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Michele Ann Kliem

# Approval Sheet

Effects of Dopamine D1-Like Receptor Activation in the Internal Pallidal Segment and the Substantia Nigra Pars Reticulata of Normal and Parkinsonian Primates

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# Effects of the Activation of Dopamine D1-Like Receptors in the Internal Pallidal Segment and the Substantia Nigra Pars Reticulata of Normal and Parkinsonian Primates

By

Michele Ann Kliem
B.S. Ashland University, 1992
M.S. University of Toledo, 1994

Adviser: Thomas Wichmann, M.D.

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Graduate Division of Biological and Biomedical Sciences

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## Abstract

This work characterizes the effects of dopamine D1-like receptors (comprised of D1 and D5 receptor subtypes; D1LRs) in the basal ganglia output nuclei and tests the hypothesis that D1LR activation modulates neuronal discharge in the internal pallidal segment (GPi) and the substantia nigra pars reticulata (SNr) under normal and parkinsonian conditions. Most studies have focused on the function of D1LRs in the striatum, the primary input structure of the basal ganglia. However, GPi and SNr also contain a preponderance of D1LRs, occupy a key position in the basal ganglia circuitry and convey information from the striatum and other basal ganglia structures to the thalamus and brainstem.

To test the hypothesis that local injections of D1LR ligands modulate neuronal activity, a recording-injection device was developed that allows the infusion of drugs within 50 to 100 microns of a recorded neuron and the assessment of changes in firing rate and pattern of individual GPi and SNr cells, before, during and after drug injections in awake monkeys. Microinjections of the D1LR agonist (SKF82958) significantly reduced firing rates and enhanced bursts and oscillatory activity in both structures in normal and parkinsonian monkeys. D1LR blockade (SCH23390) had the opposite effect in GPi (not SNr), suggesting a tonic dopaminergic tone in normal monkeys. These data suggest that the previously described pattern changes in parkinsonian animals and human patients are not the result of dopamine loss in GPi or SNr. Microdialysis measurements of GABA levels in GPi and SNr showed that activation of D1LRs increases GABA levels, a finding that is compatible with the notion that D1LR activation inhibits neuronal firing secondary to GABA release from striatal afferents in GPi and SNr. These results suggest

that D1LR activation may have powerful effects on GPi and SNr activity and mediate some of the effects of dopaminergic therapies in Parkinson's disease.

To characterize the potential targets whereby dopamine may mediate its effects in the basal ganglia output nuclei, a series of immunocytochemical studies was carried out to examine the ultrastructural location of D1 and D5 receptors in GPi and SNr. The majority of D1 and D5 receptor labeling was found in unmyelinated axons or putative GABAergic terminals in both GPi and SNr in both conditions. Immunogold studies demonstrate that axonal D1 receptor immunoreactivity was bound to the plasma membrane in normal and parkinsonian monkeys, providing evidence that D1LRs may directly influence basal ganglia outflow under both conditions.

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# Chapter 1

## General Organization of the Basal Ganglia

# 1. A Neuroanatomical Model of Basal Ganglia Function

## 1.1 General Organization of Basal Ganglia Circuits

The basal ganglia (figure 1.1) are components of larger circuits involving the cortex and the thalamus (Alexander et al., 1990) and include the striatum (comprised of the caudate and putamen), external globus pallidus (GPe), internal globus pallidus (GPi), subthalamic nucleus (STN), substantia nigra pars reticulata (SNr) and the substantia nigra pars compacta (SNc). Interactions within the basal ganglia structures are complex. Cortical information is processed in a segregated, but parallel fashion in both the striatum and the STN. It reaches the basal output nuclei, GPi and SNr through a 'direct' monosynaptic GABAergic projection from the striatum, and through an 'indirect' polysynaptic pathway that involves the GABAergic GPe and the glutamatergic STN. GPi and SNr provide tonic inhibition to thalamus and brainstem.

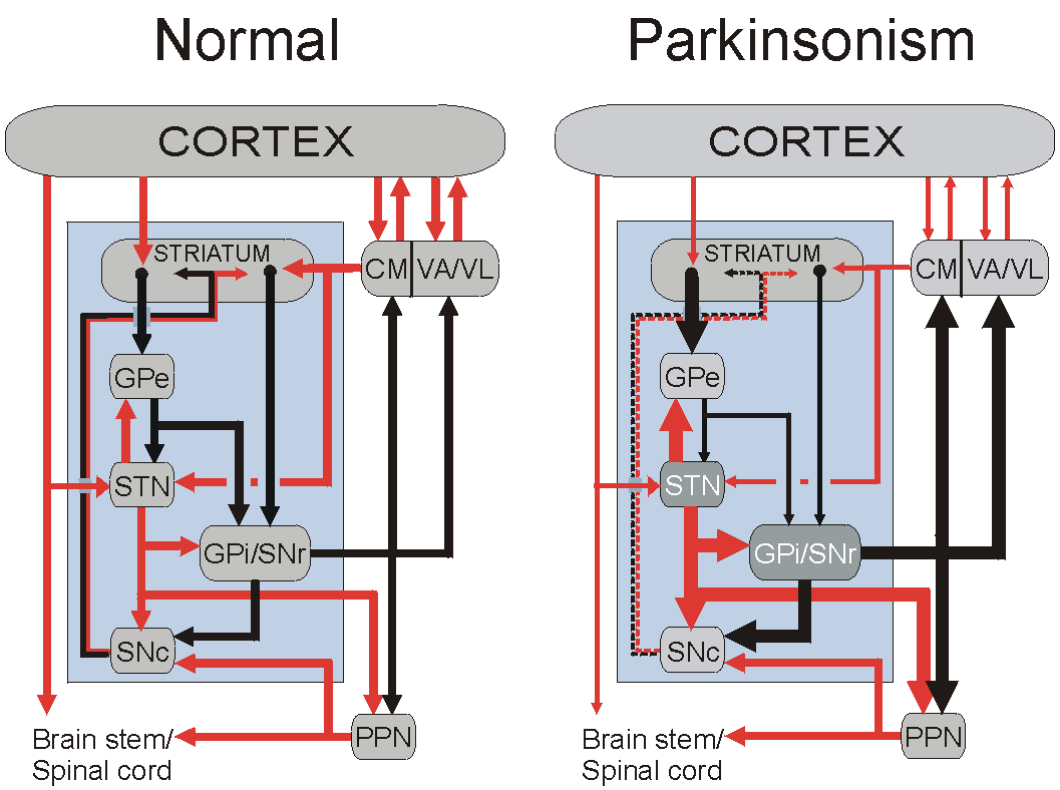



**Figure 1.1** Schematic illustration of the cortico-basal ganglia-cortical circuit under normal (left) conditions and parkinsonism (right). Inhibitory connections are shown in black, and excitatory connections are shown in red. Input to the basal ganglia through cortex and STN connect to the basal ganglia output nuclei, GPi/SNr. GPi/SNr project to thalamic and brainstem nuclei. Abbreviations: CM, centromedian nucleus of the thalamus; VA, ventral anterior nucleus of the thalamus; VL, ventrolateral nucleus of the thalamus; PPN, pedunculopontine nucleus; see text for other abbreviations.

The basal ganglia output nuclei are in a critical position to shape thalamocortical information processing. The involvement of GPi and SNr in executive, motor and limbic functions will be discussed later.

### 1.1.1 Projections to the basal ganglia

The striatal organization of motor, limbic, associative and oculomotor territories is maintained at the level of the basal ganglia output nuclei (Alexander et al., 1986). The direct and indirect pathways are comprised of inputs from distinct functional cortico-striatal domains. While the striatum sends a direct monosynaptic projection to the GPi and SNr, the GPe conveys cortico-striatal information to the basal ganglia output nuclei either directly or via the STN, and this input has been shown to be highly specific (Shink et al., 1996b). It has been demonstrated that neurons within sensorimotor, cognitive or limbic regions project to the same functional territory in GPi. Other studies in monkeys, in contrast, have shown different populations of STN projection neurons based on their collateralization and termination site in GPe, GPi and/or SNr (Sato et al., 2000b).

The focus of this dissertation is to understand the role of dopamine and D1LRs in GPi and SNr. Therefore, anatomical connectivity differences at the level of the basal ganglia output nuclei may be important to explain the functional role of dopamine in GPi and SNr. For instance, anatomic studies have demonstrated that GPi receives input from separate cortical regions, predominantly the pre-and post-central sensorimotor fields, while the SNr receives inputs primarily from associative and limbic regions (Middleton and Strick, 2000). These findings are compatible with functional studies. In task-performing monkeys, GPi neurons show movement-related responses specific for

kinematic parameters (Georgopoulos et al., 1983; Mitchell et al., 1987; Turner and Anderson, 1997) while neurons in the SNr show movement preparation- and attention-related responses in the SNr (Wichmann and Kliem, 2004). Recently, a PET study revealed a link between dopamine levels in the GPi and the pre-supplementary motor area providing evidence for a motor-learning interaction between GPi and the cortex in humans (Garraux et al., 2007). Thus, pallidal D1LRs may be involved primarily in the control of movement, while nigral D1LRs may be more important for associative and limbic functions.

Although this model of basal ganglia circuitry has helped us to understand basal ganglia disorders, especially Parkinson's disease, anatomical and physiological studies have challenged this classical view. Most notably, anatomic connectivity studies particularly of the GPe and STN suggest that they are likely more involved in the control of the intrinsic basal ganglia circuitry than originally thought (Parent and Hazrati, 1995b). For instance, the classic model suggests that GPe is a relay station that provides tonic inhibitory control over the STN. However, track-tracing studies have demonstrated that GPe efferents to the STN are largely separate from the STN projections to GPi and SNr (Parent et al., 2000). Although some of the early track-tracing studies were limited because of technical challenges and resulted in very large injections of the tracers, strong evidence suggests that the GPe-STN projection is specific (Sato et al., 2000a; Shink et al., 1996a). However, the specificity of the STN-GPe projection is less clear. In favor of the 'diffuse' view, anterograde tracer injections into the STN profusely labeled the monkey GPe (Sato et al., 2000b). In contrast, a similar tracing study revealed a highly ordered and specific relationship between neurons of GPe and STN, supporting a 'specific' view

of the STN-GPe projection (Shink et al., 1996a). However, electrophysiology studies in the parkinsonian state are not consistent with a specific STN-GPe interaction since increased activity of the STN is not consistently associated with a reliably less active GPe (Levy et al., 1997). Furthermore, the STN receives additional inputs aside from the GABAergic input from the GPe such as glutamatergic input from the cortex and the parafascicular nucleus and dopaminergic input from the SNc (Brown et al., 1979; Francois et al., 2000; Hassani et al., 1997; Lavoie et al., 1989). Similar to GPe projections, the STN also projects to the GPi and SNr (Sato et al., 2000a; Shink et al., 1996a).

### 1.1.2 Direct and indirect outflow pathways

The striatum is the largest and most complex structure of the basal ganglia. It is composed of mostly GABAergic medium-size spiny neurons (90%) that can be divided into two populations based on their neuropeptide content, dopamine receptor expression and their projection site. Striatal projection neurons that contain substance P and express D1/D5 receptors target GPi or SNr and form the 'direct' pathway, while the other population expresses enkephalin and D2 receptors and targets the STN via GPe to make-up the 'indirect' pathway. This traditional model suggests that coordinated movements are a result of finely tuned balance between these two pathways. For instance, activation of the direct pathway would increase GABA release in GPi and SNr, followed by a reduction in tonic inhibition of the GABAergic neurons that project to the thalamus. This results in the disinhibition of thalamic projection neurons, thereby leading to an excitation of cortical neurons. Because of the multi-stage set-up in the indirect pathway,

activation results in a different outcome. Stimulation of GPe neurons results in tonic disinhibition that ultimately leads to increased STN activity and tonic inhibition of thalamocortical circuits. Thus coordinated movement requires coordination between the direct and indirect pathways.

It is thought that dopamine plays an important modulatory role in movement. In situ hybridization studies support dopamine receptor segregation at the striatal level (Gerfen et al., 1990; LeMoine et al., 1990) with D1 receptors preferentially expressed by direct pathway neurons and D2 receptors by indirect pathway neurons. Thus, this segregation of dopamine receptors supports the idea that dopamine acts to facilitate movement mediated by D1LRs, and inhibit movement via D2 receptors (Gerfen et al., 1990). However, other functional and anatomical studies argue to the contrary, and suggest that D1LRs and D2LRs coexist in nearly all striatal projection neurons (Aizman et al., 2000; Surmeier et al., 1992; Surmeier et al., 1993).

The traditional basal ganglia circuitry model explains that the absence of dopamine induces changes that ultimately lead to an increase in the firing rate of GPi and SNr neurons. The model proposes that increased activity of striatal neurons of the indirect pathway result in reduced activity of GPe neurons and increased activity of STN neurons, along with reduced activity of striatal neurons of the direct pathway, resulting in a generalized increase of activity of GPi and SNr neurons. This increase in activity of the basal ganglia output neurons results in disinhibition of thalamocortical neurons thereby producing akinesia and bradykinesia, two of the primary symptoms of Parkinson's disease. However, studies in parkinsonian animals and human cannot be fully explained by the traditional basal ganglia circuitry model. For instance, electrophysiologic

experiments in rodents (Allers et al., 2000; Ruskin et al., 2002) and primates (Bergman et al., 1998; Filion and Tremblay, 1991; Miller and DeLong, 1987; Murer et al., 1997) have demonstrated prominent changes in discharge patterns of basal ganglia neurons, which can not be easily addressed with the traditional model. In addition, biochemical studies have suggested that the observed changes in GPe discharge, and changes in the direct pathway may not be sufficient to explain the altered discharge patterns reported in STN, GPi and SNr (Chesselet and Delfs, 1996). Together, these data imply that our knowledge of the basal ganglia circuitry continues to evolve, and the loss of dopamine in GPi and/or SNr is among several possibilities to explain these discrepancies. In fact, extrastriatal dopamine in the basal ganglia is a key component of many of the new attempts to explain the pathophysiology of PD.

### 1.1.3 Intrinsic organization of the GPi and SNr

GPi and SNr are considered one structure in whales and porpoises (Riese, 1924), and are often thought to be one nucleus separated by the internal capsule (DeLong et al., 1983; Mirto, 1896). Similarities have indeed been described for GPi and SNr neurons in the monkey. For instance, both populations of neurons use GABA as a neurotransmitter, and have a common morphologic structure. In the coronal plane, Nissl-stains reveal that both GPi and SNr contain large (~25 μm) and irregularly shaped neurons with the greatest number of Nissl-stained neurons located in the anterior portion of GPi, and rostral SNr sections (Hardman et al., 2002). However, the dendritic arborizations of GPi and SNr neurons were shown to be different using Golgi impregnation techniques. For instance, GPi neurons are disc-shape and lie perpendicular to striatal afferents (Yelnik et

al., 1984), while SNr neurons show a variety of shapes such as spindle or polygonal (Yelnik et al., 1987).

Other structural differences have been described for GPi and SNr neurons. For instance, nigral dendrites are generally thinner than pallidal dendrites and therefore can reach the narrowest regions of striatal axon bundles, suggesting that SNr neurons can receive inputs from different functional striatal territories (Francois et al., 1987). Even though the size of their dendritic arborizations may differ, the spatial organization of their dendrites is similar. In other words, both nuclei have dendrites deep within striatal axon bundles in such a way that one dendritic tree can envelop a striatal bundle (Francois et al., 1987; Percheron et al., 1984a; Percheron et al., 1984b).

Both GPi and SNr are comprised of GABAergic projection neurons that primarily target the thalamus and to a lesser extent the brainstem. Different classes of projection neurons have been identified in both nuclei based on their target nucleus. The primary thalamic target of GPi is the ventroateral nucleus (Arecchi-Bouchhioua et al., 1997; Hazrati and Parent, 1991; Parent et al., 2001). Two types of projection neurons have been identified in GPi using anterograde tracing techniques and three-dimensional reconstruction of serial sections in monkeys (Parent et al., 2001). Centrally located GPi neurons preferentially target the thalamus, terminating in the ventrolateral nucleus and sending collaterals to the centromedian and parafascicular nucleus, but they also send projections to the pedunculopontine tegmental nucleus (Parent et al., 2001). To a lesser extent, neurons located peripherally target the anterior thalamic nuclei with some collaterals to the lateral habenular nucleus (Parent et al., 2001). Interestingly, neither type of projection neuron targeted the ventromedial thalamus, which is the primary target

of the SNr (Ilinsky et al., 1985). In rats, four types of SNr projection neurons have been identified using the same single-axon tracing methods described for GPi. One type of projection neuron targets the thalamus almost exclusively. For instance, arborizations were found in ventromedial, ventrolateral, mediodorsal and the parafascicular/centromedial complex. Two other neuronal populations target the thalamus and either the superior colliculus, pedunculopontine tegmental nucleus or periaqueductal gray matter. The fourth type of SNr projection neuron innervated the superior colliculus and deep mesencephalic nucleus (Cebrian et al., 2005). Axon collaterals are a common feature in the rat SNr, and have been shown to be specific and topographically organized (Mailly et al., 2003). A similar system has not been studied in monkeys.

The variation and volume of GPi and SNr projection neurons that target thalamus and brainstem favors a parallel mode of information processing before being conveyed to the cerebral cortex. Additionally, this type of organization suggests that single neurons may have substantial influence on functionally different neuronal systems (e.g. thalamus, cortex and brainstem regions).

### 1.1.4 SNr projections

In the rat SNr, striatal inputs are conserved into two functional territories: dorsolateral sensorimotor and a ventromedial associative territory (Deniau and Thierry, 1997), supporting the idea of segregated channels that represent multiple cortical areas. The thalamus is the main recipient of projections from the SNr. In monkeys, the medial SNr sends a projection to the ventroanterior nucleus and the mediodorsal nucleus (Ilinsky

et al., 1985), while the lateral SNr innervates the lateral portion of the ventroanterior nucleus and the mediodorsal thalamus. Each thalamic projection innervates a distinct functional area of cortex including premotor, oculomotor, prefrontal and inferotemporal areas (Middleton and Strick, 2000). In addition, SNr also innervates the PPN (Granata and Kitai, 1991; Parent and Hazrati, 1995b), superior colliculus (Jayaraman et al., 1977), the medullary reticular formation (von Krosigk et al., 1993) and parafascicular nucleus (Sidibe et al., 2002). Through these connections, SNr can transmit oculomotor information including the control of saccades (Wurtz and Hikosaka, 1986) and orofacial movements (Chandler et al., 1990).

### 1.1.5 GPi projections

Similar to the SNr, GPi can be divided into functionally distinct territories based on corticostriatal innervation. The ventrolateral portion of GPi receives input from sensorimotor areas while the dorsal and medial portions of GPi contain inputs from associative and limbic areas, respectively (Parent, 1990). GPi strongly innervates the thalamus. In monkeys, the sensorimotor GPi projects to the ventrolateral nucleus while associative and limbic information reaches the ventroanterior and ventrolateral nucleus (Sidibe et al., 1997). Through the thalamus, information from the GPi is conveyed back to distinct functional cortical regions including supplementary motor area, motor cortex, ventral premotor cortex, and prefrontal cortex that are involved with processing primarily cognitive and skeletomotor functions (Middleton and Strick, 2000). Other important targets of GPi innervation include the PPN (Shink et al., 1997), the habenular nucleus (Parent et al., 2001) and centromedian and parafascicular nuclei (Sidibe et al., 2002).

### 1.1.6 Role of GPi and SNr in Motor Control

As described above, coordinated activity between the direct and indirect pathways are required for coordinated movements. Skeletomotor cortical regions likely activate striatal neurons that project to the direct pathway which in turn inhibit GPi and SNr neurons, facilitating movement. Activation of indirect pathway neurons, in contrast, would lead to the opposite effect or disinhibit GPi and SNr neurons, thereby suppressing movement. Evidence from recording studies in task-performing monkeys has demonstrated support for the involvement of both GPi and SNr in at least some aspects of movement control. The monkey GPi has been more widely studied in this regard. Changes in discharge during movement tasks suggest that GPi activity is related to movement kinematics (Georgopoulos et al., 1983; Mitchell et al., 1987; Turner and Anderson, 1997), movement involving sequential segments (Mushiake and Strick, 1995), movement preparation (Brotchie et al., 1991; Georgopoulos et al., 1983; Jaeger et al., 1993) and the expectation of reward (Gdowski et al., 2001). In contrast, strong anatomic evidence in monkeys suggests that SNr activity is more concerned with non-motor functions. Electrophysiologic studies in task performing monkeys have demonstrated that individual SNr cells respond to memory, attention and movement preparation (Wichmann and Kliem, 2004).

## 1.2 Projections of the Substantia Pars Compacta

### 1.2.1 Nigro-striatal Pathway

The mesotelencephalic dopaminergic system is comprised of three main groups of neurons. The ventral tegmental and the retrorubal areas (Dahlstrom and Fuxe, 1964)

project primarily to the nucleus accumbens and to the cortex, and are important in reward, emotion and memory functions (Willner and Scheel-Kruger, 1991). The tubular-infundibular pathway, originating in the hypothalamus is involved in neuroendocrine regulation, while the substantia nigra pars compacta (SNc) neurons are involved with motor and non-motor functions. The compact arrangement of the large SNc neurons can be visualized using tyrosine hydroxylase immunocytochemistry techniques in rats (Bjorklund and Lindvall, 1984; Ungerstedt, 1971) and monkeys (Felton and Sladek, 1983; Garver and Sladek, 1975). The ascending fibers of the SNc run dorsolaterally to the striatum making up the nigrostriatal dopaminergic system. It is this set of dopamine neurons that degenerates in Parkinson's disease (PD, Hornykiewicz and Kish, 1987).

Dopamine neurons of the SNc can be divided into two groups based on morphological and chemical characteristics. Dorsal tier neurons are arranged loosely and have low tyrosine hydroxylase concentrations. Dendrites from dorsal tier dopamine neurons extend in a mediolateral direction and dorsal to the densocellular area of the SNc. These densely packed cellular clusters lie dorsal to the SNr. The ventral tier neurons are large and polygonal in shape, are also tightly packed, but their dendrites extend deep into the SNr and contain high concentrations of the dopamine transporter (DAT), which serves as a site of dopamine re-uptake (Gerfen et al., 1985; Haber and Fudge, 1997; Haber et al., 1995). Ventral tier neurons are particularly vulnerable to the neurodegenerative processes leading to PD.

The striatum is the main target of nigral dopaminergic neurons, and this pathway can be organized into functional domains in monkeys (Haber and Fudge, 1997). The ventral tier dopamine neurons send projections to the sensorimotor striatum, and show

the highest levels of dopamine transporter labeling in rats (Ciliax et al., 1995; Freed et al., 1995). The associative region of the striatum receives dopaminergic input mainly from the densocellular region, while the limbic portion of the striatum is innervated by both dorsal and ventral tier neurons (Haber and Fudge, 1997). Although SNc projection neurons preferentially target a functionally specific striatal region, they are also found broadly distributed within the nigra (for review, see Haber and Fudge, 1997).

Parkinson's disease is a progressive neurodegenerative disorder caused by the loss of melanin-containing neurons of the substantia nigra pars compacta (Ehringer and Hornykiewicz, 1960). The resulting loss of dopamine in the striatum from the SNc is thought to be associated with the hallmark motoric symptoms of PD, such as akinesia, tremor, rigidity and bradykinesia. However, the link between striatal dopamine loss and the behavioral signs of PD remains uncertain. Generally, the motor symptoms of PD appear when about 70-80% of the striatal dopamine has been depleted (Bernheimer et al., 1973). Degeneration of SNc projections to other basal ganglia structures may also contribute.

### 1.2.2 Dendritic dopamine release in the substantia nigra pars reticulata

Although the SNc sends a massive projection to the striatum, dopamine also reaches the basal ganglia output nuclei. Dendrites from ventral tier SNc neurons extend dorsoventrally into the SNr, and often form bundles (Francois et al., 1987). Additionally, they contain the highest concentration of DAT and are vulnerable to neurodegeneration in PD and to MPTP-induced toxicity (Haber and Fudge, 1997). Golgi staining and electron microscopy studies show that dopamine is stored in vesicles of dendrites (Bjorklund and

Lindvall, 1975; Cajal, 1955; Rinvik and Grofova, 1970). However, the question whether dopamine release from SNc dendrites is vesicular remains unanswered.

Evidence of dopamine release from dendrites of SNc neurons into the SNr comes mainly from studies conducted in rodents and cats. Similar to axons, dendrites of SNc neurons are capable of initiating action potentials (Bergquist et al., 1998; Grace and Bunney, 1983; Hounsgaard et al., 1992) which can lead to the release of dopamine, an effect that can be blocked by TTX (Elverfors et al., 1997; Robertson et al., 1991a; Santiago and Westerink, 1992) and is calcium dependent (Bergquist et al., 1998; Chen and Rice, 2001). Local application of a DAT inhibitor (nomifensine) into the SNr significantly increases dopamine levels in the rat SNr, with no effect on striatal dopamine concentrations, (Robertson et al., 1991a).

Although the release of dopamine from vesicles may be a matter of debate (Groves and Linder, 1983; Nirenberg et al., 1996a), studies have demonstrated that reserpine an inhibitor of vesicular monoamine transport, significantly depletes dopamine stores and decreases its release (Bjorklund and Lindvall, 1975; Heeringa and Abercrombie, 1995). However, additional mechanisms may account for somatodendritic release that differ compared to terminal dopamine release. For example, some studies have shown that dopamine release from dendrites may also occur through calcium-independent mechanisms (Bergquist et al., 1998; Bjorklund and Lindvall, 1975), from the smooth endoplasmatic reticulum (Mercer et al., 1979; Nirenberg et al., 1996a), or by reversal of the dopamine transporter (Falkenburger et al., 2001). Another difference between dendritic and terminal dopamine release was studied using microdialysis in rats demonstrating that somatodendritic release occurs independent of synaptobrevin, a

vesicle associated membrane protein found in both dendrites and axon terminals (Bergquist et al., 2002).

Input to the SNc influences the release of dopamine from nigral dendrites. For instance, activity of GABAergic inputs may reduce the spontaneous release of dopamine from dendrites, an effect likely mediated through GABA-A receptors, since GABA-A receptor blockade in the rat SN significantly increased dopamine concentrations in the SN (Cobb and Abercrombie, 2002). In contrast, increased activity of glutamatergic input from the STN to the SN had the opposite effect on dendritic dopamine release after systemic administration of haloperidol, a dopamine receptor antagonist (Cobb and Abercrombie, 2002; Cobb and Abercrombie, 2003b). Although the precise mechanism of action of haloperidol is unknown, it is thought to preferentially block D2 receptors. These studies suggest that somatodendritic dopamine release is differentially regulated, such that, under normal conditions, GABA regulates dendritic release of dopamine in the SN, and when the dopaminergic system is acutely disrupted with systemic injections of haloperidol, glutamate receptor functions are important. In addition, exposure to high concentrations of potassium (Robertson et al., 1991a) or amphetamine (Gerhardt et al., 2002; Hoffman and Gerhardt, 1999; Robertson et al., 1991a) can also increase dendritic dopamine release.

### 1.2.3 SNc-GPi pathway

The SNc also sends a direct dopaminergic projection to the GPi that is largely separate from the striatum (Jan et al., 2000; Smith et al., 1989). Consistent with the idea that dopamine reaches the GPi, immunocytochemical studies have revealed TH-positive

terminals forming symmetrical synapses in the monkey GPi (Smith and Kieval, 2000). Studies in MPTP-treated monkeys have revealed that the SNc-GPi projection is relatively spared in the face of massive destruction of the nigrostriatal tract (Parent et al., 1990), supporting the idea that the SNc-GPi pathway is separate from the SNc-striatal pathway. The resilience of the SNc-GPi pathway may be due to the failure of MPTP to induce overt parkinsonian symptoms in some monkeys. For instance, MPTP-treated monkeys displaying parkinsonian motor symptoms show significant declines in dopamine (Pifl et al., 1990; Pifl et al., 1992) or dopamine fibers in GPi (Jan et al., 2000), suggesting the importance of dopamine actions in this nucleus under normal conditions. Although the functional role of dopamine in GPi is unknown, a recent PET study demonstrated a relative reduction of dopamine levels in GPi with learning (Garraux et al., 2007).

### 1.2.4 SNc projections to GPe and STN

The STN and to a lesser extent the GPe receives significant dopaminergic input from the SNc (Brown et al., 1979; Cragg et al., 2004; Jan et al., 2000; Lavoie et al., 1989; Meibach and Katzman, 1979; Parent and Smith, 1987; Prensa et al., 2000). Injections of retrograde tracers into either the GPe or STN retrogradely label SNc cells (Francois et al., 2000; Hassani et al., 1997; Smith and Bolam, 1989), which is consistent with injections of antergrade tracers into the SNc that anterogradely label fibers and terminals in the STN (Francois et al., 2000; Gauthier et al., 1999; Hanley and Bolam, 1997). Immunocytochemistry studies revealed GABAergic inputs from the GPe reach the SNc in rats, but were significantly fewer compared to the inputs to the SNr (Smith and Bolam, 1989).

The presence of dopamine in the STN was confirmed using voltammetry techniques in which local electrical stimulation of the STN significantly increased STN dopamine levels in rat slices. Furthermore, the stimulus-evoked release of dopamine was markedly extended after local application of a DAT blocker, suggesting that DAT acts to regulate STN dopamine concentrations (Cragg et al., 2004). In the parkinsonian state, immunocytochemistry studies have demonstrated significant dopaminergic loss in the GPe (Jan et al., 2000) and STN (Francois et al., 2000) of humans and monkeys. However, the consequences of dopamine loss to these structures are not clear, but may contribute to the pathophysiology and/or the motor signs associated with Parkinson's disease.

# Chapter 2

Dopamine Receptors

# 2. Dopamine Receptors

## 2.1 Introduction

Dopamine is important in motor control, cognition, emotion, neuroendocrine regulation, reward and cardiovascular regulation. The dopaminergic systems have been implicated in various diseases such as schizophrenia, tardive dyskinesia, hyperprolactinemia, dystonia and Parkinson's disease.

Physiological actions of dopamine are mediated by two families of G protein-coupled receptors (Table 2.1, for review see Emilien et al., 1999; Table 2.1, for review see Gingrich and Caron, 1993; Huang et al., 2001), D1-like receptors (D1LR, comprised of D1 and D5, see Clark and White, 1987; Neve, 1997) and D2-like receptors (D2LR, comprised of D2, D3 and D4, see Neve, 1997). Activation of D1LRs stimulates adenylate cyclase activity, while D2LRs usually have the opposite effect (Garau et al., 1978; Kebabian and Calne, 1979; Kebabian et al., 1972; Neve et al., 1989; Sunahara et al., 1991). Five distinct genes corresponding to the five receptor subtypes have been identified, all of which are members of the G protein coupled receptor (GPCR) family (Jenner and Demirdemar, 1997; Neve, 1997). Both receptor families are homologous within their transmembrane regions, but they show divergence in their coding regions, in which D1LR genes lack introns, a typical feature of GPCRs (Civelli et al., 1993; O'Dowd, 1993). Introns within the coding regions permit the formation of receptor variants, which is the case for two D2 receptor isoforms. The focus of the present thesis is the D1LRs.

**Table 2.1. Comparison of Dopamine Receptor Subtypes**

| | D1 Family | | D2 Family | | |
|---|---|---|---|---|---|
| | D1 | D5 | D2 | D3 | D4 |
| Molecular Structure | | | | | |
| (Size: amino acids) | 446[a] | 477[a] | 444[g] | 446[g] | 387[g] |
| Molecular Weight (kD) | 46[b] | 53[g] | 47[k] | ~50[o] | 41[q] |
| Function | | | | | |
| Adenylate cyclase | Stimulates[c] | Stimulates[h] | Inhibits[c,l] | Inhibits[g,p] | Inhibits[g,p] |
| Phosphoinositol turnover | Stimulates[d] | Stimulates[i] | Inhibits[g] | | |
| Protein kinase A | Stimulates[e,f] | Stimulates[j] | | | |
| Phospholipase C | Stimulates[d] | | | | |
| Phosphoprotein DARPP-32 | Stimulates[g] | | Inhibits[g] | | |
| K+ conductance | | | Enhances[m] | | |
| Ca2+ conductance | | | Inhibits[g] | | |
| L-type Ca2+ channels | Stimulates[e] | | | | |
| [Ca2+]i | Stimulates[d] | | Reduces[n] | | |

Abbreviations: kD, kilodalton; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kD

References: a, Huang et al., 2001; Jarvie et al., 1989; Kebabian and Calne,1979; d, Undie et al., 1990,1994, e, Surmeier et al., 1995; f, Liu et al., 1992; g, Cooper et al., 2003; h, Sunahara et al., 1991; i, Friedman et al., 1997; j, Yan and Surmeier, 1997; k, Bunzow et al., 1988; l, Albert et al., 1990; m, Einhorn et al., 1990; n, Surmeier et al., 2007; o, Nimchinsky et al., 1997; p, Neve et al., 1989; q, Van Tol et al., 1991

## 2.2 D1LRs

The genes for the D1 and D5 receptors are located on chromosome 5 (Grandy et al., 1991; Sunahara et al., 1991), and chromosome 4 (Gusella, 1989), respectively. The degree of homology in the transmembrane region for the D1 and D5 receptors is about 78%. Although it is thought that the intracellular domains are in large part responsible for the dopamine binding and the coupling of dopamine receptors to G proteins, D1 and D5 receptors show the highest level of homology outside this region (Dohlman et al., 1991).

D1 and D5 receptors both couple to $G\alpha_s$ (Kimura et al., 1995; Uh et al., 1998), and may also couple to other G proteins (Herve et al., 1993; Sidhu and Niznik, 2000). It is also been proposed that D1 receptors may influence intracellular calcium concentrations. One possible mechanism is through the stimulation of phosphatidylinositol (PI) hydrolysis by phospholipase C which increases 1,4,5-triphosphate levels and modulates intracellular calcium. Although evidence exists that supports this hypothesis (Liu et al., 1992b; Undie and Friedman, 1990; Undie and Friedman, 1994), other studies have failed to show changes in PI hydrolysis (Dearry et al., 1990; Pedersen et al., 1994; Tiberi et al., 1991). D1 receptors may also couple to phosphokinase A to modulate calcium channels. For example, D1 receptor stimulation increases L-type and reduces N- and P/Q-type calcium currents in rat medium spiny neurons (Surmeier et al., 1995).

Although the mechanism in which G proteins interact with D1LRs is not clear, it is thought that the third intracellular loop and the carboxy tail may be important sites of action since they show significant divergence (Civelli et al., 1992; Gingrich and Caron,

1993; O'Dowd, 1993). An interesting finding when the D5 receptor was first identified was that it had a 10-fold higher affinity for dopamine than the D1 receptor (Sunahara et al., 1991). Studies in cell lines support this finding. For instance, chimeras of both receptor subtypes were prepared in which a conserved transmembrane domain of the D1 receptor was replaced with the D5 receptor. In this preparation, the D1 receptor displayed similar affinity for the D5 receptor (Iwasiow et al., 1999). Similarly, behavioral studies show different functional effects in rats treated with D1LR agonist that shows a higher affinity for the D5 receptor (Nergardh et al., 2005), suggesting that D1 and D5 receptors may have different functions.

Another difference between D1LRs is their pattern of expression in the brain. In situ hybridization studies have shown that D1 receptors are more abundant and widely distributed than all dopamine receptors (Dearry et al., 1990; Fremeau et al., 1991b; Sunahara et al., 1991; Tiberi et al., 1991; Weiner et al., 1991). A preponderance of D1 receptors are expressed in regions that are associated with dopaminergic functions such as the striatum. Additionally, D1 mRNA and protein are located in the cerebral cortex, hypothalamus, thalamus and olfactory tubercle. Other basal ganglia structures also contain high concentrations of D1LRs. For instance, binding studies have shown that the SNr contains the densest binding, second only to that of the striatum, in monkeys (about 40-50%, D1LR binding in the SNr compared to D1LR binding in the striatum; (Besson et al., 1988; Richfield et al., 1987). The GPi also contains fairly high levels of D1LR binding in monkeys as compared to the striatum (~20-30%, Richfield et al., 1987). Because a ligand that can reliably distinguish between the D1 receptor and the D5 receptor is not available, the results from binding studies are pooled together. However,

immunocytochemical studies that use receptor specific antibodies have demonstrated that D1 receptors in GPi and SNr are preferentially localized on striatal GABAergic afferents. The lack of an mRNA signal in these nuclei along with evidence from animals with striatal lesions that showed marked reductions in D1LR binding are consistent with the finding that high concentrations of D1LRs are presynaptically located in both the monkey and rat basal ganglia output nuclei (Barone et al., 1987; Caille et al., 1996; Fremeau et al., 1991b; Levey et al., 1993; Yung et al., 1995a).

D1LRs are also found outside the central nervous system. For example, they are expressed in the kidney, vascular beds of the heart (Amenta et al., 1999; Amenta et al., 1995) retina, lymphocytes (Ricci et al., 1999; Takahashi et al., 1992), the superior cervical sympathetic ganglia and dorsal root ganglia (Xie et al., 1998), the adrenal gland (Aherne et al., 1997), parathyroid, and the gastrointestinal tract (Vaughan et al., 2000). D1LRs in the kidney and heart are not linked to adenylate cyclase activity (Felder et al., 1989; Niznik et al., 1989; Sandrini et al., 1986), but to other signaling systems such as phospholipase C. In the heart, D1LR activation increases contractility and cardiac output, natriuresis and vasodilatation. However, the concentration of dopamine may lead to different effects in the cardiovascular system (Girbes and Hoogenberg, 1998). For instance, at higher concentrations, dopamine can affect other neurotransmitter systems such as $\alpha$- and $\beta$-adrenergic receptors (Smit, 1989). Although radioligand and autoradiographic studies have confirmed the presence of D1LR on the walls of systemic arteries, other studies have demonstrated that D5 receptors are preferentially expressed on systemic blood vessels in the heart, suggesting a site of action to influence cardiac functions (Amenta et al., 1993). Similarly, D5 receptors are also preferentially expressed

on systemic blood vessels in the kidney. D1LR stimulation in the kidney dilates blood vessels and leads to diuresis and natriuresis (Lokhandwala et al., 1990). Endogenous dopamine secreted by the renal tubules regulates sodium secretion, and disruption of this peripheral dopaminergic system has been thought to be associated with genetic hypertension (Jose et al., 1992). However, other factors may influence dopamine's actions to regulate natriuretic control in the circulatory system including the administration of dopamine agonists. The exact role of dopamine in these areas is not clear. D1LR pharmacology and function in these regions is currently being investigated.

