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Neural Pathways and Receptor Mechanisms Mediating Stimulation-Evoked Striatal Dopamine Release: Relevance to Deep Brain Stimulation as a Treatment for Parkinsons Disease

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NEURAL PATHWAYS AND RECEPTOR MECHANISMS MEDIATING STIMULATION-EVOKED STRIATAL DOPAMINE RELEASE: RELEVANCE TO DEEP BRAIN STIMULATION AS A TREATMENT FOR PARKINSON'S DISEASE

by

Deranda Brewer Lester

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DEDICATION

First of all, I dedicate this work to my husband Caleb for his endless support, optimism, and patience. He has over and over again proven to be my biggest fan, and I appreciate that.

Secondly, I dedicate this work to my daughter Darcy who will one day inevitably rule the world, as she already rules mine. She serves as constant inspiration for all that I aspire to do and learn.

Finally, I dedicate this work to my parents, who taught me the value of education, instilled within me a desire to learn, and continuously provided me with the best academic opportunities. As a parent, I strive to emulate your devotion and encouragement.

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ABSTRACT

Lester, Deranda Brewer. Ph.D. The University of Memphis. May 2011. *Neuronal Pathways and Receptor Mechanisms Mediating Stimulation-Evoked Striatal Dopamine Release: Relevance to Deep Brain Stimulation as a Treatment for Parkinson's Disease.* Major Professor: Charles D. Blaha, Ph.D.

Dopaminergic neurons of the nigrostriatal dopaminergic system, projecting from the substantia nigra compacta (SNc) to the striatum, serve a critical role in mediating voluntary motor control. Parkinson's disease is a neurological disorder characterized by progressive degeneration of these dopamine neurons, which leads to dopaminergic deficiencies in the striatum. Reduced striatal dopamine transmission is thought to increase inhibitory basal ganglia output to the thalamus and subsequently reduce excitation of cortical motor areas, resulting in impaired motor functioning. Despite unclear mechanisms, deep brain stimulation (DBS) is an established neurosurgical approach for effectively treating the parkinsonian motor symptoms. Currently the subthalamic nucleus (STN) is the most commonly targeted site in these procedures, while the pedunculopontine tegmental nucleus (PPT) is emerging as a therapeutically beneficial target when stimulated alone or in combination with the STN. Thus, the connectivity between these nuclei and the nigrostriatal dopamine system is the focus of the present paper, with the overarching hypothesis being that the therapeutic benefits of STN/PPT DBS are mediated, at least in part, by activation of surviving nigrostriatal neurons, resulting in striatal dopamine release. The present study investigated several neural pathways and receptor mechanisms involved in mediating STN or PPT stimulation-evoked striatal dopamine release using *in vivo* fixed potential amperometry with carbon-fiber recording microelectrodes in the striatum of urethane-anesthetized mice. Overall, results indicate that STN stimulation evokes striatal dopamine release directly via

excitatory glutamatergic inputs to SNc dopamine cells as well as indirectly by activating excitatory glutamatergic and cholinergic STN-PPT-SNc pathways, while PPT stimulation evokes striatal dopamine release directly by activating glutamatergic and cholinergic pathways to SNc dopamine cells as well as indirectly via activation of glutamatergic and cholinergic PPT-STN-SNc projections. Understanding the influence of the STN and PPT on SNc dopamine cell activity and output of the basal ganglia-thalamocortical motor circuit may lead to novel pharmaceutical therapies as well as a better understanding of the underlying mechanisms of clinical DBS, which could then improve the therapeutic efficacy of treatments for Parkinson's disease.

PREFACE

This dissertation has been formatted to allow for the separate publication of Chapter 5 and Chapter 6. As such, this dissertation and reference list are written following the *Neuroscience* style guidelines.

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Chapter 1. Overview of the Nigrostriatal Dopamine System

The nigrostriatal dopaminergic system projects predominantly from the substantia nigra compacta (SNc) of the midbrain to the caudate putamen (striatum in the rat) of the forebrain (Albanese and Minciacchi, 1983). Stimulation of the SNc elicits fast excitatory responses in striatal neurons (Plenz and Kitai, 1996), while lesions of the SNc reduce basal levels of extracellular striatal dopamine concentrations (Dentresangle et al., 2001). In addition, striatal extracellular dopamine concentration is positively correlated with the degree of dopamine cell loss in the SNc (Altar et al., 1987). Dopamine transmission in the striatum is most commonly associated with voluntary movements and has been linked to the selection and initiation of contextually appropriate motor patterns (Hauber, 1998; Redgrave et al., 1999; Wickens, 1990). Reduced dopamine in the striatum is associated with motor symptoms of Parkinson's disease such as difficulty initiating and terminating movements, gait impairments, and muscular rigidity (Knott et al., 1999; Lev et al., 2003; Wolters and Francot, 1998), whereas excess dopamine release in the striatum can lead to repetitive motoric behaviors such as stereotypy, with the degree of intensity of stereotypical behaviors (e.g. body rearing, head bobbing, and gnawing) being positively correlated with striatal dopamine release (Sharp et al., 1987).

Physiology of the Basal Ganglia

The basal ganglia are comprised of the striatum, substantia nigra, subthalamic nucleus (STN), and globus pallidus. Anatomists have made further distinctions based on structure and function. The substantia nigra has been divided into the SNc and substantia

nigra reticulata (SNr), while the globus pallidus comprises lateral segments, namely the globus pallidus externus (GPe) and the globus pallidus internus (GPi). The putative role of the basal ganglia is to synthesize multiple sources of information from sensual, emotional, associative brain areas in order to produce a contextually appropriate response (Bolam et al., 2000). The major input station for the basal ganglia is the striatum, with the majority of neurons within this area being spiny γ-aminobutyric acid (GABA) containing projection neurons, 2% of striatal neurons being large cholinergic interneurons, and the rest being aspiny GABAergic interneurons (Hauber, 1998; Parent and Hazrati, 1995). The spiny GABAergic neurons are the main targets for most projections to the striatum (Parent and Hazrati, 1995), with dopamine receptor subtypes of these cells being both dopamine D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors (Wooten, 2001). As illustrated in Fig. 1, the neurons of the striatum project to other areas within the basal ganglia complex, the GPi and the SNr, via two pathways, a direct (monosynaptic) connection and an indirect pathway through the external segment of the globus pallidus (GPe) and the subthalamic nucleus (STN). Striatal neurons in the direct pathway utilize D1 receptors, whereas those in the indirect pathway utilize D2 receptors (Galvan and Wichmann, 2008; Gerfen et al., 1990). Activation of D1 receptors stimulates adenylate cyclase activity, thus activating the GABAergic medium spiny output neurons, whereas activation of D2 receptors inhibits adenylate cyclase, thus inhibiting GABAergic output neurons (Wooten, 2001). Therefore, the direct (via D1) and indirect (via D2) pathways have opposing actions, but may reach the same net outcome of activating motor regions of the cortex. For example, activation of D1 receptors in the direct striatal GABAergic pathway leads to inhibition of GPi/SNr

inhibitory GABAergic projections to the thalamus, subsequently increasing activity in the thalamus that, in turn, excites motor areas in the cortex (Gerfen et al., 1990; Mink, 1996; Wooten, 2001). Alternatively, activation of D2 receptors in the indirect pathway inhibits striatal inhibitory GABAergic neurons, resulting in disinhibition (excitation) of GPe inhibitory GABAergic neurons that project to the STN. As a consequence, decreased activity of the STN excitatory glutamatergic neurons that innervate the GPi/SNr, GPe, and SNc leads to a reduced inhibitory drive of these nuclei to the thalamus, thereby indirectly increasing excitation of the motor areas in the cortex (Gerfen et al., 1990; Mink, 1996; Wooten, 2001). In sum, the net effect of striatal dopamine release from the nigrostriatal pathway increases thalamocortical activity via direct or indirect reduction of GPi/SNr activity consequently facilitating voluntary movements (Gerfen et al., 1990).

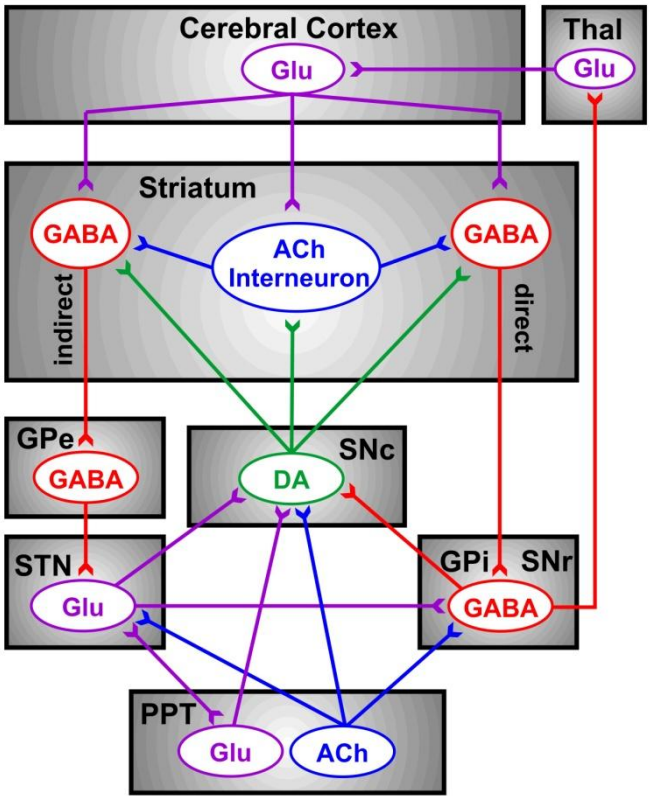


Fig. 1. Simplified thalamocortical basal ganglia circuitry depicting the innervation of the striatum by the nigrostriatal dopaminergic system and its excitatory and inhibitory influence on the direct (via D1 receptors) and indirect (via D2 receptors) GABAergic striatal output pathways to the globus pallidus internus/substantia nigra reticulata (GPi/SNr). The glutamatergic and cholinergic neurons of the pedunculopontine tegmental nucleus (PPT) connect with the basal ganglia via excitatory projections to the subthalamic nucleus (STN), substantia nigra compacta (SNc), and GPi/SNr. ACh: acetylcholine; DA: dopamine; GABA: γ-aminobutyric acid; Glu: glutamate; GPe: globus pallidus externus; Thal: thalamus.

STN and PPT Connectivity and Modulation of Striatal Dopamine Release

The STN comprises a relatively small bilateral pair of brain nuclei, located in the diencephalon close to the dorsal forebrain bundle (Hauber, 1998; Lee et al., 2006). The majority of STN neurons are projection neurons which are glutamatergic in nature (Albin et al., 1989; Smith and Parent, 1988; Van der Kooy and Hattori, 1980). The STN projects to many areas of the basal ganglia, with high amounts of collateralization, including the globus pallidus, SNr, and the SNc (Deniau et al., 1978; Hauber, 1998). These connections, specifically the direct excitatory efferent to the SNc, place the STN in a critical position to regulate dopamine activity in the striatum (Groenewegen and Berendse, 1990; Hammond et al., 1978; Kita and Kitai, 1987). The STN also projects to the pedunculopontine tegmental nucleus (PPT), offering an alternate route of mediating activity of the nigrostriatal dopamine system via the PPT's connectivity with the basal ganglia (Groenewegen and Berendse, 1990; Morrizumi and Hattori, 1992). The PPT, located in the mesopontine region of the hindbrain, contains a heterogeneous population of cholinergic and glutamatergic neurons. PPT projections to the STN, GPi, SNc, cortex, and thalamus have been identified, with the densest of these projections going to the SNc and STN (Charara et al., 1996; Clarke et al., 1997; Lee et al., 2000; Pahapill and Lozano, 2000). This prompts interest in the question of exactly how the STN and PPT may interact to differentially modulate nigrostriatal dopaminergic neurotransmission given their extensive interconnectivity and high degree of collateralization with many important nigrostriatal related structures.

Research supports a contribution of the STN in modulating functional activity of the nigrostriatal dopamine system. Stimulation of the STN has been shown to alter

neuronal activity within the SNc of rodents generating both excitatory and inhibitory postsynaptic potentials (Lee et al., 2004; Nakanishi et al., 1987) and increased firing of SNc neurons (Benazzou et al., 2000; Hammond et al., 1978; Iribe et al., 1999). Electrical stimulation of the STN has also been shown to increase dopamine extracellular levels in the striatum (Lee et al., 2006). Pharmacological activation via microinfusion of the GABA antagonist bicuculline into the STN produced enhancements in not only STN neuronal firing, but also in SNc and globus pallidus neuron activity (Chergui et al., 1994; Robledo and Feger, 1990). Intra-STN infusion of kynurenate, which non-selectively antagonizes ionotropic glutamate receptors, attenuates spontaneous activity of SNc neurons (Robledo and Feger, 1990). Most of the aforementioned studies utilized rodents; however, changes in STN activity have also been shown to significantly affect discharge patterns of SNc neurons and striatal dopamine release similarly in primates (Charara et al., 1996; Futami et al., 1995). However, the monosynaptic pathway between the STN and SNc has shown to be sparse in primates compared to rodents (Sato et al., 2000; Smith et al., 1990). Thus, changes in SNc discharge patterns following pharmacological stimulation and inhibition of the STN in primates have been suggested to be mediated primarily by excitatory SNc afferents from the PPT (Charara et al., 1996; Futami et al., 1995). The STN and PPT are reciprocally connected with excitatory projections (Futami et al., 1995; Lee et al., 2000), which have been shown to be both cholinergic and glutamatergic from the PPT to the STN (Moon-Edley and Graybiel, 1983; Oakman et al., 1999). *In vivo* electrochemical studies have previously shown that electrical and chemical stimulation of the PPT enhances dopamine efflux in the striatum (Forster and Blaha, 2003; Miller and Blaha, 2004); thus, stimulation of the STN may be increasing

discharge patterns of SNc dopaminergic neurons and generating striatal dopamine release indirectly through activation of the PPT.

Evidence illustrating the functional importance of the PPT supports a critical modulatory role of this brain region in the modulation of nigrostriatal dopaminergic activity. As noted above, excitatory glutamatergic and cholinergic neuronal cells in the PPT directly project to dopamine-containing cell bodies in the SNc (Blaha and Winn, 1993; Forster and Blaha, 2003; Moon-Edley and Graybiel, 1983; Oakman et al., 1999). Pharmacological activation of the PPT with ionotropic glutamate receptor agonists increases both the firing rate of SNc dopamine neurons (Clarke et al., 1987) and dopamine metabolism within the striatum as measured by *in vivo* voltammetry (Hernandez-Lopez et al., 1992). Electrical stimulation of the PPT has also been shown to activate STN neurons via cholinergic and glutamatergic projections (Hammond et al., 1983; Woolf and Butcher, 1986). Therefore, in addition to direct activational inputs to SNc dopaminergic cells, the PPT may also modulate nigrostriatal dopamine activity in an indirect manner, through PPT glutamatergic and cholinergic inputs to STN glutamatergic neurons that, in turn, innervate dopamine-containing cells in the SNc (Bevan and Bolam, 1995; Lee et al., 2000). An understanding of how these brain regions functionally interact to mediate nigrostriatal dopamine release is essential in enhancing our knowledge on how these pathways normally function to affect sensory-motor gating in the striatum. Such an understanding will give insight and greater clarity into neurological disorders such as Parkinson's disease which arise as a result of abnormal functioning of the nigrostriatal dopamine system.

Behavioral Correlates of Striatal Dopamine Release

Dopamine transmission in the striatum is most commonly associated with normal voluntary ballistic movements (Wickens, 1990). Increasing striatal dopamine levels with psychostimulants such as the amphetamines in animals leads to the production of repetitive and contextually redundant stereotypic behaviors, which includes behaviors such as repetitive rocking, self-grooming, sniffing, and gnawing. Indeed, early *in vivo* microdialysis studies have demonstrated that the presentation of these behaviors is correlated with abnormally high levels of striatal dopamine release (Sharp et al., 1987). Furthermore, stereotypy may be induced and subsequently attenuated by microinfusions of dopamine receptor agonists and antagonists, respectively, into the striatum (Canales and Graybiel, 2000; Presti et al., 2003). It is thought that dopaminergic receptor agonists and antagonists infused in the striatum may enhance or reduce GABAergic medium spiny neurons activity, respectively, ultimately resulting in an enhancement or reduction in communication to motor cortical areas. Thus, excessive striatal dopamine levels are thought to alter the output of striatal projection neurons (via the direct or indirect output pathways) leading to reduced activity of the GPi/SNr, as seen in hyperkinetic disorders such as Huntington's disease (Mink, 1996).

In contrast, marked reduction or absence of dopamine in the striatum leads to an overall increase in activity of the GPi/SNr, which in turn reduces neurotransmission in motor cortical areas and impairs motor control (Mink, 1996; Wooten, 2001). Animals treated with 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which are commonly used chemicals for inducing degeneration of nigrostriatal dopamine neurons, demonstrate significantly low levels of

locomotion as well as muscular rigidity, slowness of movement, and abnormal posture (Langston et al., 1984; Truong et al., 2006). Thus, these animal models mimic the neuropathology as well as behavioral symptomology seen in Parkinson's disease. In clinical cases of Parkinson's disease, as well as 6-OHDA-lesioned rats and MPTP-lesioned monkeys, administration of indirect dopamine agonists such as levodopa, dramatically ameliorates motor symptoms (Konitsiotis et al., 2000; Murer et al., 1998; Olanow et al., 2006). Furthermore, chronic treatment or acute high doses of indirect dopamine agonists such as levodopa can induce dyskinesias, which can be eliminated by either lowering the levodopa dose or pharmacologically reducing activity of SNc dopamine neurons (Obeso et al., 2002). In sum, the ultimate effect of dopamine release in the striatum, arising from SNc dopamine neurons is to facilitate movement and regulate motor patterns.

Chapter 2. Parkinson's Disease

Motor Symptoms and Neuropathology of Parkinson's Disease

Parkinson's disease is a neurological disorder affecting up to 3 percent of people aged 65 and over worldwide (Lang and Lozano, 1998; Zhang and Roman, 1993). Mean age of onset is now thought to be in the early-to-mid 60s, but has in some cases occurred as early as mid 40s (Inzelberg et al., 2002). Parkinson's disease is characterized primarily by motor symptoms that include bradykinesia (slowness in movement), tremor, rigidity, postural instability, and gait impairments, with nonmotor symptoms such as sleep disturbances and cognitive impairment appearing also (Jankovic, 2008). The principal pathology associated with Parkinson's disease is the degeneration of dopamine-containing neurons in the substantia nigra compacta (SNc), a critical component of the nigrostriatal dopamine system (Wolters and Francot, 1998). Degeneration of SNc dopamine neurons subsequently results in dopamine deficiencies within the caudate-putamen (striatum) of the forebrain (Lev et al., 2003). Reduced dopamine levels in the striatum disrupts the normal functioning of the basal ganglia-thalamocortical motor circuit, which plays a critical role in regulating motor activity (Knott et al., 1999; Lev et al., 2003; Wolters and Francot, 1998). Specifically, a reduction in striatal dopaminergic transmission, as in the parkinsonian condition, is thought to increase inhibitory output from the basal ganglia to the thalamus leading to a reduction in excitation of primary motor areas of the cortex, resulting in impaired motor functioning. Fig. 1 depicts changes in the overall activity of basal ganglia-thalamocortical motor circuit related to Parkinson's disease (modified from Galvan and Wichmann, 2008). In Parkinson's

disease, the degeneration of SNc dopamine neurons and their projections to the striatum is a slowly evolving process occurring over decades, a very heinous aspect of this disease. SNc projections to the areas of the striatum related to motor function degenerate earlier than projections to associative or limbic portions of the striatum; therefore, the motor symptoms of Parkinson's disease develop and are often detectable before the non-motor symptoms. Clinical motor symptoms are observed with at least 80% decrease in striatal dopamine content and at least 50% or greater loss of dopaminergic neurons in the SNc (Fearnley and Lees, 1991; Samii et al., 2004).

