CORTICOSTRIATAL REGULATION OF MEDIUM SPINY NEURON DENDRITIC REMODELING IN MODELS OF PARKINSONISM

By

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LIST OF ABBREVIATIONS

ACH	Acetylcholine
BDA	Biotin Dextran Amine
CAMKIIα	Calcium/Calmodulin-dependent Protein Kinase II-alpha
DIV	Days In Vitro
DA	Dopamine
FG	FluoroGold
FJC	FluoroJade C
GABA	γ-aminobutyric acid
GP	Globus Pallidus
HD	Huntington's Disease
HVA	Homovanillic Acid
IA	Ibotenic Acid
IT	Intratelencephalic
L-dopa	Levodopa
M1	Primary Motor Cortex

M2	Secondary Motor Cortex
mGluR	Metabotropic Glutamate Receptor
MPP^+	1-methyl-4-phenyl-2,3-dihydropyridinium Ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	Medium Spiny Neuron
NMDA	N-methyl-D-aspartate
NR1	NMDAR1 Subunit
NR2	NMDAR2 Subunit
6-OHDA	6-hydroxydopamine
PC	Pyramidal Cell
PD	Parkinson's Disease
PET	Positron Emission Tomography
PND	Postnatal Day
PT	Pyramidal Tract
SCI	Slow Conducting Ipsilateral
SMA	Supplemental Area
SN	Substantia Nigra

TH	Tyrosine Hydroxylase
VGLUT1	Vesicular Glutamate Transporter 1
VGLUT2	Vesicular Glutamate Transporter 2

CHAPTER I

INTRODUCTION

The striatum and movement. The collection of nuclei referred to as the "basal ganglia" was previously called the great cerebral nucleus by David Ferrier in 1876 (see Swanson 2000). The basal ganglia are an ill-defined group of subcortical nuclei, which consist of the neostriatum (caudate nucleus and putamen), globus pallidus, substantia nigra, and subthalamic nucleus. The striatum is the largest nucleus of the basal ganglia and is so named because it has a striated appearance due to the dense fiber bundles of axons that course through the region (Willis T et al. 1965). This "striped" structure was first illustrated by Andreas Vesalius in 1543 and later described by the seventeenth century physician, Thomas Willis, who designated the structure "corpus striatum" in 1664 (Sarikcioglu et al. 2008). Willis noted:

> "These bodies, if they should be dissected along the middle, appear marked, with medullar streak, as it were rays or beams; which sort of chamferings or streaks have a double aspect or tendency; to wit, some descend from the top of this body, as if they were tracts from the brain into the oblong marrow; and others ascend from the lower part, and meet aforesaid, as if they were paths of spirits from the oblong marrow into the brain. And it is worth observation, that in the whole head besides there is no part found chamfered or streaked after the like manner."

In primates the striatum is composed of the caudate nucleus and the putamen, the two nuclei being separated by the white matter of the internal capsule. However in rodents a single structure is observed, with fascicles of

myelinated corticostriatal axons being dispersed throughout the structure. The striatum is functionally divided into the dorsal striatum and the ventral striatum (see Figure 1). Whereas the dorsal striatum is implicated in voluntary movement, as well as habit-based learning, the ventral striatum plays an important role in the translation of motivation to movement.

Modulation of movement processes is mainly what the striatum is known for, but it also plays important roles in procedural learning and reward pathways. The basal ganglia were suggested to be involved in movement at the beginning of the 20th century, based on the observation that damage to these structures resulted in movement disorders (Wilson SAK 1914; Mettler R and Mettler C 1942; Mettler FA 1945; Divac et al. 1967; Denny-Brown D and Yanagisawa N 1972). Simplistically, the motor cortex sends information to the basal ganglia and the cerebellum; both areas of the brain send information back to the cortex via the thalamus.