## 2.3 D2LRs

In contrast to D1LRs, the genes encoding D2LRs contain introns. The D2 receptor coding region is interrupted by six introns, the D3 receptor contains five introns and the D4 receptor three (Giros et al., 1989; Grandy et al., 1989; Sokoloff et al., 1990; Van-Tol et al., 1991). The presence of introns in the coding regions allows for splice variants of a receptor. Indeed, two splice variants have been described for the D2 and D3 receptors (Gingrich and Caron, 1993; Giros et al., 1991). Other differences exist between the locations of the genes encoding the D2 family of receptors. For instance, the human D2 and D4 receptor genes are located on chromosome 11 (Grandy et al., 1989; Van-Tol et al., 1991), while the D3 receptor gene is found on chromosome 3. Another difference between D2LRs (and D1LRs) is the degree of conservation within the transmembrane region. The D2 and D3 receptors are ~75% homologous, while the D4 receptor is about 50% similar (Gingrich and Caron, 1993). It is worth mentioning that all dopamine

receptors contain several conserved residues in the transmembrane region, which is important for dopamine binding (Strader et al., 1989).

As mentioned for the D1LRs, the intracellular domains between the transmembrane regions are important for specificity and coupling of dopamine receptors to G proteins. Unlike the D1LRs, D2 and D3 receptors are fairly homologous in these regions. However, the D4 receptor is less so (Gingrich and Caron, 1993). Consistent with this observation, the pharmacological properties of D2 and D3 receptors are fairly similar, while the D4 receptor has shown lower affinities for most dopamine antagonists, suggesting this receptor may have different functions. The second messenger system of the D2 receptor has been extensively studied. The D2 receptor inhibits adenylyl cyclase (Albert et al., 1990; Dal-Toso et al., 1989; Neve et al., 1989; Vallar et al., 1990) and activates potassium channels (Einhorn et al., 1990). The intracellular mechanism(s) for the D3 and D4 receptors have not been well characterized.

In situ hybridization and mRNA studies have shown that D2 receptors are preferentially expressed in brain areas that receive massive dopaminergic input, such as the striatum and the olfactory tubercle. They are also located in nuclei that give rise to dopamine projections such as the SN and VTA, suggesting that D2 receptors are important in regulating the activity of dopamine neurons.

In contrast, D3 receptors are mostly found in brain regions innervated by mesolimbic dopamine neurons, such as the olfactory tubercle, islands of Calleja and nucleus accumbens (Landwehrmeyer et al., 1993; Meador-Woodruff et al., 1994). Also, a strong D3 receptor mRNA signal was demonstrated in the olfactory tubercle, hypothalamus and nucleus accumbens. In monkeys, D3 receptor mRNA was also found

in basal ganglia regions such as the caudate and putamen in monkeys. Additionally, D3 receptor mRNA was detected to a lesser degree in SN and VTA, suggesting that this receptor may also have autoreceptor functions. Binding studies have demonstrated D3 receptors in the basal ganglia, cortex, SN, hippocampus and amygdala (Lahti et al., 1995). In the same brain regions, D3 receptor expression is one order of magnitude lower than that observed for the D2 receptor. Pharmacological studies suggest that the D3 receptor may be involved with hypothermia, penile erection, agonist-induced yawning and ejaculation (Ferrari and Giuliani, 1995; Kurashima et al., 1995). D2 and D3 receptors are found in the pituitary gland (Herroelen et al., 1994), and are important in the regulation of prolactin release (Kelly et al., 1997). D3 receptor activation has also been shown to block the reinforcing effects of cocaine and amphetamine (Kling-Petersen et al., 1994).

The D4 receptor is even less widely distributed in the brain compared to the other D2LRs. Although the pattern of expression for the D4 receptor has not been well characterized, mRNA studies have shown it is predominantly located in the medulla, amygdala, midbrain, frontal cortex and striatum. A D4 receptor signal is also found in the olfactory tubercle and hippocampus. The D4 receptor has been implicated in schizophrenia, attention deficit hyperactivity disorder and delusional disorder (Swanson et al., 1998; Zenner et al., 1998).

Similar to D1LRs, D2LRs are also expressed in the peripheral nervous system, and may play a role in the cardiovascular system. For instance, activation of D2LRs inhibits norepinephrine and aldosterone release which results in vasodilatation and sodium excretion (Girbes et al., 1992; Smit, 1989). Radioligand and autoradiography studies have shown the presence of D2LRs in the walls of systemic arteries (Amenta et

al., 1993). Molecular biology studies have revealed the presence of D4 receptors in atrial tissue (O'Malley et al., 1992). In the kidney, the D3 receptor subtype predominates, although D2 and D4 receptors are also expressed. D3 receptors are preferentially located in the tubular region, while D4 receptor mRNA and light microscope autoradiography studies suggest this subtype is confined within glomerular arterioles (Matsumoto et al., 1995). Stimulation of D2LRs inhibits renin release (MacDonald et al., 1988), and is most likely a function of D3 receptors since they are found in juxtaglomerular cells. In mice, disruption of D3 receptor functions may result in renin-dependent hypertension (Asico et al., 1998).

## 2.4 Dopamine Receptor Distribution in the Basal Ganglia

Dopamine plays a major role in regulating the activity of basal ganglia neurons through D1LRs and D2LRs. Dopaminergic inputs from the SNc also reach movement-related areas in the basal ganglia (see above), and thus changes to this system can affect dopamine receptor functions. The distribution of dopamine receptors in the basal ganglia is shown in Table 2.2, and described below.

**Table 2.2. Distribution and subcellular localization of dopamine receptors in the basal ganglia.**

| Structure | Receptor Sub-Type | Subcellular Location | Projection Origin | References |
|---|---|---|---|---|
| Striatum | D2 | Presynaptic | SNc, cortex | Bamford et al., 2004; Dani et al. 2004 |
| | D2 | Postsynaptic | Medium spiny neurons (indirect pathway) | Bouyer et al., 1990; Freund et al. 1984; Smith et al., 1990 |
| | D2, D5 | Postsynaptic | Cholinergic interneurons | Lemoine et al. 1990; Bergston et al., 1995 |
| | D1 | Postsynaptic | Medium spiny neurons (direct pathway) | Bouyer et al., 1990; Freund et al. 1984; Smith et al., 1990 |
| | D3, D4 | Postsynaptic | Limbic striatum | Hurley et al., 1996; Morissette et al. 1998; Khan et al., 1998 |
| GPe | D2 | Presynaptic | Striatum | Levey et al., 1993; Yung et al., 1995; Surmeier et al., 1996 Gerfen et al., 2000 |
| | D2, D3, D4, D5 | Postsynaptic | | Khan et al., 1998, 2000; Ciliax et al., 2000 |
| STN | D1, D2, D4 | Presynaptic | Cortex | Flores et al., 1999; Smith et al., 2000; Smith et al., 2008 |
| | D1, D2, D3, D5 | Postsynaptic | | Flores et al., 1999; Ciliax et al., 2000; Bouthenet et al., 1991 |
| GPi | D1, D5 | Presynaptic | Striatum | Levey et al., 1993; Yung et al., 1995; Khan et al., 2000 |
| | D4, D5 | Postsynaptic | | Mrzljak et al., 1996; Khan et al., 2000; Ciliax et al., 2000 |
| SNr | D1, D5 | Presynaptic | Striatum | Levey et al., 1993; Yung et al., 1995 |
| | D3, D4, D5 | Postsynaptic | | Mrzljak et al., 1996; Khan et al., 1998, 2000; Ciliax et al., 2000 |
| SNc | D2, D5 | Postsynaptic | | Filloux et al., 1987; Mengod et al., 1989; Choi et al., 1995; Meador-Woodruff et al., 1991; Svenningsson et al. 2002 |

### 2.4.1 Striatum

Striatal projection neurons contain both D1LRs and D2LRs. Generally, postsynaptic D1LR expression is predominately associated with direct pathway neurons, while D2 receptors are commonly expressed in indirect pathway neurons. Postsynaptic D1 and D2 receptors are preferentially found in dendrites and spines of striatal projection neurons, suggesting they may modulate corticostriatal transmission (Bouyer et al., 1984; Freund et al., 1984; Smith and Bolam, 1990). However, presynaptic D2 and postsynaptic D5 receptors may also play an important role in modulating corticostriatal processing (Bamford et al., 2004b; Dani and Zhou, 2004). Dopamine D5 receptors are concentrated in dendritic shafts of medium spiny neurons in monkeys (Bergson et al., 1995) although rat studies show that D5 receptors in the caudate are mostly confined to spines making asymmetric synapses (Khan et al., 2000).

Dopamine may also regulate intrinsic striatal activity through postsynaptic D2 and D5 receptors found on cholinergic interneurons (Bergson et al., 1995; Lemoine and Bloch, 1990). For instance, activation of D5 receptors has been shown to increase acetylcholine release (Hersi et al., 2000), which may be important in learning (Wang et al., 2006).

Few studies have examined the expression of the less widely distributed dopamine D3 and D4 receptors in the striatum. While the ultrastructural location of D3 and D4 receptors is unclear, autoradiography, and *in situ* hydridization studies in monkeys (Hurley et al., 2005; Morissette et al., 1998) and immunoctyochemical analysis in rats (Khan et al., 1998) have revealed high levels of these subtypes in limbic regions of the striatum. Thus, dopamine may mediate its effects through a variety of pre- and postsynaptic receptors.

### 2.4.2 GPe and STN

Neurons in GPe and STN express both pre- and postsynaptic D1LRs and D2LRs. In GPe, D2 receptors are preferentially expressed on axons of striatal projection neurons (Gerfen et al., 1990; Levey et al., 1993; Surmeier et al., 1996; Yung et al., 1995a), while D3 and D4 receptor mRNA (Ariano et al., 1997; Mauger et al., 1998; Mrzljak et al., 1996; Surmeier et al., 1996) and immunoreactive cell bodies (Khan et al., 1998) have also been described. In the STN, the exact location of the receptors for dopamine is still a matter of debate. While binding and mRNA studies have demonstrated D1, D2 and D3 receptor expression in the rat STN (Bouthenet et al., 1987; Bouthenet et al., 1991; Boyson et al., 1986; Flores et al., 1999; Mansour et al., 1992; Martres et al., 1985; Savasta et al., 1986), other studies did not confirm these findings (Mansour et al., 1990; Mansour et al., 1992; Weiner et al., 1991; Yung et al., 1995b). Dopamine may also exert some of its effects through D5 receptors in both GPe and STN. Immunocytochemistry studies have shown D5 receptor labeling in GPe and STN of rats, humans and monkeys (Ciliax et al., 2000; Khan et al., 2000).

### 2.4.3 GPi and SNr

D1LRs, and to a lesser extent the D2LRs, are strongly expressed in GPi and SNr (see figure 2.1, Barone et al., 1987; Fremeau et al., 1991b; Richfield et al., 1987). Immunocytochemical studies have shown that D1 receptors are predominantly located on presynaptic axons and axon terminals of striatal projections (Yung et al., 1995b). The labeled terminals show characteristic ultrastructural features of striatal GABAergic boutons (Caille et al., 1996; Levey et al., 1993; Yung et al., 1995b), suggesting that the

striatum is the main source of D1 receptors in GPi and SNr. In constrast, immunocytochemical and *in situ* hybridization studies have described pre- and postsynaptic locations for D5 receptors in the GPi and SNr of rats and monkeys (Ciliax et al., 2000; Khan et al., 2000). The location of D2LRs in GPi and SNr is less well studied. Postsynaptic D4 receptors have been described in monkey GPi and SNr neurons (Mrzljak et al., 1996) while both D3 and D4 receptors have been reported the rat SNr (Khan et al., 1998).

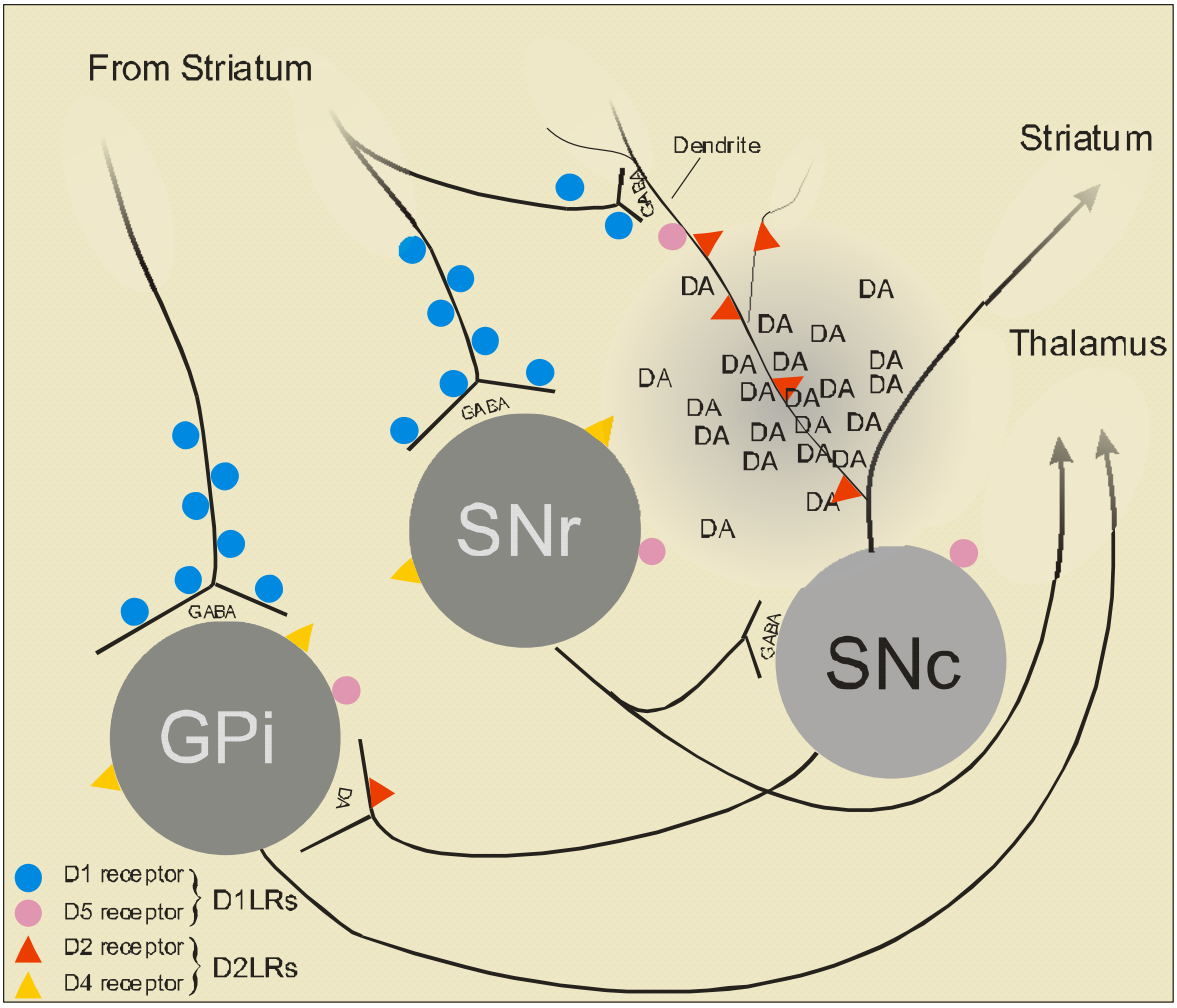



**Figure 2.1.** A schematic diagram of the location of dopamine receptors in monkey SN and GPi. Dopamine released from SNc dendrites and/or SNc terminals in GPi, may mediate neuronal activity through actions on D1LRs and D2LRs in SNr, GPi, and SNc, respectively.

### 2.4.4 SNc

In the SNc, both D1LRs and D2LRs are present (see figure 2.1), and likely contribute to mediating local effects of dopamine and regulating dopamine release and uptake. Several studies using *in situ* hybridization techniques revealed D2 receptor mRNA in the SNc (Choi et al., 1995; Mengod et al., 1989; Svenningsson and Le Moine, 2002). Binding studies confirm D2LR expression (Filloux et al., 1987; Meador-Woodruff et al., 1991) on cell bodies. Electron microscopic studies have also described postsynaptic D5 receptors in the SNc (Khan et al., 2000) however, their exact role is not clear.

## 2.5 Dopamine Autoreceptors

Because autoreceptors can be found on any part of a dopamine neuron, they can regulate both axonal and dendritic release of dopamine. For example, activation of autoreceptors located on the somatodendritic domain of SNc neurons reduces the discharge rate of dopamine neurons and inhibits dopamine release (Cragg and Greenfield, 1997), while activation of autoreceptors located on dopamine terminals inhibits dopamine synthesis and release (Anden, 1972; Anden et al., 1972; Arbilla and Langer, 1981; Imperato and Di Chiara, 1988; You et al., 1994b). Both D2 and D3 receptors serve as autoreceptors and are often more sensitive to dopamine concentrations (and dopamine agonists) than postsynaptic dopamine receptors (Bergstrom et al., 1986; Carlson et al., 1987; Skirboll et al., 1979).

## 2.6 Dopamine Transporter

The dopamine transporter (DAT) primarily serves as a site of dopamine re-uptake which recovers the released dopamine from the extracellular space and transports it into a presynaptic dopamine terminal, thereby terminating dopamine's actions and maintaining cellular homeostasis. However, reversal of the transport mechanism may also result in release of dopamine under some circumstances (Falkenburger et al., 2001). DAT is found only in dopamine neurons and is a unique marker for dopamine terminals (Kuhar, 1998). However, the tuberoinfundibular dopamine neurons lack DAT mRNA and protein (Cooper et al., 2003). Immunohistochemical studies revealed that DAT is located at extrasynaptic sites of axon terminals, suggesting that diffusion is an important method for dopamine neurotransmission.

A high level of the DAT is found in the basal ganglia, particularly the striatum, consistent with the large concentration of dopamine in this brain region, and its important role in regulating can strongly influence the tissue dopamine concentration (Amara and Kuhar, 1993; Giros and Caron, 1993). Light and electron microscopic studies documenting the distribution of the DAT further support this concept. Electron microscopic studies in rats have shown that DAT is found in distal regions of SNr dendrites (Ciliax et al., 1999; Hersch et al., 1997; Nirenberg et al., 1996b), while light microscopic studies in humans have revealed DAT-positive axons throughout the GPi (Ciliax et al., 1999). These studies further support the idea that dopamine reaches the basal ganglia output nuclei, and may act on D1LRs to influence basal ganglia outflow.

Binding studies have demonstrated that other compounds may bind the DAT, and disrupt dopaminergic function. The selectivity of MPP+, the neurotoxic metabolite of

MPTP, for DAT results in the degeneration of dopamine neurons, leading to parkinsonism (see below). Differences in DAT expression in the brain correspond with differential degree of dopaminergic cell loss after MPTP administration or in Parkinson's disease. For instance, *in situ* hybridization studies have demonstrated a marked reduction in DAT mRNA levels of dopamine neurons (Uhl et al., 1994), a finding that corroborates the significant decrease in striatal DAT binding in MPTP-treated monkeys (Bezard et al., 2001). In line with these observations, pretreatment with DAT inhibitors prevents the destruction of dopaminergic neurons by MPTP (Javitch et al., 1985), and mice lacking the DAT show no significant reduction in SNc TH labeling (Bezard et al., 1999), suggesting that MPTP-induced dopamine cell loss is related to DAT levels. The notion that a threshold exists for the onset of parkinsonian symptoms is not new (Bernheimer et al., 1973), but the time interval of the asymptomatic phase remains open for debate (Fearnley and Lees, 1991; Hoehn and Yahr, 1967; Morrish et al., 1996) It is accepted that, the loss of DAT is generally associated with the degree of motor disability associated with parkinsonism, although it cannot be used as a measure of motor impairment because increases in striatal D2 receptors may act as a compensatory mechanism since D2 receptor binding changes are not correlated with nigrostriatal degeneration (Bezard et al., 2001).

## 2.7 Effects of Dopamine Receptor Activation in the Basal Ganglia

### 2.7.1 Dopaminergic effects in the striatum

The dopaminergic innervation of the striatum is important in movement and cognitive activities. A primary dopamine-mediated function is to control corticostriatal

transmission involving pre- and postsynaptic D1LRs and D2LRs. Glutamatergic terminals of the corticostriatal pathway contact the head of dendritic spines of medium spiny neurons, while dopaminergic terminals contact the neck of spines and dendritic shafts. Dopamine, through activation of both D1LRs and D2 receptors, is therefore in a position to regulate glutamatergic inputs from the cerebral cortex (Bamford et al., 2004a; Freund et al., 1984; Surmeier et al., 2007). Activation of D2 receptors also regulates glutamate release (Centonze et al., 2003b) through the retrograde endocannabinoid system.

Dopamine is also an important regulator of synaptic transmission and modulation of plasticity at glutamatergic synapses is thought to underlie learning and memory functions. Long-term depression at corticostriatal synapses requires D2 receptor activation (Calabresi et al., 1997; Surmeier et al., 2007). D2 receptor-mediated plasticity may also involve M1 muscarinic receptors (Yan et al., 2001). In contrast, the mechanisms that regulate long-term potentiation involve co-activation of D1 and NMDA receptors (Centonze et al., 2003a; Kerr and Wickens, 2001).

Co-activation of D1LRs and D2LRs is thought to control some types of motor behaviors. The link between D2LRs and movement comes mainly from experiments in in 6-hydroxydopamine (6-OHDA)-treated animals. For instance, intrastriatal injections of a D2LR agonist induced rotations in unilateral dopamine-depleted rats (Herrera-Marschitz et al., 1985). Some studies suggest that rotational behaviors in the dopamine-depleted state may involve synergistic activities of D1LR and D2LRs when the agonists are administered systemically (Robertson and Robertson, 1986), while other studies involving intrastriatal injections do not support this idea (LaHoste and Marshall, 1990),

suggesting that the synergistic effects may involve nigral D1LRs and striatal D2LRs (Robertson and Robertson, 1987). As predicted based on their opposite biochemical responses, *in vitro* studies demonstrate that local activation of D2 receptors inhibits striatal activity in slices, reducing activity along the 'indirect' pathway, while D1 receptor activation does the opposite, leading to increased activity along the 'direct' pathway (Albin et al., 1989; Surmeier et al., 2007), supporting the idea that coordinated movement requires cooperative interactions between the two receptors. However, both D1LR and D2LR antagonists block locomotor activity (Vallone et al., 2000) and long-term depression (Calabresi et al., 1992a; Calabresi et al., 1992b). Evidence from studies using D1 knock-out mice suggests that D1 and D5 receptors may have distinct roles. For instance, endogenous dopamine stimulates D1 and D5 receptors to induce long-term potentiation and long-term depression, respectively (Centonze et al., 2003a). The same group also described separate motoric functions for the D1 and D5 receptor subtypes, in which D1 receptor stimulation facilitates locomotion, while activation of the D5 receptor has the opposite effect. Together, these findings suggest that both D1 and D5 receptors may synergistically function with D2 receptors in the striatum to fine tune motor activity and regulate long-term synaptic plasticity.

Other evidence suggests that D1LRs and D2LRs in the caudate may work together to process reward-related information (Nakamura and Hikosaka, 2006). However, the specific sub-types involved have not been identified. The use of receptor knock-out mice may prove useful to clarify their roles in this regard. Finally, postsynaptic D5 receptors located on cholinergic interneurons regulate acetylcholine and GABA transmission (Berlanga et al., 2005; Yan and Surmeier, 1997). Together,

dopamine, through D1LRs and D2LRs, can regulate a variety of functions either by direct activation of its receptors or by regulation of other neurotransmitter systems.

Lastly, dopamine concentrations, and thus striatal dopamine actions, are regulated by presynaptic D2 receptors. For instance, intrastriatal injections of a D2LR agonist (quinpirole) reduce striatal dopamine levels (You et al., 1994b). Similarly intranigral activation of D2LRs reduces dopamine release in the striatum (You et al., 1994b). These studies are in agreement with an autoreceptor role for D2 receptors.

### 2.7.2 Dopaminergic effects in GPe and STN

In agreement with anatomical descriptions, dopamine actions have been demonstrated in both the GPe and STN. In the rat GP, dopamine modulates neural discharge and *c-fos* expression (Pan and Walters, 1988; Ruskin and Marshall, 1997). For instance, local D2LR activation reduces GABA release in slices (Floran et al., 1997) and in awake rats (Querejeta et al., 2001), supporting a presynaptic location of D2LRs on striatopallidal terminals. Consistent with changes in discharge, behavioral studies show that local bilateral infusions of D1LR or D2LR antagonists into the rat GP result in akinesia (Hauber and Lutz, 1999). Both of these effects likely involve D2LRs (at pre- and postsynaptic sites) and D5 receptors (Ciliax et al., 2000; Khan et al., 2000; Surmeier et al., 1996), since D1 receptors are sparsely expressed in the GPe (Besson et al., 1988; Fremeau et al., 1991a; Levey et al., 1993; Richfield et al., 1987; Yung et al., 1995a). Studies aimed at elucidating the role of the D4 receptor have provided inconsistent results. Using D2 and D4 receptor knock-out mice, studies have shown postsynaptic D4 receptor-mediated suppression of GABAergic inhibitory post-synaptic currents (Shin et al., 2003),

supporting another mechanism in which dopamine may regulate GABA neurotransmission. However, infusions of a selective D4 receptor agonist were without effect in a recent slice study, suggesting that presynaptic D2/D3 receptor-mediated events predominate (Baufreton and Bevan, 2007).

In the STN, dopamine receptor expression and ultrastructural location is unclear. Because a variety of dopamine receptors are located in this nucleus, understanding the precise role of dopamine is complex. For instance, intrasubthalamic administration of either a mixed D1LR/D2LR agonist or selective D1LR or D2LR agonists significantly decreased STN cell firing in normal rats although no change in *cfos* expression was reported, while apomorphine and a D2LR agonist increased neuronal discharge and D1LR activation had the opposite effect in 6-OHDA-treated rats (Hassani and Feger, 1999). In contrast, local infusions of a D1LR agonist increased STN activity in normal rats (Kreiss et al., 1996). The inconsistent findings in the later study may be explained by the use of systemic administration of anesthetics such as chloral hydrate or ketamine, both glutamate antagonists. A slice study using D1 receptor knock-out mice provides evidence for a postsynaptic role for D5 receptors in regulating burst firing in STN neurons (Baufreton et al., 2003).

### 2.7.3 Dopaminergic effects in GPi and SNr

#### *2.7.3.1 D1LR activation in GPi and SNr*

The effects of D1LR activation has been extensively studied in the rodent SNr. The available evidence is somewhat contradictory, and most likely reflects the use of different experimental techniques, and the use of anesthetics.

One approach has been to examine the effects of D1LR on GABAergic transmission. Almost all of these studies have shown that D1LR activation enhances GABA release. Various *in vitro* studies have shown that D1LR activation potentiates evoked GABA efflux in slices of the SNr in both normal (Aceves et al., 1995; Floran et al., 1990; Starr, 1987) and (entopeduncular nucleus and SNr) dopamine-depleted rats (Aceves et al., 1995; Floran et al., 1990), but another study using a similar approach did not (Mayfield et al., 1999). Other slice studies have demonstrated that the administration of dopamine in the normal situation leads to an increase in GABA release (Reubi et al., 1977) or biphasic effects (van der Heyden et al., 1980), which is likely a result of both D1LR and D2LR activation. In vivo microdialysis studies in rats have consistently shown that D1LR activation increases GABA concentrations in the SNr (Matuszewich and Yamamoto, 1999; Rosales et al., 1997b; Timmerman and Westerink, 1995; Trevitt et al., 2002; You et al., 1994a) and entopeduncular nucleus (Ferre et al., 1996), data that support the findings of this dissertation.

Most *in vivo* electrophysiologic studies have focused on the effects of D1LR activation in the SNr. For instance, dopamine administration by iontophoresis increases neuronal firing (Ruffieux and Schultz, 1980) and/or attenuates the inhibitory effects of GABA in the SNr (Waszczak and Walters, 1983; Waszczak and Walters, 1986). However, D2LRs may have also contributed to these effects. Other more recent studies show that SNr cells are inhibited by endogenous dopamine, released by application of amphetamine in anesthetized (Timmerman and Abercrombie, 1996) and awake rats (Windels and Kiyatkin, 2006). These effects were blocked by the selective D1LR

antagonist, SCH23390 (Timmerman and Abercrombie, 1996; Windels and Kiyatkin, 2006), suggesting they are likely mediated through D1LRs.

Recording studies using brain slices have also yielded conflicting results. Although most *in vitro* studies have demonstrated that D1LR activation increases GABA concentrations (Floran et al., 2002; Misgeld, 2004; Radnikow and Misgeld, 1998), some studies have shown D1LR-mediated excitatory effects in SNr (Martin and Waszczak, 1994; Miyazaki and Lacey, 1998). Although strong evidence suggests that the majority of D1LRs are likely found on striatonigral terminals (see above), activation of D1LRs found on subthalamonigral terminals may also affect glutamatergic transmission (Ibanez-Sandoval et al., 2006).

#### *2.7.3.2 D1LR blockade in GPi and SNr*

Similar to D1LR activation studies, most behavioral studies have focused on D1LR-mediated effects in the rodent SNr in the normal state. In fact, most of the evidence supporting a role of the SNr in motor control comes from studies of D1LR blockade. For instance, intranigral infusions of SCH23390 inhibit amphetamine-induced behaviors (Timmerman and Abercrombie, 1996; Yurek and Hipkens, 1993), impair performance on a rod-balancing task (Bergquist et al., 2003), increase EMG activity and rigidity (Hemsley and Crocker, 2001) and decrease lever presses and locomotion (Trevitt et al., 2001). Since dopamine reaches both the SNr (Cobb and Abercrombie, 2003b; Gerhardt et al., 2002; Heeringa and Abercrombie, 1995; Robertson et al., 1991a; Santiago and Westerink, 1991) and GPi (Pifl et al., 1990) in rats and monkeys, dopamine loss at

these sites may contribute to the development of some of the motor symptoms of Parkinson's disease.

However, the behavioral effects of D1LR activation in GPi or SNr have not been well characterized in parkinsonism. It has been shown that dopamine loss in the SNr impairs motor functions, while increased nigral dopamine release can counteract motor impairment in rats with partial dopaminergic depletion (Andersson et al., 2006). However, few studies have evaluated the consequences of dopamine loss in GPi in parkinsonism. Parkinsonian patients (Bernheimer et al., 1973) and monkeys (Pifl et al., 1992) show reduced levels of dopamine in the GPi, and PET studies have also demonstrated a decrease in $^{18}$F-dopa uptake in GPi of patients with advanced parkinsonism (Whone et al., 2003). These studies suggest that dopamine loss in GPi may contribute to the development of behavioral abnormalities associated with parkinsonism. D1LR activation may also improve parkinsonian motor symptoms.

# Chapter 3

Parkinson’s Disease

# 3. Introduction

James Parkinson described in his "Essay on the Shaking Palsy" (Parkinson, 1817) the main clinical symptoms of a movement disorder that today affects approximately one million people in the United States. Now, the hallmark signs of parkinsonism, defined as a slowness of movement (bradykinesia), paucity of movement (akinesia), tremor at rest, muscle rigidity and shuffling gait are attributed to the loss of neurons in the substantia nigra pars compacta. Research in this area accelerated after the 1958 discovery by Arvid Carlsson of the neurotransmitter dopamine. Two significant findings followed. First, SNc dopamine neurons send a direct projection to the striatum and the degeneration of this pathway is associated with motor signs of Parkinson's disease. Second, most of the motor signs can be alleviated after treatment with the dopamine precursor levodopa.

PD is progressive and the first symptoms usually appear in the sixth or seventh decade of life. The incidence of the illness increases with age. The course of the disease may range from 10 to 25 years (DeLong and Juncos, 2004). Although there is no cure for PD, risk factors include family history, head injury, and pesticide exposures, while exposure to nicotine or caffeine appears to reduce the risk of developing the condition.

## 3.1 Clinical Characteristics of Parkinson's Disease

The core symptoms of parkinsonism have an insidious onset and progress gradually. The disease usually starts asymmetrically, but eventually affects both sides of the body. Other symptoms include reduced facial expression, decreased eye blinks, stooped posture and decreased arm swing. Early in the course of the disease, the motor symptoms predominate, while the non-motor symptoms and signs gain importance in

later phases. These non-motor symptoms include depression, cognitive impairment, anxiety, sleep disturbances, loss of smell, and gastrointestinal disturbances.

## 3.2 Neurochemical and Neuropathological Features of Parkinson's Disease

Parkinson's disease is a heterogeneous entity. In a minority of cases, a genetic origin can be demonstrated. At the present time, 11 different monogenetic forms of Parkinson's disease are recognized, usually resulting in young-onset disease with somewhat unusual features, such as dystonia. There is little doubt that, with time, additional genetic diseases, including polygenetic forms or diseases due to mutations in susceptibility genes, will be identified. In the majority of PD patients, however, a genetic cause cannot be identified. These so-called 'sporadic' cases are characterized by their typical clinical symptoms, the older age of onset, and by the fact that the motor symptoms in these patients readily respond to dopaminergic medications.

Pathologically, the disease is characterized by the presence of intracytoplasmic proteinaceous inclusion bodies ('Lewy bodies'), which are found in many brain regions, particularly in advanced cases of PD. This may explain the emergence of the myriad clinical manifestations of the disorder, though the exact role of Lewey bodies remains unknown. The prominent motor signs of the disease, however, are very likely caused by degeneration of SNc neurons, resulting in striatal dopamine depletion.

The pattern of degeneration of the nigrostriatal pathway is associated with changes in mRNA expression for DAT in the striatum (Uhl et al., 1994). The depletion of dopamine in the dorsolateral putamen is greater than that in the caudate nucleus (Bernheimer et al., 1973; Price et al., 1978). This is explainable by the fact that ventral

SNc projection neurons more heavily target the putamen, than the caudate, while the ventral tegmental area and dorsal SNc projection neurons, which tend to be less affected in PD, send projections to the nucleus accumbens and to the caudate, respectively.

Morphological and biochemical changes in the striatum as a result of degeneration of the nigrostriatal pathway have been the focus of many studies. For instance, loss of dendritic spines has been reported in animal models and human PD (Day et al., 2006; Ingham et al., 1989; Ingham et al., 1993; Villalba et al., 2006; Zaja-Milatovic et al., 2005), and may have a significant impact on corticostriatal transmission. Striatal dopamine loss is also associated with significant changes in dopamine receptor density and sensitivity. Studies in parkinsonian patients and animals have shown consistently that mRNA levels and D2 receptor binding sites increase in the dopamine-depleted state (Aubert et al., 2005; Bezard et al., 2001; Creese et al., 1977; Guigoni et al., 2005; Marshall et al., 1989), while both increases and decreases have been demonstrated for D1LRs (Buonamici et al., 1986; Marshall et al., 1989). However, some studies have reported no significant changes for D1LR mRNA expression and binding in MPTP-treated monkeys (Aubert et al., 2005; Betarbet and Greenamyre, 2004; Bezard et al., 2001; Guigoni et al., 2005). In 6-OHDA-treated rats, striatonigral projection neurons express less D1 receptor mRNA, while striatopallidal neurons show increased levels of D2 receptor mRNA (Gerfen et al., 1990). Since D1 mRNA is absent in both the SNr and GPi, it is difficult to interpret how changes in D1 receptor mRNA levels in the striatum correspond to D1 receptor expression in GPi and SNr. In either case, changes in the dopamine supply to extrastriatal basal ganglia sites may contribute to the development of parkinsonism.

Although age is a risk factor for PD, the pattern of SNc dopaminergic degeneration associated with PD is different from that seen during the course of normal aging. For instance, during normal aging, dopamine loss is associated preferentially with the dorsomedial portion of the SNc rather than ventrolateral and caudal areas (Fearnley and Lees, 1991), suggesting that age-related dopamine cell death occurs through a different process than the degeneration linked with PD.

## 3.3 MPTP Model of Parkinsonism

Parkinsonism induced by MPTP resembles the clinical, biochemical and pathological features of human Parkinson's disease. MPTP was discovered after intravenous injections of meperidine analogs by young drug addicts resulted in severe parkinsonism (Davis et al., 1979; Langston et al., 1983). The syndrome persisted and the patients' symptoms responded well to dopamine agonists. Post-mortem brain analysis showed specific structural damage of the SN (Davis et al., 1979; Langston et al., 1999). The discovery of the neurotoxin MPTP from a makeshift chemical laboratory, has led to the development of a monkey model of Parkinson's disease which has facilitated research in this field.