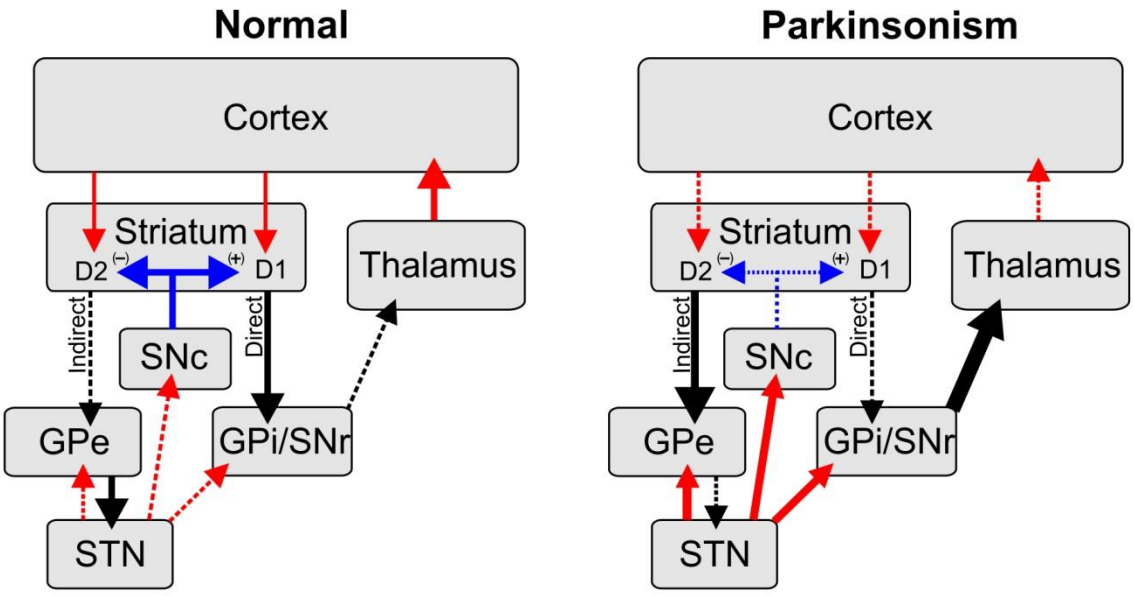


Fig. 1. Simplified depiction of Parkinsonism-related changes in overall activity of the thalamocortical basal ganglia motor circuitry. Blue arrows indicate dopaminergic projections. Red arrows indicate excitatory glutamatergic projections, and black arrows indicate inhibitory GABAergic projections. The thickness of the arrows corresponds to their presumed activity; such that the thicker lines indicate more activation, and the

dotted lines indicate less activation. GPe: globus pallidus externus; GPi: globus pallidus internus; SNc: substantia nigra compacta; SNr: substantia nigra reticulata; STN: subthalamic nucleus.

Evidence implicating nigrostriatal dysfunction in Parkinson's disease arises from a number of sources including postmortem brain analysis and functional imaging techniques. Post mortem analysis of Parkinsonian brains have demonstrated a marked degeneration of dopamine-containing cells in the SNc, as well as reduced expression of dopamine transporters and synaptic vesicle amine transporter gene expression (Fearnley and Lees, 1991; Knott et al., 1999; Zweig et al., 1989). Interestingly research also demonstrates significant reductions in neurons within the pedunculopontine tegmental nucleus (PPT), a hindbrain region which as discussed in Chapter 1 is known to critically contribute to the functioning of the nigrostriatal dopamine system via its glutamatergic and cholinergic projections to the SNc (Blaha and Winn, 1993; Chapman et al., 1997; Jellinger, 1988; Zweig et al., 1989). Additionally, significant loss of neurons within the PPT has also been found to correlate with the extent of neuronal loss of dopamine cells in the SNc (Zweig et al., 1989). Structural magnetic resonance imaging (MRI) studies have noted decreased width of the SNc in Parkinson's patients (Duguid et al., 1986; Hutchinson and Raff, 2000), and as expected, volumetric MRI analysis of parkinsonian brains have shown diminished volumes in subcortical nuclei including the striatum (Lisanby et al., 1993; Oneill et al., 2002). Functional neuroimaging is mainly used experimentally but has become useful in clinical trials aimed at measuring the

progression of Parkinson's disease (Whone et al., 2003). As measured by positron emission tomography (PET), Parkinson's disease is characterized by decreased striatal 6-[^{18}F]-fluoro-L-dopa (F-DOPA) uptake (Vingerhoets et al., 1994), which is highly correlated with reduced dopamine cell counts measured in post mortem brains (Snow et al., 1993).

Animal Models of Parkinson's Disease

Animal models of Parkinson's disease have also yielded strong supporting evidence for a neuropathology of the nigrostriatal dopamine system in this disorder. Studies that have selectively lesioned components of the nigrostriatal dopamine system (e.g. SNc, striatum) through the application of specific neurotoxins, one of the most common being the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA), have reported close approximations of the extent of the neurodegeneration seen in Parkinson's disease (Cousins and Salamone, 1996; Deumans et al., 2002). At a behavioral level, 6-OHDA lesioned animals demonstrate motor abnormalities in skilled and fine movements, as well as deficits in locomotor activity. Furthermore, such animal models demonstrate that significant lesions of the nigrostriatal dopamine system that reduce dopamine striatal tissue content by approximately 80% produce motor difficulties akin with Parkinson's disease. Interestingly, excitotoxic lesions of the PPT have also been found to produce parkinsonian type postural deficits, hypokinesia and locomotor deficits in primates (Kojima et al., 1997; Pahapill and Lozano, 2000).

Another neurotoxin commonly used to mimic Parkinson's disease neuropathology in animals is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). During the early

1980's, a number of individuals unwittingly injected a potent pyridine derivative related to the non-opioid analgesic Demerol which was contaminated with MPTP by virtue of a sloppy synthesis and sold on the streets as "China White", a synthetic heroin (Ballard et al., 1985; Langston et al., 1983). Exposure to MPTP produced selective neurodegeneration of the nigrostriatal dopamine system resulting in the development of severe bradykinesia, postural deficits, and motor rigidity similar to that seen in Parkinson's disease (Langston et al., 1999; Snow et al., 2000). The serendipitous discovery of MPTP and its parkinsonian symptoms offered new avenues in researching Parkinson's disease, and new MPTP animal models of Parkinson's disease emerged. The MPTP primate model of human Parkinson's disease has also provided additional evidence that the nigrostriatal dopamine system is particularly important in the etiology of this disease. MPTP treated primates develop motor abnormalities closely resembling those seen in humans with Parkinson's disease, with deficits including bradykinesia, rigidity, postural abnormalities and postural tremor, and rest tremor in some primate species (Kanda et al., 2000; Maratos et al., 2001; Schapira, 2002). For this reason, MPTP administration in primates is considered the most predictive model for antiparkinsonian efficacy of novel drugs in humans (Gerlach and Riederer, 1996). In sum, animal models such as these suggest that interfering with neural areas that importantly influence nigrostriatal dopamine activity may contribute to the severe motor abnormalities associated with Parkinson's disease. Also, these animal models have been very useful in studying the therapeutic strategies for motor symptom treatment and potential neuroprotection (Betarbet et al., 2002; Schober, 2004; Terzioglu and Galter, 2008).

Etiology of Parkinson's Disease

The etiology for the vast majority of Parkinson's disease cases is largely unknown and thus classified as idiopathic. Controversy exists as to how much of the disease results from a strictly genetic cause, a purely environmental factor, or a combination of the two (Di Monte, 2003; Farrer, 2006). Thus, despite the overwhelming evidence implicating nigrostriatal dysfunction in Parkinson's disease, the precise cause for neuronal loss and deficient dopaminergic activity within the nigrostriatal dopamine system remains unclear. Several mechanisms have been proposed for the cell death associated with Parkinson's disease, including oxidative stress and excitotoxicity. Oxidative stress is an adverse effect that occurs when the generation of highly reactive free radicals exceeds the system's ability to neutralize and eliminate them, resulting in damage to the cellular membrane lipids, proteins, and DNA (for review see Simonian and Coyle, 1996). Post mortem brains of Parkinson's patients have shown increased amounts of free radical damage indicators, such as lipid peroxidation and oxidized DNA (Alam et al., 1997; Dexter et al., 1989). 6-OHDA is selectively taken up by dopaminergic neurons (and other catecholaminergic neurons near site of infusion) and causes oxidative stress and ultimately cell degeneration (Cohen and Heikkila, 1974). Oxidative stress is also thought to participate in MPTP-induced toxicity of dopamine neurons (Zang and Misra, 1993). For this reason, antioxidant approaches for neuroprotective therapies seem warranted and have shown preclinically to protect against MPTP toxicity and 6-OHDA lesioning in animal models. It is important to note however, clinical trials assessing the effectiveness of antioxidants as neuroprotective agents for Parkinson's disease have been inconclusive, with transient results at best (Alexi et al., 2000).

It has also been proposed that the neurodegeneration associated with Parkinson's disease may result from increased glutamatergic transmission in the SNc, most likely due to overactivity and burst firing of STN neurons (Johnson et al., 2009). Glutamate receptors, specifically N-methyl-D-aspartic acid (NMDA) receptors, are known to mediate excitotoxicity caused by high levels of glutamate. Therefore, activation of these receptors in the SNc may contribute to the degeneration of dopamine neurons in this region (Waxman and Lynch, 2005). In support of this argument, NMDA antagonists have been noted to reduce or delay SNc degeneration and motor deficits caused by MPTP administration or 6-OHDA lesioning (Johnson et al., 2009). These results support the hypothesis that NMDA receptor activation contributes to neurodegeneration in Parkinson's disease and suggest that blockade of NMDA receptors may be a useful strategy for slowing disease progression. However, the widespread expression and diverse functional roles of NMDA receptors raise concern that targeting these receptors would lead to serious unwanted side effects. Clinical studies have therefore used weak NMDA receptor antagonists and have generally failed to find any therapeutic benefit when administered alone (without levodopa) (Johnson et al., 2009). More promising studies suggest that selectively targeting NMDA receptor subtypes specific to regions relevant to Parkinson's disease pathophysiology may represent safer neuroprotective options (Jin et al., 1997). As such, further clinical studies using more selective drugs targeting NMDA receptors are needed. In sum, the specific factors that contribute to or initiate overly active NMDA mechanisms in excitotoxicity are poorly understood, and the potential contribution of other types of glutamate receptors to the development and progression of Parkinson's disease symptoms remains unclear.

Treatments for the Motor Symptoms of Parkinson's Disease

The most effective pharmaceuticals for treating the motor symptoms of Parkinson's disease are drugs that restore dopaminergic function in the striatum, with the most commonly prescribed being the dopamine precursor levodopa (Olanow et al., 2004). Levodopa is usually combined with carbidopa (Lodosyn) or benserazide (Serazide) as Sinemet or Prolopa, respectively, to prevent peripheral conversion of levodopa to dopamine by dopa-decarboxylase (Olanow et al., 2004). Dopamine synthesized from levodopa activates both D1 and D2 receptors in the striatum, which is important therapeutically as antiparkinsonian drugs with high D2 and low D1 affinity have been shown to be less effective in reversing motor symptoms compared to levodopa (Wooten, 2001). Conjoint use of levodopa with drugs that inhibit dopamine-degrading enzymes (e.g. monoamine oxidase inhibitors) within surviving dopamine nerve terminals have further been shown to enhance the therapeutic effects of levodopa alone, presumably by slowing the metabolic breakdown of dopamine, while the conjunctive use of dopaminergic agonists with levodopa has also proved therapeutically beneficial during later stages of Parkinson's disease (Hurtig, 1997). In fact, levodopa has been used to distinguish Parkinson's disease from other conditions that may resemble Parkinson's disease, a true testament to the reliability of levodopa for treating the motor symptoms of Parkinson's disease. Reduced motor symptoms following a single administration of levodopa can help to confirm the diagnosis of Parkinson's disease, and a negative response is thought to be an indication for alternative diagnosis (D'Costa et al., 1995).

While levodopa treatment provides relief of motor symptoms for several years in most patients, complications occur with long-term use. As dopaminergic neurons

continue to deteriorate, the levodopa dose is effective for a shorter time, and the patient experiences "wearing off" sooner. Motor fluctuations can also become unpredictable with sudden switches between good therapeutic response (i.e. mobility) and no therapeutic response (i.e. immobility), referred to as the "on-off" phenomenon (Marsden and Parkes, 1976). Also, increased doses of levodopa leads to abnormal involuntary movements (e.g. dyskinesia and leg dystonia), which can be lessened by reduction of the dose, but the dose decrease then generally leads to loss of control of the disease. These motor complications have an incidence of 10% per year, so that after taking levodopa 5 years roughly 50% of patients experience these detrimental side effects (Rajput et al., 1984; Rascol et al., 2000). Patients therefore become increasingly disabled even with treatment, which is a particular problem given that levodopa remains the most effective treatment for Parkinson's disease despite these serious drawbacks. Thus, novel pharmaceuticals as well as interventive neurosurgical treatments, such as deep brain stimulation, are continuously being explored and refined for better management of the motor symptoms associated with Parkinson's disease. Advances in our understanding of the connectivity and function of the basal ganglia circuitry will continue to open the door for novel therapeutic strategies.

Chapter 3. Deep Brain Stimulation as a Treatment for Parkinson's Disease

Parkinson's disease is a neurological disorder characterized by a progressive degeneration of the dopamine neurons in the substantia nigra compacta (SNc) and a subsequent reduction in striatal dopamine levels (Obeso et al., 2002). Parkinson's disease treatments attempt to alleviate symptoms by restoring dopamine transmission in the striatum (Clarke, 2004). Although oral administration of the dopamine precursor levodopa, the most commonly prescribed pharmaceutical for ameliorating the motor symptoms of Parkinson's disease, is highly effective for several years in most patients, as the disease progresses with time chronic levodopa treatment is associated with the development of complications, such as motor fluctuations and dyskinesias, which can be just as problematic as the disease itself (Marsden and Parkes, 1976; Rascol et al., 2000). When patients reach this stage, interventive neurosurgery such as deep brain stimulation (DBS) is an option to consider. Because of the limitations of the current available pharmaceutical treatments and the efficacy and favorable safety profile of DBS, this interventive neurosurgical treatment approach is now approved by the US Food and Drug Administration and is routine in clinical use for treatment of Parkinson's disease (Krack et al., 2003). The application and substantial progress of functional neurosurgery rank amongst the most significant of therapeutic advances in Parkinson's disease, perhaps second only to the introduction of levodopa.

DBS involves implanting electrodes with four contacts into the target area of the brain and connecting it to an implanted pulse generator usually located in the chest area, much like a pacemaker for the heart. Conventional DBS systems use a relatively high-

frequency (100-250 Hz) pulse train applied continuously at the site of electrode implantation (McIntyre et al., 2006). One key aspect permitting reliable therapeutic benefits of this procedure is that stimulation parameters provided by the implanted pulse generator can be adjusted postoperatively to improve efficacy, reduce side-effects, and adapt to the course of the disease. Results from clinical trials have repeatedly shown that DBS plus medical therapy improves patient quality of life as well as clinical scores on the Unified Parkinson's Disease Rating Scale more than the best medical therapy alone (Deuschl et al., 2006; Weaver et al., 2009; Williams et al., 2010). The most common target for DBS in Parkinson's disease is the subthalamic nucleus (STN) as this ameliorates the cardinal symptoms of the disease (i.e. bradykinesia, rigidity, and tremor) while at the same time reducing medication needs for the patient (Limousin et al., 1998; Molineuvo et al., 2000; Moro et al., 1999; Volkmann et al., 2001).

Hypotheses of the Mechanism of Action of DBS

Despite the acceptance of DBS as a well qualified therapeutic tool for treating the motor symptoms of Parkinson's disease, the mechanism of action of DBS remains poorly understood and debated in research. Because the therapeutic effects of DBS are similar to those of a lesion of targeted nuclei, whether it is the STN (for Parkinson's disease), globus pallidus interna (for generalized dystonia), or ventrointermedial nucleus of the thalamus (for essential tremor), DBS has been thought to silence neurons at the site of stimulation (Benazzouz et al., 1995; Lozano et al., 2002). However, emerging evidence is beginning to discredit neuronal silencing hypothesis and, as such, implicates additional mechanisms of DBS, which involve activation of local neuronal terminals at the site of

stimulation that inhibit and/or excite efferent outputs. In turn, this has been postulated to enhance efferent neurotransmission, which may ultimately normalize activity within structures of the basal ganglia complex (see Benabid, 2003; Lozano and Eltahawy, 2004; McIntyre et al., 2004; Uc and Follett, 2007). Specifically, recent studies have shown that DBS results in excitation and altered firing patterns of neurons in the STN (Carlson et al., 2010; Garcia et al, 2003; Lee et al., 2007), increased activity in dopaminergic neurons of the SNc (Lee et al., 2003, 2004), as well as enhanced dopamine release in the striatum (Lee et al., 2006). These findings have lead to the "dopamine release" hypothesis which proposes that STN DBS improves motor symptoms related to Parkinson's disease by activating surviving nigrostriatal dopaminergic neurons and subsequent increases in striatal dopamine release (Lee et al., 2009). Several studies using *in vivo* microdialysis have shown that STN DBS increases dopamine metabolites DOPAC and HVA in the striatum of normal and 6-hydroxydopamine (6-OHDA) lesioned rats (Meissner et al., 2001, 2002, 2003; Paul et al., 2000). Furthermore, DBS of the STN decreases or eliminates the need for levodopa (Molineuvo et al., 2000; Moro et al., 1999) and is most effective in patients who have responded well to levodopa (Breit et al.,2004), suggesting that effective DBS requires endogenous dopamine production. Also consistent with the notion that STN DBS activates surviving nigrostriatal dopamine neurons are clinical observations that DBS can generate dyskinesias resembling those seen when excess levodopa is given (Frank et al., 2007).

While supporting evidence from basic research is available, the hypothesis that DBS of the STN contributes to symptom relief in Parkinson's disease due to an evoked increase in striatal dopamine release remains controversial. Two major techniques in

basic research studies have provided the majority of findings that oppose the dopamine release hypothesis. First, most basic studies using *in vivo* microdialysis do not report an increase in striatal dopamine release during stimulation of the STN in intact or 6-OHDA lesioned animals (Bruet et al., 2001; Meissner et al., 2001; Paul et al., 2000). Although *in vivo* monitoring of slow (min-hrs) changes in dopamine release is easily accomplished using conventional microdialysis methods, analysis of more rapid changes in dopamine release that may result from STN DBS requires an equally rapid "real-time" detection and monitoring system. Second, several positron emission tomography (PET) studies using [11C]-raclopride binding to measure dopamine release have failed to demonstrate significant raclopride displacement despite improvements in motor performance following STN DBS (Abosch et al., 2003; Hilker et al., 2003; Strafella et al., 2003). However, PET scanning with raclopride has relatively poor temporal resolution and requires an increase of greater than 90% of baseline measures in order to detect a change in dopamine receptor populations (Hilker et al., 2003; Volkow et al., 1993). Thus, it seems likely that inconsistencies in the literature may be due to technical difficulties in measuring striatal dopamine release. Whether STN DBS improves Parkinson's disease motor symptoms via increased release of dopamine in the striatum is the overall focus connecting the experimental studies in Chapters 5 and 6. For this reason, in order to avoid the issues of temporal resolution and sensitivity seen in other techniques, these experimental studies involve real-time monitoring of striatal dopamine release following electrical stimulation using fixed potential amperometry (FPA), which offers the highest temporal resolution and sensitivity to monitor changes in dopamine release evoked by

electrical stimulation of all *in vivo* neurochemical recording methods to date (Venton et al., 2002).

Candidate Pathways Mediating DBS-evoked Striatal Dopamine Release

There are several neuronal pathways by which STN DBS could elicit dopamine release in the striatum. First, stimulation of the glutamatergic input that projects from the STN to the SNc has been shown to activate nigrostriatal dopaminergic pathways directly (Meltzer et al., 1997). Second, stimulation of the glutamatergic neurons of the STN projecting to the pedunculopontine tegmental nucleus (PPT) indirectly activates nigrostriatal dopamine neurons via reciprocal excitatory innervation back to the STN which leads to subsequent SNc activation by the aforementioned glutamatergic inputs (Lee et al., 2000) and by activating excitatory cholinergic and glutamatergic inputs from the PPT to the SNc (Blaha and Winn, 1993; Forster and Blaha, 2003). As is mentioned in Chapter 1 and expanded upon in Chapter 5, the interconnectivity between the PPT and nuclei within the basal ganglia potentially provides the PPT an interesting position in which to mediate nigrostriatal dopamine activity (Mena-Segovia et al., 2008).

