the agonist dose needs to be increased. However,
patients experiencing severe DID are rarely controlled with this regimen in the long-term.
Apomorphine is a parenteral solution that can be used for acute “off” phenomenon in PD and may
have some benefit in some patients with DID. Apomorphine being water-soluble can be injected subcutaneously or applied intranasally. The use of continuous subcutaneous apomorphine infusion has been found to abort “off” periods, reduce dyskinesias, and improve PD motor scores, with the added benefit of an LD-sparing effect (Deleu, Hanssens et al. 2004). Apomorphine can cause severe nausea and vomiting due to its fast onset of action and stimulation of D₂ DA receptors involved in emesis in the area postrema (Hinson 2010).

2.3. Surgical Management Of DID

DID in some patients can be a complication that limits the ability to pharmacologically control the symptoms of advanced PD. In such patients, surgical intervention becomes necessary. Ablative surgeries in the past had a relatively limited role due to the nature of the procedure, irreversibility and the inability to modulate the therapy according to the need of the patient. Because of these limitations, the advent of deep brain stimulation (DBS) with its numerous advantages over ablative surgeries described in the following sections has made surgical management of PD and DID more accepted in patients (Rezai, Machado et al. 2008). Such surgical procedures are effective in the treatment of LD-induced motor complications, such as DID, that cannot be satisfactorily controlled with medical therapies (Guridi, Obeso et al. 2008). The modalities by which surgical interventions reduce dyskinesias are multifold: 1) Reduction in daily DA intake; 2) Increasing on-time and thus reducing the repetitive LD dosing schedules; 3) Direct anti-dyskinesia effect.

Ablative Procedures

Ablative procedures effective in controlling DID include:

Thalamotomy
The anti-dyskinesia effect of thalamotomy has been variable. Ventral intermedia nucleus (VIM) of thalamus is not a part of the pallidal receiving area and hence does not have an anti-dyskinesia effect (Tasker, Munz et al. 1997). A lesion in the pallidal receiving area of thalamus (nucleus ventralis oralis and ventralis posterior) has been shown to have profound anti-dyskinesia effects (Narabayashi, Yokochi et al. 1984). In a study of thalamotomy in MPTP monkeys, Page et al., found that a lesion of the pallidial outflow receiving areas of the thalamus had a significant anti-dyskinesia effect but similar lesions in cerebellar or nigral outflow receiving areas (VIM) had no anti-dyskinesia effect (Page 1992; Page, Sambrook et al. 1993).

Pallidotomy

Posteroventral pallidotomy has been shown to have a significant and sustained anti-dyskinesia effect (Lozano, Lang et al. 1995; Baron, Vitek et al. 1996). A randomized, controlled trial by Vitek et al. (Vitek, Bakay et al. 2003) comparing unilateral pallidotomy with medical therapy showed improvement in contralateral dyskinesias in all patients with a significant reduction in ipsilateral dyskinesias. Several earlier studies confirm the significant and sustained anti-dyskinesia effect of pallidotomy (de Bie, de Haan et al. 1999; Merello, Nouzeilles et al. 1999). The mechanism of action of pallidotomy in reducing DID is more complex. Pallidotomy improves PD symptoms by reducing pallidal neuronal activity, which in turn restores thalamocortical excitability. This should theoretically worsen the DID. The anti-dyskinetic effect of pallidotomy is considered the function of normalizing the pattern of firing of GPI (Guridi, Obeso et al. 2008). The optimal lesion location within the GPI has been variously argued to be anteromedial (Gross, Lombardi et al. 1999) and posteroventral (Krauss, Desaloms et al. 1997). Some of these aspects have been discussed in Chapter 1. This result from pallidotomy argues that there is deficiency in current basal ganglia models to explain dyskinesias. While pallidotomy would be expected to worsen dyskinesias, it does not. The electrophysiological “hallmark” of
DID is electrographic suppression/silence. Perhaps inactivity in GPI has an alternative pathophysiological mechanism besides the release of the thalamocortical pathways. This issue needs to be further evaluated. One putative hypothesis from this finding is the notion that interhemispheric pathways play a significant role in modulating electrophysiological changes in downstream basal ganglia structures from the striatum (discussed in Chapter 4 and 5).

Subthalamotomy

Subthalamotomy has been performed in only a small number of patients due to the risk involved in the procedure. Alvarez et al. (Alvarez, Macias et al. 2001) reported no anti-dyskinesia effect of unilateral subthalamotomy in the short-term or long-term (Alvarez, Macias et al. 2009) follow-up. Around 15% of patients (14 patients) with unilateral subthalamotomy in this study developed postoperative hemichorea-ballism which required an additional pallidotomy in eight patients (Alvarez, Macias et al. 2009). On the other hand, Su et al. (Su, Tseng et al. 2003) reported a significant reduction (75%) in dyskinesias after unilateral subthalamotomy in their study. They also state that the lesions in patients with anti-dyskinesia effect were larger and probably affected the pallidofugal fibers. With a significant risk of developing postoperative hemichorea-ballism and variable anti-dyskinetic response, subthalamotomy is probably the least useful procedure for treating dyskinesias.

All of these ablative procedures are associated with an increased risk of hemorrhage and bilateral ablative procedures are associated with further risks, including speech, swallowing, and cognitive problems. With the advent of DBS, ablative lesions are now rarely performed.

Deep Brain Stimulation (DBS)
DBS is routinely performed on patients with medically intractable PD. The targets for DBS in PD have included a number of nodal points in the basal ganglia thalamocortical circuit. These include the VIM of the thalamus, the GPI and the STN (Rezai, Machado et al. 2008). VIM DBS predominately improves tremor; GPI and STN have been the primary targets for the treatment of the motor symptoms associated with PD. Though GPI and STN DBS both improve PD symptoms (e.g., tremor, rigidity and bradykinesia), there is a continued debate over which site is more effective in improving motor symptoms, reducing PD medications and controlling medication associated side effects such as DID and motor fluctuations (Krack, Pollak et al. 1998; Burchiel, Anderson et al. 1999; Limousin-Dowsey, Pollak et al. 1999; Allert, Volkmann et al. 2001; Krause, Fogel et al. 2001; Volkmann 2004; Anderson, Burchiel et al. 2005). Another area of interest is stimulation of the pedunculopontine nucleus (PPN) for PD.

**VIM DBS**

VIM DBS provides excellent tremor relief, but does not have anti-dyskinesia effect, as shown in various studies (Benabid, Pollak et al. 1996; Tasker, DeCarvalho et al. 1996; Limousin-Dowsey, Pollak et al. 1999). However, some anti-dyskinesia effect is observed in VIM DBS when the electrode is more posterior, medial, and deeper, probably modulating the centromedian and parafascicular complex (Caparros-Lefebvre, Blond et al. 1993).

**GPI DBS**

Most major studies have reported that GPI DBS is effective in reducing all the cardinal motor signs of PD as well as improving motor fluctuations, reducing dyskinesias and increasing on time (DBS for Parkinson's Disease Study Group 2001). Ghika et al. (Ghika, Villemure et al. 1998) reported that the mean off time decreased from 40% to 10%, and the mean dyskinesia scores were reduced to one-third. Burchiel et al. reported a significant reduction in dyskinesias
(Burchiel, Anderson et al. 1999), while Kumar et al. reported that the reduction in the total "on" dyskinesias score was 66% (Kumar, Lang et al. 2000). Volkmann et al. reported a sustained reduction in dyskinesias at 5 years follow up of 64% (Volkmann, Allert et al. 2004). Rodrigues et al. reported a reduction in dyskinesia scores by 76% (Rodrigues, Walters et al. 2007) out 4 years. Several such studies have confirmed that pallidal stimulation is associated with a marked reduction in contralateral DID in addition to improvements in “off”-period. The duration of benefit on motor complications following DBS in GPI is sustained. The location of the DBS lead has an effect on the anti-dyskinetic effect of GPI DBS. Bejjani et al. (Bejjani, Damier et al. 1997; Bejjani, Damier et al. 1998) have demonstrated different clinical effects after stimulation of the dorsal and the posteroventral part of the globus pallidus (GP). With stimulation in the more dorsal portions of the pallidum, they reported improvement in akinesia and rigidity, but an exacerbation of dyskinesias. Stimulation in the posteroventral portion of GP had a pronounced anti-dyskinetic effect, but worsened bradykinesia.

**STN DBS**

STN DBS has been the established modality of therapy for advanced PD patients since the initial studies by the group of Dr. Benabid in Grenoble (Limousin, Pollak et al. 1995; Krack, Limousin et al. 1997). STN DBS has shown a dramatic and sustained anti-dyskinesia effect in various major studies (Limousin, Pollak et al. 1995; Krack, Limousin et al. 1997; Limousin, Krack et al. 1998; Benabid, Benazzouz et al. 2000). The effect of STN DBS on DID is homogeneous and well accepted. Patients undergoing STN DBS have a significant antidyskinetic effect that can be closely correlated with a reduction in LD dose (Guridi, Obeso et al. 2008). STN DBS improves peak-dose as well as diphasic dyskinesia (Krack, Limousin et al. 1997). It also results in significant reduction (47%) in LD dose (Krack, Limousin et al. 1997). The reduction in dose and DID is sustained over a long period. In a survey published by Hamani et al. of multiple
studies involving 737 patients in 34 neurosurgery units, STN DBS improved DID by 73% at 6 months and 94% at 12 months in the on-stimulation on-medication state in comparison to the preoperative on-medication scores (Hamani, Richter et al. 2005). Long-term studies of bilateral DBS-STN in patients with advanced PD demonstrate the stability of this therapeutic efficacy.

**PPN DBS**

PPN DBS has been used in a number of PD patients for gait and postural impairment (Tsang, Hamani et al. 2010). Early studies suggested that PPN DBS could be utilized in patients who respond poorly to anti-PD medications or other neurosurgical treatments (Plaha and Gill 2005). This was later confirmed in patients receiving both PPN and STN DBS (Stefani, Lozano et al. 2007). Although the authors suggest that this procedure is appropriate for treating parkinsonian symptoms, they indicate that PPN DBS is not suitable in targeting DID. In 2010, Ferraye and colleagues found that PPN DBS only provided modest amelioration of parkinsonian symptoms. According to their findings, dyskinesias were not alleviated with PPN stimulation (Ferraye, Debu et al. 2010).

**DBS Conclusion**

It is evident that DBS is an effective therapy for PD patients with motor complications like DID. The primary benefit of DBS is a reduction in both dyskinesias and “off” time. Stimulation of both the GPI and the STN are effective in treating the motor features of PD and LD related motor complications like DID, but the preferable target remains a controversial topic. It is possible that stimulation of the GPI has a more direct effect in blocking dyskinesias, while reduction in dyskinesias with STN DBS may primarily relate to a reduction in LD dose. A recent study compared the effects of STN DBS and GPI DBS (Follett, Weaver et al. 2010). Subthalamic and pallidal DBS resulted in improvement in motor function, reduction in dyskinesias and
reduction in dose of dopaminergic medications. Effects on motor function and dyskinesias did not differ significantly between the two groups. Patients undergoing subthalamic stimulation required a significantly lower dose of dopaminergic agents than did those undergoing pallidal stimulation. The difference may be an important consideration in patients having side effects, as a reduction in medications may lead to a better quality of life.

Although there is success with surgical treatment for ameliorating DID, surgical intervention has major complications such as morbidity and death (up to 4%). Since DBS involves electrical stimulation and hardware, frequent replacement of malfunctioning electrodes (up to 19%) and infection (up to 2.5%) are a major issues (Bronstein, Tagliati et al. 2011). Patients also need post-operative visits to optimize treatment. Further, most patients still require anti-PD medications in conjunction with DBS. DBS treatment for DID is mainly palliative and not curative. Hence, it is important to understand the other pathophysiological mechanisms associated with DID and to develop other antidyskinetic therapies. Despite these drawbacks, surgical therapies for PD and DID has given us insights into the pathophysiological mechanisms that underly DID. For example, it is clear that VIM is not involved in controlling the genesis of DID. On the other hand, other thalamic nuclei seem to be significantly involved. It is also clear that certain portions of the GPI are involved in the genesis of DID. It also appears that STN stimulation primarily works by reduction of LD dose and not necessarily the reduction of basal ganglia circuit abnormalities that are causative of DID. This could be validated by increasing LD dose to evaluate if higher doses still cause DID during STN stimulation. Several studies have shown that this indeed is the case and that LD dose reduction is the primary mechanism. Turning off the DBS after the acute lesion effect of STN-DBS show that LD doses need to be increased to provide sufficient anti-PD effects and such increase is accompanied by DID reemergence.

In this context, if there is a drug that does not cause DID, this would be highly desirable for many advanced PD patients. Discovery of novel drugs that provide that same anti-PD benefit
as LD without causing DID would mitigate the need for surgical intervention or adjunct therapies. Hence, investigators continue to examine this option. This would also provide insights into possible mechanisms involved in DID. In light of this, we performed the following studies and propose two novel therapies for the treatment of DID.

2.4. Novel Therapies For DID – *Mucuna pruriens*

*Mucuna pruriens* (*M. pruriens*) is a legume plant indigenous to South Asia. The dried endocarp powder of the *M. pruriens* bean is used to treat parkinsonism (*Kampavata*) in the Ayurvedic Indian medical system (Manyam 1990; Manyam and Sanchez-Ramos 1999). Review of the Ayurvedic literature and current Ayurvedic practitioners indicate that PD patients treated with *M. pruriens* do not develop DID. *M. pruriens* has been shown to contain LD (Damodaran and Ramaswamy 1937; Bell and Janzen 1971; Vaidya, Aloorkar et al. 1978; Vaidya, Sheth et al. 1978; Pras, Woerdenbag et al. 1993; Mahajani, Doshi et al. 1996; Modi, Patel et al. 2008), which has been presumed to be the mechanism of its action in PD. Under this presumption, many experimental studies have concomitantly administered *M. pruriens* with a dopa-decarboxylase inhibitor (DDCI) like benserazide (BZ, (Kasture, Pontis et al. 2009)) or carbidopa (CD, (Hussian and Manyam 1997)); or used subjects that concurrently took a DDCI as part of their treatment regimen without adequate washout (Katzenschlager, Evans et al. 2004). However, no DDCI is utilized in Ayurveda with *M. pruriens* treatment. Many of these previous studies have also suggested that the anti-PD effects of *M. pruriens* could not be entirely explained by the 4-5% of LD, and that there are other natural ingredients present that have anti-PD and antidyskinetic effects. Therefore, we conducted a comprehensive set of experiments in the HP rat and primate models of PD to assess the anti-parkinsonian and anti-dyskinetic effects of *M. pruriens*. In these studies, we demonstrate that *M. pruriens* has superior anti-PD and anti-dyskinetic effects when compared to LD treatment. Furthermore, our studies prove that LD is unlikely to be the only
active agent in *M. pruriens*, and that other compounds in combination with LD accounts for its mechanism of action.

**Study #1** - A water extract of *Mucuna pruriens* provides long-term amelioration of parkinsonism with reduced risk for dyskinesias (Lieu, Kunselman et al. 2010).

**Materials and Methods**

**Animals**

Female Sprague-Dawley rats (250-400 g) were used. Procedures were conducted in compliance with institutional protocols, and in accordance with the NIH for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). The diagram of the experimental paradigm is shown in Figure 2-1.
Figure 2.1: Diagram for MPE experiments

- Normal Rats
- MFB 6-OHDA Lesioning
  - 3-4 wks
- Lesion Validation
  - (> 245 apomorphine rotation/35 mins)
  - 2 wks washout
- Experimental Design
  - Baseline, Post-lesion Behavior

**Experiment 1 (N=21)**
- LD+BZ/MPE+BZ
- Group 1: 6 mg/kg
- Group 2: 4 mg/kg
- Group 3: 2 mg/kg
- Stepping/DID – 30 mins

**Experiment 2 (N=7)**
- MPE Alone
- Dose 1: 240 mg/kg
- Dose 2: 400 mg/kg
- Stepping/DID – 30 mins

**Experiment 3 (N=6)**
- Long-term MPE Alone
- Dose: 400 mg/kg
- Stepping/Vibrissa/Body Axis/Cylinder/DID – 90 mins

**Experiment 4 (N=3)**
- Group 1: LD alone
- Group 2: MPE alone
- Group 3: LD+BZ
- Stepping/Vibrissa/DID – 90 mins
- DID – 90 mins

**Experiment 5 (N=10)**
- Group A: LD+BZ then MPE alone
- Group B: MPE alone then LD+BZ
- DID – 90 mins
6-OHDA lesion and rotational validation for HP state

Rats were unilaterally lesioned using techniques we have previously described (Subramanian, Marchionini et al. 2002). Briefly, rats were anesthetized with ketamine/xylazine and placed in a stereotactic frame. 6-OHDA was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 2.0 mg/ml. The skull was exposed, small burr holes made, and stereotaxic injections at a rate of 1.0 μl/min for 2 min were made into the medial forebrain bundle (AP: -4.4, ML: 1.2, DV: -7.5) and the substantia nigra (SN) (AP: -5.3, ML: 2.0, DV: -7.5) in relation to bregma and dural surface. After completion of surgery, the incision was sutured and rats were allowed to recover. Apomorphine was administered at 0.2 mg/kg (s.c.) at 3 and 5 weeks after 6-OHDA exposure to measure apomorphine-induced rotations. Only HP rats with >245 rotations over 35 min were used in the study. A prolonged drug holiday was taken before treatments were performed.

Treatment with LD and MPE

A simple water extract of *M. pruriens* endocarp powder (MPE) was developed based on the presence of approximately 4-5% LD content in *M. pruriens* (Mahajani, Doshi et al. 1996) and HPLC estimates of the *M. pruriens* endocarp powder that we used in this study (HPLC data on file, Zandu Pharmaceuticals, Mumbai, India). *M. pruriens* endocarp powder was obtained by Zandu Pharmaceuticals, Mumbai, India, and grown with no batch-to-batch variability, utilizing standardized HPLC techniques. *M. pruriens* endocarp powder was mixed in sterile water for 30 min then centrifuged at 15,000 RPM for 15 min. The supernatant was extracted, filtered and stored in sterile containers at 4°C for up to 1 week and dispensed daily. MPE was agitated briefly before each animal exposure and remained stable and clinically efficacious as a refrigerated solution for 1 week. Treatments were given IP on alternating sides of the abdomen. In experiment 1, we administered benserazide (BZ) at a dose of 15 mg/kg along with LD (LD methyl ester) or
MPE. In experiment 2 and 3, we used parenteral MPE alone without any additives. In experiment 4, we used LD alone, MPE alone and LD+BZ. In experiment 5, we used LD+BZ and MPE alone.

**Experimental design**

*Experiment #1 - comparison between equivalent doses of LD+BZ and MPE+BZ*

Twenty-one HP rats separated into three groups received LD for 10 days b.i.d. at 6 mg/kg (Group 1), 4 mg/kg (Group 2), and 2 mg/kg (Group 3) with 15 mg/kg of BZ (LD+BZ). After 7 day drug-washout, animals received the equivalent LD dose of 120 mg/kg MPE (Group 1), 80 mg/kg MPE (Group 2), and 40 mg/kg MPE (Group 3) with 15 mg/kg of BZ (MPE+BZ) for 10 days b.i.d. Behavioral assessments (stepping test and DID ratings) were taken 30 min after treatment.

*Experiment #2 - exposure to high doses of MPE alone*

HP rats (N=7) were exposed to MPE alone at 240 mg/kg (equivalent to 12 mg/kg LD) for 10 days b.i.d. After drug-washout for 7 days, rats were exposed to 400 mg/kg (equivalent to 20 mg/kg LD) for 10 days b.i.d. Behavioral assessments (stepping test and DID ratings) were taken 30 min after treatment.

*Experiment #3 - long-term efficacy of MPE alone*

In this experiment, HP rats (N=6) received MPE alone at 400 mg/kg (equivalent to 20 mg/kg LD) b.i.d. for 10 days to evaluate long-term effects up to 90 min post-treatment. Behavioral assessments for amelioration of parkinsonism (stepping test, vibrissae-evoked forelimb placement test, body axis bias test and cylinder test) were taken at 30 min, 60 min, and 90 min after treatment. DID and contralateral turning was assessed at 5 min, 15 min, 30 min, 60 min, and 90 min after treatment.
Experiment #4 - long-term anti-parkinsonian efficacy comparison for LD alone, MPE alone and LD+BZ

In the following experiment, we directly assessed whether equivalent doses of LD alone or MPE alone without BZ could induce equivalent behavioral benefit. HP rats were treated with LD alone (24 mg/kg), MPE alone at 480 mg/kg (equivalent to 24 mg/kg LD), or LD+BZ (6 mg/kg LD + 15 mg/kg BZ) for an average of 4 days b.i.d. (N=5 per group). Behavioral assessments for parkinsonism alleviation (stepping test and vibrissae-evoked forelimb placement test) were taken at 30 min, 60 min, and 90 min after treatment exposure. Our preliminary blinded studies showed that LD+BZ treatment induced DID that appeared to interfere with the animal’s performance of the cylinder and body axis bias tests at 60 min. The stepping test and vibrissae-evoked forelimb placement tests were not significantly affected by DID. Therefore, we excluded the cylinder and body axis bias tests in this experiment.

Experiment #5 - long-term drug-induced dyskinesia priming effects of LD+BZ and MPE alone

In a cross-over experimental design to examine dyskinesia priming effects, Group A was first treated chronically with LD+BZ (6 mg/kg LD + 15 mg/kg BZ) for 10 days. After 10 days of LD+BZ, animals were then treated chronically with MPE alone at 480 mg/kg (equivalent to 24 mg/kg LD) (N=5) for another 10 days with no drug-washout between treatments. Group B was first treated chronically with MPE alone at 480 mg/kg (equivalent to 24 mg/kg LD) for 10 days. After 10 days of MPE, animals were then treated chronically with LD+BZ (6 mg/kg LD + 15 mg/kg BZ) (N=5) for another 10 days with no drug-washout between treatments. DID ratings were taken at 5 min, 15 min, 30 min, 60 min, and 90 min after treatment.

Behavioral assessment
**Stepping test**

A modified version of the stepping test (Olsson, Nikkhah et al. 1995) was used to measure forelimb akinesia and rigidity. The animal is held by the experimenter restraining both hindlimbs and one forelimb. The unrestrained forelimb touches the tabletop. The number of adjusting steps was counted while the rat is moved sideways along the surface at a rate of 90 cm/5 s in the direction of the unrestrained forelimb. The test was done separately for both forelimbs. Stepping test scores are expressed as the difference between the unaffected (ipsilateral to lesion) and affected forelimb (contralateral to lesion). Trials where animals struggled were not recorded.

**Vibrissae-evoked forelimb placement test**

This test was performed as described by Dr. Schallert (Schallert, Fleming et al. 2000; Schallert 2006). Animals were gently held at the torso. The hindlimbs and forelimb being tested were allowed to hang freely while the forelimb not being tested was carefully restrained. Each forelimb was evaluated by bringing the rat towards the edge of the tabletop to elicit a forelimb reaching behavior towards the surface. Each forelimb was tested independently for 10 trials. The number of successful forelimb placements for each forelimb onto the tabletop was scored.

**Body axis bias test**

A modified version of the body axis bias test (curling behavior) was used to measure posturing activity (Borlongan and Sanberg 1995; Warraich, Allbutt et al. 2009). The rat was placed individually in a plastic testing cage. After attaining a neutral position with all four limbs touching the bottom of the cage, the rat was vertically lifted by the base of the tail so its head was approximately 1 inch from the cage bottom. The first direction of body deviation away from vertical axis of $\geq 10^\circ$ was recorded during a 5s interval. An ipsilateral turn towards the lesioned side was scored with a +1, contralateral turn away from lesioned side scored as -1, and no bias as
0 during the 5 s interval. This behavior was recorded twice at each time point.

**Cylinder test**

A modified version of the cylinder test was used to measure forelimb usage preference in spontaneous, exploratory behavior (Schallert, Fleming et al. 2000; Schallert 2006). Rats were placed into a clear cylinder (25 cm tall, 16 cm ID) and videotaped for 3-10 min depending on the activity of each animal. Time spent in the cylinder was either limited to approximately 20 total contacts or scored for the entire 10 min trial period. The number of unaffected, affected, and both forelimb placements were counted. Limb asymmetry scores were calculated using the following formula: (# of unaffected limb contacts + 0.5 x both forelimb contacts)/total contacts x100.

**DID ratings**

DID were rated using a scale similar to previous reports (Steece-Collier, Collier et al. 2003; Steece-Collier, Soderstrom et al. 2009). The term “dyskinesia” is used to describe abnormal involuntary movements of the forelimb, neck, and trunk characterized by dystonic posturing, hyperkinesias and stereotypies. Animals were placed in individual cages and sessions videotaped for 2 min. Intensity rating scores were 0-none, 1-mild, 2-moderate, and 3-severe. Frequency ratings ranged from 0-none, 1-present < 50% of observation session, 2-present > 50% of observation session, and 3-present for entire session. Frequency and intensity were multiplied to attain individual severity scores. Profiles are the sum of limb, neck and trunk severity scores. Behavioral assessments were done in a blinded fashion such that the investigators were unaware of treatment in the animals.

**Tyrosine hydroxylase (TH) immunohistochemistry**

Animals were deeply anesthetized with pentobarbital and euthanized via transcardial
perfusion with heparinized saline and paraformaldehyde-lysine-periodate fixative (McLean and Nakane 1974). Brains were removed, cryoprotected and sectioned coronally at 60 µm. Sections were processed using a modified procedure previously described for TH immunohistochemistry (Subramanian, Emerich et al. 1997; Subramanian, Marchionini et al. 2002). Briefly, sections were treated with anti-TH antibody (1:350, Pel-freeze) for 72 h, washed and treated with biotin-donkey anti-rabbit IgG (1:300). Subsequently, sections were treated with Avidin-HRP (1:500), developed through a DAB reaction, mounted and coverslipped before microscopic evaluation and photography. Stereological counts using optical fractionator was used to estimate TH+ SN pars compacta (SNC) neurons to confirm that there was >92% loss on the lesioned hemisphere.

Statistical analysis

Repeated measures or one-factor ANOVA was used with post-hoc Tukey-Kramer Multiple Comparisons Test for behavioral comparisons. Data are expressed as mean ± SEM. Significance was set at p < 0.05.

Results

Experiment #1 - comparison between equivalent doses of LD+BZ and MPE+BZ

Stepping test

Scores for Group 1 (equivalent to 6 mg/kg LD) were 10.3 ± 2.9 post-lesion which significantly decreased to -1.9 ± 2.3 with LD+BZ (p < 0.05 vs. post-lesion) and -1.2 ± 2.5 with MPE+BZ (p < 0.05 vs. post-lesion). Group 2 (equivalent to 4 mg/kg LD) post-lesion scores were 11.6 ± 3.3 which decreased to 1.7 ± 2.1 with LD+BZ (p < 0.05 vs. post-lesion) and -1.8 ± 2.5 with MPE+BZ (p < 0.01 vs. post-lesion). The scores for Group 3 (equivalent to 2 mg/kg LD) were 6.9 ± 2.0 post-lesion. Scores decreased to 3.0 ± 0.96 with LD+BZ but were not significant (p > 0.05 vs. post-lesion). However, there was a significant decrease to 1.1 ± 1.8 with MPE+BZ.
(p < 0.05 vs. post-lesion) in these animals (Fig. 2-2A).

**Dyskinesia profile**

Profiles for Group 1 were 18.3 ± 2.3 for LD+BZ and 19.0 ± 2.1 for MPE+BZ. Dyskinesia profiles for Group 2 were 11.7 ± 3.5 for LD+BZ and 12.9 ± 3.4 for MPE+BZ. Profile scores for Group 3 with LD + BZ were 0.93 ± 0.24 and 2.4 ± 0.87 for MPE+BZ. Group 3 showed significantly less DID than Group 1 and 2 for both LD+BZ and MPE+BZ (p < 0.05) (Fig. 2-2B).

These results show that LD+BZ and MPE+BZ at high and medium doses provide significant anti-parkinsonian effects but induce severe dose-dependent DID. However, only the lowest MPE+BZ dose was able to significantly ameliorate parkinsonism, whereas the equivalent synthetic LD+BZ dose did not provide significant behavioral benefit.
Figure 2-2. Comparison of LD+BZ and MPE+BZ. (A) Group 1 (equivalent to 6 mg/kg LD) and Group 2 (equivalent to 4 mg/kg LD) showed significant amelioration of parkinsonism as shown by the stepping test when exposed to both LD+BZ and MPE+BZ (*p < 0.05 vs. post-lesion, **p < 0.01 vs. post-lesion). However, doses given to Group 3 (equivalent to 2 mg/kg LD) indicate that only MPE+BZ had significant relief of parkinsonism. (B) There was a dose-dependent severity of DID among the 3 groups. There was no significant difference within groups (**p < 0.001 Group 1 vs. Group 3; **p < 0.01 Group 2 MPE+BZ vs. Group 3; *p< 0.05 Group 2 LD+BZ vs. Group 3 LD+BZ).