Because of the strong resemblance to the human disease, the MPTP monkey model is currently considered the gold standard for studying parkinsonism. MPTP is a lipid soluble molecule that easily crosses the blood brain barrier (Markey et al., 1984). Once inside the brain it is oxidized to MPP+ by the enzyme monoamine oxidase-B in glia and serotonin neurons, and released into the extracellular space. Then MPP+, which has a high affinity for DAT, enters nigral neurons through axon terminals in the striatum

followed by retrograde transport along nigrostriatal axons (Javitch et al., 1985; Mayer et al., 1986). Evidence suggests that MPP+ may interfere with mitochondrial oxidation and redox reactions (Nicklas et al., 1985), and other cellular enzymes (Klaidman et al., 1993). MPP+ can also bind to the monoamine vesicular monoamine transporter-2 and become sequestered inside synaptic vesicles (Liu et al., 1992a). In humans and monkeys, MPTP intoxication mimics the clinical and neuropathological aspects of idiopathic Parkinson's disease. In Rhesus macaques, MPTP produces most of the motoric symptoms of Parkinson's disease such as muscle rigidity, bradykinesia, akinesia, balance problems and freezing. Most monkey species do not develop a resting tremor, one of the early symptoms of the human disease, after MPTP administration. However, the African green monkeys reliably show a resting tremor. MPTP-treated monkeys also respond favorably to dopamine agonists and develop extra-pyramidal side effects with chronic exposure to levodopa. MPTP administration also produces a similar pattern of damage to midbrain dopaminergic neurons. For instance, ventral tier SNc neurons are more vulnerable than dorsal tier SNc and ventral tegmental area neurons (Sirinathsinghji et al., 1992; Varastet et al., 1994). However, two characteristic features of PD are not found in the MPTP monkey model. First, locus coeruleus neurons are relatively spared (Forno et al., 1993; Forno et al., 1986), and second, Lewy bodies are absent (Forno et al., 1993). However, nigral Lewy bodies have been demonstrated in mice implanted with mini-osmotic pumps for the slow administration of MPTP (Fornai et al., 2005). Then again, this has not been shown in monkeys using a similar method of MPTP delivery. The MPTP monkey model of PD highlights the importance of dopamine in the development of behavioral, and biochemical and structural brain changes. However, studies of idiopathic PD point to a

multi-stage progression in which lesions are located throughout the brain and suggests that dopamine loss is a contributing factor, but not a central feature of the neuropathology (Braak et al., 2003).

## 3.4 Pathophysiology of Parkinsonism

While the link between dopamine loss and behavioral signs of parkinsonism is unclear, electrophysiologic changes in basal ganglia after MPTP-treatment and in patients with Parkinson's disease have been well documented. For instance, dopamine loss in the basal ganglia is associated with changes in firing rate, increased incidence of burst discharges, oscillatory activity and synchrony between neurons.

### 3.4.1 Changes in Firing Rate

Recording studies in the extrastriatal basal ganglia of MPTP-treated monkeys have provided strong evidence for reduced neuronal activity in GPe and an increase in activity in STN and GPi (Bergman et al., 1994; Filion et al., 1985; Hassani et al., 1996; Miller and DeLong, 1987; Raz et al., 1996; Soares et al., 2004; Wichmann et al., 1999). Similar changes have been documented in the monkey SNr after MPTP (figure 3.1), but they are less pronounced (Wichmann et al., 1999). Recordings performed in patients with Parkinson's disease support these findings in animals (Lozano et al., 1996; Taha et al., 1997).

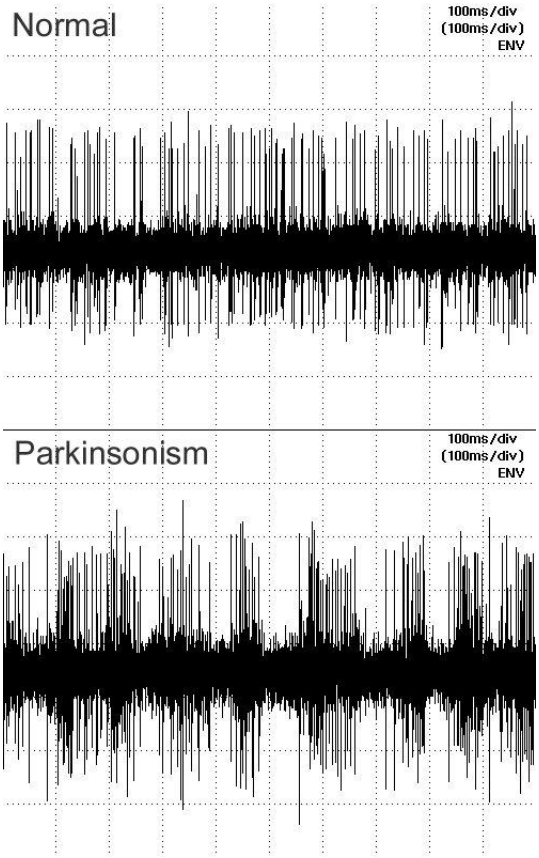



**Figure 3.1.** Changes in discharge pattern of an SNr unit under normal (top) conditions and parkinsonism (bottom). Shown is a 1 second data segment. The neuronal discharge rate is increased in parkinsonism. Also, the obvious changes in firing pattern, such as prominent oscillatory activity, are displayed.

Evidence from metabolic studies supports this view (Brownell et al., 2003; Crossman et al., 1985; Mitchell et al., 1986; Schwartzman and Alexander, 1985). Traditionally, these discharge rate changes have been interpreted as consequences of striatal dopamine loss, which may influence other basal ganglia structures (see figure 1.1). Briefly, loss of striatal dopamine is thought to result in increased inhibition of GPe through the 'indirect' pathway, which leads to disinhibition of the STN, GPi and SNr. Also, striatal dopamine loss leads to reduced activity through the 'direct' pathway, further disinhibiting GPi and SNr. Increased GPi and SNr activity may result in enhanced inhibition of thalamocortical neurons, which ultimately results in the development of parkinsonian motor signs (DeLong, 1990).

However, this traditional model only takes into account the role of dopamine acting in the striatum, and assumes that other functional changes throughout the basal ganglia in the dopamine-depleted state, namely changes in the function of GABAergic and glutamatergic pathways are secondary to striatal dopamine depletion. Consistent with the current model, microdialysis studies in parkinsonian animals have demonstrated increased levels of GABA in GPe (Galeffi et al., 2003; Robertson et al., 1991b), and reductions in GABA levels in the STN, in which GPe is the primary source of GABAergic input (Soares et al., 2004). However, it is thought that the bulk of GABA in the GPe comes from axon collaterals originating in the GPe, not from striatal afferents (Parent and Hazrati, 1995a; Windels et al., 2005), a notion that is in stark contrast to the prediction that GPe activity is reduced in parkinsonism. Moreover, studies in the rat SNr show GABA concentrations to be either increased (Windels et al., 2005) or unchanged (Galeffi et al., 2003; Ochi et al., 2004).

Studies have also measured mRNA or protein levels of the GABA-synthesizing enzyme glutamate decarboxylase (GAD). It is thought that GAD levels correspond to GABA activity. GAD mRNA levels in GPi and SNr are increased in parkinsonism (Kincaid et al., 1992; Salin et al., 2002; Soghomonian et al., 1994) corroborating the increased neuronal activity in these structures. Similar results have been shown for indirect pathway neurons. For instance, GAD mRNA levels are increased in striatopallidal projection neurons (Laprade and Soghomonian, 1999; Soghomonian and Laprade, 1997). In contrast, other studies have shown no change in GAD mRNA levels or GAD immunoreactivity in GPe and STN (Pedneault and Soghomonian, 1994; Schneider and Wade, 2003; Soares et al., 2004), a finding that is at odds with the traditional model.

Another way to assess the degree of change in GABAergic transmission is to measure binding densities of GABA receptors. Changes in receptor binding and protein levels are in line with the traditional model. For instance, GABA-A and GABA-B receptor binding or mRNA levels were decreased in the GPe and increased in GPi and SNr of parkinsonian patients or animals (Calon et al., 1999; Chadha et al., 2000a; Gnanalingham and Robertson, 1993; Griffiths et al., 1990; Katz et al., 2005; Pan et al., 1985; Robertson et al., 1990), supporting the notion that GABA levels are increased leading to a reduction in firing rate of GPe neurons, resulting in reduced GABAergic inhibition and an increase in firing rate of GPi and SNr neurons.

In addition to changes in the GABAergic system, the glutamatergic system has also been studied in the dopamine-depleted state. In the striatum, the available data are inconsistent in regards to changes in receptor binding or protein expression of ionotropic

glutamate receptors in parkinsonism (Bernard et al., 1997; Betarbet and Greenamyre, 2004; Betarbet et al., 2000; Dunah et al., 2000). On the other hand, binding studies have demonstrated a reduction in both NMDA and AMPA receptors in GPi and SNr of parkinsonian patients and animals (Bernard et al., 1997; Betarbet et al., 2000; Dunah et al., 2000), likely due to the increased activity of STN neurons. In contrast, changes in metabotropic glutamate receptors have been less explored. In GPi and SNr, the mGluR1$\alpha$ receptor is decreased in parkinsonian monkeys (Kaneda and Kita, 2005; Samadi et al., 2007). Studies of the mGluR2/3 receptors in the striatum have provided conflicting results (Samadi et al., 2007; Testa et al., 1998) while mGluR5 receptor changes appear regionally specific in MPTP-treated monkeys (Samadi et al., 2007).

The changes in receptor density and neurotransmitter levels are only partially in line with the predictions of the current model of basal ganglia function in parkinsonism. It appears that the link between the observed biochemical changes and the well-known changes in firing rate in the dopamine-depleted state cannot be fully explained by this traditional model of the basal ganglia (see above). Together, these findings suggest that dopamine loss in the striatum along with firing rate changes in the basal ganglia are not enough to explain the development of the behavioral symptoms associated with Parkinson's disease.

### 3.4.2 Burstiness

Prominent burst discharges are well documented in GPe, GPi and STN neurons of MPTP-treated monkeys (Bergman et al., 1994; Filion, 1979; Soares et al., 2004; Wichmann et al., 1999; Wichmann and Soares, 2006) and parkinsonian patients

(Hutchison et al., 1994; Magnin et al., 2000). However, the contribution of dopamine in the generation of bursts in the basal ganglia is not clear. Although the loss of dopamine in the striatum likely contributes to the development of bursts, other dopaminergic pathways may play a role. For instance, in the STN, dopamine reduces inhibitory synaptic input (Shen and Johnson, 2000), and the loss of dopamine in the STN enhances GABAergic inputs, resulting in rebound bursting (Bevan et al., 2007; Shen and Johnson, 2005). Other studies have shown that D2 receptor activation reduces bursting in STN slices from 6-OHDA treated rats (Zhu et al., 2002), while D5 receptor stimulation increases bursts in STN neurons of normal animals (Baufreton et al., 2003).

Another point to consider is the strong connection between GPe and STN. In a dopamine-free medium, organotypic cultures of GPe and STN neurons form connections and produce bursts which are eliminated when the connections between the two nuclei are severed (Plenz and Kitai, 1999), suggesting that dopamine loss to GPe and STN as well as the interaction between these two structures may be important in the development of bursts. However, it is difficult to translate the findings from these artificial cultures to the realistic *in vivo* situation. Other connections such as striatal and cortical inputs may be involved. Then again, GPe activity hyperpolarizes STN neurons resulting in rebound bursts in STN slices (Beurrier et al., 1999; Bevan et al., 2007), supporting the hypothesis that pattern and rate of inhibitory inputs are important factors influencing whether STN neurons discharge in single-spike mode or bursting pattern. In the *in vivo* situation, the temporal structure of bursts in MPTP-treated monkeys was significantly altered in GPe, GPi and STN (Wichmann and Soares, 2006), compared to bursts recorded in normal animals.

The contribution of bursts to the behavioral manifestations of parkinsonism is not clear. Similar to the findings of this dissertation, dopaminergic agonist treatment does not reduce bursting activity in GPi and SNr in parkinsonian monkeys or patients. Although intrastriatal injections of a D1LR agonist reduced burst firing in the SNr of 6-OHDA-treated rats (Tseng et al., 2000), most studies have shown opposite effects after systemically administered drugs in both parkinsonian animals (Heimer et al., 2006; Lee et al., 2001) and patients (Levy et al., 2001). Comparable to our findings in normal monkeys, D5 receptor activation induced burst firing in rat STN neurons (Baufreton et al., 2003), suggesting that endogenous dopamine may contribute at least in part to burst firing.

The link between burst firing in the basal ganglia and movement is unknown. Other brain areas such as the cortex (Evarts, 1966; Georgopoulos et al., 1986; Goldberg et al., 2002) and thalamus (Steriade et al., 1993) display bursting activities during normal movement and carry stimulus-related information, respectively. Although the cause of burst firing is not known, it is likely that the generation of bursts involve a refined interaction between intrinsic cellular properties, sensory information and network characteristics (for review, see (Krahe and Gabbiani, 2004). Evidence supports two types of burst firing in the brain. First, presynaptic bursts may facilitate synaptic transmission (Thomson, 1997). Second, bursts may carry stimulus-related information. This theory suggests that bursts carry the same information as tonic spikes, but increase the signal-to-noise ratio. Thus, if the mechanisms that underlie the signal-to-noise ratio of bursts are corrupt, both intrinsic cellular properties and network dynamics may be affected. In the basal ganglia, single cells fire in bursts, although the function of these bursts and their relationship to behavior is not known. However, the appearance of widespread burst

firing in the basal ganglia circuitry is a common pathological feature associated with parkinsonism in both animal models and patients. Although the origin of these bursts is unknown, it is thought that the emergence of prominent bursting activity interferes with processing of cortical information, which ultimately disrupts normal movements perhaps through a disruption of intrinsic cellular properties or at the network level.

### 3.4.3 Oscillations

Alterations in oscillatory activity in basal ganglia neurons is another common electrophysiologic feature associated with parkinsonism. The emergence of abnormal oscillatory activity in the alpha (8-15 cycles/s) and beta (3-8 cycles/s) frequency ranges has been reported in GPe, GPi and STN of MPTP-treated monkeys (Gatev et al., 2006) and patients (Levy et al., 2002a; Rivlin-Etzion et al., 2006) with Parkinson's disease. Since striatal projection neurons discharge typically at low frequencies and generally do not display rhythmic firing patterns under normal and parkinsonian conditions, it is unclear how degeneration of the nigrostriatal pathway would contribute to the changes in oscillatory activity seen in the other basal ganglia structures in the dopamine-depleted state. Therefore, changes in extrastriatal basal ganglia structures likely have a strong influence in the generation of oscillatory activity. Many studies have focused on the role of the STN in this regard. It appears that the interaction between the GPe and STN may play an important role in oscillatory activities. For example, transient inhibitory inputs from GPe lead to rebound bursts in STN neurons (Plenz and Kitai, 1999) which may precipitate bursting activity in GPe, generating a pacemaker system. Cortical projections, however, may influence this network (Hartmann-von Monakow et al., 1978; Nambu et al.,

2002). In addition, field potential studies have recently shown that abnormal oscillatory activity in the STN is conveyed to the GPi in parkinsonian patients (Brown et al., 2004). It is thought that field potentials represent synaptic activity produced by many neurons covering extensive areas, but the mechanism in which field potential oscillations are generated is not clear. Regardless of the mechanism involved, these data suggest that the STN and/or the GPe-STN network may be important in regulating the basal ganglia-thalamocortical system. Although it is currently not known whether dopamine loss in the GPi or the SNr contributes to abnormal oscillations in parkinsonism, the findings of this dissertation suggest that these pattern changes are likely not due to dopamine loss at these sites.

### 3.4.4 Abnormal Synchrony

Individual basal ganglia neurons discharge independently under normal conditions. However, in the dopamine-depleted state, basal ganglia cells become synchronous. For instance, synchronous units are prominent within individual basal ganglia structures as well as neighboring nuclei, and often with oscillations (Hammond et al., 2007). These coordinated changes in the electrical activity are likely due to dopamine loss in the basal ganglia. Evidence from parkinsonian monkeys and humans support this notion since systemically administered dopaminergic drugs reduce synchronous activity in the basal ganglia (Heimer et al., 2002; Levy et al., 2002b). Although most studies suggest that intrinsic changes in the striatum most likely contribute (Berretta et al., 2001; Cepeda et al., 1989; O'Donnell and Grace, 1993; Onn and Grace, 2000), other changes in the basal ganglia network may be responsible. For instance, computational studies

suggest that reduced striatal activity may influence GPe discharge which could cause synchronous activity throughout the basal ganglia (Terman et al., 2002). However, the relationship between dopamine and the emergence of synchronous activity has not been specifically studied.

### 3.4.5 Conclusion

The traditional view of the basal ganglia has led to profound insights into the pathophysiology of the basal ganglia in parkinsonism (and other basal ganglia related disorders; (DeLong, 1990), and to current neurosurgical treatments (Benabid et al., 2006). However, findings in parkinsonian animals and humans cannot be fully explained by the simplified view of the basal ganglia (see above). It is not known how the loss of extrastriatal dopamine in GPi and SNr contributes to the pathophysiology of PD. The aim of my dissertation was to assess the role of dopamine actions through D1LRs in the GPi and SNr in normal function and in parkinsonism.

## 3.5 Treatment for Parkinson's disease

The most common strategy in the treatment of PD is pharmacotherapy (see Table 3.3). Since dopamine does not cross the blood brain barrier, other approaches to replace dopamine involve either the administration of the precursor to dopamine (levodopa) or drugs that mimic the action of dopamine (dopamine agonists). Levodopa (in combination with drugs that improve its pharmacokinetics) is the best drug therapy to date. Its actions are dependent on the conversion of levodopa to dopamine by residual dopamine neurons followed by activation of dopamine receptors. It is highly effective in alleviating all of

the motor symptoms of Parkinson's disease, and can activate D1LRs and D2LRs equally well. However, levodopa becomes less effective due to side effects, dyskinesias and a narrowing therapeutic window.

Other approaches for the treatment of Parkinson's disease are neurosurgical ablation (e.g., pallidotomy, see Baron et al., 1996; e.g., pallidotomy, see Lozano et al., 1995) and deep brain stimulation (DBS; (Benabid et al., 2006; Mendis et al., 1999). Surgery is generally indicated in patients with debilitating pharmacotherapy-related motor fluctuations or dyskinesias. Lesioning the motor portion of the GPi is effective for the treatment of dyskinesias, while DBS alleviates all parkinsonian motor symptoms. Today, DBS surgeries are more common than pallidotomies, which destroy brain tissue, and are permanent. DBS requires the implantation of a neurostimulator to deliver electrical stimulation to the target area (GPi or STN). It is reversible and fairly easy to adjust the stimulation parameters in individual patients, and thus has the advantage over surgical ablation. The physiologic mechanisms underlying the effectiveness of DBS are currently debated.

GPi remains a major target for treatment of movement disorders in humans. However, this does not lessen the potential importance of the SNr as a target for therapeutic interventions. Infusions of glial-derived neurotrophic factor (GDNF) into the monkey SN have been shown to alleviate all of the cardinal parkinsonian motoric signs in MPTP-treated monkeys (Gash et al., 1996b). Similarly, dopaminergic cell grafts aimed at the SNr provided effective symptomatic improvement (Starr et al., 1999b).

**Table 3.3. Ki-values of commonly used dopaminergic compounds.**

| | | D1 Family | | D2 Family | | |
|---|---|---|---|---|---|---|
| | | D1 | D5 | D2 | D3 | D4 |
| | Dopamine | 1450-26000[a,b] | 228[f] | 210-8924[a,b] | 23[p] | 28[t] |
| D1-Like | SKF38393 | 18[c] | 100[f] | >10000[e] | >10000[e] | 1800[t] |
| Agonists | Dihydrexidine | 6.46[d] | 16[k] | 43-660[d,g] | 94[k] | 13[k] |
| | SKF82958 | 4.56[e] | ? | 264[e] | 77.3[e] | ? |
| D1-Like Antagonists | SCH23390 | 0.12[e] | 0.3[f] | 1210[e] | >10000[e] | 3560[t] |
| D2-Like | Bromocriptine | 672[f] | 454[f] | 2.8[m] | 2-7[i,p,q] | 285-372[i,u] |
| Agonists | Quinpirole | >1000[g] | >10000[i] | 8[n] | 24[r] | 0.23-124[i,r] |
| | Apomorphine | 371-680[f,h,i] | 14-363[f,i] | 15[n] | 16-73[i,p,q,s] | 4.4[i] |
| | Pergolide | 338-1363[f,i] | 33-918[f,i] | 7.8[o] | 2.3-5.5[i,s] | 59[i] |
| | Ropinirole | >10000[i] | >10000[i] | 1148[i] | 37[i] | 851[i] |
| | Pramipexole | >10000[i] | >10000[i] | 3.1[p] | 4-11[i,p] | 129[i] |
| D2-Like | Clozapine | 170 | 330 | 230 | 170 | 21 |
| Antagonists[j] | Haloperidol | 80 | 100 | 1.2 | 7 | 2.3 |
| | Raclopride | 18000 | | 1.8 | 3.5 | 2400 |
| | Spiperone | 350 | 3500 | 0.06 | 0.6 | 0.08 |
| | Sulpiride | 45000 | 77000 | 15 | 13 | 1000 |

Dissociative constants (Ki, nM) of ligands are compared between the dopamine receptor subtypes.References: a, Billard et al., 1984; b, Neve, 1990; c, Andersen et al., 1992; 2004; d, Salmi et al.; e, Neumeyer et al., 2003; f, Sunahara et al., 1991; g, Mottola et al., 2002; h, toll et al., 1998; i, Millan et al., 2002; j, Seemen & Van Tol 1994; k, Quandil et al., 2003; l, Zahniser et al., 1983; m, Seeman et al., 2001; n, Levantet al., 1992; o, Andersen et al., 198 p, Sautel et al., 1995; q, Freedman et al., 1994; r, Tang et al., 1994; s, Sokoloff et al., 1992; t, Van Tol et al., 1999; u, Seeman et al., 1993

## 3.6 Dopaminergic Agonists

Dopaminergic agonists are used both experimentally and in clinical applications. Early studies focused on agents acting at D2LRs (Creese et al., 1977; Goldstein et al., 1980; Heikkila et al., 1981; Mishra et al., 1980). However, it wasn't until 1978 that the first specific tool to study D1LR receptors was discovered. SKF38393 (Pendleton et al., 1978) although a partial agonist, represented a breakthrough to study D1LR function. Soon after, reports of the compound SCH23390 (Cross et al., 1983; Iorio et al., 1983), a D1LR antagonist, followed. The discovery of these two compounds led to the identification of important structural components that confer selective D1LR binding affinity. Using this information, dihydrexidine was developed as the first high-affinity full D1LR agonist (Lovenberg et al., 1989). This agent ameliorated parkinsonian signs in MPTP-treated monkeys (Taylor et al., 1991). Although dihydrexidine has some affinity for D2LRs (Mottola et al., 1992), the antiparkinsonian effects were not affected by a selective D2LR antagonist but were blocked completely by SCH23390, demonstrating the importance of D1LR activity in parkinsonism. Similarly, SKF82958, the D1LR agonist used in this dissertation, also has antiparkinsonian effects in MPTP-treated monkeys (Blanchet et al., 1996). SKF82958 was also shown to be more effective in advanced, relative to mild, motor impairment induced by MPTP in monkeys (Goulet and Madras, 2000), implying that the dopaminergic tone at D1LRs may be more important in the advanced stages of PD.

Despite the advancement in our knowledge of D1LR function, the parallel between parkinsonism and catalepsy induced by D2LR antagonists led to the idea that the beneficial effects of levodopa were mediated via D2LRs (Cederbaum and Schleifer,

1990). Several selective D2LR and mixed D1LR/D2LR agonists have been developed for clinical use. None of the selective agents are as effective as levodopa, but they are often used as an adjunct therapy to levodopa or as a monotherapy in the early stages of the disease. In general, dopamine agonists readily cross the blood brain barrier, and compared to levodopa, are longer acting and provide a more uniform stimulation of dopamine receptors. Ropinirole and pramipexole, commonly used D2LR selective agonists, are non-ergot alkaloid derivatives. In clinical trials, ropinirole is less efficacious relative to levodopa with a 20-fold higher affinity for the D3 over D2 receptors, similar to dopamine (Coldwell et al., 1997). Comparatively, pramipexole shows a similar pharmacological profile. Although studies have demonstrated that pramipexole is effective as a monotherapy in patients in the early stage of the disease (Dooley and Markham, 1998), other clinical trials, as mentioned by DeLong and Juncos (DeLong and Juncos, 2004), show less complete symptomatic control than levodopa. Another non-ergot dopamine receptor agonist, apomorphine is a fairly potent and short-acting agent that has been shown to be markedly effective when administered subcutaneously (Poewe et al., 1988). It is a full agonist at D2LR receptors and a partial agonist at D1LRs, which may explain its effectiveness in the later stages of PD. However, its oral use is limited due to pharmacokinetics and azotemia (Colzi et al., 1998; Poewe et al., 1993). Other agents with activity at D1LR and D2LR sites (Fuller and Clemens, 1991; Olanow and Koller, 1998) include the ergot derivatives pergolide and bromocriptine. These agents are effective for the treatment of bradykinesia and gait problems, but less effective in treating tremor, and are not without side effects.

One of the most debilitating side effects associated with chronic dopamine receptor stimulation is the development of dyskinesias. Dyskinesias are exaggerated movements of the choreiform and dystonic type that generally occur at the peak dose effect or at the beginning or end of a dose. After about five years of levodopa treatment, more than half of the PD patients develop dyskinesias (DeLong and Juncos 2004). The development of dyskinesias has in large part been attributed to the sensitization of denervated dopamine receptors. However, it is likely that the mechanism behind dyskinesias is complicated. Evidence suggests that dyskinesias may be mediated via D1LRs (Boyce et al., 1990; Gerfen, 2000; Mehta et al., 2000) or D2LRs (Gomez-Mancilla and Bedard, 1991), while other studies imply that both family of receptors are involved (Gomez-Mancilla and Bedard, 1992). Despite the possibility that D1LRs may be involved with the generation of dyskinesias in levodopa-primed patients, the use of a selective D1LR agonist alone under a carefully monitored situation may provide symptomatic relief without dyskinesias.

### 3.6.1 Summary

Based on the data from D1LR studies in monkeys and apomorphine use in humans, developing a selective D1LR agonist remains a good idea. The results from D1LR studies in parkinsonism may also explain why selective D2LRs are more effective in the early stages of PD, but not the later stages when dopamine loss is more extensive, underlining the importance of D1LRs in parkinsonism. However, the development of selective D1LR agonists has been difficult due to low bioavailability and side effects (although all dopaminergic drugs currently available are not without side effects and

often need to be discontinued). For example, pergolide was very efficacious, but it was recently removed from clinical use because it induced cardiac valvular fibrosis in some patients (Pritchett et al., 2002; Schade et al., 2007). In fact, many of the untoward effects associated with dopamine receptor agonist activity are likely due to stimulation of peripheral D1LRs. Because D1LRs use different signaling pathways (see above) it may be possible to develop selective agonists to treat disorders (e.g. PD, attention-deficit, addiction) or functions of the central nervous system (e.g. memory, cognition,) or peripheral conditions (e.g. vasculature, heart, kidney, lung), thereby limiting the potential for side effects. Additionally, the design of agents that could selectively target either the D1 or D5 receptor or activate both subtypes in a manner similar to dopamine may be the ideal strategy.

# Chapter 4

## Current Research and Significance

### 4. Goals of This Dissertation

Substantial anatomic evidence has demonstrated that dopamine reaches the basal ganglia output nuclei, GPi and SNr, in monkeys. Therefore, dopamine may be important in regulating neuronal discharge at these sites. D1LRs are the predominant dopamine receptor type in GPi and SNr. Most evidence suggests that D1LRs are located presynaptically on GABAergic striatal afferents to these nuclei, although recent data has revealed a postsynaptic location for the D5 receptor in the rat SNr. These studies will help us understand the contribution of dopamine loss in GPi and SNr, and whether drugs with D1LR activity and restorative treatments aimed at GPi or SNr may be effective in the management of Parkinson's disease. Although most of the drugs used to treat patients with Parkinson's disease have minimal activity at D1LR sites, current drug therapies with significant D1LR affinity (e.g. levodopa) may be associated with the improvement of motor symptoms as well as untoward effects. Also, the effectiveness of drugs with D1LR activity, such as apomorphine, suggests that D1LRs represent an important target for the treatment of parkinsonism.

The primary goal of this work was to understand the effects of activating D1LRs in the GPi and SNr in normal and dopamine-depleted monkeys and correlate these findings with clinical relevance in the treatment of Parkinson's disease. Additionally, it is important to note that one original aim of this dissertation included a behavioral component that involved assessing movement after local administration of the D1LR agonist in GPi and SNr in MPTP-treated monkeys. However, the results from preliminary experiments were difficult to interpret due to confounding factors such as a lesion-induced improvement from the insertion of the injection cannula, especially in GPi (see figure 4.1). Therefore, these experiments were not carried out.

This dissertation has focused on the neuronal effects of D1LR-mediated events and the location of D1 and D5 receptors in GPi and SNr. Thus, the goals of the proposed studies are: 1. Assess GPi and SNr neuronal activity after local injections of a D1LR agonist (in normal and MPTP-treated monkeys) and a D1LR antagonist (in normal monkeys); 2. Measure GABA levels in GPi and SNr under basal conditions and after exposure to D1LR ligands in normal monkeys; 3. Examine the location and distribution of D1 and D5 receptors in GPi and SNr in normal and parkinsonian monkeys.

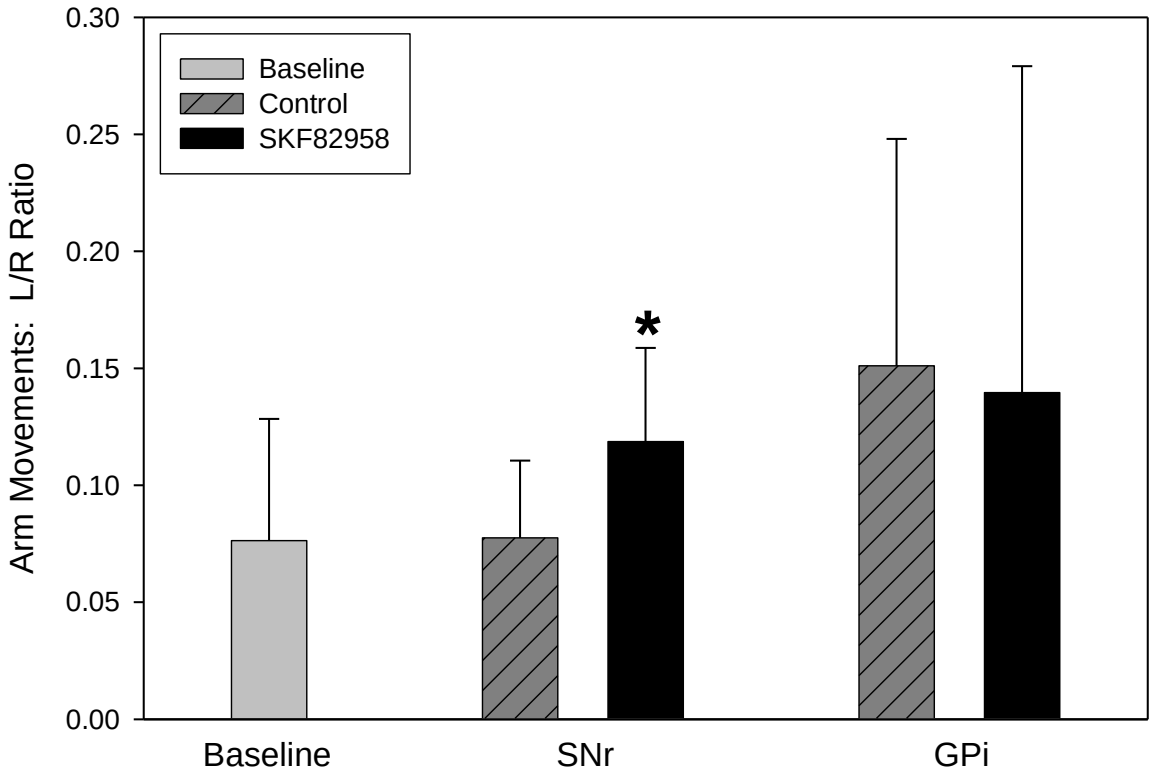



**Figure 4.1.** The effect on arm movements in hemiparkinsonian monkeys after D1LR activation in SNr and GPi. A comparison of arm movements of the parkinsonian (or drug-injected; L) side of the body with the non-affected (or untreated; R) side were calculated as a ratio over a 20-min time period. The baseline observations (n=42) were carried out in the parkinsonian state and prior to drug injections. Vehicle (n=6 in both cases) was infused for control experiments, and the D1LR agonist, SKF82958 (n=6 in both cases; 3 μg/μl; total volume, 3 μl) was infused (rate, 0.2 μl/30 sec) for drug experiments. Testing began 20 minutes after the start of the injections.

**4.1 Hypothesis One: Local injections of D1LR agonists and antagonists into GPi and SNr modulate neuronal activity**.

Extracellular single-unit recordings were completed in awake monkeys with a novel recording-injection device (Chapter 6). GPi and SNr neurons were recorded before, during and after the infusion of small quantities of D1LR ligands into the immediate vicinity of the recorded neuron in normal (Chapter 7) and parkinsonian monkeys (Chapter 8). The use of the recording-injection device necessitated extensive testing to minimize potential structural damage as well as optimize cellular recording. Also, to perfect the arrangement between the injection cannula and the electrode, I tested different configurations by analyzing the quality of the recording and the timing of the drug response by infusing muscimol into GPe. Next, I optimized other injection parameters such as the rate and volume of drug infusions using muscimol. Based on previous literature reports in slices and rats, I expected that D1LR stimulation would reduce neuronal activity while D1LR blockade would have the opposite effect.

**4.2 Hypothesis Two: D1LR activation will increase GABA release in GPi and SNr**.

Microdialysis was used to measure GABA levels in GPi and SNr under basal conditions and after exposure to D1LR ligands in normal monkeys (Chapter 7). Current anatomic models of the basal ganglia show that D1LRs in GPi and SNr are found at presynaptic sites. Therefore, I expected that GABA release would increase in response to infusions of the D1LR agonist in both structures while the D1LR antagonist would have the opposite effect. These experiments are important to help us understand how D1LR stimulation may lead to changes in discharge rate.

**4.3 Hypothesis Three: The distribution of the D1 and D5 receptors in the GPi and SNr will not change after MPTP treatment. The ultrastructural localization of the D1 receptor will predominately be found on plasma membranes in both nuclei after MPTP treatment.**

These experiments compared the distribution and ultrastructural location of D1 and D5 receptors in the GPi and SNr in normal animals to that in dopamine-depleted animals (Chapter 8). These experiments complemented the electrophysiological data to yield a functional and anatomic basis for changes associated with parkinsonism at the level of GPi and/or SNr.

# Chapter 5

Methods and Technical Considerations

## 5. Introduction

The methods used in this dissertation to study the effects of D1LR activation in GPi and SNr in normal and parkinsonian monkeys have been used extensively in Drs. Thomas Wichmann's and Yoland Smith's labs. Each technique used here served as a practical and useful tool to address specific questions however, none are without limitations. By combining techniques to address a central question and careful experimental design, the final outcome has provided a solid framework that, I think, contributes to our basic understanding of basal ganglia circuitry.

## 5.1 Methods

### 5.1.1 Electracellular recording

Recording the activity of single cells in the *in vivo* situation is challenging, but it offers a unique way to eavesdrop on the communication between neighboring neurons. Recording techniques were first used to study the activity of single nerve fibers (Adrian, 1926; Adrian and Zotterman, 1926), and have developed to make it possible to study the responses of single cells to the local application of substances or neurotransmitter in the vicinity of active neurons. However, our knowledge of how single neurons relate to a particular behavior was limited due to the need for anesthesia in the *in vivo* situation. These challenges were overcome by the pioneer recording studies in the cortex of behaving monkeys (Jasper, 1958; Jasper et al., 1960). Another neuroscientist developed a microdrive (Hubel, 1957) to advance microelectrodes into the brain of an unrestrained cat sitting quietly in the researcher's lap. The contribution of these investigators and others has provided us the opportunity to study the brain at work. In fact, characterizing the electrophysiologic properties of single cells and brain nuclei has improved our

understanding of neuronal circuitry of the basal ganglia and has led to therapeutic treatments in Parkinson's disease.

Today, extracellular electrophysiology serves as an excellent tool to record electrical activity of a single neuron in an awake animal. These high-resolution recordings are performed by placing an electrode that is insulated up to its tip into the brain. Action potentials are recorded from cells near the tip of the electrode at the millisecond temporal resolution in which they spontaneously occur, and represent changes in membrane potential. These brief fluctuations in membrane potential reflect the movement of ions across the extracellular space in response to the rapid opening and closing of voltage-gated ion channels, and are measured between the electrode and a reference connection in volts, generating a small signal. For detection of this signal, an amplifier is used. A filter is also part of the set-up to eliminate other electrical interferences.

Although recording techniques are not easy, the amount of information that can be obtained from a single animal during a recording session outweighs the difficulties. One limitation of recording experiments is that it can be difficult to define the precise location of a neuron and its connections. In this regard, combining electrophysiology experiments with anatomic and biochemical methods has provided a better understanding of how D1LR effects may be mediated in the basal ganglia output nuclei in normal and parkinsonian monkeys.

### 5.1.2 Recording/injection device

The development and use of a technique that combines single unit recordings with precisely timed, small microinjections of drugs in the vicinity of the recorded cell has provided us with the ability to address more specific functional questions in awake, resting monkeys. Since the combination recording-injection system is capable of producing a high quality neuronal signal and simultaneous and accurate delivery of small volumes of drugs, we are able to assess changes in neuronal discharge before, during, and after drug infusions. More specifically we can analyze directly the effect of a drug on discharge rates and patterns of neurons located close to the injection site.

The injection-recording system used in this study has been widely used by Dr. Thomas Wichmann's laboratory (Galvan et al., 2005a; Galvan et al., 2005b; Kliem et al., 2007; Kliem and Wichmann, 2004). About the same time, another group of researchers independently designed a similar combination injection-recording device (Kita et al., 2006; Kita et al., 2004). Although the outer diameter of both injection-recording systems are similar (~ 0.5 mm), several differences also exist between the two systems. First, Kita's group uses a two-barrel system for injections combined with a recording wire while our model is more simplistic, and combines a microelectrode with a single tube for drug/vehicle infusions. Second, and the most significant difference, is the distance between the tip of the electrode and the site of infusion. The assembly of the system used in this dissertation maintains a close proximity between recorded neurons and the site of infusion (~ 0.1 mm), and is generally associated with a reliable onset of drug responses (within 120 s post injection). The distance between the tips of the recording wire and the silica tubing used for injections by Kita's group is significantly greater (~ 0.6 - 0.7 mm), resulting in significant variability in the onset of drug responses ranging between 3 and

10 minutes, and in some cases even longer. This variabililty makes it difficult to interpret their findings and reconcile whether the changes in neuronal firing observed by Kita's group are due to receptors in the vicinity of the recorded neuron or those in close proximity to the drug infusion site or due to drug diffusion at distance locations.