The improvement in motor symptoms in Parkinson's patients has been correlated with the location and electrical intensity of chronic stimulation (Garcia et al., 2005), and therapeutic outcomes of DBS have suggested that the best improvement in symptoms is obtained when stimulating the white matter corresponding to myelinated axons of passage in the region of the zona incerta just immediately dorsal to the STN (Saint-Cyr et al., 2002; Voges et al., 2002). DBS in this region likely results in stimulation of dopaminergic axons within the medial forebrain bundle (MFB) projecting from the SNc

to the striatum. Thus, thirdly, DBS of the STN may be activating surviving nigrostriatal dopamine neurons in Parkinson's patients via direct stimulation of the MFB. In fact, it has been postulated that DBS of the MFB may be superior to DBS of the STN in enhancing dopamine release in the striatum (Lee et al., 2006). These candidate neural pathways are expanded upon and investigated systematically in the experimental studies in Chapters 5 and 6. Understanding the underlying mechanisms of DBS and the neural pathways affected could lead to improvements in stimulation locations and parameters, which may prove invaluable in improving DBS interventive neurosurgical procedures and enhancing the clinical efficacy for the treatment of Parkinson's disease.

Chapter 4. Fixed Potential Amperometry Methodology

The experimental studies in Chapters 5 and 6 utilize *in vivo* fixed potential amperometry (FPA), an electrochemical method that has been valuable in elucidating the modulation of forebrain dopamine activity by other neurotransmitter systems (Blaha et al., 1996, 1997; Floresco et al., 1998; Lester et al., 2008). Like many i*n vivo* amperometric recording techniques, FPA uses a three-electrode configuration that incorporates an auxiliary electrode (typically platinum, chrom-alloy or stainless-steel wire), reference electrode (normally silver/silver chloride) and recording electrode (see Fig. 1) (Blaha and Phillips, 1996). In the experiments of Chapters 5 and 6, the procedure specifically involves the implantation of a carbon fiber recording electrode and the placement of a silver/silver chloride reference and stainless-steel auxiliary electrode combination in contact with brain tissue. An electrometer and analog to digital chart recorder (EA162 Picostat and ED401 e-corder 401, eDAQ Inc., Colorado Springs, CO, USA; simply referred to as the electrometer in all other chapters) creates a circuit between the three electrodes, allowing the application of a fixed continuous potential (+0.8 V) to the recording electrode via the auxiliary electrode, while maintaining a potential difference between the recording and reference electrode (Blaha and Phillips, 1992). Continuously applying a potential to the recording electrode allows dopamine to be continuously oxidized at the electrode surface. As such, FPA allows a high temporal resolution (10,000 samples/sec or higher dependent on the analog to digital converter of the recording device) for the analysis of dopamine neurotransmission *in vivo*. Pharmacological studies have validated the selectivity of FPA as a measurement of electrically-stimulated dopamine efflux *in vivo* (Dugast et al., 1994;

Forster and Blaha, 2003; Lee et al., 2006). For example, this has been demonstrated by significant increases in laterodorsal tegmentum stimulation-evoked oxidation current in the nucleus accumbens and subthalamic nucleus stimulation-evoked oxidation current in the striatum of rats in response to systemic administration of the dopamine reuptake inhibitor nomifensine, but not following serotonin or norepinephrine reuptake blockade with fluoxetine or desipramine (Lee et al., 2006). Consequently, this *in vivo* technique has been utilized commonly to evaluate the kinetics of stimulation-evoked dopamine release and reuptake and drug-induced changes in the magnitude and temporal pattern of dopamine neurotransmission, as well as the biochemical basis of dopaminergic cell burst firing in anesthetized rats and mice (Benoit-Marand et al., 2000; Dugast et al., 1994; Forster and Blaha, 2003; Lee et al., 2006; Lester et al., 2008, 2010; Suaud-Chagny et al., 1995).

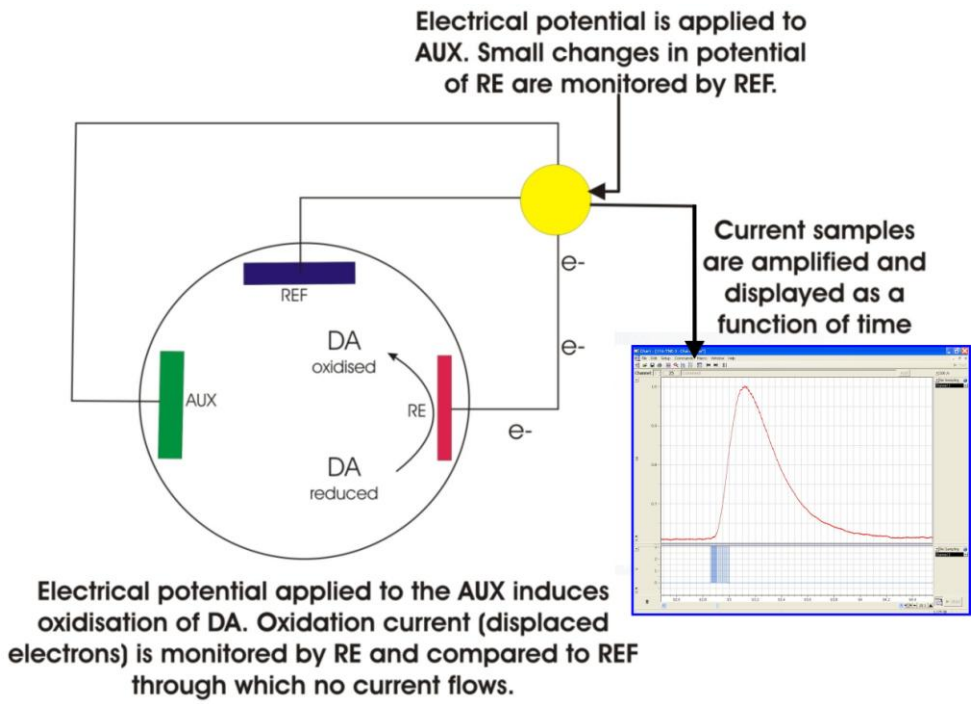


Fig. 1. Simplified diagram of a three-electrode system used in amperometric electrochemical recordings. The electrometer applies an electrical potential to the auxiliary (AUX) electrode that is suitable to oxidize dopamine (DA) at the surface of the recording electrode (RE), which is held constant relative to the reference electrode (REF). Oxidized dopamine molecules transfer electrons to the RE which are measured as current flow via the electrometer (EA162 Picostat) and passed as an analog signal to the analog to digital chart recorder and (ED401 e-corder 401) where it is converted to a digital signal for display via Chart software on a computer monitor in near real time. Adapted from Blaha and Phillips (1996).

Electrochemical Recording Electrodes Used in FPA

Polyacrylic nitrile, pitch, or pyrolytic-based carbon provides an electrochemically inert surface covered with oxygen-containing functional groups that facilitate electron transfer from compounds undergoing oxidation or reduction at the electrode surface (Kawagoe et al., 1993). Carbon fiber microelectrodes to record stimulation-evoked dopamine release *in vivo* were fashioned by threading a single carbon fiber (10 μm o.d.) through a borosilicate glass capillary tube. The tube was then heat-pulled to form a tip through which the carbon fiber protruded. Carbon paste was packed into the bore of the electrode and a wire inserted to make contact with the fiber. The wire was secured in place with a carbon paste (super glue mixed with carbon powder) (see Fig. 2). The protruding carbon fibers were cut under a stereomicroscope so that the active recording electrode surface was approximately 250 μm long. A new carbon fiber recording electrode was used in each animal. As a consequence of its small size, the carbon fiber recording electrode results in minimal tissue damage at the site of insertion and allows for high degree of local specificity for assessment of regional differences in neurochemical efflux (Stamford, 1989). Moreover, the small size enables faster sampling of the dopamine oxidation current as there is less depletion of the neurochemical at the electrode surface (Forster and Blaha, 2003; Kawagoe et al., 1993).

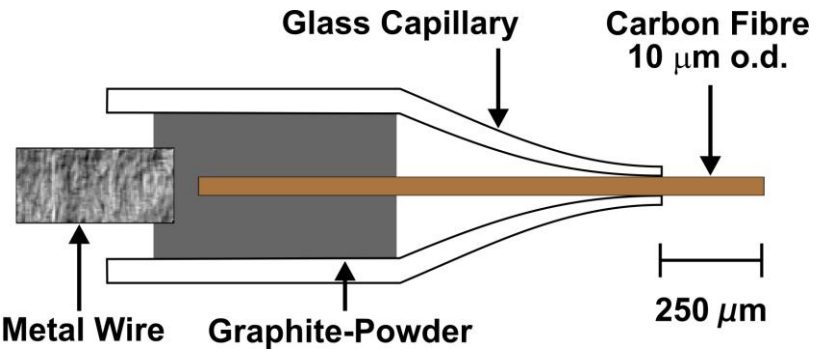


Fig. 2. Illustration of the carbon fiber recording electrode fabricated for use in fixed potential amperometry to monitor the oxidation of dopamine (corresponding to dopamine efflux) *in vivo*.

Stereotaxic Surgery and Recording Set-up for FPA

Anesthetized mice are mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) within a mouse head-holder adaptor (Stoelting, Wood Dale, IL, USA). Stereotaxic coordinates for each of the target sites, which include the subthalamic nucleus (STN), medial forbrain bundle (MFB), pedunculopontine tegmental nucleus (PPT), substantia nigra compacta (SNc), and striatum in Chapters 5 and 6, are determined from the mouse atlas of Paxinos and Franklin (2001). As shown in Fig. 3, in each mouse a concentric bipolar stimulating electrode (SNE-100; Rhodes Medical Co., CA, USA), a 31 g stainless-steel guide infusion cannula, and a carbon fiber recording microelectrode is implanted into the desired brain sites. A silver/silver chloride reference and stainless-steel auxiliary electrode combination is also placed on contralateral cortical tissue.

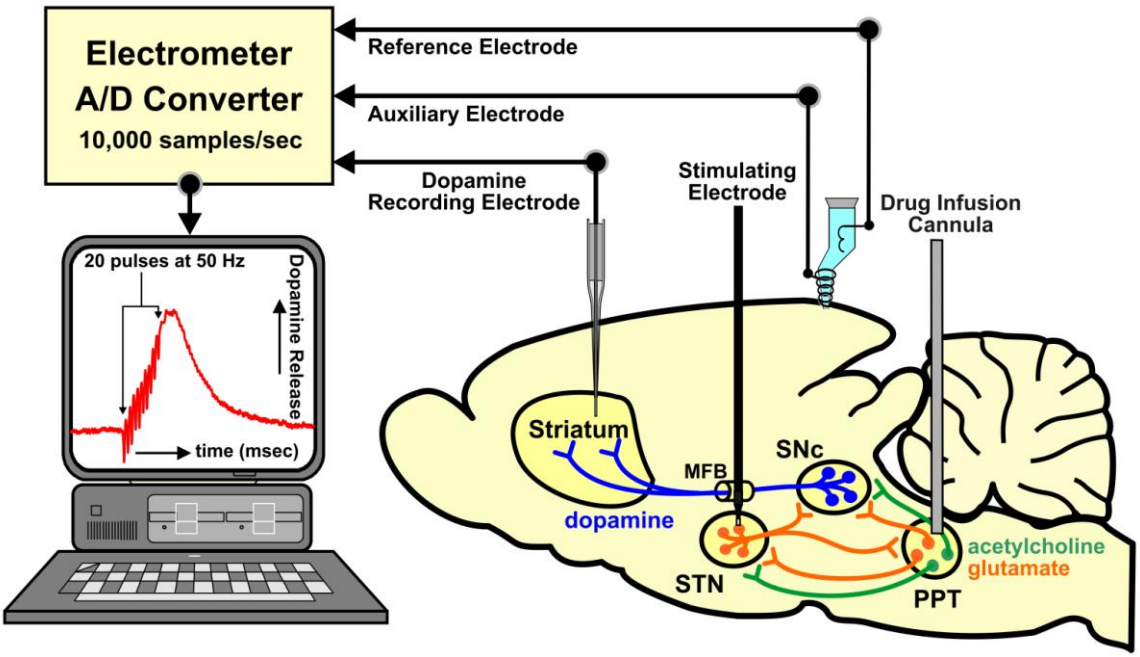


Fig. 3. Schematic diagram of the mouse brain illustrating a typical setup for *in vivo* fixed potential amperometric recording of stimulation-evoked striatal dopamine release. In the experiments in Chapters 5 and 6, a carbon fiber recording electrode is positioned in the striatum. A stimulating electrode is positioned in the dorsal portion of the medial forebrain bundle (MFB), subthalamic nucleus (STN), or pedunculopontine tegmental nucleus (PPT). A silver/silver chloride reference and stainless-steel auxiliary electrode combination is placed in contact with contralateral cortical tissue, and a drug infusion cannula is implanted into the PPT, STN, or substantia nigra compacta (SNc).

Amperometric recordings are made within a Faraday cage to increase the signal to noise ratio (Forster and Blaha, 2003). The stimulation site varies (MFB, STN, or PPT) to accommodate the aim of each project, but the stimulation protocol consists of twenty 0.5 msec duration pulses at 50 Hz delivered every 30 sec over a 1 hour testing period,

delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). With the addition of microinfusions of drugs, FPA lends itself to the investigation of the role of receptor subtypes in stimulation-evoked phasic dopamine efflux. Intracerebral infusions of the local anesthetic lidocaine is an effective means of temporarily blocking all axonal transmission to or from specific areas and pathways with recovery approximately 10 min post-infusion (Blaha et al., 1997; Floresco et al., 1998). This drug procedure offers a unique means to determine the functional neuroconnectivity of DBS-mediated striatal dopamine release and the relationship between DBS target sites, such as the MFB, STN, or PPT. By temporarily blocking transmission through one of these sites, it is possible to determine whether DBS of these structures evokes striatal dopamine transmission via direct or indirect routes to the SNc. Furthermore, infusions of specific receptor antagonists help determine the neurochemical nature of the neuronal pathways involved in mediating stimulation-evoked striatal dopamine release. To confirm that the observed drug effects are not attributable to non-specific effects of the microinfusion procedure, microinfusions of sterile phosphate-buffered saline (PBS, pH~7.4) served as a control.

Post-Experiment Procedures

Upon the completion of each FPA session, the stimulation, recording, and infusion sites are marked, either by lesioning or stain infusion. After euthanasia, the brains are removed and properly stored until sectioning. Coronal sections are sliced in a cryostat and observed under a light microscope to confirm that the placements of stimulating

electrodes, recording electrodes, and drug infusion cannulae are within the anatomical boundaries of the target site.

The mean change in dopamine oxidation current, corresponding to stimulation-evoked dopamine efflux, is converted to mean dopamine concentration (μM) by post-experiment *in vitro* calibration of the carbon fiber electrode in solutions of dopamine (2-10 μM) using a flow injection system (Michael and Wightman, 1999). For each animal, changes in stimulation-evoked dopamine concentration after infusion were expressed as mean percent changes with respect to pre-infusion baseline responses (100%) and are subsequently averaged across animals. The appropriate statistical tests are then performed, either comparing between experimental groups or before and after drug infusion.

Chapter 5. Neural Pathways Mediating Striatal Dopamine Release following High Frequency Stimulation: Relevance to DBS as a Treatment for Parkinson's Disease

Deep brain stimulation (DBS) is an established interventive neurosurgical approach for effectively treating the motor symptoms of Parkinson's disease (Benabid, 2003; Krack et al., 2003). The most common stimulation site for DBS as a treatment for Parkinson's disease is the subthalamic nucleus (STN); however, the pedunculopontine tegmental nucleus (PPT) is emerging as a therapeutically beneficial target when stimulated by itself or in combination with the STN (Stefani et al., 2007). Thus, the connectivity between these two nuclei and the basal ganglia is the focus of the present paper. Despite the acceptance of DBS as a therapeutic tool for treating parkinsonian motor symptoms, which onset with at least 80% decrease in striatal dopamine content and 50% or greater loss of dopaminergic neurons in the substantia nigra compacta (SNc) (Fearnley and Lees, 1991; Samii et al., 2004), the mechanism of action of DBS remains poorly understood and debated in research. Because the therapeutic effects of DBS are similar to those of a lesion, DBS has been thought to act by silencing neuronal activity at the site of stimulation (Benazzouz et al., 1995; Lozano et al., 2002). However, emerging evidence implicates additional mechanisms, which involve activation of local neuronal terminals at the site of DBS that inhibit and/or excite efferent outputs. In turn, this has been postulated to enhance efferent neurotransmission, which may ultimately normalize activity within structures of the basal ganglia complex (Benabid, 2003; Lozano and Eltahawy, 2004; McIntyre et al., 2004; Uc and Follett, 2007). Specifically, recent studies in rodents have shown that electrical stimulation of the STN results in excitation of

neurons in the STN (Garcia et al, 2003; Lee et al., 2007), increased activity in dopaminergic neurons of the SNc (Lee et al., 2003, 2004), as well as enhanced dopamine release in the striatum (Lee et al., 2006). These findings have led to the "dopamine release" hypothesis which proposes that STN DBS improves motor symptoms related to Parkinson's disease, in part, by activating surviving nigrostriatal dopamine neurons and subsequently increasing striatal dopamine release (Lee et al., 2009). DBS of the STN has been shown to decrease or eliminate the need for levodopa (Molineuvo et al., 2000; Moro et al., 1999) and is most effective in patients who have responded well to levodopa (Breit et al., 2004). Thus, clinical findings support the dopamine release hypothesis by suggesting endogenous dopamine production is required for DBS to be therapeutically successful. Furthermore, DBS can generate dyskinesias resembling those seen when excess levodopa is given (Frank et al., 2007).

STN DBS could elicit dopamine release in the striatum through activation of a number of neural pathways. First, stimulation of the glutamatergic neurons that project from the STN to the SNc have been shown to activate nigrostriatal dopaminergic neurons directly (Meltzer et al., 1997). Second, stimulation of glutamatergic neurons of the STN projecting to the PPT may indirectly activate nigrostriatal dopamine neurons via both reciprocal excitatory innervation back to the STN which leads to subsequent SNc activation by the aforementioned glutamatergic inputs (Lee et al., 2000) and/or by activating excitatory cholinergic and glutamatergic PPT neuronal projections to the SNc (Blaha and Winn, 1993; Forster and Blaha, 2003). Thirdly, DBS of the STN may activate nigrostriatal dopamine neurons via direct stimulation of the dorsal portion of the medial forebrain bundle (MFB) within the zona incereta. Therapeutic outcomes of DBS

have suggested that the best symptom improvements result when stimulating the white matter just dorsal to the STN (Saint-Cyr et al., 2002; Voges et al., 2002). DBS in this region likely results in stimulation of dopaminergic axons within the MFB passing directly from the SNc to the striatum. Thus, it is conceivable that DBS directly aimed at the MFB may be superior to STN DBS in enhancing striatal dopamine release (Lee et al., 2006). Therefore, the present study also conducted experiments designed to determine the neuronal pathways involved in evoking striatal dopamine release via stimulation of the MFB.

In regards to DBS of the PPT, the interconnectivity between the PPT and nuclei within the basal ganglia complex allows the PPT to play a critical role in the modulation of nigrostriatal dopaminergic activity (Forster and Blaha, 2003; Miller and Blaha, 2004; Mena-Segovia et al., 2008), which may explain the findings from recent clinical trials showing that DBS of the PPT is effective in ameliorating parkinsonian motor symptoms, particularly gait and postural disabilities (Stefani et al., 2007). Thus, the present study also investigated the relative influence of cholinergic and glutamatergic PPT projections in eliciting striatal dopamine release. Previous work from our lab using *in vivo* chronoamperometry has shown that PPT stimulation elicits striatal dopamine release, in which dopamine release could be blocked by intra-SNc infusions of nicotinic and muscarinic acetylcholine receptor (nAchR and mAchR, respectively), and ionotropic glutamate receptor (iGluR) antagonists (Forster and Blaha, 2003). However, in addition to direct excitatory inputs to SNc dopaminergic cells, as noted above, the PPT may also indirectly activate these dopaminergic cells via cholinergic and glutamatergic projections

to excitatory glutamatergic neurons in the STN that, in turn, innervate SNc dopaminergic cells (Bevan and Bolam, 1995; Lee et al., 2000).