Experiment #2 - exposure to high doses of MPE alone

Stepping test

Scores were 13.1 ± 1.1 at post-lesion. Scores after treatment with 240 mg/kg of MPE significantly decreased to 5.0 ± 1.3 (p < 0.01 vs. post-lesion) and 4.1 ± 1.6 (p < 0.01 vs. post-lesion) with 400 mg/kg of MPE (Fig. 2-3A).
Dyskinesia profile

Dyskinesia profiles for animals exposed to 240 mg/kg of MPE were 0.69 ± 0.16. Profiles for exposure to 400 mg/kg of MPE were 1.6 ± 0.87. These scores were significantly different from dyskinesia profiles of Group 1 (LD+BZ and MPE+BZ) from experiment #1 (p < 0.001) (Fig. 2-3B).

These results suggest that MPE without any add-on DDCI (BZ) can provide behavioral benefit with a reduction in DID severity.
Figure 2-3. High doses of MPE alone. (A) Animals treated with MPE alone at 240 mg/kg MPE and 400 mg/kg MPE (equivalent to 12 mg/kg and 20 mg/kg LD) showed significant amelioration of parkinsonism in the stepping test (**p < 0.01 vs. post-lesion). (B) MPE caused significantly less DID than doses of LD+BZ and MPE+BZ (***p < 0.001 vs. LD+BZ and MPE+BZ).

Experiment #3 - long-term efficacy of MPE alone

Stepping test

Scores at post-lesion were 15.6 ± 1.5 which significantly improved after treatment with 400 mg/kg of MPE alone with scores of 6.9 ± 1.2 at 30 min, 5.1 ± 0.98 at 60 min, and 8.7 ± 0.71 at 90 min (p < 0.001 vs. post-lesion) (Fig. 2-4A).
**Vibrissae-evoked forelimb placement test**

Post-lesion scores showed significant deficit with values of 0.67 ± 0.40. The unaffected forelimb showed no behavioral deficits. However, significant amelioration of parkinsonism was evident after MPE alone at 30 min with a score of 7.1 ± 0.77, 9.1 ± 0.36 at 60 min and 9.9 ± 0.06 at 90 min after treatment (p < 0.001 vs. post-lesion) (Fig. 2-4B).

**Body axis bias test**

Body axis bias significantly decreased after administration of MPE from post-lesion to 0.28 ± 0.48 at 30 min, 0.56 ± 0.61 at 60 min, and 0.33 ± 0.49 at 90 min after MPE treatment (p < 0.05 vs. post-lesion) (Fig. 2-4C).

**Cylinder test**

Post-lesion cylinder test scores were 87.7 ± 2.4. There was a significant decrease with MPE alone with scores at 53.3 ± 7.7 at 30 min after drug exposure (p < 0.01 vs. post-lesion) showing almost equivalent usage of both forelimbs. Scores at 60 min (74.2 ± 3.7) and 90 min (85.4 ± 4.3) showed a decreasing trend but were not significant from post-lesion scores (p > 0.05) (Fig. 2-4D).

**Dyskinesia profile and contralateral turning**

At 5 min and 15 min after treatment with 400 mg/kg MPE alone, animals displayed both contralateral turning and DID. Four animals displayed consistent contralateral rotations with an average of 49.9 ± 5.8 rotations at 5 min and 22.0 ± 4.9 at 15 min after treatment. Dyskinesia profiles were 7.3 ± 1.3 at 5 min, 4.8 ± 1.8 at 15 min and 2.6 ± 0.58 at 30 min after treatment. These profiles were significantly less than Group 1 dyskinesia profiles in experiment #1 with BZ (p < 0.01 and p <0.001) (Fig. 2-4E). There was no contralateral turning at 30 min, 60 min, and 90
min and DID were non-existent at 60 min and 90 min after treatment.

The results of this experiment suggest that MPE alone can provide significant long-term behavioral benefit while reducing the severity of DID.
Figure 2-4. Long-term effects of MPE alone at 400 mg/kg (equivalent to 20 mg/kg LD). (A) Significant amelioration of parkinsonism up to 90 min post-treatment in the stepping test (**p < 0.001 vs. post-lesion). (B) Almost complete restoration of forelimb usage with the vibrissae-evoked forelimb placement test after treatment (**p < 0.001 vs. post-lesion). (C) Posturing behavior as measured by the body axis bias test shows less bias after drug exposure (*p < 0.05 vs. post-lesion). (D) Spontaneous forelimb usage bias was significantly decreased as shown by the cylinder test at 30 min but not at 60 min and 90 min (**p < 0.01 vs. post-lesion). (E) Dyskinesia profiles were significantly less with MPE alone at 5 min, 15 min, and 30 min after drug exposure compared to LD+BZ and MPE+BZ (**p < 0.01 vs. LD+BZ, ***p < 0.001 vs. MPE+BZ and LD+BZ at 15 min and 30 min).
Experiment #4 - long-term anti-parkinsonian efficacy comparison for LD alone, MPE alone and LD+BZ

Stepping test

LD alone (24 mg/kg)

Scores were 15.0 ± 0.71 at post-lesion. LD alone treatment did not provide significant long-term amelioration of parkinsonism with scores of 8.2 ± 2.87 at 30 min, 9.4 ± 1.40 at 60 min and 8.6 ± 1.17 at 90 min (p > 0.05 vs. post-lesion).

MPE alone (480 mg/kg - equivalent to 24 mg/kg LD)

Post-lesion scores were 15.6 ± 0.60. There was significant improvement after treatment with MPE alone with scores of 1.0 ± 0.45 at 30 min, 3.2 ± 1.28 at 60 min and 3.4 ± 1.81 at 90 min (p < 0.001 vs. post-lesion).

LD+BZ (6 mg/kg LD + 15 mg/kg BZ)

Scores were 15.0 ± 0.71 at post-lesion. After treatment with LD+BZ, there was significant alleviation with scores of 1.2 ± 0.97 at 30 min, 6.8 ± 2.08 at 60 min and 0.2 ± 3.22 at 90 min (p < 0.05 vs. post-lesion) (Fig. 2-5A).

Vibrissae-evoked forelimb placement test

LD alone (24 mg/kg)

There was significant deficit at post-lesion with scores of 0.10 ± 0.10. The unaffected forelimb had no behavioral deficits. There was no significant amelioration of parkinsonism with LD alone at 30 min with scores of 5.4 ± 2.27, 2.2 ± 1.36 at 60 min and 4.0 ± 2.45 at 90 min after treatment (p > 0.05 vs. post-lesion).
**MPE alone (480 mg/kg - equivalent to 24 mg/kg LD)**

Post-lesion scores showed significant deficit with values of 0.20 ± 0.20. The unaffected forelimb was normal with this test and had no deficits. However, significant amelioration of parkinsonism was evident after MPE alone at 30 min with a score of 8.0 ± 1.14, 9.0 ± 0.63 at 60 min and 10.0 ± 0.00 at 90 min after treatment (p < 0.001 vs. post-lesion).

**LD+BZ (6 mg/kg LD + 15 mg/kg BZ)**

Post-lesion scores had significant deficit with values of 0.10 ± 0.10. The unaffected forelimb was normal. There was significant amelioration of parkinsonism after LD+BZ only at 30 min (6.8 ± 1.77; p < 0.05 vs. post-lesion) and 90 min (8.0 ± 2.0; p < 0.01 vs. post-lesion). Scores at 60 min were not significant when compared to post-lesion (4.8 ± 2.17; p > 0.05 vs. post-lesion) (Fig. 2-5B).

Taken together, these results demonstrate that MPE alone provides significant behavioral benefit and that the equivalent synthetic LD dose when given without BZ is unable to provide significant equivalent anti-parkinsonian effects.
Figure 2-5. Comparison of LD alone (24 mg/kg), MPE alone at 480 mg/kg (equivalent to 24 mg/kg LD) and LD+BZ (6 mg/kg LD + 15 mg/kg BZ). (A) Significant amelioration of parkinsonism in the stepping test for MPE alone and LD + BZ (*p < 0.05 vs. post-lesion, ***p < 0.001 vs. post-lesion) but not with LD alone. (B) Significant restoration of forelimb usage with the vibrissae-evoked forelimb placement test after MPE alone and LD + BZ but not with LD alone (*p < 0.05, **p < 0.01, ***p < 0.001 vs. post-lesion).

Experiment #5 - long-term drug-induced dyskinesia priming effects of LD+BZ and MPE alone

Group A: first treatment - LD+BZ, second treatment - MPE alone

Group A first received LD+BZ (6 mg/kg LD + 15 mg/kg BZ) then MPE alone (480 mg/kg - equivalent to 24mg/kg LD). LD+BZ dyskinesia profile scores were 0.05 ± 0.05 at 5 min, 3.7 ± 2.06 at 15 min, 14.3 ± 5.48 at 30 min, 12.0 ± 4.25 at 60 min and 11.1 ± 4.55 at 90 min. MPE
alone dyskinesia profile scores were 5.0 ± 2.48 at 5 min, 3.7 ± 1.81 at 15 min, 1.3 ± 0.58 at 30 min and no DID at 60 min and 90 min. Comparisons between various treatment time points showed significant differences between MPE alone and LD+BZ treatments (p < 0.05) (Fig. 2-6A). These results show that MPE alone substantially ameliorates the occurrence of DID in animals that had previously exhibited robust DID in response to intermittent LD+BZ treatments.

*Group B: first treatment - MPE alone, second treatment - LD+BZ*

Group B first received MPE alone (480 mg/kg - equivalent to 24 mg/kg LD) then LD+BZ (6 mg/kg LD + 15 mg/kg BZ). MPE alone dyskinesia profile scores were 2.0 ± 0.75 at 5 min, 1.5 ± 0.97 at 15 min, 1.1 ± 0.48 at 30 min, 0.25 ± 0.25 at 60 min and 0.05 ± 0.05 at 90 min. LD+BZ dyskinesia profile scores were 0.0 at 5 min, 0.85 ± 0.56 at 15 min, 12.5 ± 1.60 at 30 min, 19.2 ± 3.23 at 60 min and 12.9 ± 4.72 at 90 min. Comparisons between various treatment time points showed significant differences between MPE alone and LD+BZ treatments (p < 0.01) (Fig. 2-6B). MPE alone pretreatment did not appear to ameliorate the intensity of LD+BZ-induced dyskinesias.
Confirmation of HP nigrostriatal degeneration

Apomorphine-induced rotations, TH immunohistochemistry and unbiased stereological counts of TH+ SNC neurons confirmed the HP state and unilateral degeneration of TH+ nigrostriatal neurons.

Discussion

Our study is the first to demonstrate that a simple water extract of *M. pruriens* endocarp powder with no additives has a superior effect to the combination of MPE+BZ on parkinsonism and that MPE alone is superior to LD alone or LD+BZ combinational therapy in terms of efficacy.
of ameliorating parkinsonism with dramatically reduced risk for DID. This result is consistent with the observations by Ayurvedic practitioners that PD patients treated with M. pruriens alone do not exhibit any significant DID. Interestingly, we found that addition of BZ to MPE induced severe DID. The most probable explanation for our findings is that the inhibitory effect of BZ on peripheral DDC allowed the abrupt and rapid increased transport of natural LD contained in MPE across the blood brain barrier without being inactivated in the peripheral blood. This increased availability of LD to the brain is the most likely cause of the severity of DID in MPE+BZ treated animals. An alternative explanation is that the natural form of LD contained in MPE is in combination with one or more natural agents that protect it from rapid decarboxylation by DDC and allow gradual protected transport across the blood brain barrier. A third possibility is that MPE may have natural antidyskinetic agents (such as choline or serotonin – see Table 2-2) that prevents or mitigates DID and that the addition of BZ to MPE negates these beneficial anti-DID compounds.

Previous reports in the rat have suggested that chronic M. pruriens treatment has no significant effect on LD content or dopamine and its metabolites in the nigra or striatum (Manyam, Dhanasekaran et al. 2004). However, these experiments were performed in a manner that did not immediately test whether LD levels in the brain were acutely altered after administration of M. pruriens. In these studies, animals were fed orally M. pruriens powder mixed with rat chow nightly for 52 weeks then sacrificed in the morning several hours after the last drug exposure. Therefore, it is possible that nigrostriatal catecholamine content was higher immediately after exposure than it was at the time of brain examination. Future studies that measure nigrostriatal LD and dopamine content in a strict time course post-drug administration will be necessary to further delineate whether addition of BZ or other DDCI to MPE will cause a rapid increase of LD and DA in the striatum. Moreover, unlike other preparations derived from M. pruriens that required significant proprietary processing, we utilized a simple water extract of
M. pruriens dried endocarp that can be stored up to 1 week in a refrigerator and show that such an extract can provide significant behavioral amelioration of parkinsonism with minimal risk for DID in a sustained fashion.

We observed an apparent bimodal time course of behavioral benefit in the stepping and vibrissae-evoked forelimb placement tests when HP rats were treated with LD+BZ (Fig. 2-5A and B) and LD alone (Fig. 2-5B) with an initial benefit noted at 30 min post-treatment, followed by a reduction in benefit at 60 min post-treatment and resumption of benefit at 90 min post-treatment. The behavioral rater and independent blinded evaluations of the tapes did not reveal any DID that significantly interfered with the ability of the animals to perform these behavioral tasks. A previous study evaluating adjusting steps (modified step test) in 6-OHDA lesioned rats at a time course of 15 min and 45 min post-LD (6 mg/kg) treatments reported more behavioral benefit at the 45 min time point than the 15 min time point (Kasture, Pontis et al. 2009). The differences in results between this study and the present study may be due to the use of different evaluation time points (45 min vs. 60 min), shorter stepping test procedure (70 cm/4 s vs. 90 cm/5 s) and/or a lower dose of BZ (6 mg/kg vs. 15 mg/kg). Thus, the reasons for this apparent bimodal time course are unclear. One possibility is that neurocognitive toxic effects of LD and LD+BZ that we did not specifically test interfered with the ability of these animals to perform the stepping and vibrissae-evoked forelimb placement tests at 60 min. Future studies that include neurocognitive testing may help with comparison of MPE to LD and LD+BZ in the non-motor aspects of PD.

Previous studies using a variety of formulations of M. pruriens have suggested that there are anti-PD compounds besides LD in this naturally occurring seed (Vaidya, Rajagopalan et al. 1978; Manyam 1990; HP-200 in Parkinson's Disease Study Group 1995; Hussian and Manyam 1997; Nagashayana, Sankarankutty et al. 2000; Katzenschlager, Evans et al. 2004; Manyam, Dhanasekaran et al. 2004; Kasture, Pontis et al. 2009). However, these previous studies did not
recognize the water-soluble nature of these compounds and the notion that MPE is best when used by itself with no additives of DDCI. Early clinical trials of *M. pruriens* for PD (Vaidya, Rajagopalan et al. 1978; HP-200 in Parkinson's Disease Study Group 1995) suggested the presence of other compounds besides LD in *M. pruriens* endocarp powder provide anti-PD effects. In the HP-200 (a proprietary *M. pruriens* formulation) study, two separate and distinctive populations of PD patients were recruited; the first group of patients that had PD for several years and had used anti-PD medications for several years, and a second group of patients who were drug naïve newly diagnosed PD patients. The first group of patients did not have any drug-washout prior to enrollment. Therefore, the effects of HP-200 by itself could only be assessed in the second group of drug naïve early PD patients, who do not develop DID. Hence the effects of *M. pruriens* on DID could not be properly assessed in this study (HP-200 in Parkinson's Disease Study Group 1995). However, they noted that the drug naïve cohort got substantial benefits despite being on very low doses of HP-200 that contained very small quantities of natural LD, leading them to conclude that additional anti-PD agents were contained in HP-200. Vaidya et al. also made a similar observation in their PD patients treated with *M. pruriens* (Vaidya, Rajagopalan et al. 1978). However, these investigators bought *M. pruriens* from multiple local Ayurvedic drug providers that are known to vary in quality and used a clinical rating method that has subsequently been shown to be deficient. Moreover, these investigators allowed the use of concomitant medications without any restrictions. Therefore, critics had attributed the anti-PD effects of *M. pruriens* in these studies mainly to the naturally present LD in the seed and not to other compounds found in *M. pruriens*.

A subsequent preclinical behavioral study evaluated the rotational effects of *M. pruriens* in the 6-OHDA lesioned HP rat by exposing animals orally to equivalent doses of *M. pruriens* and LD with and without CD (Hussian and Manyam 1997). In this study, *M. pruriens*+CD treated HP rats displayed significantly more contralateral rotations than equivalent doses of synthetic LD
+CD. When animals were exposed to *M. pruriens* alone, minor contralateral rotations were noted. However, these investigators did not evaluate any other behavioral effects of *M. pruriens*. A recent study of a proprietary formulation of *M. pruriens* that reportedly contains a higher (12.5%) concentration of natural LD was tested in combination with BZ in HP rats using behavioral tests. These investigators did not test spontaneous behaviors (e.g. cylinder test) and did not use standardized DID scales. These investigators noted that their *M. pruriens* formulation (in combination with BZ) was more efficacious and produced a lower incidence of involuntary movements than the equivalent LD+ BZ (Kasture, Pontis et al. 2009). However, they did not test their formulation without added BZ.

Nagashayana et al. evaluated *M. pruriens* mixed with other Ayurvedic treatments in PD patients (Nagashayana, Sankarankutty et al. 2000) who were taken off all medications 15 days prior to enrollment. The first group of patients underwent cleansing and palliative therapy while the second group underwent only palliative treatment. Only the first group exhibited significant symptomatic improvement. These findings support our results that *M. pruriens* treatment may be more effective when the patients do not have other competing DDCI in the system and a complete washout of any DDCI may enhance the effectiveness of *M. pruriens*. Katzenschlager and colleagues also report effectiveness of a proprietary formulation of *M. pruriens* in 8 advanced PD patients (Katzenschlager, Evans et al. 2004). They showed that *M. pruriens* treated patients had a more rapid onset of action and longer “on” time when compared to LD+CD treatment of comparative doses. This single dose study was a 4-h evaluation of this formulation of *M. pruriens* that contained several additives. These patients were not completely washed off their existing anti-PD medications that included DDCI. Thus, this 4-h study could not truly evaluate the effects of *M. pruriens* (without any DDCI) on PD symptoms including the risk of causing DID. This could be an explanation for why they did not find any differences in DID between *M. pruriens* treatment and the LD+CD treatment in their patients. Moreover, withdrawal of anti-PD
medications in patients with advanced disease has morbidity and can lead to potential fatality. Therefore, a clinical research trial of *M. pruriens* or MPE in advanced PD patients without any confounding DDCI would be extremely difficult to execute.

The present study addresses several drawbacks of previous studies by using a well-characterized rat model of PD, treatment dosing similar to what is used in PD patient population, single drug treatment with no confounding concomitant medications or additives, a full battery of validated behavioral tests including DID assessments for the parkinsonian rat, and histological confirmation of uniform nigrostriatal deficits in all animals. Furthermore, we demonstrate that MPE is effective with duration of action that exceed oral *M. pruriens* and is advantageous over conventional oral anti-parkinsonian medications. Gastrointestinal dysfunction is a common problem in PD and may cause issues with absorption of oral treatments (Goetze, Nikodem et al. 2006). To avoid problems with oral consumption and potential issues with gastrointestinal absorption, we used a water extract of the *M. pruriens* seed powder parenterally administered. Future studies are necessary to identify the water-soluble anti-dyskinetic and antiparkinsonian compounds that are present in MPE.

**Study # 2 – The anti-parkinsonian and anti-dyskinetic mechanisms of *Mucuna pruriens* in the non-human primate and rat**

**Materials and Methods**

**Primate studies**

Fourteen adult (6-9 kg) rhesus (Macaca mulatta) and two cynomolgus (Macaca fascicularis) monkeys received either intracarotid (ICA) MPTP to induce an HP state, ICA+systemic (IV) MPTP to induce an overlesioned HP (OHP) model, or systemic (IM or IV)
injections of MPTP to induce bilateral parkinsonism (Oiwa, Eberling et al. 2003; Subramanian, Lieu et al. 2010; Gilmour, Lieu et al. 2011; Lieu, Deogaonkar et al. 2011). Each animal was operant conditioned, behaviorally trained (Gilbert, Leszczynski et al. 2004) to accept medications to ensure proper consumption and clinical oral simulation as described in detail below. Clinical assessments were taken post-MPTP to ensure stability of parkinsonism and at subsequent treatment exposure using the modified version of Unified Parkinson’s Disease Rating scale for primates (mUPDRS) (see Appendix B) (Lieu, Deogaonkar et al. 2011). Electrophysiological recordings before and after treatments were done in awake, behaving parkinsonian animals. Standard extracellular single cell recording techniques were used, as described in detail in Chapter 5 and in our previous report (Gilmour, Lieu et al. 2011).

**Administration of MPTP to induce parkinsonism**

For ICA administration of MPTP to create an HP state, animals were placed under deep general anesthesia, the left common carotid artery was exposed, and the internal carotid artery was isolated followed by manual retrograde injection of MPTP solution (0.5 mg/kg body weight at a concentration of 1 mg/ml) over a period of 15 minutes. The animal was allowed to recover and assessed for stability of HP. Depending on stability of HP state (see below for behavioral testing details), exposure to ICA MPTP was performed up to 4 times in each animal. Repeat surgeries were not performed before 2 weeks of observation had been completed and surgical scar from the previous surgery had healed. The cumulative ICA MPTP dose ranged from 0.5 – 2.5 mg/kg. A subset of animals was rendered overlesioned HP (OHP). To achieve an OHP state, animals were initially treated with ICA MPTP. Once HP state was stable, the animal received subsequent injections of IV MPTP (0.2 mg/kg), inducing mild parkinsonism in the previously unaffected side. Another set of animals was rendered bilaterally parkinsonian with systemic IV or IM injections of MPTP (0.2 mg/kg). Cumulative doses of systemic MPTP ranged from 0.2 mg/kg
– 1.0 mg/kg. Drug treatments were then given only when animals were stable parkinsonian for > 3 months as determined by no changes in the mUPDRS ratings performed twice each month separated by a minimum of 15 days and operant conditioned for a minimum of 6 months such that they were compliant with oral dosing of medications (see sections below).

Operant conditioning for oral medication compliance without compromising enrichment protocols in parkinsonian primates

The following protocol was used for operant conditioning and behavioral training in each animal to ensure compliance and complete consumption of antiparkinsonian medications. Each animal was individually housed such that visual and olfactory contact with conspecifics was maintained at all times. Various types of toys were placed in each cage and rotated every other week. At any one time, every cage contained a hanging toy, such as a ball or a mirror and at least one (usually two) chewing toys, such as a Hercules dental chew toy, Dental ball, Kong toys, nylabones or pieces of wood (Bio-Serv). In some instances, certain animals showed adverse stress reactions to certain types of toys. In this case, that toy was removed from the animal’s cage and replaced with another toy. In addition to the regular feed, monkey diet was supplemented with fruits, vegetables, nuts, or other types of “treats” everyday. The size, quantity, and time of day that these “treats” were given (always in the late afternoon after training) was monitored carefully so as not to interfere with our behavioral training. Also, each day, Monday – Friday, the animals were presented with an enrichment activity. These activities included watching cartoons, “novel” food day, foraging devices and special activities such as air-popped popcorn or bowls of water to play with. On weekends, the animals were given extra food treats. Cages were checked for any remaining monkey biscuits or treats each evening; any remaining food was removed in order to maintain the appropriate level of food scheduling necessary for operant training of a food-picking task or voluntary consumption of medications. All animals were observed for stereotypical
(pacing, rocking, digit-sucking) and self-injurious behaviors (self-biting, head banging). If any such behaviors were seen, enrichment for those animals was increased. Oral LD and *M. pruriens* endocarp powder (MPEP) were successfully administered twice daily (AM and PM) by hiding powdered drug in food treats. MPE was given orally without the need of hiding it in any additional foods or liquids. Animals appeared to enjoy MPE and voluntarily consumed it completely at each dose. Improvement in parkinsonism was then assessed by using the mUPDRS and confirmed through analysis of blood plasma levels in representative animals at the same time as the mUPDRS exams. Animals were continuously monitored to ensure complete consumption and drug compliance during treatments by investigators. Depending on the desired task (consumption of treatment or interaction with investigator to evaluate mUPDRS), any of the enrichment protocols in **Table 2-1** below could be detrimental to operant conditioning. Similarly, any of the training conditions could cause behavioral problems in monkeys due to lack of appropriate enrichment. To overcome these barriers to operant conditioning, we limited the time when enrichment was given (all enrichment was given after training session to maintain food scheduling), scheduled enrichment only in the afternoon or following the end of a testing session, and maintained visual, sound and olfactory stimuli without interfering with operant conditioning.
Table 2-1. Conditions for operant conditioning in parkinsonian primates

<table>
<thead>
<tr>
<th>Ideal conditions for training</th>
<th>Ideal conditions for enrichment</th>
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</thead>
<tbody>
<tr>
<td>Single-housed animals</td>
<td>Group-housed animals</td>
</tr>
<tr>
<td>Supplemental toys without food (mirrors, chew toys)</td>
<td>Supplemental toys containing food (foraging devices)</td>
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<tr>
<td>No visual contact with conspecifics</td>
<td>Visual contact with conspecifics</td>
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<tr>
<td>No sound</td>
<td>Sound (movies, radio, wildlife sounds)</td>
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Clinical assessment

Behavioral ratings were performed using the mUPDRS in a blinded fashion. The mUPDRS consists of subjective rater dependent but validated and reliable blinded evaluations of vocalization/hooting, facial expression, tremor (rest or action), muscle tone/rigidity, hypokinesia, finger dexterity, foot agility, balance/postural instability, spontaneous gait, dystonia and circling/dyskinesia. Each item on the mUPDRS has a range from 0 (no motor deficits) to 4 (very severe impairment) for each limb or body part and is modeled after the UPDRS (Appendix A and B) used to rate PD patients in clinical trials. Animals were also further assessed for DID using a modified Abnormal Involuntary Movements Scale (AIMS) previously described for primates (Blanchet, Konitsiotis et al. 1998; Konitsiotis, Blanchet et al. 2000). AIMS scores are represented as the total sum of dystonic posturing and choreiform movements in the face, trunk and each limb. Severity was evaluated using the following scale: 0=none; occasional, mild=1; intermittent, moderate=2; continuous, severe=3. The entire clinical rating session was videotaped for a minimum of 4 hours after each dose of medication for a minimum of 8 hours of video. mUPDRS and AIMS scores were taken at stable parkinsonian baseline state, placebo and at an average time
of 75 minutes post drug treatments. We established the optimal LD+CD dose for each animal using blinded testing every 2 weeks at monthly intervals starting at a dose of 50 mg LD + 12.5 mg CD b.i.d. (i.e. LD 100mg + CD 25mg/day) and escalated by 100 mg LD every 2 weeks to achieve no further improvements in mUPDRS scores despite dose escalation. Thereafter, the lowest dose of LD that produced the largest improvement in mUPDRS scores was chosen as the optimal dose of LD in each animal. Thus, animals were tested on LD treatment for a minimum of 3-4 months to determine their optimal LD dose. All animals were washed out of LD treatments for 1 month before initiating MPEP or MPE treatments.

Electrophysiology

The recordings were sorted by offline principal component analysis, and interspike intervals (ISIs) were generated. Acceptable records were comprised of at least 400 spikes and had duration between 60 and 120 sec. Firing rates and seven measures of the firing patterns were employed: the coefficient of variation (CV) of the ISIs, the burst index (mean of the ISI distribution divided by the mode, (Hutchinson, Levy et al. 1997)) the percent of spikes in bursts and percentage of time in bursts calculated by the Poisson-surprise method (Legendy and Salcman 1985), the density discharge histogram (DDH) compared to the DDH of a random Poisson spiketrain (Kaneoke and Vitek 1996), the range of the DDH, and the sample entropy (Richman and Moorman 2000). The seven numeric firing pattern metrics were compared using the Wilcoxon-Mann-Whitney rank-sum test, and the categorical DDH classification was compared using Fisher’s 2x2 exact test (grouping together “Poisson” and “bursty” categories).

Experiments in parkinsonian primates

Experiment 1 - Effects of MPEP, MPEP+CD and LD+CD in the parkinsonian primate
We first completed a preliminary dose-finding study to determine optimal doses and toxicity adverse effects of MPEP and MPEP+CD. HP monkeys were tested on MPEP and MPEP+CD (25mg) to find optimal dosing after attaining stable HP state (N=3). Each treatment epoch was followed by 2 weeks of washout. Two separate mUPDRS scores 14 days apart were obtained on placebo and for each treatment epoch. MPEP was titrated in these studies from 6g/day to the highest dose of 18g/day (N=11) to evaluate gastrointestinal effects, DID or behavioral correlates of psychiatric symptoms. Blood draw was performed 90 minutes after administration and consumption of medications (placebo, LD+CD (250mg/62.5mg) and MPEP+CD (4.5g/25mg)) to test the bioavailability of orally administered LD and MPEP at approximately equivalent doses for pharmacological estimation of DA levels.

After finding preliminary optimal doses of MPEP, we examined the effects of 4.5g MPEP alone (~225mg LD) (N=5), 4.5g MPEP (~225mg LD)+25mg CD (N=6) and 100-200mg LD+25-50mg CD (i.e., daily doses were: 9g MPEP alone (N=5), 9g MPEP+50mg CD (N=6) and 200-400mg LD+50-100mg CD (N=6)) in HP and OHP primates. In a subset of animals, cranial recording chambers were surgically implanted to permit chronic single cell extracellular neuronal recording from the left STN and SNR in the stable HP state (N=3), with LD+CD treatment (N=2) and with MPEP+CD treatment (N=1) (a portion of this study has been presented elsewhere (Gilmour, Lieu et al. 2011) and in detail in Chapter 5).