Although the use of such combination systems allows changes in neuronal discharge parameters to be evaluated in response to local activation or blockade of receptors near or in the vicinity of the recorded neuron, a few technical issues should be pointed out. The primary concern of evaluating pharmacologic effects using a combination injection-recording system *in vivo* is the lack of control over the extent of drug diffusion and drug concentration at the receptor. This may explain the variation in the degree and latency of drug responses. Although the location of the dopamine receptors under study is mostly pre-synaptic, we cannot assess the drug concentrations at the receptor or the number of receptors activated. Also, the microenvironment (e.g. glial processes, oligodendrocytes, myelinated axons) may have an impact on the outcome of the drug responses by potentially interfering with drug diffusion. To minimize the variability between experimental sessions, the configuration of the injection-recording device, the rate and volume of each injection and the drug concentration was kept constant.

### 5.1.3 Drug selectivity

Another point to consider in the electrophysiologic experiments is the selectivity of the ligands used. Both dopaminergic ligands (SKF82958, D1LR agonist; SCH23390, D1LR antagonist) used in this study are specific for D1LRs (Neumeyer et al., 2003; Toll

et al., 1998) but neither can be used to distinguish potential responses from D1 and D5 receptors. Of potential concern is the use of SCH23390 because it also has relatively high affinity for serotonin 5-HT2A (Neumeyer et al., 2003) and 5-HT2C receptors (Briggs et al., 1991; Millan et al., 2001; Woodward et al., 1992). Serotonin 5-HT2A receptors are not a major concern since *in situ* hybridization studies revealed that they are found in low concentrations in the monkey pallidum and nigra (Lopez-Gimenez et al., 2001). Serotonin 5-HT2C receptors, on the other hand, are found in lateral portion of the monkey SNr, but the not the GPi (Lopez-Gimenez et al., 2001). Since SCH23390 is a partial or full agonist at postsynaptic serotonin 5-HT2C receptor sites (Hoyer et al., 1989; (Millan et al., 2001), it would be expected to increase neuronal discharge rates of SNr or GPi neurons. In GPi, this expectation is of particular concern for my study, since infusions of SCH23390 significantly increased pallidal firing rates. However, a recent electrophysiologic study in monkeys demonstrated that GPi infusions of serotonergic ligands were without significant effect on pallidal discharge (Kita et al., 2007). Together, the electrophysiologic results and mRNA findings suggest that the effects observed in this dissertation were likely mediated through blockade of D1LRs.

Another concern with the use of the D1LR agonist is that D1LR activation may lead to desensitization of the D1 and/or D5 receptors. Although each dopamine receptor subtype contains a different array of potential regulatory phosphorylation sites that may influence the rate and extent of desensitization (Gingrich and Caron, 1993), only the D1 (not D5) receptor has been studied in this regard. Desensitization, however, is unlikely to be a contributing factor since the rate of infusion in this study was short followed by drug diffusion (Lewis et al., 1998).

### 5.1.4 Microdialysis

The microdialysis technique provides a means to sample neurotransmitter tissue concentrations in intact animals (see figure 5.1). Although it is not clear what proportion of the measured GABA concentrations is derived from synaptic sources (Westerink and de Vries, 1989), our results clearly demonstrate that the method is sensitive enough to measure changes in ambient GABA levels after local administration of dopaminergic ligands in GPi and SNr. As the majority of D1LRs are located on axons or terminals of GABAergic neurons in these structures, it is most likely that the measured changes in GABA levels reflect changes in synaptic transmitter release. The basal levels of GABA measured in GPi and SNr in this study were similar to those reported in previous rodent and nonhuman primate experiments (Galvan et al., 2005b; Matuszewich and Yamamoto, 1999; Robertson et al., 1991b; Trevitt et al., 2002).

Another constraint of microdialysis is the probe size since it may contribute to low spatial resolution of extracellular transmitter measurements (Parsons and Justice, 1994) that may be derived from neighboring nuclei. This in unlikely, however, to be an issue since the active region of the probes were in the target nuclei indicating that in this study we measured local effects of dopaminergic ligands in either GPi or SNr. Also, D1LR desensitization is not likely to be a concern since selective D1LR agonists were infused over a relatively short-time period (see above; (Lewis et al., 1998). Finally, the concentrations of SKF82958 and SCH23390 used in this study may be considered high. It is thought that only a small portion (10-20%) of the ligand will cross the dialysis membrane and enter the brain (Parsons and Justice, 1994), so large concentrations are often required to elicit a measurable response. To minimize the variability of the

responsiveness to drug infusions, the rate and concentration of drug delivery, and the collection volume of microdialysate were kept constant throughout this series of studies. Despite the constraints of the available techniques I was able to measure responses of GPi and SNr neurons to local infusions of D1LR ligands.

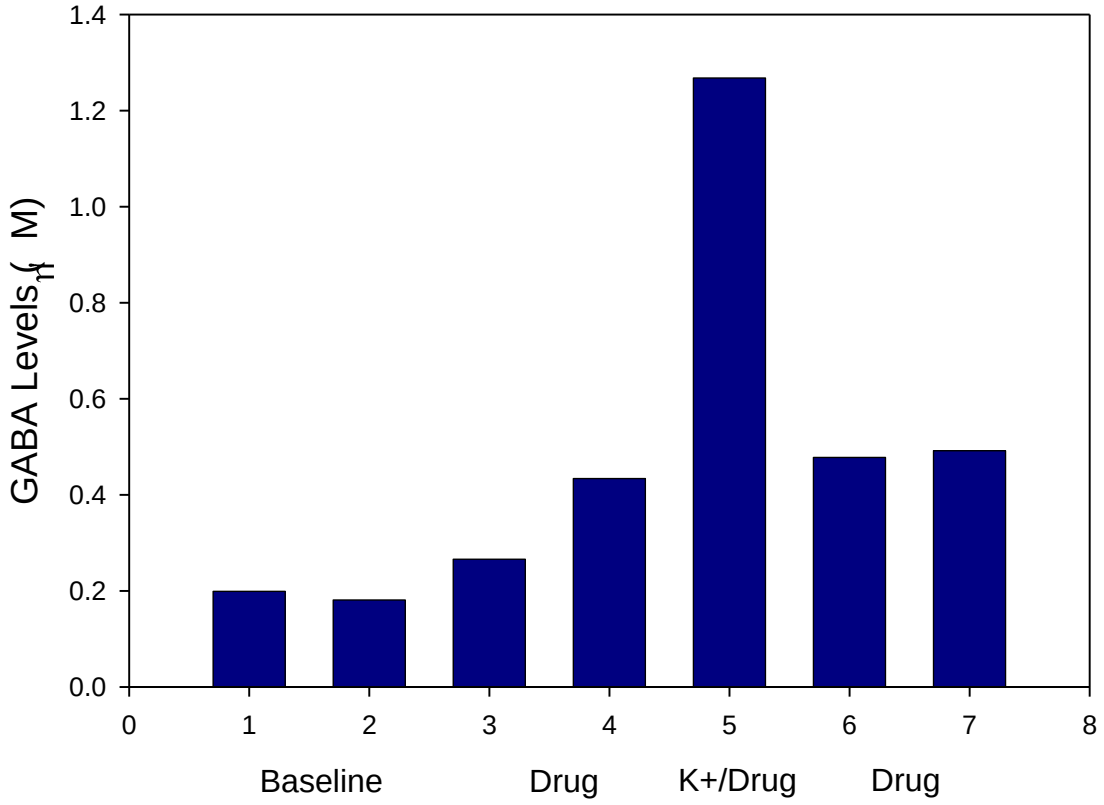



**Figure 5.1.** Example of changes in GABA concentrations in SNr after SKF82958 (0.411 μg/μl) infusion. The probes were perfused with aCSF for two hours at a rate of 2 μl/min for stabilization of GABA levels before the start of sample collection. Samples 1-2 served as the baseline. SKF82958 (0.1 μg/μl) was added to the perfusate for the remainder of the experiment. Samples were collected every 10 minutes. A high concentration of potassium (80 mM) was added to the drug solution for the duration of sample 5 to assess the viability of the tissue.

### 5.1.5 Immunocytochemistry

Although the D1 and D5 receptor subtypes share a high homology in the transmembrane regions, they have significant divergence at the third intracellular loop and carboxy terminus (Sunahara et al., 1991; Tiberi et al., 1991) which has led to the development of selective antibodies that can discriminate D1 and D5 receptors reliably. The D1 and D5 receptor antibodies used in this study have been well characterized using immunoprecipitation, immunoblots and immunocytochemistry methods that reveal the specific reactivity of each antibody is selective for the corresponding receptor subtype (Khan et al., 2000; Levey et al., 1993).

Immunocytochemical techniques are useful to detect the ultrastructural location of receptors in the brain. I examined the subcellular location of the D1 and D5 receptors in GPi and SNr of normal and MPTP-treated monkeys using two different immunocytochemical methods that provide complementary results. The immunoperoxidase and immunogold pre-embedding methods are well established (Charara et al., 2004; Hubert and Smith, 2004a), and the similar results for the ultrastructural and subcellular localization of the D1 receptor using both techniques verifies the consistency of the labeling. One problem associated with both pre-embedding methods is the penetration of immunoreagents and access to antigenic sites which can be solved only by using post-embedding methods.

A few other constraints should be addressed. First, although the immunogold technique provides a more specific location of the receptor on plasma membranes, the silver-intensified gold particles do not diffuse widely and the chemicals used in this process may damage the membranes. Also, one gold particle likely represents many

receptors, and it is possible that only sites that correspond to a cluster of receptors are detected. Because of these limitations, the pre-embedding peroxidase approach was used in parallel. However, the immunoperoxidase technique is not without limitations. Because the immunoperoxidase reactivity often fills the entire element, quantification of receptor sites within each nucleus or at the elemental level is not permitted. Also, double-labeling experiments were not carried out since the majority of D1 and D5 receptor labeling was located in axons. Another interesting experiment would have been to directly compare the density of the D1 receptor to that of the D5 receptor. However, this approach is not feasible because it is likely that the binding affinities of the antibodies to the respective receptor subtype differ, thus not permitting a direct comparison in receptor concentration.

### 5.1.6 Behavioral assessment of MPTP-treated monkeys

A variety of behavioral methods were used to measure the degree of impairment induced by MPTP. Some animals spontaneously recover over a period of a few weeks. In these cases, subsequent MPTP injections were carried out to reach a moderate, stable state of parkinsonism (see figures 5.2, 5.3), as judged by repeated behavioral observations. Therefore, the animals used for the recording experiments underwent the most extensive testing with all methods described below to confirm a stable hemi-parkinsonian state before and during recording sessions.

Most animals were taught to perform a food retrieval task to quantify bradykinesia. A food treat was placed in a well. The entry and exit from the well was

monitored by two infrared beams. The timing of the infrared beam disruptions was recorded to computer disk. Ten trials per side were completed daily (figure 5.2).

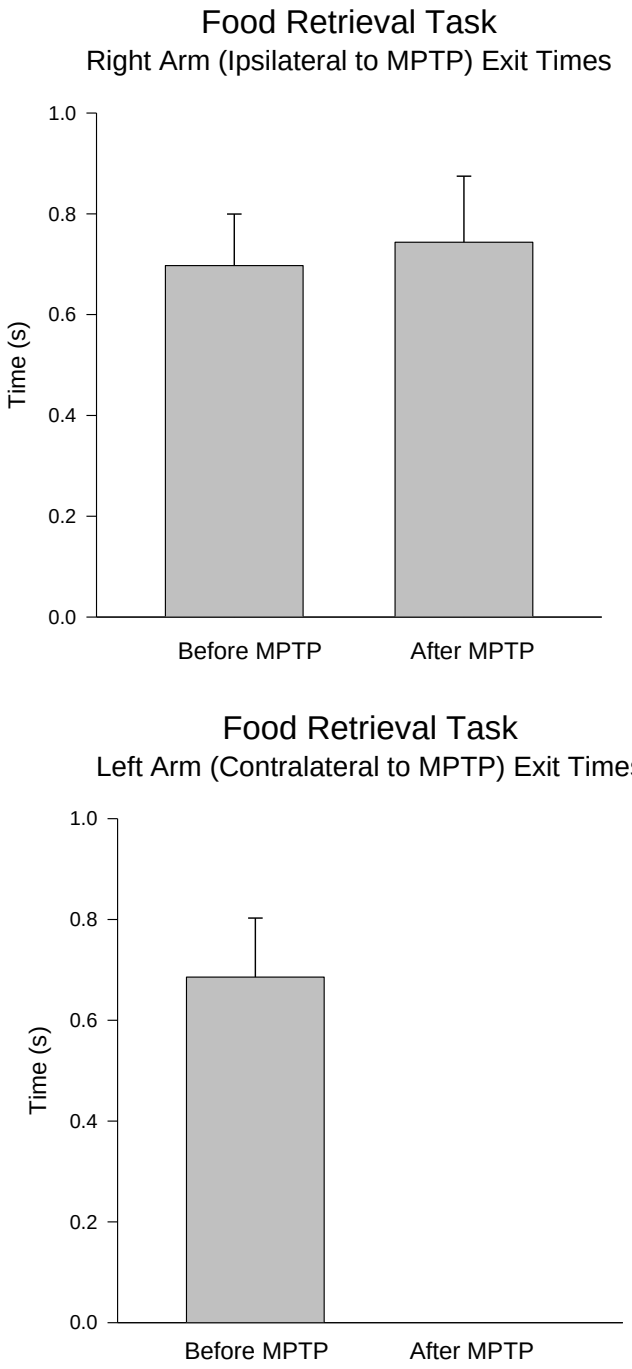



**Figure 5.2.** Quantification of bradykinesia after unilateral intracarotid injections of MPTP. Data represent the average exit time ±SD of the ipsilateral (top panel) and contralateral (bottom panel) arms during a food retrieval task. Exit time refers to the time (s) for the animal to retrieve a food treat from a well. The precise exit time from the well was monitored by two infrared beams, and recorded to computer disk. Ten trials were completed before (n=22) and after MPTP (n=42) injections in one monkey.

Parkinsonian motor signs were documented through observations of spontaneous cage behavior. A computer-assisted behavioral scoring system was used to quantify behavioral changes in animals treated unilaterally with MPTP. For this, limb movements were documented over a 20-min time period. This system has been used and validated in other studies (Bergman et al., 1990; Soares et al., 2004; Wichmann et al., 2001). Briefly, a computer keyboard key was assigned to a limb, and each time the animal moved a limb, the key was pressed for the duration of the movement. The ratio of the arm movements was calculated as an index of the degree of disability (figure 5.3).

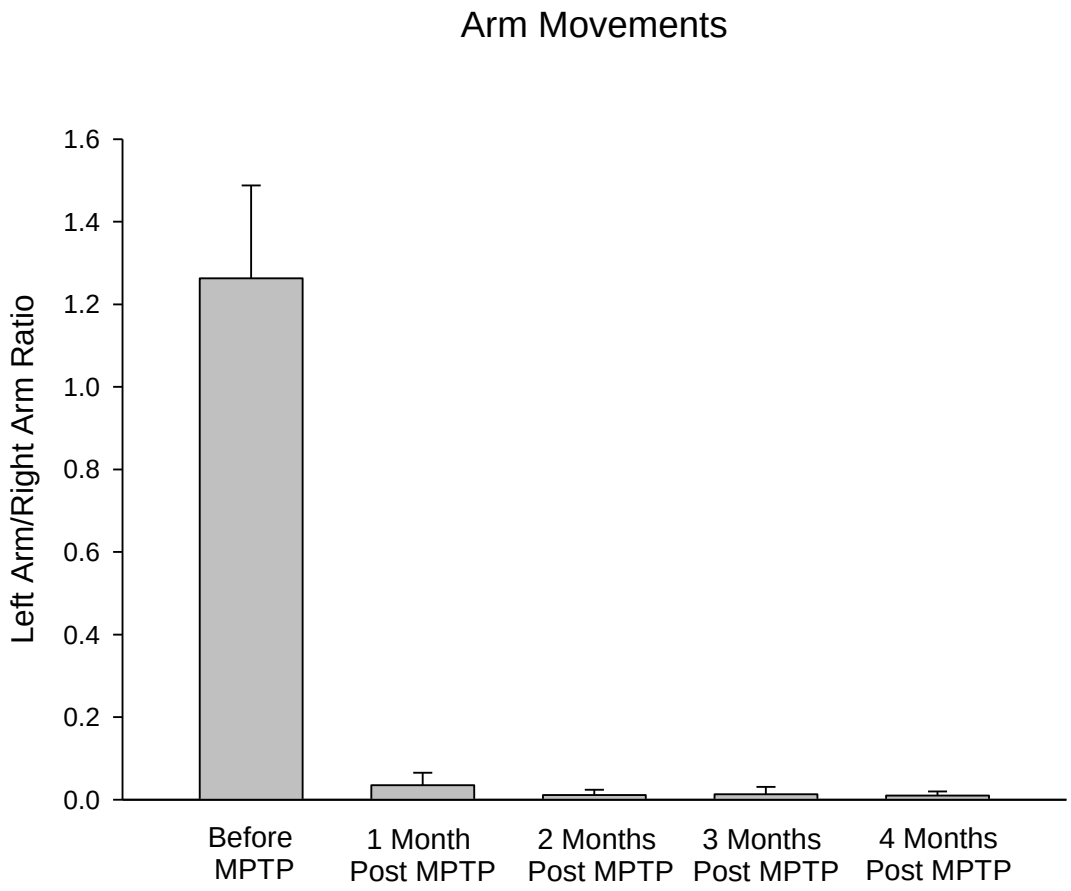



**Figure 5.3.** A comparison of arm movements of the parkinsonian side (left) of the body with the non-affected (right) side were calculated as a ratio (L/R) over a 20-min time period. The data are separated into time blocks that represent one (n=3) and two (n=8) months before and one (n=11), two (n=12), three (n=10) and four (n=5) months after MPTP injections. Data are shown as the mean ±SD.

An automated activity monitoring system was also used. The observation cage was equipped with eight infrared beams (Banner Engineering Corp., Minneapolis, MN) arranged in a square formation on two adjacent sides of the cage (back and side) of the cage. The animal's behavior was also videotaped. A computer system was attached and logged the timing of beam crossings. Off-line, the total activity counts within a 20-min period were calculated (figure 5.4).

Finally, a 15-point rating scale was used to determine the degree of behavioral change induced by MPTP treatment. Nine criteria were used to assess parkinsonian motor signs (gross motor activity, balance, posture, arm bradykinesia, arm hypokinesia, leg bradykinesia, leg hypokinesia, arm tremor, leg tremor), each on a scale of 0-3 (normal/absent to severe), yielding a maximum score of 27. Dystonia and chorea were rated separately with a 4-point scale (arm dystonia, leg dystonia, dyskinetic arm movements, dyskinetic leg movements), yielding a maximum score of 12. Stereotypy and rotational behaviors were rated, yielding a maximal score of 6.

All observations were performed biweekly at the same time of day. The behavioral assessment methods were used before and after MPTP injections, and throughout the recording experiments.

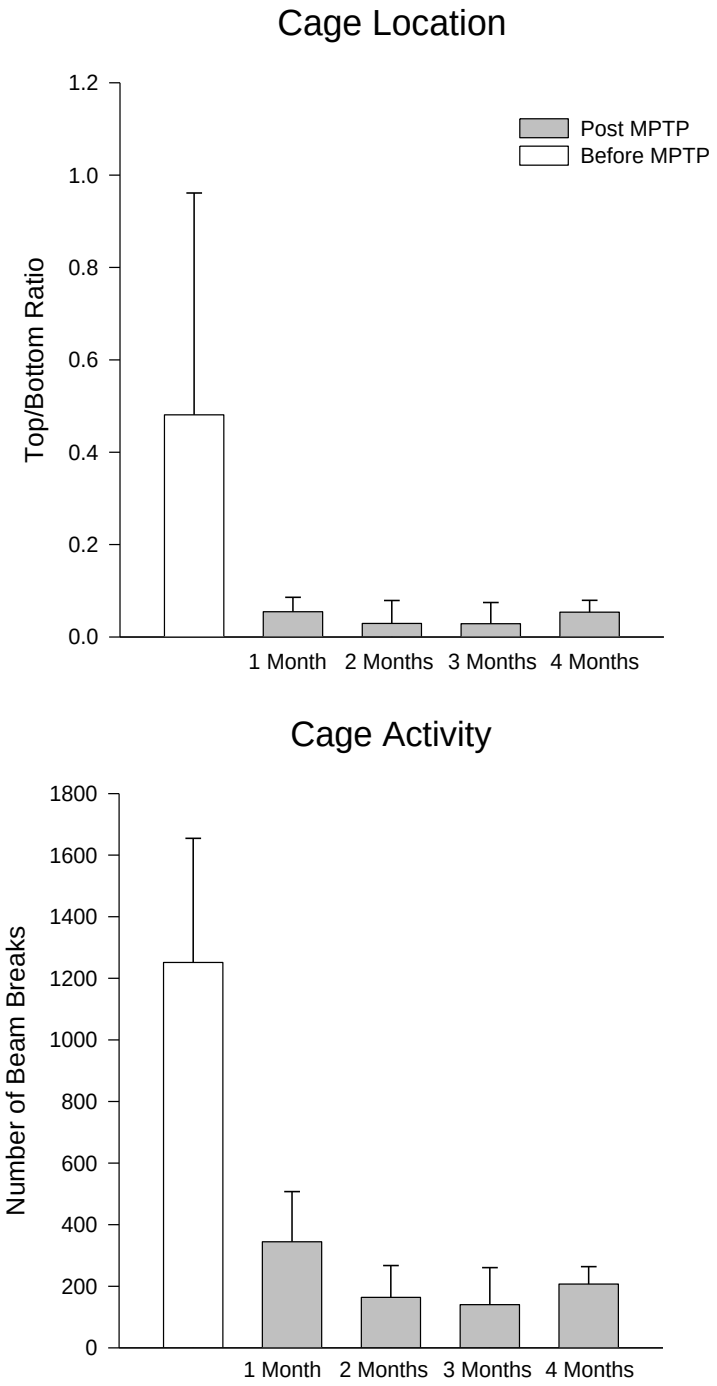



**Figure 5.4.** Degree of disability after unilateral intracarotid injections of MPTP in one monkey. Using the information from the infrared beam emitter/receiver systems located on the two adjacent sides of the observation cage, a comparison of the time spent in the top portion of the cage to the bottom of cage was calculated as a ratio (top panel). Beam crossings were monitored (20 min) and serve as an indicator of spontaneous cage activity. Total numbers of counts are represented on the y-axis (bottom panel). The data are separated into time blocks that represent two (n=11) months before and one (n=11), two (n=12), three (n=10) and four (n=5) months after MPTP injections. Data are shown as the mean ±SD.

## 5.2 Analysis

### 5.2.1 Electrophysiology

#### *5.2.1.1 Spike sorting*

A template-matching spike sorting routine with subsequent principal component analysis (Spike2) was used off-line to determine inter-spike intervals (ISIs). The ISI data were used to generate a discharge rate read-out in one-second intervals in Matlab. Originally, I used the Multi Spike Detection (MSD) program to detect and sort spikes as well as calculate ISIs. However, I found this system to be inferior to the routines available from Spike2. In my experience, the MSD device was not reliable in differentiating two (or more) similar waveforms firing at similar frequencies.

For quality control, ISI distribution histograms were constructed in Spike2 and examined for each unit to detect the possible inclusion of multiple cells. If mistakes or multiple cells or units were found in the sampling, the record was either re-sampled or discarded. Needless to say, I spent a significant portion of time analyzing cells. For the analysis, I felt it was important to distinguish changes in firing rate from interference in the record due to abrupt movements made by the monkeys and units that showed dramatic reductions in firing rate. Although decreases in firing rate were expected in response to D1LR agonist infusions, I wanted to be sure that these reductions were real and that I did not simply lose the cell. To ensure changes in firing rate were due to drug infusions, some units were inspected spike by spike. Only those units in which I was confident that the recording was stable were included in the final analysis.

The behavioral state of the animal is a concern in recording studies. To ensure a constant behavioral state (resting quietly, but awake) of the monkeys used in my study,

video cameras were installed in the monkey testing lab. Since I was situated with the recording equipment in a separate, but adjacent lab, it was vital for me to monitor closely any change in the animal's behavioral state. If I detected signs of drowsiness, I took steps to maintain a constant vigilant state of the monkey. In some cases, some hand clapping worked well, and in other cases singing. One of my monkeys had a finite period of time in which she would cooperate, and to accommodate her and maintain the integrity of the experimental design, I performed only one penetration per experimental session.

#### *5.2.1.2 Drug injections*

The parameters chosen to classify a neuron as having an 'effect' took careful consideration. Based on previous studies (Kliem and Wichmann, 2004), it was rare for a cell to respond immediately upon drug infusion using the rate and volume of the drug injections in my set-up. The start of a response generally occurred between 60 - 120 s after the start of the drug infusion. It was important to set a cut-off for a cell to show a response, and therefore I defined a time window for classifying drug responses. Thus, an injection was classified effective if the discharge rate significantly differed from the baseline for at least 60 s, with an onset within 240 s from the start of the injection. Although the cut-off of 4 minutes is arbitrary, it was necessary to include cells that were responding to the drug infusions rather than other influences. In fact, I did not encounter any cells that significantly changed their firing rate outside of this window.

For subsequent analysis, Matlab was used. Medians with percentile boundaries rather than means with SDs were calculated since the discharge rates and burst values

during the baseline periods varied and could not be expected to be normally distributed. Medians are also less affected by extreme values and are more representative of the data. Changes in drug-induced bursts and oscillatory activity were also examined. Bursts are spikes or action potentials that are closely spaced. For this portion of the analysis, I calculated burst indices (the number of spikes in bursts divided by the total number of spikes) during the pre-injection period and after drug injections for all cells tested. The method for burst detection used in this dissertation was similar to the algorithm developed by Legendy and Salcman (Legendy and Salcman, 1985) called the 'surprise' method. This method assumes that the ISIs in a data segment follow the Poisson distribution, and makes a prediction about the expected ISIs in a given data segment. If a series of unexpectedly short ISIs is encountered, this series is called a 'burst'. Such series of ISIs can be assigned a 'surprise' number, reflecting the levels of (statistical) unexpectedness. The algorithm by Legendy et al. goes through a variety of steps to optimize the surprise value of each burst. Formation of the burst index represents a normalization step in which the number of spikes within bursts is set in proportion to the overall number of spikes in the entire data stream. This serves to minimize the dependence of the burst detection algorithm on the cell's firing rate.

To assess changes in oscillatory activity, two frequency bands (3-8 Hz range and 8-15 Hz range) were analyzed. These two bands were chosen because spectral power in these bands increases in the firing patterns of STN and GPi neurons in parkinsonian monkeys. ISIs were converted to frequency readouts, with a 10 ms bin width. Using these numbers as input values, power spectra were calculated. According to the Nyquist criterion, the chosen bin width allowed us to evaluate spectral frequencies up to 50 Hz.

The sums of the power spectral values in the 3-8 Hz and 8-15 Hz ranges were divided by the total spectral power, resulting in proportional values that could be compared across cells or experimental conditions.

### 5.2.2 Statistics

Non-parametic methods were used for the analysis of electrophysiological and biochemical data. Mann-Whitney tests were used to compare the discharge rates in the baseline period with those in the drug effect period for individual experiments. The Wilcoxon signed rank-test was used to compare the data collected before and after drug or vehicle injections for neurons (recording-injection experiments) and to compare data collected from drug experiments against those from the control experiments (microdialysis experiments) in GPi and in SNr. A minimum *p*-level below 0.05 was accepted as indicating a significant difference.

### 5.2.3 Immunocytochemistry

The analysis for this portion of the study consisted of counting and comparing the number of labeled elements in ultra-thin sections. To visualize the locations of D1 and D5 receptors, first tissue blocks were cut (60 μm slice thickness) using a vibrating microtome and processed for electron microscopic immunocytochemistry and photographed. Then, from a series of 30-50 electron micrographs, immunoreactive elements were categorized as axons, terminals, dendrites or glial processes. I calculated the relative proportion of these elements and expressed them as a percent of all labeled elements in the tissue areas that were examined as described above. Photographs were

taken randomly of tissue sections that contained immunoreactivity, without a specific bias for a particular type of tissue element. However, this technique is open to several potential biases, caused by the relative abundance of tissue elements in the sections, and, of course, the possibility of uneven tissue penetration of the antibodies. I was very careful to systematically photograph tissue sections regardless of the element labeled or the density of the immunoreativity in the sections. For the D1 receptor, the majority of immunoreactivity was found in unmyelinated axons, and was similar between animals in both states examined. In contrast, D5 receptor labeling was found in all cellular elements. To prevent a bias for sampling one element over another, I increased the number of photographs taken in each structure to provide a more accurate assessment of the distribution of the labeling. In other words, I examined a larger surface area to adequately evaluate the pattern of labeling in normal and parkinsonian monkeys.

# Chapter 6

# A Method to Record Changes in Local Neuronal Discharge in Response to Infusion of Small Drug Quantities in Awake Monkeys

**Authors**: Michele A. Kliem and Thomas Wichmann

Kliem MA and Wichmann T. A Method to Record Changes in Local Neuronal Discharge in Response to Infusion of Small Drug Quantities in Awake Monkeys, *Journal of Neuroscience Methods*, 138:45-49, 2004.

## 6. Abstract

A newly designed combination microelectrode-injection system is described which can be used to record electrophysiological responses of individual neurons in the primate brain to local administration of small quantities of drugs or other compounds. The assembly of the system is simple, and the materials used are inexpensive. The system consists of a standard tungsten microelectrode alongside fused silica tubing within a polyimide sleeve. The major advantage of this device is the ability to record with excellent quality, changes in the activity of single cells *in* vivo within 50-100 μm of the location of drug injections.

## 6.1 Introduction

In recent years, several groups have described injection devices that can be used to deliver drugs and record the electrical activity of the target structures in primates (Chen et al., 2001; Crist et al., 1988; Hamada and DeLong, 1992; Tokuno et al., 2002; Tokuno et al., 1998; Tsai et al., 1997). These injection systems help to precisely inject drugs under electrophysiologic guidance, but in most cases the recording quality is not sufficient to reliably record changes in neuronal discharge of single neurons. In order to assess electrophysiological effects of pharmacologic compounds at the single cell level in monkeys, we developed a new technique that allows evaluation of electrophysiological changes in response to the infusion of small quantities of ligands in awake primates. The advantage of the proposed system is the improved recording quality, providing the means to evaluate discharge changes of individual cells to nearby drug infusions. Data obtained through use of this system can be used to directly compare neuronal firing rates, the incidence of bursts and other parameters of neuronal discharge at the single-cell level before, during, and after drug injections.

The poor quality of single-unit activity recorded with existing combination systems (Hamada and DeLong, 1992; Tokuno et al., 2002) is primarily due to the fact that a large diameter, low impedance wire is used for recording. The main use of this type of combination system is to guide the injection cannula to a particular brain region, relying on changes in multiunit background activity in pre-mapped brain regions. In addition, the physical arrangement of electrode and injection device may also limit the quality of recording. For instance, the design used by Chen et al. (Chen et al., 2001) aligns the electrode tip flush with the tip of the 'injectrodes'. This is an arrangement

which we have found to impede neuronal recording. One combination recording-injection model (Tsai et al., 1997) has been reported to yield good neuronal recording. This method combines a microinjection capillary with a glass-insulated microwire. Similarly, a glass-coated tungsten wire can be combined with multibarreled glass micropipettes (Li et al., 1990) for neuronal recording and iontophoresis in primates. In both cases, the recording quality is superior to that of low-impedance recording wires, but the assembly of these systems is laborious and difficult, and the resulting electrodes are fragile.

We set out to develop a simpler combination microelectrode recording-injection system that would allow us to record changes in neuronal activity of individual units in the immediate vicinity (within 50-100 μm) of the injection site *in vivo*. The primary goal of this effort was to evaluate neurons with the same high quality recording that could be obtained by standard single-unit recording with high impedance metal microelectrodes, for several minutes before, during and after infusion of small quantities of drugs.

## 6. 2 Methods

### 6.2.1 Assembly of recording-injection system

The process to assemble the recording-injection device is illustrated in Figure 6.1. The individual steps of assembly are shown in the figure, and will be discussed here in turn. To begin the assembly process, fused silica tubing (I.D. = 40 μm; O.D. = 103 μm; Polymicro Technologies, Phoenix, AZ) is broken free from the spool resulting in a beveled tip (Step 1). The choice of this particular type of injection tubing was guided by several considerations. It is advantageous that fused silica is sturdy, rigid, and chemically

durable. It does not kink easily, and air bubbles do not become trapped inside the tubing as often occurs with other types of small-bore tubing (for instance, polyethylene tubing). To reduce dead space, fused silica tubing with the smallest inner diameter may initially appear ideal. However, we have found that fused silica tubing with a diameter less than 40 μm is more rigid, more susceptible to breaking, and clogs more easily.

In the second step, a 10.5 mm segment of polyimide tubing (O.D. = 0.5 mm; MicroLumen, Tampa, FL) is prepared to serve as a sleeve for protection of the injection tubing and recording electrode. The sleeve adds stability to the injection/recording assembly, and protects it, so that it is easier to manipulate in the experimental session. One end of the polyimide tubing is beveled using dissecting scissors. The beveled end of the silica tubing is threaded through the polyimide 'sleeve'. Alternative options (i.e., the use of metal tubing or manual application of epoxy to fix the electrode to the silica tubing) would significantly increase the outer diameter of the system, and were therefore not further explored.

Next, a tungsten microelectrode (Frederick Haer Co., Bowdoinham, ME; Z = 0.7-2.5 MΩ; shaft diameter = 250 μm; length = 12.5 cm; expolylite insulation) is inserted into the polyimide tubing under a stereomicroscope to configure the distal portion of the system as shown in Figure 6.1, Step 3. Glass-coated electrodes (Dias and Segraves, 1997) or fine-tip electrodes (Chen et al., 2001) can also be used for recording, however, we found glass-coated electrodes do not slide smoothly through the polyimide sleeve, and to break more easily while fine-tip electrodes are expensive and are more difficult to handle.

In our experience, the spatial relationship between the microelectrode tip and the beveled end of the fused silica is critical to ensure high quality neuronal recording. The

best configuration between the injection portion of the system and recording characteristics is accomplished by rotating the opening of the beveled end of the silica tubing so that it is opposite the microelectrode tip to support infusion of compounds in the direction of the microelectrode. The tip of the silica tubing is adjusted to project 1 mm from the polyimide sleeve, and the electrode tip is placed so that it extends 50-100 μm further (Step 3). The tips of the silica and electrode are secured in place adjacent to each other by applying small quantities of epoxy glue at the end of the polyimide sleeve. Figure 6.1 illustrates the configuration of the microelectrode and beveled silica tubing inside the polyimide sleeve.

At the proximal end of the silica tubing, a 1 mm 23-ga stainless steel tube (Small Parts, Miami Lakes, FL) is placed over the fused silica and glued in place with epoxy as shown in Step 4. The metal tubing provides a leak-free connection to the liquid switch (CMA, Solna, Sweden) through a 'flexible connector' (CMA), as shown in Figure 6.1, Step 5. The liquid switch is configured with three ports to deliver ligands, and allows continuous monitoring of all substances.

Once the epoxy has cured, excess silica is removed from the proximal end of the recording-injection system and the 'flexible connector' is attached to the outlet of the liquid switch. The inlets of the liquid switch are connected to 1 ml gastight syringes (CMA), operated by an infusion pump (Harvard Instruments) for pressure infusion of sub-microliter quantities of drugs or artificial cerebral spinal fluid (aCSF, comprised of (in mM concentrations) 143 NaCl, 2.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1 $Na_2HP0_4$, pH=7.2-7.4) to the injection site. The combination system is fixed in a microdrive (MO-95B, Narishige; Tokyo, Japan) so that it can be lowered into the brain with precision. The

proximal end of the electrode is connected to standard preamplifiers for electrophysiologic recording. The total assembly time of the combination recording-injection system is less than 15 minutes.