Diseases of the basal ganglia. Disturbances in the basal ganglia result in a myriad of movement disorders, both hypokinetic and hyperkinetic. Hyperkinetic disorders, or disorders of increased motor function, include Huntington's disease (HD), dystonia, and hemiballismus. HD is a genetic, neurodegenerative disease in which striatal MSNs degenerate as a result of a mutation in the Huntington protein. Dystonia involves sustained muscle contractions that cause twisting and abnormal postures. Hemiballismus ("half jumping") is a rare disorder usually

seen after strokes that result in unilateral lesions in the vicinity of the subthalamic nucleus.

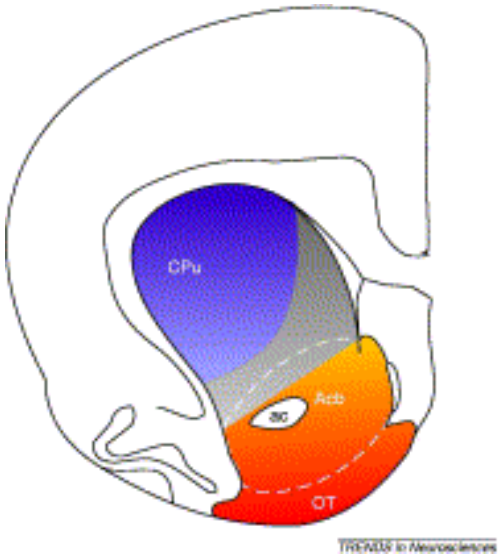

Figure 1. The striatum is divided into the dorsal striatum (caudate and putamen) shown in blue and the ventral striatum (nucleus accumbens) shown in orange. Although illustrated is a distinct border between dorsal and ventral striatum, no such delineation truly exits *in vivo*. Abbreviations: CPu, caudate and putamen; Acb, accumbens. Image from *Voorn et al. 2004.*

Probably the best known basal ganglia disorder is Parkinson's Disease (PD). Parkinson's Disease is a hypokinetic disorder, in which there is reduced motor function. The pathology of PD involves the degeneration of the pigmented dopamine cells in the substantia nigra (black substance, referring to the heavily pigmented dopamine neurons). The degeneration of the substantia nigra (SN) dopamine neurons results in a decrease in the amount of striatal dopamine (see Figure 2), and the appearance of the cardinal symptoms of PD: bradykinesia, resting tremor, and rigidity. Postural instability is also observed, but usually presents somewhat later in the course of the disease.

The gold standard of treatment for PD is administration of the dopamine precursor L-dihydroxyphenylalanine (levodopa, L-DOPA). Direct dopamine agonists have increasingly been used in the treatment of PD. Although levodopa is incredibly beneficial in treating the symptoms of PD, after 3-7 years patients develop on-off effects and abnormal involuntary movements (dyskinesias). Later in the course of PD, the full symptomatic responsive to L-DOPA treatment is decreased.