Experiment 2 – Effects of MPE and LD in the parkinsonian primate

HP, OHP and bilateral parkinsonian animals (N=5) were treated with placebo, LD+CD or MPE b.i.d. for 3-11 days. See section above for MPE technique description. The dosage concentration of MPE was based on 4-5% of natural occurring LD in MPEP such that the MPE solution had approximately 24 mg LD per ml.
mUPDRS scores were obtained on placebo, oral LD (1 to 3.5 tablets LD/CD -100/25) and escalating doses of 4ml - 36ml MPE orally (~96mg - 864mg LD, respectively) until optimal doses were found using a blinded, randomized design with 2 weeks washout between treatments. When compliance was an issue with oral LD+CD, animals received systemic injections of LD at the equivalent optimal doses of oral LD (LD methyl ester with BZ). DID were assessed using the AIMS rating scale in animals that displayed clear LD-induced dyskinesias similar in advanced PD (Blanchet, Konitsiotis et al. 1998; Konitsiotis, Blanchet et al. 2000; Lieu, Deogaonkar et al. 2011). Data was analyzed using ANOVA with Tukey post-test (mean ± SEM).

**Rat Studies**

*Drug treatment with LD and MPE with LD removed in the HP rat*

In this study, we used HP rats to test our MPE treatment with LD removed using conventional HPLC and standard elution techniques. HP rats (N=3) were initially primed with LD methyl ester+BZ (6 mg/kg + 15 mg/kg) b.i.d. IP for 2 days. After LD washout for 2 weeks, animals were treated with MPE without LD (MPE(-)LD) alone without BZ with 3 doses. Using conventional HPLC and standard elution techniques, we removed LD from optimal MPE dose equivalent to 480 mg/kg MPE previously used to create MPE(-)LD. Conventional HPLC run were accomplished over a 45-minute period and 3 fractions based on elution time (early phase elute 0-15 minutes, mid phase elute 16-30 minutes and late elute 31-45 minutes) were collected into MPE fractions 2, 3 and 4. These 3 fractions were tested and compared to MPE. Comparison of HPLC chromatogram of MPE with HPLC of MPE(-)LD demonstrated elimination of LD peak. The collected fractions from HPLC were evaporated and re-dissolved into sterile water and injected IP. DID and vibrissae-evoked forelimb placement tests were evaluated after both treatments. Vibrissae-evoked forelimb placement test was taken at post-lesion and post-treatment. DID were analyzed up to 120 min post-treatment.
Results - Primate Studies

Experiment 1 - MPEP and LD effects on parkinsonism and basal ganglia electrophysiology

For the initial, preliminary dose finding experiment, mean mUPDRS scores improved by 4% (change from 35 to 33.5), 24.2% (35 to 26.5), and 27.1% (35 to 25.5) respectively with 3g, 6g, and 9g (total daily dose) of MPEP alone. Optimal dose for MPEP+CD was determined to be 9g of MPEP + 50mg of CD/day. In this experiment, mean mUPDRS score showed no improvements for the placebo treatments (mean mUPDRS score of 35 to 35.2) compared to a 49.1% improvement (mean mUPDRS score changed from 35 to 17.8) at this optimal MPEP+CD dose. No observable adverse events were evident at these doses of MPEP alone or MPEP+CD.

Doses at 12g/day and 18g/day caused compliance issues and severe adverse effects with successful consumption. These included nausea, retching, vomiting, behavior that mimicked hallucinations and increased aggression. Serum peak dose estimation of DA levels were 180 pg/ml after placebo treatment, 27,600 pg/ml after MPEP+CD treatment (4.5g MPEP+25mg CD; ~225mg of LD) and 22,220 pg/ml after LD+CD administration (250/62.5).

For the optimal dose experiment, mUPDRS scores on placebo were 15.6 ± 2.6 decreased to 8.0±1.6 with MPEP alone, 7.7 ± 1.5 with MPEP+CD and 4.5 ± 1.1 with LD+CD optimal doses (Fig. 2-7). These doses caused no observable adverse effects.
Figure 2-7. Comparison of mUPDRS scores in parkinsonian primates with placebo, MPEP alone, MPEP+CD and LD+CD demonstrate significant amelioration of parkinsonism after treatments. *p < 0.05, **p < 0.01 compared to placebo.

A portion of this electrophysiological data has been presented in a previously published report (Gilmour, Lieu et al. 2011) and in Chapter 5. SNR firing rate showed significant reduction in SNR on both LD+CD and MPEP+CD. STN firing rate showed no significant difference, but a trend toward reduction on MPEP+CD (Fig. 2-8A). SNR firing pattern became more bursty on LD+CD, measured by Poisson DDH comparison. SNR patterns changes on MPEP+CD did not reach statistical significance, but showed a trend toward increased burstiness, but not as pronounced as LD+CD. STN patterns did not show a statistical change, although both LD+CD and MPEP+CD showed a trend toward reduction of the number of bursty neurons (Fig. 2-8B). Median SNR normalized coefficient of variation was higher on LD+CD than baseline HP state. On MPEP, the SNR showed a trend toward increased normalized CV, but it was not significant. There were no significant changes in the STN (Fig. 2-8C). The proportion of spikes in bursts and proportion of time in bursts (measured from the Poisson-surprise method) did not show any statistically significant changes. However, there was a trend for both LD+CD and MPEP+CD to
make the SNR more bursty, with MPEP+CD showing a smaller effect than LD+CD. There was a trend for LD+CD to make the STN less bursty, and MPEP+CD showed the same trend to an even larger degree (Fig. 2-8D and E). DDH Range Counts were not statistically different in the different conditions. However, there was a trend for LD+CD to make the SNR more bursty. MPEP+CD showed a trend toward making the SNR more bursty but it was not significant (Fig. 2-8F). The burst index was not statistically significant between groups. However, there was a trend for LD+CD to make the SNR more bursty, which was not replicated on MPEP+CD. In fact, there was a trend for MPEP+CD to reduce the burstiness of SNR. In the STN, the trends were reversed, but again neither were significant (Fig. 2-8G). Sample Entropy did not show any significant differences, although the MPEP+CD treatment slightly reduced the SNR sample entropy and slightly increased the STN sample entropy (Fig. 2-8H).
Figure 2-8. Electrophysiological recordings on LD and MPEP. (A) Firing rates of SNR and STN in the HP monkey in stable HP state (baseline) and on LD+CD (Levodopa) and MPEP+CD (Mucuna) (Kruskal-Wallis p<0.01, ** p<0.01 rank-sum using Tukey’s HSD correction, compared to baseline HP state). (B) Poisson comparison of SNR and STN neurons PreLD (stable baseline HP state) (Fisher’s 2x2 two-sided exact test grouping “poisson” category together with “regular”, * p=0.0164), PostLD (LD treatments) and PostMP (MPEP+CD). (C) Coefficient of variation at HP state and with treatments (Kruskal-Wallis p<0.05, * p<0.05 rank-sum using Tukey’s HSD correction, compared to baseline HP) (D-G) Measures of firing patterns in the SNR and STN in HP state and on treatments. (H) Sample entropy of SNR and STN. CV-coefficient of variation, DDH-density discharge histogram.

Experiment 2 - MPE ameliorates parkinsonism without causing DID
mUPDRS scores on placebo were 18.0 ± 5.6, which significantly decreased with optimal doses of MPE treatments (5.4 ± 0.4) and LD+CD treatments (5.3 ± 1.9) (Fig. 2-9A). Average optimal dose of LD was 250 mg and optimal dose of MPE was 20 ml (~480mg LD) b.i.d. in this experiment that included HP, OHP and bilateral parkinsonian animals. MPE caused no apparent gastrointestinal problems or drug-induced hallucinations. However, LD+CD treatments produced significant DID (AIMS score = 7.3±1.3) in two bilaterally parkinsonian animals, whereas no apparent DID were observed with MPE treatments (AIMS score = 0) (Fig. 2-9B).

Figure 2-9. Behavioral effects of M. pruriens water extract (MPE) in the parkinsonian primate. (A) A water extract of MPEP (MPE) significantly reduces parkinsonism in the parkinsonian primate at optimal doses similar to LD+CD (B) and does not cause dyskinesias. *p < 0.05.
Results - Rat Studies

Drug treatment with LD and MPE with LD removed

LD (6 mg/kg + 15 mg/kg BZ) significantly reduced parkinsonism as demonstrated by the vibrissae-evoked forelimb placement test comparing the HP state (0.33±0.33) to 30, 60 and 90 min post-LD (10.0 ± 0.0 for each timepoint) (Fig. 2-10A). However, severe dyskinesias were present. Dyskinesia scores were 13.3 ± 4.1 at 15 min, 17.5 ± 7.4 at 30 min 8.3 ± 1.9 at 60 min, 9.2 ± 2.9 at 90 min and 2.9 ± 1.1 at 120 min (Fig. 2-10B). Interestingly, MPE(-)LD (equivalent to 480 mg/kg MPE) provided partial amelioration of parkinsonism with no dyskinesias in animals primed with LD treatments (Fig. 2-10B). Vibrissae-evoked forelimb placement test scores with MPE(-)LD were 5.7 ± 2.2 at 30 min, 5.0 ± 2.1 at 60 min and 6.3 ± 1.7 at 90 min (Fig. 2-10A).
Figure 2-10. Removal of LD from MPE (MPE(-)LD) ameliorates parkinsonism in the rat model of PD. (A) LD (6 mg/kg + 15 mg/kg BZ) caused long-term amelioration of parkinsonism. MPE(-)LD (equivalent to 480 mg/kg MPE) provided partial amelioration of parkinsonism. (B) LD produced severe, disabling dyskinesias, whereas none were seen with MPE(-)LD. *p < 0.05, **p < 0.01.

Discussion

In the present study, we demonstrate that *M. pruriens* in powder and water extract form can significantly ameliorate behavioral deficits in the primate model of PD. We also demonstrate that the mechanistic actions of *M. pruriens* cannot be attributed to LD alone, and that *M. pruriens* has a unique mechanism of action on the basal ganglia electrophysiology that is different from that of LD when tested at equivalent doses. Furthermore, we show that complete removal of LD from *M. pruriens* does not remove its anti-PD effects. This is a confirmation of our earlier suggestions and a complete rebuff of previous contentions that the anti-PD effects of *M. pruriens*
were simply due to natural LD. Indigenous medicines based on natural products like *M. pruriens* are often unique in that they contain several constituents in perfect combination. *M. pruriens* has over 50 known constituents that have been identified to date and perhaps others to be identified (Table 2-2) (Burgess, Hemmer et al. 2003; Manyam, Dhanasekaran et al. 2004; Manyam, Dhanasekaran et al. 2004). Identifying the single component or combination of components in *M. pruriens* responsible for its anti-parkinsonian/anti-dyskinetic effects is daunting. Although identification of each individual component and its exact quantity required to reproduce these effects is theoretically possible, such a task is time consuming and expensive. *M. pruriens* is widely farmed in many countries as an inter-crop and is exceedingly inexpensive to produce as a standardized natural product with uniform efficacy. Thus, this renewable, natural product may represent a paradigm shift in contemporary drug discovery methods where identification of active ingredients, synthetic manufacture, safety and efficacy testing followed by mass marketing of the synthesized compounds is replaced by a strategy that focuses on identification of safety and efficacy of a standardized natural product (that has several dozen constituents in unique combination) and its mechanism of action when used as a whole. While such an approach may sound counterintuitive, archaic and confrontational to the current wisdom of scientific advancement, it is pragmatic and has the potential to revolutionize the therapy of PD with the possibility of worldwide availability of *M. pruriens* formulations. Such a modified approach to drug discovery requires a shift in conventional dogma and acceptance of mechanistic explanations that are based on the use of the natural compounds as a whole.
Table 2-2. Known components of *M. pruriens*

<table>
<thead>
<tr>
<th>Component</th>
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<tbody>
<tr>
<td>Arachidic acid</td>
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</tr>
<tr>
<td>Arginine</td>
<td>Lysine</td>
</tr>
<tr>
<td>Ash</td>
<td>Methionine</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6-Methoxyharman</td>
</tr>
<tr>
<td>Behenic acid</td>
<td>1-Methyl-3-carboxyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolone</td>
</tr>
<tr>
<td>Beta carbolne</td>
<td>Mucunadine</td>
</tr>
<tr>
<td>Beta sitosterol</td>
<td>Mucunain</td>
</tr>
<tr>
<td>Bufotamine</td>
<td>Mucunine</td>
</tr>
<tr>
<td>Calcium</td>
<td>Myristic acid</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Niacin</td>
</tr>
<tr>
<td>Choline</td>
<td>Nicotine</td>
</tr>
<tr>
<td>Cystine</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Coenzyme Q-10</td>
<td>Oleic acid</td>
</tr>
<tr>
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<td>5-Oxyindole-3-alkylamine</td>
</tr>
<tr>
<td>N,N-Dimethyltryptamine-N-oxide</td>
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</tr>
<tr>
<td>L-Dopa</td>
<td>Palmitoleic acid</td>
</tr>
<tr>
<td>Cis-12,13-epoxyoctadec-trans-9-cis-acid</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Cis-12,13-epoxyoctadec-trans-9-enoic-acid</td>
<td>Proline</td>
</tr>
<tr>
<td>5-Methoxy-N,N-dimethyltryptamine-N-Oxide</td>
<td>Protein</td>
</tr>
<tr>
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<td>Prurienidine</td>
</tr>
<tr>
<td>Fiber</td>
<td>Prurienine</td>
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<tr>
<td>Gallic acid</td>
<td>Riboflavin</td>
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<tr>
<td>Glutamic acid</td>
<td>Saponins</td>
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<tr>
<td>Glutathione</td>
<td>SD</td>
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<tr>
<td>Glycine</td>
<td>Serine</td>
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<tr>
<td>Histidine</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>Stearic acid</td>
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<tr>
<td>Indole-3-alkylamine</td>
<td>Thiamin</td>
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<tr>
<td>Iron</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
<td>Valine</td>
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<tr>
<td>Linoleic acid</td>
<td>Vernolic acid</td>
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**Effects of *M. pruriens* endocarp powder**

In experiment 1, we found that MPEP had to be dosed at higher quantities to get a maximal effect when compared to LD (100-200mg synthetic LD versus 225mg natural LD in MPEP). The large volume of MPEP powder (6g to 18g/day) in experiment 1 were very difficult to successfully administer in monkeys due to gastrointestinal side-effects, similar to that of earlier reports (Vaidya, Rajagopalan et al. 1978; HP-200 in Parkinson's Disease Study Group 1995; Behari, Bhatnagar et al. 2002; Katzenschlager, Evans et al. 2004). These gastrointestinal effects could be in part due to the large protein content in this leguminous cotyledon powder, a well-known cause of abdominal bloating, flatulence and gastrointestinal irritability. Serum DA measurements demonstrate that bioavailability and peak plasma pharmacokinetics of natural LD contained in MPEP and synthetic LD are quite similar. This finding further strengthens the notion that MPEP contains additional anti-PD and anti-dyskinetic agents beyond the 4-5% natural LD content.

Previous reports have demonstrated that LD and other DA replacement therapies can significantly alter firing properties of basal ganglia nuclei. This has been discussed in Chapter 1, in Chapter 5 and our recently published paper in detail (Albin, Young et al. 1989; DeLong 1990; Hutchinson, Levy et al. 1997; Boraud, Bezard et al. 1998; Lozano, Lang et al. 2000; Levy, Dostrovsky et al. 2001; Heimer, Rivlin-Etzion et al. 2006; Giannicola, Marceglia et al. 2010; Lafreniere-Roula, Darbin et al. 2010; Gilmour, Lieu et al. 2011). We demonstrate that SNR firing rate is significantly decreased after treatment with both LD and MPEP. However, LD treatment did not decrease STN firing rate. Interestingly, MPEP did cause a trend in decreasing STN firing rate. We also found differential firing patterns between the two treatments. LD caused a significant increase in SNR bursting activity but this increase was not seen with MPEP. We also found slight differences between LD and MPEP in the other measures of burstiness. Various pharmacological treatments are known to alter basal ganglia firing patterns, which include
serotonin, N-methyl-D-aspartate, and DA agonists (Tseng, Riquelme et al. 2000; Lee, Shin et al. 2001; Levy, Dostrovsky et al. 2001; Shen, Kozell et al. 2007), which may account for the
differential bursting firing patterns of MPEP.

Effects of *M. pruriens* endocarp powder water extract

The ameliorative effects of oral MPE treatments are similar to the anti-PD effects of
LD+CD treatment, a gold-standard for pharmacological therapeutic efficacy in PD, sans its
deleterious side-effects in the parkinsonian primate. MPE provides a simple and inexpensive
solution to these problems with gastrointestinal intolerance of MPEP, and demonstrates that the
anti-PD and anti-dyskinetic compounds contained in *M. pruriens* are water-soluble and effective
without the need for concomitant DDCI. This suggests that even monkeys “primed” to develop
DID from repeated exposure to LD+CD treatments can be successfully treated with MPE without
causing dyskinesias. Furthermore, the anti-PD and anti-dyskinetic effects of MPE were not
diminished by chronic exposure, drug washout and re-exposure. LD does not cause dyskinesias in
HP rhesus monkeys as described in detail in Chapter 3. In this context, we demonstrate the anti-
PD properties of MPE in HP and OHP primates, models that represent restricted nigrostriatal
dopaminergic loss; and the anti-PD and anti-dyskinetic effects in the bilateral parkinsonian
monkey, a model that represents more severe PD and readily exhibits DID. These findings in the
MPTP-treated monkey models are similar to what we have shown in the parkinsonian rat. Some
investigators have argued that drug-induced abnormal involuntary movements displayed by the
parkinsonian rat are not equivalent of DID seen in PD, and believe that the phenomenology of
DID in the primate model more closely resemble clinical DID (Langston, Quik et al. 2000). We
address this in the current primate study, confirming the preclinical relevance of the anti-PD and
anti-dyskinetic effects of MPE without a DDCI in parkinsonian primates. Taken together, these
studies in the rodent and primate models of PD provide compelling preclinical evidence of the
efficacy and safety for MPE. Biochemical measurements from MPEP-treated primates as previously mentioned provide proof that LD+CD treatments were appropriately dosed in animals that developed DID in the MPE experiments. We hypothesize that the improved safety profile of MPE may be due to additional beneficial compounds as speculated in previous studies (HP-200 in Parkinson's Disease Study Group 1995; Manyam, Dhanasekaran et al. 2004; Manyam, Dhanasekaran et al. 2004).

We escalated the dose of MPE more than what was needed to match the optimal anti-PD effects obtained from LD+CD treatments (MPE doses up to >1600mg LD equivalent dose per day). Nonetheless, these animals tolerated these large doses without adverse effects. MPE contains 4-5% LD that is identical to the 4-5% LD content reported for MPEP powder. Since MPE was administered without a DDCI, we hypothesize that the effects cannot be entirely due LD content in MPE because LD would be metabolized via peripheral DDCI, suggesting that MPE may have DDCI-like activities. Thus, our rodent studies and primate studies collectively provide behavioral evidence that the anti-PD effects of MPE and MPE(−)LD cannot be explained by the presence of 4-5% natural LD alone. Other water-soluble compounds that remain unidentified contained in MPE have to be implicated for the anti-PD and anti-dyskinetic effects observed in these studies.

Oral administration of antiparkinsonian treatments to parkinsonian monkeys

This is the first report of any phytomedicine that has been tested in primates using operant conditioned methods for oral voluntary consumption to simulate clinical PD pharmacotherapy, using placebo controls and a blinded prospective study design. This study design could represent an ideal method to perform future preclinical studies of phytomedicines in PD. We found compliance with oral consumption easier with MPE compared to MPEP, MPEP+CD or LD+CD. Previous studies with various MPEP formulations have a number of
disadvantages that include variable behavioral assessments, use of concomitant medications, inadequate washout, lack of LD dose controls and excess variability in study populations. In the present study, we overcame these disadvantages by 1) using a well-established primate model of PD that exhibits motor fluctuations and DID that closely resemble its phenomenology to patients with PD, 2) ensuring drug compliance to replicate the clinical experience of PD patients, 3) utilizing the same behavioral rater for all mUPDRS assessments to eliminate inter-rater variability, 4) ensuring that our animals received no concomitant medications.

*M. pruriens* as a novel treatment for PD and DID

We demonstrate that *M. pruriens* and MPE have unique mechanistic properties that are differential from LD, and that the unique combination of constituents within *M. pruriens* contributes to both its anti-PD and anti-dyskinetic effects. This will be advantageous to PD patients who currently take LD-containing formulations and have to experience its long-term side effects that often require invasive surgical intervention. This study also shows that MPE contains a yet-to-be investigated portfolio of anti-PD and anti-dyskinetic agents that could open up new therapeutic avenues for PD, yet, constitute a daunting and expensive conventional drug discovery approach. While additional scientific studies to identify these individual anti-PD and anti-dyskinetic components contained in MPE may be warranted, parallel studies to evaluate the clinical use of MPE as a safe and effective alternative to LD therapy in PD is also immediately indicated with our demonstration of its unique beneficial mechanisms of action.
2.5. Novel Therapies For DID - D1 DA Full Agonist EFF0311

Since LD is accepted as being a prodrug for DA, there has been an impetus to find DA agonists that could replace LD, yet LD remains the gold standard of treatment. For several decades, there was a prevailing view that the indirect activation of D2 receptors was the principal mechanism by which LD worked (Cederbaum, Schleifer et al. 1990). In the early 1980’s, the involvement of D1 receptors in motor control, and their interaction with D2 receptors was first demonstrated (Mailman, Schulz et al. 1984). Although this suggested a possible role for D1 agonists in the treatment of PD, studies with the two D1 agonists then available (SKF38393 and CY208-243) had modest or no antiparkinsonian action in the bilateral MPTP primate model (Close, Marriott et al. 1985; Falardeau, Bouchard et al. 1988; Nomoto, Jenner et al. 1988; Temlett, Chong et al. 1988; Bedard and Boucher 1989; Temlett, Quinn et al. 1989; Boyce, Rupniak et al. 1990; Gomez-Mancilla and Bedard 1991; Elliott, Walsh et al. 1992; Blanchet, Bedard et al. 1993; Gomez-Mancilla, Boucher et al. 1993; Nomoto and Fukuda 1993), HP monkeys (Domino and Sheng 1993), or in humans (Braun, Fabbrini et al. 1987; Temlett, Quinn et al. 1989; Tsui, Wolters et al. 1989; Emre, Rinne et al. 1992).

It was not widely recognized, however, that both SKF38393 and CY208-243 were only partial, D1 agonists. When dihydrexidine, the first high affinity full D1 DA receptor agonist, became available (Lovenberg, Brewster et al. 1989; Brewster, Nichols et al. 1990; Mottola, Brewster et al. 1992), it was found to cause dramatic antiparkinson effects (Taylor, Lawrence et al. 1991), with similar results later reported for other full D1 agonists in primates, both non-human (Kebabian, Britton et al. 1992; Gnanalingham, Hunter et al. 1995; Asin, Domino et al. 1997; Grondin, Bedard et al. 1997; Goulet and Madras 2000) and human (Blanchet, Fang et al. 1998; Rascol, Blin et al. 1999; Rascol, Nutt et al. 2001). Unfortunately, these earlier drugs were plagued with a variety of problems, ranging from very short duration of action, rapid tolerance, or several types of toxicity (Mailman, Huang et al. 2001; Mailman, Huang et al. 2007). Indeed,
dihydrexidine, despite its very short duration of action, is the only one of these D₁ agonists that is currently available for clinical experimentation.

Recently, Vishu et al., discovered that the 2-methyl-analog of dihydrexidine (known as EFF0311) has high D₁ affinity and intrinsic activity, is somewhat more D₁:D₂ selective than dihydrexidine, and has no marked off-target activity. Most importantly, EFF0311 has a duration of action at least four times longer than dihydrexidine at equivalent molar doses (Murthy, Gowdahalli et al. 2011). In the present study, we further examined the antiparkinsonian and antidyskinetic effects of single doses of this novel D₁ full agonist EFF0311 in the hemiparkinson rat and primate model of PD.

Materials and Methods

Rat experiments

Animals

Female Sprague-Dawley rats (250-400 g) were used in the present study and had free access to standard food chow and water. All surgical and behavioral procedures were conducted in compliance with institutional protocols, and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978).

6-OHDA lesioning and rotational validation of HP state

Sprague-Dawley rats (Charles River Laboratories) were rendered HP by unilateral exposure of 6-OHDA using a modified procedure previously described and detailed above (Subramanian, Marchionini et al. 2002; Lieu, Kunselman et al. 2010).

Drug treatments with EFF0311 and LD
Animals received single injections (SQ or IP) of either 0.3 mg/kg EFF0311 (Group 1 - N=5), 0.6 mg/kg EFF0311 (Group 2 - N=5), or 6 mg/kg LD + 15 mg/kg benserazide (BZ) (Group 3 - N=5). EFF0311 was dissolved in 0.1% ascorbate solution. This dose of LD+BZ was chosen based on our previously published data (Lieu, Kunselman et al. 2010). Parkinsonian behaviors (stepping test and vibrissae-evoked forelimb placement test) were evaluated at HP post-lesion baseline, and at 30, 60 and 120 min post-treatment. DID ratings were taken at 30, 60, and 120 min post-treatment.

**Behavioral Assessments**

We utilized the stepping test and vibrissae-evoked forelimb placement test to evaluate parkinsonism and rated severity of DID (see details for behaviors above). All behaviors were evaluated by a blinded investigator not aware of treatment to the animals.

**Primate experiments**

To further validate the effectiveness of EFF0311 in PD, we evaluated the effects of EFF0311 in the bilaterally parkinsonian primate (N=1). A cynomologus macaque was rendered parkinsonian by systemic IM injection of MPTP. After a stable parkinsonian state had been achieved for at least 6 months, we evaluated the effects of placebo and single IM dose of EFF0311 (1 mg/kg) in a randomized, blinded studied. The animal was exposed to either placebo or single IM injection of EFF0311 on two separate occasions. The animal was then evaluated using the mUPDRS at 1.5 hours and 5.5 hours post-treatment (Appendix B). The two mUPDRS evaluations were then averaged for each treatment epoch.

**Statistical Analysis**
Behavioral data was analyzed using a one-way or repeated measures analysis of variance with post-hoc Tukey-Kramer multiple comparisons test. Data are expressed as mean ± SEM. Significance was set at $p < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

**Results**

**Rat experiments**

*Stepping test*

As can be seen in **Figure 2-11A**, the higher dose of EFF0311 and LD were equieffective in attenuating this measure of parkinsonism. There was a trend for the lower dose of EFF0311 at 120 min, but this was not significant. It is noteworthy that 0.6 mg/kg EFF0311 was effective at all time points, whereas LD was not effective until 120 min. As has been previously reported for rotations in this model (Murthy, Gowdahalli et al. 2011), pretreatment with the selective D$_1$ antagonist SCH39166 (0.1 mg/kg) completely blocked the actions of EFF0311 in all of our experiments (data not shown).

*Vibrissae-evoked forelimb placement test*

Unilateral nigrostriatal lesioning prevented placement behavior in the contralateral forelimb. The forelimb ipsilateral to lesion showed no deficit in this test. As can be seen in **Figure 2-11B**, the results of this experiment were generally similar to that in the stepping test. In this case, both doses of EFF0311, as well as LD, were effective in attenuating this measure of parkinsonism. The response to EFF0311 was somewhat greater than that caused by LD but was not significantly different. Importantly, EFF0311 was effective at the first (30 min) time point, whereas LD was not.

*Drug-induced dyskinesia ratings*
As is shown in Figure 2-11C, both LD and the higher doses of EFF0311 caused a significant increase in the dyskinesia profile rating scale we used. There was no significant difference between the effects of the 0.6 mg/kg dose of EFF0311 and LD as both caused large changes at all three time points. Interestingly LD caused a significant increase at 30 min in this dyskinesia scale, whereas it was ineffective in the stepping and vibrissae-evoked forelimb placement test where no effects were seen until 60 min. The lower doses of EFF0311 that had caused marked antiparkinson effects at 0.3 mg/kg in the vibrissae-evoked forelimb placement test only caused minor dyskinesias in this paradigm.

A) Stepping Test

B) Vibrissae-Evoked Placement Test

C) Dyskinesias

Figure 2-11. EFF0311 behavioral results in the parkinsonian rat. (A) Amelioration of parkinsonism - Stepping test. **\textit{p} < 0.01 vs. baseline, ***\textit{p} < 0.001 vs. baseline. (B) Amelioration of parkinsonism - Vibrissae-evoked forelimb
placement test. *p < 0.05 vs. baseline, ** p < 0.01 vs. baseline, ***p < 0.001 vs. baseline (C) Drug-induced dyskinesia severity scores. At 30 min, **p < 0.01 vs 0.3 mg/kg EFF0311, at 60 min, **p < 0.01 vs 0.3 mg/kg EFF0311, and at 120 min, **p < 0.01 vs 0.3 mg/kg EFF0311

Primate experiments

After placebo treatment, the animal displayed significant parkinsonism. With single IM treatment of EFF0311 (1 mg/kg), the animal demonstrated significant amelioration of parkinsonism. Average mUPDRS scores during placebo were 13.5 ± 1.5 at 1.5 hours and 11.8 ± 2.3 at 5.5 hours post-placebo. With EFF0311, the animal showed complete amelioration of parkinsonism (mUPDRS score = 0) at 1.5 and 5.5 hours post-EFF0311 treatment without dyskinesias (Fig. 2-12).