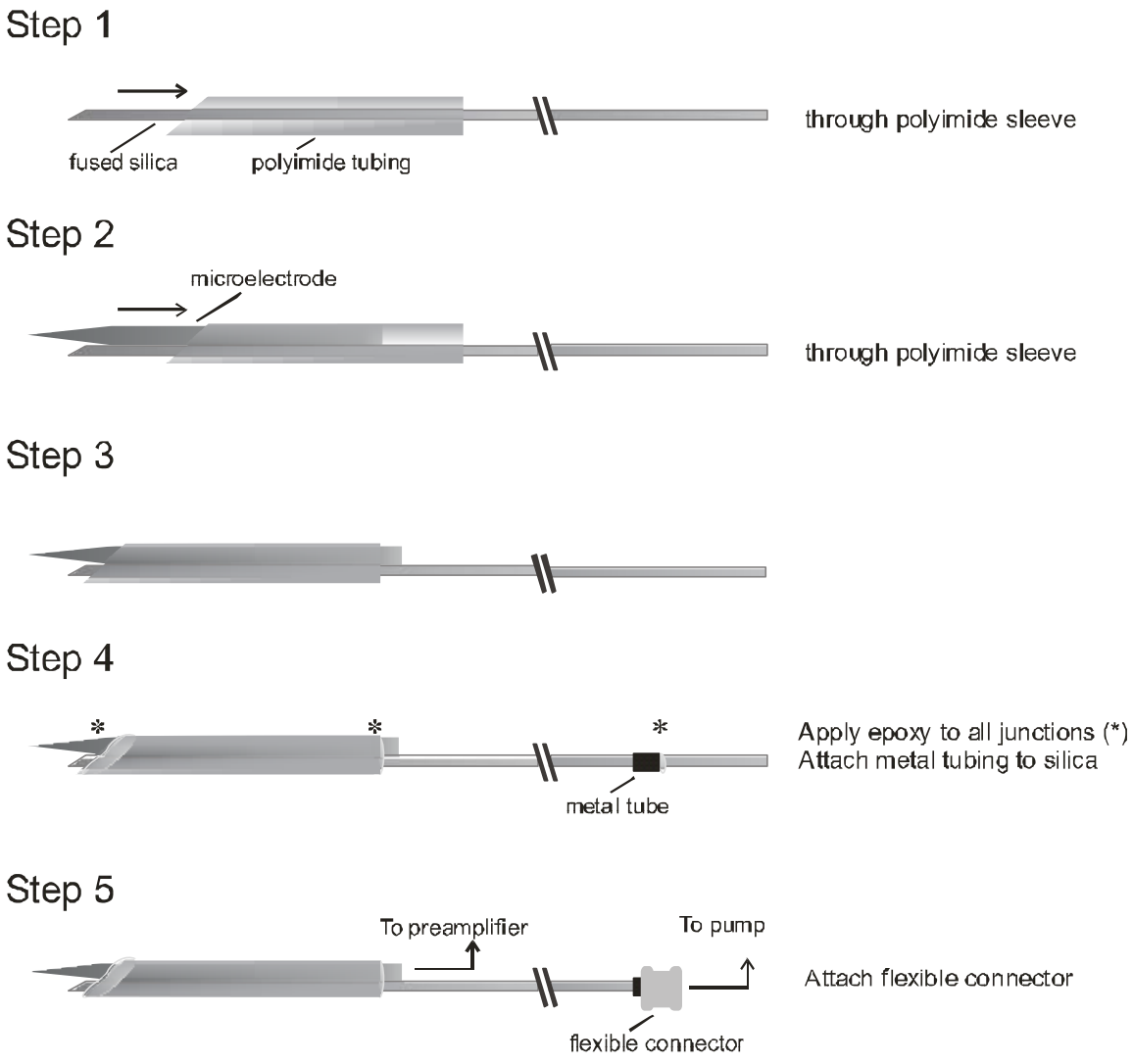
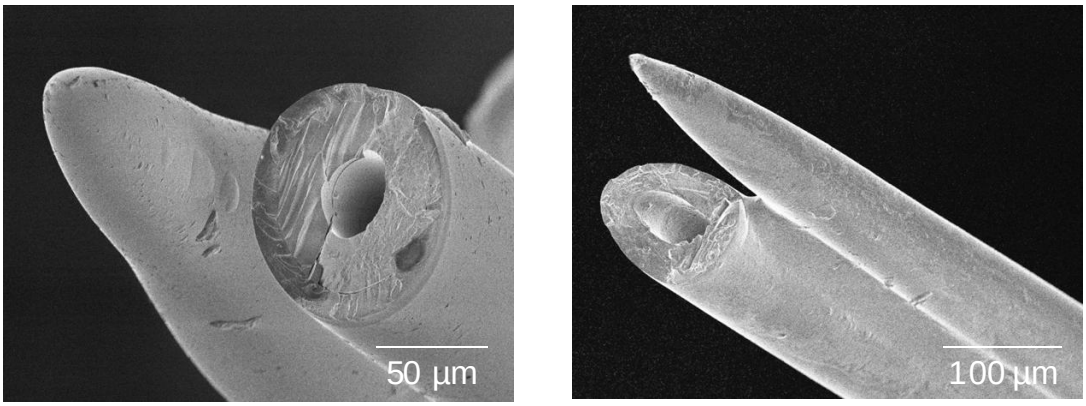



**Figure 6.1** Assembly of microelectrode-injection system.

A.




B.


**Figure 6.2.** Arrangement of the microelectrode tip and fused silica tubing inside a polyimide sleeve. A. Electron photomicrographs taken with a scanning electron microscope (Emory University) at 3.0 kV. B. A photomicrograph showing the end result of the assembly process.

### 6.2.2 Use of recording-injection system

All solutions that are perfused through the system are filtered using a 0.250 μm micropore filter (Fisher Scientific, Hampton, NH) before use to remove any particles from being incorporated into the final injection solution, and to prevent contamination of the system. Solutions are loaded into individual syringes, while carefully avoiding air bubbles. The syringes to be used in a given experiment are then fixed to the infusion pump, and connected to tubing which links them to the liquid switch. The pump is switched on for the remainder of the experiments, so that there is continuous flow of all of the solutions through the liquid switch.

Flow through the injection system is established with repeated flushes with filtered distilled water at a rate 0.5-1.0 μl/min before being positioned into the microdrive. Once the system is fixed to the microdrive, one of the three liquid switch ports is connected to it, and from this point on until the end of the drug injection, there is continuous flow of either aCSF or drug solution through the injection system. This strategy was chosen so as to prevent clogging of the injection system. In addition, a simple mechanism is provided by which the integrity of the system can be tested. Any clogging of the infusion lines will result in increased pressure throughout the injection tubing, which is easily recognized as a leak of injectate at the liquid switch. Prior to insertion of the system into the brain, the injection tubing is flushed (1.0 μl/min) with filtered aCSF. At the conclusion of each experiment, the device is again visually checked for proper flow of solutions, providing further confirmation that, in fact, drug solution was infused. This is followed by a thorough cleaning of the system with filtered distilled water to prevent crystal formation. The impedance of the recording electrode is checked

before and after each use to ensure that the recording quality remains satisfactory. After use, the microelectrode-injection tip is cleaned thoroughly with alcohol, and stored for reuse. It is possible to use gas sterilization as a means to condition the system for reuse.

### 6.2.3 Other methods used for preliminary experiments

#### *6.2.3.1 Animal and surgery*

All experimental protocols were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Anonymous, 1996), and the PHS Policy on Humane Care and Use of Laboratory Animals (amended 2002), and were approved by the Institutional Animal Care and Use Committee at the Institutional Biosafety Committee at Emory University. For the pilot experiments described here, a Rhesus monkey received a stainless steel metal chamber which was stereotactically aimed at the globus pallidus at an angle of 50º in the coronal plane for chronic recording under aseptic conditions and isoflurane anesthesia. The recording chamber, together with a standard metal head holder (Crist Instruments, Hagerstown, MD), was embedded into a dental acrylic 'cap' which was affixed to the animal's skull. The experimental sessions began one week after surgery.

#### *6.2.3.2 Recording and injection procedures*

All recordings and injections were made using the above-mentioned microdrive. The probes were slowly lowered with the microdrive through the dura, using a 20-ga guide tube.

The boundaries of the target nuclei (external and internal segment of the globus pallidus) were first delineated with electrophysiological mapping procedures using a standard tungsten microelectrode. We then used the electrophysiologic target information to place the recording-injection system to record single pallidal neurons. For each of the recorded neurons, the electrical activity in the 60 s segment prior to drug infusion served as the control period (Figure 6.2A). After this control period we switched the liquid switch to infuse the GABA-A receptor agonist muscimol (1 mg/ml; Sigma, St. Louis, MO) at a rate of 0.5 µl/min. After 1 µl of drug was delivered, we switched the infusion system back to regular aCSF which was infused until the end of recording. Neuronal activity recorded prior to muscimol infusion was compared with the activity of the same neuron during and after drug infusion to identify changes in discharge rates and patterns.

The recorded neuronal activity was amplified (DAM80, WPI, Sarasota, FL), displayed on a digital oscilloscope (DL-1540, Yokogawa, Tokyo, Japan), and audio-amplified. The recorded activity was stored to computer disk using a data acquisition system (Spike2, CED, Cambridge, UK) for later off-line analysis.

#### *6.2.3.3 Analysis*

Action potentials were detected using a spike sorting routine (provided by the Spike2 system) to isolate action potential waveforms for individual units, and compute interspike intervals (ISIs). The ISI information was used to calculate average discharge rates, ISI distributions, and the incidence of burst discharges for each neuron before, during, and after injections using algorithms in the Matlab programming environment

(Mathworks, Natick, MA). The post-drug discharge rates (binned in 15 s-intervals) of the neuron were considered different from the baseline activity if the discharge rate of the recorded neuron changed by 2 S.D from the average of the baseline activity.

## 6.3 Results

Figure 6.3 shows the response of a single pallidal neuron before, during, and after (Figure 6.3A and B) infusion of 1 μl of muscimol. Immediately after completion of the muscimol infusion (as indicated by the solid black line), the pallidal cell was completely inhibited. The neuronal activity recovered and returned to baseline after several minutes (Figure 7.3B). Reductions in activity were seen in all of the pallidal cells ($n$ = 6 cells), and the maximal effect was similar for all injections.

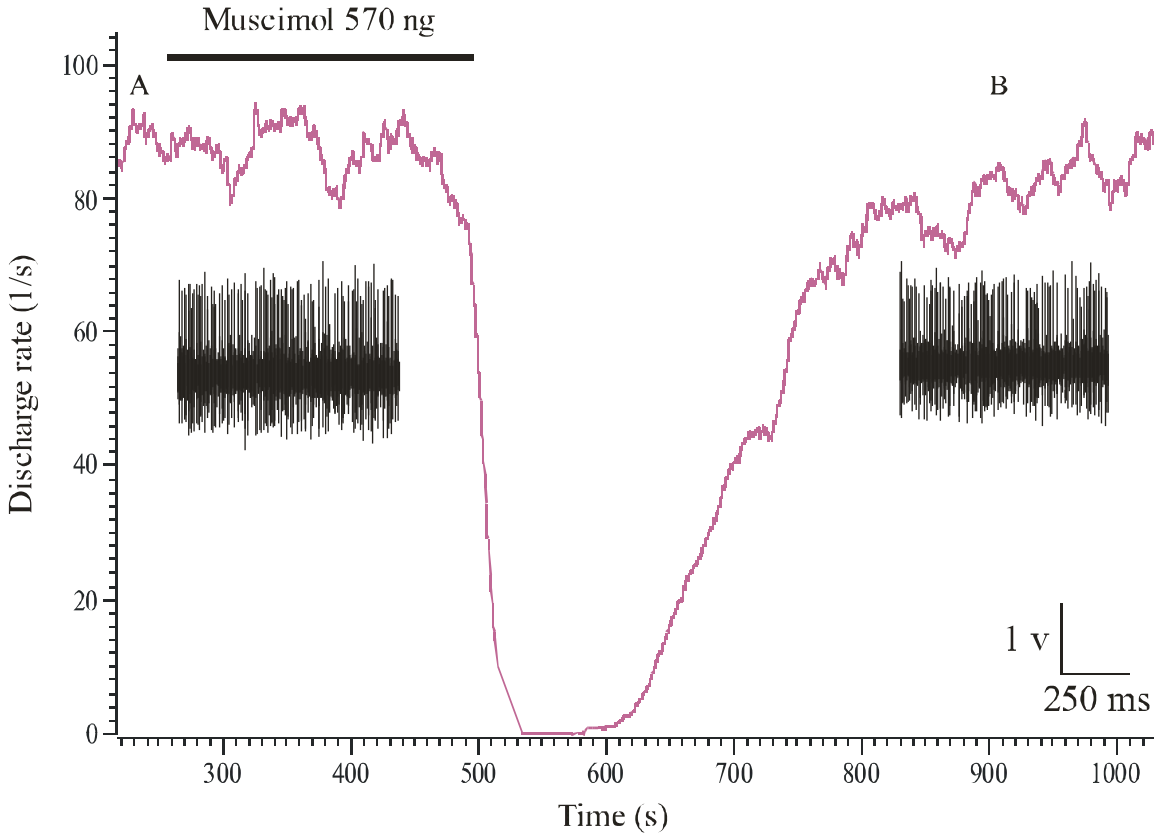



**Figure 6.3.** Changes in single-unit extracellular recording of a pallidal neuron using the combination recording-injection system to deliver drug. A. Baseline neuronal discharge. B. Return to baseline neuronal discharge. The black line represents the length of drug infusion period (corrected for the deadspace of the infusion system).

## 6.4 Discussion

Most of the available recording/injection devices were developed because of the need for electrophysiologic guidance for the delivery of substances into defined brain targets. The existing designs often use low-impedance wire electrodes that serve to distinguish white and gray matter, and are sufficient to place substances into brain tissue. However, they cannot be used to examine the effects of drugs on the discharge activity of single cells near the injection site. Because of these limitations, we sought to develop a combination microeletrode recording-injection system that is capable of high quality neuronal recording and accurate delivery of small volumes of substances which would enable changes in neuronal discharge to be examined before, during, and after injections of drugs.

There are several important differences between the new system and existing devices. One of these differences is that a high-impedance microelectrode is used for recording of single cell activity. The quality of the resulting electrophysiologic records is good enough to analyze directly the effect of a drug on discharge rates and patterns of neurons located close to the injection site. Secondly, fused silica tubing is used to inject drugs. This miniaturizes the injection portion of the system so that drugs can be injected within very short distances from the recording electrode. Finally, we use a liquid switch to change the infusion medium. With this approach control injections can be recorded, as well as responses to both an agonist and antagonist of the same cell. Similar results could be also obtained by combining multiple injection tubes with one microelectrode. However, the size of the resulting system, and, hence, the tissue damage, would be substantially larger than the size of our system. The system presented here is of

comparable size to several others reported in the literature, whose outer diameters range from 0.366 mm (Hamada and DeLong, 1992) to 0.462 mm (Tokuno et al., 1998).

Overall, the system is easy to build, and, with the proper care, can be reused several times. Although designed for use in primates, the dimensions given here can be adjusted for use in other animal species.

In conclusion, the simple recording-injection device presented here provides the capability to detect changes in neuronal discharge in response to small amounts of infused compounds within 100 μm of the injection site. This technique can be used for pharmacologic experiments at the single cell level in awake animals.

# Chapter 7

# Activation of Nigral and Pallidal Dopamine D1-Like Receptors Modulates Basal Ganglia Outflow in Monkeys

**Authors**: Michele A. Kliem, Nigel T. Maidment, Larry C. Ackerson, Sugong Chen, Yoland Smith and Thomas Wichmann

**Kliem, M.A**., et al. Activation of Nigral and Pallidal Dopamine D1-Like Receptors Modulates Basal Ganglia Outflow in Monkeys, *Journal of Neurophysiology*, 98:1489-1500, 2007.

## 7. Abstract

Studies of the effects of dopamine in the basal ganglia have focused on the striatum, while the functions of dopamine released in the internal pallidal segment (GPi) or in the substantia nigra pars reticulata (SNr) have received less attention. Anatomic and biochemical investigations have demonstrated the presence of dopamine D1-like receptors (D1LRs) in GPi and SNr, which are primarily located on axons and axon terminals of the GABAergic striato-pallidal and striato–nigral afferents. Our experiments assessed the effects of D1LR ligands in GPi and SNr on local GABA levels and neuronal activity in these nuclei in Rhesus monkeys. Microinjections of the D1LR receptor agonist SKF82958 into GPi and SNr significantly reduced discharge rates in GPi and SNr, while injections of the D1LR antagonist SCH23390 increased firing in the majority of GPi neurons. D1LR activation also increased bursting and oscillations in neuronal discharge in the 3-15 Hz band in both structures, while D1LR blockade had the opposite effects in GPi. Microdialysis measurements of GABA concentrations in GPi and SNr showed that the D1LR agonist increased the level of the transmitter. Both findings are compatible with the hypothesis that D1LR activation leads to GABA release from striato-pallidal or striato-nigral afferents, which may secondarily reduce firing of basal ganglia output neurons. The antagonist experiments suggest that a dopaminergic 'tone' exists in GPi. Our results support the finding that D1LR activation may have powerful effects on GPi and SNr neurons, and may mediate some of the effects of dopamine replacement therapies in Parkinson's disease.

## 7.1 Introduction

The basal ganglia are a group of subcortical structures which interact with the cerebral cortex and thalamus (Alexander and Crutcher, 1989). According to the traditional model of basal ganglia connectivity and function (Albin et al., 1989; Alexander and Crutcher, 1990; Nambu et al., 1996), cortical information reaches the basal ganglia primarily through the striatum, and is then conveyed to the basal ganglia output structures, the internal pallidal segment (GPi) and the substantia nigra pars reticulata (SNr), via monosynaptic GABAergic projections, as well as polysynaptic routes. Basal ganglia output provides inhibitory input to the thalamus and brainstem. These circuits are highly topographic in their organization (Middleton and Strick, 2002; Wichmann and Delong, 2006). At the level of the basal ganglia output circuitry, movement-related circuits predominately pass through GPi, while associative and limbic circuits traverse the SNr.

Dopamine is thought to modulate neuronal activity throughout this system. Released in the striatum from terminals of the nigrostriatal projection (Bernheimer et al., 1973; Hornykiewicz and Kish, 1987), dopamine is known to alter the activity of striatal projection neurons (Gerfen, 1995), resulting down-stream in an overall reduction of inhibitory basal ganglia output from GPi and SNr (Wichmann and Delong, 2006). Dopamine may also act in the extrastriatal basal ganglia (Bernheimer et al., 1973; Cheramy et al., 1981; Geffen et al., 1976; Robertson et al., 1991a; Schneider and Rothblat, 1991; Smith and Bolam, 1989; Whone et al., 2003). These actions of dopamine, specifically within SNr and GPi, are less well explored. Dopamine is supplied to GPi via the nigro-pallidal projection which arises from a population of substantia nigra pars compacta (SNc) neurons which is different from that giving rise to the nigrostriatal

projection in primates (Jan et al., 2000; Smith et al., 1989), while in the SNr, dopamine is released from dendrites of SNc neurons that descend dorsoventrally and arborize profusely along the basis of the SNr (Arsenault et al., 1988; Bjorklund and Lindvall, 1975; Nieoullon et al., 1978). It is unclear whether the neurons that provide dopamine to the SNr via dendritic release are the same as those that provide dopamine to the striatum or GPi.

Dopamine acts at D1-like receptors (D1LRs, including D1- and D5-receptors, (Clark and White, 1987; Neve, 1997) and D2-like receptors (D2LRs, including D2-, D3-, and D4-receptors, (Neve, 1997). Receptor binding studies in monkeys have demonstrated that GPi and SNr contain predominately D1LRs (Richfield et al., 1987). Most of these receptors are located on presynaptic axons and axon terminals of GABAergic striatopallidal and striatonigral projections (Barone et al., 1987; Fremeau et al., 1991b; Kliem et al., 2006; Levey et al., 1993; Mengod et al., 1991; Yung et al., 1995b). The aim of the present study was to explore the effects of dopamine receptor activation and of locally released dopamine on the rate and patterns of neuronal spiking in the non-human primate GPi and SNr. Changes in spontaneous neuronal discharge were examined before, during and after local infusions of selective D1LR ligands into GPi or SNr in awake monkeys. Microdialysis methods were used to assess whether the local administration of these agents alters GABA concentrations.

## 7.2 Materials and Methods

### 7.2.1 Animals

Five Rhesus monkeys (Macacca mulatta, 3-5 kg) were used for these studies. They were housed under conditions of protected contact housing, with free access to standard primate chow and water. Prior to the other procedures, the animals were trained to sit in a primate chair, to adapt to the laboratory, and to permit handling by the experimenter. The experimental protocols were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Anonymous, 1996) and the PHS Policy on Humane Care and Use of Laboratory Animals (amended 2002), and were approved by the Animal Care and Use Committee of Emory University.

### 7.2.2 Surgical procedures

After completion of behavioral training, two stainless steel chambers for chronic access (inner diameter, 16 mm) were stereotactically positioned over trephine holes under aseptic conditions and anesthesia with isoflurane (1 - 3%). A chamber directed at the pallidum was placed at an angle of 40° from the vertical in the coronal plane (A = 12, L = 10, D = 4; Horsley-Clarke coordinate system) and a chamber aimed at the SN was placed at an angle of 25° posterior to the vertical in the sagittal plane (A = 9, L = 5, D = 0). The chambers were affixed to the skull with dental acrylic. Metal head holders were also embedded into the acrylic cap to permit head stabilization during the recording and microdialysis procedures. The animals were allowed to recover for one week after the surgery. They were awake throughout all of the subsequent experiments.

### 7.2.3 Electrophysiologic recording experiments

Electrophysiologic recording techniques were used to generate maps and to explore the effects of locally applied drugs on the spiking activities of neurons in SNr and GPi.

#### *7.2.3.1 Mapping procedures*

For initial electrophysiologic mapping, the neuronal activity in GPi and SNr was recorded extracellularly with tungsten microelectrodes (Frederick Haer Co., Bowdoinham, ME; impedance 0.5-1.0 MΩ at 1 kHz). A microdrive (MO-95B, Narishige, Tokyo, Japan) was used to lower the electrodes through the dura into the brain, using a 20-gauge guide tube (Small Parts, Miami Lakes, FL). The microdrive carried an X-Y stage that allowed us to determine the penetration coordinates inside the recording chamber. In addition, the microdrive was equipped with a linear potentiometer which was used to generate digital depth readouts, reflecting the position of the tip of the electrode with 10-µm accuracy. The electrical neuronal signal was amplified (DAM-80 amplifier, WPI, Sarasota, FL), filtered (400-10,000 Hz, Krohn-Hite, Brockton, MA), displayed on a digital oscilloscope (DL1640, Yokogawa, Tokyo, Japan), and made audible via an audio amplifier. GPi and SNr neurons were identified by their characteristic high frequency discharge rates. Surrounding nuclei have distinctly different firing rates and patterns (Soares et al. 2004; Starr et al. 1999).

#### *7.2.3.2 Injection/recording experiments*

We used combined microelectrode recording-injection probes (Kliem and Wichmann, 2004) to examine the effects of D1LR-selective ligands on SNr and GPi neuronal activity in three monkeys. A standard polyimide-coated tungsten

microelectrode (see above) alongside fused silica tubing (Polymicro Technologies, Phoenix, AZ; inner diameter, 40 µm; outer diameter, 102 µm) was enclosed by a protective polyimide sleeve (MicroLumen, Inc., Tampa, FL; outer diameter, 0.5 mm), and held in place with epoxy glue. The tip of the electrode extended about 75 µm beyond the tip of the silica tubing (range 50-100 µm). At the proximal end of the silica tubing, a 10 mm 23-ga stainless steel tube (Small Parts) was placed over the fused silica and glued in place with epoxy. The metal tubing provided a leak-free connection to a micro-T connector (CMA, Solna, Sweden) through a 'flexible connector' (Saint-Gobain, Akron, OH; Tygon tubing; inner diameter, 0.020"). The inlets of the micro-T were connected to 1 ml gas-tight syringes (CMA), driven by a remotely controlled dual-syringe infusion pump (CMA/102) for pressure infusion of sub-microliter quantities of drug or vehicle solutions.

To test the integrity of the injection system, it was flushed with artificial cerebrospinal fluid (aCSF-comprised of (in mM): 143 NaCl, 2.8 KCl, 1.2 CaCl2, 1.2 MgCl2, 1 Na2HPO4, pH=7.2-7.4) at 5 µl/min prior to insertion into the brain. Prior to the experimental sessions, the selective D1LR agonist, SKF82958 (Sigma-Aldrich Co, St. Louis, MO; 3 µg/µl), and the D1LR antagonist, SCH23390 (Sigma-Aldrich Co; 5 µg/µl) were dissolved in aCSF and 0.1% ascorbic acid solution, followed by sonication. All solutions were filtered prior to infusion (Fisher Scientific, Hampton, NH; pore size = 0.2 µm).

In a typical experiment, one syringe was filled with aCSF, while a second one was filled with the drug solution or vehicle (for control injections). The system was then lowered into the brain through a 20-gauge guide tube. Each cell encountered with the

system's microelectrode was recorded for at least one minute pre-infusion (pump off), during the infusion (pump on, running at 0.25 µl/min for 2 minutes), and for at least four minutes thereafter (pump off). The amplified signal, as described above, was stored to computer disk using the CED data acquisition system (Spike2, CED, Cambridge, UK). Each animal underwent several recording-injection procedures, separated by at least 24 hours. Neurons throughout both nuclei were sampled. In most experimental sessions, single drug injections were carried out, although in a few, a second injection was done, spatially separated from the first by at least 500 µm, and injected at least thirty minutes apart. In our experience, drug effects (with the injection volume chosen here) rarely extend more than 200-300 µm beyond the tip of the recording electrode (personal observation and(Kita et al., 2004), and cumulative drug effects were not seen in these studies.

### 7.2.4 Microdialysis experiments

Microdialysis experiments were carried out to assess the effects of D1LR ligands on GABA release in the SNr and GPi in two monkeys. The dialysis probes were custom-made (CMA; length: 135 mm). They consisted of a cuprophane membrane (length, 2 mm; outer diameter, 0.24 mm; cutoff molecular weight, 6 kD). The diameter of the outer steel shaft was 0.38 mm. The inflow line of the microdialysis system was connected to a 1 ml gas-tight glass syringe (CMA). Prior to use, the probes were flushed in succession with 70% ethanol, distilled water and aCSF (all delivered at a rate of 5 µl/min).

Each animal underwent several microdialysis procedures, separated by at least 24 hours. For each microdialysis session, a new probe track was used in order to sample

undisturbed tissue. Prior to insertion, a metal tube with a fitting stylet was lowered to the appropriate stereotaxic coordinates through a guide tube into the brain. The distal end of the tube was adjusted, according to depth data obtained through electrophysiologic mapping experiments, so that the tube ended one millimeter above the target. The stylet was removed and the microdialysis assembly was inserted into the inner tube, advanced into the target area. Before the start of sample collections, the probes were perfused with aCSF for two hours at a rate of 2 μl/min for stabilization of GABA levels. Two ten-minute microdialysate samples were then collected into glass centrifuge vials. These samples were used to establish the baseline GABA levels. The perfusate was then switched for ten minutes to one containing SKF82958 (0.411 μg/μl), SCH23390 (0.01 μg/μl), or vehicle (for control experiments), using a liquid switch syringe selector (CMA). In one set of experiments, the perfusate was then switched back to normal aCSF for the next three samples. In a second set of experiments, the D1LR ligands or vehicle were perfused for the remainder of the experiment. In these experiments, a high concentration of potassium (80 mM) was added to the drug/vehicle solution for the duration of sample #5 to assess the ability of the tissue to release GABA when stimulated. During high-potassium exposure, the concentration of NaCl was lowered to maintain osmolality (67.8 mM NaCl, 80 mM KCl, 1.2 mM MgCl2, and 1.2 mM CaCl2). At the end of each microdialysis session, the probe was removed, and placed into aCSF containing a known concentration of GABA (10 ng/ml) to determine transmitter recovery. The recovery values clustered closely around 15-20%. The values reported in this paper were not further adjusted for recovery.

All samples were immediately placed on dry ice and later stored at -80ºC until the time of analysis. The concentration of GABA in the dialysate samples was determined by high performance liquid chromatography with fluorometric detection as described previously (Murphy and Maidment, 1999). Briefly, automated precolumn o-phthaldialdehyde (OPA) derivatization of amino acids was achieved by adding 30 µl of derivatization reagent, containing OPA (3 µg) and ß-mercaptoethanol (0.125% v/v) in 0.125M boric acid buffer (pH 10), to each sample 2 min before injection onto a C18 reverse-phase column (150 X 3.0 mm; 3 µm particle size; Thermo-Fisher). GABA was eluted with an acetonitrile/methanol gradient. The limit of detection for GABA was 5 fmol (3:1 signal to noise ratio).

### 7.2.5 Histology

At the conclusion of the experiments, the monkeys were sedated with ketamine (10 mg/kg, i.m.) and then injected with an overdose of sodium pentobarbital (25 mg/kg, i.v.), followed by transcardial perfusion with 500 ml of saline and 4% paraformaldehyde/0.1% glutaraldehyde fixative in 0.1 M phosphate buffer (PB), pH 7.2. The brains were removed, cryoprotected in a 30% sucrose solution in 0.1 M PB, cut at 50 □ m with a freezing microtome, collected serially and stored at-20º C in an anti-freeze solution. Tissue blocks containing GPi were sectioned in the coronal plane, while those containing the SNr were sectioned in the parasagittal plane. One of every four sections was stained with cresyl violet. These sections were later used to verify probe and electrode locations.

### 7.2.6 Data analysis

#### *7.2.6.1 Electrophysiologic data*

For the electrophysiologic analysis, data from three monkeys (F, I, and A) were examined. Only those recordings in which the reconstruction of the location of recorded neurons, based on probe position measurements during the experimental sessions, and postmortem histological analyses, showed that they were located in GPi or SNr were used. Forty-five neurons were included in this study (monkey F, 10 neurons; monkey I, 31 neurons; monkey A, 4 neurons). There were no clear differences in neuronal activity or the responses to drug infusions between the animals and the data were pooled for the subsequent analysis. A template-matching spike sorting routine with subsequent principal component analysis (Spike2) was used for off-line measurements of inter-spike intervals (ISI). ISI distribution histograms and autocorrelograms were carefully examined for evidence of erroneous sampling of multiple cells, or inclusion of noise in the data. If such evidence was found, the data records were either re-sorted or discarded.

All of the subsequent analysis steps were carried out, using custom-written algorithms in the Matlab software environment (MathWorks, Natick, MA). The ISI data were binned in one-second intervals to generate frequency readouts. The median of these values in the pre-infusion data segment was defined as the baseline firing rate of the neuron. An injection was considered to have an effect if the rate of discharge significantly differed from baseline for a minimum duration of 60 s, with an onset within 240 s from the beginning of drug injection (see below). The parameters chosen to define a drug effect were based on a previous study using the same recording-injection device (Kliem and Wichmann, 2004). The ‘effect period’ was defined as the time period in

which the discharge rate of a neuron was maximally affected by the injection. This epoch consisted of a 60 s data segment which started 30 s before, and ended 30 s after the maximal effect. In most cases, drug effects lasted much longer than the minimum of 60 s, usually until the end of the record. For neurons in which the injection had no effect on the firing rate, a 60 s data segment starting 120 s after the beginning of the injection was used as a surrogate.

To examine whether drugs affected the activity of a cell within a given experimental group (e.g., SNr cells tested after injection of the D1LR agonist), the discharge rates during the effect epoch were statistically compared with the respective median baseline discharge rates (see below). In each case, the median discharge rate during the effect epoch was also expressed as a percentage of the baseline discharge rate. Medians, as well as 25th and 75th percentiles of these percentages are reported in the Results section.

In addition, burst indices were calculated for the baseline and effect periods. Bursts were detected using the Poisson 'surprise' method developed by Legendy and Salcman (Legendy and Salcman, 1985). For the present analysis, a 'surprise' value of 3 was used (Aldridge and Gilman, 1991; Wichmann and Soares, 2006), and burst indices were calculated as the ratio between the number of spikes found in bursts and the total number of spikes for the record. Finally, changes in oscillatory firing of SNr and GPi cells were assessed with power spectral methods. As described in previous publications (Soares et al., 2004), we converted the ISI data into frequency readouts, and calculated power spectra. The algorithm was implemented in Matlab (Welch's method, mean detrending). For each cell, the raw spectra were integrated in the 1-3 Hz, 3-8 Hz, 8-15

Hz and > 15 Hz ranges, and normalized to the total power in the spectrum. Although the evaluation of slow oscillations would also have been of interest (Ruskin et al., 1999; Wichmann et al., 2002), this type of analysis was not possible because the duration of the evaluated response periods was too short.

#### *7.2.6.2 Analysis of microdialysis data*

In each experiment, the basal level of GABA was calculated as the average of microdialysis samples 1 and 2. The magnitudes of drug (or vehicle) effects on GABA measurements were calculated by subtracting the basal level (as defined above) from sample 3. A similar calculation was performed to assess changes in GABA levels after stimulation with a high concentration of potassium. For this, the basal GABA level was subtracted from sample #5. The changes in GABA levels were expressed as a percent change from baseline for each experiment.

#### *7.2.6.3 Statistics*

Non-parametric statistics were used throughout this study. Within individual electrophysiologic drug-injection experiments, Mann-Whitney tests were used to compare the frequency values in the baseline period with those in the drug effect period. The Wilcoxon signed rank-test was used to compare paired recording or microdialysis data collected before and after drug injections. p-values below 0.05 were accepted as indicators of significance.

## 7.3 Results

### 7.3.1 Electrophysiologic effects of D1LR ligand injections

We examined the effects of local micro-injections of D1LR-selective ligands on the neuronal activity of 22 SNr neurons and 23 GPi neurons in the immediate vicinity of the injection sites. The median firing rate for all recorded SNr cells at baseline was 37.6 spikes/s (30.6 - 47.1 spikes/s, 25th - 75th percentile), while the median rate for all GPi cells at baseline was 64.4 spikes/s (44.2 – 81.6). Control injections of aCSF in SNr (n = 6 cells) or GPi (n = 5) had no effect on the neuronal activity (data not shown). Although we tested neurons throughout the extent of both nuclei, the relative small sample size does not allow us to examine whether the drug effects which are reported below were regionally specific within GPi or SNr.

#### 7.3.1.1 Effects of D1LR activation

The infusion of the selective D1LR agonist SKF82958 (3 µg/µl) reduced the discharge rates of the majority of recorded neurons in SNr (10/13, figure 7.1) and GPi (8/13), while a minority of cells in both structures (2/13 in SNr, 1/13 in GPi) responded with an increase in neuronal discharge. Only a few cells did not respond (1/13 in GPi, 4/13 in GPi) to the drug injections. The median discharge rate of SNr cells tested decreased from 38.3 spikes/s (30.1 – 60.1) at baseline to 12.4 spikes/s (2.3 – 46.5) after drug exposure. Infusions of the D1LR agonist reduced the firing of SNr cells by a median of 61% (37 – 93 %; $p < 0.05$, Wilcoxon signed-rank test; figure 7.2). The maximal effect was seen in the SNr with a median latency of 357.8 s (198.4 – 520.3). In GPi, the median discharge rate was 75.5 spikes/s (59.2 - 96.9) at baseline, and 56.1 spikes/s (29.5 - 93.7) after exposure to the D1LR agonist. Infusion of the D1LR agonist

reduced the firing of GPi cells by a median of 13% (5 – 59 %; $p < 0.05$, Wilcoxon signed-rank test; figure 7.2). In this case, the maximal effect was seen at a median latency of 296.2 s (146.8 – 376.0). In most SNr cells (6/10), the reduced neuronal discharge was preceded by a brief increase in neuronal discharge as shown in figure 1A (arrow). Such biphasic effects were not seen in GPi.

Analysis of burst discharges (figure 7.3) revealed that the median proportion of spikes within bursts increased in SNr from 12.3% (4.5 – 18.5) to 18.5% (6.1 – 34.5), and in GPi from 12.8% (9.4-16.0) to 13.3% (7.4 – 21.4). Compared to the cell's baseline burst index, the median increase amounted to 39.0% (-5.2 – 155.0) in SNr and 15.4% (0 – 34.7) in GPi. These changes achieved significance only in GPi ($p < 0.05$, Wilcoxon signed rank test).

Statistical comparisons of the normalized integrated power spectra showed that in SNr, the median power in the 3-8 Hz band increased with the exposure to the D1LR agonist from 3.0% (2.1 – 4.2) to 4.2% (3.0 – 4.8). Compared to the cell's baseline power in this band, the drug exposure produced a median increase of 27.9% (12.3 – 42.4, $p < 0.05$). The median power in the 8-15 Hz band increased from 4.5% (2.7 - 5.6) to 6.7% (3.3 - 6.8). Compared to the cell's baseline power in the 8-15 Hz band, the drug exposure produced a median increase of 17.8% in this band (9.4 – 72.8, $p < 0.05$). There was a concomitant decrease in the frequencies above 15 Hz, from 89.9% (87.6 – 93.0) to 86.4% (85.7 – 90.3). In GPi, the proportional distribution of power was qualitatively similar to that in SNr, but did not reach significance (figure 7.3).

#### *7.3.1.2 Effects of D1LR blockade*

The infusion of the D1LR antagonist SCH23390 had different effects on SNr and GPi neurons. The responses of SNr neurons to the administration of this drug varied. Although a proportion of recorded SNr cells (3/9) showed an increase in firing after the injections, the median change in firing rates from 36.2 spikes/s (28.9 – 45.9) at baseline, to 47.5 spikes/s (25.9 – 55.7) after drug application, was not significant when compared to the cell's baseline firing rate. Two SNr cells showed a reduction of activity and four did not respond to drug application (figure 7.2). The median latency of the drug effect was 486.7 s (356.4 – 525.9) after the start of the drug infusion. The majority of GPi neurons (7/10) showed a significant increase in their discharge rate after SCH23390 administration (figure 1), while two cells showed a reduction in discharge and one cell did not respond. The median discharge rate increased from 44.9 spikes/s (24.8 – 69.3) to 69.5 spikes/s (28.8 – 91.4; figure 8.2). Compared to the cell's baseline firing rate, the median change amounted to an increase of 30.7% (1.8 – 62.4, $p < 0.05$). The median latency between the beginning of the injection and the maximal effect was 182.6 s (160.5 – 325.0).

Analysis of burst discharges (figure 7.3) revealed that the median proportion of spikes within bursts in SNr was not significantly changed, while it decreased from 19.9% (17.4-23.1) to 11.8% (4.4 – 22.2) in GPi. Compared to each cell's baseline burst index, the median reduction amounted to a decrease of 37.8% (66.1 – 8.0; $p < 0.05$) in GPi.

As shown in figure 7.3, the median integrated power in the 3-8 Hz band changed from 3.9% (3.5 – 4.7) to 3.7% (3.1 – 4.4) in GPi. Compared to the cell's baseline power in this band, the drug exposure produced a median reduction of 7.8 % (3.7 – 15.5; $p <$

0.05). The median power in the 8-15 Hz band did not change. There was no significant change in the proportional distribution of power in the SNr.