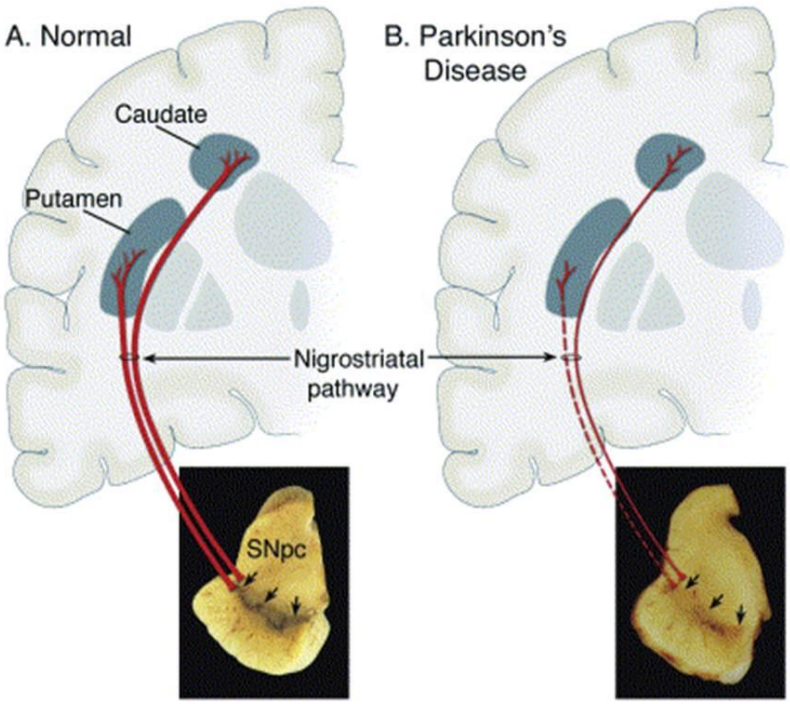

Figure 2. Dopaminergic innervation of the striatum (caudate and putamen). A.) Normal nigrostriatal innervation is schematized in red. B.) In Parkinson's disease the substantia nigra dopamine cells degenerate with a resultant loss of striatal dopamine levels illustrated by the hatched and thinned red lines). Image from *Dauer et al. 2003*.

Striatal cell morphology. Two major types of neurons are found in the striatum: medium-sized cells and large cells (interneurons). The cells of medium size are the projection neurons of the striatum. These cells were subsequently termed the medium spiny neurons (MSNs) by Kemp and Powell (1971), which are richly invested with dendritic spines. MSNs account for approximately 90-95% of all striatal neurons and utilize γ-aminobutyric acid (GABA) as their classical neurotransmitter (Gerfen 1992). As the name suggests, MSNs have a medium-sized soma (8-17 μm in diameter) possess dendrites that radially emanate and of which are densely studded with dendritic spines (see Figure 3), the sites of excitatory synapses.

The geometries of dendritic spines suggest that they are independent compartments that "protect" dendrites from sharp, rapid rises in intracellular calcium (Segal M 1993, 1995). Segal noted:

> "I should like to take this a step further, and propose a novel function for spines: by isolating the synapse from the dendrite, the spine protects the neurons from toxic insults associated with the raised [Ca^{2+}], that follows synaptic activity."

For example spines with large heads and thin necks sequester calcium in the spine head, whereas in spines with a low spine head: neck diameter ratio calcium may frequently invade the neck of the spine and the dendritic shaft (Sabatini et al. 2002; Noguchi et al. 2005).

As originally suggested by Vogt and Vogt (1920), there are several types of MSNs. Studies of Golgi-impregnated MSNs have revealed subtle differences in the location and density of dendritic spines on MSNs, with five different classes

of MSNs defined (Chang et al. 1982). By far the most common of MSNs are the so-called type I class, which possess aspiny proximal dendrites and somata with distal dendrites that are densely studded with dendritic spines. Type II MSNs differ in that their somata occasionally possess spines and their dendrites have significantly fewer spines compared to that of the type I class. Type III MSNs have less branched dendrites that are relatively aspiny and smooth. Type IV MSN somata are aspiny and have dendrites that branch repeatedly with a very sparse labeling of spines. Finally type V MSNs are similar to type IV in having aspiny somata, but differ in that the secondary dendrites branch significantly less and are very long (Chang et al. 1982). There have been no studies examining functional, genetic, or neurochemical differences between the five types of MSNs.