Figure 2-12. Amelioration of parkinsonism in the MPTP-treated primate with EFF0311. Single injection of EFF0311 (1 mg/kg) produced long-term behavioral benefit in the parkinsonism primate as demonstrated by mUPDRS scores above.

Discussion

Recent work (Murthy, Gowdahalli et al. 2011) has shown that the selective EFF0311 will cause rotations in the unilateral 6-OHDA rat that last many times longer than those caused by
most experimental DA agonists like dihydrexidine or SKF82959. This is the first study to evaluate more subtle effects of this drug, ones that might be relevant to its clinical use, in a murine and primate model. Our data suggest that EFF0311 is as effective as LD in attenuating the deficits induced by the dopaminergic lesion. There are two noteworthy findings in our rodent data. First, rats treated with EFF0311 had a more rapid “therapeutic” response, consistent with the fact that as a direct D₁ agonist does not have to be bioconverted before working. Second, some of the antiparkinson effects could be elicited by doses of EFF0311 that did not induce any dyskinesia. In the primate model of PD, we found that EFF0311 can completely abolish parkinsonism, and has long-term effectiveness after a single dose.

The contribution of which DA receptors are important in the genesis of dyskinesia has been controversial. There are data, however, suggesting that D₁ agonists may have beneficial effects on pre-existing dyskinesias, either in animals (Blanchet, Bedard et al. 1993) or humans (Rascol, Blin et al. 1999), sometimes improving motor symptoms without concomitant dyskinesia. This may depend, however, on the severity of the pre-existing dyskinesias (Rascol, Nutt et al. 2001). Several studies have shown that D₁ agonists were somewhat superior in abolishing dyskinesias while retaining antiparkinsonian activity (Pearce, Jackson et al. 1995; Pearce, Jackson et al. 1999).

It is noteworthy that of the many experimental leads that have utilized preclinical models, full D₁ agonists are the only class of drug whose activity has translated to LD-equalling effects in both MPTP-treated primates and PD patients (Shiosaki, Jenner et al. 1996; Blanchet, Fang et al. 1998; Rascol, Blin et al. 1999; Rascol, Nutt et al. 2001). Because no adverse effects were seen in these preliminary studies described above, as well as in those of Murthy et al. (Murthy, Gowdahalli et al. 2011), a foundation has been provided to study the effects of EFF0311 when given chronically to animal PD models in which both the propensity to induce dyskinesias and
the ability to elicit them in primed animals can be studied. Such data could provide a rationale to consider EFF0311 as a drug candidate.

Our data suggests that the decreased severity of dyskinesias with EFF0311 may be due to the longer duration of action via activation of D₁ receptors when compared to the effects of LD. This novel D₁ agonist with its desirable pharmacological properties and longer duration of action may make this compound very useful for studies that examine the relative role of different DA receptor subtypes in the induction and/or reversal of DID.

2.6. Conclusion

It is evident that there are numerous adjunct drug therapies for DID in PD that can lessen DID severity. When these pharmacological treatments fail, patients can receive functional neurosurgical therapies to mitigate DID. However, these treatments are either out of reach for many patients or are only moderately effective in treating DID. We describe two potential alternatives to these currently available treatments (the Ayurvedic medication M. pruriens water extract and the D₁ full agonist EFF0311) that have superior anti-PD effects and reduced risk for dyskinesias when compared to LD. In Figure 2-13, we summarize some of the antidyskinetic treatments described in this chapter and its target basal ganglia nuclei.
Figure 2-13. Summary of anti-dyskinetic treatments in PD. DBS has been targeted at the VIM (ventral intermediate nucleus), STN (subthalamic nucleus), CM/Pf (centromedian/parafascicular complex) and GPI (globus pallidus interna). EFF0311 targets D₁ receptors located in the striatum. M. pruriens contains many different compounds and may affect a number of different basal ganglia nuclei as well as cortical connections to the basal ganglia (original artwork by Andrew Gillies).

Despite these significant advances, increasing our understanding of the mechanisms associated with DID can improve how we treat dyskinesias. In the next chapter, we describe a retrospective evaluation of the lack of dyskinesias in the HP rhesus monkey after chronic LD treatment. Although the exact reason as to why such animals do not develop dyskinesias is unclear, the results of our study led us to hypothesize about the lack of dyskinesias in these animals and the potential mechanisms that may be associated with this phenomenon: the interhemispheric pathways of the striatum.
Chapter 3

Levodopa therapy in clinically hemiparkinsonian rhesus monkeys: the potential role of interhemispheric connections in drug-induced dyskinesias

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3 This chapter is a modified version of the journal article “Dyskinesias do not develop after chronic intermittent levodopa therapy in clinically hemiparkinsonian rhesus monkeys” published by Lieu et al., 2011 in Parkinsonism and Related Disorders, Volume 17, pp. 34-39. Authors: C Lieu, M Deogaonkar, R Bakay, T Subramanian. All authors wrote and revised the article. All authors were involved in conducting the experiments and analyzing data.
3.1. Abstract

The stable MPTP-induced HP rhesus monkey model of PD has been frequently used to test preclinical experimental therapeutics targeted to treat patients with advanced PD who suffer from motor fluctuations and DID. We retrospectively analyzed data from 17 stable HP rhesus monkeys treated long-term with chronic intermittent dosing of LD (LD) in an attempt to induce choreoathetoid and dystonic dyskinesias. Rhesus monkeys in stable HP state for greater than 6 months as confirmed by multiple blinded behavioral ratings and 18F-dopa Positron Emission Tomography (PET) were treated with optimal doses of LD to provide maximal amelioration of unilateral clinical parkinsonism without any adverse effects. Thereafter, each animal was given chronic intermittent daily challenge with doses of LD up to 700 mg/day orally or with 300 mg/kg/day parenteral injections. LD treatments failed to induce choreoathetoid and dystonic dyskinesias in these animals despite chronic intermittent high dose administration. These results suggest that the stable strictly unilateral HP rhesus monkey model of PD may not be a suitable animal model to test experimental therapeutics targeted against dyskinesias, and that bilateral parkinsonian rhesus models that readily demonstrate DID and clinically relevant motor fluctuations are more appropriate for preclinical experimental testing of therapies designed to treat patients with advanced PD. The mechanisms by which these HP rhesus monkeys do not develop dyskinesias is currently unclear. One possible mechanism that may account for the lack of dyskinesias in this monkey model is the inhibition from the crossed interhemispheric nigrostriatal pathway. We investigated this hypothetical mechanism in experiments described in Chapter 5 and 6.
3.2. Introduction

Rhesus monkeys rendered HP by MPTP have been a commonly used model of PD to test preclinical experimental therapeutics. Many investigators utilize the HP rhesus monkey because of the convenience of animal husbandry, built-in control that can be used for side-to-side behavioral comparisons, and the availability of considerable neuroscientific literature (Subramanian, Emerich et al. 1997; Andringa, Vermeulen et al. 1999; Starr, Wichmann et al. 1999; Kordower, Emborg et al. 2000; Emborg, Shin et al. 2001; Gilbert, Leszczynski et al. 2004; Collier, Dung Ling et al. 2005; Soderstrom, O'Malley et al. 2006; Emborg 2007; Subramanian, Lieu et al. 2010). However, we and others have anecdotally observed that strictly unilateral HP monkeys remain resistant to DID. To examine this systematically, we retrospectively reviewed 10 year data from stable HP rhesus monkeys treated with chronic intermittent doses of LD using a standardized protocol to determine the occurrence of choreoathetoid or dystonic LID in such animals.

3.3. Materials and methods

Animals

Seventeen adult female rhesus monkeys (Macaca mulatta, 3-6 kg) used as “negative controls” (no treatment or placebo) over the period from 1997 to 2007 were evaluated in this study. Behavioral observations reported in this retrospective study were done prior to enrollment into any experimental therapeutics protocol. In all cases, randomization into the “negative control” arm had been completed with no consideration of the hypothesis presented in this paper. Procedures were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978), and approved by the institutional animal care and use committee. All efforts were made to minimize pain and discomfort to the animals.
Behavioral evaluations

The timeline for the experimental design is shown in Figure 3-1. Through operant conditioning, the movement time of a fine motor task (FMT) was evaluated using a modified food picking test done twice weekly utilizing two sets of 3x2 matrix of food wells embedded in a Plexiglass board (Kordower, Emborg et al. 2000). Each set of food wells can be accessed by the monkey only using the arm that is being tested. Six raisins were placed on the board for each trial. The time required to retrieve all six raisins was taken for each arm over 10 trials.

A clinical rating scale based on the motor portion of the Unified Parkinson’s Disease Rating Scale modified for primates (mUPDRS) (Subramanian, Lieu et al. 2010) as described in Chapter 2 (Appendix B) was performed by a physician-scientist in a blinded fashion twice weekly and videotaped. The rater was blinded to the hypothesis presented in this paper at the time the ratings were performed.

![Figure 3-1](image)

**Figure 3-1.** Experimental procedure for MPTP exposure, LD administration, mUPDRS, FMT, and observation of LD-induced dyskinesias (LID).

Intracarotid injection of MPTP

Each animal received intracarotid injection of MPTP to induce hemiparkinsonism. See details in Chapter 2.
LD responsiveness in stable HP animals

The stability of the right HP state was confirmed using FMT and mUPDRS for a minimum of 6 months before testing the animal for responsiveness to LD therapy and for the genesis of LID. Animals were treated for 3-12 months with LD/dopa-decarboxylase inhibitor. Oral LD treatments were initiated at 100 mg LD/25 mg carbidopa (CD) b.i.d. and increased by 100 mg LD every 72 h until no further improvement in mUPDRS and FMT (each taken 1.5 h after LD) to determine the optimal dose. Animals were operant conditioned to accept medications, visually monitored and videotaped for 12 h thus ensuring complete drug consumption using methods described in Chapter 2 and elsewhere (Gilbert, Leszczynski et al. 2004). During a drug washout period for 4 weeks, behavioral evaluations continued. Plasma DA levels were measured using gas chromatography at pre-LD and post-optimal LD (Mizuno 1977).

To test the effects of intermittent high dosing of LD to cause dyskinesias, animals received single daily doses of LD/CD at 700/175 mg/day orally or escalating single injections of LD/benserazide starting at 50/12.5 mg/kg/day and up to 300/12.5 mg/ kg/day for 3-4 weeks. Animals were carefully observed and videotaped for a minimum of 12 h to further evaluate the beneficial effects of LD and determine onset of LID. Thereafter, each 12 h video was de-identified, scrambled such that they were in random order and carefully evaluated by a blinded rater using a standard dyskinesia rating scale. This dyskinesia rating scale ranged from 0 (no dyskinesias) to 4 (severe dyskinesias) and took into account only unequivocal limb dyskinesias in the form of chorea, ballismus, flinging of arms, violent jerks or athetosis and dystonias modeled after the scale described by Kurlan et al. (Kurlan, Kim et al. 1991).

Evaluation of dopaminergic nigrostriatal loss

Dihydroxy-6-$^{18}$F-fluorophenylalanine ($^{18}$F-dopa) PET imaging was done after attaining a stable HP state in randomly chosen animals and Ki values (sec$^{-1}$) obtained to confirm unilateral
nigrostriatal deficit (N=8). Post-mortem DA transporter immunoautoradiography (DAT-IAR) was also used to quantitatively assess the loss of dopaminergic fibers in the striatum in randomly chosen animals (N=12, anonymous numbers were assigned for each brain and randomization table was used). Tyrosine hydroxylase (TH) immunohistochemistry was used to assess the loss of dopaminergic substantia nigra (SN) neurons in all monkeys. Details for these techniques have been previously described (Subramanian, Emerich et al. 1997).

Quantitative estimates of SN TH-positive (TH+) neurons were performed using the optical fractionator probe (Stereo Investigator, MBF Bioscience) using systematic random sampling as determined by unbiased stereological counting procedures (N=3). Every 12th section through the SN was evaluated. Briefly, both the left (ipsilateral to intracarotid MPTP) and right (contralateral to intracarotid MPTP) SN was first outlined at low magnification (4x). A sampling site grid was then computer-generated with unbiased counting frames (125x125 mm) and superimposed onto the SN outline. Top and bottom guard zones were applied to each site, and a dissector height of 25 mm was used. Section thickness was measured at each site and counts for SN TH+ neurons were performed at high magnification. Total SN TH+ neurons for each hemisphere were then calculated using the Stereo Investigator software.

Statistics

Fisher’s exact test and Student’s t-test were used to measure the change in mUPDRS and FMT. One-way analysis of variance with Bonferroni’s multiple comparison post-test was used to compare stereological counts. Data are expressed as mean ± SEM. Significance was set at $p < 0.05$.

3.4. Results

Confirmation of stable HP state and LD responsiveness
FMT and mUPDRS behaviorally confirmed HP status. Right FMT significantly increased from 6.54 ± 0.17 s pre-MPTP to 23.0 ± 2.4 s post-MPTP (p < 0.001). Left FMT remained unaffected, 6.68 ± 0.25 s pre-MPTP and 6.19 ± 0.18 s post-MPTP (p > 0.05) (Fig. 3-2A). The mean dose of LD that produced optimal resolution of parkinsonism as measured by the mUPDRS was 200 mg b.i.d. The mean mUPDRS score post-MPTP was 12.6 ± 1.3 which improved to 3.6 ± 0.39 (p = 0.02) after optimal LD. At washout, the mUPDRS returned to 11.7 ± 1.0 (p = 0.02) (Fig. 3-2B). Additionally, FMT performance post-LD improved on the right side following optimal dose and returned to baseline when LD was washed-out. The mean post-MPTP FMT during this trial was 29.9 ± 1.7 s, which improved to 9.1 ± 1.2 after LD, worsening back to 21.4 ± 1.6 after LD washout (p = 0.045) (Fig. 3-2C). Median plasma DA levels pre-LD were 180 pg/ml and increased to 22,220 pg/ml on optimal LD dose.
Figure 3-2. Confirmation of stable HP state and LD responsiveness. (A) FMT before and after MPTP. Contralateral side remains unaffected post MPTP (***p < 0.001) (Seconds = time to complete FMT). Improvement in the mUPDRS (B) and FMT on the affected side (C) while on LD therapy which reverses back after LD washout (*p < 0.05).
**LD-induced dyskinesia assessment**

No unequivocal limb dyskinesias in the form of chorea, ballismus, flinging of arms, violent jerks or athetosis and no dystonias were observed in any of the animals for up to 12 months of LD treatments. Furthermore, deliberate chronic over-dosing up to 700 mg/day (approximately 140 mg/kg/day) of oral LD or 300 mg/kg/day of injectable LD caused no apparent limb dyskinesias. Some animals did show ill-sustained contraversive circling, occasional motor tics or minor stereotypy on both sides of the body for less than 1 min/h of video after this high dose LD treatment (700 mg/day). We did not include circling, rare simple motor tics or minor stereotypies in our study as indicators of dyskinesias. Tics and stereotypies were defined by comparison against 12 h videos obtained from these stable HP animals before treatments with any anti-PD medication.

**Evaluation of dopaminergic nigrostriatal loss in stable HP monkeys**

$^{18}$F-dopa PET imaging showed a significant reduction of $^{18}$F- dopa uptake and metabolism in the striatum ipsilateral to the side of MPTP administration and preservation of signal on the contralateral side (Fig. 3-3A). Animals demonstrated an 87% reduction in uptake in the ipsilateral caudate and a 79% reduction in uptake in the ipsilateral putamen. For example, Ki values (sec$^{-1}$) were 1.32 $\times$ 10$^{-5}$ and 2.025 $\times$ 10$^{-5}$ in the ipsilateral caudate and putamen, respectively. The Ki values (sec$^{-1}$) were 10.0 $\times$ 10$^{-5}$ and 9.53$\times$10$^{-5}$ in the contralateral caudate and putamen, respectively. The average DAT-IAR values of the lesioned side ipsilateral to MPTP injection in the caudate were 603 dpm/mg and 486 dpm/mg in the putamen. The contralateral side was 4936 dpm/mg and 3951 dpm/ mg in caudate and putamen, respectively. The dopaminergic fiber loss was less marked in the ventromedial striatum and the tail of caudate (Fig. 3-3B). TH immunohistochemistry showed extensive loss of TH staining in the striatum and nigra on the lesioned side as compared to the contralateral side (Fig. 3-3C). Unbiased stereological counts of
SN TH+ neurons from previous studies using normal rhesus monkeys aged 3-10 years old report an average of 178,198 ± 51,802 SN TH+ neurons unilaterally (Gerhardt, Cass et al. 2002; Kanaan, Kordower et al. 2007). TH+ counts from our animals were 18,418 ± 4425 in the left SN (ipsilateral to intracarotid MPTP) ($p < 0.05$) and 99,667 ± 11,711 in the contralateral hemisphere (CE =0.14) (Fig. 3-3D). There was no anatomical or histological evidence of placebo-induced changes either in the striatum or nigra in these animals.
Figure 3-3. Confirmation of HP state. (A) Trans-axial 18F-dopa PET scans from HP rhesus monkeys. (B) DAT-IAR to estimate quantitative dopaminergic fiber loss in the striatum. (C) View of TH staining in the striatum and nigra in the HP rhesus monkey. (D) Unbiased stereological counts of unilateral SN TH+ neurons. Right nigra is contralateral to intracarotid MPTP and left nigra is ipsilateral to intracarotid MPTP-expressed as percentage (*p < 0.05 vs. normal).

3.5. Discussion

Our results confirm that strictly unilateral HP rhesus monkeys do not develop LID. The experiments described here closely resemble the clinical dosing regimen of dopaminergic medications in PD patients. Animals were exposed to chronic intermittent optimal oral doses of
LD then very high doses of LD both orally and parenterally in sufficient amounts that should have lead to the genesis of choreoathetoid and dystonic LID. However, despite careful assessments for LID, none were observed for up to 12 months of testing. Several other investigators using the strictly HP rhesus monkey have anecdotally observed the lack of choreoathetoid and dystonic LID, but to the best of our knowledge not reported in the literature though systematic investigation. It was previously reported that high doses of LD produced dyskinesias in “HP” rhesus monkeys (Kurlan, Kim et al. 1991), but careful read of this previous literature indicate that these animals in fact had clinical and pathological evidence of bilateral parkinsonism. This was acknowledged in that article by the authors themselves (see page 116-117 of (Kurlan, Kim et al. 1991)). Our systematic blinded retrospective review is the first of its kind to report the lack of LID in HP rhesus monkeys despite exposure to large intermittent doses of LD.

LID has been attributed to nigrostriatal degeneration and non-physiological pulsatile stimulation of striatal DA receptors from chronic intermittent LD (Mouradian, Heuser et al. 1989; Bibbiani, Costantini et al. 2005). This theory would predict that HP rhesus monkeys should develop unilateral (on the contralateral limbs) dyskinesias when given chronic intermittent high dose LD treatments. However, this did not occur. It is unclear what mechanisms are involved in the lack of LID in these animals. We discuss here the potential mechanisms that may explain our findings.

One possibility is that there is an interhemispheric inhibitory influence that prevents the genesis of unilateral dyskinesias from the less affected hemisphere in the HP rhesus monkey. The nature of this inhibitory influence is unclear but could be mediated via the small percentage of interhemispheric fibers of the nigrostriatal pathway that immediately crossover at the mesencephalon from the opposite hemisphere. Such crossover fibers represent an estimated 5% of SN dopaminergic fibers in the rat and up to 13% in monkeys (Fass and Butcher 1981;
Collingridge 1982; Francois, Percheron et al. 1984). This theory would suggest that a threshold of bilateral nigrostriatal denervation is essential for the genesis of dyskinesias. This theory is supported in part by the finding that HP rhesus monkeys when overlesioned (with additional doses of MPTP), to cause bilateral but asymmetric parkinsonism, readily develop dyskinesias that are identical to LID seen in PD. These animals have bilateral but markedly asymmetric nigrostriatal denervation (Oiwa, Eberling et al. 2003). Similarly, systemic MPTP causing bilateral parkinsonism in rhesus monkeys also readily develop LID (Papa, Desimone et al. 1999; Bankiewicz, Daadi et al. 2006; Liang, DeLong et al. 2008). Thus, one possibility is that HP monkeys that we report here have interhemispheric inhibition of LID. As a corollary, rats with less than 95% unilateral loss of nigrostriatal innervation as a result of striatal 6-hydroxydopamine (6-OHDA) lesioning (e.g., Sauer and Oertel model) and monkeys with <85% unilateral nigrostriatal denervation from intracarotid injections of MPTP are likely to have intact (on both sides) interhemispheric dopaminergic fibers. Moreover, the Ungerstedt 6-OHDA lesioned rat model of PD that causes >95% unilateral loss of striatal DA innervation do develop involuntary movements that are similar to some degree to LID (Ungerstedt and Arbuthnott 1970), whereas rats that receive a partial lesion that cause only 50% striatal denervation do not develop LID (Sauer and Oertel 1994). Similarly, as discussed earlier, the over-lesioned HP rhesus and bilateral parkinsonian rhesus monkeys readily develop LID. We are not aware of similar reports on the status of interhemispheric nigrostriatal fibers in humans, but we would speculate that humans have at least as many dopaminergic nigrostriatal crossover fibers as in monkeys. Another possible pathway that may modulate dyskinesias is the interhemispheric and intrahemispheric corticostriatal connections (Wilson 1986; Reiner, Jiao et al. 2003). It is well known that the cortical synaptic connections to the striatum are altered in response to DA depletion that pathophysiologically alters the output of medium spiny neurons and subsequent basal ganglia nuclei (Raju, Ahern et al. 2008; Soderstrom, O'Malley et al. 2010). Future studies are necessary
to evaluate if changes to these corticostriatal connections influence the genesis of LID.

The second possibility is that the protection against LID in our HP animals is simply a lack of sufficient unilateral nigral denervation and do not involve the crossover fibers or interhemispheric inhibition. This theory would suggest that extensive unilateral nigrostriatal denervation is sufficient for the genesis of contralateral LID. However, this theory appears to be unlikely in the animals we report here as they had near complete loss of all ipsilateral nigrostriatal fibers as demonstrated by DAT-IAR (>80%, mean DAT-IAR drop from 4500 to 540), $^{18}$F-dopa PET imaging showing near complete loss of $^{18}$F-dopa uptake in the lesioned left hemisphere (>80% reduction demonstrated by striatal Ki values) and histology (SN TH+ stereology 178,000 to 18,000-proof of 90% reduction). This degree of denervation represents almost complete denervation of the ipsilateral nigrostriatal pathway (the remaining 10% representing fibers that crossover to the opposite hemisphere). Thus, the lack of sufficient ipsilateral nigrostriatal denervation in the 17 monkeys we report here is unlikely to explain their resistance to develop LID despite chronic 12 month intermittent exposure to LD. As reported in the literature, any additional intracarotid injections in these HP animals either fail to cause additional clinical parkinsonism (so-called MPTP resistance) or cause parkinsonism in the previously asymptomatic side such that these animals are now clinically bilaterally parkinsonian (Kurlan, Kim et al. 1991).

As a clinical correlate to our observations in the HP monkey, idiopathic PD patients with strictly unilateral symptoms (stage I Hoehn and Yahr) do not develop LID until they progress to stage II (bilateral parkinsonism) despite clearly demonstrating neurodegeneration (on PET imaging and in autopsy studies) in the SN bilaterally even in stage I disease. This clinical observation is true even among young onset PD patients who have the highest (>80%) risk of developing LID (Kumar, Van Gerpen et al. 2005). Moreover, it is now clear from numerous early PD clinical trials including those that exclusively used LD as a treatment, that PD patients in stage I disease do not develop LID despite imaging studies in these early PD patients confirming
the asymmetric bilateral dopaminergic denervation (Fahn 2006). Although many clinicians are aware of this phenomenon in idiopathic PD patients, we are unaware of any systematic study that has examined the lack of LID in stage I patients (requiring LD withdrawal and examination in the “on” and “off” states to confirm their clinical stage over an extended period of longitudinal follow-up). Rare case reports of patients with secondary parkinsonism (non-idiopathic PD patients) or with hemiatrophy-HP syndromes who developed LID had severe near complete destruction of the SN and the adjacent mesencephalon where crossover fibers reside (Alves, Barbosa et al. 1992; Ruzicka, Urgosik et al. 2005), suggesting that crossover fibers were destroyed in these patients. Future anatomical studies in post-mortem brains of patients who had developed LID or hemi-LID are needed to validate this hypothesis in humans.

The third, but much less likely possibility to explain the lack of LID in our animals is that this is a species-specific finding that somehow protects HP rhesus monkeys from LID. Investigators report the genesis of “dyskinesias” in normal and partially lesioned monkeys other than rhesus macaques (Sassin, Taub et al. 1972; Clarke, Boyce et al. 1989; Schneider 1989; Boyce, Rupniak et al. 1990; Pearce, Jackson et al. 1995; Di Monte, McCormack et al. 2000; Pearce, Heikkila et al. 2001; Togasaki, Tan et al. 2001). The discrepancies with these reports and the present study most likely stems from how these investigators chose to define LID (e.g., circling counted as dyskinesias), variations in the amount of LD administered (e.g., LD dose 10 times of what is required for optimal anti-parkinsonian benefits) and routes of administration (e.g., intraperitoneal). In the present study, we strictly defined LID to include only choreoathetoid and dystonic movements that are readily seen in the severely bilateral parkinsonian rhesus monkey and are identical to what is seen in humans with advanced PD on LD therapy. We did not include circling, simple motor tics or minor stereotypies in our study. Circling was rarely (<1 event/hour of video) noted in our study, and only with very high doses of LD.

The risk of developing LID has been attributed to the degree of lesion in both SN (and
potentially the locus coeruleus), dosing regimen of LD, and sensitivity of detecting dyskinesias
(Kurlan, Kim et al. 1991; Mavridis, Degryse et al. 1991; Fahn 2006; Wang, Zhang et al. 2010).

We demonstrate that HP monkeys reported here had stable unilateral nigrostriatal degeneration
through blinded clinical ratings and fine motor performance testing, quantitative analysis of
histological sections, DAT-IAR and \(^{18}\text{F}\)-dopa PET imaging. Previous studies have demonstrated
an 86% decrease in SN TH+ neurons ipsilaterally and 25% decrease in SN TH+ neurons
contralaterally in HP young rhesus monkeys in comparison to historical data in normal rhesus
monkeys using unbiased stereological counts with the optical fractionator method (Gerhardt, Cass
et al. 2002; Collier, Lipton et al. 2007; Kanaan, Kordower et al. 2007). Our stereological SN TH+
counts demonstrate a 90% decrease in the ipsilateral SN and 44% decrease in the contralateral SN
compared to previous reports in normal rhesus monkeys. Taken together, these studies
demonstrate a near complete unilateral loss of nigrostriatal neurons (except for the 10% of fibers
that crossover to the opposite striatum) was present in the HP rhesus monkeys reported in our
study.

The extent of SN lesioning in this animal model is at its maximum unilaterally. Any
additional lesioning with MPTP causes bilateral symptoms. The regimen of LD dosing we used
simulated the clinical experience in PD patients with chronic, high intermittent doses. Identical
dosing regimen readily induces LID in bilateral parkinsonian monkeys. These animals were
carefully observed by an experienced clinician scientist for a minimum of 12 h each day for
several months using randomized blinded video rating to detect LID. Thus, lack of sufficient
unilateral lesioning, inadequate LD dosing or the lack of careful clinical observation could not
explain the lack of LID in these strictly unilateral HP monkeys. In the present study, we did not
collect biochemical data because each animal was perfused and brains fixed for histological
analysis. We did not obtain stereological counts of neurons in the locus coeruleus that are known
to play a role in PD (Mavridis, Degryse et al. 1991; Wang, Zhang et al. 2010), although its role in
the genesis of LID in HP rhesus monkeys is unknown. Future studies are warranted utilizing DA biochemical analysis techniques and stereological counts of the locus coeruleus in strictly unilateral HP rhesus monkeys that are chronically exposed to LD to further strengthen our findings.

The strictly unilateral stable HP rhesus monkey model of PD has been extensively used by researchers to test various preclinical therapies including cell transplantation and gene therapy (Subramanian, Emerich et al. 1997; Starr, Wichmann et al. 1999; Kordower, Emborg et al. 2000; Soderstrom, O'Malley et al. 2006; Emborg, Carbon et al. 2007). Our results suggest this strictly unilateral rhesus monkey model of PD, which has a proven track record of excellent utility in pathophysiological and electrophysiological studies, is not a suitable model to test experimental therapeutics targeted to improve advanced PD. In particular, treatments that seek prophylactic and palliative treatments for LID or pathophysiology of LID cannot be meaningfully tested in this model. The severely parkinsonian bilateral rhesus monkey model or the overlesioned HP monkey model that readily exhibit all the classic hallmarks of LID seen in PD patients may be a more appropriate model to test experimental therapeutics in advanced PD and to examine the pathophysiology of LID.