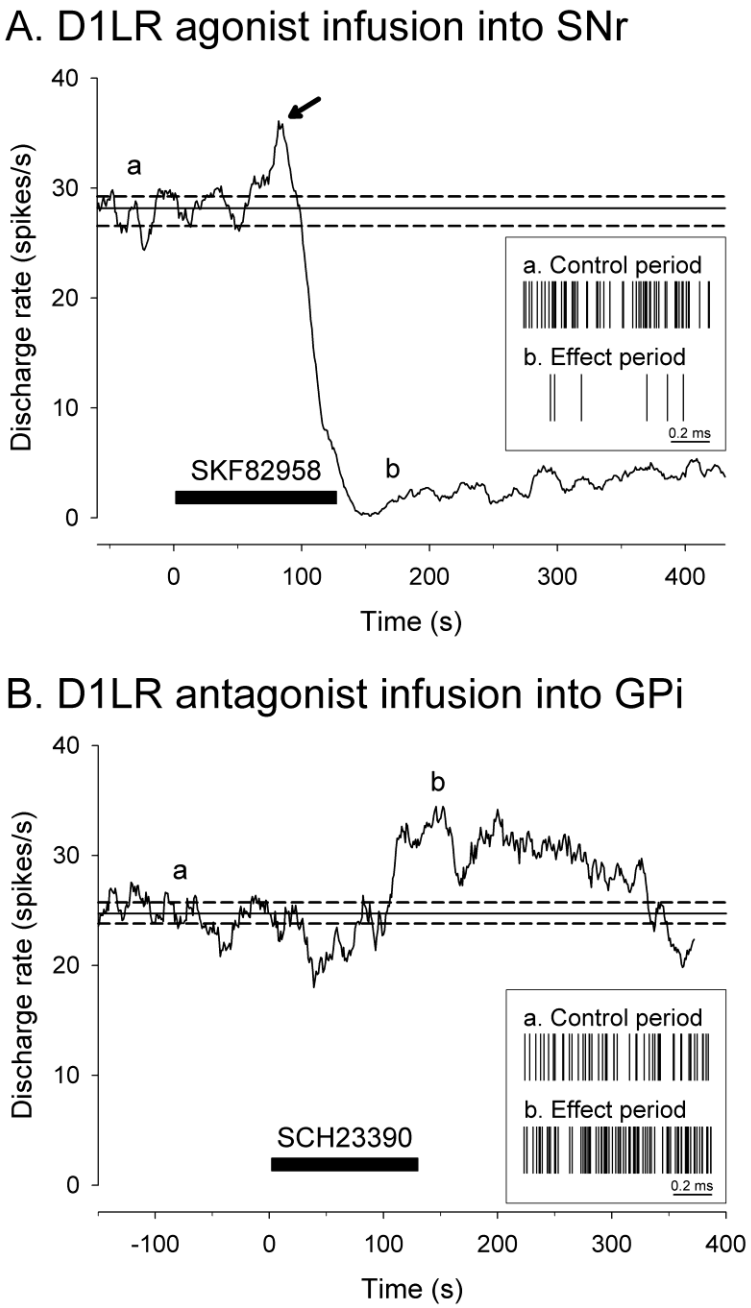


**Figure 7.1.** Responses of an SNr and a GPi neuron to infusions of D1LR ligands. The figure shows changes in neuronal discharge rates of an SNr neuron (A) after infusion of a D1LR agonist (SKF82958, 3 µg/µl), and a GPi neuron (B) after infusion of a D1LR antagonist (SCH23390, 5 µg/µl). Both drugs were infused for two minutes at 0.25µl/min. The thin solid line represents the median discharge rate during the baseline period, while dashed lines indicate 25th and 75th percentiles. The thick solid line at the bottom of each plot represents the time and duration (2 minutes) of the drug infusion (0.25 µl/min). The black arrow in A. shows the brief increase in neuronal discharge that often preceded the D1LR-induced decrease in firing in the SNr. Insets in both parts of the figure show 1 s segments of neuronal discharge during the pre-injection period (a) and effect period (b).

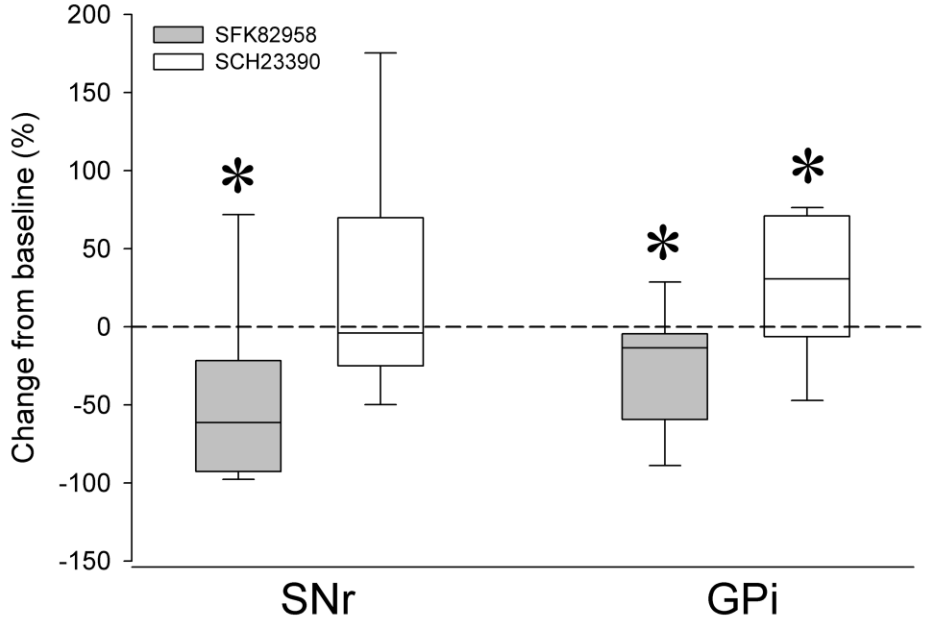



**Figure 7.2**. Changes in discharge rate of SNr and GPi neurons to infusions of D1LR ligands. For each cell in an experimental group, the discharge rate during the effect epoch was calculated and compared with the baseline discharge rate. The changes in discharge rate were expressed as a percent change from baseline for each experiment. Medians of these percentages are reported here along with the 25th and 75th percentiles (top and bottom of boxes), and minima and maxima (top and bottom of error bars). * $p < 0.05$, Wilcoxon signed-rank test.

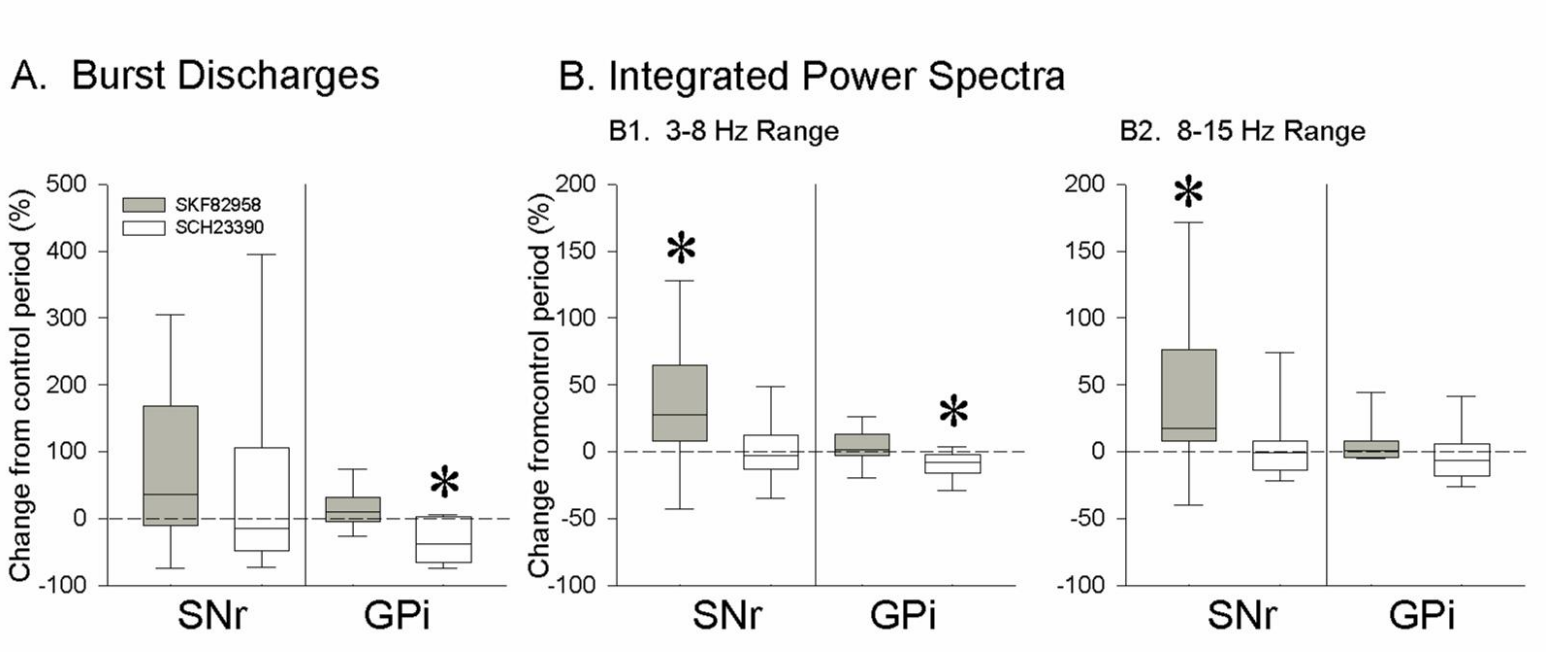



**Figure 7.3**. Changes in discharge patterns in GPi and SNr after infusions of D1LR ligands. The changes in burst incidence, and changes in integrated power spectra in the 3-8 Hz, and the 8-15 Hz bands are expressed, in each case, as a percent change from baseline for each experiment. Medians of these percentages are reported here along with the 25th and 75th percentiles (top and bottom of boxes), and minima and maxima (top and bottom of error bars). *, $p < 0.05$, Wilcoxon signed-rank test.

### 7.3.2 Microdialysis experiments

The median basal levels of GABA were 0.2 μM in SNr (0.12 – 0.40, n = 26) and 0.35 μM in GPi (0.25 – 0.79, n = 26). Exposure of the tissue to 80 mM K+ was done to assess the viability of the tissue and resulted in at least a three-fold increase in GABA efflux in all cases tested (data not shown). As shown in figure 7.4, reverse microdialysis of SKF82958 (0.411 μg/μl) induced a significant increase in the GABA concentration in GPi ($p < 0.05$; Wilcoxon test) and SNr ($p < 0.05$; Wilcoxon test). The median increase in GABA levels was 16.8% (5.7 – 40.1; n = 9) in SNr, and 12.0% (4.3 – 20.3; n = 10) in GPi. Infusion of SCH23390 (0.01μg/μl; SNr, n = 11; GPi, n = 10), or of vehicle (SNr, n = 6; GPi, n = 5) had no significant effect on GABA concentrations in GPi and SNr.

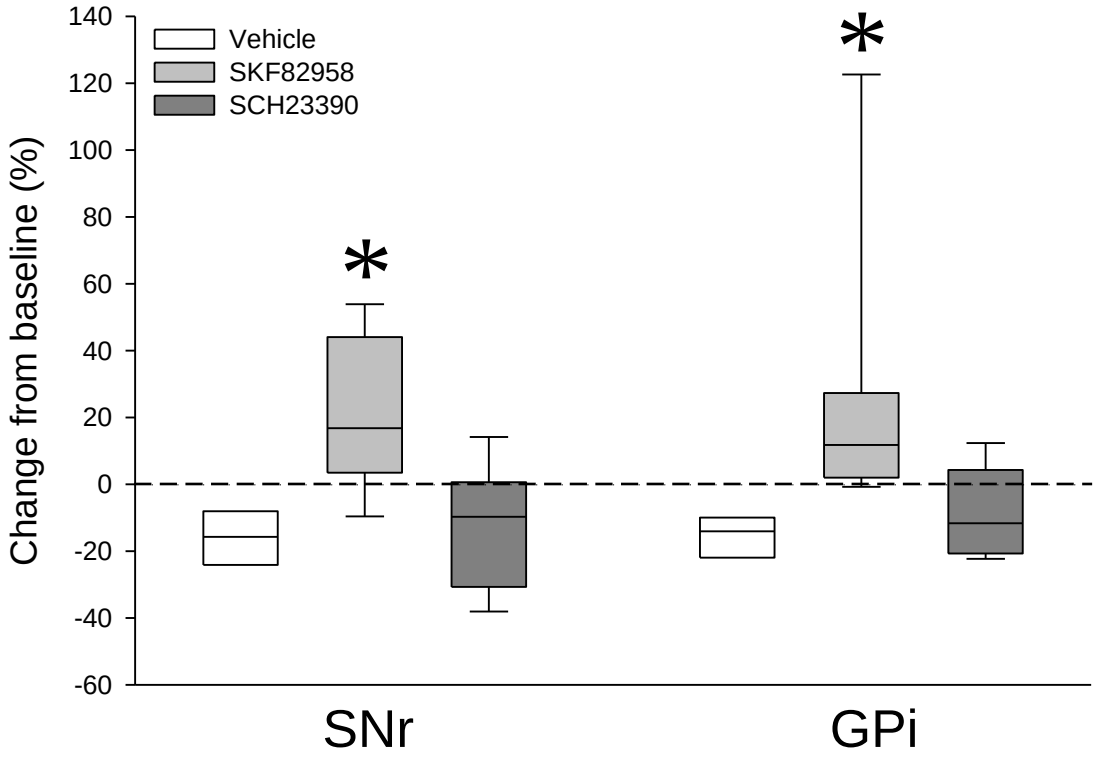



**Figure 7.4.** Changes in GABA levels in response to local infusion of the D1LR agonist SKF82958 (0.411 µg/µl) or the D1LR antagonist SCH23390 (0.01 µg/µl), as determined by microdialysis. The drugs were delivered via reverse microdialysis. The changes in GABA levels were expressed as a percent change from baseline for each experiment. The percentage values from drug experiments were compared against those from the control experiments. Medians of these percentages are reported here along with the 25th and 75th percentiles (top and bottom of boxes), and minima and maxima (top and bottom of error bars). * $p < 0.05$, Wilcoxon signed-rank test.

## 7.4. Discussion

Our results demonstrate that the activation of D1LRs reduces neuronal discharge rates in SNr and GPi, along with changes in oscillatory and burst discharge patterns. The microdialysis data suggest that at least some of these effects could be secondary to increased GABA release from terminals of the striato-nigral and striato-GPi projections. The effects of local infusion of the D1LR antagonist provide evidence that activation of D1LRs by endogenous dopamine acts to reduce GPi activity.

### 7.4.1 Technical considerations

Although the methods used in this report are well established in the literature, several technical issues need to be addressed. One concern is that the pharmacologic effects seen with the combined injection/recording device are more variable in terms of the maximal amplitude and the latency of the observed effects than those seen with in vitro methods (see also, (Galvan et al., 2005b; Kita et al., 2006; Kita et al., 2004; Kliem and Wichmann, 2004). This is due in part to the greater biological complexity of the in vivo system, but is also caused by technical constraints, such as differences in the exact dimensions of the injection system, the variable extent or direction of drug diffusion, or the presence of drug diffusion barriers (see also (Galvan et al., 2005b).

A second issue is that in vivo sampling of tissue concentrations of GABA with microdialysis is an imperfect technique, due to its low time resolution, and because it is not clear what proportion of the measured GABA concentrations is derived from synaptic sources (Westerink and de Vries, 1989). Despite this shortcoming, however, our results demonstrate that the method is sensitive enough to detect changes in ambient GABA

levels after local administration of D1LR ligands in GPi and SNr. Because most D1LRs are located on axons or terminals of GABAergic neurons in these structures, it is likely that the measured changes in GABA levels, in fact, reflect changes in transmitter release at synaptic sites. The basal levels of GABA measured in SNr and GPi in our study were similar to those reported in previous rodent and nonhuman primate experiments (Galvan et al., 2005b; Matuszewich and Yamamoto, 1999; Robertson et al., 1991b; Trevitt et al., 2002; Windels et al., 2005).

With regard to the pharmacologic agents used, it is important to consider the specificity of the D1LR active compounds that were used in this study. SKF82958 binds with much higher affinity to D1LRs than to D2LRs (Neumeyer et al., 2003; Toll et al., 1998), but does not reliably distinguish between D1 and D5 receptors. It has low affinities at serotonin- and α2-adrenergic receptors (Neumeyer et al., 2003; Toll et al., 1998). Likewise, SCH23390 binds with high affinity to D1LRs, but does not distinguish between D1 and D5 receptors. Its affinity for adrenergic alpha receptors (Bogeso et al., 1995; Neumeyer et al., 2003), D2LRs or serotonin 5-HT1 or 5-HT3 receptors is low (Markstein et al., 1986; Neijt et al., 1988; Waeber et al., 1988). It binds, however, with relatively high affinity to 5-HT2A (Neumeyer et al., 2003) and 5-HT2C receptors (Briggs et al., 1991; Millan et al., 2001; Woodward et al., 1992). Because 5-HT2A receptors are present only in relatively low density in the monkey GPi, SNr and SNc, the affinity of SCH23390 does not represent a significant problem (Lopez-Gimenez et al., 2001). The situation is different for 5-HT2C receptors. These receptors are expressed in the rat SNr and GPi (Eberle-Wang et al. 1997), and mRNA for them has been found in lateral portions of the monkey SN (not in GPi) (Lopez-Gimenez et al., 2001). SCH23390 acts as

a partial or full agonist at 5-HT2C receptors (Hoyer et al., 1989; Millan et al., 2001), and could therefore increase pallidal or nigral activity through postsynaptic 5-HT2C receptor activation. In our experiments, SCH23390 effects at 5-HT2C receptors in GPi would be particularly important, because the drug acted to increase firing in this area. However, in distinction to rodent studies where activation of postsynaptic 5-HT2C receptor increases SNr firing (Di Giovanni et al., 2006a; Di Giovanni et al., 2006b; Fox and Brotchie, 2000a; Fox and Brotchie, 2000b; Fox et al., 1998; Invernizzi et al., 2007; Rick et al., 1995), a recent study of serotonin effects on neuronal activity of pallidal neurons in awake primates did not find evidence for significant postsynaptic 5-HT2 receptor-mediated effects (Kita et al., 2007). Other studies have failed to show mRNA for 5-HT2C receptors in the primate GPi (Lopez-Gimenez et al., 2001). Because of these findings, it seems most likely that SCH23390 in our study acted via D1LRs blockade rather than as a 5-HT2C receptor agonist.

Another concern related to the drugs used in this study is the possibility that D1LR activation may have led to desensitization of D1 receptors. This is, however, not likely to be a significant confounding factor, since the selective D1LR ligands were infused over a short-time period, followed by a gradual diffusion of ligand, limiting the degree of desensitization (Lewis et al., 1998).

### 7.4.2 D1LR agonist effects

D1LRs are strongly expressed in SNr and GPi (Barone et al., 1987; Fremeau et al., 1991b; Richfield et al., 1987), supporting the hypothesis that dopamine released at these locations may activate D1LRs. Electron microscopic studies have shown that in the rat

SNr and entopeduncular nucleus (rodent homologue of monkey GPi) D1 receptors are predominately located presynaptically on unmyelinated axons and axon terminals (Yung et al., 1995b). These receptors are expressed in putative GABAergic terminal boutons that form symmetric synapses on dopaminergic dendrites arising from nearby SNc neurons, or from dendrites of non-dopaminergic SNr neurons (Caille et al. 1996). In both SNr and GPi, the labeled terminals show ultrastructural features typical for striatal GABAergic boutons, suggesting that the striatum is the main source of the D1-containing GABAergic terminals of basal ganglia output nuclei in rodent (Levey et al., 1993; Yung et al., 1995b) and monkey (Caille et al., 1996).

The effects of dopamine receptor activation in the SNr, and specifically, the effects of D1LR activation have been extensively evaluated in rodents. These studies have resulted in conflicting evidence, which is very likely due to the use of different experimental techniques and different pharmacologic approaches. In addition, the in vivo studies on this topic differ in the use of anesthetics.

One approach has been to study GABA release in response to either dopamine or D1LR ligands. Dopamine was shown to increase GABA release from rat brain slices in early studies (Reubi et al., 1977), or to induce biphasic effects (van der Heyden et al., 1980), perhaps due to the fact that D1LRs and D2LRs receptors are activated by dopamine. Other studies of GABA release from brain slices showed that D1LR activation potentiates evoked GABA efflux in the SNr (Aceves et al., 1995; Floran et al., 1990; Starr, 1987), although this was not true in other studies (Mayfield et al., 1999). Published reports on the effects of D1LR activation on GABA release in vivo, studied in awake rats with microdialysis techniques, have consistently demonstrated that D1LR

activation increases GABA levels in the SNr (Matuszewich and Yamamoto, 1999; Rosales et al., 1997a; Timmerman and Westerink, 1995; Trevitt et al., 2002; You et al., 1994b) and entopeduncular nucleus (the rodent homologue of the primate GPi; (Ferre et al., 1996).

In vivo electrophysiologic studies reported that dopamine acts to increase the activity (Ruffieux and Schultz, 1980), and/or attenuated the inhibitory effects of GABA (Waszczak and Walters, 1983; Waszczak and Walters, 1986) on SNr neurons. At least some of these effects may have been mediated via D2LRs (Waszczak, 1990). More recent in vivo studies in which the electrophysiologic effects of D1LR-specific ligands were investigated have shown that SNr neurons are inhibited by endogenous dopamine, released by application of amphetamine in anesthetized (Timmerman and Abercrombie, 1996) or in awake unrestrained rats (Windels and Kiyatkin, 2006). This effect was blocked by SCH23390, and was, thus, likely due to D1LRs activation (Timmerman and Abercrombie, 1996; Windels and Kiyatkin, 2006).

Brain slice recording studies have also led to inconsistent results. Although D1LR-mediated excitatory effects on SNr neurons were seen in some studies (Martin and Waszczak, 1994; Miyazaki and Lacey, 1998), the majority of the in vitro recording studies has demonstrated the opposite effect, i.e., an increase in GABAergic transmission in response to D1LR activation (Floran et al., 2002; Misgeld, 2004; Radnikow and Misgeld, 1998). While there is substantial evidence that most D1LRs are located on striatonigral terminals, a recent study has suggested that portions of the D1LR-mediated modulation of SNr activity may involve D1LR effects on glutamate release from the STN-SNr projection (Ibanez-Sandoval et al., 2006).

There is obviously no clear consensus on the effects of D1LR activation in the rodent SNr, but most of the studies are compatible with the notion that D1LR activation results in increased GABA release from striatonigral terminals, and in subsequent inhibition of SNr neurons. Our electrophysiologic and microdialysis studies are in line with this view.

The exact mechanism by which D1LR activation increases ambient GABA levels in the primate GPi and SNr, and leads to a reduction of firing in these brain areas remains unclear, however. Under rest conditions, such as those chosen for our study, the striatal output neurons that give rise to these monosynaptic pathways have a low intrinsic activity (Kimura, 1992), so that D1LR effects on action-potential induced GABA release would be small. It is therefore likely that a substantial component of the ambient GABA levels measured with microdialysis in the SNr and GPi originates from other sources, such as projections from GPe (Parent and Hazrati, 1995a; Windels et al., 2005). Local axon collaterals of neurons intrinsic to GPi or SNr may also play a role, although the anatomy and the extent of such axon collaterals have not been conclusively studied in the primate GPi or SNr. The activity of GPe projections would not be expected to change with activation of D1LRs in GPi or SNr.

Given the predominant anatomical location of D1-receptors on striatal afferents to SNr and GPi, it is possible that the modulation of GABA levels through D1LR activation at rest may have occurred largely through action-potential-independent mechanisms, for instance through an influence of the D1LR agonist on spontaneous GABA release from nerve terminals. In favor of this idea, electrophysiologic studies in rat SNr slices have indicated that the frequency of miniature inhibitory post-synaptic potentials, elicited in

the presence of the sodium channel blocker tetrodotoxin and the calcium channel blocker Cd++, increases with D1LR receptor stimulation (Radnikow and Misgeld, 1998). Similarly, some of the effects of D1LR activation on GABA release persisted in the presence of tetrodotoxin (Timmerman and Westerink, 1995).

As mentioned above, SKF82958 may have also acted via activation of D5 receptors. Immunohistochemical and in situ hybridization studies have demonstrated that D5 receptors are present in the SNr and GPi of rats and monkeys (Bergson et al., 1995; Choi et al., 1995; Ciliax et al., 2000; Khan et al., 2000), and in electron microscopic studies D5 receptors are found postsynaptically, on dendrites and cell bodies of GPi, SNr and SNc neurons (Ariano et al., 1997; Bergson et al., 1995; Ciliax et al., 2000; Kliem et al., 2006). Stimulation of these postsynaptic receptors may induce direct activation of basal ganglia output neurons through postsynaptic depolarization (Baufreton et al., 2003), which would tend to counteract the GABA-mediated inhibitory effects of presynaptic D1 receptor activation (see above). However, the overall result of D1LR activation in our experiments was dominated by the inhibition that was presumably produced through effects on presynaptic D1receptors. It is possible that D5 receptor activation contributed to the excitatory effects on SNr and GPi neurons which were seen in a subset of neurons. Unfortunately, no pharmacologic tools are available at this time to reliably distinguish between D1 and D5 receptors.

### 7.4.3 D1LR antagonist effects

We carried out D1LR antagonist experiments to investigate whether D1LRs are activated by endogenous dopamine. Previous studies in rodents have suggested that

D1LR antagonist may increase GABA levels in the SNr under some circumstances (Garcia et al., 1997; Starr, 1987), although this has not always been seen (Mayfield et al., 1999). In our experiments, exposure to the D1LR antagonist increased the electrophysiologic activity of the majority of GPi cells, and of a subset of SNr cells. GABA levels were not significantly changed in either structure. The most likely explanation for this discrepancy is that the effect size on GABA levels was too small to be detected with the number of observations used in our microdialysis experiments. An alternative explanation would be that SCH23390 exerted its frequency-increasing effects through activation of postsynaptic 5-HT2C receptors rather than modulation of GABA levels in this structure. Because the available evidence is in favor of a predominant action of this drug at D1LRs rather than 5-HT2 receptors (see discussion above), we will consider the drug effects only in terms of its known effects on dopaminergic transmission.

In behavioral experiments in rodents, unilateral intranigral injections of SCH23390 inhibit amphetamine-induced behaviors (Timmerman and Abercrombie, 1996; Yurek and Hipkens, 1993) and impair performance on a rod-balancing task (Bergquist et al., 2003), while bilateral injections diminish lever pressing behavior and locomotor activity (Trevitt et al., 2001). Several studies have confirmed the presence of dopamine in rat (Cobb and Abercrombie, 2003a; Heeringa and Abercrombie, 1995; Robertson et al., 1991a; Santiago and Westerink, 1991) and monkey SNr (Gerhardt et al., 2002) as well as monkey GPi (Pifl et al., 1990). The question whether dopamine has a physiologic role in the regulation of activity in GPi and SNr is particularly relevant, because it has been suggested that dopamine depletion at these sites may contribute to the development of some of the behavioral abnormalities in Parkinson's disease (Crocker, 1995; Forno, 1996;

Gash et al., 1996b; Gerhardt et al., 1999; Hemsley and Crocker, 2001; Hornykiewicz, 1998; Parent and Cossette, 2001; Starr et al., 1999b; Wichmann and DeLong, 2003).

Our study provides evidence that many GPi cells may be tonically inhibited by ambient dopamine, while the responses of SNr neurons were more variable, perhaps because sources of endogenous dopamine in GPi and SNr differ substantially. GPi receives a direct projection from the SNc (Parent et al., 1990; Schneider and Dacko, 1991; Smith et al., 1989), while dopamine in the SNr originates from dendritic release from SNc cells (Bjorklund and Lindvall, 1975; Cheramy et al., 1981; Geffen et al., 1976; Wassef et al., 1981). Compared to GPi, the degree of tonic activation of D1LRs in the primate SNr appears to be low under physiologic conditions, either because less dopamine is released, or because the released dopamine is rapidly cleared from the extracellular space (Suaud-Chagny et al., 1995). The latter possibility is suggested by binding studies in human and monkey GPi and SNr which have demonstrated that dopamine uptake sites are present in higher concentration in SNr compared to GPi (Gnanalingham et al., 1995; Marcusson and Eriksson, 1988).

The behavior of the animal under study may also have a strong influence on the level of ambient dopamine in these structures. It is well established that the activity of dopaminergic neurons, and, thus, potentially also dopamine concentrations, change during the performance of rewarded tasks (Hollerman and Schultz, 1998; Ljungberg et al., 1992; Mirenowicz and Schultz, 1994; Schultz, 1998; Schultz et al., 1997; Waelti et al., 2001). The lack of effect of the D1LR antagonist in the SNr, measured during behavioral 'idling', as in our study, may not reflect the true behavioral importance of the neuromodulator. It remains to be seen whether locally injected dopamine receptor

antagonists would interfere with behavioral performance, or influence neuronal spiking during the performance of tasks, as was shown for D1LR antagonist injections into frontal cortex (Nakamura and Hikosaka, 2006; Williams and Goldman-Rakic, 1995).

### 7.4.4 Activation of D1LRs influences discharge patterns of SNr and GPi neurons

D1LR activation increased the proportion of spikes found in bursts in SNr and GPi, while blockade of these receptors had the opposite effect. We also found that in both structures, oscillations in the 3-8 Hz band and 8-15 Hz bands were enhanced by D1LR activation, while D1LR blockade had the opposite effect in GPi (no effect was observed in the SNr). The changes in bursting may be explained by the fact that GABA, released after D1LR activation, may hyperpolarize SNr and GPi cells which, in turn, may have increased their tendency to discharge in bursts. This phenomenon has been described for multiple brain regions, including GP, the rodent equivalent of the primate GPe, and STN, (Beurrier et al., 2000; Beurrier et al., 1999; Bevan et al., 2002; Kass and Mintz, 2006; Nambu and Llinas, 1994; Overton and Greenfield, 1995), but has not been described for GPi or SNr in rodents or primates. It is interesting that the increase in bursting and oscillatory activity in the basal ganglia output nuclei are seen not only after D1LR activation, but also in chronically dopamine-depleted animals and humans (Bergman et al., 1998; Levy et al., 2001; Murer et al., 1997; Wichmann et al., 1999; Wichmann and Soares, 2006). Our findings suggest therefore that reduced activation of D1LRs in GPi or SNr does not contribute to, and may even counteract, the parkinsonism-related differences in firing. In the parkinsonian state, network interactions between the striatum and other basal ganglia structures may be more important in determining the

pattern abnormalities in GPi and SNr than the reduced activation of D1LRs by endogenous dopamine (see also, (Gatev et al., 2006).

### 7.4.5 Functional and clinical implications

Our study demonstrates both similarities and differences between GPi and SNr. Based on their position within the basal ganglia circuitry, similarities in their neuronal composition, and basic similarities in neuronal firing characteristics (Wichmann et al., 1999), these nuclei are often seen as homologous, with GPi subserving motor functions, and SNr subserving in large part associative and limbic functions. Some of our results, such as the finding of an inhibitory response to D1LR receptor activation in most neurons in both GPi and SNr support this view. However, the examination of dopaminergic functions in these nuclei also demonstrates clear differences between them which are apparent in our results, including the lack of D1LR antagonist effects in SNr, the finding that SNr neurons often showed biphasic responses upon infusion of D1LR agonists (an initial increase followed by a decrease), and the more pronounced (median) inhibition seen after D1LR infusion in SNr. These differences may be related to one another, for instance through the possibility that low levels of background activation may result in (moderate) desensitization of D1LRs in GPi, whereas no such desensitization is at work in the SNr. While not explained at a mechanistic level, the different responses of GPi and SNr neurons to D1LR ligands are most likely related to the fact that dopamine is released synaptically in GPi, while it is released dendritically in the SNr (Bjorklund and Lindvall, 1975; Cheramy et al., 1981; Geffen et al., 1976; Nieoullon et al., 1978). The differences between the responses of GPi and SNr neurons to D1LR activation may also have

behavioral consequences. The motor circuit of the basal ganglia which passes through the primate GPi may be under tighter (and perhaps more precisely timed) dopaminergic control than the limbic and associate circuits that are prominent in the SNr.

The behavioral effects of D1LR activation in SNr or GPi have not been extensively studied. The available evidence suggests that D1LR activation in the SNr results in behavioral activation in rats (Timmerman and Abercrombie, 1996), and may be involved in startle responses (Meloni and Davis, 2004). Consistent with these results, D1LR antagonist injections in the SNr impair motor activities in rats (see above, (Bergquist et al., 2003; Trevitt et al., 2001). It remains an open question whether dopamine depletion in GPi and SNr contributes to the development of parkinsonism and the changes in firing rates and patterns seen in the basal ganglia output nuclei that have been identified in parkinsonism (see above). Combined microdialysis and behavioral studies using a rod-balancing task have indicated that partial depletion of dopamine in the SNr significantly impairs motor functions and that dopamine release in the SNr can compensate for some of the impairments induced by striatal dopamine loss (Andersson et al. 2006). Further evidence for the potential role of local dopamine loss at the level of the SNr in the generation of abnormal basal ganglia output in the dopamine-depleted state comes from studies in which electrophysiologic abnormalities in SNr activity were identified in brain slices that included the SN (but not the striatum) in reserpinized rats (Wittmann et al., 2002). It has also been suggested that changes in the dopaminergic system in GPi may be involved since dopamine concentrations are lowered in GPi in the brains of patients with Parkinson's disease as compared to controls (Bernheimer et al., 1973), and the finding of (presumably adaptive) changes in the uptake of the dopamine

precursor 18F-dopa in GPi in positron emission studies in parkinsonian patients (Whone et al., 2003).

Our studies do not support the hypothesis that loss of nigral D1LR activation in Parkinson's disease contributes significantly to firing abnormalities in the majority of basal ganglia output neurons. This, however, does not diminish the potential for a functional impact of dopamine replacement strategies aimed at the these nuclei, specifically the SN. Infusions of glial-derived neurotrophic factor into the SN have been demonstrated to ameliorate parkinsonism in MPTP-treated primates, most likely through an increase in nigral dopamine levels (Gash et al., 1996b), and dopaminergic mesencephalic cell grafts into the SN area substantially improve motor signs of parkinsonism in this animal model (Gerhardt et al., 1999; Starr et al., 1999b).

The finding that drugs acting at D1LRs exert pharmacologic effects in SNr or GPi also implies that some of the side effects of dopaminergic treatments may be explainable through actions at these sites. For instance, because the SNr is strongly involved in associative and limbic basal ganglia circuits (Haber and Fudge, 1997; Middleton and Strick, 2002; Parent and Hazrati, 1994), some of the cognitive or emotional side effects of D1LR-active drugs may be mediated through activation of the nigral dopamine receptors.

# Chapter 8

# Ultrastructural Localization and Function of Dopamine D1-Like Receptors in the Substantia Nigra Pars Reticulata and the Internal Segment of the Globus Pallidus of Parkinsonian Monkeys

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## 8. Abstract

The motor symptoms of Parkinson's disease are commonly attributed to striatal dopamine loss, although reduced dopamine innervation of extrastriatal basal ganglia nuclei, such as the internal pallidal segment (GPi) and substantia nigra pars reticulata (SNr) may also contribute. Anatomic and biochemical studies have shown the presence of dopamine D1 and D5 receptors in both of these structures under normal conditions, and we have recently demonstrated that local activation of D1-like receptors reduces neuronal discharge rates and enhances bursts and oscillatory activity in GPi and SNr of normal monkeys (Kliem et al., 2007). Here, we present experiments assessing the location and function of D1-like receptors in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated parkinsonian monkeys. In normal and MPTP-treated monkeys, the majority of D1 and D5 receptor immunoreactivity was associated with unmyelinated axons or putative GABAergic terminals in GPi and SNr, but significant D5 receptor immunostaining was also found postsynaptically in dendrites. Plasma membrane-bound axonal D1 receptor immunoreactivity was found in both normal and MPTP-treated monkeys. Local microinjections of the D1/D5 receptor agonist SKF82958 significantly reduced discharge rates in GPi and SNr neurons, while it increased burst firing and oscillatory activity in the 3-15 Hz band in SNr, but not GPi. Together with our recent observations, these findings provide evidence that functional D1/D5 receptors are expressed in GPi and SNr in the normal and parkinsonian states. Physiological activation of these receptors by endogenous dopamine or dopamine agonist therapies to treat parkinsonism may, therefore, regulate basal ganglia outflow in normal and pathological conditions.

## 8.1 Introduction

The substantia nigra pars compacta (SNc) projects to the striatum (Bernheimer et al., 1973; Hornykiewicz and Kish, 1987), but also provides dopamine to the basal ganglia output nuclei, the internal pallidal segment (GPi) and substantia nigra pars reticulata (SNr, Bernheimer et al., 1973; Cheramy et al., 1981; Geffen et al., 1976; Pifl et al., 1990; Robertson et al., 1991a; Schneider and Rothblat, 1991; Smith et al., 1989; Whone et al., 2003). In GPi, dopamine is released from terminals of the SNc-GPi projection which, in monkeys, arises from a population of SNc neurons mainly separate from that giving rise to the nigrostriatal projection (Jan et al., 2000; Smith et al., 1989). Dopamine reaches SNr cells through dendritic release from ventral tier SNc neurons (Arsenault et al., 1988; Bjorklund and Lindvall, 1975; Nieoullon et al., 1978).