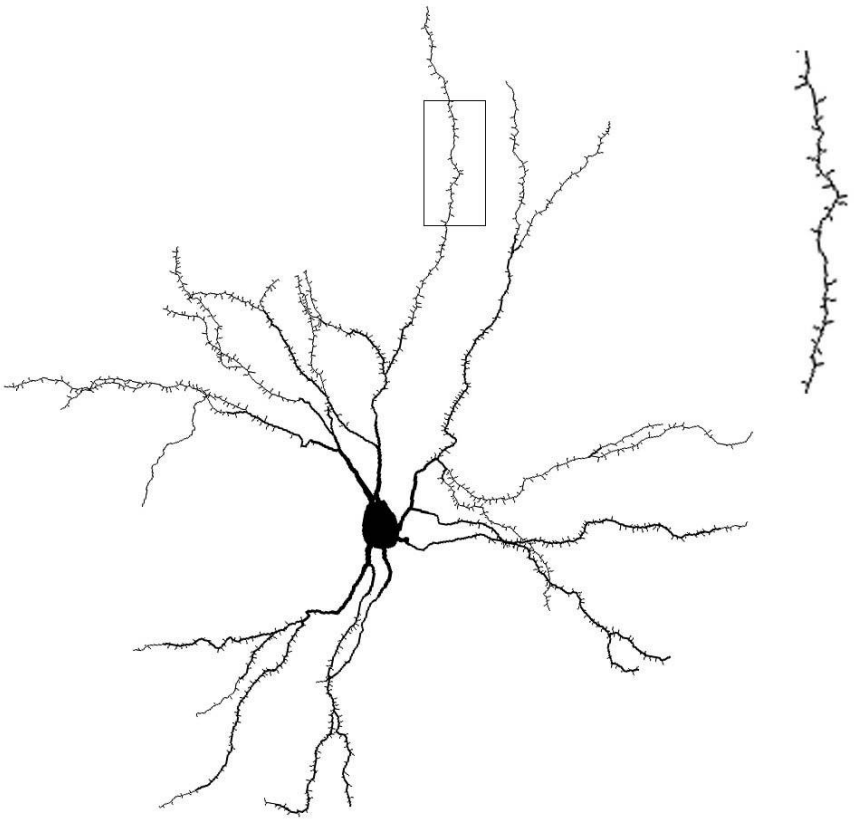

Figure 3. Medium spiny neuron in the dorsolateral striatum of a sham rat reconstructed using the computer software program Neurolucida. A magnified segment of dendrite is shown in the inset.

As noted above, most (80-84%) MSNs are type I (Kita and Kitai 1988). There are no data on physiological differences across the five morphologically defined MSNs. We will discuss MSNs as a single class.

Defining MSNs by non-morphological criteria. MSNs can also be defined on the basis of efferent projections, peptide content, and receptor expression. Two populations of MSNs can be defined on the basis of their projection targets: MSNs that project to the substantia nigra (SN) form the so-called "direct" pathway (striatonigral), while MSNs that contribute to the "indirect" pathway (striatopallidal) innervate the globus pallidus (GP). The name "indirect" pathway MSNs came about because these cells project to the GP and then the SN, unlike the direct pathway MSNs which project directly to the SN. Although it is clear that direct and indirect pathway neurons are functionally distinct (Gerfen et al. 1990; Gerfen 2000; Day et al. 2006), the strict segregation of direct and indirect pathway MSNs projecting to the SN and GP is an oversimplification: MSNs project to both SN and GP but with markedly different degrees of terminal arborization (Wu et al. 2000; Levesque and Parent 2005). For example, the direct pathway MSNs emit small axon collaterals to the GP that do not take up retrograde tract tracers efficiently, with the axon terminals in the SN being much more perfuse. These data help explain why retrograde tracer studies have found small percentages of MSNs that are retrogradely labeled from both the GP and SN. These target-defined MSNs can also be distinguished by the dopamine receptors and peptide co-transmitters they express: direct pathway MSNs

express the D_1 receptor and the peptide co-transmitter substance P, whereas indirect pathway MSNs express the D_2 receptor and enkephalin (Gerfen et al. 1990). *In situ* hybridization techniques have provided evidence for the lack of colocalization of D_1 and D_2 receptors on striatal MSNs (Gerfen et al. 1990; Le Moine et al. 1990, 1991). Evidence for co-localization of D_1 and D_2 receptor expression has also been shown using immunocytochemistry (Aizman et al. 2000), although conclusive data for D_2 receptor antibody specificity is lacking. Pharmacological treatment with D_1 or D_2 agents selectively change the abundance of peptide transmitters in direct and indirect MSNs, suggesting functional distinctions between these two types of cells (Pollack and Wooten 1992; Engber et al. 1992; Granata et al. 1996; Steiner and Gerfen 1999).

More recent data examining the physiological responses from D_1 versus D_2 receptor BAC transgenic mice suggest the presence of differences in MSN somatodendritic morphology as well as excitability. Direct pathway (striatonigral) MSNs possess more primary dendrites and as a result have greater total dendritic length as compared to indirect pathway (striatopallidal) MSNs. The resting membrane potential of striatonigral MSNs is also more hyperpolarized than that of the striatopallidal MSNs, which can be attributed to the increase in total dendritic length of D1 expressing MSNs (Gertler et al. 2008).