It had been previously shown that there is an asymmetric presence of DA between hemispheres. The left striatum has been reported to have higher levels of DA when compared to the right. Haaxma and colleagues (Haaxma, Helmich et al. 2010) evaluated motor disability in right-handed PD patients with either predominant left or right hemisphere DA depletion. They found that patients with predominant right hemisphere DA loss had more motor dysfunction than patients with predominant left hemisphere DA loss. The authors suggest that the right hemisphere (non-dominant for right handed patients) may be more susceptible to DA denervation, and may account for higher motor dysfunction. In the context of our study, it is unclear if non-human primates have a hand preference since animals were able to perform the FMT with both hands at
the same speed pre-MPTP. We had only administered MPTP via the left carotid artery. This would affect the left striatum, which as stated above, contains higher levels of DA. Future studies that compare right versus left ICA MPTP as it correlates to motor disability in HP Rhesus monkeys could further enhance our understanding of side of symptom onset in relation to severity of parkinsonism and possibly DID.

3.6. Conclusion

Taken together, our study demonstrates that despite chronic LD treatment and extensive unilateral nigrostriatal degeneration, clinically HP rhesus monkeys do not develop DID. Based on these results, we hypothesized that the interhemispheric pathways may play a role in the prevention of dyskinesias in this animal model and potentially in early stage I PD patients. To further expand on the role that interhemispheric connections play in PD, we review the currently available literature about interhemispheric connections to the striatum in Chapter 4.
Chapter 4

The interhemispheric connections of the striatum: implications for Parkinson’s disease and drug-induced dyskinesias

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This chapter is a modified version of the journal article manuscript “The interhemispheric connections of the striatum: implications for Parkinson’s disease and drug-induced dyskinesias” published by Lieu and Subramanian, 2011 in Brain Research Bulletin (in press). Authors: C Lieu and T Subramanian. CL and TS developed the entire manuscript.
4.1. Abstract

In Chapter 3, we show that strictly, clinically HP rhesus monkeys do not develop LD-induced dyskinesias despite chronic intermittent exposure and significant unilateral loss of nigrostriatal neurons and DA. It is currently unclear what mechanisms prevent the onset of dyskinesias in these animals. However, based on our study and results from previous lesioning studies in both the rat and monkey models of PD, we hypothesize that one potential mechanism that may prevent the genesis of dyskinesias in these animals is interhemispheric neuromodulation. Two potential interhemispheric connections that may modulate dyskinesias are the interhemispheric nigrostriatal and corticostriatal pathways. Few investigators have examined the interhemispheric nigrostriatal and corticostriatal connections and the functional role they may play in DID in PD. In this chapter, we assess the neuroanatomical, electrophysiological, neurochemical and behavioral properties of these interhemispheric connections and discuss how they may play a role in DID.
4.2. Introduction

In Chapter 3, we show that strictly clinically HP rhesus monkeys do not develop dyskinesias despite chronic intermittent treatments with LD. Based on the prevailing hypothesis for the development of dyskinesias described above, these animals should display dyskinesias at least on the hemi-body contralateral to the hemisphere that has nigrostriatal degeneration. However, this was not evident in our study. The mechanisms by which these animals remain resistance to dyskinesias are unclear. We hypothesize in our study that one possible mechanism that prevents dyskinesias in HP rhesus monkeys is via interhemispheric neuromodulation mediated by the interhemispheric nigrostriatal pathway.

The human relevance of this data as described in Chapter 3 (Lieu, Deogaonkar et al. 2011) is the notion that idiopathic PD also begins unilaterally (Hoehn and Yahr stage 1), and such patients in stage I disease never develop any dyskinesias. We are unaware of any known reports of unilateral PD (H&Y stage I) patients developing any DID. Published literature to date in which early stage I patients were given large doses of LD as part of placebo-controlled randomized studies did not exhibit DID while they were in stage I disease (Fahn 2006). It is well known that in patients with idiopathic PD at the time of initial onset of symptoms, > 50% of the nigrostriatal dopaminergic neurons have succumbed to neurodegeneration. However, this neurodegeneration is asymmetric and the onset to disease symptoms of PD on one side of the body is a well established diagnostic criterion for PD. Therefore, despite the lack of disease progression in the HP MPTP-treated monkey model, there are many phenomenological similarities between the HP rhesus monkey model and stage I idiopathic PD. As a corollary, the MPTP-treated bilateral animal faithfully develops in all cases LD-induced dyskinesias that is virtually identical to dyskinesias seen in PD patients. Thus, the MPTP-treated monkey model of PD, despite its static nature, allows adequate modeling of the human disease at different stages and investigation of pathophysiological questions regarding the origin of dyskinesias. Most interestingly, as MPTP
doses are increased in the creation of the HP model, the animal develops bilateral disease despite
the deliberate injection of the toxin unilaterally via a single internal carotid artery. This finding
further supports the notion that the loss of interhemispheric nigrostriatal connections as
hypothesized in our study is mandatory to cause DID. The observation that stage I PD patients
remain resistant to drug induced dyskinesias while stage II (bilateral) disease patients begin to
exhibit DID may be the clinical correlate to the dyskinesia symptoms seen in the MPTP-treated
monkey models of PD.

In context to Figure 4-1, we retrospectively reviewed the literature to support this
interhemispheric neuromodulation theory in both the monkey and rat model of PD based on data
from various lesioning studies. There are two different rat models of PD that are currently
utilized. The first was described by Ungerstedt, with lesioning of the MFB (Ungerstedt and
Arbuthnott 1970). The other by Sauer and Oertel, with lesioning at the striatum (Sauer and Oertel
1994). The Ungerstedt model develops dyskinesias with LD and is used to model DID in many
studies, whereas the Sauer and Oertel striatal lesion model does not develop dyskinesia. We
suggest that the dyskinetic Ungerstedt model of PD which causes > 95% unilateral nigrostriatal
degeneration by 6-OHDA neurotoxin injection into the medial forebrain bundle leads to loss of
interhemispheric nigrostriatal neurons (Ungerstedt and Arbuthnott 1970; Lieu, Kunselman et al.
2010). In contrast, the non-dyskinetic striatal lesioned model (Sauer and Oertel 1994; Winkler,
Kirik et al. 2002) with approximately 50-70% nigrostriatal loss by 6-OHDA injection into the
striatum retains these interhemispheric nigrostriatal neurons. Similarly in our non-dyskinetic HP
rhesus monkey model, we suggest that interhemispheric nigrostriatal neurons are retained but lost
in the bilateral parkinsonian rhesus monkey that readily develops dyskinesias (Papa, Desimone et
al. 1999; Liang, DeLong et al. 2008).

In this context, it is worthwhile to explain the differences between the unilateral 6-OHDA
lesioned HP rat and MPTP-treated HP monkey. The injection of the neurotoxin in the case of 6-
OHDA (Ungerstedt model) (Ungerstedt and Arbuthnott 1970) is adjacent to the site of crossing of
the interhemispheric nigrostriatal fibers (see discussion below under tracer studies) in the
mesencephalon. This location causes the lesioning of the interhemispheric nigrostriatal fibers and
its neurodegeneration. In addition, most 6-OHDA-treated rats have near complete lesion of
virtually all dopaminergic nigrostriatal neurons in the ipsilateral nigra. On the other hand,
injection of MPTP to create the HP rhesus monkey model of PD is performed via a substantially
different technique of neurotoxin administration (see details in Chapter 2). MPTP is injected
intracarotid unilaterally repeatedly on the same side. MPTP administered in this manner
predominantly remains in the ipsilateral hemisphere and to a lesser extent crosses over into the
contralateral hemisphere via the Circle of Willis. The effects of MPTP (MPP+) is primarily at the
level of the striatum where it is taken up via the DA transporter into the nigrostriatal fibers and
retrogradely transported to cause delayed toxicity of the cell bodies located in the SN. Due to this
very different route of administration, the nigrostriatal degeneration is predominantly ipsilateral,
and as noted in Chapter 3 (Lieu, Deogaonkar et al. 2011), the lesion is about 90% complete. We
hypothesize that in this scenario, the interhemispheric nigrostriatal fibers are spared, allowing the
animal to be resistant to LD-induced dyskinesias.

In this chapter, we closely examine the previous literature that has used neuroanatomical,
neurochemical, electrophysiological, and behavioral techniques to highlight the interhemispheric
connections. Although we hypothesize that the most likely candidate for interhemispheric
neuromodulation in DID is by the nigrostriatal pathway, it is also possible that interhemispheric
corticostriatal neurons play a role in dyskinesias, which we also describe below. These
interhemispheric connections could provide important information about the pathophysiological
basis of PD and DID, and may prove to be a novel target for future anti-PD and anti-dyskinetic
treatments.
Figure 4-1. Hypothesized degeneration of interhemispheric nigrostriatal pathway in dyskinesias. (A) Retention of the interhemispheric nigrostriatal pathway in the striatal 6-OHDA lesioned non-dyskinetic rat which is lost in the medial forebrain bundle 6-OHDA lesioned dyskinetic rat (B). Similarly, retention of the interhemispheric nigrostriatal pathway in the non-dyskinetic HP (lesioned by intracarotid MPTP) rhesus monkey (C) which is lost in the bilateral parkinsonian dyskinetic rhesus monkey (lesioned by systemic MPTP) (D). Solid, black lines represents intact nigrostriatal pathway and dotted, gray lines represents lost, degenerated nigrostriatal pathway.

4.3. Interhemispheric connections in the normal state

4.3.1. Interhemispheric nigrostriatal connections

*Tracer Studies in the Rat*
A number of investigations have examined the presence and topographic organization of interhemispheric nigrostriatal projections using various labeling techniques in the rat. Most studies have used retrograde tracing techniques to identify the presence of these projections using horse-radish peroxidase (HRP). In 1980, Veening and colleagues note the presence of retrogradely labeled cells in the contralateral SNc after unilateral microiontophoretic injections of HRP into the striatum (Veening, Cornelissen et al. 1980). Similarly, unilateral injection of wheat germ agglutinin-HRP into two anterior regions of rat striatum lead to sparsely labeled nigral neurons in the opposite hemisphere at the middle rostrocaudal regions of the SN (Consolazione, Bentivoglio et al. 1985). Additional experiments in adult Long-Evans rats with unilateral striatal injections of HRP confirm these findings. This study suggested that these interhemispheric nigrostriatal connections account for approximately 3% of the ipsilateral pathway (Douglas, Kellaway et al. 1987). Control injections in the cortex and nucleus accumbens did not label contralateral SN, validating the presence of specific crossing nigrostriatal projections. To further delineate the topography of these projections, it has been suggested that these neurons originate mainly in the middle and caudal parts of the SN (Morgan, Steiner et al. 1986), whereas the ipsilateral nigrostriatal projections are distributed more in the rostral SN. This suggests that there is an inverse distribution between ipsilateral and contralateral projecting nigrostriatal neurons in the rostrocaudal plane. In another study using 10 normal male Wistar rats, investigators only found 1-5 contralaterally HRP-labeled SN neurons after unilateral striatal injection of HRP in 6 out of 10 rats (Pritzel, Huston et al. 1983). These differences in results found with HRP striatal injection and the presence of contralaterally labeled SN neurons may be attributed to site of injection and survival period of the animal.

Anterograde tracer (3H)leucine injected unilaterally into the SNc in male albino rats leads to the presence of interhemispheric nigrostriatal axonal projections discretely terminating in the medial and lateral portions of the contralateral striatum (Morgan and Huston 1990). With infusion
of Evans Blue (a retrograde fluorescent tracer) unilaterally into the striatum of Sprague-Dawley rats, Fass and Butcher report 2-14 neurons positively labeled in the contralateral SN per brain (Fass and Butcher 1981). Similar to studies with HRP, control injections with this fluorescent tracer to the cortical regions directly above the striatum did not cause contralateral SN labeling, and thus confirm that the presence of contralateral SN labeling is entirely due to crossing interhemispheric nigrostriatal projections. In another study, nuclear yellow was injected into one striatum and granular blue into the opposite striatum (Loughlin and Fallon 1982). One to two retrogradely labeled neurons of each tracer were found in the SN and ventral tegmental area (VTA) contralateral to injection through each SN section examined. Investigators did not observe any double-labeled cells. These results were further confirmed using striatal injections of either propidium iodide or Granular Blue and demonstrate that SN contralateral projections to the striatum account for 1-2% of the ipsilateral projections (Fallon, Wang et al. 1983). Similar findings were observed in young rats (6 and 30 day old) that had received Nuclear Yellow into one striatum and granular blue in the contralateral striatum (Fig 4-2), finding that crossed cells approximated 1% of ipsilateral cells with 6-day old rats having slightly more crossed cells than the 30 day old group (Altar, Neve et al. 1983). However, dispersion of dyes was more extensive in 6 day old than 30 day old, and thus may account for differences in number of crossing cells observed in the two groups. In a different study, neonatal rats received striatal lesion in one striatum then subsequent retrograde tracer in the opposite hemisphere in adulthood and found neurons retrogradely-labeled in the contralateral nigra. The authors suggest that these projections are most likely not branching since they were not affected by contralateral striatal lesion (Jaeger, Joh et al. 1983), and that ipsilateral and contralateral projecting nigrostriatal neurons are two distinct separate populations. However, this finding is different to those found by Pritzel and colleagues (Pritzel, Sarter et al. 1983). Injection of Nuclear Yellow in one striatum and Fast Blue into the other striatum lead to both contralaterally labeled SNc neurons at 5% of ipsilaterally
labeled neurons with a small number of nigral neurons double-labeled with both tracer. This particular observation demonstrates bifurcation of single nigrostriatal neurons between the two striatum. Differences in these studies may be due to procedural differences such as tracer used, survival time and analysis of tissue. Nonetheless, these studies validate the presence of interhemispheric nigrostriatal neurons in the rat which approximate < 1-10% of the ipsilateral nigrostriatal connections. Table 4-1 summarizes the tracer studies in the rat.

Figure 4-2. (A) Experimental paradigm for retrograde striatal tracer injection described by Altar, Neve et al. 1983. Left striatum – granular blue, Right striatum – nuclear yellow. (B). Coronal section of striatum with left granular blue and right nuclear yellow injections (top). SN sections showing distribution of labeled neurons contralateral to injection site; dark dots – granular blue, white dots – nuclear yellow (middle and bottom). Reproduced with permission (Altar, Neve et al. 1983).
Furthermore, investigators have examined the location of where these interhemispheric nigrostriatal neurons decussate as well as the neurochemical properties of these cells. One group of investigators suggests that these fibers cross in the diencephalon, specifically near the thalamus, based on unilateral, striatal injection of HRP (Pritzel, Sarter et al. 1983). However, others have more convincingly shown that the interhemispheric nigrostriatal neurons most likely cross near the ventral tegmental region. In these studies, transection of the corpus callosum and thalamic region in rats does not prevent labeling of contralateral neurons after unilateral striatal injection of HRP or Evans Blue, whereas midsagittal transection at the ventral mesencephalon prevents contralateral labeling of interhemispheric nigrostriatal projections (Fass and Butcher

Table 4-1. Summary of interhemispheric nigrostriatal studies in the rat

<table>
<thead>
<tr>
<th>Reference</th>
<th>Tracer</th>
<th>Injection Site</th>
<th>Survival Period</th>
<th># of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vining et al., 1980</td>
<td>HRP</td>
<td>Striatum - Unilateral</td>
<td>1-3 days</td>
<td>N/A</td>
</tr>
<tr>
<td>Fass and Butcher, 1981</td>
<td>Evens Blue</td>
<td>Striatum - Unilateral</td>
<td>2 days</td>
<td>2-14/brain</td>
</tr>
<tr>
<td>Leightin and Fallon, 1982</td>
<td>Granular Blue Nuclear Yellow</td>
<td>Striatum - Unilateral</td>
<td>24 h</td>
<td>5-10% of ipsi</td>
</tr>
<tr>
<td>Altar et al., 1982</td>
<td>Nuclear Yellow</td>
<td>Striatum - Unilateral</td>
<td>20-20 h</td>
<td>~1% of ipsi</td>
</tr>
<tr>
<td>Pritzel et al., 1983a</td>
<td>HRP</td>
<td>Striatum</td>
<td>24 h</td>
<td>1 to 5</td>
</tr>
<tr>
<td>Pritzel et al., 1983b</td>
<td>Fast Blue Nuclear Yellow</td>
<td>Striatum</td>
<td>1-7 days (dependent upon tracer transport)</td>
<td>~5% of ipsi</td>
</tr>
<tr>
<td>Fallon et al., 1993</td>
<td>Fospidium Solide, Granular Blue</td>
<td>Medially and Laterally in Anterior 2/3s of Striatum</td>
<td>24-30 h</td>
<td>1-2% of ipsi</td>
</tr>
<tr>
<td>Jeepser et al., 1985</td>
<td>HRP</td>
<td>Striatum - Unilateral</td>
<td>1 day</td>
<td>2 to 12</td>
</tr>
<tr>
<td>Mintz et al., 1985</td>
<td>HRP</td>
<td>Striatum - Unilateral</td>
<td>2-3 days</td>
<td>3 to 6xction</td>
</tr>
<tr>
<td>Consolazio et al., 1990</td>
<td>WGA-HRP</td>
<td>Striatum - Unilateral (basal and pal surface) S1 - AP: 2.2; L: 2.2; V: 4.2 S2 - AP: 2.0; L: 2.5; V: 5.0</td>
<td>18-24 h</td>
<td>0 to 2</td>
</tr>
<tr>
<td>Morgan et al., 1996</td>
<td>HRP</td>
<td>Striatum - Unilateral</td>
<td>24 h</td>
<td>See article</td>
</tr>
<tr>
<td>Douglas et al., 1997</td>
<td>HRP</td>
<td>Striatum - Unilateral</td>
<td>2-3 days</td>
<td>~3% of ipsi</td>
</tr>
</tbody>
</table>

Anterograde Studies
Morgan and Hudson, 1990 | [3H]Lesone | SNpr - Unilateral (intercalary line) R: 2.2; L: 1.7; D: 1.9; focal site; 2.3 below intercalary line | 7 days |
1981; Mintz, Douglas et al. 1985; Douglas, Kellaway et al. 1987). Additionally, there is evidence that most of these interhemispheric nigrostriatal neurons are dopaminergic neurons based on their morphological similarities to ipsilateral projecting nigrostriatal neurons and as demonstrated through various techniques such as tyrosine hydroxylase immunocytochemistry (Altar, Neve et al. 1983; Jaeger, Joh et al. 1983; Pritzel, Sarter et al. 1983; Consolazione, Bentivoglio et al. 1985).

Vibrissae usage in the rat is important for sensorimotor function. Using HRP injections, removal of vibrissae in the rat has shown to induce plastic asymmetrical changes to the number of HRP-positive ipsilateral and crossing nigrostriatal projections (Steiner, Weiler et al. 1989; Steiner, Weiler et al. 1992). On the other hand, the nigrostriatal interhemispheric connections do not seem to undergo plastic changes during other types of sensorimotor behavior, such as the reinforcement of operant turning movement (Morgan, Rosenkranz et al. 1985). These results demonstrate that the interhemispheric nigrostriatal connections are subject to plasticity changes in response to sensorimotor alterations.

**Tracer Studies in the Cat**

Early cat studies have also been utilized to characterize the presence of interhemispheric SNc neurons to the caudate/striatum. Injection of HRP (ranging from 0.05-0.6µl) into the caudate of cats found dense staining in the ipsilateral SNc in addition to labeling in the SNc contralateral to the injection site (Royce 1978). Similarly, a double-labeling study using Nuclear Yellow or bisbenzamide into one caudate and HRP in the opposite caudate demonstrate interhemispheric nigrostriatal neurons at < 1% of labeled SN neurons (Fisher, Shiota et al. 1984). According to the authors, double labeling (SNc neurons labeled with two different tracers) was minor and to a lesser extent than singly labeled interhemispheric nigrostriatal neurons. They argue that the results of double-labeled neurons may be due to technical issues. However, these findings
indicate sparse divergent, branched projections of the interhemispheric nigrostriatal projections in the cat, similar to results found in the rat.

Tracer Studies in the Primate

Data from primates has also shown the existence of crossing nigrostriatal neurons. Studying multiple projections from the SN, Francois and associates injected HRP in the putamen and rostral caudate nucleus of one macaque (Francois, Percheron et al. 1984). They found that 13% of all labeled neurons were located in the SN of the opposite hemisphere. Another macaque in that study received HRP in the head of the caudate and the superior portion of the rostral putamen and showed similar labeling results in the contralateral nigra.

Taken together, these animal studies confirm the presence of interhemispheric nigrostriatal neurons in different species using a variety of retrograde and anterograde tracer techniques. They have also characterized the topography of these projections and demonstrated that they are dopaminergic in phenotype, indicating potential significance to PD.

Neurophysiological and neurochemical functionality studies of the interhemispheric nigrostriatal projections

Additional studies using electrophysiological and chemical techniques validate the functional coupling properties of the interhemispheric nigrostriatal pathway. Striatal stimulation in one hemisphere can antidromically activate a small proportion of nigral neurons in the contralateral hemisphere as demonstrated by extracellular recordings in albino rats. Furthermore, these interhemispheric neurons exhibit electrophysiological properties similar to dopaminergic neurons (Collingridge 1982). Also, these cells can demonstrate collision cancellation with a timed
action potential before stimulation. Castellano and Diaz similarly suggest that the SN can
influence crossing nigrostriatal dopaminergic cells via stimulation (Castellano and Rodriguez
Diaz 1991), specifically the electrophysiological action of A9 cells being influenced by the
contralateral substantia nigra. When these interhemispheric connections are transected in the rat,
firing rates and discharge patterns change, with a more regular firing pattern and decrease in burst
firing when compared to normal animals (Castellano, Rivero et al. 1993). In terms of chemical
modulation, unilateral lesion to the nigra can affect DA levels in the contralateral striatum
(Nieoullon, Cheramy et al. 1977). Additionally, unilateral administration of dopaminergic drugs
in the SN can cause alterations in the release of DA in the opposite caudate in the cat (Nieoullon,
Cheramy et al. 1979). Taken together, these studies demonstrate that there is neurophysiological
and neurochemical coupling of the two nigrostriatal systems and that the nigra in one hemisphere
can modulate the electrophysiological and neurochemical activity of the contralateral striatum.

4.3.2. The interhemispheric corticostriatal connections

Cortical connections to the striatum are known to play an important role in modulating
the activity of striatal medium spiny neurons. The neuroanatomical morphology and topography
of intrahemispheric corticostriatal connections have been extensively studied. However, a number
of labeling studies have identified the presence of interhemispheric corticostriatal connections.

Tracer Studies in the Rat

Similar labeling studies in the rat have found interhemispheric corticostriatal connections
originating in the sensorimotor/primary motor regions of the cortex and projecting to the
dorsolateral striatum (Cospito and Kultas-Illinsky 1981; Alloway, Lou et al. 2006; Alloway, Smith
et al. 2009). Cheng and colleagues have identified that lesion sprouting occurs from the
contralateral cortex to the deafferented striatum, indicating the presence of interhemispheric
corticostriatal connections (Cheng, Tong et al. 1998). Moreover, Neuropeptide Y in the striatum seems to be influenced by the contralateral cortex (Salin and Nieoullon 1996). These studies further suggest a discrete corticostriatal connection that spans between the two hemispheres.

**Tracer Studies in the Cat**

An early study in the cat demonstrated that single cortical neurons from the sensorimotor region send decussated projections to the contralateral striatum or collaterals to both the ipsilateral and contralateral striatum (38% of corticostriatal projections) (Fisher, Boylan et al. 1986). Single cortical neurons can send collaterals to up to three different areas; the contralateral cortex, ipsilateral striatum and contralateral striatum. However, these only accounted for 2% of all labeled cells. These pyramidal corticostriatal neurons originate in laminae III – V. Further findings in the cat demonstrate that there is topographic collateralization of interhemispheric corticostriatal connections from the motor, cingulate and prefrontal cortex using other types of retrograde labels, such as Fast Blue and Diamidino Yellow (Rosell and Gimenez-Amaya 2001).

**Tracer Studies in the Primate**

An early study by Fallon and Ziegler using silver staining identified that prefrontal cortical regions project to the contralateral striatum in the rhesus monkey through the corpus callosum (Fallon and Ziegler 1979). To our knowledge, we are unaware of any studies that have examined the relative density of intrahemispheric and interhemispheric corticostriatal connections in response to DA denervation in animal models of PD.

4.4. Interhemispheric connections in the DA depleted state

4.4.1. Interhemispheric nigrostriatal labeling studies in lesioned animals
It has been suggested that lesions to the nigrostriatal dopaminergic pathway can induce a short-term compensatory increase of interhemispheric nigrostriatal neurons (Pritzel, Huston et al. 1983). In this study using kainic acid or 6-OHDA before unilateral labeling with HRP in rats, an increase in interhemispheric nigrostriatal neurons at 7 and 21 days was found when compared to controls. However, the number of interhemispheric nigrostriatal neurons 90 days after lesion was decreased, similar to that of control normal animals. A similar increase in interhemispheric nigrostriatal projections after nigral lesioning was also found using the retrograde fluorescent tracers Fast Blue and Nuclear Yellow. In another study, 6-OHDA injected at the region of the ventral tegmental region and SN in rats prevented HRP labeling of interhemispheric nigrostriatal neurons (Douglas, Kellaway et al. 1987). Similarly, a decrease in fluorescent retrograde-labeled crossing nigrostriatal cells was found in young rats after exposure to 6-OHDA (Altar, Neve et al. 1983) when compared to normal controls. These two studies demonstrate that the interhemispheric nigrostriatal neurons are susceptible to degeneration via dopaminergic neurotoxin, providing further evidence that these neurons are dopaminergic and their potential role in PD. The variations in results in terms of increase/decrease of interhemispheric nigrostriatal projections in response to 6-OHDA may arise from differences in neurotoxin injection site and type of tracer used. However, these changes to interhemispheric nigrostriatal connections in response to neurodegeneration warrant further research as they may provide further insight into patterns of nigrostriatal degeneration in PD and onset of dyskinesias.

4.4.2. 6-OHDA behavioral studies and interdependence of the nigrostriatal pathways

A study comparing behavioral deficits in the unilateral striatal lesioned rats to bilaterally lesioned rats with 6-OHDA suggest that there is interdependence between the two nigrostriatal systems (Roedter, Winkler et al. 2001). Animals with bilateral striatal lesions had significantly more behavioral deficits than unilateral striatal lesions. For example, using a food-picking task,
unilateral striatal lesioned animals showed no deficit in taking and eating food pellets. However, the bilateral striatal lesioned animals were able to take food pellets to a lesser degree than unilaterally lesioned animals. The bilaterally lesioned animals also had significant difficulty eating food pellets due to additional deficits in voluntary motor function when compared to unilateral lesioned animals. Bilaterally lesioned animals showed other behavioral problems in spontaneous activity not evident in the unilateral lesioned animals. These findings are interesting since both groups of animals had similar partial hemispheric nigrostriatal degeneration. Thus, it would be expected that bilateral lesioned animals would show similar behavioral deficits to the unilateral animals but in a bilateral manner. However, this was not the case. In another study, intrastriatal 6-OHDA in rats caused a decrease motor function in the contralateral forelimb (Faraji and Metz 2007). However, subsequent exposure to 6-OHDA into the opposite striatum worsened the functionality of the same forelimb that was initially impaired due to the unilateral lesion. This suggests pathways from not only ipsilateral but interhemispheric contralateral nigrostriatal connections may control a single forelimb. These findings support the hypothesis that the functioning of both nigrostriatal systems is important for movement. These findings also support our hypothesis that interhemispheric nigrostriatal connections may play an important role in the behavioral aspects of parkinsonism and perhaps in the prevention of dyskinesias in the HP rhesus monkey and striatal lesioned rat.

4.4.3. Dissociation of interhemispheric connections in 6-OHDA-lesioned animals

Hemispheric transection studies in rats have been used to examine if interhemispheric connections play a role in 6-OHDA nigrostriatal lesioning and rotational behavior, a test utilized to examine nigrostriatal asymmetry. In an early study, Mintz and colleagues severed callosal connections located at the thalamic region in HP 6-OHDA lesioned rats. They found that transection did not influence amphetamine-induced rotations in these lesioned animals (Mintz,
Douglas et al. 1985). Although cutting the connections near the thalamus does not influence amphetamine-induced rotations, transection of the corpus callosum after 6-OHDA lesioning has been shown to significantly alter behavioral open field exploration, turning behavior and apomorphine-induced rotations (Sullivan, Parker et al. 1993). Another study dissecting interhemispheric connections in the forebrain region found that transection at this region did not prevent apomorphine-induced rotations in 6-OHDA treated animals (Steiner, Morgan et al. 1985). Another lesion study identified changes in striatal DA receptor activity after transection of the corpus callosum. The authors suggest that these changes may be through the regulation of interhemispheric connections (Lawler, Gilmore et al. 1995). Taken together, these transection studies demonstrate that interhemispheric connections can influence behavioral responses to nigrostriatal lesioning as well as affect response to DA therapies for PD. Future studies that correlate these behavioral changes to transection/inhibition of specific interhemispheric nigrostriatal or corticostriatal connections are warranted to validate the role that interhemispheric connections play in parkinsonism.