To-date no studies have systematically examined these candidate pathways as to their relative involvement in mediating the effects of MFB, STN, or PPT DBS on striatal dopamine release. Therefore, the present studies investigated these potential pathways *in vivo* using fixed potential amperometry (FPA) with carbon fiber microelectrodes positioned in the striatum to record striatal dopamine efflux evoked by DBS-like high frequency electrical stimulation (HFS) of the MFB, STN, or PPT. By temporarily blocking transmission through various nuclei via microinfusions of the local anaesthetic lidocaine, we were able to determine whether HFS of the MFB, STN, or PPT evokes striatal dopamine transmission via direct or indirect routes to the SNc and their relative importance in mediating these effects. Furthermore, infusions of specific receptor antagonists helped to uncover the neurochemical nature of the pathways that mediate MFB, STN, and PPT stimulation-evoked striatal dopamine release.

Experimental Procedures

The following experiments were approved by the Institutional Animal Care and Use Committee at the University of Memphis and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize animal pain and discomfort.

Animals and surgery

Seventy-six male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), 8-11 weeks of age and weighing 20-27 g at the time of surgery, were used. Animals were housed five per cage in a temperature controlled environment (21 ± 1ºC) with a 12 h light: 12 h dark cycle (lights on at 0600 h). Food and water were available *ad libitum*. Mice were anesthetized with urethane (1.5 g/kg, i.p.) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) within a mouse head-holder adaptor (Stoelting, Wood Dale, IL, USA), ensuring the skull was flat. Body temperature was maintained at 36 ± 0.5°C with a temperature-regulated heating pad (TC-1000; CWE Inc., New York, NY, USA). Determined from the mouse atlas of Paxinos and Franklin (2001), stereotaxic coordinates (AP from bregma, ML from midline, and DV from dura, all in mm) for each target site were as follows: striatum: AP +1.4, ML +1.4, DV -2.5; MFB: AP -2.0, ML +1.1, DV -4.0; STN: AP -2.0, ML +1.6, DV -4.0; SNc: AP -3.1, ML +1.5, DV -3.8; PPT: AP -4.7, ML +1.25, DV -2.7). In each mouse a concentric bipolar stimulating electrode (SNE-100; Rhodes Medical Co., CA, USA) was implanted into the left MFB, STN, or PPT of each mouse. A 31 g stainless-steel guide infusion cannula was implanted into the left SNc, STN, or PPT with the tip of the guide cannula positioned 2 mm above site. An Ag/AgCl reference and stainless-steel auxiliary electrode combination was placed in surface contact with contralateral cortical tissue approximately 2.0 mm posterior to bregma, and a carbon fiber recording microelectrode with an active recording surface of 250 μm (length) by 10 μm (o.d.) (Thornel Type P, Union Carbide, Pittsburgh, PA, USA) was then implanted into the left striatum.

FPA and electrical stimulation

All amperometric recordings were made within a Faraday cage to increase the signal to noise ratio (Forster and Blaha, 2003). A fixed potential (+0.8 V) was applied to the recording electrode, and oxidation current was monitored continuously (10K samples/sec) with an electrometer filtered at 50 Hz. Approximately 10 min following implantation of the recording electrode, a series of 0.5 ms duration cathodal monophasic current pulses (20 pulses at 50 Hz applied every 30 sec over a 1 hr recording period) was delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). Intensity levels were set at 800 μAmps in the MFB and PPT and 400 μAmps in the STN as determined by preliminary studies to be optimal for each target site. Intensity levels were also lower in the STN in an attempt to conclusively limit the stimulation region to the STN. FPA coupled with carbon fiber microelectrodes has been confirmed as a valid technique for real-time monitoring of changes in striatal dopamine oxidation current evoked by brief electrical stimulation of afferent inputs to midbrain dopamine neurons (Dugast et al., 1994; Forster and Blaha, 2003; Lester et al., 2008).

Drug microinfusions

Microinfusions were performed by first backloading the drug into a fibreglass cannula (80 μm o.d., Polymicro Tech. Inc., AZ, USA) connected via PE10 tubing to a 5 μl microsyringe (Scientific Glass Engineering Inc., Austin, TX, USA) mounted on a microinfusion pump (Stoelting, Wood Dale, IL, USA). After a 10 min electrical stimulation baseline recording period, the cannula was inserted 2 mm beyond the tip of

the implanted guide cannula, and 1.0 µl infusions were made over a 1.5 min period. Infusions of the local anesthetic lidocaine (4%) were performed to temporarily block axonal transmission to or from the infusion sites (Blaha et al., 1997; Floresco et al., 1998). By temporarily blocking transmission through the SNc, STN, or PPT, we were able to determine whether HFS of the MFB, STN, or PPT evokes striatal dopamine transmission via direct or indirect routes to the SNc. Furthermore, in order to assess the relative contributions of GluRs and AchRs in the SNc and STN in mediating stimulation-evoked striatal dopamine release, MFB, STN, or PPT-evoked striatal dopamine release was monitored in separate groups of mice before and during intra-SNc or STN infusions of the iGluR antagonist kynurenate (1 µg), the metabotropic GluR (mGluR) antagonist (+)-methyl-4-carboxyphenylglycine (MCPG; 1 µg), the mAchR antagonist scopolamine (10 µg), the nAchR antagonist mecamylamine (1 µg), or a combination of these drugs. To confirm that observed drug effects were not attributable to non-specific effects of the microinfusion procedure, microinfusions of 1.0 µl of sterile phosphate-buffered saline (PBS, pH~7.4) were also performed. See Table 1 for the complete list of experimental groups. The microinfusion cannula was left in place over the duration of the experiment. All drugs were prepared immediately before use at doses determined by preliminary studies in this laboratory.

Table 1. Drugs used to assess the contributions of cholinergic and glutamatergic projections on stimulation-evoked changes in striatal dopamine concentrations.

Stimulation Site	Infusion Site	Drug	Target receptors	% Change from baseline
MFB	SNc	PBS	–	- 2% ± 1
MFB	SNc	Lidocaine	axonal	+ 62% ± 12
MFB	SNc	Kynurenate	iGluRs	- 3% ± 4
MFB	PPT	PBS	–	- 3% ± 1
MFB	PPT	Lidocaine	axonal	- 4% ± 4
STN	SNc	PBS	–	- 4% ± 2
STN	SNc	Lidocaine	axonal	- 84% ± 3
STN	PPT	PBS	–	- 1% ± 3
STN	PPT	Lidocaine	axonal	- 46% ± 6
STN	SNc	Kynurenate	iGluRs	- 46% ± 4
STN	SNc	MCPG	mGluRs	- 3% ± 1
STN	SNc	Scopolamine	mAchRs	- 28% ± 5
STN	SNc	Mecamylamine	nAchRs	- 28% ± 5
PPT	SNc	PBS	–	- 2% ± 2
PPT	SNc	Lidocaine	axonal	- 90% ± 3
PPT	STN	PBS	–	- 5% ± 3
PPT	STN	Lidocaine	axonal	- 50% ± 8
PPT	STN	Kynurenate + MCPG	iGluRs + mGluRs	- 23% ± 6
PPT	STN	Scopolamine + mecamylamine	mAchRs + nAchRs	- 26% ± 3

Data collation and statistical analysis

MFB, STN, or PPT stimulation-evoked dopamine efflux were quantified by extraction of data points occurring within the range of 0.25 sec pre- and 2.0 sec post-stimulation from the recorded oxidation current in the striatum at 30 sec intervals over the course of the recording period. The mean change in dopamine oxidation current, corresponding to stimulation-evoked dopamine efflux, was converted to a mean dopamine concentration (μM) by post-experiment *in vitro* calibration of the carbon fiber electrode in solutions of dopamine (2-10 μM) using a flow injection system (Michael and Wightman, 1999). For each animal, changes in stimulation-evoked dopamine concentration after infusion were expressed as mean percent changes with respect to pre-infusion baseline responses (100%). Mean peak levels in dopamine concentration following the infusion were statistically compared to pre-infusion baseline responses using paired two-tailed t-tests with the alpha level set at 0.05.

Histology

Upon the completion of each experimental session, an iron deposit was made in the stimulation and recording site by passing direct anodic current (100 μA and 1 mA, respectively) for 10 sec through the stimulating and recording electrodes, and 1.0 μl cresyl violet stain was infused into the cannula site. Mice were then euthanized with a 0.25 ml intracardial injection of urethane (0.345 g/ml). Brains were removed, immersed overnight in 10% buffered formalin containing 0.1% potassium ferricyanide, and then stored in 30% sucrose/ 10% formalin solution until sectioning. After fixation, 30 μm coronal sections were sliced in a cryostat at -30°C, with a Prussian blue spot resulting

from a redox reaction of the ferricyanide marking the stimulation site. Placements of stimulating electrodes, recording electrodes, and drug infusion cannulae were determined under a light microscope and recorded on representative coronal diagrams (Paxinos and Franklin, 2001).

Chemicals

Urethane, lidocaine, kynurenate, MCPG, scopolamine, and mecamylamine were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All chemicals, with the exception of urethane (distilled water), were dissolved in sterile PBS (pH~7.4).

Results

Stereotaxic placements of infusion cannulae, recording and stimulating electrodes

Recording electrode placements (n = 56) were confined to the striatum (range in mm: 1.34 to 1.54 anterior to bregma, 1.30 to 1.60 lateral to midline, and 2.40 to 2.70 ventral to dura; Fig. 1A). Stimulating electrode tips (n = 20) were localized within the MFB (range in mm: 1.94 to 2.18 mm posterior to bregma, 1.00 to 1.20 mm lateral to midline, and 3.80 to 4.10 mm ventral to dura; Fig. 1B). Stimulating electrode tips (n = 28) and infusion cannula tip placements (n = 16) were accurately positioned within the STN (range in mm: 1.94 to 2.18 posterior to bregma, 1.50 to 1.70 lateral to midline, and 3.90 to 4.20 ventral to dura; Fig. 1B), and infusion cannula tip placements (n = 28) were localized within the SNc (range in mm: 2.92 to 3.16 posterior to bregma, 1.40 to 1.60 lateral to midline, and 3.60 to 3.90 ventral to dura; Fig. 1C). The tips of stimulating electrodes (n = 24) and infusion cannula (n = 8) were confined within the PPT (range in mm: 0.39 to 0.63

posterior to lambda, 1.15 to 1.40 lateral to midline, and 2.60 to 2.90 ventral to dura; Fig. 1D).

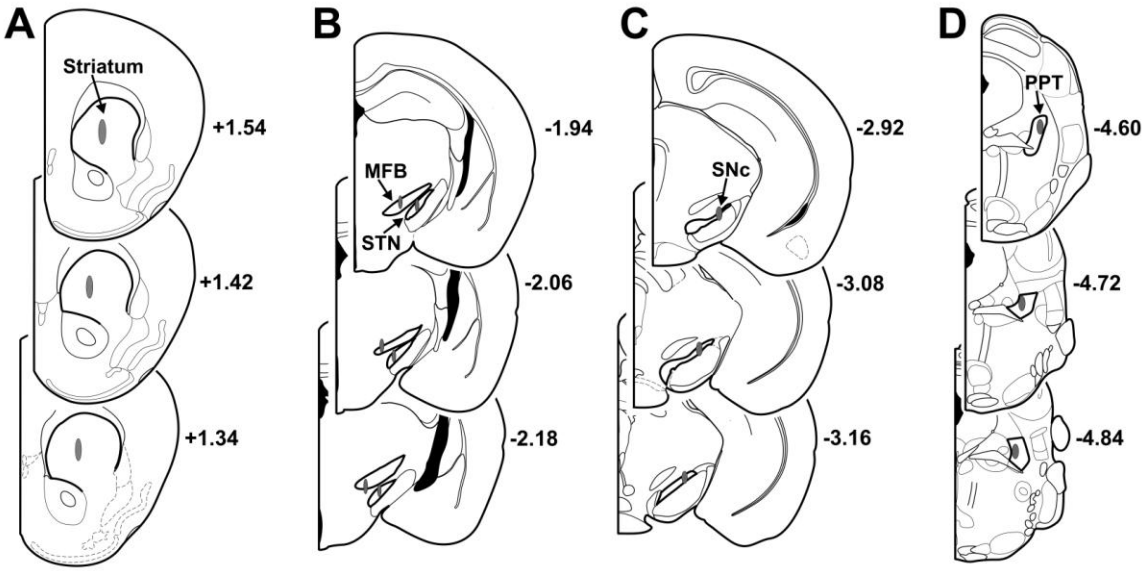


Fig. 1. Representative coronal sections of the mouse brain (adapted from the atlas of Paxinos and Franklin, 2001), with dark gray shaded areas indicating the placements of carbon fiber recording electrodes in the striatum (A), stimulating electrodes or drug infusion cannulae in the medial forebrain bundle (MFB) (B), subthalamic nucleus (STN) (B), substantia nigra compacta (SNc) (C), or pedunculopontine tegmental nucleus (PPT) (D). Numbers correspond to mm from bregma.

Effects of intra-SNc or PPT lidocaine or SNc GluR blockade on MFB stimulation-evoked dopamine efflux

With respect to pre-infusion baseline levels, MFB stimulation-evoked striatal dopamine efflux was not significantly altered at 5 min following PBS infusion into the SNc (n = 4; 98.1% ± 0.8, $p = 0.09$; Fig. 2A and C) or PPT (n = 4; 97.4% ± 1.1, $p = 0.10$; Fig. 2B and D). Intra-SNc infusion of lidocaine significantly increased MFB stimulation-evoked striatal dopamine levels from pre-infusion baseline levels (n = 4; 161.6% ± 12.4, $p = 0.03$; Fig. 2A and C) with the peak increase occurring 5 min post-infusion. However, lidocaine infused into the PPT did not significantly alter MFB stimulation-evoked dopamine efflux in the striatum assessed at 5 min following infusion (n = 4; 95.8% ± 3.9, $p = 0.35$; Fig. 2B and D). Infusion of the iGluR antagonist kynurenate into the SNc also had no significant affect on MFB stimulation-evoked striatal dopamine efflux assessed at 5 min following infusion (n = 4; 96.8% ± 3.9, $p = 0.472$; Fig. 2A and C).

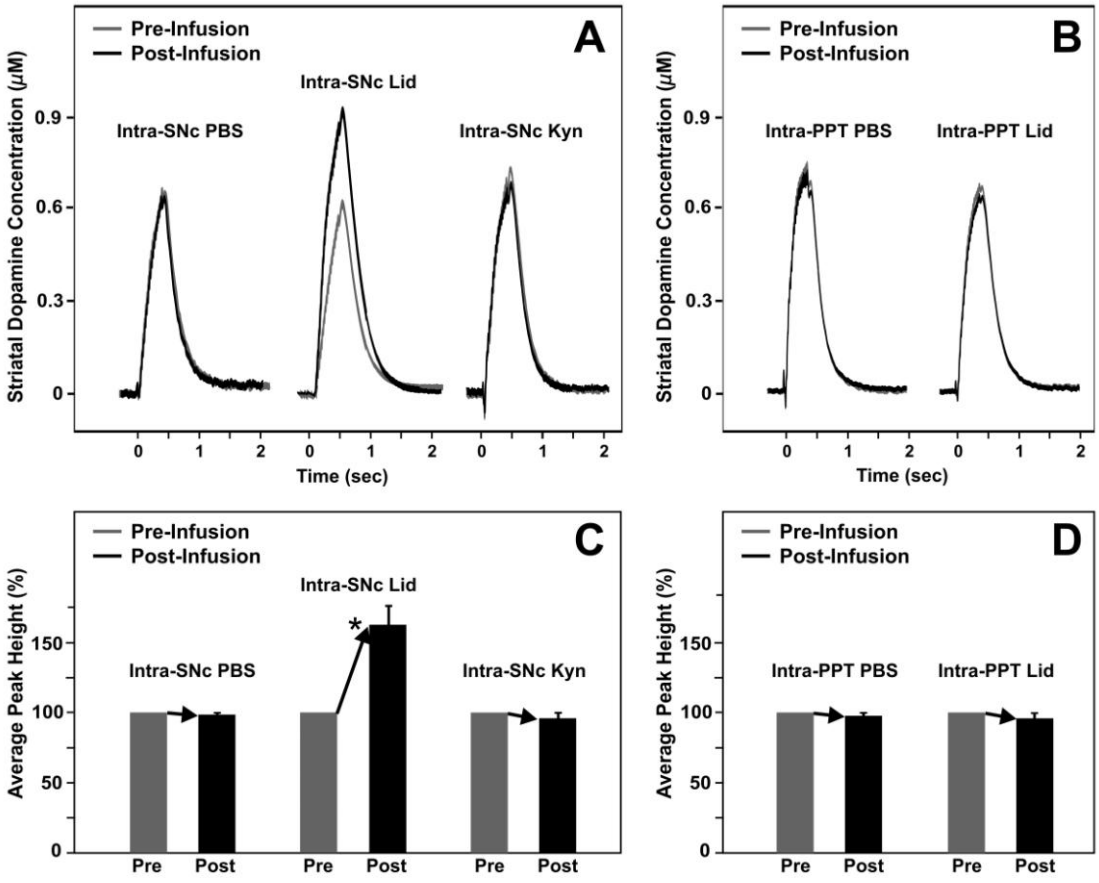


Fig. 2. Mean amperometric recordings of dopamine release in the striatum evoked by electrical stimulation of the dorsal portion of the medial forebrain bundle (A and B) and corresponding mean peak percentages (C and D). Profiles illustrate mean peak effects in response to substantia nigra compacta (SNc) (A) or pedunculopontine tegmental nucleus (PPT) (B) microinfusions of sterile phosphate-buffered saline (PBS, pH~7.4) or lidocaine (lid) or the ionotropic glutamate receptor antagonist kynurenate (kyn). Time zero indicates the start of the train of 20 pulses at 50 Hz. * Significant change in striatal dopamine concentration after infusion compared to pre-infusion baseline responses (100%).

Effects of intra-SNc or PPT lidocaine on STN stimulation-evoked dopamine efflux

With respect to pre-infusion baseline levels, STN stimulation-evoked striatal dopamine efflux was not significantly altered at 5 min following PBS infusion into the SNc (n = 4; 96.2% ± 1.7, $p = 0.11$; Fig. 3A and C) or PPT (n = 4; 99.5% ± 3.1, $p = 0.87$; Fig. 3B and D). STN stimulation-evoked striatal dopamine efflux was significantly attenuated by lidocaine (4%) infused into either the SNc (n = 4; 15.9% ± 3.9, $p < 0.01$; Fig. 3A and C) or the PPT (n = 4; 54.2% ± 6.4, $p = 0.02$; Fig. 3B and D) compared to pre-infusion baseline levels, with the peak decrease occurring 5 min post-infusion.

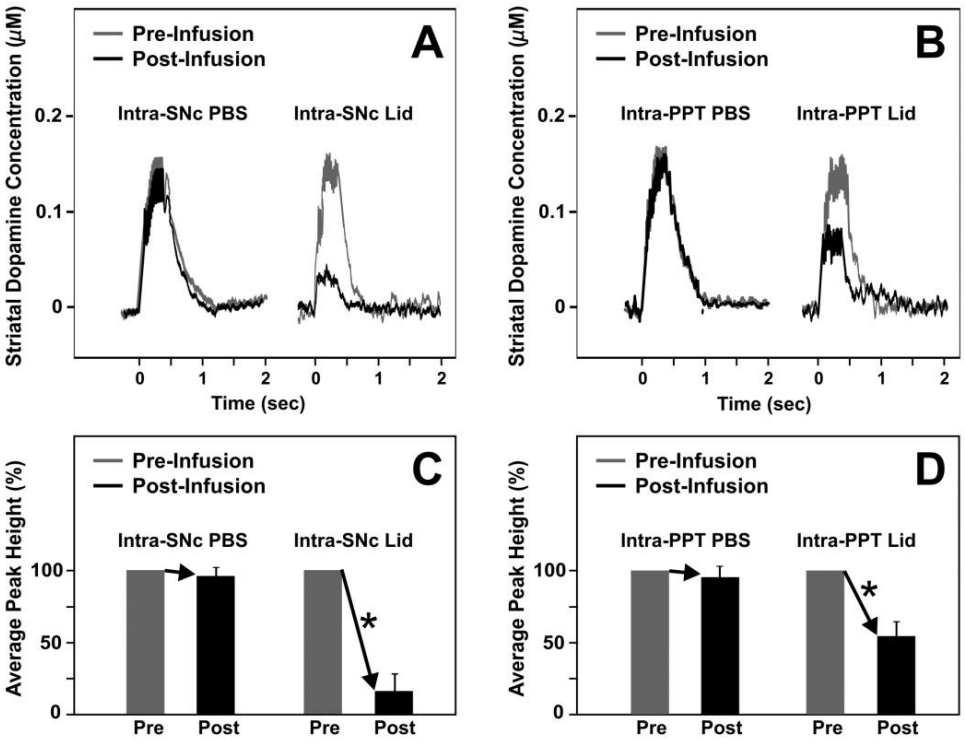


Fig. 3. Mean amperometric recordings of dopamine release in the striatum evoked by electrical stimulation of the subthalamic nucleus (A and B) and corresponding mean peak percentages (C and D). Profiles illustrate mean peak effects in response to substantia

nigra compacta (SNc) (A) or pedunculopontine tegmental nucleus (PPT) (B) microinfusions of sterile phosphate-buffered saline (PBS, pH~7.4) or lidocaine (lid). Time zero indicates the start of the train of 20 pulses at 50 Hz. * Significant change in striatal dopamine concentration after infusion compared to pre-infusion baseline responses (100%).