Interneurons. In addition to MSNs, the striatum contains several different classes of interneurons. Among these are cholinergic interneurons, calretinin-positive GABAergic interneurons, parvalbumin-positive GABAergic interneurons, and

GABAergic interneurons that express somatostatin, neuropeptide Y and nitric oxide synthase (Kawaguchi et al. 1995). The large aspiny cholinergic interneurons account for ~1-3% of striatal cells. These interneurons express D_2 receptors that regulate acetylcholine release (LeMoine et al. 1990), but also may express in very low abundance D1-like (D_5) receptors that potentiate $GABA_A$ receptors, resulting in enhanced hyperpolarization and slower discharge rates of these cells (Yan and Surmeier 1997; Bennett and Wilson 1998). Equal in number to the cholinergic interneurons are the somatostatin producing interneurons, and GABA-ergic interneurons. The GABA-ergic interneurons consist of 1) fast-spiking parvalbumin-positive, 2) low threshold spiking nitric oxide synthase-expressing, and 3) calretinin-positive interneurons. Interneurons clearly play important functions regulating MSNs. However, we have limited our studies to evaluating the corticostriatal regulation of MSN dendritic spines and will not discuss further interneurons.

Synaptic architecture of MSNs. Despite the above mentioned differences in MSNs, these cells have a simple characteristic synaptic architecture of afferents onto MSN dendritic spines. MSNs receive inputs from the cortex, the thalamus and the SN, all of which synapse primarily onto distal dendrites (Kemp and Powell 1971). In contrast, inputs to MSNs from axon collaterals of other MSNs and striatal interneurons tend to occur onto the soma or proximal dendrite (See Figure 4). Both the cortex and the thalamus provide potent sources of excitation (glutamate) onto MSN dendritic spines (Kemp and Powell 1971). Dopamine

synapses from the SN occur most frequently onto the neck of dendritic spines (72.1%, Smith et al. 1994), which also receive a glutamatergic input on their head (Smith et al. 1994). Both DA and ionotropic glutamate receptors are localized to MSN dendritic spines.

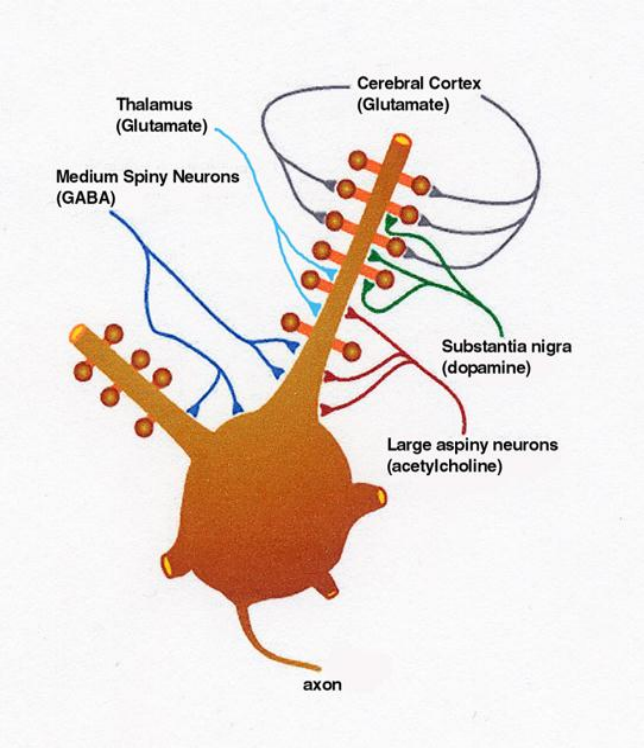

Figure 4. Medium spiny neuron afferents. Schematic illustrates the distribution of glutamatergic (from cortex and thalamus), dopaminergic (substantia nigra), and cholinergic (large aspiny interneurons) inputs to the MSN, as well as GABA-ergic afferents from recurrent axon collaterals of other MSNs. *Image from Mink 1999.*