4.4.4. Neurophysiological changes in the HP rhesus monkey with LD: implications for interhemispheric connections

PD is accompanied by alterations to basal ganglia electrophysiology. Previous studies in the LD-treated bilateral parkinsonian monkey show that neuronal activity from the globus pallidus interna (GPI) is almost entirely suppressed during dyskinesias as described in Chapter 1 (Papa, Desimone et al. 1999). Based on this data and our current understanding of the classical rate model of the basal ganglia, it would be hypothesized that LD would have a similar effect on the neuronal firing properties of the subthalamic nucleus (STN) and substantia nigra reticulata (SNR) in HP non-dyskinetic monkeys. We had recently demonstrated that LD treatment in the HP rhesus monkey does not completely normalize basal ganglia electrophysiology in the STN.
and SNR described in detail in Chapter 5 (Gilmour, Lieu et al. 2011). We found SNR firing rate decreased with LD treatment while firing pattern showed a trend towards increased burstiness. Interestingly, in the STN, we found no significant changes in firing rate or pattern between the HP state and LD-treated state. These findings suggest that intermittent LD treatments, while providing sufficient behavioral benefits in ameliorating parkinsonism, does not provide “normalization” of basal ganglia neurophysiology in terms of the discharge rates or discharge patterns. Further, in the case of the SNR we found that LD treatments in the HP monkey caused a trend towards increased burstiness of discharge patterns. These findings in one sense are the exact opposite of “electrophysiological silence or suppression”, a proposed electrophysiological “hallmark” of DID. We hypothesize that the intact interhemispheric dopaminergic innervation from the “less affected” hemisphere is instrumental in “preventing” the downstream neurophysiological “suppression/silence” in the STN/SNR and GPi.

4.5. Conclusion

This chapter identifies the existence of the interhemispheric connections of the striatum and some of their functional properties in the normal and DA depleted state (Rohlfs, Nikkhah et al. 1997; Pelled, Bergman et al. 2002). The progressive nigrostriatal degenerative properties of PD and the onset of dyskinesias have been associated with multiple pathological changes to the basal ganglia system at molecular, chemical and electrophysiological levels (Deogaonkar and Subramanian 2005). The classic pathophysiological explanation for the genesis of LD-induced dyskinesias is the notion that nigrostriatal dopaminergic neuronal degeneration and striatal denervation is associated with upregulation of dopaminergic sensitivity of D₁ subtype and D₂ subtype receptors in the striatum as described in Chapter 1. Such supersensitivity of D₁ and D₂ receptors are potentiated by pulsatile stimulation of these receptors via infrequent dosing of dopaminergic medications. This combination of nigrostriatal D₁ and D₂ receptor supersensitivity
combined with pulsatile dopaminergic oral medications is the hypothesized mechanism for DID. Additional mechanisms that have been postulated are the lack of DA buffering capacity and alterations in non-dopaminergic neurotransmitter systems in response to pulsatile dopaminergic stimulation in PD. A few studies in the non-human primate have suggested that pulsatile stimulation with LD even in the intact striatum can provoke “dyskinesias”. However, it is clear upon review of these studies that choreiform dyskinetic movements and dystonia as seen in humans do not occur in the unlesioned intact non-human primate. As described above, the strictly unilateral HP monkey model of PD does not show DID despite >90% loss of ipsilateral nigrostriatal neuronal connectivity and prolonged high-dose dopaminergic treatment. By contrast, the bilaterally parkinsonian monkey model is observed to exhibit classic choreiform dyskinesias and dystonia similar to dyskinesias seen in PD patients. The human correlate to this experimental primate finding is the notion that stage I disease patients also remain resistant to DID until they reach stage II (bilateral) disease, when they begin to exhibit DID. These findings suggest that the interhemispheric nigrostriatal dopaminergic fibers have a significant influence on the genesis of dyskinesias.

We hypothesize two possible mechanisms for the role of interhemispheric pathways to influence the genesis of DID in PD. The first possibility is that the synaptic connectivity between the crossed interhemispheric nigrostriatal neurons to host striatal medium spiny neurons are more robust than that of the ipsilateral nigrostriatal pathway. The experimental result that interhemispheric nigrostriatal dopaminergic fibers resist early neurotoxicity from both 6-OHDA (Sauer and Oertel Model) and MPTP (unilateral intracarotid model); acting via striatal DA transporter mediated uptake and retrograde transport to the SN argue in support of such a hypothesis. Additional histological studies are warranted to explore this hypothesis, such as electron microscopic techniques using specific labels that identify various connections between the interhemispheric nigrostriatal and corticostriatal pathways and the striatum (Meredith, Farrell
et al. 1999; Soderstrom, Meredith et al. 2008; Soderstrom, O'Malley et al. 2010). The second possibility is that there is synaptic and biochemical plasticity in the connections between the interhemispheric fibers and the partially denervated striatum. In this scenario, we hypothesize that when there is unilateral neurodegeneration, the interhemispheric nigrostriatal pathway originating from the contralateral SN become upregulated, providing partial amelioration of DA deficiency until more advanced stages of the disease ensues. Studies in the 6-OHDA rat that suggest that SN dopaminergic cell counts are increased in the contralateral SN following unilateral lesioning indicate that such plasticity may be at play in the interhemispheric pathways. Additional studies to measure the levels of DA secreted by the interhemispheric pathways at various stages of parkinsonism are warranted to prove this hypothesis. Alternately, the interhemispheric pathways may exhibit synaptic plasticity in response to hemiparkinsonism resulting in increased synaptic connectivity between the partially denervated striatum and crossed interhemispheric fibers. Thus, we hypothesize that the role of interhemispheric fibers in the genesis of DID is complementary to existing pathophysiological hypothesis of D₁/D₂ subtype receptor supersensitivity and pulsatile dopaminergic stimulation. Our hypothesis requires the additional step of the loss of interhemispheric nigrostriatal pathway besides the need for D₁/D₂ receptor supersensitivity and pulsatile dopaminergic stimulation for the genesis of DID. As a corollary, we hypothesize that the preservation of the interhemispheric connections will prevent the genesis of DID.

It is expected that the interhemispheric nigrostriatal fibers that originate on the side that is lesioned in the 6-OHDA rat and the MPTP treated monkey will undergo a near complete lesioning and subsequent neurodegeneration. We hypothesize that the loss of interhemispheric nigrostriatal fibers going from the lesioned nigra to the less affected striatum should have no consequence for the genesis of DID until the less affected striatum itself undergoes neurodegeneration to reach the appropriate threshold for developing D₁/D₂ subtype receptor supersensitivity (Fig. 4-1). In other words, our hypothesis predicts that unilateral lesioning or
natural loss of ipsilateral nigrostriatal fibers and the associated crossed interhemispheric fibers that originate in the SN that is primarily affected in itself is insufficient to cause DID, and there needs to be additive loss of interhemispheric nigrostriatal fibers that originate in the opposite hemisphere for the genesis DID. Future experiments that allow targeted lesioning of the interhemispheric nigrostriatal pathway along with unilateral lesioning of the nigrostriatal pathway, we believe, would readily cause LD-induced dyskinesias. By contrast, our hypothesis predicts that any animal with intact interhemispheric nigrostriatal fibers would remain resistant to dyskinesias.

In summary, data from Chapter 3 demonstrating that HP rhesus monkeys do not develop dyskinesias after chronic LD treatment (Lieu, Deogaonkar et al. 2011) in combination with previous reports described in this chapter suggests that preservation of interhemispheric inhibition may account for the lack of dyskinesias in stage I of PD. As a corollary, preservation of interhemispheric fibers from further degeneration may be a useful therapeutic approach to prevent DID in advancing PD.
Chapter 5

The effects of chronic levodopa treatments on neuronal firing properties of the subthalamic nucleus and substantia nigra reticulata in hemiparkinsonian rhesus monkeys: implications for interhemispheric connections

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5 This chapter is a modified version of the previously published article “The effects of chronic levodopa treatments on neuronal firing properties of the subthalamic nucleus and substantia nigra reticulata in hemiparkinsonian rhesus monkeys” in the journal Experimental Neurology (Year 2011, Issue 228, pp.53–58). Authors: T Gilmour, C Lieu, M Nolt, B Piallat, M Deogaonkar, T Subramanian. TG and CL contributed equally to this work. All authors contributed to experimental procedures, data collection and analysis. MN analyzed RMS data and assisted in recordings from monkey B. BP and MD recorded from monkey A and analyzed data from monkey A. All authors assisted in writing and finalizing the manuscript.
5.1. Abstract

As briefly described in Chapter 4, we hypothesize that the presence of interhemispheric nigrostriatal connections may account for the resistance of DID in HP rhesus monkeys. Additional indirect evidence was also briefly presented to support the notion that interhemispheric connections plays a significant role in preventing the neurophysiological changes in the subthalamic nucleus (STN) and substantia nigra reticulata (SNR) associated with DID. In this chapter, we detail the electrophysiological studies in HP nonhuman primates with chronic LD therapy. We found that the STN and SNR had a mean firing rate of $42.6 \pm 3.5$ Hz (mean ± SEM) and $52.1 \pm 5.7$ Hz, respectively in a stable HP state. Chronic intermittent LD exposure induced marked amelioration of parkinsonism with no apparent drug-induced motor complications. LD treatments did not significantly change the mean firing rate of STN neurons ($41.3 \pm 3.3$ Hz) or bursting neuronal firing patterns. However, LD treatments induced a significant reduction of the mean firing rates of SNR neurons to $36.2 \pm 3.3$ Hz ($p < 0.05$) and a trend toward increased burstiness. The entropy of the spike sequences from STN and SNR was unchanged by LD treatment, while there was a shift of spectral power into higher frequency bands in local field potentials (LFP). The results of this study demonstrate that there are distinct electrophysiological changes that occur in the non-dyskinetic HP rhesus monkey after chronic LD treatments and changes that have been previously reported in basal ganglia recordings that are hallmarks for DID do not occur in such animals. Since our results support the idea that HP monkeys do not develop dyskinesias and the electrophysiological hallmarks (“electrophysiological silence”) of DID, we hypothesize that this is related to the preservation of interhemispheric nigrostriatal connections in HP rhesus monkeys. Future studies in the dyskinetic and non-dyskinetic parkinsonian monkey that evaluate both the electrophysiological changes to basal ganglia downstream nuclei and the presence or absence of interhemispheric nigrostriatal connections are warranted.
5.2. Introduction

The loss of DA due to nigrostriatal degeneration subsequently leads to pathophysiological alterations in basal ganglia electrophysiology. The models of normal and parkinsonian basal ganglia function have been supported by many electrophysiological studies (Albin, Young et al. 1989; DeLong 1990). In the MPTP-lesioned primate, Bergman and colleagues demonstrated an increase in STN firing rate from normal to parkinsonian state (Bergman, Wichmann et al. 1994), and a similar increase in SNR neuronal firing rate (Wichmann, Bergman et al. 1999). Bergman and Wichmann and colleagues also showed that the primate STN and SNR became more bursty after MPTP treatment (Bergman, Wichmann et al. 1994; Wichmann, Bergman et al. 1999). The same increases of rate and burstiness in STN and SNR have been confirmed in a rat model of PD (Breit, Bouali-Benazzouz et al. 2001; Breit, Lessmann et al. 2006; Breit, Bouali-Benazzouz et al. 2007). The synchronized oscillations represented by the LFP have also been shown to have characteristic patterns in the normal and parkinsonian states. Brown and colleagues showed that normal rats have a LFP peak in the 70 Hz region, similar to human PD patients medicated with LD and undergoing intraoperative recordings (Brown, Oliviero et al. 2001; Brown, Kupsch et al. 2002). Mallet and colleagues showed that anesthetized HP rats showed a prominent beta LFP peak in the activated state which normal rats did not have (Mallet, Pogosyan et al. 2008). Together, studies demonstrate that the neurophysiological properties of STN and SNR are altered in response to nigrostriatal denervation and DA depletion.

While no studies to date have examined the effects of chronic LD therapy on STN or SNR neuronal firing rates or patterns, the effects of acute DA agonist therapy have been examined in the human STN. Recordings in PD patients undergoing functional neurosurgery for deep brain stimulation show that apomorphine (a DA agonist) treatment increases burst discharges in the STN but does not alter firing rate at optimal doses (i.e. provided significant
amelioration of parkinsonism without dyskinesias). However, apomorphine treatments decrease STN firing rates in the dyskinetic state (Lozano, Lang et al. 2000). Also, the prominent beta peak in STN LFP has been shown to be greatly reduced when the patient is acutely given apomorphine or LD treatments (Brown, Oliviero et al. 2001; Giannicola, Marceglia et al. 2010).

Based on studies to date, electrographic silence or near complete reduction of single cell neuronal discharges in the STN and GPI have been hypothesized as “hallmarks” of dyskinesias. Specifically, LD-induced dyskinesias have been associated with “suppression/silencing” of GPI neuronal firing in the bilateral parkinsonian rhesus monkey (Papa, Desimone et al. 1999). Based on these data, we hypothesized that HP monkeys receiving LD would have a reduction in STN and SNR firing rate, and a decrease in burstiness neuronal discharges, but would not develop electrographic silence in the STN and SNR. In the present study, we evaluated the basal ganglia electrophysiological changes of the STN and SNR with LD treatment in non-dyskinetic, clinically HP Rhesus monkeys to address this hypothesis.

5.3. Materials and methods

Two adult female Macaca mulatta were housed according to standards set forth in the NIH ‘Guide for the Care and Use of Laboratory Animals.’ All procedures were carried out in strict compliance with the “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985) and were approved by the local institutional animal care and use committee.

Assessing parkinsonism and chronic LD therapy

Detailed information on intracarotid injections to create an HP model and behavioral paradigm using the mUPDRS can be found in Chapter 2 (see Appendix B) as previously published (Lieu, Deogaonkar et al. 2011). Once behavioral stability was documented, recording chambers were surgically implanted to permit chronic single cell extracellular neuronal recording
from the left STN and SNR in the stable HP state and on chronic LD treatment. LD/carbidopa therapy was initiated at an oral dose of 100 mg/25 mg twice a day and gradually escalated by 100 mg/25 mg every 72 h until no further improvement in mUPDRS scores was observed. This was defined to be the optimal dose and was subsequently held constant throughout the dosing period. Serum DA levels were measured in a single monkey to check that LD was being successfully delivered, showing DA concentrations of 1.022 ng/mL before starting LD dosing and 17.04 ng/mL while on optimal LD dosing. Occasionally, the monkeys would refuse oral intake (defined by missing three successive doses). When this occurred, injectable benserazide and methyl ester of LD (25/100) were administered SQ/IM. At the point of stable mUPDRS score improvement and steady twice daily LD/carbidopa dosing for at least 1 week, extracellular neuronal recordings were resumed. Dyskinetic activity was not seen on this stable dosing regimen as demonstrated in Chapter 3 (Lieu, Deogaonkar et al. 2011).

**Electrophysiology**

All recordings were performed in awake, behaving animals. Wakefulness was monitored by eye blink reflex and responsiveness of animal to investigators while in the restraint chair. Animals were trained to allow passive limb movements by experimenters in order to examine somatotopic responses during recordings (Starr, Subramanian et al. 2000). Extracellular single cell recordings were carried out using glass coated platinum–iridium microelectrodes (impedance 0.5–1.0 Mega-ohms, FHC) or tungsten microelectrodes (0.5–2.0 Mega-ohms, FHC). The STN and SNR nuclei were systematically sampled, recording each neuron encountered at the target depth range, with tracts typically separated laterally from each other by 1 mm. The electrical signal was amplified (MDA-41 BAK or ISO-80 WPI), filtered (200–10,000 Hz, Krohn-Hite), monitored on an audio loudspeaker, and displayed on a digital oscilloscope to ensure good signal
isolation. The signals were digitally sampled at 25000 samples per second (Spike2, CED). Simultaneously, LFP were filtered (3–500 Hz) and digitized at 1000 samples per second.

Localization within the nuclei was confirmed in five ways. First, the depth of the electrode tip was correlated to a rhesus brain atlas. Second, firing characteristics of landmarks in the brain were monitored as reported previously (Starr, Subramanian et al. 2000). Third, on some tracts, after a neuron had been recorded for at least 60 s for later analysis, somatotopic responses were examined by flexing and extending the monkey's arm or leg during the recording (Fig. 5-1). Fourth, recording tracts were histologically confirmed in the STN and SNR at the end of the study after the primate was euthanized and brain analyzed as described previously (Lieu, Deogaonkar et al. 2011). Fifth, the root mean square (RMS) was calculated on the activity recorded along each track. RMS was used to confirm accurate dorsal-ventral progression during each track from thalamus, to STN, to SNR. RMS has been used clinically to determine the borders of STN, as the overall activity in STN is higher than that superior and inferior to STN (Moran, Bar-Gad et al. 2006; Snellings, Sagher et al. 2009).
Figure 5-1. Somatotopic neuronal response to elbow movement during a STN neuronal recording session. Black bar shows period during which the monkey’s arm was being manipulated by the researcher. During flexion of the monkey’s arm, there is an increase in firing rate in this STN neuron (black bar). During opposing contraction (no black bar), the STN neuron's firing rate decreases.

Data analysis

During offline analysis, interspike intervals (ISIs) were generated using Spike2's template matching spike sorting algorithm. Neuron sorting and isolation was further refined using principal component analysis on the spike waveforms. First, raw data was imported into Spike2 software. Second, to ensure isolation of single neurons, amplitude thresholds were set depending on characteristics of a specific neuronal action potential. Third, action potentials with similar amplitudes were then clustered using principal component analysis and then grouped together as firing activity for a single neuron. In each case, records were comprised of at least 400 spikes and had duration between 60 and 120 s.
In addition to firing rates, seven measures of the firing patterns were employed. First, the coefficient of variation (CV) of the ISIs was computed for each recording. A low CV indicates a regularly firing cell. Second, the burst index was computed as the mean of the ISI distribution divided by the mode of the ISI distribution (Hutchinson, Levy et al. 1997). A higher burst index indicates a cell that tends to fire in bursts. Third and fourth, the percent of spikes in bursts and percentage of time in bursts were calculated using the Poisson-surprise method (Legendy and Salcman 1985). Bursts were confirmed if their Poisson-surprise value was greater than or equal to 3 (Aldridge and Gilman 1991; Wichmann and Soares 2006). This provided a sliding time-window view of the burstiness of each spike train, a different perspective than the other burstiness metrics that process the entire recording at once. Fifth, the density discharge histogram (DDH) (Kaneoke and Vitek 1996) of each recording was computed and compared to the DDH of a random Poisson spike train. If the DDH of the recording was not significantly different from the Poisson DDH by a chi-square test, the firing pattern was classified as ‘Poisson.’ If the pattern was significantly different, the pattern was classified as ‘regular’ or ‘bursty’ depending on whether the variance of the DDH was less than one or greater than one, respectively (Levy, Dostrovsky et al. 2001). Sixth, the range of the DDH was computed. Bursty neurons had a larger DDH range because some bins contained large numbers of spikes.

Seventh, the sample entropy was computed as a measure of spike randomness. The sample entropy is based on the approximate entropy measure introduced by Pincus, and is modified to reduce estimation bias (Pincus 1991; Richman and Moorman 2000). We used an embedding dimension of 2 and a tolerance of 0.2 times the standard deviation, based on empirical optimization and on previous literature. Darbin and colleagues have reported nonlinear features of monkey basal ganglia neuronal firing and Lafreniere-Roula and colleagues have recently reported an entropy reduction in STN recordings from human PD patients following apomorphine treatment (Darbin, Soares et al. 2006; Lafreniere-Roula, Darbin et al. 2010). Thus, we chose this
additional metric to examine whether our chronic LD treatment might have a similar effect. The seven numeric firing pattern metrics were compared using the Wilcoxon–Mann–Whitney rank-sum test, and the categorical DDH classification was compared using Fisher's $2 \times 2$ exact test (grouping “Poisson” and “bursty” categories together).

Spectra were generated from LFP signals using the Welch periodogram method, using 512-point discrete Fourier transforms with 128-point overlap windows. The periodograms were normalized using the power in the 65–80 Hz band to eliminate any bias due to 60 Hz line noise. The spectral power was then summed in 5-Hz bins. Sums were compared in each frequency band between the On-LD and Off-LD conditions using an unpaired two-sided T-test. The centroids of power spectral density (PSD) segments cropped between 10 and 50 Hz were computed to test for spectral power shifts in this region. The centroids were compared using an unpaired two-sided T-test. The spike-triggered average (STA) of the LFP signal was computed and compared to the STA of a randomly shuffled version of the same ISI sequence. STA is used to measure coherence and coupling between LFP and neuronal single cell recordings. A rank-sum test was used to compare the ratio of the peak-to-trough difference of the STA to the peak-to-trough difference of the randomized STA.

5.4. Results

Chronic LD treatment substantially improved parkinsonism in both monkeys without causing DID. The mUPDRS score significantly decreased in the LD-treated state (77.7% for Monkey A and 79.1% for Monkey B, $p < 0.001$ unpaired two-sided T-test on pooled data, Fig. 5-2).
Figure 5-2. mUPDRS behavioral scores for each monkey separately and the pooled scores (**p < 0.01, ***p < 0.001, two-sided unpaired T-test). Black columns represent tests taken during off-LD periods (Monkey A: n=6, Monkey B: n=6, Combined: n=12), and gray columns represent tests taken during on-LD periods (Monkey A: n=3, Monkey B: n=5, Combined: n=8).

During microelectrode recordings, we calculated the RMS of the activity recorded along multiple tracks, one of many measures that allowed us to determine location within the various nuclei. The RMS increased as the electrode traveled out of the thalamus and into STN, and decreased upon exiting STN and entering SNR. Figure 5-3 shows normalized RMS values of recordings in thalamus, STN and SNR.
Figure 5-3. Normalized root mean square (RMS) power in neuronal recordings from nuclei at different depths. Each tract was normalized by the STN power. A rank-sum test was performed to test the difference between the Thalamus and STN recordings and between SNR and STN recordings (n=7 tracks, *p < 0.05, **p < 0.01).

From the microelectrode recordings, we examined the firing rate and firing pattern of neurons in both nuclei before and after LD treatment. Although Monkey B showed an increase in STN firing rate, the pooled mean STN firing rate (± SEM) showed no significant change (42.6 ± 3.5 Hz to 41.3 ± 3.3 Hz, Fig. 5-4). The SNR pooled mean firing rate on LD decreased (52.1 ± 5.7 Hz to 36.2 ± 3.3 Hz, p<0.013, Fig. 5-4). In both the STN and the SNR Monkey B showed a larger effect than Monkey A, but both monkeys showed the same trends.
Figure 5-4. Effects of LD on neuronal firing rates. Top and bottom rows: firing rates of SNR and STN neurons, respectively (mean ± SEM). Black columns represent data recorded before LD treatment (SNR: n=30; STN: n=50), and gray columns represent data recorded during LD treatment (SNR: n=39; STN: n=76) (*p < 0.05, T-test).

The quantification of the firing patterns did not show a significant difference in most measures in either nucleus (Table 5-1). The STN did not show any trend toward increasing or decreasing burstiness. The SNR showed a trend toward an increase in burstiness in all measures of burstiness, and this trend was statistically significant in the DDH range (p < 0.05).
Table 5-1. Firing pattern metrics (mean ± SEM) comparing the SNR and STN in the off-LD and on-LD conditions. Only the DDH range showed a significant difference in SNR, with increased burstiness in the LD-treated condition (*p < 0.05, rank-sum test). Sample size is shown in pattern classification (number of neurons, SNR LD-off = 30; SNR LD-on = 50; STN LD-off = 39; STN LD-on = 76).

<table>
<thead>
<tr>
<th></th>
<th>SNR Off-LD</th>
<th>SNR On-LD</th>
<th>STN Off-LD</th>
<th>STN On-LD</th>
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<tbody>
<tr>
<td>CV</td>
<td>0.80 ± 0.06</td>
<td>0.86 ± 0.06</td>
<td>0.96 ± 0.06</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Burst Index</td>
<td>4.93 ± 1.42</td>
<td>9.17 ± 1.71</td>
<td>3.74 ± 0.68</td>
<td>3.23 ± 0.33</td>
</tr>
<tr>
<td>Sample Entropy</td>
<td>1.48 ± 0.07</td>
<td>1.58 ± 0.06</td>
<td>1.46 ± 0.63</td>
<td>1.48 ± 0.45</td>
</tr>
<tr>
<td>DDH Range</td>
<td>4.60 ± 0.21</td>
<td>5.44 ± 0.26*</td>
<td>4.87 ± 0.19</td>
<td>4.95 ± 0.14</td>
</tr>
<tr>
<td>Percent of time in bursts</td>
<td>0.51 ± 0.16</td>
<td>1.08 ± 0.29</td>
<td>1.36 ± 0.34</td>
<td>1.26 ± 0.23</td>
</tr>
<tr>
<td>Percent of spikes in bursts</td>
<td>2.05 ± 0.64</td>
<td>5.4 ± 1.61</td>
<td>4.96 ± 1.28</td>
<td>4.84 ± 0.91</td>
</tr>
<tr>
<td>Poisson DDH pattern classification</td>
<td>Regular: 28</td>
<td>Regular: 39</td>
<td>Regular: 29</td>
<td>Regular: 58</td>
</tr>
<tr>
<td>(number of neurons)</td>
<td>Poisson: 1</td>
<td>Poisson: 4</td>
<td>Poisson: 5</td>
<td>Poisson: 10</td>
</tr>
<tr>
<td>Bursty: 1</td>
<td>Bursty: 7</td>
<td>Bursty: 5</td>
<td>Bursty: 8</td>
<td></td>
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</tbody>
</table>

LFP were also analyzed from the neural recordings. LFP spectra in SNR showed a decrease in the low beta frequencies (20–25 Hz) and an increase in higher beta frequencies (30–35 Hz) on LD (Fig. 5-5). In the STN, there was a similar shift of spectral power into higher frequency bands, seen as a reduction in the 15–20 Hz band and an increase in the 35–55 Hz band on LD. The centroids of spectral power in the 10–50 Hz band were significantly higher in both the STN and SNR (p < 0.05). No significant differences in the spike-triggered averages were seen between the on-LD and off-LD groups (Fig. 5-6).
Figure 5-5. LD effects on spectral power of local field potentials (Off-LD SNR: n=28; On-LD SNR: N=16; Off-LD STN: n=36; On-LD STN: n=22). Left and center: power spectral densities (PSDs) were computed using Welch periodograms, normalized using the power in the 65–80 Hz band, and summed into 5-Hz bins. These graphs display the normalized power in each bin, labeled at the lower edge of each bin, with SE error bars (*p < 0.05, two-sided unpaired T-test). Right: centroids of PSD segments cropped between 10 and 50 Hz to focus on the changes in this region (*p < 0.05, two-sided unpaired T-test).
Figure 5-6. Mean spike-triggered average (STA) of the local field potential signal across all neurons recorded together with LFP in the SNR and STN both before and during LD treatment, in the 12-25 Hz band (A) and the 25-40 Hz band (B). Data shown are the ratio of the STA peak-trough difference to the randomized STA peak-trough difference. Plots show the median (red line), quartiles (box), mean (green triangle), SEM (green whiskers), extent up to 1.5 times the interquartile distance (black whiskers) and outliers beyond 1.5 times the interquartile distance for all analyzed neurons. The rank-sum nonparametric test showed no significant differences between the groups.
5.5. Discussion

In the present study, we found no significant changes in firing rate or pattern in the STN between HP state and with LD treatment. We did find a significant decrease in SNR firing rate with an increase in bursting firing patterns. Our findings support the notion that one or multiple mechanisms prevent the electrophysiological “silencing” in these monkeys as proposed in previous dyskinetic studies. Chronic LD treatments seem to have almost no effects in the STN (indirect pathway), but has a slight effect in the SNR (combination of indirect/direct pathway), suggesting that LD is more associated with changes in the direct pathway than the indirect pathway. Because we did not see a trend towards the electrophysiological “hallmark” of “silencing” in either nuclei, our hypothesized rate and burstiness reduction support the notion that there must be some inherent mechanism that prevent such changes. One possible insight from these studies is the idea that clinically discernible behavioral improvements do not necessarily cause remediation of electrophysiological changes in the basal ganglia. This underlying lack of remediation of electrophysiological changes may be the biological basis for a number of drug-induced phenomena including on-off, dyskinesias, and end-of-dose wearing off.

In this study, we focused on the firing rates and patterns in the STN and SNR in HP rhesus nonhuman primates in the stable parkinsonian state and when exposed to chronic intermittent LD treatment in order to mimic the clinical scenario of LD administration in PD patients. This initial study was performed in the context of continuing these studies in the dyskinetic parkinsonian monkey to compare the effects of chronic LD treatment in both the non-dyskinetic and dyskinetic parkinsonian monkey models. In the sections below, we further discuss our electrophysiological results in the context of previous published data.