Effects of SNc GluR or AchR blockade on STN stimulation-evoked dopamine efflux

With respect to pre-infusion baseline levels, intra-SNc infusion of the iGluR antagonist kynurenate (1 µg) significantly attenuated STN stimulation-evoked striatal dopamine levels (n = 4; 54.1% ± 3.0, $p < 0.01$; Fig. 4A and B) with the peak decrease occurring 5 min post-infusion; however, intra-SNc infusion of the mGluR antagonist MCPG (1 µg) had no significant effect on STN stimulation-evoked striatal dopamine levels (n = 4; 97.1% ± 1.1, $p = 0.09$; Fig. 4A and B). STN stimulation-evoked striatal dopamine was also significantly reduced by intra-SNc infusion of the muscarinic AchR antagonist scopolamine (10 µg; n = 4; 72.2% ± 4.6, $p < 0.01$; Fig. 4A and B) or the nicotinic AchR antagonist mecamylamine (1 µg; n = 4; 71.8% ± 4.8, $p = 0.01$; Fig. 4A and B), with the peak decrease occurring 5 min after infusion of each drug.

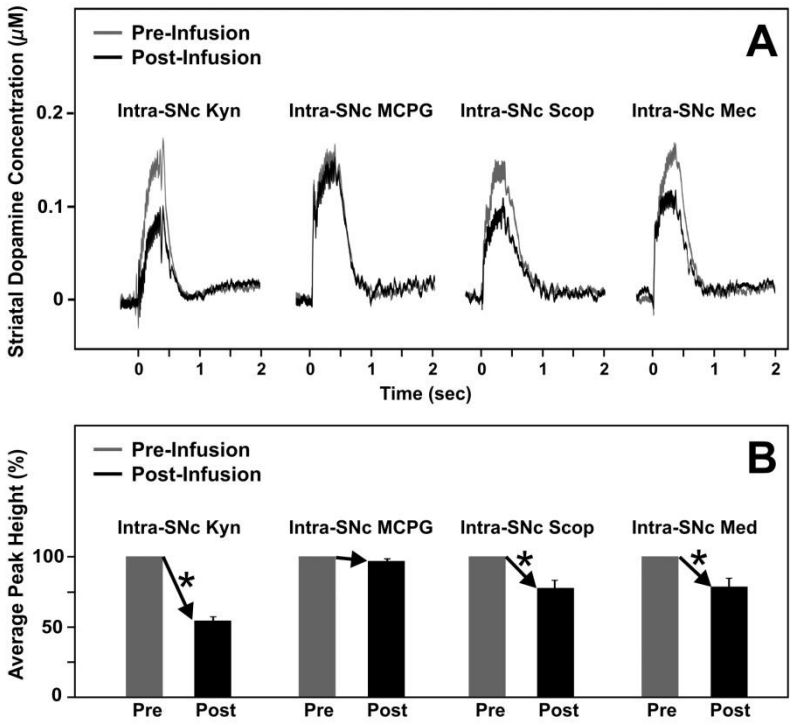


Fig. 4. Mean amperometric recordings of dopamine release in the striatum evoked by electrical stimulation of the subthalamic nucleus (A) and corresponding mean peak percentages (B). Profiles illustrate mean peak effects in response to substantia nigra compacta (SNc) microinfusions of the ionotropic glutamate receptor antagonist kynurenate (kyn), the metabotropic glutamate receptor antagonist (+)-methyl-4-carboxyphenylglycine (MCPG), the muscarinic acetylcholine receptor antagonist scopolamine (scop), or the nicotinic acetylcholine receptor antagonist mecamylamine (mec). Time zero indicates the start of the train of 20 pulses at 50 Hz. * Significant change in striatal dopamine concentration after infusion compared to pre-infusion baseline responses (100%).

Effects of intra-SNc or STN lidocaine on PPT stimulation-evoked dopamine efflux

With respect to pre-infusion baseline levels, PPT stimulation-evoked striatal dopamine efflux was not significantly altered at 5 min following PBS infusion into the SNc (n = 4; 98.2% ± 2.2, $p = 0.50$; Fig. 5A and C) or STN (n = 4; 95.7% ± 3.3, $p = 0.28$; Fig. 5B and D). PPT stimulation-evoked dopamine in the striatum was completely abolished following intra-SNc lidocaine (4%) infusion (n = 4; 9.9% ± 2.6, $p < 0.01$; Fig. 5A and C) and significantly, but less dramatically, reduced following intra-STN lidocaine infusion (n = 4; 50.0% ± 7.7, $p < 0.01$; Fig. 5B and D), with the peak decrease at each site occurring 5 min post-infusion.

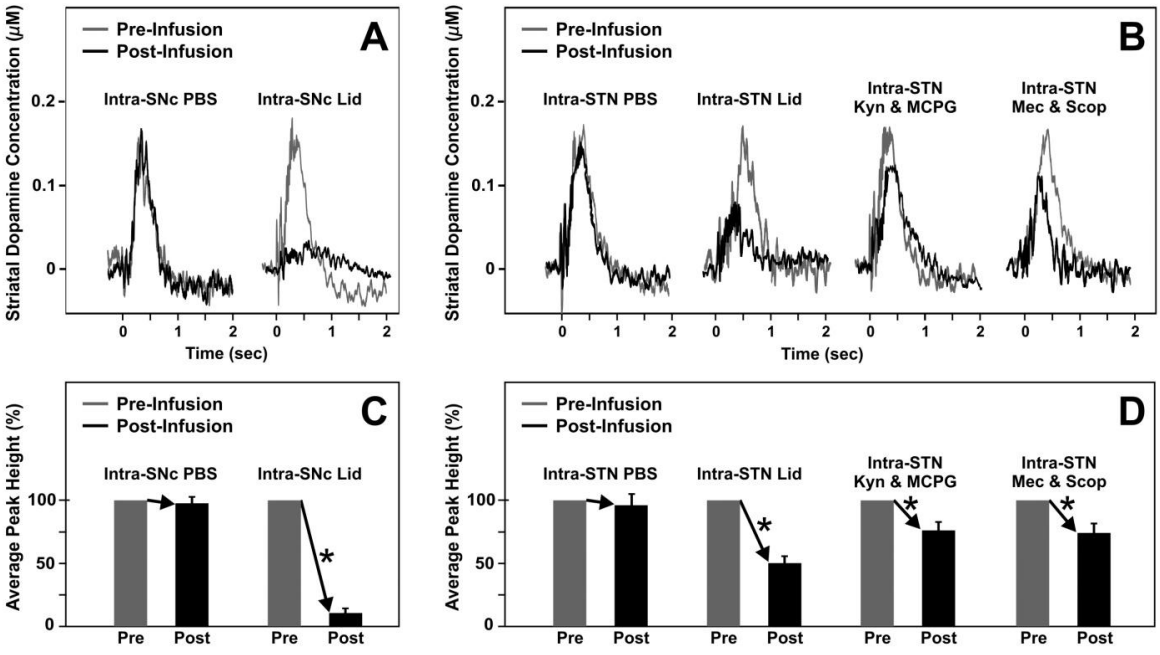


Fig. 5. Mean amperometric recordings of dopamine release in the striatum evoked by electrical stimulation of the pedunculopontine tegmental nucleus (A and B) and corresponding mean peak percentages (C and D). Profiles illustrate mean peak effects in

response to substantia nigra compacta (SNc) (A) or subthalamic nucleus (STN) (B) microinfusions of sterile phosphate-buffered saline (PBS, pH~7.4), lidocaine (lid), a combination of the ionotropic glutamate receptor antagonist kynurenate (kyn) with the metabotropic glutamate receptor antagonist (+)-methyl-4-carboxyphenylglycine (MCPG), or a combination of the nicotinic acetylcholine receptor antagonist mecamylamine (mec) and the muscarinic acetylcholine receptor antagonist scopolamine (scop). Time zero indicates the start of the train of 20 pulses at 50 Hz. * Significant change in striatal dopamine concentration after infusion compared to pre-infusion baseline responses (100%).

Effects of STN GluR or AchR blockade on PPT stimulation-evoked dopamine efflux

With respect to pre-infusion baseline levels, infusion of a combination of the iGluR antagonist kynurenate (1 µg) and the mGluR antagonist MCPG (1 µg) into the STN significantly attenuated PPT stimulation-evoked dopamine efflux in the striatum (n = 4; 77.5% ± 6.2, $p = 0.03$; Fig. 5B and D) with the peak decrease occurring 5 min post-infusion. PPT stimulation-evoked striatal dopamine was also significantly reduced by blockade of STN AchRs (n = 4; 74.2% ± 3.4, $p < 0.01$; Fig. 5B and D) via infusion of a combination of the mAchR antagonist scopolamine (10 µg) and the nAchR antagonist mecamylamine (1 µg), with the peak decrease occurring 5 min post-infusion.

Discussion

As measured by *in vivo* FPA, stimulation of either the MFB, STN, or PPT elicits dopamine release in the dorsal striatum. STN stimulation-evoked striatal dopamine release was markedly attenuated by inactivation of either the SNc or the PPT, and PPT stimulation-evoked dopamine release in the striatum was significantly reduced following inactivation of either the SNc or the STN. Therefore, neural interactions between these nuclei are likely involved in the underlying mechanisms of DBS as a treatment for the motor symptoms of Parkinson's disease. The present findings provide a glimpse at the relative significance of the glutamatergic and cholinergic connections between the SNc, STN, and PPT in regards to striatal dopamine neurotransmission.

Neuronal pathways mediating striatal dopamine release following MFB stimulation

Lee et al. (2006) used a monoclonal antibody to TH to demononstrate in rats that the axons of ascending dopamine neurons from the SNc align closely along the dorsal surface of the STN with some fibers potentially passing through the nucleus itself. A similar pattern of TH staining of catecholaminergic neurons has been shown in both monkeys and humans (Lee et al., 2005). Thus, it is conceivable that clinical DBS of the STN is activating these axons of the MFB due to the close proximity and that HFS of the MFB may optimally enhance dopamine transmission in the basal ganglia (Lee et al., 2006). The present findings suggest that MFB stimulation is mediated predominately by activation of ascending SNc dopamine neurons, as lidocaine infusions into the SNc did not reduce MFB-evoked striatal dopamine release. Previous studies have shown that pharmacological denervation of dopamine axonal transmission promotes a rapid

compensatory mechanism that dramatically enhances synthesis and storage of dopamine in terminal vesicles (Brown et al., 1991). These findings are consistent with our observation of an enhancement in MFB stimulation-evoked striatal dopamine release following lidocaine inactivation of the SNc. The present results show that stimulation of the MFB is not dependent upon SNc iGluRs, as kynurenate infused into the SNc had no effect on MFB-evoked dopamine, again suggesting that MFB stimulation is mediated predominately by activation of ascending SNc dopamine neurons rather than neurochemical activities within the SNc. Altogether, these data may help to explain the clinical improvements in motor symptoms of Parkinson's patients following stimulation of the border and white matter dorsal to the STN (Herzog et al., 2004; Saint-Cyr et al., 2002; Voges et al., 2002).

Neuronal pathways mediating striatal dopamine release following STN stimulation

The present study examined several neuronal circuits in the mid- and hindbrain by which clinical DBS of the STN could increase striatal dopamine transmission. Stimulation of the STN has been shown to alter neuronal activity within the SNc of rodents generating initial transient inhibitory (via activation of GABAergic interneurons in the SN reticulata) and more prolonged excitatory (via direct activation of dopaminergic neurons in the SNc) postsynaptic potentials (Lee et al., 2004), leading to increased firing of SNc neurons (Hammond et al., 1978; Iribe et al., 1999; Benazzou et al., 2000). Changes in STN activity have also been shown to significantly affect discharge patterns of SNc neurons and striatal dopamine release in primates. Shimo and Wichmann (2009) concluded that increases in the firing rate of SNc neurons following intra-STN injections of carbachol,

and decreases in the firing rate of SNc neurons after intra-STN injections of muscimol, may have resulted from changes in activity along the connections between the STN and SNc via an excitatory glutamatergic pathway well documented in rodent research (Hammond et al., 1978; Kita and Kitai, 1987; Smith and Parent, 1988). However, the monosynaptic pathway between the STN and SNc has been shown to be relatively sparse in primates compared to rodents (Smith et al., 1990; Sato et al., 2000). Therefore, the observed changes in SNc discharge patterns in primates may have been mediated primarily by excitatory SNc afferents from the PPT (Futami et al., 1995; Charara et al., 1996). The STN and PPT are reciprocally interconnected with excitatory projections (Futami et al., 1995; Lee et al., 2000), which have been shown to be both cholinergic and glutamatergic from the PPT to the STN (Moon-Edley and Graybiel, 1983; Oakman et al., 1999). *In vivo* electrochemical studies in rodents have previously shown that electrical and chemical stimulation of the PPT enhances dopamine efflux in the striatum (Forster and Blaha, 2003; Miller and Blaha, 2004); thus, it is highly probable that stimulation of the STN in primates may be increasing discharge patterns of SNc dopaminergic neurons to elicit striatal dopamine release indirectly through STN activation of the PPT.

The present results confirm that STN stimulation is dependent on activities of both the SNc and PPT, as inactivation of either of these nuclei decreased STN-evoked striatal dopamine efflux by 84.1% and 45.8%, respectively, compared to pre-infusion baseline responses. Thus, these findings suggest that HFS of the STN works by activating the SNc directly via excitatory STN-SNc projections and indirectly via excitatory STN-PPT projections that, in turn, provide excitatory PPT inputs to SNc dopamine cells. However, these findings cannot distinguish the extent to which STN

stimulation is activating dopamine cells in the SNc via reciprocal excitatory innervation between the STN and PPT. Future experiments incorporating intra-STN infusions of selective glutamate and acetylcholine receptor antagonists would address this issue. In addition, STN stimulation-evoked striatal dopamine release was significantly diminished by inactivation of the SNc; however, it was not completely abolished. This is not surprising considering the relatively wide medial to lateral distribution of dopamine cell bodies in the midbrain comprising the SNc (see Fig. 1C), such that a single infusion of drug into the SNc likely fails to inactivate all of these cells. Thus, the small remaining response (15.9%) can be attributed either to incomplete drug inactivation of the SNc. Alternatively, unavoidable stimulation of some of the dopamine axons within the medial forebrain bundle that project immediately dorsal to STN on their way to the striatum may also have contributed to the response following intra-SNc lidocaine inactivation. This observation would support the notion that STN DBS may act by directly stimulating SNc dopamine axons passing near or through the STN (Lee et al., 2006).

The STN and PPT are the only subcortical nuclei in the basal ganglia complex whose glutamate-containing neurons directly innervate nigrostriatal dopaminergic neurons (Kitai et al., 1999; Overton and Clark, 1997). Stimulation of the STN has been shown to alter neuronal activity within the substantia nigra of rodents resulting in elevated glutamate release in the SNc (Windels et al., 2000). Glutamate release in the SNc activates dopamine neurons that project to the striatum. The present results show that STN stimulation is dependent upon iGLuRs, but not mGluRs, in the SNc as intra-SNc kynurenate significantly decreased STN-evoked striatal dopamine release (54.1%) while intra-SNc MCPG had no effect. These findings are consistent with our previous

findings and others (Balon et al., 2003; Forster and Blaha, 2003; Lavoute et al., 2006). The remaining STN stimulation-evoked striatal dopamine release (45.9%) following intra-SNc kynurenate infusion can be attributed to a major extent to excitatory cholinergic SNc inputs from the PPT. However, as mentioned, it cannot be completely discounted that a small portion of the signal may have been due to unavoidable stimulation of a small number of dopamine axons immediately dorsal to the STN within the medial forebrain bundle. Intra-SNc infusions of scopolamine or mecamylamine decreased STN-evoked striatal dopamine release by 27.8% and 28.2%, respectively. Accordingly, these results suggest that cholinergic projections from the PPT to the SNc mediate roughly half of the STN stimulation-evoked dopamine release in the striatum. In sum, the present data confirms the significance of the PPT in STN stimulation-evoked striatal dopamine release and suggest that glutamatergic projections (acting on iGluRs in the SNc) and cholinergic projections (acting on mAchRs and nAchRs in the SNc) mediate approximately half of the striatal dopamine release following STN stimulation.

Neuronal pathways mediating striatal dopamine release following PPT stimulation

Excitatory glutamatergic and cholinergic inputs from the PPT directly project to dopamine-containing cell bodies in the SNc (Blaha and Winn, 1993; Forster and Blaha, 2003; Moon-Edley and Graybiel, 1983; Oakman et al., 1999). Our previous work using *in vivo* chronoamperometry to measure basal changes in dopamine release has shown that PPT stimulation elicits an initial transient increase in striatal dopamine release, in which this rapid increase in dopamine release could be blocked by a combination of intra-SNc infusions of nAchR and iGluR antagonists. This transient stimulation time-locked

increase was followed by a delayed, prolonged increase in striatal basal dopamine release that could be selectively blocked by mAchR antagonists infused into the SNc (Forster and Blaha, 2003). However, electrical stimulation of the PPT has also been shown to activate STN neurons via cholinergic and glutamatergic projections (Woolf and Butcher, 1986; Hammond et al., 1983). Therefore, in addition to direct cholinergic activational inputs to SNc dopaminergic cells, the PPT may also enhance striatal dopamine release via indirect PPT glutamatergic and cholinergic inputs to STN glutamatergic neurons that, in turn, innervate directly dopamine-containing cells in the SNc (Bevan and Bolam, 1995; Lee et al., 2000). The present results show that PPT stimulation-evoked striatal dopamine release is significantly dependent on activities of the SNc and STN, as inactivation of the SNc and STN lead to decreases in PPT-evoked striatal dopamine efflux of 90.1% and 50.0%, respectively, compared to pre-infusion baseline responses. Thus, these findings suggest that clinical DBS of the PPT may involve activation of the SNc directly via excitatory PPT-SNc projections and indirectly via excitatory PPT-STN projections that, in turn, provide excitatory inputs to SNc dopamine cells. However, these findings cannot distinguish the extent to which PPT stimulation is activating dopamine cells in the SNc via reciprocal excitatory innervation between the STN to the PPT. Future experiments incorporating intra-PPT infusions of selective GluR antagonists would address this issue.

The present study further examined the excitatory glutamatergic and cholinergic pathway from the PPT to the STN and found that PPT-evoked striatal dopamine release is dependent upon both GluRs and AchRs in the STN, as intra-STN GluR antagonists or AchR antagonists both significantly decreased PPT-evoked striatal dopamine release by

22.5% or 25.8%, respectively. Together, the present data confirms the significance of the SNc, as well as the STN, in PPT stimulation-evoked striatal dopamine release and suggests that combined glutamatergic and cholinergic projections from the PPT to the STN are mediate approximately half of PPT-evoked dopamine release in the striatum.

Conclusions

The present study shows that electrical stimulation of the MFB, STN, or PPT elicits dopamine release in the striatum. MFB stimulation evokes striatal dopamine through direct stimulation of dopamine axons and is independent of neurochemical activity within the SNc and PPT, while STN or PPT stimulation elicits striatal dopamine through several neural routes. The relative contributions of the direct and indirect projections to the SNc that are involved in mediating STN or PPT stimulation-evoked dopamine release are summarized in Fig. 6A and B. STN-evoked dopamine release in the striatum is almost fully dependent upon (84.1%) activation of dopamine cells in the SNc, while also partially dependent upon projections from the PPT (45.9%). Fig. 6A also illustrates the relative involvement of SNc iGluRs (45.9%) and mAchRs and nAchRs (27.8% and 28.2%, respectively) in STN-evoked striatal dopamine release. PPT-evoked striatal dopamine release was highly dependent upon activation of dopamine cells in the SNc (90.1**%**), and partially dependent upon activation of STN cells that project to the SNc (50.0**%**), equivalent to the relative involvement of STN GluRs or AchRs (22.5% + 25.8%).