MSNs possess two membrane states, an “up” state and a “down” state (Wilson & Kawaguchi 1996). During the up state, the membrane potential of MSNs is depolarized to -60 mV, which enables the generation of spikes. In the down state, the potential is -85 mV, and inactivation of the transient potassium channel current occurs and MSNs fire a short burst of action potentials. These up and down states are determined by potent convergent cortical inputs onto MSN dendrites. Dopamine overall modulates transitions between states. Specifically, D_1 receptors on direct MSNs increase depolarization by enhancing the opening of L-type calcium channels, which leads to the potentiation of excitatory effects (Hernandez-Lopez et al. 1997). Dopamine D_2 receptors on indirect pathway MSNs tonically inhibit the L-type calcium channel (Hernandez-Lopez et al. 2000; Day et al. 2006).

Regulation of medium spiny neurons. The dopamine innervation of the striatum arises from neurons in the SN and contributes to a characteristic synaptic triad involving SN and cortical afferents and the dendrites of striatal MSNs. The synaptic arrangement of these three elements typically involves a dopamine synapse with the neck of a MSN dendritic spine, and a corticostriatal terminal synapsing onto the spine head (Bouyer et al. 1984; Freund et al. 1984; Smith et al. 1994). This synaptic arrangement suggests that dopamine is in a position to modulate the influence of corticostriatal glutamatergic axons on MSNs (see Figure 5). Additionally there are data indicating that D2-like (but not D_4) heteroreceptors on the terminals of corticostriatal axons tonically inhibit release

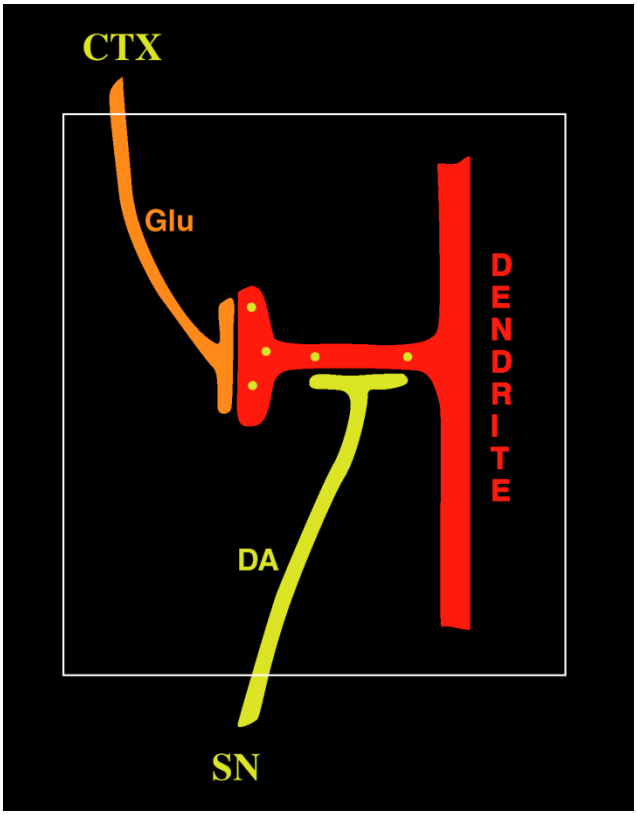

Figure 5. Schematic illustrating the medium spiny neuron synaptic arrangement in the striatum. Shown in red is an MSN dendritic spine receiving a cortical glutamatergic input onto the spine head (orange) and a substantia nigra dopaminergic input onto the spine neck (yellow).

of glutamate from these axons (Rubinstein et al. 2001; Bamford et al. 2004b; see Figure 6). Even if dopamine axons do not synapse onto spines directly, they are always present within 1.0 µm of dendritic spines (Arbuthnott and Wickens 2007), offering a potent paracrine (volume transmission) mode of regulation. Thus, in the dopamine-depleted striatum excess glutamatergic drive from corticostriatal terminals, coupled with other mechanisms intrinsic to the MSN that are normally regulated by dopamine (Day et al. 2006), contributes to hyperexcitable MSNs (Florio et al. 1993; Colwell and Levine 1996; Surmeier and Kitai 1997; Meshul et al. 1999; Cepeda et al. 2001; Canales et al. 2002).