Dopaminergic transmission is mediated through D1-like (D1LRs, Clark and White, 1987; Neve, 1997) and D2-like (D2LRs, Neve, 1997) G protein-coupled dopamine receptors. The D1LRs, the focus of this paper, are comprised of D1 and D5 subtypes and are positively coupled to adenylyl cyclase (Gingrich and Caron, 1993; Kebabian and Calne, 1979). These receptors are expressed in high concentrations in the monkey GPi and SNr (Besson et al., 1988; Richfield et al., 1987). Previous studies have demonstrated that D1 receptors are presynaptically located in axons and axon terminals of the GABAergic striatopallidal and striatonigral projections (Barone et al., 1987; Caille et al., 1996; Fremeau et al., 1991b; Levey et al., 1993; Yung et al., 1995a). Less is known about the ultrastructural location of D5 receptors in the basal ganglia (Bergson et al., 1995; Ciliax et al., 2000; Khan et al., 2000). In our recent electrophysiologic and pharmacologic studies, we showed that local activation or blockade of D1LRs modulates

neuronal discharge in the basal ganglia output nuclei of normal monkeys (Bergson et al., 1995; Kliem et al., 2007), likely due to modulation of GABA release from striatal projections to these nuclei.

Studies of D1LR expression in the basal ganglia of dopamine-depleted animals have provided conflicting and unclear results. For instance, some authors showed that the level of D1 receptors mRNA is decreased in striatonigral projection neurons of dopamine-depleted rats (Gerfen et al., 1990) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys (Morissette et al., 1996), while other *in situ* hybridization studies in MPTP-treated monkeys resulted in opposite findings along with no significant change in D1LR binding in these animals (Betarbet and Greenamyre, 2004; Gnanalingham et al., 1993). To our knowledge, no studies have assessed potential changes in D1LR protein immunoreactivity or compared the ultrastructural location and function of D1LRs in the basal ganglia output nuclei between normal and parkinsonian states. This knowledge is important to better understand the physiologic significance and pharmacotherapeutic relevance of extrastriatal dopamine innervation of GPi and SNr in normal and pathological conditions. The aim of the present study was, therefore, to examine the physiologic effects of local activation of D1LRs and compare the ultrastructural location of D1 and D5 receptors immunoreactivity in GPi and SNr between normal and MPTP-treated monkeys.

## 8.2. Materials and Methods

### 8.2.1 Animals

Sixteen Rhesus monkeys (*Macacca mulatta*, 3-10 kg) were used for this study. Three of these animals were used for electrophysiological recording of GPi and SNr neurons, while brain tissue from the remaining animals was used for immunocytochemical studies. Eight of these animals were normal, and five were treated with MPTP. The animals were housed under conditions of protected contact housing, with free access to standard primate chow and water. All experimental protocols were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Anonymous, 1996) and the PHS Policy on Humane Care and Use of Laboratory Animals (amended 2002), and were approved by the Animal Care and Use Committee of Emory University.

### 8.2.2 General outline of procedures

In vivo electrophysiologic recording methods were used to locate the basal ganglia and characterize changes in discharge properties of GPi and SNr neurons after microinjections of a selective D1/D5 receptor agonist in monkeys treated with MPTP.

Electron microscopic immunoperoxidase studies of D1 and D5 receptor immunoreactivity were carried out on GPi and SNr sections from normal (n=8) and MPTP-treated monkeys (n=5). In addition, immunogold studies were done in both structures to assess the degree of plasma membrane expression of D1 receptor immunoreactivity. These were successfully completed in four normal and one parkinsonian monkey.

### 8.2.3 Electrophysiology experiments

#### *8.2.3.1 Recording chamber placement*

The animals were first trained to sit in a primate chair, to adapt to the laboratory, and to permit handling by the experimenter. We then placed two stainless steel chambers (inner diameter = 16 mm, Crist Instruments, Hagerstown, MD) for chronic access over trephine holes under aseptic conditions and isoflurane anesthesia (1-3%). One chamber was stereotactically aimed at the SNr in the parasagittal plane (A=9, L=5, D=0; Horsley-Clarke coordinate system) at a 25º angle, posterior to the vertical. A second chamber was directed at the GPi in the coronal plane (A=12, L=10, D=4) at a 40º angle. The chambers were both positioned over the right hemisphere and affixed to the skull with dental acrylic (Atlanta Dental Supply, Duluth, GA). A metal head holder (Crist Instruments) was embedded in the acrylic cap to permit head stabilization during recording procedures.

#### *8.2.3.2 MPTP administration*

The MPTP-treated monkeys used in the electrophysiological experiments have previously been used in a similar study in the normal state (Kliem et al., 2007). MPTP was administered by injection into the right common carotid artery after occlusion of the external carotid artery on the same side (0.5 - 0.7 mg/kg per injection, 1 mg/ml, infused over 10 minutes, Sigma, St. Louis MO) under sterile conditions and isoflurane gas anesthesia. Monkey F received one injection, monkey I received two injections 14 days apart, and monkey E received 3 injections spaced by at least 14 days. Monkey E also received additional doses of MPTP systemically (0.25 mg/kg i.m.; over the course of two months), 4 months after the intracarotid treatment (Eberling et al., 1998). Recording

experiments started at least 3 weeks after the last MPTP injection. At the time of recording, all three animals showed moderate to severe parkinsonian motor signs on the side contralateral to the MPTP injections, including bradykinesia, rigidity, postural instability and flexed limb posture. They continued to feed and groom themselves. The stability of their parkinsonian motor signs was documented with biweekly observations throughout the recording sessions (for details, see Soares et al., 2004).

#### *8.2.3.3 Mapping procedures*

After stabilization of parkinsonian motor signs, the boundaries of GPi and SNr were confirmed using extracellular single-cell recordings with tungsten microelectrodes (Frederick Haer Co., Bowdoinham, ME; impedance 0.5-1.0 MΩ at 1 kHz). For each electrode penetration, a 20-gauge steel guide tube (Small Parts, Miami Lakes, FL) was used to penetrate the dura, and a microdrive (MO-95B, Narishige, Tokyo, Japan) to subsequently lower electrodes into the brain. The microdrive has an X-Y platform to determine the coordinates inside the recording chamber, and a linear potentiometer coupled to the vertical cylinder that moves the electrode and provides us with a position read-out of the electrode tip. The electrical neuronal signal was amplified (DAM-80 amplifier, World Precision Instruments, Sarasota, FL), filtered (400-10,000 Hz, Krohn-Hite, Brockton, MA), displayed on a digital oscilloscope (DL1640, Yokogawa, Tokyo, Japan), and made audible via an audio amplifier. GPi and SNr neurons were easily identified by their characteristic high frequency discharge rates (Starr et al., 1999a).

### *8.2.3.4 Injection/recording procedures*

A combination microelectrode recording-injection system (Kliem and Wichmann, 2004) was used to assess the effects of a D1LR agonist on GPi and SNr neurons. This system allows us to study the effects of drugs on the activity of neurons in the vicinity of the injection site (Galvan et al., 2005b; Kita et al., 2004), (Kita et al., 2006; Kliem et al., 2007; Kliem and Wichmann, 2004). A standard tungsten microelectrode (see above) was positioned alongside fused silica tubing (Polymicro Technologies, Phoenix., AZ; inner diameter = 40 μm; outer diameter = 120 μm) inside a polyimide sleeve (MicroLumen, Inc., Tampa, FL; outer diameter = 0.5 mm) and secured with epoxy glue. The tip of the microelectrode extended 50 – 100 μm beyond the tip of the silica tubing. A 10 mm section of 23-gauge stainless steel tubing (Small Parts) was held in place over the silica tubing at the proximal end with epoxy glue, to provide a tight link to a micro-T connector (CMA, Solna, Sweden) via a 'flexible connector' (Saint-Gobain, Akron, OH; inner diameter = 0.020', Tygon tubing). Gas-tight syringes (CMA; 1 ml) were connected to the inlets of the micro-T, and controlled remotely by a dual syringe infusion pump (CMA/102) to infuse sub-microliter quantities of solutions.

Prior to the injections, the selective D1LR agonist, SKF82958 (Sigma-Aldrich; 3 μg/ μl) was dissolved in artificial cerebrospinal fluid (aCSF; comprised of (in mM): 143 NaCl, 2.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1 $Na_2HPO_4$, pH=7.2-7.4) and 0.1% ascorbic acid solution, placed in a sonicator and filtered (Fisher Scientific; pore size = 0.2 μm). Vehicle was infused for control experiments. The injection-recording device was lowered into the brain using the microdrive and guide tube (see above). Cells were recorded for at least one minute with the pump in the 'OFF' position serving as a baseline.

While we continued to record, the pump was switched to the 'ON' position for drug infusion (rate = 0.25 μl/min; 2 minutes) and then switched off again. The post-infusion period lasted for at least four minutes. The recorded neuronal signals were amplified and stored to computer disk using the CED data acquisition system (Spike2, CED; Cambridge, UK). Given the very small volume of drug infused, the drug effects are not likely to extend more than 200-300 μm beyond the tip of the recording electrode (personal observation and (Kita et al., 2004).

The animals were awake throughout all of the recording-injection experiments. Recording sessions were separated by at least 24 hours. In some sessions, more than one injection was done. In these rare instances, the injections were spatially separated by at least 500 μm, and at least thirty minutes apart. Overt cumulative effects were not seen with these injections.

#### *8.2.3.5 Histology*

After completion of the recording experiments, the animals were sacrificed with an overdose of sodium pentobarbital (100 mg/kg, i.v.), and then transcardially perfused with oxygenated Ringer solution followed by 2 liters of fixative (4% paraformaldehyde,0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.2)). The brains were then removed from the skull and cut into 10 mm-thick blocks containing GPi or SNr. The tissue blocks were cut into 60 μm-thick sections with a freezing microtome, and stained with cresyl violet to verify electrode locations. Other sections were immunostained for tyrosine hydroxylase (see procedure below) to confirm the loss of

dopaminergic neurons in the SNc, and the loss of dopamine in the striatum. The sections were mounted on slides and analyzed under the light microscope.

#### *8.2.3.6 Analysis of electrophysiologic neuronal data*

Only neurons in which reconstruction of the electrode track, based on recording tip position measurements during the experimental sessions and postmortem histology, showed that they were within the GPi or SNr were used. An off-line template-matching spike sorting routine with subsequent principal component analysis (Spike2, CED) was used to determine inter-spike intervals (ISIs). ISI distribution histograms were examined for quality control.

Matlab (MathWorks, Natick, MA) was used for all steps of the subsequent analysis. The ISI data were used to generate a discharge rate read-out in one-second intervals. The median of these values in the pre-infusion data segment was defined as the baseline discharge rate of the neuron. An injection was classified as having had an effect if the discharge rate significantly differed from the baseline for at least 60 s, with an onset within 240 s from the start of the injection. These parameters were chosen based on previous studies (Kliem et al., 2007; Kliem and Wichmann, 2004). The 'effect period' was defined as the time period in which the discharge rate of a neuron was maximally affected by the drug injection. For statistical analysis, this epoch contained a 60 s data segment which started 30 s before, and ended 30 s after the maximal effect. The majority of drug effects usually continued until the end of the record. For non-responding cells, a 60 s data segment starting 120 s after the beginning of the injection was selected for analysis.

We statistically compared the median baseline discharge rate with the median discharge rate during the effect epochs. The median discharge during the effect epoch was also expressed as a percentage of the baseline discharge rate. We report medians and $25^{th}$ and $75^{th}$ percentiles of these percentages in the summary analyses in the Results.

We also calculated burst indices for the baseline and effect epochs, using the Poisson 'surprise' method (Legendy and Salcman, 1985). A 'surprise' value of 3 was chosen (Aldridge and Gilman, 1991; Wichmann and Soares, 2006) to calculate burst indices (i.e., the number of spikes found in bursts divided by the total number of spikes for the record). In addition, changes in oscillatory discharges were assessed with power spectral methods. Power spectra were calculated from the ISI data, and described elsewhere (Soares et al., 2004). The raw spectra was integrated in the 1-3 Hz, 3-8 Hz, 8-15 Hz and >15 Hz ranges for each cell.

#### *8.2.3.7 Statistics*

Non-parametric methods were used throughout this study. Mann-Whitney tests were used to compare the discharge rates in the baseline period with those in the drug effect period for individual experiments. The Wilcoxon signed rank-test was used to compare the data collected before and after drug or vehicle injections for neurons in SNr and in GPi. p-values of less than 0.05 indicated significance.

### 8.2.4 Immunocytochemical experiments

#### *8.2.4.1 Tissue preparation and histology*

For this portion of the study, tissue from thirteen rhesus monkeys, different from those used in the electrophysiologic sessions, was used. Five animals received MPTP. Three animals received only systemic injections of MPTP (i.m., 0.2 mg/kg/week) while two other monkeys received an intracarotid injection of MPTP (see above) followed by a series of i.m. injections (0.2 mg/kg) until they reached stable parkinsonian motor signs. The total amount of MPTP injected per animal ranged between 3-7 mg/kg. Both normal and MPTP-treated monkeys that showed stable parkinsonism, were later perfused with a mixture of paraformaldehyde (4%) and glutaraldehyde (0.1%) as described above. Tissue blocks were cut in 60 μm-thick sections with a vibrating microtome and processed for light (LM) or electron microscopic (EM) immunocytochemistry to visualize D1 and D5 receptor immunoreactivity (see below and Charara et al., 2004). Some sections were also processed to reveal tyrosine hydroxylase (Millipore, Temecula, CA; 1:1000) immunostaining at the light microscopic level to confirm striatal dopamine denervation.

Before immunocytochemical processing, sections prepared for EM or LM procedures were first rinsed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), then incubated in 1% sodium borohydride solution in PBS (20 minutes), rinsed in PBS, treated with a cyroprotectant solution (EM only), frozen at -80º C, thawed, and finally rinsed again (PBS).

### *8.2.4.2 Immunoperoxidase localization of D1 and D5 receptors*

**8.2.4.2.1 Primary antisera**. Two affinity-purified monoclonal antibodies were used. Although the transmembrane regions of D1 and D5 receptors are highly homologous, these receptors differ significantly at the third intracellular loop and carboxy-terminus (Sunahara et al., 1991; Tiberi et al., 1991). We used highly specific monoclonal D1 receptor antibodies (Sigma-Aldrich Co, St. Louis, MO, 1:75, Levey et al., 1993) which were raised against 97 amino acids in the carboxy-terminus. This antibody has been extensively used and well characterized in both rodents and primates (Betarbet and Greenamyre, 2004; Levey et al., 1993; Paspalas and Goldman-Rakic, 2005). We also used a selective and thoroughly characterized D5 receptor antiserum (1:500, made by one of the authors [ZUK]) raised against a ten amino acid peptide in the carboxy-terminus of D5 receptor protein (residues 428-438, Khan et al., 2000; residues 428-438, Sunahara et al., 1991; Tiberi et al., 1991).

**8.2.4.2.2 Immunoperoxidase procedure.** Sections were pre-blocked in a solution containing 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS at room temperature for 1 hour. Next, the sections were incubated for two days in primary antibody solution at 4º C. For LM processing, 0.3% triton-X-100 was added to the pre-incubation and primary antibody solutions, and kept over night at room temperature. The sections were then incubated in a solution containing biotinyated goat anti-rat (D1 receptor immunohistochemistry), anti-rabbit (D5 receptor immunohistochemistry), or anti-horse (TH visualization) IgG (Vector Labs, Burlingame, CA; 1:200), and avidin-biotin complex solution (Vectastain Standard Kit, Vector Labs;

Hsu et al., 1981; 1:100). Immunoreactive agents were diluted in PBS containing 1% NGS, 1% BSA, and 0.3% Triton (LM only). Sections were rinsed in PBS and Tris buffer (0.5 M, pH 7.6) before being transferred to a solution containing imidazole (0.01M; Fisher Scientific, Hampton, NH), hydrogen peroxide (0.006%) and 3-3'-diaminobenzidine tetrahydrochloride (0.025%; Sigma-Aldrich) for 10 minutes.

After washes in PBS and phosphate buffer (PB; 0.1 M, pH 7.4), the sections were postfixed in 1% osmium tetroxide (20 minutes), rinsed in PB and dehydrated in an increasing gradient of ethanol. Uranyl acetate (1%) was added to the 70% alcohol step within the gradient to improve contrast in the EM analysis. Next, the sections were exposed to propylene oxide before being embedded in epoxy resin (Durcupan, ACM; Fluka, Ft. Washington, PA), mounted on microscope slides and placed in an oven (60º C) for two days.

Blocks of GPi and SNr were taken from the microscope slides and glued with cyanoacrylate to the top of resin blocks. Ultrathin sections (60 nm) were taken from the surface of the blocks using an ultramicrotome (Leica Ultracut T2, Nussloch, Germany), collected onto Pioloform-coated single copper grids, stained with lead citrate (5 minutes; (Reynolds, 1963), and examined with an electron microscope (Zeiss EM 10C, Thornwood, NY).

**8.2.4.2.3 Immunogold procedure.** To further characterize the subcellular location of axonal D1 receptor immunoreactivity, we used the pre-embedding immunogold technique. Although slightly less sensitive, this method offers a higher level of spatial resolution than the immunoperoxidase method.

Tissue from five animals was used for the immunogold localization of the D1 receptor. Sections were processed for pre-embedding immunogold method as described in previous studies from our laboratory (see Galvan et al., 2006; see Hubert and Smith, 2004b). Briefly, they were preincubated for 1 hour in a solution containing 5% milk and 1% BSA in PBS, followed by several rinses in Tris-buffered saline-gelatin. Next, the sections were exposed to solutions containing 1% milk, 1% BSA and the primary antibody. Afterwards, the sections were rinsed and transferred to a solution containing goat anti-rat IgGs (1:100) conjugated to 1.4 nm gold particles before exposure to the HQ kit (Nanoprobes) for silver intensification of the gold particles (5-10 minutes). The sections were then rinsed with 2% aqueous sodium acetate buffer and PB, and treated with osmium tetroxide (0.5% in PB, 0.1 M, pH 7.4) for 10 minutes. The remainder of the tissue preparation was the same as described above for the immunoperoxidase reaction.

**8.2.4.2.4 Qualitative analysis**. Sections immunolabeled for the D5 receptor were examined with a Leica DMRB microscope. Images were photographed at a magnification of 1.6 – 20X-1.25 (camera: Leica DC500; Varshaw Scientific, Inc. Atlanta, GA) and captured with computer software (Leica IM50; v.1.20). They were then transferred to Adobe Photoshop (Adobe Systems, San Jose, CA) and adjusted for brightness and contrast.

### *8.2.4.3 Quantitative ultrastructural analysis*

Ultrathin sections were examined with a Zeiss EM10C electron microscope. Randomly selected areas containing immunoperoxidase or gold particles were scanned

and photographed at a magnification of 16,000 – 25,000X (camera: DualView 300W; Gatan, Pleasanton, CA; controller software: Digital Micrograph Software; Gatan, Inc., Warrendale, PA; v. 3.6.5). Some micrographs were adjusted for brightness and contrast with either Digital Micrograph or Adobe Photoshop.

**8.2.4.3.1 Analysis of immunoperoxidase data.** From a series of 30-50 electron micrographs per block, immunoreactive elements were categorized as axons, terminals, dendrites or glial processes based on ultrastructural criteria as described by Peters et al. (Peters et al., 1991). The relative proportion of immunoreactive structures was calculated and expressed as a percent of total labeled elements in each brain region examined. The total surface area (SA) of tissue examined in each structure was calculated for the different animals and the mean percentages (+/-SD) of labeled elements in GPi and SNr of normal and MPTP-treated monkeys were calculated.

**8.2.4.3.2 Analysis of immunogold data.** Silver-intensified gold labeling was identified as large (10-30 nm) electron dense particles that were classified as plasma membrane-bound if they were apposed to or in close contact with the plasma membrane or intracellular if they were not in direct contact with the plasma membrane.

## 8.3 Results

### 8.3.1 Electrophysiologic effects of D1LR agonist injections

We assessed the effects of local micro-injections of the SKF82958 on the activity of 19 SNr and 25 GPi neurons in the immediate vicinity of the injection site. The data collected from the three animals used in this part of the study were pooled. We tested neurons throughout the full rostrocaudal and mediolateral extents of both nuclei. Because of the relatively small sample size, we did not determine whether the drug-mediated effects were regionally specific within GPi or SNr. Control injections had no effect on neuronal activity (n = 5; data not shown).

#### *8.3.1.1 Effects of D1LR activation*

The infusion of SKF82958 (3 µg/µl) reduced the discharge rate of the majority of neurons recorded in SNr (14/19) and GPi (19/25), while a few cells did not respond (5/19 in SNr, 4/25 in GPi; figures 8.1 and 8.2). The remaining 2 cells in GPi responded with an increase in neuronal discharge. The median discharge rate of SNr cells decreased from 38.1 spikes/s (25.1 - 43.7; $25^{th}$ - $75^{th}$ percentile) at baseline to 22.0 spikes/s (13.0 – 33.7) after drug exposure, reducing the firing of individual SNr cells by a median of 36% (12 – 72 %; $p < 0.005$, Wilcoxon signed-rank test; figure 8.1). The effect reached its maximum with a median latency of 373 s (215.3 – 446.6). In GPi, the median discharge rate was 64.6 spikes/s (41.9 – 98.1) at baseline, and 28.5 spikes/s (19.7 - 58.1) after exposure to SKF82958, reducing the firing of GPi cells by a median of 62% (25 – 79 %; $p < 0.001$, Wilcoxon signed-rank test; figure 8.2). In this case, the maximal effect was seen at a median latency of 257.2 s (227.7 – 409.2).

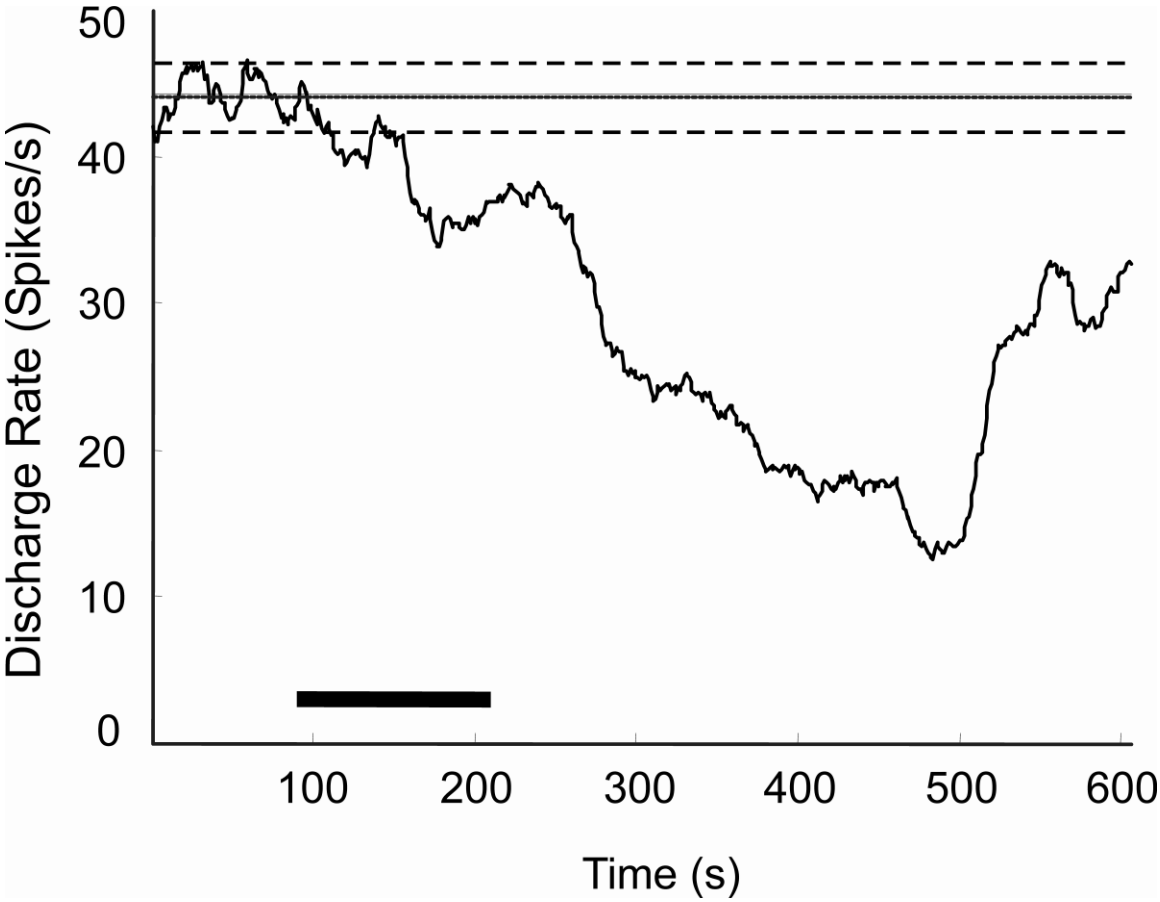


**Figure 8.1.** D1LR activation reduced the firing rate of a GPi neuron. The solid line represents the median discharge rate during the baseline period, while dotted lines indicate 25$^{th}$ (bottom) and 75$^{th}$ (top) percentiles. The thick line at the bottom of the plot indicates the time and length of drug infusion.

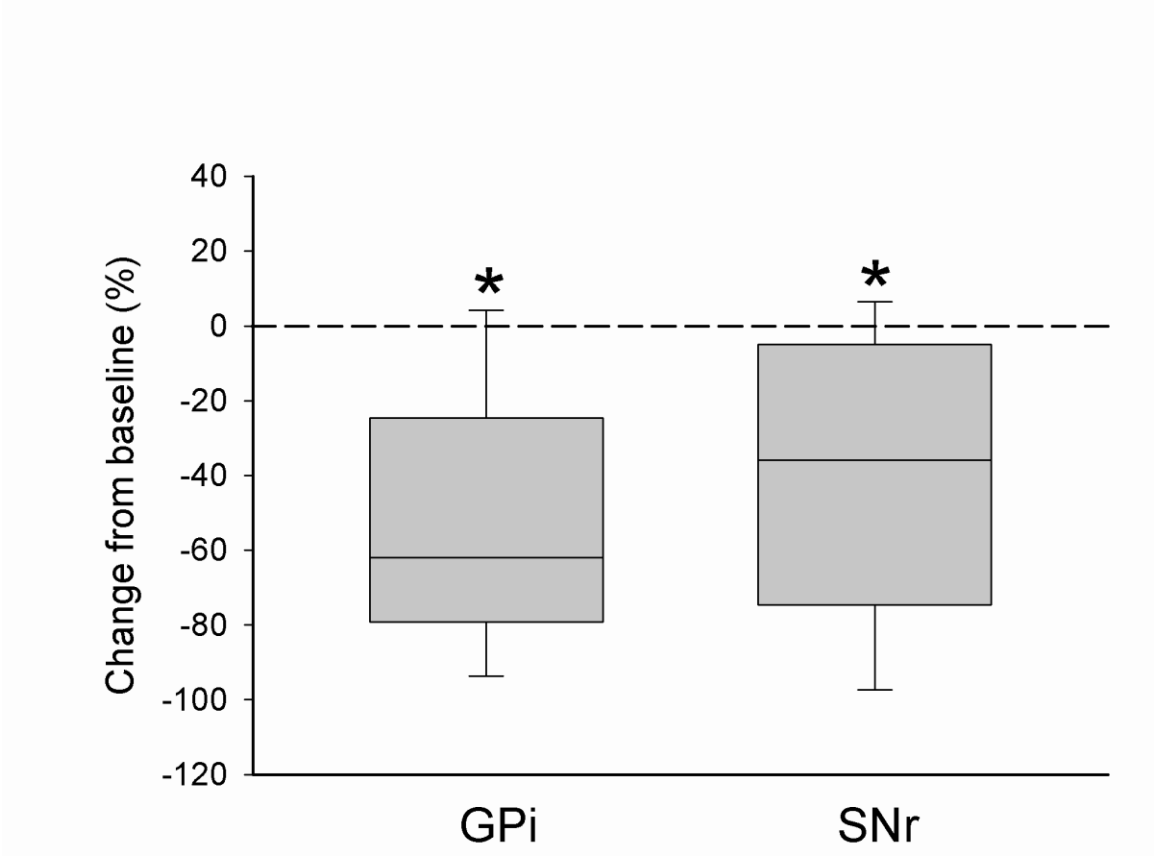


**Figure 8.2.** Effects of SKF82958 on the firing rate of GPi and SNr neurons. For each cell in an experimental group, the discharge rate during the effect epoch was calculated and compared with the baseline discharge rate. The changes in discharge rate are expressed as a percent change from baseline for each experiment. Medians of these percentages are reported here along with the 25$^{th}$ and 75$^{th}$ percentiles (top and bottom of boxes), and minima and maxima (top and bottom of error bars). * $p < 0.05$, Wilcoxon signed-rank test.

Analysis of burst discharges revealed that the median proportion of spikes within bursts increased in GPi from 14.8% (8.3-19.4) to 21.0% (14.3 – 24.8), and in SNr from 15.2% (9.3 – 21.5) to 18.1% (13.3 – 28.7; figure 8.3). Compared to the cell's baseline burst index, the median increase amounted to 31.9% (9.6 – 94.2%; $p < 0.001$) in GPi and no change (-42.7 – 121.1%, n.s.) in SNr.

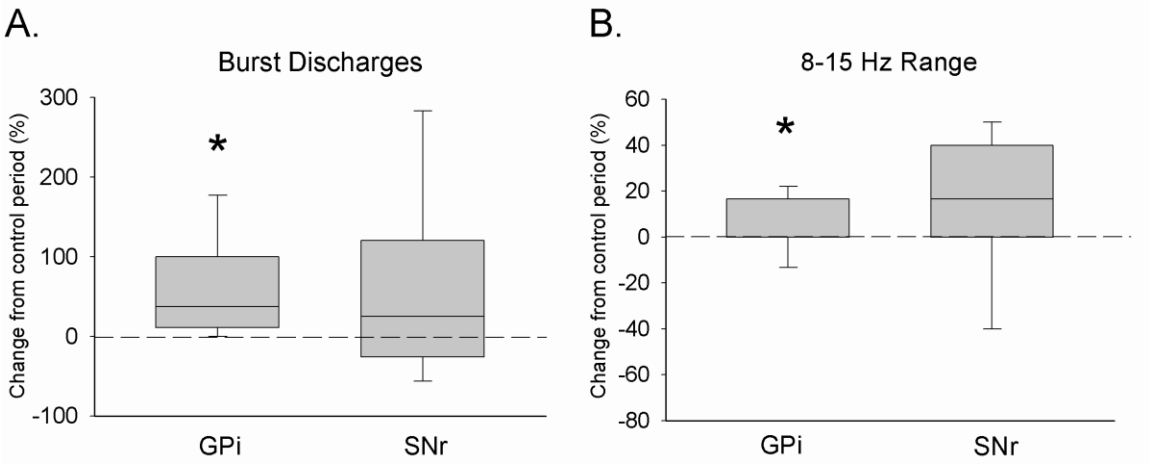


**Figure 8.3.** Changes in discharge patterns in GPi and SNr after infusions of SKF82958. The changes in burst incidence (A), and changes in integrated power spectra in the 8-15 Hz band (B) are expressed as a percent change from baseline for each experiment. Medians of these percentages are reported here along with the 25$^{th}$ and 75$^{th}$ percentiles (top and bottom of boxes), and minima and maxima (top and bottom of error bars). * $p < 0.05$, Wilcoxon signed-rank test.

Statistical comparisons of the normalized integrated power spectra showed that exposure to the SKF82958 did not change the median power in GPi discharge in the 3-8 Hz band (4.7% (4.2 – 5.0) at baseline; 4.8% (4.5 – 5.1) after SKF82958). The median power in the 8-15 Hz band increased slightly after drug exposure from 6.3% (5.3 - 6.8) to 6.6% (5.9 - 7.2; $p < 0.05$; figure 8.3). No change was observed in the frequencies above 15 Hz (85.6% (84.0 – 86.6) to 85.7% (83.8 – 86.5)). In SNr, the proportional distribution of power was qualitatively similar to that in GPi, but did not reach significance.

### 8.3.2 Localization of D1 and D5 receptors

In order to provide a substrate for the interpretation of physiological data obtained in this study, we analyzed the ultrastructural localization of D1 and D5 receptor immunoreactivity in GPi and SNr of normal and MPTP-treated monkeys. Because there was no obvious regional heterogeneity in the distribution of either receptor subtype in GPi and SNr, data collected from different regions within these structures were pooled.

#### *8.3.2.1 Ultrastructural localization of D1 and D5 receptors*

The overall of distribution of D1 receptor immunoreactivity in GPi and SNr did not differ significantly between normal and parkinsonian monkeys (figures 8.4 and 8.7). D1 receptors were almost exclusively found in unmyelinated, likely pre-terminal, axonal segments in the SNr of normal (89%, ± 5.2; n=4; SA, 2936.4 $\mu m^2$), and MPTP-treated monkeys (88.8%, ± 5.9; n=3; SA, 2202.3 $\mu m^2$). A similar pattern of labeling was found in GPi (90.4%, ± 2.2; n=3 in normal vs 90.2%, ± 0.40; n=3 in MPTP-treated monkeys; SA, 2202.3 $\mu m^2$). No significant difference was found in the relative distribution of D1

receptor labeling between normal and MPTP-treated monkeys (figure 8.7). Glial processes did not display immunoreactivity for D1 receptors in either condition.

At the light microscopic level, the GPi and SNr contained a significant number of D5-immunoreactive neurons and a moderately labeled neuropil in both normal and MPTP-treated monkeys (figure 8.5), with no obvious difference in the overall pattern of labeling between the two groups of animals. However, a dramatic loss of D5-immunoreactive neurons was found in the ventral tier of the substantia nigra pars compacta (SNc), while neurons in the dorsal tier of the SNc were relatively spared in MPTP-treated monkeys (figure 8.5). When examined at high magnification, the D5 receptor immunoreactivity was localized mainly in cell bodies and proximal dendrites of GPi and SNr neurons, without any obvious change in the pattern of staining between the normal and MPTP conditions (figure 8.5). No specific elements could be recognized in the neuropil of either structure at the light microscopic level.

At the electron microscopic level, the distribution pattern of D5 receptor labeling resembled that of D1 receptors, i.e. the majority of D5 receptor immunoreactivity was confined to unmyelinated axons (figures 8.6 and 8.7) in GPi (72.0%, ± 8.5; n=3 in normal vs 67.0% ± 3.7; n=3 in MPTP-treated monkeys; SA, 3670.5 $\mu m^2$) and SNr (74.3%; ± 17.6; n=3 in normal vs 74.8% ± 5.8; n=3 in MPTP-treated monkeys; SA, 3450.3 and 3670.5 $\mu m^2$, respectively) with no significant difference between normal and MPTP conditions (figure 8.7). However, in both experimental groups, a significantly larger proportion of D5 receptor-immunoreactive elements were accounted for by dendrites, terminals and glial processes than in D1 receptor-immunostained tissue (figure 8.6). In some cases, the immunoperoxidase labeling of dendrites was confined to a small sector of

the labeled element, while in others, a dense and diffuse immunoreactivity completely filled the labeled dendrites (figure 8.6). Labeled glial processes were often found enfolding the rosettes of striatal-like terminals around single dendrites (figure 8.6C).

#### *8.3.2.2 Immunogold localization of D1 receptors*

The pattern of D1 receptor immunogold labeling was consistent with the immunoperoxidase data. Most gold particles were found in small unmyelinated axons (figure 8.4), with only scarce postsynaptic labeling. In both normal and MPTP-treated monkeys, about half of the gold particles were bound to the plasma membrane of immunoreactive axonal processes in GPi (47.8%, n=3 in normal; 62.1%, n=1 after MPTP) and SNr (47.7%, n=3 in normal; 60.1%, n=1 after MPTP).

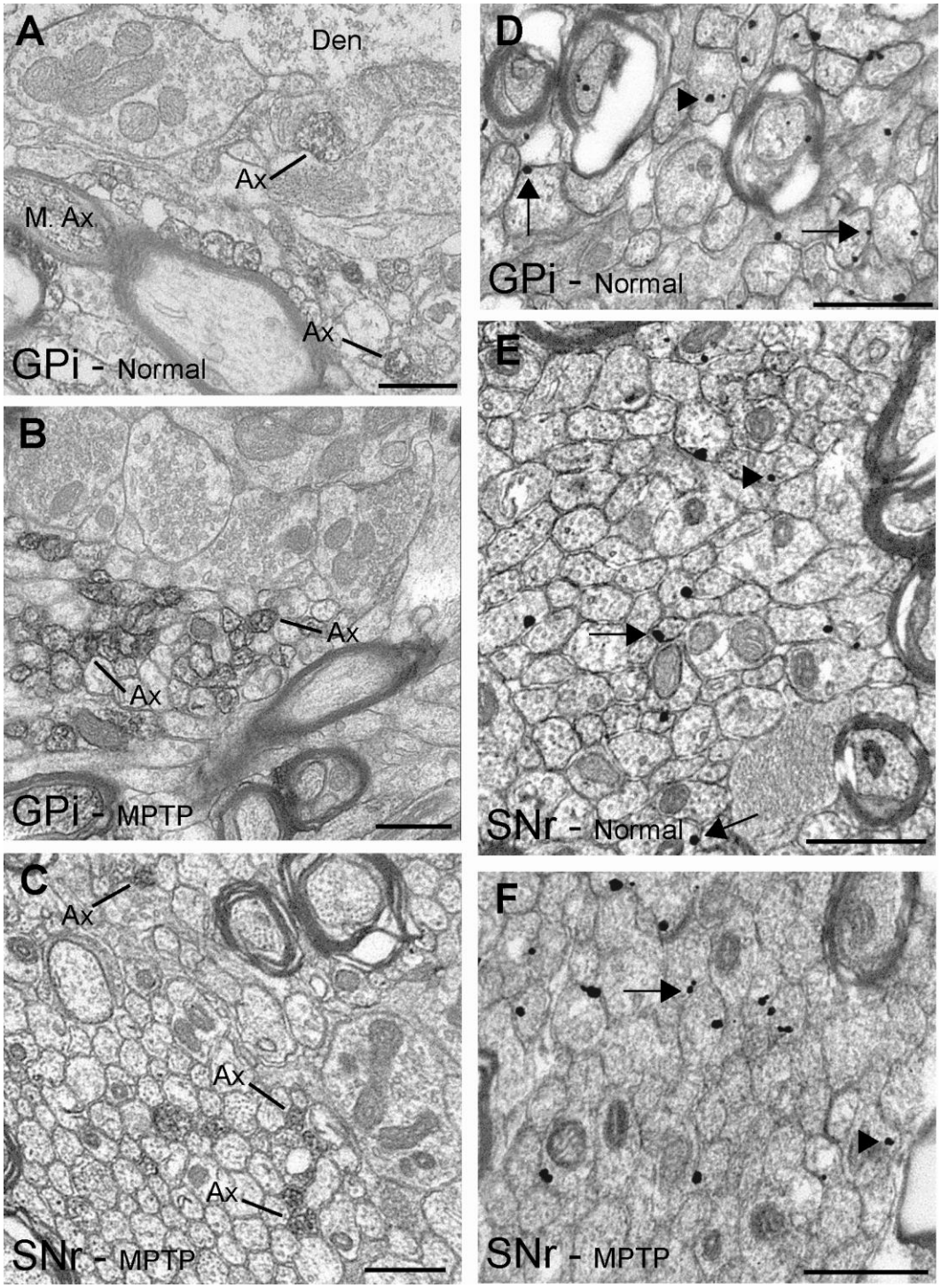


**Figure 8.4.** Localization of D1 receptors in GPi and SNr of normal and MPTP-treated monkeys. D1 receptor-immunolabeled unmyelinated axons (Ax) are shown in the GPi and SNr of normal and MPTP-treated monkeys (A-C). An immunoreactive myelinated axon (M.Ax) is also depicted in GPi (A). D1 receptor immunogold labeling is apposed to the plasma membrane (arrows in D-F) or intracellular (arrowheads in D-F) in unmyelinated axons that travel through GPi and SNr of normal and MPTP-treated monkeys (D-F). Scale bars: 0.5 μm.