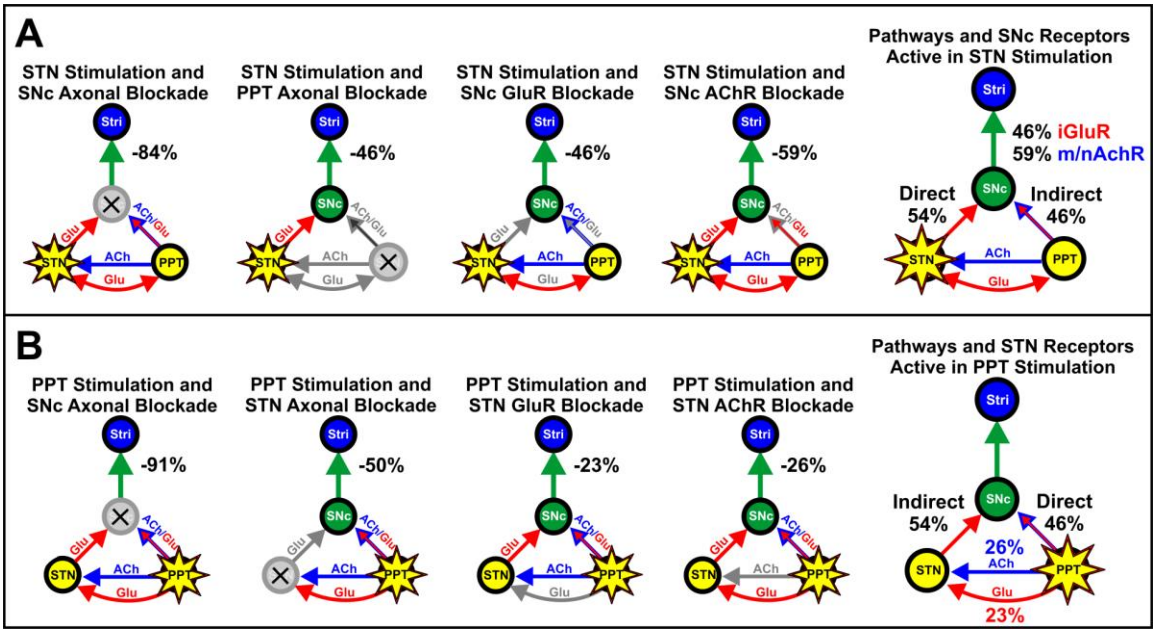


Fig. 6. Summary of relative contributions of neuronal projections mediating subthalamic nucleus (STN) (A) or pedunculopontine tegmental nucleus (PPT) (B) stimulation-evoked dopamine release in the striatum (stri). ACh: acetylcholine; Glu: glutamate; m/nAchR: muscarinic/nicotinic acetylcholine receptor; iGluR: ionotropic glutamate receptor; SNc: substantia nigra compacta.

In relation to DBS as a treatment for Parkinson's disease, these findings support research indicating that DBS of the STN, as well as the PPT, provides therapeutic benefits due to increased extracellular levels of dopamine in the striatum (Lee et al., 2009). Although these studies were conducted in intact animals, future experiments using the present neurochemical recording procedures in 6-OHDA lesioned mice would provide knowledge of the involvement of these pathways in an animal model of Parkinson's disease. Another intriguing future experiment could be monitoring dopamine concentrations while stimulating the STN and PPT simultaneously, as clinical

studies have shown combined STN and PPT DBS to be more therapeutically efficacious than DBS of the STN or PPT alone on certain symptoms, such as the control of axial motor impairments (Stefani et al., 2007). Understanding the underlying mechanisms of DBS of the STN and the interconnected PPT could lead to improvements in stimulation locations and parameters, which may prove invaluable in improving DBS procedures and enhancing its clinical efficacy for the treatment of Parkinson's disease.

Chapter 6. Substantia Nigra Compacta Glutamate Receptors Modulate Dopamine Release in the Striatum

The nigrostriatal dopamine pathway, comprised of dopamine-containing neurons in the substantia nigra compacta (SNc) and their projections to the striatum, is integral in motor functioning, including the selection and initiation of contextually appropriate motor patterns (Hauber, 1998; Redgrave et al., 1999). The dopaminergic neurons of the SNc degenerate in Parkinson's disease, leading to reduced dopamine levels in the striatum and, subsequently, clinical symptoms such as bradykinesia, tremor, and rigidity (Fearnley and Lees, 1991; Olanow and Tatton, 1999). Identifying receptors that modulate the activity of dopamine neurons in the SNc may help in the development of novel therapeutic strategies for treating the symptoms of Parkinson's disease and perhaps even slowing the progression of the disease. The subthalamic nucleus (STN) and the pedunculopontine tegmental nucleus (PPT) provide significant glutamatergic excitatory inputs to the SNc, which induce burst firing of SNc dopamine neurons resulting in sustained release of dopamine in the striatum (Grillner and Mercuri, 2002; Kitai et al, 1999; Overton and Clark, 1997). Glutamatergic input to the SNc has received a great deal of attention based on findings that suggest overactivity of STN glutamatergic projections in Parkinson's disease and the potential contributory role of long-lasting glutamate receptor stimulation in the degeneration of dopaminergic neurons (Rodriguez et al., 1998). The present study expands on the involvement of these projections in Parkinson's disease by focusing on their modulatory control of dopamine release in the striatum.

Previous studies have shown that the effects of glutamate in the SNc are mediated by ionotropic glutamate receptors (iGluRs), which activate ion-gated channels, and metabotropic glutamate receptors (mGluRs), which activate slow and more complex effects mediated by G-coupled protein secondary messenger systems (Chatha et al, 2000; Valenti et al, 2005). Three iGluR subclasses have been identified based on their definitive agonist and include N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate, with the latter 2 sometimes collectively referred to as non-NMDA receptors (Hollmann and Heinemann, 1994). The relative contribution of these glutamate receptor subtypes in the SNc in mediating STN glutamatergic activation of fast phasic activity of the nigrostriatal dopamine system remains largely unknown. Therefore, the present study investigated the extent to which each of these GluR subtypes is involved in mediating striatal dopamine release by infusing NMDA, AMPA/kainate, and mGluR antagonists into the SNc while recording STN stimulation-evoked dopamine efflux in the striatum using *in vivo* fixed potential amperometry with carbon fiber microelectrodes.

Experimental procedures

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Memphis and conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize animal pain and discomfort.

Animals and surgery

Sixteen male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), 8-11 weeks of age and weighing 20-27 g at the time of surgery, were used. Mice were housed four per cage at 21 ± 1ºC with a 12 h light: 12 h dark cycle (lights on at 0600 h). Food and water were available *ad libitum*. Mice were anesthetized with urethane (1.5 g/kg, i.p.) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with a mouse head-holder adaptor (Stoelting, Wood Dale, IL, USA). A temperature-regulated heating pad (TC-1000; CWE Inc., New York, NY, USA) maintained body temperature at 36 ± 0.5°C. All stereotaxic coordinates (AP from bregma, ML from midline, and DV from dura, in mm) were determined from the mouse atlas of Paxinos and Franklin (2001). In each mouse, a concentric bipolar stimulating electrode (SNE-100; Rhodes Medical Co., CA, USA) was implanted into the left STN (coordinates: AP -2.0, ML +1.6, DV -4.0). A 31 g stainless-steel guide infusion cannula was implanted into the left SNc, with the guide cannula tip 2 mm above site (coordinates: AP -3.1, ML +1.5, DV -3.8). An Ag/AgCl reference and stainless-steel auxiliary electrode combination was placed on contralateral cortical tissue approximately 2.0 mm posterior to bregma, and a carbon fiber recording electrode (250 μm length x 10 μm o.d.; Thornel Type P, Union Carbide, Pittsburgh, PA, USA) was then implanted into the left striatum (coordinates: AP +1.4, ML +1.4, DV -2.5).

Fixed potential amperometry and electrical stimulation

Amperometric recordings in a Faraday cage consisted of applying a fixed potential (+0.8 V) to the recording electrode and monitoring dopamine oxidation current continuously

(10K samples/sec) with an electrometer filtered at 50 Hz. Approximately 10 min following implantation of the electrodes, a series of 0.5 ms duration cathodal monophasic current pulses (20 pulses at 50 Hz applied every 30 sec at 800 μAmps) was delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). Fixed potential amperometry with carbon fiber electrodes has been confirmed as a valid technique for real-time monitoring of changes in striatal dopamine oxidation current evoked by electrical stimulation of afferent inputs to midbrain dopamine neurons (Dugast et al., 1994; Forster and Blaha, 2003; Lester et al., 2008).

Drug microinfusions

Intra-SNc infusions were performed by backloading each drug into a fibreglass cannula (80 μm o.d., Polymicro Tech. Inc., AZ, USA) connected via PE10 tubing to a 5 μl microsyringe (Scientific Glass Engineering Inc., Austin, TX, USA) mounted on a microinfusion pump (Stoelting, Wood Dale, IL, USA). After a 10 min baseline recording period, the cannula was inserted 2 mm beyond the guide cannula tip, and 1.0 μl infusions were made over a 1.5 min period. Separate groups of mice received intra-SNc infusions of the following drugs: the mGluR antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG) (2.0 μg), the NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (1.0 μg), and the AMPA/kainate receptor antagonist 6-cyano-7-1fitroquinoxaline-2,3-dione (CNQX) (0.2 μg). Drugs were prepared immediately before use at doses determined by preliminary studies in this laboratory.

Separate phosphate-buffered saline (PBS, pH~7.4) infusions served as drug effect controls.

Data collation and statistical analysis

STN stimulation-evoked dopamine efflux were quantified by extraction of data points occurring within the range of 0.25 sec pre- and 2.0 sec post-stimulation at 30 sec intervals from the recorded oxidation current. Mean changes in striatal dopamine oxidation current, corresponding to STN stimulation-evoked dopamine efflux, were converted to mean dopamine concentrations (μM) by post-experiment *in vitro* calibration of the carbon fiber electrode in solutions of dopamine (2-10 μM) using a flow injection system (Michael and Wightman, 1999). For each animal, changes in stimulation-evoked dopamine concentration after infusion were expressed as mean percent changes with respect to pre-infusion baseline responses (100%). Mean peak levels in dopamine concentration following the infusion were statistically compared to pre-infusion baseline responses using paired two-tailed t-tests. In order to compare the relative contributions of NMDA and AMPA/kainate SNc receptors, mean peak percentage changes in dopamine concentration were compared between the mice receiving intra-SNc infusion of CPP and those receiving intra-SNc CNQX using independent two-tailed t-tests. The alpha level for all analyses was set at 0.01.

Histology

At the end of each experiment, an iron deposit was made by passing direct anodic current (100 μA for 10 sec) through the stimulating and recording electrodes, and 1.0 μl cresyl

violet stain was infused through the cannula. Mice were then euthanized with a 0.25 ml intracardial injection of urethane (0.345 g/ml). Brains were removed, immersed overnight in 30%/10% sucrose/formalin plus 0.1% potassium ferricyanide until sectioning. After fixation, 30 µm coronal sections were sliced in a cryostat at -30°C, and placements of stimulating electrodes, recording electrodes, and drug infusion cannulae were determined under a light microscope and recorded on representative coronal diagrams (Paxinos and Franklin, 2001).

Chemicals

Urethane, CPP, CNQX, and MCPG were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). All chemicals, with the exception of urethane (distilled water), were dissolved in sterile PBS (pH~7.4).

Results

Stereotaxic placements of infusion cannulae, recording and stimulating electrodes

Recording electrode placements (n = 16) were confined to the dorsal striatum (range in mm: 1.34-1.54 anterior to bregma; 1.30-1.60 lateral to midline; 2.40-2.70 ventral to dura) (Fig. 1A). Stimulating electrode tips (n = 16) were positioned within the STN (range in mm: 1.94-2.18 posterior to bregma; 1.50-1.70 lateral to midline; 3.90-4.20 ventral to dura) (Fig. 1B). Cannula tip placements (n = 16) were localized within the SNc (range in mm: 2.92-3.16 posterior to bregma; 1.40-1.60 lateral to midline; 3.60-3.90 ventral to dura) (Fig. 1C).

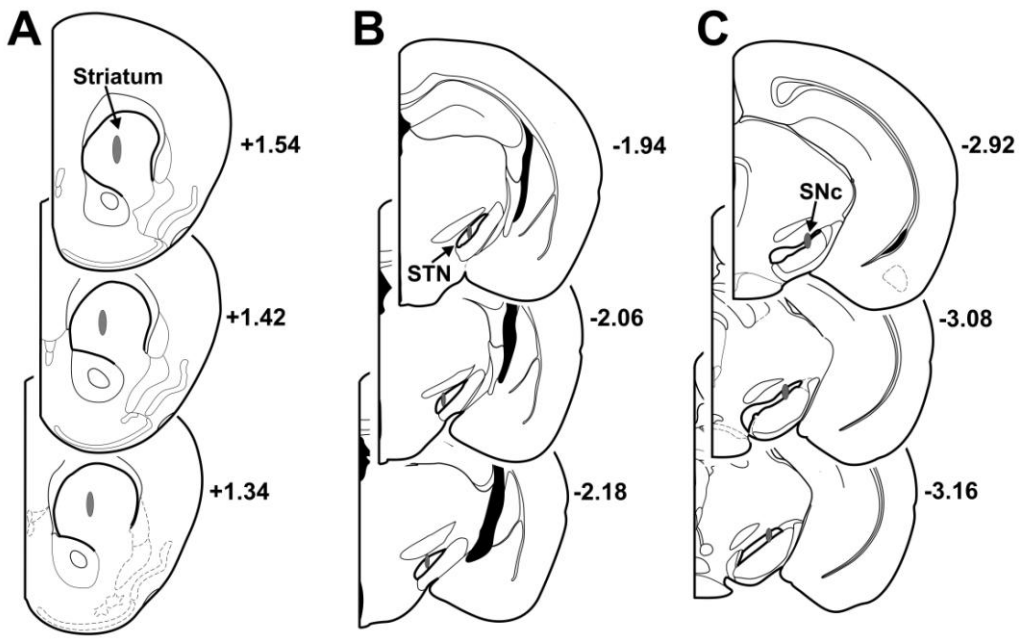


Fig. 1. Representative coronal sections of the mouse brain (adapted from the atlas of Paxinos and Franklin, 2001), with shaded areas indicating the placements of (A) amperometric recording electrodes in the striatum, (B) stimulating electrodes in the subthalamic nucleus (STN), (C) drug infusion cannulae in the substantia nigra compacta (SNc). Numbers correspond to mm from bregma.

Effects of GluR blockade on STN stimulation-evoked dopamine efflux

Intra-SNc infusion of PBS (n = 4) did not significantly alter STN stimulation-evoked striatal dopamine efflux from pre-infusion baseline levels (96.2% ± 1.7 at 5 min post-infusion, $p = 0.110$) (Fig. 2A and B). Infusion of the mGluR antagonist MCPG (2.0 µg; n = 4) also had no significant effect on STN-evoked striatal dopamine efflux (97.1% ± 1.1 at 5 min post-infusion, $p = 0.084$) (Fig. 2A and B). However, intra-SNc infusion of the NMDA receptor antagonist CPP (1.0 µg; n = 4) or the AMPA/kainate receptor antagonist

CNQX (0.2 µg; n = 4) significantly decreased evoked dopamine efflux, reaching a maximum of 60.1% ± 5.2 and 67.6% ± 2.3 respectively ($p < 0.01$) at 5 min post-infusion (Fig. 2A and B). The peak percent change of STN stimulation-evoked dopamine striatal release following intra-SNc CPP were not significantly different compared to the percent change following CNQX infusion into the SNc ($p = 0.23$).

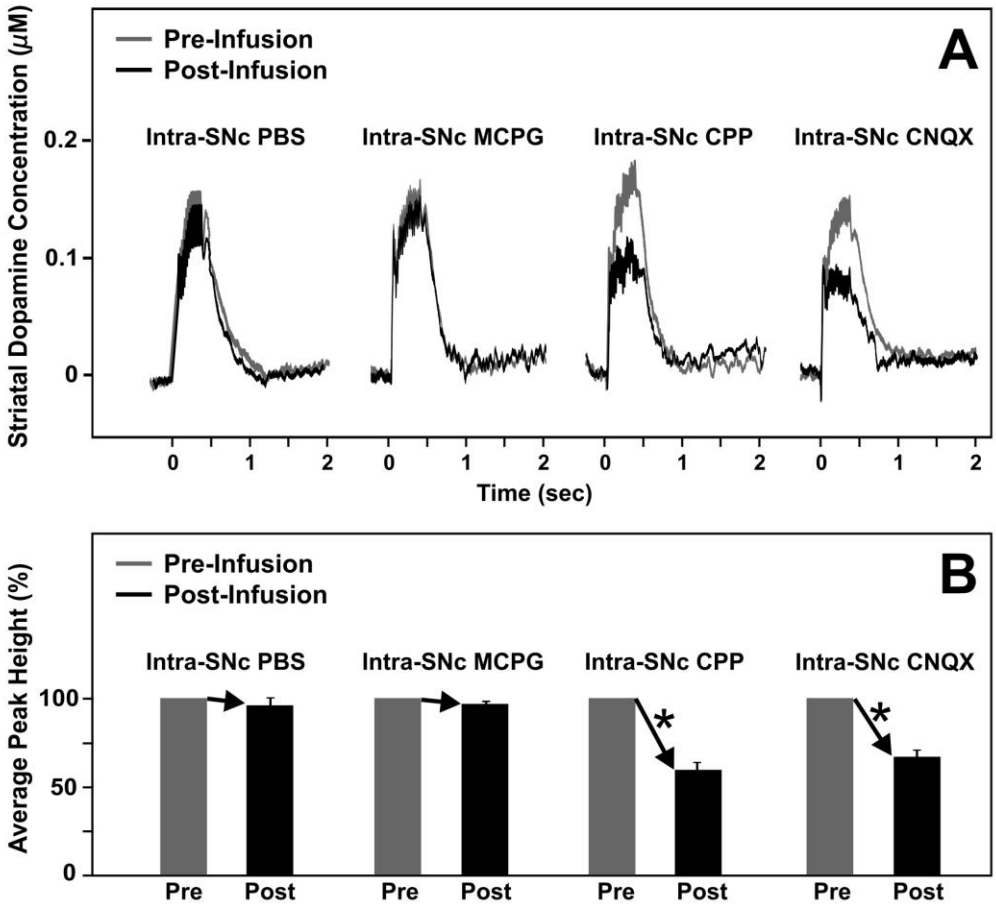


Fig. 2. Mean amperometric recordings of dopamine release in the striatum evoked by electrical stimulation of the subthalamic nucleus (A) and corresponding mean peak percentages (B) following substantia nigra compacta (SNc) microinfusions of phosphate-

buffered saline (PBS, pH~7.4), the metabotropic glutamate receptor antagonist (+)-methyl-4-carboxyphenylglycine (MCPG), the NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), the AMPA/kainate receptor antagonist 6-cyano-7-1fitroquinoxaline-2,3-dione (CNQX). Time zero indicates the start of the stimulation (20 pulses at 50 Hz). * Significant change in striatal dopamine concentration after infusion compared to pre-infusion baseline responses (100%).

Discussion

STN stimulation-evoked dopamine release in the striatum as measured using *in vivo* fixed potential amperometry was significantly attenuated following infusion of an NMDA or AMPA/kainate antagonist into the SNc. In contrast, intra-SNc infusion of an mGluR antagonist, including drug vehicle (PBS), had no effect on STN stimulation-evoked dopamine release in the striatum. The present results suggest that iGluRs in the SNc, compared to mGluRs, play a more critical role in mediating relatively brief excitatory glutamatergic activation of SNc dopamine neurons.