The thalamus is the second major source of glutamatergic input to the striatum. Thalamostriatal terminals are found not only on dendritic spines, but also on the dendritic shaft. We will restrict this discussion to the effects of cortico-striatal interactions, although it is clear that other players contribute to MSN function and regulation.

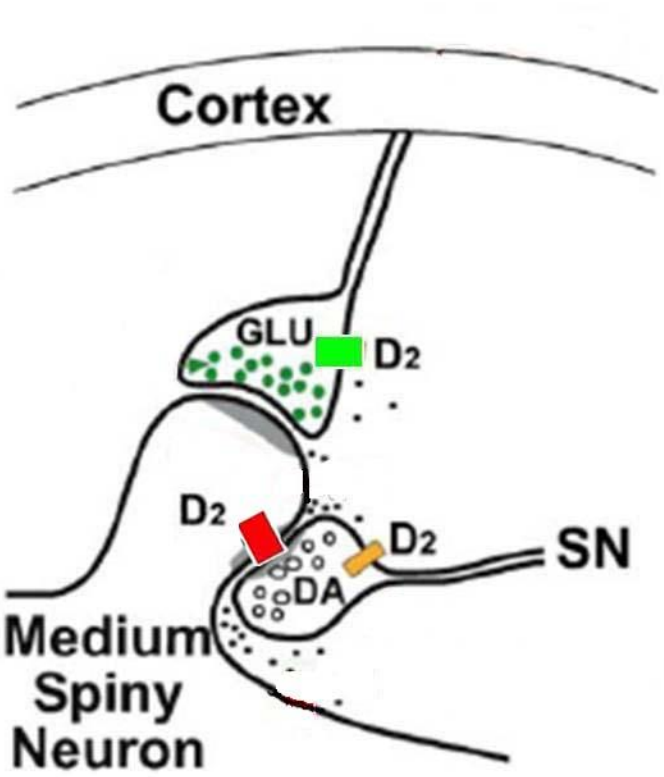

Figure 6. Dopamine D_2 receptor localization on the striatal triad. D_2 receptors are evident on the dendritic spine (post-synaptic), and on the corticostriatal and dopaminergic terminals (presynaptic). Image modified from *Bamford et al. 2004.*

The experiments in this dissertation set out to elucidate specifically whether cortical lesions that disrupt the excitatory corticostriatal innervation or pharmacological suppression of corticostriatal glutamate release attenuates MSN spine loss in the DA-denervated striatum in rats. Our studies began with elucidating the precise striatal region receiving innervation from the M1 motor cortex using neuroanatomical tract tracing techniques.

CHAPTER II

CORTICOSTRIATAL PROJECTION

In his drawings of the connections of the nervous system from Golgi-stained material, Cajal observed that certain pyramidal cells (PCs) in the cortex possess an axon that extends into the striatum. Anterograde degeneration methods such as the Marchi technique subsequently allowed other scientists to bolster the evidence for a cortico-striatal projection (Glees 1944; Carman et al 1965; Webster 1965). With later advances in neuroanatomical tract-tracing techniques, including the development of anterograde and retrograde tracers, more information on the corticostriatal projection became known (Wilson 1987; McGeorge and Faull 1987, 1989; Wright et al. 2001, and Reiner 2003).

The corticostriatal fibers from nearly the entire ipsilateral neocortex project in a highly organized manner onto all of the neostriatum. In all species examined the corticostriatal projection exhibits a distinct topographical organization in which the medial cortices project onto the more medial striatum (caudate nucleus) and the more lateral cortices preferentially innervate the more lateral striatum (putamen). In addition to the ipsilateral corticostriatal innervation, a modest striatal innervation arises from the contralateral cortex.