Firing rates
The firing rates we observed in the parkinsonian state are consistent with what has been reported in MPTP-treated primates and PD patients. Neuronal discharge rates in the parkinsonian state range from 26 to 50 Hz in the STN, and 45–86 Hz in the SNR (Bergman, Wichmann et al. 1994; Hutchison, Allan et al. 1998; Wichmann, Bergman et al. 1999; Bejjani, Dormont et al. 2000; Levy, Hutchison et al. 2000; Magnin, Morel et al. 2000; Theodosopoulos, Marks et al. 2003; Wichmann and Soares 2006).

The pooled mean STN firing rate did not show a significant change on LD. This fits with a previous report in human PD patients which showed that apomorphine treatments did not change the STN firing rate in the human (Lozano, Lang et al. 2000; Levy, Dostrovsky et al. 2001). However, this result does not fit the rate model, which predicts that amelioration of PD symptoms would correlate with a reduced STN firing rate. Our result also contrasts with a previous study which showed a reduction in STN firing rate after apomorphine treatment (Kreiss, Mastropietro et al. 1997). However, LD has important differences from apomorphine. Apomorphine can act upon DA receptors anywhere, whereas LD mediates its effect only through surviving dopaminergic neural pathways. This may explain the difference.

In contrast to the STN, the firing rate of the SNR decreased significantly on LD. This result is similar to a previous report of acute apomorphine treatment in the normal primate demonstrating significant SNR decreases of firing rate but no significant changes in firing pattern after apomorphine treatment (Nevet, Morris et al. 2004). Our result is also similar to the report by Park and colleagues that DA D₁ receptor agonist SKF38393 and D₂ receptor agonist Quinpirole reduced the SNR firing rates (Park, Jeon et al. 2007).

One possible explanation for why the effects of chronic LD treatment and acute DA agonist treatment are similar in the SNR but different in the STN would be that based on the classic rate model of Albin and DeLong, chronic LD treatment in the parkinsonian state affects the direct pathway more than the indirect pathway (Albin, Young et al. 1989; DeLong 1990).
Another possible explanation may be related to better preservation of the substantia nigra pars compacta (SNC)–SNR connectivity than the SNC–STN connectivity in these animals. Surviving dopaminergic connections from the SNC would putatively enable exogenous LD mediated DA conversion and synaptic release into the SNR that would mimic the effects of apomorphine acting directly on DA receptors located in the SNR. Supporting this notion, Prescott and colleagues recently showed that in the SNR of human patients, LD increased stimulation-evoked plasticity (Prescott, Dostrovsky et al. 2009). They noted in passing that LD decreased SNR firing rates, but they did not systematically examine this decrease. Our results confirm their firing rate observation in a systematic way in the primate model, and further suggest that treatment-induced increases in available DA may have a larger effect on SNR firing rates than on STN firing rates in the parkinsonian condition. This may be due to a specific role of the small dopaminergic connection between the SNC and the SNR in regulating neuronal firing (Kliem, Maidment et al. 2007).

Firing patterns

Previous studies have shown that cells in the primate STN and SNR become more bursty after MPTP-induced parkinsonism (Bergman, Wichmann et al. 1994; Wichmann, Bergman et al. 1999; Wichmann and Soares 2006). In our study we did not try to replicate this well-documented change between normal and parkinsonian monkeys, but instead focused on the difference between the parkinsonian state off-LD and on-LD treatments.

In the STN, we did not observe any significant changes in the bursting spike patterns of cells from the LD treatment. This contrasts with previous studies that showed that apomorphine treatment increased the burstiness (Levy, Dostrovsky et al. 2001) and reduced the entropy (Lafreniere-Roula, Darbin et al. 2010) of STN neurons. This may again illustrate the difference between LD and DA agonists. Apomorphine directly acts upon the DA receptors, so it exerts its
effect whether or not the normal DA pathways are operational. LD requires the operation of remaining dopaminergic neurons in physiological DA pathways to be converted to DA and exert its effect.

In the SNR there was a slight trend toward increasing burstiness on LD, which was significant in one out of the seven firing pattern measures we examined. This lack of reduction of the burstiness fits with the finding by Boraud and colleagues that the neuronal firing patterns in the related GPI nucleus do not become less bursty on LD (Boraud, Bezard et al. 1998). Although our study design prevents conclusions about the “normalizing” effect of LD or lack thereof, we clearly demonstrate that in both the SNR and STN, LD did not reduce the bursting patterns that previous studies have associated with parkinsonism.

Local field potentials

Our LFP measurements show that LD decreased the STN LFP power in the 15–20 Hz band and increased power in the 35–55 Hz band. This fits with similar previous literature reports (Gatev, Darbin et al. 2006; Hammond, Bergman et al. 2007; Galvan and Wichmann 2008). Similarly, Sharott and colleagues showed that apomorphine treatment reduced the beta LFP peak in awake behaving rats (Sharott, Magill et al. 2005). In humans, several studies have shown that LD treatments reduce the amplitude of the LFP beta peak in patients undergoing deep brain stimulator implants (Brown, Oliviero et al. 2001; Alonso-Frech, Zamarbide et al. 2006; Kuhn, Kupsch et al. 2006; Giannicola, Marceglia et al. 2010; Lopez-Azcarate, Tainta et al. 2010). Some human studies have reported seeing a peak in the high gamma band (60–90 Hz) on LD treatment (Brown, Oliviero et al. 2001; Lopez-Azcarate, Tainta et al. 2010), but we did not see any significant increase in the LFP power above 60 Hz (data not shown). It is possible that the increase we observed in low gamma band activity (35–55 Hz) in the STN may be a primate analogue to the human and rodent high gamma band increases (Hammond, Bergman et al. 2007).
Another explanation is suggested by the report by Alonso-Frech and colleagues that high gamma band activity was increased during dyskinesias in human patients (Alonso-Frech, Zamarbide et al. 2006). The fact that our monkeys did not become dyskinetic may explain why we did not see an increase in STN high gamma activity.

To our knowledge, the current study is the first to examine the effects of LD on SNR LFP in the primate. Our results show that LD treatment reduces SNR low beta LFP activity and increases the high beta LFP activity, similar to the changes in the STN. Like the STN, we did not see increases in SNR high gamma activity (above 60 Hz, data not shown). We did not see specific SNR increases in the 35–55 Hz power in 5-Hz bins on LD, but the centroid of the LFP spectral power in the 10–50 Hz band was shifted significantly higher in both the SNR and STN. Thus, overall, the synchronous synaptic input to the SNR changed along with the STN input in response to chronic intermittent LD treatments, although perhaps not quite as robustly. The differences in the STN LFP may be due to the direct cortical input pathway to the STN, which has recently been shown by optogenetic experiments to be highly important in the activation of basal ganglia pathways (Gradinaru, Mogri et al. 2009).

**Basal ganglia electrophysiology and interhemispheric inhibition**

Based on the notion that LD completely “suppresses/silences” GPI firing in dyskinetic monkeys (Papa, Desimone et al. 1999), we would expect to see a similar trend of decreased activity in STN and SNR firing in the HP rhesus monkey after LD treatment. However, in the STN, we found no significant changes in the firing rate or pattern after chronic LD treatment. In the SNR, we found that LD treatments induced a decrease in firing rates and caused a trend towards increased burstiness of discharge patterns. Our findings do not support the notion that chronic intermittent LD treatment causes the hypothesized reduction in rate and burstiness of STN and SNR. In addition, our study also did not show the electrophysiological “hallmark” of
DID which is “electrophysiological silence or suppression” with LD treatment. We hypothesize that intact interhemispheric dopaminergic innervation from the “less affected” hemisphere is instrumental in “preventing” the downstream neurophysiological “suppression/silence” in the STN/SNR and GPi.

In Table 5-2, we show our results in the context of our hypothesis that intact interhemispheric nigrostriatal connections account for our electrophysiological findings (firing discharge properties and LFP) in the STN and SNR in HP rhesus monkeys in the HP state (A) and after chronic LD treatment (B). We also show the expected results of how loss of interhemispheric nigrostriatal connections may lead to further increases in firing rate and bursting firing pattern in the more severe parkinsonian state (C). We then show our expected results in the dyskinetic state where interhemispheric fibers are lost (D). Based on this model, we would expect little to no firing in all nuclei as proposed by previously published reports. We also show how LFP would be affected in these two hypothesized conditions. Future studies that evaluate the presence or absence of interhemispheric inhibition in the HP model and in the dyskinetic model in combination with electrophysiological recordings from downstream basal ganglia nuclei are warranted.
Table 5-2. Electrophysiological changes in the STN and SNR/GPI. Direction of arrow indicates increase or decrease. Number of arrows indicates extent of increase or decrease. In the first two conditions, we propose our findings in the HP monkey are associated with intact interhemispheric nigrostriatal connections. In the last two conditions, we describe predicted changes after loss of interhemispheric nigrostriatal (IH NS) connections and with dyskinesias. LFP – local field potentials, FR – firing rate, FP – firing pattern (burstiness).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hemiparkinsonian State &amp; IH NS connections</th>
<th>HP State with LD &amp; IH NS connections</th>
<th>Parkinsonian State No IH NS connections</th>
<th>Dyskinetic Parkinsonian State No IH NS connections</th>
</tr>
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Chapter 6

Loss of interhemispheric nigrostriatal connections in the dyskinetic hemiparkinsonian rat
6.1. Abstract

The exact neuroanatomical correlates that modulate DID still remain unclear. In this context, we described in Chapter 4 the potential role that interhemispheric nigrostriatal connections have in the dyskinetic monkey and rat. More specifically, we hypothesized that intact interhemispheric nigrostriatal connections prevent the onset of dyskinesias, and that the genesis of dyskinesias is due to degeneration of these interhemispheric connections. In this chapter, we describe tracer and lesioning experiments that directly examine this hypothesis. In experiment 1, we first labeled the ipsilateral and interhemispheric nigrostriatal pathways by injection of retrograde tracer unilaterally into the striatum in normal rats. Rats then received one of three different injection paradigms of 6-OHDA ipsilateral to tracer injection: single intrastriatal injection of 6-OHDA (S-STR), multiple intrastriatal injection of 6-OHDA (M-STR), and unilateral injection of 6-OHDA into the medial forebrain bundle (MFB). A control group received only retrograde tracer injection and no 6-OHDA. A battery of behavioral tests demonstrated moderate hemiparkinsonism in the S-STR group and severe hemiparkinsonism in the M-STR and MFB group. LD treatment caused robust dyskinesias in the M-STR and MFB group but not in the S-STR or normal group. Histological analysis showed moderate ipsilateral loss of nigrostriatal neurons in the S-STR group, and severe ipsilateral loss of nigrostriatal neurons in the M-STR and MFB groups when compared to controls. Tracer-labeled neurons were found in the substantia nigra pars compacta (SNC) contralateral to injection site in the control and S-STR groups, indicating intact interhemispheric nigrostriatal connections. However, there were no tracer-labeled SNC neurons found in the M-STR and MFB groups, indicating loss of interhemispheric nigrostriatal connections. In experiment 2, we utilized a labeling technique with an optogenetic dual viral vector system to discretely label the nigrostriatal interhemispheric connections in normal rats. In this system, one vector was injected into pre-synaptic cell bodies (right nigra) and the other viral vector injected at the post-synaptic terminal site (left striatum). Using a “switch-
gene” in this system, we were able to discretely label interhemispheric nigrostriatal neurons without labeling other extraneous neuronal projections. Our data show that unilateral M-STR and MFB exposure to 6-OHDA results in loss of both ipsilateral and interhemispheric nigrostriatal connections. This suggests that loss of interhemispheric nigrostriatal neurons is correlated to the genesis of LD-induced dyskinesias in the parkinsonian rat.

6.2. Introduction

The prevailing hypothesis for the development of DID in advanced PD is striatal DA receptor supersensitivity due to DA depletion and chronic intermittent exposure to pharmacological DA replacement therapies (Chase, Baronti et al. 1989). We have recently shown that clinically HP rhesus monkeys do not develop dyskinesias regardless of chronic high doses of LD administration as described in Chapter 3 (Lieu, Deogaonkar et al. 2011). We demonstrated that these animals had extensive unilateral nigrostriatal degeneration. Based on the prevailing hypothesis (Chase, Baronti et al. 1989; Bibbiani, Costantini et al. 2005), this would suggest that such animals should develop dyskinesias with chronic intermittent LD treatment. However, none of the animals displayed choreoathetoid or dystonic dyskinesias. We therefore hypothesized that interhemispheric neuromodulation may prevent the onset of dyskinesias in both the non-dyskinetic parkinsonian monkey and rat (Chapter 4), and that loss of this neuromodulation leads to the genesis of dyskinesias and alterations to basal ganglia circuitry. One possibility for interhemispheric inhibition in these animals is by the small percentage of interhemispheric nigrostriatal connections (Fass and Butcher 1981; Collingridge 1982) as described in detail in Chapter 4. To our knowledge, the role this pathway plays in PD and DID has not yet been investigated. To investigate this, we used two different techniques (labeling and lesioning experiment and optogenetics experiment).
6.3. Materials and Methods

Experiment 1 – Comparison of retrograde labeling in normal and parkinsonian rats

Animals and surgical procedures

Twenty-one adult Sprague-Dawley rats were utilized in the present study. The following procedures were conducted in compliance with institutional protocols and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). Animals received stereotactic injections of the retrograde tracer fluorogold (FG) or true blue (TB) unilaterally into the striatum (AP: +1.0, ML: +3.0, DV from dura: -5.0). Retrograde tracers were injected at 0.05µl/min for a total of 0.5-0.7 µl of tracer solution. A subset of rats received unilateral injections of both tracer or received tracer injection at multiple striatal sites (Table 6-1).

After one week, animals underwent a second surgery for 6-OHDA injection(s). The rats were separated into 4 groups (N=4-6 per group – Table 6-1). Group 1 were normal controls which received no 6-OHDA. Group 2 received single unilateral intrastriatal injection of 6-OHDA (S-STR) (the Sauer and Oertel model) (Sauer and Oertel 1994). Group 3 received multiple unilateral intrastriatal injection of 6-OHDA (M-STR) (Winkler, Kirik et al. 2002), Group 4 received unilateral injection of 6-OHDA into the MFB (MFB) (the Ungerstedt model) (Ungerstedt and Arbuthnott 1970; Lieu, Kunselman et al. 2010). Doses for intrastriatal 6-OHDA were 20µg/4µl per site and injected at a rate of 0.5µl/min. Doses for MFB 6-OHDA were 12µg/4µl per site and injected at a rate of 0.67µl/min. After each injection, the needle was left in the brain for 10 min then slow retracted over 2.5 min. After each surgery, the wound was sutured and the animal was monitored for recovery.

**Behavioral Tests**

After three weeks post-lesion, all animals were tested for parkinsonism to measure behavioral deficit (stepping test and cylinder test). All groups were then treated for 6 days BID IP with LD methyl ester (6 mg/kg) and benserazide (15 mg/kg) to evaluate onset of DID (see Chapter 2 for details).
Experiment 2 – Dual viral vector injection system in normal rats

We used a dual viral vector injection system into normal rats to label interhemispheric nigrostriatal neurons as previously described (Gradinaru, Zhang et al. 2010). This novel system allows for the labeling of discrete sets of neurons without labeling extraneous nuclei. In this system, we injected WGA-Cre gene (AAV2-EF1a-mCherry-IRES-WGA-Cre) into the left striatum and the Cre-dependent opsin virus DIO-eNpHR-EYFP (AAV5-EF1a-DIO-eNpHR3.0-EYFP) (EYFP – enhanced yellow fluorescent protein) into the right nigra. The left striatum received WGA-Cre at 3 sites: Site 1: AP: +1.7; ML: +2.5; DV: -5.5, Site 2: AP: +0.7; ML: +3.0; DV: -5.5, Site 3: AP: -0.8; ML: +4.0; DV: -5.5. Each striatal site received 1 μl of AAV-WGA-Cre. The right nigra received 2 μl of eNpHR-EYFP at site: AP: −5.3, ML: 2.0, DV: −7.5. Injections were done at a rate of 0.5 μl/min. After injection, needles were left in the brain for 10 min then slowly retracted. Figure 6-1 summaries our optogenetic experimental paradigm. At the 3 weeks, animals were euthanized and brain sections histologically examined.
Figure 6-1. Optogenetic dual viral vector system. WGA-Cre was injected into the left striatum (STR) and eNpHR was injected into the right substantia nigra compacta (SNC). Only connections between the two nuclei will express yellow fluorescent protein (interhemispheric nigrostriatal pathway).

Histological Analysis

At the end of the experiment, all animals were euthanized via transcardial perfusion with heparinized saline and 4% paraformaldehyde. Brains were removed and cryoprotected, then cut on a freezing microtome at 60 μm. Serial sections through the substantia nigra were evaluated for tracer-positive nigral cells (FG or TB) and stained for tyrosine hydroxylase (TH) (Pelfreez 1:250) as previously described (Subramanian, Marchionini et al. 2002; Lieu, Kunselman et al. 2010). Animals with misplaced retrograde tracer injection were excluded from histological analysis (N=6).

Representative animals with the same tracing injection parameters were utilized to obtain tracer-positive nigral cell counts using unbiased stereological optical fractionator procedures or the Abercrombie method (Abercrombie 1946; Ishiyama, Geiger et al. 2011; Lieu, Deogaonkar et al. 2011). Quantitative estimates of tracer-labeled neurons in the nigra were performed with the optical fractionator probe (Stereo Investigator, MBF Bioscience) in the control and S-STR group.
ipsilateral hemispheres. This utilizes systematic random sampling using unbiased stereological counting methods. The SN was outlined under low magnification on serial sections (every 8\textsuperscript{th} - 10\textsuperscript{th} section). A computer-generated unbiased sampling site grid counting frame was overlaid on the SN outline. Section thickness was measured, and tracer+ SN neurons were counted at high magnification. Top and bottom guard zones were applied at every sampling site, and only neurons within the optical dissector region (15 $\mu$m – top of section to bottom of section) were counted. Total FG/TB+ SN neurons were calculated using Stereo Investigator computer software. The Abercrombie correction factor method was utilized if counting procedures did not meet the criteria for optical fractionator procedures (e.g. less than 100 total cells present) in the contralateral hemisphere of the control and S-STR groups, and in both hemispheres of the M-STR and MFB groups. Every section was counted and correction factor was implemented. All behavioral and histological data was analyzed using analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test, and expressed as mean ± SEM.

6.4. Results

Experiment 1 – Retrograde labeling studies in normal and parkinsonian rats

Control animals showed no behavioral deficits as demonstrated by the stepping test (Fig. 6-2A) and cylinder test (Fig 6-2B). The S-STR group displayed mild parkinsonism in the stepping test and severe parkinsonism as shown in the cylinder test, similar to the M-STR and MFB groups. M-STR and MFB groups had major deficits in both behaviors. LD exposure to control and S-STR groups caused no evident DID, whereas the M-STR group and the MFB group displayed robust DID (Fig 6-2C).
6-OHDA induces significant deficit in forelimb usage in the stepping test (A) and cylinder test (B). LD exposure leads to dyskinesias in the M-STR and MFB groups but not in control or S-STR groups (C). ***p < 0.001 compared to control, **p < 0.01, *p < 0.05 compared to control.

Histological analysis of representative nigral brain sections showed neurons labeled with tracer in the ipsilateral nigra in addition to labeled neurons in the contralateral nigral in control animals (Fig. 6-3). In this group, ipsilateral and contralateral neurons had similar morphological characteristics with multiple processes projecting from the cell body. Cell bodies were pyramidal-like with elongated cell bodies with extensive neuritic processes. In the S-STR group, labeled nigral neurons were also found ipsilaterally and contralaterally. However, some neuronal cell
bodies were smaller in diameter but were still pyramidal in shape, with fewer neuritic processes extending from the perikarya compared to the control group with the same tracer injection coordinate (Fig. 6-3). We did not identify any region-specific labeling of these interhemispheric nigrostriatal neurons in the nigra. In animals with one hemisphere labeled with FG and the opposite with TB, we did not identify any dual-labeled nigral neurons, suggesting that there are no nigral neurons that cross to both striata. In the M-STR and MFB group, there was only a few clearly labeled neurons in the ipsilateral nigra, but none labeled in the contralateral nigra. Similar to previous reports using 6-OHDA to degenerate FG-labeled nigrostriatal neurons (Sauer and Oertel 1994; Sautter, Kupsch et al. 1997), we found secondary FG labeling of non-neuronal, phagocytic cells in the nigra which was evident in the S-STR, M-STR and MFB groups. These cells had truncated processes, were crescent or ovoid-shaped cells with microglia-like morphology, and were not included in the counts below (Fig. 6-4).
Figure 6-3. Retrograde tracer-labeled neurons in the nigra. Control animals had clearly labeled ipsilateral and contralateral labeling of the nigra (yellow neurons). TB labeled neurons were found ipsilaterally next to contralateral FG-labeled neurons. Animals in the S-STR group had fewer neurons ipsilaterally compared to control as well as labeled contralateral nigral neurons.

Figure 6-4. FG-labeled non-neuronal cells in substantia nigra not included in counts.

In control animals, the nigral neurons were clearly labeled by striatal tracer injection. Stereological counts in the substantia nigra (SN) of tracer-labeled neurons in the control group were $11909 \pm 958.0$. In the S-STR group, the number of nigral neurons was $4649 \pm 416$. In the
M-STR group, there were 101 ± 19 nigral neurons labeled. In the MFB group, there were 8.3 ± 5.0 nigral neurons labeled (Table 6-2, expressed as percentage). For these numbers, we only counted cells with a neuronal phenotype, and not cells with secondary FG labeling as described above.

For interhemispheric tracer-labeled neurons in the nigra (Abercrombie method), the counts were 26.7 ± 6.1 for the normal group, and 29.3 ± 4.0 for the S-STR group. In the M-STR and MFB groups (the two groups that developed DID), no interhemispheric nigrostriatal neurons could be identified (Table 6-2, express as percentage). Furthermore, the subset of animals in the M-STR group that received multiple retrograde tracer injections had no evidence of labeled contralateral nigral neurons. TH staining demonstrated moderate loss of dopaminergic neurons in the nigra and striatum of the S-STR group, and severe loss in the M-STR and MFB groups when compared to control. Fluorescence immunohistochemical staining for TH was overlaid with labeled interhemispheric nigrostriatal neurons, suggesting that they are dopaminergic (Fig. 6-5).

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<tr>
<th>Group</th>
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<th>Contralateral SN</th>
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<tr>
<td></td>
<td>(% Control)</td>
<td>(% Control)</td>
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<tr>
<td>Control</td>
<td>100 ± 8.0</td>
<td>100 ± 22.8</td>
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<tr>
<td>S-STR</td>
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<td>109 ± 15.0</td>
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<tr>
<td>M-STR</td>
<td>0.85 ± 0.2 *<strong>,</strong></td>
<td>0 <strong>,</strong></td>
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<td>MFB</td>
<td>0.07 ± 0.04 *<strong>,</strong></td>
<td>0 *<strong>,</strong></td>
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**Table 6-2. Summary of tracer-positive nigral counts (mean ± SEM).**

***p < 0.001 compared to control, **p < 0.01 compared to S-STR for Ipsilateral SN

***p < 0.001 compared to S-STR, **p < 0.01 compared to control and S-STR for Contralateral SN
Figure 6-5. Tyrosine hydroxylase (TH) staining in the substantia nigra compacta of dopaminergic neurons (red) overlaid with FG labeled (yellow) interhemispheric nigrostriatal neurons at low and high magnification. Dual labeling of neurons with FG and TH indicate that these neurons are dopaminergic, and have implications for PD and DID.

Experiment 2 – Labeling of interhemispheric nigrostriatal pathway using viral vectors

There was successful infection of medium-spiny neurons with the WGA-Cre gene as demonstrated by mCherry expression (red cells) (Fig. 6-6). We also found eNpHR+ cells (1-2 cells per animal) (yellow) within the right nigra (Fig. 6-6). These cells will only express the EYFP if there is synaptic connectivity between WGA-Cre infected medium-spiny neurons (in the left striatum) and eNpHR infected nigral neurons (in the right nigra). In these neurons, we found similar morphological characteristics as shown with our FG and TH labeling. These preliminary results demonstrate the capability to accurately label the interhemispheric nigrostriatal pathways using optogenetics labeling techniques.

By using three different techniques (retrograde tracing/lesioning, TH immunohistochemistry and optogenetic tracing viral vector systems, we were able to demonstrate the presence and characteristics of interhemispheric nigrostriatal connections in the normal and parkinsonian rat.
Figure 6-6. Optogenetic dual viral vector labeling of medium spiny neurons in the left striatum (red – top row) and interhemispheric nigrostriatal neurons (yellow – bottom row).

6.5. Discussion

In Chapter 4, we hypothesized that intact interhemispheric nigrostriatal connections may prevent the onset of DID in the rat and monkey models of PD. In this study, normal rats exposed to LD had no dyskinesias. Similarly, animals that had received single injections of intrastriatal 6-OHDA (S-STR – Sauer and Oertel model) did not develop dyskinesias. In both these groups, we found tracer-labeled SNC neurons from the less affected contralateral hemisphere. Furthermore, we found a slight increase in the number of these interhemispheric nigrostriatal neurons labeled in the S-STR nigra compared to the normal group. This supports data from previous reports
suggesting that interhemispheric nigrostriatal neurons have a plastic response to 6-OHDA (see Chapter 4). Interestingly, we found that multiple, unilateral injections of 6-OHDA into the striatum or MFB (M-STR and MFB groups) causes significant tracer-labeled SNC degeneration in both the ipsilateral and contralateral nigra. These animals displayed clear dyskinesias after LD administration. Taken together, these results suggest that loss of interhemispheric nigrostriatal connections (cell bodies and neurites) is correlated to the genesis of dyskinesias in the parkinsonian rat.

As described in Chapter 4, there are two possible mechanisms by which these interhemispheric nigrostriatal connections prevent DID. One mechanism is by increased activity of the interhemispheric nigrostriatal connections in response to DA depletion, thus preventing DA receptor supersensitivitiy. To a certain extent, our study supports this notion due to the fact that we noticed an increase in the number of interhemispheric nigrostriatal connections in the S-STR group compared to normals as shown in Table 6-2. Another possible mechanism is that the axonal arborization of interhemispheric nigrostriatal connections are more extensive than the ipsilateral nigrostriatal connections. Future experiments to evaluate these hypothesis are discussed in Chapter 7.

There are several technical issues of this study that is worth discussing here. One problem is that TH immunohistochemistry may not stain all dopaminergic cells if a cell is compromised (6-OHDA-treated). This could lead to incorrect identification of all TH+ dopaminergic cells in the nigra (Bjorklund, Rosenblad et al. 1997). Another issue is the difficulty of combining TH and FG. In our experience, FG can quench TH staining using conventional immunohistochemistry. Another issue is that FG depends on retrograde transport. If targeting is incorrect or cells are compromised (as with the administration of 6-OHDA), then FG does not label all the cells. Also, as shown above, FG can leak out of cells during processing or after neuronal degeneration of FG+ cells. This leads to false positive cells. Further, cell counts with un-biased stereology versus
Abercrombie method is an issue. Stereology utilizes a set of pre-determined rules that are necessary to give correct cell estimates. If these conditions are not met (specifically number of available cells to count), then cell count estimates are unlikely to be accurate. In cases where the number of cells to count is scarce, stereology cannot be used and Abercrombie can be utilized. It has been acknowledged by multiple investigators that the Abercrombie method provides less accurate counts than stereology when sample size is adequate to perform stereology. However, the Abercrombie method is the next best available technique for quantitative counting when stereology cannot be utilized as in this case when sample sizes of cells to be counted are too few. Lastly, appropriate microscope filters need to be utilized to ensure that no false positive identification of FG occurs.

Optogenetics is a novel technique which uses genetic and optical methods to control the activity of a specific set of neurons in vivo, using a tethering method as shown in Figure 6-7. By controlling these specific neurons, investigators can correlate neuronal activity to behavioral responses. In this context, we utilized a novel optogenetic dual viral vector system to label the interhemispheric nigrostriatal pathway. Unlike other labeling techniques that label all incoming connections to a specific structure, this dual viral vector system allows investigators to discretely label only certain pathways. Our ability to label the interhemispheric nigrostriatal pathway with this optogenetic viral vector system indicates that we could potentially control the activity of these interhemispheric nigrostriatal connections to determine their exact role in parkinsonism and DID.

In our experiment, we successfully infected interhemispheric nigrostriatal neurons with the opsin gene eNpHR (which is tagged with EYFP). Briefly, this eNpHR opsin gene encodes light-sensitive chloride channels which integrates into interhemispheric nigrostriatal neurons and their connections. Light delivery using fiberoptics to these neurons opens these channels and causes an influx of chloride into the cell, thus inactivating these cells. The ability to utilize
optogenetics tracing techniques to label interhemispheric pathways may provide novel insights on
the role of interhemispheric connections in PD and DID, which is described further in Chapter 7.

Figure 6-7. Optogenetic tethering system in the freely-moving rat. We developed a tethering system to deliver optical
laser light into the nigra of a rat. Future experiments will allow for the optical inhibition of nigral neurons transfected
with the eNpHR gene using this system.
Chapter 7

Drug-induced dyskinesias in Parkinson’s disease: future research for novel therapies and pathophysiological mechanisms
7.1. Introduction

We have described the various alterations associated with DID ranging from biochemical to electrophysiological changes in this dissertation research. In this chapter, we discuss how the research described in this dissertation could impact the development of future therapies and propose new experiments based on our novel findings.