The role of SNc mGluRs in mediating nigrostriatal dopamine activity

The present finding that intra-SNc MCPG had no effect on STN stimulation-evoked dopamine release in the striatum warrants consideration. The mGluRI subtype, belonging to the Group I mGluRs, is the predominant mGluR subtype localized to SNc dopamine cells (Kosinski et al., 1998; Testa et al., 1994), justifying the use of the Group I and II antagonist MCPG (Pin and Duvoisin, 1995; Riedel, 1996). Furthermore, we utilized a dose of MCPG that has been effective in abolishing accumbal basal dopamine

efflux as evoked by electrical stimulation of the ventral subiculum of the hippocampus (Blaha et al., 1997). Consequently, the results of the present study imply that mGluRs in the SNc are not employed by STN glutamatergic afferents to mediate striatal dopamine efflux. Previous studies on the role of SNc mGluR receptors in nigrostriatal dopamine activity are somewhat conflicting. The activation of mGluR1 subtype in the SNc has been shown to both excite and inhibit dopamine neurons (Fiorillo and Williams, 1998; Guatteo et al, 1999; Meltzer et al., 1997), while the activation of Group II mGluRs at STN-SNc synapses, most likely located presynaptically on glutamatergic terminals, inhibits glutamatergic transmission in SNc dopamine neurons (Bonci et al, 1997; Wang et al., 2005). Our utilization of fixed potential amperometry allows for the measurement of phasic dopamine release, rather than basal extracellular levels of dopamine. Thus, the rapid responses seen with STN-stimulation, generating burst firing of SNc dopamine neurons, are most likely not affected by the complex, relatively slower, actions of mGluRs. While the findings of the present study are strengthened by similar previous findings that intra-SNc MCPG had no effect on brief PPT stimulation-evoked striatal dopamine release (Forster and Blaha, 2003), further studies involving various doses of MCPG and other mGluR antagonists are needed before the use of mGluRs by STN projections to the SNc can be excluded. In this regard, it is significant to note that prolonged stimulation of the STN has been shown to result in an initial short duration increase in phasic striatal dopamine release (~1 sec) that is followed by a lower but still elevated level of dopamine release (Lee et al., 2006). This later inhibition of stimulation-evoked phasic dopamine release was thought to reflect compensatory effects at the level of the SNc glutamate-containing terminal. Theoretically, activation of group II and III

mGluRs on glutamate-containing terminals may attenuate dopamine cell activity by reducing excessive glutamate release onto dopamine cells in the SNc, despite a continuous level of firing activity (Grillner and Mercuri, 2002; Mercuri et al., 1993).

The role of SNc iGluRs in mediating nigrostriatal dopamine activity

Both NMDA and non-NMDA receptors were shown to be involved in STN stimulation-evoked dopamine in the striatum, as evidenced by significantly attenuated responses following intra-SNc NMDA or AMPA/kainate receptor antagonists. The present results also indicate that NMDA and AMPA/kainate receptor subtypes have an equal role in modulating excitatory glutamatergic inputs arising from the STN, as the maximum percent decreases in STN-evoked striatal dopamine release post-infusion did not differ between the NMDA and AMPA/kainate receptor antagonists. Activation of AMPA and kainate receptors opens membrane ion-channels to allow the rapid influx of positively charged sodium resulting in the generation of fast moment-to-moment excitatory post-synaptic potentials (Borges and Dingledine, 1998). NMDA receptors are highly conductive to the positive ion calcium, although the entry of calcium in the resting state is blocked by magnesium (Chapman, 2009). Usually, activation of NMDA receptors requires colocalised AMPA/kainate receptors to depolarise first so that the entry of positive sodium displaces magnesium ions from the NMDA ion pore thus permitting calcium influx into the cell, consequently depolarizing it (Michaelis, 1998). Thus, a similar degree of attenuation in STN-evoked striatal dopamine following blockade of SNc NMDA or AMPA/kainate receptors in the present study is not surprising. Previous research has shown that synaptic potentials in dopamine neurons evoked by single

stimulating pulses are mediated predominantly by activation of AMPA/kainate receptors and to a smaller extent through NMDA receptor activation (Johnson and North, 1992; Mereu et al, 1991). Whereas, a preferential role for NMDA receptors, compared to non-NMDA receptors, in producing burst activity in SNc dopamine neurons has been described (Chergui et al., 1994; Overton and Clark, 1997). It is possible that activation of NMDA receptors requires a larger amount of glutamate release than non-NMDA receptors, as high frequency stimulation, perhaps corresponding with burst firing, is required to evoke synaptic potentials with a large contribution of NMDA receptors (Grillner and Mercuri, 2002). Nonetheless, it seems that glutamatergic excitation of dopamine neurons in the SNc, and subsequent striatal dopamine release, is mediated to some degree by both NMDA and AMPA/kainate receptors, as widespread distribution of both types of these subtype receptors on dopaminergic neurons in the SNc has been well established (Chatha et al., 2000).

Conclusions

The present results suggest that NMDA and AMPA/kainate receptors both play a significant role in modulating striatal dopamine release evoked by electrical stimulation of the STN. Because glutamate receptors mediate synaptic transmission in the SNc, a vital site in the extrapyramidal motor circuit, pharmacological manipulation of these receptors may be able to alter dysfunctional neurotransmission and thus provide a promising therapeutic target for treating Parkinson's disease. Animal model studies suggest that altering the activity of these receptors pharmaceutically may serve to alleviate parkinsonian motor symptoms or perhaps even slow disease progression by

delaying dopamine neuron degeneration, thought to be associated with excitotoxicity caused by relatively high extracellular levels of glutamate. For example, antagonists of NMDA and AMPA receptors have been shown to reverse motor symptoms and levodopa-induced dyskinesias in animal models (Gossel et al., 1995; Klockgether and Turski, 1990; Schwarz et al., 1996), as would be expected given the findings of the present study. Although blocking SNc mGluRs had no effect on STN stimulation-evoked striatal dopamine release in the present study, pharmaceutical modulation of mGluRs have shown promise in providing neuroprotection of SNc dopamine neurons in animal models of Parkinson's disease (Johnson et al., 2009), further suggesting a role for mGluRs, located presynaptically, in maintaining functional basal glutamate levels in the SNc (Grillner and Mercuri, 2002; Mercuri et al., 1993). Therefore, GluRs represent promising targets for the development of nondopaminergic pharmaceutical therapies for the treatment of Parkinson's disease, and more studies are necessary to determine the relative contributions of each receptor subtype in mediating afferent activation of the nigrostriatal dopamine system.

Chapter 7. Summary, Conclusions, and Implications for Future Research

The experimental studies of Chapters 5 and 6 were undertaken to investigate important neural circuits that functionally contribute to phasic dopamine neurotransmission within the nigrostriatal dopaminergic system. These investigations were conducted with the purpose of extending current knowledge of the neural connectivity of nuclei, specifically the substantia nigra compacta (SNc), subthalamic nucleus (STN), and pedunculopontine tegmental nucleus (PPT), with projections that mediate striatal dopamine release and subsequently influence activity of the basal ganglia-thalamocortical motor circuit. This chapter presents an overview of the present findings and their functional implications to pharmaceutical and surgical treatments for the motor symptoms of Parkinson's disease.

Overall, using *in vivo* fixed potential amperometry the present studies show that electrical stimulation of the dorsal portion of the medial forebrain bundle (MFB), STN, or PPT, all of which are targets for clinical deep brain stimulation (DBS) procedures as a treatment for the motor symptoms of Parkinson's disease, elicits dopamine release in the dorsal striatum. Stimulation of the MFB, which consists of axons of ascending SNc dopamine neurons that project closely along the dorsal surface of the STN to the striatum, was investigated as a control for STN stimulation, as it has been suggested that DBS of the MFB may optimally enhance dopamine transmission in the basal ganglia, thus providing better therapeutic results compared to DBS of the STN (Lee et al., 2006). The present findings confirm that MFB high frequency stimulation (HFS)-evoked striatal dopamine release is mediated predominately by activation of ascending SNc dopamine neurons, independent of activity within the SNc and PPT. Striatal dopamine release

elicited by STN or PPT stimulation, however, occurs via more complicated neural interactions, which are likely involved in the underlying mechanisms of STN or PPT DBS as a treatment for the tremor, gait and postural symptoms of Parkinson's disease. Reductions in striatal dopamine release following pharmacological blockade of axonal transmission or cholinergic/glutamatergic receptor populations was used to determine the specific involvement of the SNc, STN, and PPT in mediating HFS-evoked striatal dopamine release. The influence of these subcortical nuclei and their intrinsic receptor mechanisms on STN or PPT-driven nigrostriatal dopamine transmission is presented below.

Subcortical Involvement in STN HFS-Evoked Nigrostriatal Dopamine Transmission

The present studies examined several neuronal circuits in the mid- and hindbrain that could be involved in STN HFS-evoked dopamine release in the striatum. Results confirmed that STN stimulation is dependent on activities of both the SNc and PPT, as pharmacological blockade of axonal transmission within either of these nuclei significantly decreased STN-evoked striatal dopamine efflux compared to pre-infusion baseline responses. Thus, these findings suggest that stimulation of the STN works by activating the SNc directly via excitatory STN-SNc projections and indirectly via excitatory STN-PPT projections that, in turn, provide excitatory PPT inputs to SNc dopamine cells. The relative contributions of the direct and indirect projections to the SNc that are involved in mediating STN HFS-evoked dopamine release are summarized in Fig. 1A. The present data highlight the significance of the PPT in STN HFS-evoked striatal dopamine release as neuronal projections through the PPT mediate roughly half

(45.9%) of STN HFS-evoked dopamine release in the striatum. As expected STN-evoked striatal dopamine release is almost fully dependent upon activation of dopamine cells in the SNc; however, blockade of axonal transmission within the SNc did not completely abolish STN HFS-evoked striatal dopamine release. Under the present experimental conditions, it is likely that a single infusion of lidocaine or receptor blocking drug failed to inactivate the entire SNc, as well as unavoidable stimulation of some of the dopamine axons within the MFB that may also have contributed to the response elicited by STN stimulation. It is important to note that in previous amperometric recording of STN stimulation-evoked dopamine release in rats (Lee at al., 2006), this was avoidable given the relatively larger size of the STN in rats, compared to mice.

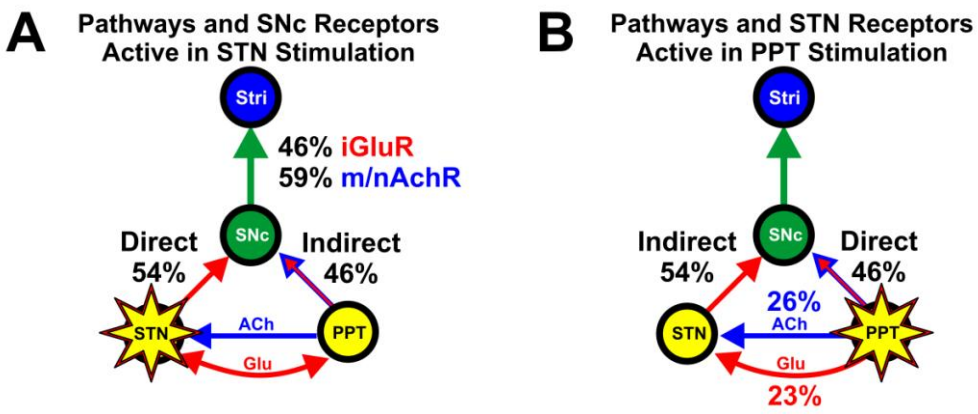


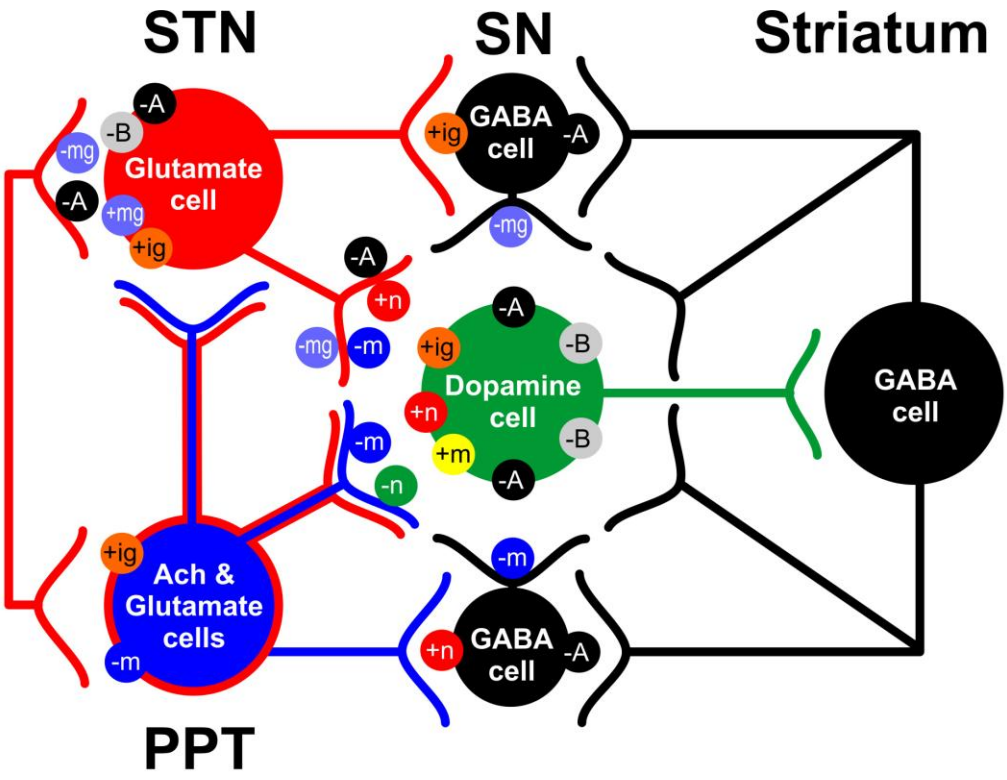
Fig. 1. Summary of relative contributions of neuronal projections mediating subthalamic nucleus (STN) (A) and pedunculopontine tegmental nucleus (PPT) (B) HFS-evoked dopamine release in the striatum (stri). ACh: acetylcholine; Glu: glutamate; m/nAchR:

muscarinic or nicotinic acetylcholine receptor; iGluR: ionotropic glutamate receptor; SNc: substantia nigra compacta.

PPT receptor mechanisms mediating STN HFS-evoked nigrostriatal dopamine transmission

Although the present studies only included intra-PPT infusions of lidocaine, rather than receptor antagonists, previous studies have shown that the PPT receives glutamatergic projections from the STN and prefrontal cortex (Kita and Kitai, 1987; Sesack et al., 1989), as well as GABAergic projections from the substantia nigra reticulata (SNr) and globus pallidus (Kang and Kitai, 1990; Moriizumi and Hattori, 1992). Both NMDA and AMPA receptors have been shown to be located on cholinergic and glutamatergic cells within the PPT, and activation of both types of iGluRs increase PPT activation (Steiniger and Kretschmer, 2003) (see Fig. 2); however, activation of both A and B GABA receptor subtypes has been shown to inhibit activity of PPT neurons, although the precise location of these GABA receptors in the PPT remain unclear (Saitoh et al., 2003). Furthermore, mAchRs (specifically of the M2 family) have been localized presynaptically on PPT cholinergic neurons (Vilaro et al., 1992). Intra-PPT infusions of the non-selective mAChR antagonist scopolamine enhances striatal dopamine release and dopamine-dependent behaviors such as locomotion and stereotypy; both of which can be blocked by the cholinergic agonist carbachol infused into the PPT (Chapman et al., 1997; Mathur et al., 1997). These mAChRs are most likely autoreceptors of the M2 family as intra-PPT infusion of the M2/4 selective mAChR antagonist methoctramine has been shown to

enhance striatal dopamine release (Miller and Blaha, 2004). Activation of M2-like mAChRs in the PPT results in hyperpolarization of mesopontine cholinergic cells (Luebke et al., 1993; Leonard and Llinas, 1994) and a net decrease in excitation to SNc dopaminergic cells resulting in lowered extracellular levels of striatal dopamine (Forster and Blaha, 2003). Therefore, M2-like mAChRs are thought to function as cholinergic autoreceptors involved in feedback inhibition at the level of PPT cholinergic cells, serving as regulators of information received by the PPT.



RECEPTOR SUBTYPES

+n	nAChR	+m	mAChR	+ig	iGluR	-A	GABA-A
-n	nAChR	-m	mAChR	-mg	mGluR	-B	GABA-B

Receptors mediating increases in Dopamine activity:

1. +n[1] and +ig[2] on SN Dopamine Cells - fast
2. +n[3] on Glutamate Terminals in SN - fast
3. -A[4] on SN GABA Cells - fast
4. +m[5] on SN Dopamine Cells - slow
5. -m[6] and -mg[6] on GABA Terminals in SN - slow
6. +ig[7] on STN Glutamate Cells - fast
7. +mg[8] on STN Glutamate Cells - slow
8. +ig[9] on PPT Ach/Glutamate Cells - fast

Receptors mediating reductions in Dopamine activity:

1. +n[10] and +ig[11] on SN GABA Cells - fast
2. -A[4] and -B[4] on SN Dopamine Cells - fast
3. -A[12] on Glutamate Terminals in the SN - fast
4. -n[13] on Ach Terminals in the SN - fast
5. -m[6] on Ach/Glutamate Terminals in the SN - slow
6. -A[14] and -B[14] on STN Glutamate cells - fast
7. -A[14] on Glutamate Terminals in the STN - fast
8. -m[15] on PPT Acetylcholine cells - slow

Fig. 2. Simplified basal ganglia circuitry depicting the muscarinic/nicotinic acetylcholine receptors (m/nAchRs), ionotropic/metabotropic glutamate receptors (i/mGluRs), and GABA-A/B receptors within the substantia nigra (SN), subthalamic nucleus (STN), and pedunculopontine tegmental nucleus (PPT) responsible for mediating nigrostriatal dopamine activity. + indicates excitatory effects upon receptor activation, and – indicates inhibitory effects upon receptor activation. Note that the GABAergic projection from the globus pallidus externus to the STN has been omitted for clarity; however, activation of mAchRs on these incoming terminals has been shown to excite STN neurons by inhibiting GABA release in the STN (Shen and Johnson, 2000). Thus, mAchRs located presynaptically on GABA terminals in the STN are receptors that mediate increases in dopamine activity not listed in this figure. References corresponding to citation numbers: 1: Picciotto et al., 1999; 2: Meltzer et al., 1997; 3: Schilstrom et al., 2003; 4: Paladini et al., 1999; 5: Forster et al., 2001; 6: Grillner and Mercuri, 2002; 7: Chergui et al., 1994; 8: Kearney and Albin, 2000; 9: Steiniger and Kretschmer, 2003; 10: Yin and French, 2000; 11: Bonci and Malenka, 1999; 12: Manzoni and Williams, 1999; 13: Prior and Singh, 2000; 14: Charara et al., 2000; 15: Miller and Blaha, 2004.

Subcortical Involvement in PPT HFS-Evoked Nigrostriatal Dopamine Transmission

The present results show that PPT HFS-evoked striatal dopamine release is significantly dependent on activities of the SNc and STN, as inactivation of the SNc or the STN both led to significant decreases in PPT-evoked striatal dopamine efflux compared to pre-drug infusion baseline responses. Thus, these findings suggest that clinical DBS of the PPT

may involve activation of the SNc directly via excitatory PPT-SNc projections and indirectly via excitatory PPT-STN projections that, in turn, provide excitatory inputs to SNc dopamine cells. The relative contributions of the direct and indirect projections to the SNc that are involved in mediating STN HFS-evoked dopamine release are summarized in Fig. 1B. As expected PPT HFS-evoked dopamine release in the striatum is almost fully dependent upon activation of dopamine cells in the SNc, either directly or indirectly. The present data again highlight the significance of the PPT-STN reciprocal connectivity, as neuronal projections through the STN mediate 50.0% of the PPT HFS-evoked dopamine release in the striatum. Thus, the present results suggest that the connectivity of the PPT and STN may be equally as important as the connectivity between the PPT and SNc; however, the neurochemical nature of this pathway has received historically less attention compared to the PPT-SNc projections. For this reason, the present studies included experiments designed to distinguish the neurotransmitters involved in activating the STN following PPT HFS.