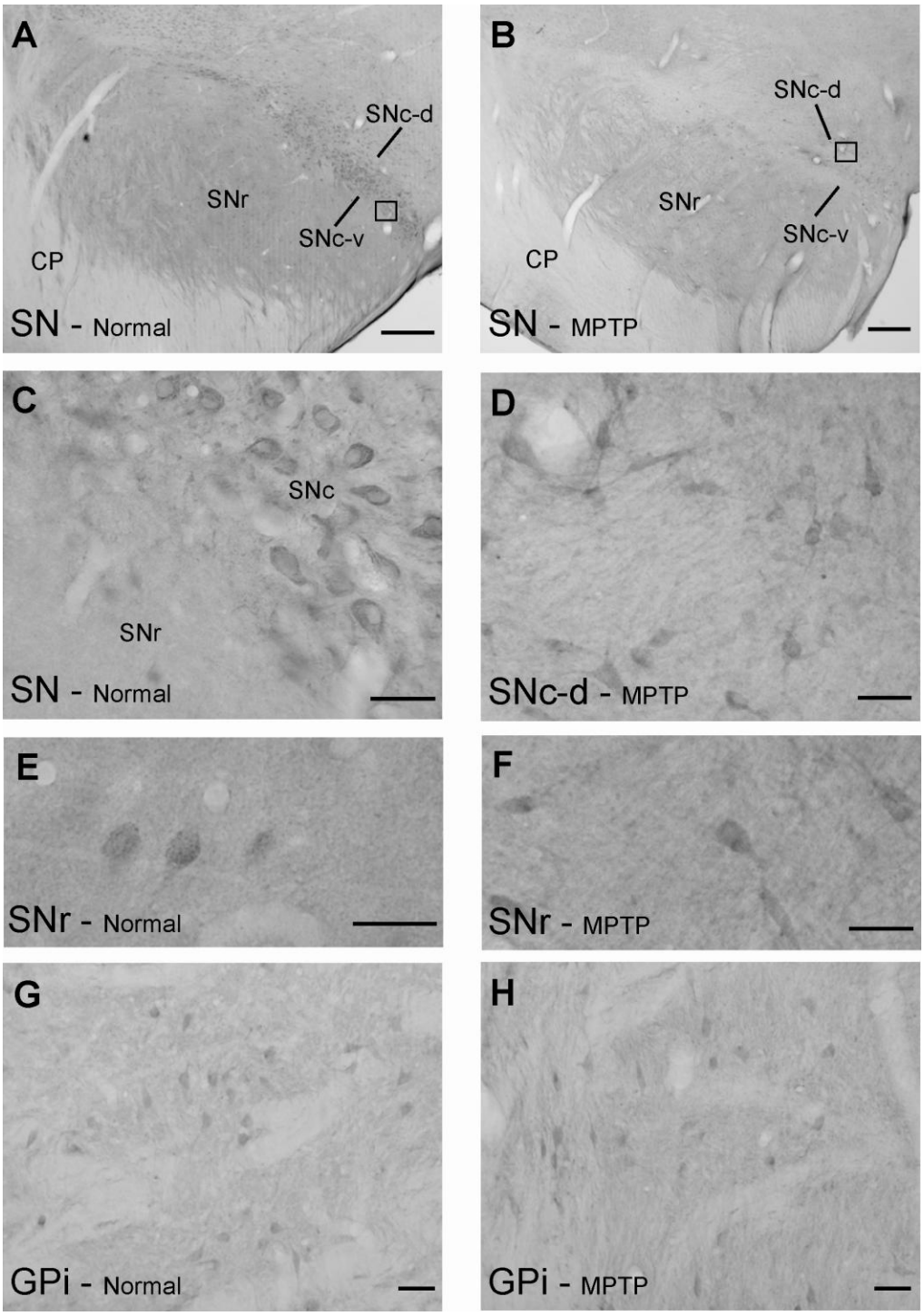


**Figure 8.5.** Light micrographs showing cellular D5 receptor immunostaining in GPi and SN of normal and MPTP-treated monkeys. Strong D5 receptor labeling is found in cell bodies of ventral tier SNc neurons in normal animals (A), which is subsequently lost after MPTP treatment (B). However, strongly labeled D5 receptor-immunoreactive neurons remain in the dorsal tier of SNc after MPTP (D). No significant change in the pattern of D5 receptor immunoreactivity was found in the SNr or GPi of normal (E, G) versus MPTP-treated (F, H) monkeys. SNc-d, substantia nigra pars compacta-dorsal; SNc-v, substantia nigra pars compacta-ventral. Scale bars = 0.5 mm (A-B), 50 μm (C-H).

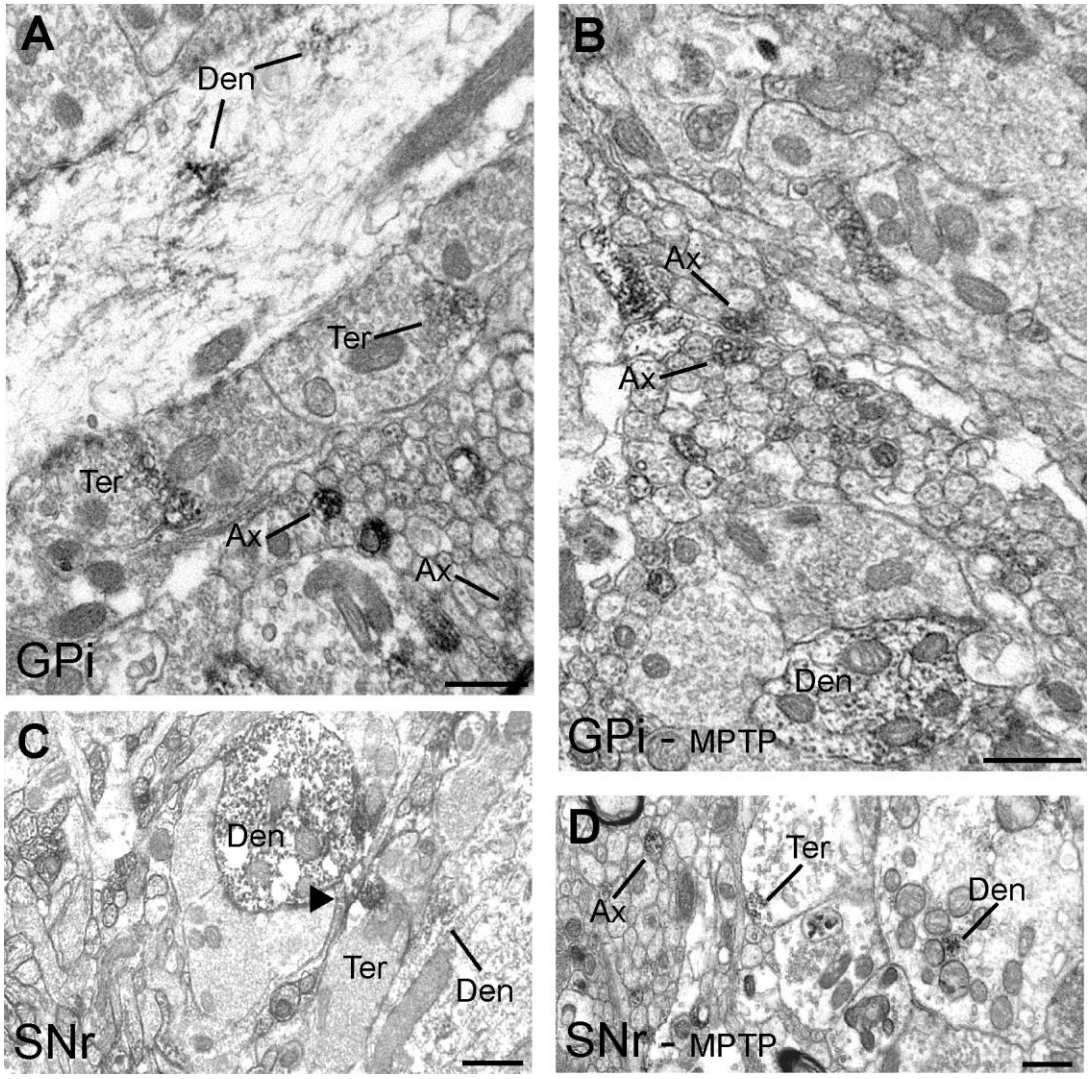


**Figure 8.6.** Localization of D5 receptor immunoreactivity in GPi and SNr in normal and MPTP-treated monkeys. (A-D) illustrate D5 receptor immunolabeling in dendrites (Den), unmyelinated axons (Ax) and terminals (Ter) in GPi (A-B) and SNr (C-D) of normal and MPTP-treated monkeys. Occasional glial labeling is also depicted in the SNr (arrowhead in C). Scale bars: 0.5 μm.

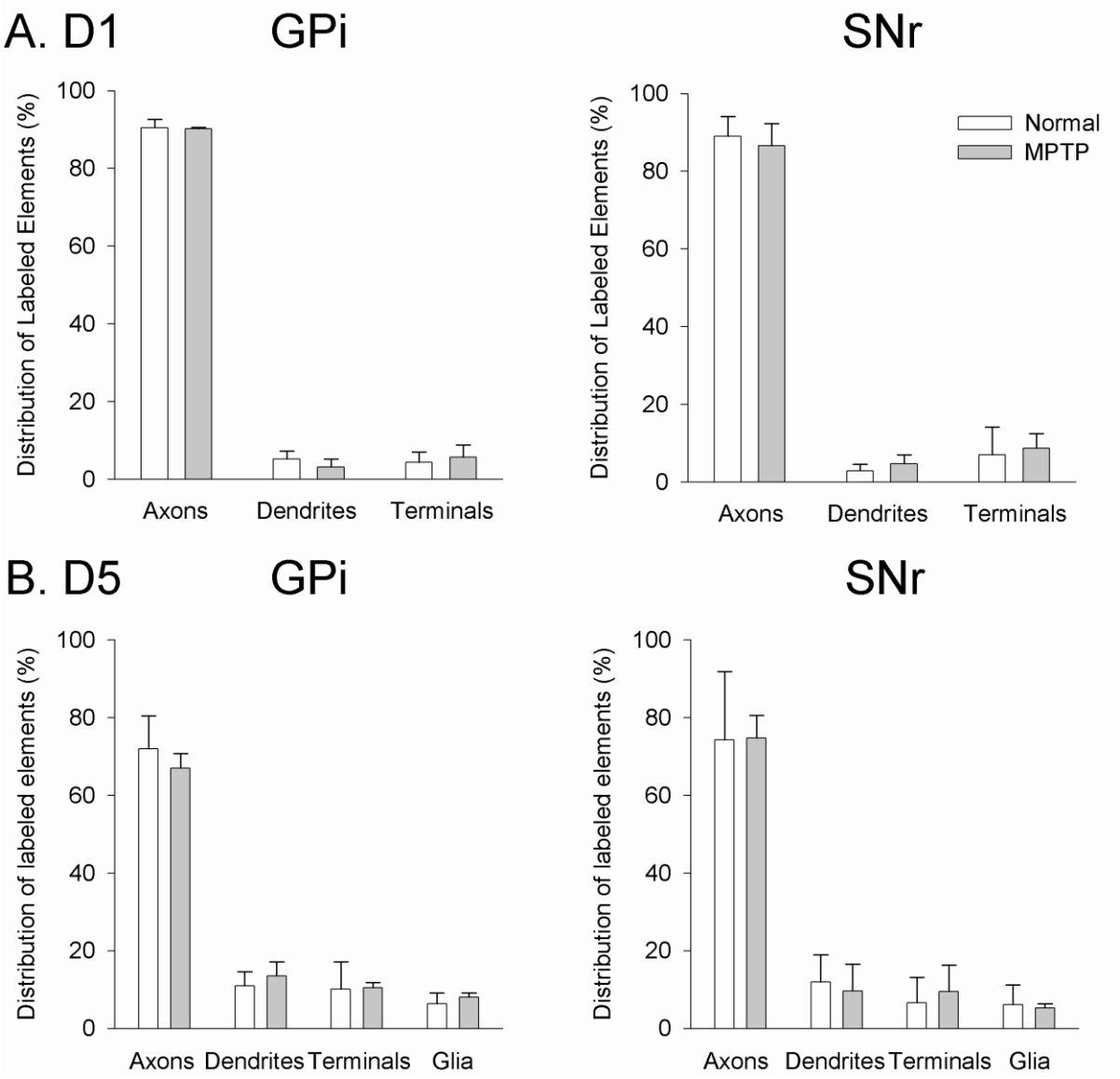


**Figure 8.7.** Comparative distribution of D1 (A) and D5 (B) receptors in axons, dendrites, terminals and glia (B only) in GPi and SNr of normal and MPTP-treated monkeys. Data are expressed as percentages of immunolabeled elements. Each bar represents the mean ± SD.

## 8.4 Discussion

These results, together with our recent study in normal monkeys (Kliem et al., 2007), demonstrate that D1LR activation reduces neuronal discharge rates in GPi and SNr, along with changes in bursts and oscillatory activity in MPTP-treated monkeys. The EM data suggest that the D1LR-mediated changes in neuronal discharge of basal ganglia output neurons are likely due to activation of presynaptic D1 and D5 receptors which are located on the plasma membrane of striato-GPi and striatonigral axons, although postsynaptic D5 receptors may also play a role.

### 8.4.1 D1 and D5 receptors in the monkey GPi and SNr

Our results show that most D1 and D5 receptors in GPi and SNr of normal and MPTP-treated monkeys are located in unmyelinated axons and putative GABAergic axon terminals. Immunoreactive axon terminals for both receptor types displayed ultrastructural characteristics of striatal GABAergic boutons (Smith et al., 1998), suggesting that the functional effects of D1 and D5 receptors in GPi and SNr are likely mediated in large part through the pre-synaptic regulation of GABAergic striatal transmission (Kliem et al., 2007).

In line with our findings, previous EM studies in normal rodents (Yung et al., 1995b) and monkeys (Caille et al., 1996; Levey et al., 1993) have also described D1 receptor expression in striatal axons in GPi and SNr. It was interesting to find that most receptor labeling was confined to unmyelinated axons instead of axon terminals, a common feature for many functional pre-synaptic G protein-coupled or ionotropic receptors, including kainate receptors, group II metabotropic glutamate receptors, and

GABA-B receptors (Chittajallu et al., 1999; Clarke et al., 1997; Fisher and Alger, 1984; Galvan et al., 2006; Huntley et al., 1993; Petralia et al., 1994; Rodriguez-Moreno et al., 1997). The significance of such axonal labeling remains poorly understood, but our immunogold data show that about half of D1 receptor immunoreactivity in axons is bound to the plasma membrane in normal monkeys, suggesting that these receptors may bind extracellular dopamine, potentially regulating striatofugal GABAergic transmission. Although we collected immunogold data from only one MPTP-treated monkey, the findings obtained in this animal are in line with those collected in controls suggesting that presynaptic D1 receptor-mediated regulation of GABA release likely occurs in both normal and parkinsonian conditions.

Little attention, however, has been paid to the localization and function of D5 receptors in GPi and SNr. The limited specificity of D5 receptor antibodies and the lack of drugs that can differentiate D1 receptors from D5 have considerably hampered progress in elucidating the respective roles of these two receptors in the basal ganglia.

However, *in situ* hybridization and immunohistochemical studies have reported D5 receptor mRNA and protein expression in the rat and monkey GPi and SNr (Bergson et al., 1995; Choi et al., 1995; Ciliax et al., 2000; Khan et al., 2000). Qualitative EM studies have found D5 receptors at both pre-and post-synaptic locations in the rodent and monkey SNr (Bergson et al., 1995; Khan et al., 2000), but none of these studies provide quantitative information on the relative distribution of D5-immunoreactive elements in basal ganglia nuclei under normal or parkinsonian conditions. We found that the majority of D5 receptor labeling was in axons and axon terminals in GPi and SNr, and

that MPTP treatment did not significantly alter the D5 receptor distribution in these structures.

### 8.4.2 Neuronal effects of D1LR activation in GPi and SNr in normal animals

Based on previous studies, it is likely that one of the effects of D1LR activation in GPi and SNr is to increase GABA release from terminals of the striatopallidal and striatonigral projections (Ferre et al., 1996; Trevitt et al., 2002), (Kliem et al., 2007; Matuszewich and Yamamoto, 1999; Rosales et al., 1997b; Timmerman and Westerink, 1995). Increased GABA release also likely underlies the prominent reduction of discharge rates in SNr and GPi neurons after local application of D1LR agonists in these nuclei (Aceves et al., 1995; Ferre et al., 1996; Floran et al., 1990; Kliem et al., 2007; Rosales et al., 1997b; Timmerman and Abercrombie, 1996; Timmerman and Westerink, 1995; Trevitt et al., 2002; Waszczak and Walters, 1983; Waszczak and Walters, 1986; Windels and Kiyatkin, 2006). However, the mechanism(s) by which D1LR-activation increases GABA release from striatopallidal and striatonigral terminals are not clear. Striatal projection neurons are minimally active at rest (Kimura, 1992), so that basal GABA release from these terminals should be small. It is difficult to understand how the large effect of D1LR agonists can be explained through modulation of such small transmitter quantities. It has been speculated that D1LR activation may affect GABA release independent of striatal activity (Kliem et al., 2007). SNr recordings in rat brain slices support this idea, showing that the frequency of miniature inhibitory postsynaptic potentials increases after D1LR activation (Radnikow and Misgeld, 1998).

We cannot rule out that D1LR activation in our experiments also affected glutamate release from terminals of subthalamic projections to SNr or GPi. D1 and D5 receptor proteins and mRNA have, indeed, been found in monkey and rodent STN neurons (Ciliax et al., 2000; Flores et al., 1999), and D1LR activation modulates glutamate release in the normal rat SNr (Hatzipetros and Yamamoto, 2006; Ibanez-Sandoval et al., 2006; Rosales et al., 1997a).

### 8.4.3 Neuronal effects of D1LR activation in GPi and SNr in parkinsonism

Previous *in vivo* studies in dopamine-depleted rats have shown that systemically administered D1LR agonists lead to a reduction of SNr activity (Waszczak et al., 1984; Weick and Walters, 1987). With the present study we show that D1LR agonists, applied locally in GPi or SNr, have the same effect in dopamine-depleted monkeys.

Although the loss of dopamine in the basal ganglia results in substantial changes in dopamine receptor function and expression, there is significant controversy regarding the exact nature of these changes. For instance, *in situ* hybridization data from unilateral 6-hydroxydopamine (6-OHDA)-treated rats, demonstrated decreased expression of D1 receptor mRNA in striato-nigral projection neurons (Gerfen et al., 1990). However, D1LR-mediated increases in GABA release in slices of the entopeduncular nucleus (rodent analogue of GPi) and SNr of unilateral 6-OHDA-treated rats is not significantly different from controls, suggesting that D1LR function in basal ganglia output nuclei is unaffected in the parkinsonian state (Aceves et al., 1995; Floran et al., 1990). In MPTP-treated monkeys, D1LR binding or protein expression either does not change or is slightly up-regulated in GPi and SNr (Aubert et al., 2005; Betarbet and Greenamyre,

2004; Gnanalingham et al., 1993). On the other hand, intranigral injections of a D1LR agonist enhance contralateral rotations in dopamine-depleted rats compared to normal animals (Yurek, 1997), suggesting D1LR system changes may contribute. However, these behavioral observations may in fact be due to changes in the GABAergic system since D1LR function and GABA levels are linked (see above, and also, Calon et al., 1995; Chadha et al., 2000a; Chadha et al., 2000b; Gnanalingham and Robertson, 1993; Pan et al., 1985). Our findings are in line with these observations, demonstrating a significantly greater magnitude of D1LR-mediated reductions of firing rate in GPi of MPTP-treated monkeys compared with controls (Kliem et al., 2007). Similar to findings from normal monkeys (Kliem et al., 2007), we found that D1LR activation increases the proportion of spikes in bursts and oscillations in the 8-15 Hz band (at least in GPi) in MPTP-treated monkeys. This result is somewhat paradoxical because it resembles pathological findings obtained from animal models and patients with Parkinson's disease (Bergman et al., 1998; Brown, 2003; Levy et al., 2001; Murer et al., 1997; Wichmann et al., 1999; Wichmann and Soares, 2006). Increased bursting after local D1LR activation may be partly mediated by rebound excitation that follows GABA-mediated hyperpolarizations, as shown in rat brain slices of GP and STN (Beurrier et al., 2000; Beurrier et al., 1999; Bevan et al., 2002; Kass and Mintz, 2006; Nambu and Llinas, 1994; Overton and Greenfield, 1995). Our findings, therefore, suggest that dopamine loss in GPi and SNr is unlikely to contribute to the increase in burst firing or oscillations that is typically seen in parkinsonism.

### 8.4.4 Activation of D1/D5 receptors in GPi and SNr may influence behavior

The behavioral effects of D1LR activation in GPi or SNr have not been well characterized in parkinsonism. It is known that systemic administration of D1LR agonists has anti-parkinsonian effects in MPTP-treated monkeys (Blanchet et al., 1996; Goulet and Madras, 2000) and that local D1LR blockade in the rat SNr impairs motor activity (Bergquist et al., 2003; Trevitt et al., 2001) and increases EMG activity and rigidity (Hemsley and Crocker, 2001). Behavioral and biochemical studies in rats with partial dopaminergic depletion have also demonstrated that dopamine loss in the SNr impairs motor functions, and that increased nigral dopamine release can counteract motor impairment (Andersson et al., 2006). Less is known about an involvement of dopamine loss in GPi in parkinsonism. A reduction of dopamine has been reported in the GPi of parkinsonian patients (Bernheimer et al., 1973) and MPTP-treated monkeys (Pifl et al., 1992). PET studies have demonstrated a decrease in $^{18}$F-dopa uptake in GPi of patients with advanced parkinsonism (Whone et al., 2003). Taken together, these studies suggest that nigral and pallidal D1LRs may play roles in regulating motor behaviors, and that D1LR activation may improve parkinsonian motor symptoms. This evidence, however, has to be weighed against our finding of an increase in burst firing and oscillations after local D1LR activation in the monkey GPi and SNr, and the disappointing preliminary clinical experience with D1LR agonist therapy in patients with Parkinson's disease (Mailman et al., 2001).

Despite the difficulty in pharmacologically discriminating D1 from D5 receptor-mediated effects, various groups have recently attempted to characterize the specific roles of these two receptor subtypes in motor behavior using mice with deletion of genes

encoding for either receptor subtype. These studies support the idea that D1 or D5 receptor activation has opposite effects on locomotor activity; D1 receptor stimulation facilitates locomotion, whereas D5 receptor activation attenuates motor behavior. For instance, intracerebroventricular administration of an antisense oligodeoxynucleotide against the D5 receptor in dopamine-depleted rats blocks D1LR agonist-induced contralateral turning (Dziewczapolski et al., 1998). Furthermore, D5 receptor knock-out mice are hyperactive (Sibley, 1999) and D1LR antagonist administration in D1 receptor knock-out mice decreases motor activity (Centonze et al., 2003a). Finally, behavioral and pharmacologic studies suggest that selective D5 receptor preferring D1LR agonists suppress locomotion in rats in open-field studies (Nergardh et al., 2005).

It is not clear how these findings specifically relate to D1 or D5 receptor-mediated effects in the GPi or SNr. However, it is known that dopamine replacement therapies aimed at the SN, such as grafts of embryonic mesencephalic tissue containing dopaminergic cells (Starr et al., 1999b) or the infusion of glia-derived neurotrophic factor ameliorate parkinsonian motor signs in MPTP-treated monkeys (Gash et al., 1996a; Gerhardt et al., 1999). Conceivably, D1LR activation plays a role in these beneficial effects. Increased GABA release induced by activation of D1 receptors on striatal efferents to GPi or SNr may facilitate movement by reducing the increased activity of output neurons in these nuclei. As activation of postsynaptic D5 receptors may have the opposite effect, the development of specific D1 receptor agonists or of compounds that combine D1 receptor agonistic and D5 receptor antagonistic effects may be a worthwhile strategy of treating parkinsonism

# Chapter 9

## General Summary and Conclusions

## 9. Summary and Significance of Findings

The box model describing the basal ganglia circuitry has laid the groundwork for basal ganglia research of the last twenty years, and it continues to be assessed and modified. The basal ganglia-thalamo-cortical loop was introduced in the 1980s to help explain both normal and abnormal function. Under normal conditions, a balance between the direct and indirect pathways influence basal ganglia output, while lesions of the SNc result in the loss of dopamine in the circuit that produces the symptoms associated with parkinsonism. This imbalance between activity in these two pathways is believed to be the origin of several motor dysfunctions which has led to the rationale leading to neurosurgical proceduces such lesioning of the GPi. In other words, this model was used to help explain akinesia and bradykinesia, major symptoms of idiopathic PD because of the overactivity of the basal ganglia output nuclei. In the 1990s, lesions of the GPi were very successful in alleviateing motor signs of PD. But, based on the above model, you would expect GPi lesions to lead to dyskinesias (which does not happen).

It appears that the interconnections of the basal ganglia are quite complex and dynamic. One shortcoming of the traditional model of basal ganglia circuitry is that it does not take into account SNc inputs to GPi and the interdigitation of SNc dendrites containing dopamine that reach the SNr. Although the traditional model explains firing rate changes in GPi and SNr induced by nigrostriatal dennervation, it does not explain the observed pattern changes in these nuclei.

Dopamine actions are mediated through D1LRs and D2LRs in the brain. The importance and function of D1LRs in the striatum is well-known. Most models of PD, however, have neglected the possible role and function of D1LR-mediated events in the

GPi and SNr. The overall goal of this dissertation was to characterize the effects of D1LR activation on the GPi and SNr activity in normal and parkinsonian monkeys as well as to explore the ultrastructural location of D1LR sites for dopamine and dopamine agonists. Knowledge of electrophysiologic effects and biochemical changes induced by local stimulation or blockade of D1LR ligands as well as the anatomic location of D1 and D5 receptors described in this dissertation supports the idea that D1LRs in SNr and GPi may mediate some of the effects of endogenous dopamine and that D1LRs in these areas may be a possible target for antiparkinsonian therapy.

Because our knowledge regarding dopamine and D1LR function in the monkey GPi and SNr was lacking, of primary importance to this dissertation was to locally stimulate or block D1LRs and measure neuronal changes in activity. Therefore, we developed a combination electrode-injection device to record changes in cellular activity of single GPi and SNr neurons after infusions of sub-microliter quantities of drugs in the vicinity of the neuron. The manuscripts on this topic outline two detailed electrophysiologic studies of D1LR-mediated changes in neuronal activity in the basal ganglia output nuclei under normal and parkinsonian conditions. In each study, three parameters were analyzed: discharge rate, incidence of bursts, and changes in oscillatory activity. In general, the responses of both GPi and SNr cells were similar in normal and parkinsonian monkeys. Specifically, D1LR activation in GPi and SNr significantly reduce discharge rates under both conditions, demonstrating that responses to D1LR activation are retained in parkinsonian monkeys. D1LR blockade, on the other hand, significantly increased neuronal firing rate in only GPi (not SNr), suggesting a dopaminergic tone in this nucleus. Overall, these findings suggest that dopamine and

dopamine agonists with D1LR activity may play a role in regulating motor and non-motor functions in GPi and SNr in normal and parkinsonian monkeys.

Changes in neuronal discharge patterns such as bursts or oscillatory activity were also evaluated. Although the mechanisms underlying bursts and oscillations are not known, it is possible that they are a result of local loss of dopamine to GPi and/or SNr. The results show that both GPi and SNr neurons tended to show more bursting activity after activation of D1LRs in normal and parkinsonian conditions, while D1LR blockade in normal monkeys significantly decreased burst discharges in GPi (not SNr). Two frequency bands (3-8 Hz range and 8-15 Hz range) were analyzed to assess potential changes in oscillatory activity. These frequencies were chosen because spectral power in these bands increases the firing patterns of subthalamic nucleus and GPi neurons in parkinsonian monkeys. Interestingly, both burst firing and oscillations also increased with D1LR activation in GPi and SNr in normal and MPTP-treated monkeys, suggesting that the previously described pattern changes in parkinsonian animals are not the result of dopamine loss in GPi or SNr.

The second aim of this dissertation examined a possible mechanism by which D1LR activation may alter discharge rates in SNr and GPi. Specifically, local changes in GABA levels were measured after infusions of D1LR ligands in GPi and SNr in normal monkeys. D1LR activation significantly increased GABA concentrations while D1LR blockade had no effect. These data suggest that ambient levels of dopamine are low and that the D1LR-mediated reductions in discharge rate are modulated, at least in part, by D1LRs on striatal afferents in GPi and SNr.

It is well established that D1 receptors are located on presynaptic axons and axon terminals of GABAergic striatal-GPi and striatonigral projection neurons in normal animals. However, our knowledge of the location of D5 receptors has been limited by the lack of a selective D5 receptor antibody. Through a collaboration with Dr. Z. Khan, we examined the distribution of D5 receptors in the monkey GPi and SNr. Thus, the third aim of my dissertation was to compare the distribution and ultrastructural location of D1 and D5 receptors in GPi and SNr in normal and MPTP-treated monkeys. I found that the overall pattern of D1 receptor labeling in GPi and SNr did not change with dopamine depletion. The majority (90%) of elements labeled under either condition were unmyelinated axons and putative GABAergic terminals. Only a small portion of dendrites and terminals showed immunoreactivity. To identify the subcellular location of D1 receptors in GPi and SNr in these monkeys, I used the pre-embedding immunogold technique and found that D1 immunoreactivity in unmyelinated axons is equally distributed between intracellular compartments, and bound to the plasma membrane in both nuclei in the normal state. The fraction of receptors bound to the plasma membrane slightly increases after MPTP treatment. This portion of the study was completed in only one monkey. Attempts to repeat them in additional MPTP-treated monkeys were not successful due to the poor preservation of ultrastructural membranes in the available material. Although this may limit the interpretation of the results, together these findings suggest that the D1 receptor is likely functional because the most of the gold particles were bound to the plasma membrane of axons.

The pattern of labeling for the D5 receptor was similar to that of the D1 receptor. Most D5 receptor labeling (60-70%) was found in unmyelinated axons in GPi and SNr

with some in axon terminals, dendrites and glia. Together with the D1 receptor findings, these results suggest that changes in neuronal discharge after the D1LR agonists (hypothesis 1) are likely mediated in large part by the pre-synaptic regulation of GABAergic striatal transmission. Therefore, D1/D5 receptor actions may fine-tune motor and non-motor functions processed in GPi and SNr. In PD, the loss of D1/D5 receptor function may underlie some aspects of parkinsonism. Thus, D1LRs remain a promising therapeutic target in PD.

## 9.1 Future Directions

This work highlights the function of D1LRs in GPi and SNr under normal and parkinsonian conditions. In addition, these findings have enhanced our knowledge of discharge properties associated with endogenous dopamine and D1LR function in these structures, evidence that was unavailable from previous studies. Also, while most studies have focused on D1LR activities in the rat SNr, the role of dopamine and D1LRs in GPi was neglected. The findings of this dissertation suggest that dopamine actions mediated by D1LRs are important in regulating neuronal discharge in GPi under normal and parkinsonian conditions, suggesting that GPi may play a more significant role in movement. Therefore, the experiments listed below are proposed to follow-up with the results of this dissertation and fill gaps in our knowledge regarding the role of endogenous dopamine and D1LRs in processing movement-related information and behavior in GPi. Also, other experiments are proposed to examine biochemical and electrophysiological changes in both GPi and SNr in the parkinsonian state.

### 9.1.1 Electrophysiological

A paradoxical and unexpected finding of the dissertation was the increased incidence of bursts after D1LR activation in GPi and SNr in the normal and parkinsonian states. In addition, D1LR blockade significantly reduced bursting in GPi neurons compared to baseline (pre-drug) recordings in normal monkeys. These findings were surprising since it is well known that burst firing tends to increase in both GPi and SNr of MPTP-treated monkeys and parkinsonian patients. It may be interesting to study whether there are differences in the bursts and peri-burst periods between recordings from the parkinsonian and D1LR-treated animals. Previous work has shown that the sequence of ISIs preceding a burst is not random, and that the temporal succession of ISIs differs between the normal and parkinsonian states. Thus I would compare bursting parameters, such as changes in ISI length before and after bursts and the proportion of time spent in bursts after exposure to a D1LR agonist, to those recorded after MPTP-treatment (drug naïve animals) in both of these structures. These findings may demonstrate significant differences between burst discharges associated with parkinsonism and those recorded in response to D1LR activation. These data may help us understand how dopamine contributes to burst firing under normal conditions and may be differentiated from bursts that may contribute to abnormal information processing in parkinsonism.

### 9.1.2 Behavior

An interesting finding of this work was that D1LR blockade increased the firing rate of GPi neurons, suggesting a dopaminergic tone in this structure. To further expand on this finding and the role of endogenous dopamine in GPi to modulate movement-

related functions, I would test the effects of local blockade of D1LRs in GPi in task-performing monkeys. Since GPi activity is associated with movement kinematics, as well as the preparation, context and execution of movement, I would specifically evaluate the movement and reaction times in a simple movement task and a delayed response task that contains a memory component. I would expect that local blockade of D1LRs would disrupt movement preparation and execution-related aspects of task performance.

Next, because of the substantial effect D1LR activation had on firing rate in the GPi in both normal and parkinsonian monkeys, I would examine the behavioral effects of locally infused current anti-parkinsonian drug therapies with D1LR activity and selective D1LR agonists in GPi of MPTP-treated monkeys. To avoid confounding factors such as 'lesion' effects (i.e. pallidotomy), I would inject into the same site in the GPi. The behavioral analysis would include observations of spontaneous cage behavior along with a detailed assessment of overall cage activity along with time spent moving individual limbs. Also, I would assess the degree of behavioral disability after each injection using a parkinsonian rating scale. Finally, I would include a thorough examination of any anti-parkinsonian effects mediated through D1LR activation such as bradykinesia by examining reaction and movement times during a food retrieval task. Since current dopaminergic therapeutic agents (levodopa, apomorphine) used to treat patients with Parkinson's disease have demonstrated reductions of GPi firing along with substantial improvements of parkinsonian symptoms, I would expect that intrapallidal injections of drugs with D1LR activity may contribute to this effect.

### 9.1.3 Function

The literature is lacking functional studies testing the effects of D1LR activation on GABAergic transmission in the dopamine depleted-state in GPi and/or SNr. Therefore, I would use microdialysis to measure GABA levels in GPi and SNr of hemi-parkinsonian monkeys under basal conditions and after local activation of D1LRs. Based on my electrophysiologic results, I expect to observe similar changes in GABA levels in response to drug exposure in both structures that were demonstrated in the normal state (e.g. increased GABA concentrations after D1LR activation). Based on my electrophysiological results in GPi after MPTP, I expect to observe substantial increases in GABA release in parkinsonian monkeys as compared to the normal state in this structure, a finding that would correspond to the dramatic reduction in firing rate after D1LR activation in MPTP-treated monkeys.

As mentioned previously, an interesting finding of this dissertation is the electrophysiologic and biochemical changes in GPi in response to infusions of D1LR ligands in normal and parkinsonian conditions, suggesting that GPi is an important site of action for endogenous dopamine and dopaminergic drugs with D1LR activity. To my knowledge, dopamine in the GPi has not been measured in awake monkeys. Since it is known that dopamine projections reach the GPi, and D1LRs are expressed in high concentrations, I would measure dopamine levels using microdialysis in resting and behaving monkeys. In resting animals, however, dopamine concentrations may be below the detection limit of the assay. In this case, I would measure dopamine concentrations in these animals after local infusions of amphetamine. This experiment would simply confirm the presence of dopamine in this structure in the normal state. The next set of

experiments would test the hypothesis that dopamine in GPi is directly involved with processing movement-related information. Using microdialysis, dopamine concentrations would be measured in task performing monkeys with the expectation that during the learning phase of a movement task, dopamine concentrations would increase. Due to the time resolution limitations of microdialysis, it would not be possible to assess changes in dopamine levels during specific aspects of the task such as cognitive, memory or attention, preparation and execution of movement.

## 10. References


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