7.2. MPE: a novel therapy for PD

Biochemical experiments of MPE: a novel therapy for PD

In Chapter 2, we performed a comprehensive set of experiments that suggest MPE as a possible novel alternative treatment for PD. In this section, we describe a set of experiments to evaluate the biochemical properties of MPE to further delineate the mechanistic actions associated with its anti-dyskinetic effects. These experiments could potentially reveal possible ingredients in MPE that play a role in the anti-PD and anti-DID effects of MPE. Such subcomponent identification could potentially open up new vistas in the therapy of PD and DID.

Experiment 1: We will test the hypothesis that removal of naturally occurring LD in MPE and substitution of synthetic LD back into MPE at equivalent doses will not provide the same anti-PD and anti-dyskinetic effects in parkinsonian rats as described in Chapter 2. We will use HPLC to remove LD from MPE as described in Chapter 2 (MPE(-)LD), then mix synthetic LD (sLD) back into the solution of equivalent amount (MPE (+) sLD). We will measure rat blood levels for LD concentration, and microdialysis to measure DA content in the striatum. We would use the proper control groups to make between and within-group comparisons (normal group, 6-OHDA-lesioned group without treatments, 6-OHDA lesioned group with MPE) for all experiments described. Our expected outcome is that replacement of synthetic LD in MPE (MPE (+) sLD) will not reproduce the same anti-PD or anti-DID efficacy as MPE. This result would suggest that there are unique properties of the natural LD found in MPE that account for its
superior behavioral effects which may be due unique formulation of natural LD or unique interactions between the natural LD and the non-LD components in MPE. An example of the former would be racemic forms of dopa that under correct conditions convert to LD and an example of the later would be the notion of certain peptides protecting natural LD from the enzymatic effects of dopa decarboxylase and thereby providing better serum and brain access of natural LD. Although we cannot be sure how this will turn out, one possible outcome would be that LD/DA content in the blood and in the striatum with synthetic LD replacement (MPE (+) sLD) will be lower than with MPE, indicating less bioavailability with synthetic LD replacement. This may suggest that natural LD in MPE exists in a racemic form that allows serum levels to go up in the right conditions. Another outcome could be that serum levels remain equitable between MPE and MPE (+) sLD, but there is a difference in the LD and dopamine microdialysis measurements from the striatum. If this outcome occurs, then the possibility of non-LD constituents within MPE providing protection of the natural LD from the effects of DDC would be more likely. It is also possible that synthetic LD replacement causes similar or better behavioral benefit than MPE. This is unlikely based on our results in Chapter 2 demonstrating that synthetic LD alone is unable to significantly ameliorate parkinsonism to the extent of MPE alone.

Experiment 2: MPE contains multiple compounds as listed in Table 2-2. However, it is unclear as to how these compounds interact with LD to provide superior anti-PD and anti-DID effects. In this experiment, we can conduct fractionation studies to further evaluate how these compounds affect parkinsonism and DID. In this experiment, small molecules (e.g. single aminoacids) can be removed via dialysis to create MPE without small molecules (MPE (-) SM) and a small molecule (SM) only fraction. Furthermore, another fractionation study to remove proteins from MPE using protease treatment can also be performed. MPE will be resuspended in appropriate buffers (based on optimal activity of proteases used) and incubated with immobilized
trypsin (sequencing grade), carboxypeptidase-A (CP-B), carboxypeptidase-B (CP-B), or elastase. Bioactivity will be performed on extracts before and after such protease treatment. We can then reconstitute each fraction and expose them to HP rats. A battery of behavioral tests (cylinder test, stepping test, DID severity, etc., a rodent battery of behavioral tests as we have described in our original MPE experiments) will be performed to evaluate the effects of these fractions for their anti-PD and anti-DID effects. Further molecular characterization of the components within MPE is likely to show that there are a number of separate anti-PD and anti-DID ingredients. It is possible to attempt to recreate the effects of MPE by combining identified components using synthetic equivalents to ultimately prove its mechanism of action. Alternate outcomes include the notion that none of our fractionation experiments are successful in identifying the ingredients in MPE.

Target identification of MPE: Second messenger systems and electrophysiology

It is evident that DID is associated with numerous changes to second messenger systems in striatal MSN (Pavon, Martin et al. 2006; Guan, Zhan et al. 2007) as shown in Fig. 1-8 (reproduced below). Identifying and comparing these changes in second messenger systems in animals treated with LD and MPE will increase our understanding of how MPE provides anti-PD and anti-DID effects. In this experiment, we will test the hypothesis that MPE will differentially alter second messenger systems when compared to LD. To test this, we will use microarray techniques to identify changes to specific second messenger systems (Vrana, Freeman et al. 2003; Cadet, Brannock et al. 2010). We will expose two groups of parkinsonian rats with either LD or MPE. After chronic exposure, we will remove the brain, take a lysate of the striatum and analyze the presence/absence and amounts of numerous second messenger system molecular changes in the striatal lysate. We expect that certain molecules (such as phosphorylated ERK and phosphorylated Thr34 on DARPP-32, which is known to increase with DID) will be decreased in
the MPE-treated group compared to the LD-treated group. By using microarray techniques and analyzing multiple changes to the striatum, we can then make specific conclusions about how MPE affects certain pathways. Furthermore, we can hypothesize as to which neurotransmitter-receptor interactions (DA, adenosine, neuropeptides, etc.) affect these striatal second messenger systems and correlate those conclusions to the anti-PD and anti-DID effects of MPE and its known components (Table 2-2). Another possible outcome is that there are no significant differences of second messenger systems between LD and MPE. This would suggest that MPE does not affect systems within the striatal MSN, and that the anti-PD and anti-DID effects of MPE more potently affect other basal ganglia nuclei.

Experiments using electrophysiological techniques would also be important to evaluate the anti-DID mechanisms of MPE and to make conclusions on whether MPE targets the direct or indirect pathways of the basal ganglia. We demonstrate in Chapter 2 that *M. pruriens* powder can
differentially alter firing properties of basal ganglia nuclei (STN and SNR) when compared to LD in the non-dyskinetic HP Rhesus monkey. This suggests that other mechanisms besides LD in *M. pruriens* affect basal ganglia neuronal firing. A similar experiment could be conducted to compare changes to neuronal firing properties in the STN, SNR and GPI in bilaterally parkinsonian monkeys after treatment with MPE and LD. We have already shown that MPE does not cause DID in this model while LD produces severe DID. In the experiment described below, we will test the hypothesis that MPE treatment would not cause the “hallmark” electrophysiological suppression/silencing of GPI/SNR neurons that is typically seen with LD treatment and onset of DID in the bilaterally parkinsonian monkey (Papa, Desimone et al. 1999). We expect that electrophysiological silencing of GPI/SNR neurons will prevented with MPE treatment but seen after LD treatment with DID. It is unclear as to which firing properties will result after MPE treatment. However, one possible outcome is that firing properties after MPE treatment would be similar to what we described in the non-dyskinetic HP Rhesus monkey after LD (Gilmour, Lieu et al. 2011). We found a decrease in GPI/SNR firing with an increase in bursting, and no significant alterations to STN firing. Again, this would suggest that MPE not only ameliorates parkinsonism without dyskinesias but also prevents the “hallmark” electrophysiological silencing associated with DID. Furthermore, if MPE does not alter STN firing, then this would suggest that MPE does not target the indirect pathway. If we find changes to SNR/GPI firing with MPE (similar to that of M. pruriens powder as shown in Chapter 2), we can conclude that MPE targets the direct pathway.

**Neuroanatomical changes with MPE**

As described in Chapter 1, dendritic spines on striatal MSN are modified in advanced PD, causing aberrant synaptic connectivity striatal inputs. It has been previously shown that certain drugs can prevent these pathophysiological synaptic changes of dendrites in PD (Soderstrom,
In this experiment, we will test the hypothesis that MPE alters synaptic connectivity to the MSN differently than LD. After chronic treatment with LD or MPE, we will evaluate the severity of DID in parkinsonian rats. We will then histological analyze alterations to dendritic spines in each treatment group using electron microscopy. We expect that MPE will prevent pathophysiological alterations to dendritic spines associated with PD and DID. These results would suggest that MPE is able to prevent pathophysiological changes to MSN and abnormal synaptic connectivity associated with advanced PD and DID. However, it is also possible that MPE does not protect from abnormal, plastic spine alterations, and that the effects of MPE act on a different basal ganglia target.

7.3. Interhemispheric nigrostriatal connections in the parkinsonian primate: implications for basal ganglia electrophysiology and DID

Based on our findings that HP rhesus monkeys are resistant to dyskinesias and our findings that electrophysiological “suppression” is not seen in basal ganglia nuclei in this model, we hypothesized that preservation of interhemispheric nigrostriatal connections prevents dyskinesias and also prevents the electrophysiological “hallmark” of DID, “suppression” of GPI/SNR. In the figures below, we summarize our findings and propose expected outcomes for basal ganglia neuronal activity in relation to interhemispheric nigrostriatal connections and the genesis of dyskinesias in the HP primate and bilateral parkinsonian primate with and without LD treatment. In this experiment, we would utilize tracing techniques to evaluate the presence or absence of interhemispheric nigrostriatal connections in combination with electrophysiological studies in the non-dyskientic (HP) and dyskinetic (bilateral parkinsonian) primate models of PD.

Figure 7-1. Expected basal ganglia firing discharge rate and pattern with loss of interhemispheric nigrostriatal connections (IH NS) and onset of dyskinesias. Number of starbursts denote firing rate (more starbursts indicate...
increase in firing rate), size of starbursts denote bursting activity (larger starbursts indicate increase in bursting discharges). Blue – putamen; Olive green – globus pallidus externa; Light green – globus pallidus interna; Purple – substantia nigra compacta; Orange – substantia nigra reticulata. Dashed thick black line – degenerated ipsilateral nigrostriatal connections; intact thin black line – retained interhemispheric nigrostriatal connections; dashed thin black line – degenerated interhemispheric nigrostriatal connections.

Fig. 7-1A) Hemiparkinsonism (+IH NS)

The figure above represents our HP rhesus monkey in the baseline HP state. In the SNR, we found that SNR firing rate was slightly higher than STN firing, which is denoted by the number of starbursts in each nuclei. In this scenario, we hypothesize that the interhemispheric nigrostriatal connections are intact (+IH NS).
In the HP rhesus monkey, we found no change in STN firing or bursting activity with chronic LD treatment. In the SNR, we found that LD decreased firing rate and increased bursting discharges. We hypothesize that the prevention of dyskinesias and the lack of basal ganglia electrophysiological “suppression” in the HP rhesus monkey are due to intact interhemispheric nigrostriatal connections (+IH NS).
We hypothesize that with progression of nigrostriatal degeneration in the bilateral parkinsonian monkey, there is loss of interhemispheric nigrostriatal connections (-IH NS). As shown in previous reports in advanced PD patients and bilateral parkinsonian monkeys, we expect that this would cause both firing rate and bursting pattern to significantly increase in this advanced parkinsonian state.
We predict that the electrophysiological “hallmark” of suppression/silence will occur with LD treatment and onset of dyskinesias and loss of interhemispheric nigrostriatal connections.

7.4. Interhemispheric nigrostriatal connections in the parkinsonian rat and DID

In Chapter 6, we described a set of experiments that suggests interhemispheric nigrostriatal connections may modulate DID in the rat model of PD. In our experiments, we were able to show that loss of interhemispheric nigrostriatal connections is correlated to onset of DID in the parkinsonian rat. In the section below, we describe a set of experiments that would further evaluate the direct effect of interhemispheric nigrostriatal connections on DID in the parkinsonian rat.

Optogenetic experiments
Experiment 1: In this experiment, we will test the hypothesis that inhibition of the interhemispheric (nigrostriatal and/or corticostriatal) connections lead to the genesis of DID in the rodent model of PD. For these experiments, we will use recombinant viral vectors that conditionally express Natronomonas halorhodopsin (NpHR) in vivo that will allow selective expression of photosensitive opsins in the interhemispheric pathways such that these pathways can be inactivated using specific laser light. The idea is to use the combination of 2 different vectors, one containing double-floxed Cre-dependent AAV5 carrying eNpHR 3.0–EYFP (AAV5-Ef1a-DIO-eNpHR 3.0-EYFP) and the second virus that provides a trans-synaptically mediated switch to turn on eNpHR 3.0 (AAV2-Ef1a-mCherry-IRES-WGA-Cre) that will selectively label the crossed nigrostriatal pathway and/or the crossed corticostriatal interhemispheric pathway. This is extensively described in Chapter 6 and depicted below (Fig. 7-2). Animals will be behaviorally characterized and then receive graded lesioning of the nigrostriatal pathway with striatal injections of 6-OHDA (Sauer and Oertel model). These animals will be repeatedly tested for parkinsonian behavior and DID. Subsequent optical inhibition of the interhemispheric nigrostriatal and/or corticostriatal connections will determine the role these connections play in the genesis of LID when treated with LD. All animals will undergo extensive histopathological analysis, SN dopaminergic neuron cell counts, and quantification of interhemispheric eNphR3.0-positive neurons.

In this paradigm, we will use AAV5-Ef1a-DIO-eNpHR 3.0-EYFP vector (henceforth referred as eNpHR 3.0) such that the neurons express the eNpHR 3.0 light-sensitive chloride pump, and AAV2-Ef1a-mCherry-IRES-WGA-Cre (henceforth referred to as WGA-Cre) to act as a switch such that only specific set of neurons conditionally express eNpHR 3.0. These vectors will enable specific and targeted labeling of the interhemispheric pathways. Group 1: Interhemispheric nigrostriatal pathway – Animals (N=10) will receive eNpHR 3.0 viral vector into the right nigra and injections of WGA-Cre in the left striatum at 3 sites to label the
interhemispheric nigrostriatal neurons originating in the right nigra and terminating in the left striatum. Group 2: Interhemispheric corticostrial pathway – Animals (N=10) will receive eNpHR 3.0 viral vector into the MI motor cortex and WGA-Cre into the left striatum sites as Group 1 in order to label the interhemispheric corticostrial pathway originating in the right cortex and terminating in the left striatum. Dosages and injection sites are based on area of expression of halorhodopsins in previous reports and viral dispersion of ~1.0 mm3 in brain regions. After 5 weeks to allow appropriate integration of eNpHR 3.0 into interhemispheric corticostrial and nigrostriatal neurons, animals will undergo a second surgery involving left 6-OHDA striatal lesion as described in Chapter 6 (S-STR and M-STR). In this surgery, cannulae implantation will be stereotactically targeted at the right MI cortex or nigra for subsequent fiber-optic implantation and optical inhibition of these brain regions. After 3 weeks to allow proper unilateral neurodegeneration, animals will characterized using a battery of behavioral tests to evaluate parkinsonism as we have previously described in Chapter 2. These include tests to determine degree of parkinsonism and measure quantitatively the extent of LID. Optical inhibition in either the right SN or right MI cortex using a 589 nm light laser source (wavelength which selectively causes inhibition of eNpHR positive neurons) will be used during LD treatments using a tethered system (as shown in previously in Figure 6-7) that allows awake animal behavioral assessments while optical stimulation is performed (Fig. 7-2). Animals will be evaluated for onset of LID when the light source is on (optical inhibition of eNpHR 3.0-expressing interhemispheric neurons) and when the light is off. Additional animals (N=30) will be used for following controls: 1) bilateral striatal injections of WGA-Cre “switch” + left SN Cre-dependent eNpHR 3.0 (light on left SN), 2) Left striatal injections of WGA-Cre “switch” + left SN Cre-dependent eNpHR 3.0 (light on left SN), and 3) left striatal injections of WGA-Cre “switch” + right SN Cre-dependent eNpHR 3.0 + striatal 6-OHDA (M-STR) + (light on right SN).
At the end of the study, animals will be euthanized via transcardiac perfusion with heparinized saline and 4% paraformaldehyde. Brains will be removed from the skull, cryoprotected and cut in coronal sections at 60 μm on a freezing microtome. TH, dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT-2) immunohistochemistry will be utilized for detection of SNC neurons and their fibers. Such SNC neurons will be counted using unbiased stereology as described in Chapters 2 and 6. All appropriate negative and positive controls will be used to ensure quality and meaningful comparisons. We will evaluate the presence of EYFP and mCherry (for eNpHR 3.0-positive neurons) in cortical and nigral neurons using fluorescence microscopy. Based on the neuroanatomical location of EYFP/mCherry-labeled neurons in the cortex, we will also utilize immunohistochemical markers to identify glutamatergic neurons.

We expect that successful inhibition of the crossed nigrostriatal fibers that originate from the right SN in partially lesioned right HP rats will cause LID (when light is on). We expect that LID will cease once these interhemispheric areas are no longer photically stimulated (optical inhibition is “turned off”). These findings will suggest that interhemispheric nigrostriatal connections play crucial role in the prevention of LID. Appropriate control experiments would further verify these results. For example, optogenetic inhibition of only the left nigrostriatal pathway (sparing the crossed interhemispheric pathways) would only result in right HP state without LID, much like what is seen with striatal 6-OHDA lesioning. Optogenetic inhibition of the left SN and its crossed innervation to the right striatum would also lead to right HP state without causing LID when exposed to LD. In animals that get graduated multisite striatal 6-OHDA as described in Chapter 6 (M-STR group), we would expect DID to occur even without optogenetic inhibition of the right SN as in this case the crossed pathway would be predicted to be lesioned by multiple doses of 6-OHDA. Furthermore, we expect, EYFP labeling to confirm our predictions and to demonstrate highly specific targeting of appropriate pathways. We expect
optogenetic inhibition of interhemispheric corticostriatal neurons to be less of a contributor and so milder LID to occur. We would expect all appropriate controls to support this conclusion. Such an outcome will suggest that crossed corticostriatal connections play a lesser influence on the pathophysiology of LID. Alternate outcomes include the notion that neither inhibition of the nigrostriatal pathway or the corticostriatal pathway cause LID. Another possibility is that both crossed pathways contribute equally or that simultaneous optogenetic inhibition of both crossed nigrostriatal and corticostriatal pathways are necessary to cause LID. Although none of these alternate outcomes can be ruled out, our preliminary data support the notion that crossed nigrostriatal pathways may be critical in the genesis of LID. If it turns out that both the crossed nigrostriatal and the corticostriatal pathways are not contributory to the genesis of LID, we will explore other less described pathways like the interhemispheric striato-striatal pathway as a possible venue of interhemispheric anti-LID influence. Finally, it is possible that short-term optogenetic inactivation of the interhemispheric pathway/s alone is not sufficient for the genesis of LID. Plastic changes that accompany neurodegeneration may be necessary. This hypothesis will be tested via the use of graduated multisite striatal 6-OHDA, one of the controls included in the rat experiments. In future experiments, we can also perform electrophysiological single-cell recordings in basal ganglia nuclei to determine if optical inhibition of interhemispheric connections causes alterations to neuronal firing properties.
Figure 7-2. Proposed optogenetic experiments and outcomes. In this experiment, we propose to utilize a dual viral vector system to optogenetically label the nigrostriatal and corticostriatal interhemispheric connections. We can then optically control these labeled neurons by delivering laser light into the cortex and nigra (orange light). In the STN and SNR/GPI table inserts, we hypothesize changes to basal ganglia electrophysiology in the HP state, HP state with LD treatment, and with interhemispheric inhibition of the pathways (IHI). Direction and number of arrows indicate an increase or decrease in firing rate (FR) or bursting activity.

Experiment 2: As we describe in Chapter 4, there are two potential mechanisms by which this small number of interhemispheric nigrostriatal neurons may prevent the onset of dyskinesias. In this experiment, we will test the hypothesis that biochemical upregulation of DA neurotransmission to the DA-denervated striatum by interhemispheric nigrostriatal connections prevents DID. For example, if the left nigrostriatal pathway is significantly degenerated, we hypothesize that nigral neurons originating in the right hemisphere and terminating in the left striatum respond to this denervation by biochemical upregulation of DA release. In this context, we hypothesize that after LD administration, the interhemispheric nigrostriatal connections increase neurotransmission of DA by providing continuous DA to the denervated striatum. This would prevent DA receptor supersensitivity and prevent the onset of dyskinesias. An experiment
utilizing microdialysis to measure DA content in the striatum and optogenetic techniques described above would provide evidence for this hypothesis.

Using the dual viral vector system as described in experiment 1 above (WGA-Cre and eNpHR), we can first optogenetically label the interhemispheric nigrostriatal connections (nigral neurons originating in the right nigra and terminating in the left striatum) in the normal rat. The same lesioning paradigm of the left nigrostriatal pathway as described in Chapter 6 (normal, S-STR, M-STR and MFB groups) would be used in these optogenetically labeled rats. A fiberoptic would be targeted to the right nigra such that the optogenetically labeled interhemispheric nigrostriatal connections can be optically controlled. After lesioning, a microdialysis probe can be inserted into the left striatum to measure DA content. DA content could be measured in four different conditions for each group: 1) post-lesion, 2) with LD treatment, 3) during interhemispheric nigrostriatal optical inhibition, and 4) during interhemispheric nigrostriatal optical inhibition with LD treatment.

We would expect a significant decrease in DA content in the left striatum after lesion in the S-STR, M-STR and MFB groups compared to normal. With LD treatment in the three lesioned groups, we expect DA content to increase. In the same groups, we would then optically inhibit the interhemispheric nigrostriatal connections. In the S-STR group, which does not develop DID, we hypothesize that optical inhibition of the interhemispheric nigrostriatal connections would cause a dramatic decrease of DA content to the left striatum, and lead to DID in these animals. In the M-STR and MFB groups, we would expect that optical inhibition of interhemispheric nigrostriatal connections would not make a difference in DA content to the denervated striatum after LD treatment since these interhemispheric connections are lost in these two groups. The results of this experiment would directly show that inhibition of interhemispheric nigrostriatal connections leads to DID. In this context, another experiment which inhibits glutamatergic interhemispheric corticostriatal connections as described in experiment 1 in
combination with measuring glutamate content using microdialysis could be performed. It is possible that the hyperactivity associated with DID can be prevented by optical inhibition of interhemispheric cortical inputs to the striatum. We would expect that inhibition of these cortical inputs would decrease glutamate to the striatum, and prevent the onset of DID.

**Neuroanatomical analysis of interhemispheric nigrostriatal connections**

The second mechanism by which interhemispheric nigrostriatal connections may modulate DID is through synaptic connectivity. Although there are only a small number of interhemispheric nigrostriatal cells (up to 10%), they may have synaptic arborization that is different than ipsilateral nigrostriatal connections. In this experiment, we will test the hypothesis that interhemispheric nigrostriatal connections have more dense synaptic connectivity than ipsilateral nigrostriatal connections, and that this interhemispheric synaptic connectivity is correlated to DID. We hypothesize that this may be the mechanism by which the S-STR group in the rat and HP rhesus monkey is resistant to DID. However, with advanced nigrostriatal degeneration (in the M-STR and MFB group in the rat, and bilateral parkinsonian primate), this interhemispheric nigrostriatal synaptic connectivity is lost which then leads to DID. Electron microscopy (to highly magnify axonal connections) in combination with tract tracing methods (to differentiate between ipsilateral and interhemispheric connections) may be useful to further evaluate the density of striatal synaptic connections of interhemispheric nigrostriatal connections.

A future experiment could be performed in the parkinsonian rat using the four groups described in Chapter 6 (control, S-STR, M-STR and MFB). We hypothesize that these interhemispheric connections in the control and S-STR groups (which do not develop dyskinesias) would be highly arborized with multiple axonal connections to MSN when compared to control ipsilateral nigrostriatal axonal connections to MSN. However, in the M-STR and MFB groups (which readily develop dyskinesias), we would expect to see impaired or no
interhemispheric nigrostriatal axonal connections. This would provide evidence that even with extensive unilateral nigrostriatal degeneration, interhemispheric nigrostriatal connections can prevent DA receptor supersensitivity and onset of DID due to its extensive synaptic connection to the MSN in the denervated striatum.

7.5. Conclusion

Findings from these experiments would enhance our understanding of the pathophysiological mechanisms associated with DID. This would also advance the development of novel, more effective treatments for DID. Treatments that can protect these interhemispheric nigrostriatal connections from degeneration such as transplantation of glial cell-derived neurotrophic factor and pigment epithelium-derived factor (Venkiteswaran, Marupudi, Lieu et al., 2011 – in preparation) would provide further evidence about how the interhemispheric nigrostriatal connections modulate DID.
Appendix A

Modified Unified Parkinson’s Disease Rating Scale for PD Patients

III. MOTOR EXAMINATION

18. Speech
   0 = Normal.
   1 = Slight loss of expression, diction and/or volume.
   2 = Monotone, slurred but understandable; moderately impaired.
   3 = Marked impairment, difficult to understand.
   4 = Unintelligible.

19. Facial Expression
   0 = Normal.
   1 = Minimal hypomimia, could be normal "Poker Face".
   2 = Slight but definitely abnormal diminution of facial expression.
   3 = Moderate hypomimia; lips parted some of the time.
   4 = Masked or fixed facies with severe or complete loss of facial expression;
      lips parted 1/4 inch or more.

20. Tremor at rest (head, upper and lower extremities)
   0 = Absent.
   1 = Slight and infrequently present.
   2 = Mild in amplitude and persistent. Or moderate in amplitude, but only intermittently present.
   3 = Moderate in amplitude and present most of the time.
   4 = Marked in amplitude and present most of the time.

21. Action or Postural Tremor of hands
   0 = Absent.
   1 = Slight; present with action.
   2 = Moderate in amplitude, present with action.
   3 = Moderate in amplitude with posture holding as well as action.
   4 = Marked in amplitude; interferes with feeding.

22. Rigidity (Judged on passive movement of major joints with patient relaxed in sitting position.
     Cogwheeling to be ignored.)
   0 = Absent.
   1 = Slight or detectable only when activated by mirror or other movements.
   2 = Mild to moderate.
   3 = Marked, but full range of motion easily achieved.
   4 = Severe, range of motion achieved with difficulty.
23. **Finger Taps** (Patient taps thumb with index finger in rapid succession.)
   0 = Normal.
   1 = Mild slowing and/or reduction in amplitude.
   2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
   3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
   4 = Can barely perform the task.

24. **Hand Movements** (Patient opens and closes hands in rapid succession.)
   0 = Normal.
   1 = Mild slowing and/or reduction in amplitude.
   2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
   3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
   4 = Can barely perform the task.

25. **Rapid Alternating Movements of Hands** (Pronation-supination movements of hands, vertically and horizontally, with as large an amplitude as possible, both hands simultaneously.)
   0 = Normal.
   1 = Mild slowing and/or reduction in amplitude.
   2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
   3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
   4 = Can barely perform the task.

26. **Leg Agility** (Patient taps heel on the ground in rapid succession picking up entire leg. Amplitude should be at least 3 inches.)
   0 = Normal.
   1 = Mild slowing and/or reduction in amplitude.
   2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
   3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
   4 = Can barely perform the task.

27. **Arising from Chair**
   (Patient attempts to rise from a straightbacked chair, with arms folded across chest.)
   0 = Normal.
   1 = Slow; or may need more than one attempt.
   2 = Pushes self up from arms of seat.
   3 = Tends to fall back and may have to try more than one time, but can get up without help.
   4 = Unable to arise without help.

28. **Posture**
   0 = Normal erect.
   1 = Not quite erect, slightly stooped posture; could be normal for older person.
   2 = Moderately stooped posture, definitely abnormal; can be slightly leaning to one side.
   3 = Severely stooped posture with kyphosis; can be moderately leaning to one side.
29. Gait
0 = Normal.
1 = Walks slowly, may shuffle with short steps, but no festination (hastening steps) or propulsion.
2 = Walks with difficulty, but requires little or no assistance; may have some festination, short steps,
or propulsion.
3 = Severe disturbance of gait, requiring assistance.
4 = Cannot walk at all, even with assistance.

30. Postural Stability (Response to sudden, strong posterior displacement produced by pull on shoulders
while patient erect with eyes open and feet slightly apart. Patient is prepared.)
0 = Normal.
1 = Retropulsion, but recovers unaided.
2 = Absence of postural response; would fall if not caught by examiner.
3 = Very unstable, tends to lose balance spontaneously.
4 = Unable to stand without assistance.

31. Body Bradykinesia and Hypokinesia (Combining slowness, hesitancy, decreased armswing, small
amplitude, and poverty of movement in general.)
0 = None.
1 = Minimal slowness, giving movement a deliberate character; could be normal for some persons. Possibly reduced amplitude.
2 = Mild degree of slowness and poverty of movement which is definitely abnormal. Alternatively, some reduced amplitude.
3 = Moderate slowness, poverty or small amplitude of movement.
4 = Marked slowness, poverty or small amplitude of movement.
Appendix B

Modified Unified Parkinson’s Disease Rating Scale for Primates

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RUL – Right Upper Limb, LUL – Left Upper Limb, RLL – Right Lower Limb, LLL – Left Lower Limb, R – Right, L – Left. (0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = unable to utilize/cannot perform task)
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Student Travel Fellowship Award from the Center of Molecular and Cellular Neuroscience of
The Penn State Neuroscience Institute
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PUBLICATIONS