STN receptor mechanisms mediating PPT HFS-evoked nigrostriatal dopamine transmission

In further examining the excitatory glutamatergic and cholinergic pathway from the PPT to the STN, the present findings suggest that PPT HFS-evoked striatal dopamine release is dependent upon both glutamate receptors (GluRs) and acetylcholine receptors (AchRs) in the STN, as intra-STN GluR antagonists or AchR antagonists both significantly decreased PPT-evoked striatal dopamine release by 22.5% or 25.8%, respectively. Thus, it seems that combined glutamatergic and cholinergic projections from the PPT to the

STN mediate approximately half of PPT-evoked dopamine release in the striatum. Although the use of broad-spectrum receptor antagonists in these studies precludes identification of specific receptor subtypes, both ionotropic and metabotropic GluRs (i/mGluRs) have been found in the STN (Gotz et al., 1997; Testa et al., 1994). All iGluR subtypes, which are N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate (with the latter 2 sometimes collectively referred to as non-NMDA receptors), have been localized postsynaptically within the STN (Albin et al., 1989; Clarke and Bolam, 1998). Activation of either NMDA and non-NMDA receptors is thought to excite STN neurons, as studies utilizing *in vitro* slice preparations show that the application of both NMDA and non-NMDA glutamatergic antagonists reduce excitatory firing of STN neurons (Chergui et al., 1994; Shen and Johnson, 2000). Future studies recording PPT HFS-evoked striatal dopamine release before and after intra-STN infusion of specific NMDA or AMPA/kainate receptor antagonists would help to determine the relative extent to which these iGluRs within the STN mediate pathways important to modulating striatal dopamine activity from PPT glutamatergic afferents.

The mGluRs are subdivided into 3 groups, I, II, and III, all of which are expressed to some degree in the STN (Testa et al., 1994). The mGluRI and mGluRIII receptors are thought to be located postsynaptically and presynpatically, respectively, while the mGluRII are thought to be located both presynaptically and postsynaptically (Cartmell and Schoepp, 2000; Wang et al., 2000). Activation of mGluRs in the STN has been shown to both increase and inhibit excitation of STN glutamate neurons (Abbott et al., 1997; Awad et al., 2000; Awad-Granko and Conn, 2001). Although the localization of

STN mGluRs and their role in mediating nigrostriatal dopamine release is unclear, it has been suggested that activation of presynaptic mGluRIII subtypes on glutamate terminals reduces STN activity while activation of postsynaptic mGluRII subtypes increases STN activity (Kearney and Albin, 2000). The effects of STN mGluR activation on STN activity and dopamine-related behaviors appears to be occurring through several mechanisms that are still unclear (Kearney and Albin, 2000); therefore, future studies recording PPT HFS-evoked striatal dopamine release before and after infusion of selective mGluR antagonists would be useful in elucidating their role in mediating nigrostriatal dopamine transmission.

The use of a combination of broad-spectrum AchR antagonists in the present studies also prevented identification of the specific STN muscarinic and nicotinic AchRs (m/nAchRs) utilized by PPT afferents to the STN. Cholinergic agonists such as carbachol have been shown to excite STN neurons (Flores et al., 1996); however, nAchR agonists alone had no apparent effect on neuronal cell activity in the STN (Feger et al., 1979). Furthermore, the mAchR antagonist scopolamine, but not the nAchR antagonist mecamylamine, have been shown to block acetylcholine-evoked STN cell excitations (Feger et al., 1979); thus, it may be postulated that STN AchRs are primarily muscarinic (see Fig. 2). The M3 mAchR subtype, in particular, is prominently expressed in the STN (Levey et al., 1994), and Shen and Johnson (2000) found that the mAchR agonist muscarine reduced the amplitude of GABA inhibitory postsynaptic currents, while the effect was blocked by the non-subtype specific mAchR antagonist scopolamine as well as an M3 specific mAchR antagonist. These investigators concluded that muscarinic agonists in the STN act at presynaptic M3 mAchRs on GABA afferents, causing

disinhibition (excitation) of STN neurons, thereby permitting afferents from the PPT to have a greater excitatory influence on STN output. Thus, STN mAchRs, particularly of the M3 subtype, may be involved in the indirect activation of SNc dopaminergic cells via PPT-STN-SNc pathways (Shen and Johnson, 2000).

SNc Receptor Mechanisms Mediating STN and PPT HFS-Evoked Nigrostriatal Dopamine Transmission

Intra-SNc infusions of specific GluR and AchR antagonists helped to uncover the SNc receptor mechanisms that mediate STN and PPT HFS-evoked striatal dopamine release. The present findings suggest that activation of both GluRs and AchRs in the SNc is involved nigrostriatal dopamine transmission elicited by STN or PPT stimulation. In regards to GluRs, the present results suggest that iGluRs in the SNc, compared to mGluRs, play a more critical role in mediating relatively brief excitatory glutamatergic activation of SNc dopamine neurons. Fig. 2 illustrates i/mGluRs, m/nAchRs, and GABA receptors within the SNc and SNr responsible for mediating nigrostriatal dopamine activity. Both NMDA and non-NMDA receptors were shown to be involved in STN HFS-evoked dopamine in the striatum, as evidenced by significantly attenuated responses following intra-SNc NMDA or AMPA/kainate receptor antagonists. These findings were not surprising given that widespread distribution of both types of these subtype iGluRs on dopaminergic neurons in the SNc has been well established (Chatha et al., 2000).

However, the present finding that infusion of an mGluR antagonist into the SNc had no effect on STN HFS-evoked dopamine release in the striatum warrants consideration. Previous studies on the role of SNc mGluR receptors in nigrostriatal

dopamine activity are somewhat conflicting, as activation of mGluRs in the SNc has elicited both excitation and inhibition of nigrostriatal dopamine activity (Bonci et al, 1997; Fiorillo and Williams, 1998; Guatteo et al, 1999; Meltzer et al., 1997; Wang et al., 2005). The mGluRI subtype is the predominant mGluR subtype localized to SNc dopamine cells (Kosinski et al., 1998; Testa et al., 1994); however, group II and III mGluRs are thought to be located presynaptically within the SNc (Mercuri et al., 1993). Activation of group II and III mGluRs on glutamate-containing terminals may attenuate dopamine cell activity by reducing excessive glutamate release onto dopamine cells in the SNc, despite a continuous level of firing activity (Grillner and Mercuri, 2002; Mercuri et al., 1993). Indeed, continuous HFS of the STN has been shown to elicit a rapid increase in striatal dopamine release that quickly abates within two seconds of stimulation to approximately one-third the peak height of the initial increase (Lee et al., 2006), possibly reflecting a delayed inhibitory presynaptic regulation of glutamate release. Thus, it is possible that the the relatively rapid (~1 sec duration) responses seen in the present studies utilizing *in vivo* fixed potential amperometry with STN-stimulation are not affected by the complex, relatively slower, actions of mGluRs. Further studies utilizing longer term stimulation and selective mGluR antagonist infusions into the SNc are needed to clarify the role of these receptors mediating STN and/or PPT activation of SNc dopamine neurons, particularly in consideration that conventional DBS involves continuous stimulation of target structures.

The use of broad-spectrum mAchR and nAchR antagonists in the present studies also did not permit identification of the specific receptor subtypes utilized by PPT afferents to the SNc. However, excitation of midbrain dopaminergic neurons by

muscarine has been shown to be mediated by M1-like receptors (Lacey et al., 1990), and given that relatively high expression levels of mRNA for the M5 mAchR subtype in the SNc and the finding that the M5 subtype is the only mAchR to be definitively localized on SNc dopaminergic cells (Reever et al., 1997; Vilaro et al., 1990), SNc mAchRs of the M5 subtype are thought to be involved in the release of striatal dopamine following PPT stimulation (Forster et al., 2001; Forster and Blaha, 2003). Nicotine administered locally into the SNc increases the firing of SNc dopaminergic neurons and enhances concentrations of dopamine metabolites in the striatum (Lichtensteiger et al., 1976, 1982). Several nAchR subunits, such as α3 to α7 and β2 to β3, have been shown to be present in the SNc (Champtiaux et al., 2002; Charpantier et al., 1998; Goldner et al., 1997; Klink et al., 2001; Wonnacott et al., 2006). In particular, cholinergic inputs from the PPT may enhance nigrostriatal dopaminergic transmission via activation of α4β2 and α7 nAchRs localized on dopaminergic cells in the SNc (Livingstone and Wonnacott, 2009).

Implications of the Current Findings to Parkinson's Disease and DBS

Furthering the current state of knowledge on the interconnectivity between important neural structures which can functionally influence nigrostriatal dopamine transmission allows for insight into potential pharmacological and surgical treatment of basal ganglia-related disorders, such as Parkinson's disease. Results from the series of experiments in Chapters 5 and 6 add to the growing body of evidence supporting an important role of the STN and PPT in modulating striatal dopamine release and subsequent output from the

basal ganglia to the thalamus and motor areas of the cortex, thus influencing motor functioning.

Glutamatergic and cholinergic receptor subtypes as targets for the treatment of Parkinson's disease

As the present results suggest that NMDA and AMPA/kainate receptors both play a significant role in modulating striatal dopamine release evoked by HFS of the STN, pharmacological manipulation of these receptors may be able to alter dysfunctional neurotransmission and thus provide a promising therapeutic target for treating Parkinson's disease. For example, antagonists of NMDA and AMPA receptors have been shown to reverse motor symptoms and levodopa-induced dyskinesias in parkinsonian animal models (Gossel et al., 1995; Klockgether and Turski, 1990; Schwarz et al., 1996). Animal model studies also suggest that altering the activity of these receptors pharmaceutically may even serve to slow disease progression by delaying dopamine neuron degeneration, thought to be associated with excitotoxicity caused by relatively high extracellular levels of glutamate. Specifically, NMDA receptors are known to mediate excitotoxicity caused by high levels of glutamate. Therefore, activation of these receptors in the SNc may contribute to the degeneration of dopamine neurons in this region (Waxman and Lynch, 2005). In support of this argument, NMDA antagonists have been noted to reduce or delay SNc degeneration and motor deficits caused by MPTP administration or 6-OHDA lesioning (Johnson et al., 2009). Thus, blockade of NMDA receptors have been suggested to be a potentially useful strategy for slowing disease progression. However, the widespread expression and diverse functional

roles of NMDA receptors raise concern that targeting these receptors would lead to serious unwanted side effects. Clinical studies have therefore used weak NMDA receptor antagonists and have generally failed to find any therapeutic benefit when administered alone (without levodopa) (Johnson et al., 2009). More promising studies suggest that selectively targeting NMDA receptor subtypes specific to regions relevant to Parkinson's disease pathophysiology may represent safer neuroprotective options (Jin et al., 1997). As such, further clinical studies using more selective drugs targeting NMDA receptors are warranted. Although blocking SNc mGluRs had no effect on relatively brief STN HFS-evoked striatal dopamine release in the present study, pharmaceutical modulation of mGluRs have shown promise in providing neuroprotection of SNc dopamine neurons in animal models of Parkinson's disease (Johnson et al., 2009), further suggesting a role for mGluRs, located presynaptically on glutamate terminals in the STN, in maintaining functional non-toxic basal glutamate levels in the SNc (Grillner and Mercuri, 2002; Mercuri et al., 1993). Therefore, GluRs represent promising targets for the development of nondopaminergic pharmaceutical therapies for the treatment of Parkinson's disease, and more studies are necessary to determine the relative contributions of each receptor subtype in mediating afferent activation of the nigrostriatal dopamine system.

Given the well known functional interactions of the cholinergic systems with the nigrostriatal dopaminergic system (for review see Lester et al., 2010), selective pharmaceutical agents acting on the various AchR subtypes existing heterogeneously at key anatomical sites in the brain also represent promising pharmaceutical targets in the treatment of Parkinson's disease. Historically, anticholinergics were the first available drugs for the alleviation of Parkinson's symptoms, and are still used as secondary

treatments for Parkinson's disease in conjunction with other antiparkinsonian drugs (Katzenschlager et al., 2003). Centrally-acting anticholinergics, all specific for mAchRs, include benztropine (Cogentin), which is widely prescribed, and biperiden (Akineton), orphenadrine (Norflex), and procyclidine (no longer prescribed in the U.S.) (Deleu et al., 2002). Anticholinergic drugs have been used mainly in tremor-predominant cases of Parkinson's disease and are thought to act by counterbalancing the reduced dopaminergic influence on the medium spiny GABAergic output neurons to the globus pallidus (Clarke, 2002; Lees, 2005). Parkinsonian symptoms in mice induced by mAchR agonists can be reduced by systemic administration of a broad-spectrum mAchR antagonist, as well as a mAchR antagonist with moderate selectivity for the M4 mAchR (Betz et al., 2007).

Furthermore, findings that parkinsonian-like symptoms in mice can be reduced by systemic administration a broad-spectrum mAchR antagonist, as well as a mAchR antagonist with moderate selectivity for the M4 mAchR suggests that blockade of M4 mAchR may be beneficial in reducing parkinsonian symptoms (Betz et al., 2007). However, systemic administration prevents the identification of the neural location at which the receptors are being blocked. Intra-PPT infusions of the non-selective mAchR antagonist scopolamine enhances striatal dopamine release and dopamine-dependent behaviors such as locomotion and stereotypy; both of which can be blocked by the cholinergic agonist carbachol infused into the PPT (Chapman et al., 1997; Mathur et al., 1997). These mAchRs are most likely autoreceptors of the M2 family (M2 and M4) as M2 receptors have been localized presynaptically on PPT cholinergic neurons (Vilaro et al., 1992, 1994), and intra-PPT infusion of the M2/4 selective mAchR antagonist

methoctramine has been shown to enhance striatal dopamine release (Miller and Blaha, 2004). Therefore, M2/4 mAchRs are thought to function as cholinergic autoreceptors involved in feedback inhibition at the level of PPT cholinergic cells, regulating information received by the PPT. Blocking these mAchR within the PPT and subsequently increasing PPT activation of SNc dopamine neurons may be therapeutically beneficial for treating the motor symptoms of Parkinson's disease as reduced excitatory cholinergic output from the PPT has been found to result in parkinsonian-like postural deficits, hypokinesia, and locomotor deficits in primates (see Pahapill and Lozano, 2000). Furthermore, cholinergic neurons in the PPT are reduced by nearly 40% in Parkinson's patients, and a significant loss of PPT neurons has been found to correlate with the extent of neuronal loss of dopaminergic cells in the SNc and the severity of Parkinson's disease symptoms (Rinne et al., 2008; Zweig et al., 1989). In sum, increasing activation of PPT neurons via blockade of mAchRs may relieve the motor symptoms of Parkinson's disease by increasing activity of the remaining PPT projection neurons to SNc dopamine neurons.

Findings from animal studies also suggest that nicotine or nAchR agonists may be an effective treatment for the motor symptoms of Parkinson's disease. Stimulation of nAchRs has been shown to modulate locomotor activity in intact nonlesioned animals as well as ameliorate motor dysfunctions in animals with nigrostriatal damage (Meshul et al., 2002; Schneider et al., 1998). Additionally, studies have shown that people who smoke, or have smoked regularly, are 50% less likely to develop Parkinson's disease than those who have never smoked, and nicotine has been found to alleviate parkinsonian cognitive and motor symptoms once Parkinson's disease has developed (see Janhunen

and Ahtee, 2007). The mechanisms underlying these therapeutically beneficial qualities of nicotine are not known. Smoking and nicotine treatment have been shown to protect the nigrostriatal dopaminergic neurons from degeneration following MPTP or 6-OHDA treatment (Costa et al., 2001; Parain et al., 2003). However, acute or short-term treatment with nicotine has shown little to no effects on motor activity in Parkinson's patients or parkinsonian animal models, suggesting that nicotine treatment may only provide a neuroprotective and/or restorative effect with chronic use (see Quik et al., 2007).

Mechanism of action of deep brain stimulation as a treatment for Parkinson's disease

The present studies show that HFS of the MFB, STN, or PPT elicits dopamine release in the dorsal striatum. In relation to DBS as a treatment for Parkinson's disease, the present findings add support for the "dopamine release" hypothesis which proposes that DBS improves motor symptoms related to Parkinson's disease, in part, by activating surviving nigrostriatal dopamine neurons and subsequently increasing striatal dopamine release (Lee et al., 2009; Shah et al., 2010). The present results indicate that MFB stimulation is mediated predominantly by activating ascending SNc dopamine axons, while STN stimulation evokes striatal dopamine release directly via excitatory glutamatergic inputs to SNc dopamine cells as well as, to a lesser degree, indirectly by activating excitatory glutamatergic and cholinergic STN-PPT-SNc pathways. PPT stimulation evokes striatal dopamine release directly by activating glutamatergic and cholinergic pathways to SNc dopamine cells as well as indirectly via activation of glutamatergic and cholinergic PPT-STN-SNc projections. These data may help to explain the clinical improvements in motor symptoms of Parkinson's patients following stimulation of the border and white

matter dorsal to the STN (notably the zona incerta) (Herzog et al., 2004; Saint-Cyr et al., 2002; Voges et al., 2002) and may further suggest that DBS dorsal to the STN (within the MFB), rather than within the STN proper, may be optimal in increasing striatal dopamine levels for therapeutic benefits of Parkinson's disease (Lee et al., 2006).

The present findings also add further support for the PPT as a potential target for DBS as a treatment for certain motor symptoms of Parkinson's disease, as PPT stimulation elicited dopamine release in the striatum similar in magnitude to that of STN stimulation. The dual stimulation of the STN and PPT in clinical DBS procedures is an interesting and promising approach given the connectivity between the two nuclei highlighted in these studies (Stefani et al., 2007). A better understanding of the neural connectivity and mechanisms involved in DBS could potentially revolutionize the procedure and lead to much greater clinical efficacy. For example, expanding on the implications of the dopamine release hypothesis could lead to the next generation of DBS devices in which the system can monitor dopamine neurotransmission during stimulation, thus providing a neurochemical sensing feedback mechanism to maintain dopamine concentrations in the striatum at optimal levels for therapeutic efficacy (Lee et al., 2009).

Future Directions and General Conclusions

Integrity of the nigrostriatal dopamine pathway is critical for the normal processing of sensory-motor information, with disruptions leading to neurological motor disorders, such as Parkinson's disease. The nigrostriatal dopamine pathway and other nuclei within the basal ganglia have many functionally critical interconnections as well as extensive connections with mesopontine glutamatergic and cholinergic pathways, to the

extent that pathology of the PPT is correlated with the motor symptoms of Parkinson's disease (Rinne et al., 2008; Zweig et al., 1989). The electrochemical technique applied in the experiments in Chapters 5 and 6 has provided a method with which to confirm and extend research investigating the neuronal pathways and receptor mechanisms involved in HFS-evoked nigrostriatal dopamine transmission. Findings have thus indicated a complex role of glutamatergic and cholinergic afferents from the STN and PPT in modulating dopamine release in the striatum via direct and indirect routes to the SNc. Together with what is known of the physiological role of i/mGluRs and m/nAchRs in the STN, PPT, and SNc, results highlight the need for further development and application of selective ligands.

The purpose of these studies was to explore the functional interconnectivity between nuclei involved in afferent regulation of nigrostriatal transmission. Therefore, it was necessary to use an intact brain, rather than *in vitro* slice preparations, so that normal neuronal influences on nigrostriatal dopamine release were maintained giving more ecological validity to the measures (Beurrier et al., 2006). However, it has been noted that a possible limitation of *in vivo* monitoring is that it requires deep anaesthesia of animals which may increase the inhibitory responses of the central nervous system (West, 1998). However, this limitation has been minimized by the use of the anaesthetic urethane which has been shown to not interfere with dopamine functioning (Sabeti et al., 2003). Still, the evaluation of STN or PPT HFS-evoked striatal dopamine release, perhaps coupled with behavioral studies, in freely-moving animals would completely eliminate the issue of anaesthetic interference while further elucidating the behaviorally functional roles of the GluRs and AchRs identified in the present studies. Furthermore,

as the present studies were conducted in intact animals, the applicability of the conclusions is limited. Importantly, we have shown that HFS of the STN can elicit measurable dopamine release in the striatum of 6-OHDA lesioned animals, and the amount of HFS-evoked dopamine release correlated with the extent of 6-OHDA-induced denervation (Blaha et al., 2008). Therefore, future experiments using the present neurochemical recording procedures in 6-OHDA lesioned mice are feasible and would provide knowledge of the involvement of these pathways in an animal model of Parkinson's disease.

Nonetheless, the findings of the present studies shed considerable light on the neural connectivity as well the receptor mechanisms involved in mediating HFS-evoked nigrostriatal dopamine transmission. Understanding the influence of the STN and PPT on SNc dopamine cell activity and output of the basal ganglia-thalamocortical motor circuit may lead to novel pharmaceutical therapies as well as a better understanding of the underlying mechanisms of clinical DBS of the MFB, STN, and the interconnected PPT; both of which could lead to improvements the therapeutic efficacy of neuroprotective and symptomatic treatments for Parkinson's disease.

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