Striatal afferents from M1 cortex. "A pyramidal cell is not a pyramidal cell is not a pyramidal cell" (Deutch AY; contrarian misquote of Gertrude Stein's "A rose is a

rose is a rose is a rose"). Corticostriatal PCs are classified into 3 distinct groups based upon morphology, physiology and hodology. Intratelencephalic (IT-type), pyramidal tract (PT-type), and more recently the slow conducting ipsilateral (SCI) pyramidal neurons all contribute to the striatal innervation (Landry et al. 1984; Wilson 1987; Cowan and Wilson 1994; Ballion et al. 2008). IT-type neurons possess small perikarya approximately 12-15 µm in diameter and are primarily found in layers III and upper V; these PCs project bilaterally within the telencephalon, including to the basal ganglia and contralateral cortical sites (Levasque et al. 1996; Levasque and Parent 1998; Wright et al. 1999, 2001; Reiner et al. 2003). The PT-type neurons are larger, with perikarya of 15-20 µm in diameter, and are primarily located in deep layer V of the cortex. The main axon of the PT-type PC traverses the striatum, where it emits axon collaterals as the axon courses to the ipsilateral pyramidal tract (Wilson 1987; Cowan and Wilson 1994; Levesque et al. 1996; Reiner et al. 2003). The SCI neurons are a subset of PT-type cells that can be distinguished from the latter on the basis of the latency of their antidromic response (Ballion et al. 2008). The IT and PT-type cells have very similar discharge rates and spike waveforms (Cowan and Wilson 1994).

It is not known if IT and PT PCs target different types of striatal MSNs or even interneurons. Despite not being able to differentiate which PCs target MSNs, it is important to note that IT PCs send a contralateral projection to the striatum that is less dense than the PT PC ipsilateral projection. However to a

certain degree, one can distinguish PT from IT cells based upon their laminar organization as well as soma size.

In order to determine the location of the striatal territory that receives an input from the M1 motor cortex, the anterograde tract tracer biotinylated dextran amine (BDA) was deposited into the M1 region, and the distribution of labeled axons in the striatum was charted. In addition, a retrograde tract tracing study was performed using the retrograde tracer FluoroGold (FG) deposited into the dorsolateral striatum. These studies were conducted to ensure that we examined MSNs in the region of the striatum that receives inputs from the cortex that will be ablated in studies described in chapter IV.

Methods

Animals. Adult male Sprague-Dawley rats (Harlan; Indianapolis, IN), were group housed on a 12:12 light dark schedule with food and water freely available. All studies were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and under the oversight of the Vanderbilt University Animal Care and Use Committee.

BDA iotophoresis into the M1 motor cortex. Animals were anesthetized with isofluorane and secured in a stereotaxic frame. A 10% BDA solution (BDA; MW 10,000; Invitrogen, Eugene, OR) prepared in 0.1M sodium phosphate was loaded into fiber-filled glass pipettes (25-30 μm outer diameter) and was iontophoretically deposited into two sites in the M1 cortex (AP: +0.7; ML: +2.0,

+3.6; DV: -2.0, -2.3) using + 5.0 µA pulsed (7 sec on/off) current for 10 min for each site. BDA was deposited into two sites in the M1 motor cortex to produce a large enough deposit encompassing all or most of the M1 cortex that was lesioned in subsequent studies. Animals survived for 7-10 days before being sacrificed. The distribution of anterogradely-labeled axons in the striatum was then determined in four animals.

FG Iontophoresis into the dorsolateral striatum. A 4% FG solution prepared in 0.1M cacodyllic acid, was loaded into fiber-filled glass pipettes (25-30 µm OD) and deposited into the dorsolateral striatum by iontophoresis delivered at a current of +5.0 µA for 10 minutes (7 seconds on and off) (AP:+1.0, ML:+3.8, DV:-4.2). The animals were allowed to recover for 7 days before being sacrificed.

Immunohistochemistry. Animals were transcardially perfused with 0.1M sodium phosphate buffer followed by ice cold 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer. Brains were removed, postfixed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose solution at 4ºC. The tissue was then sectioned using a sliding microtome at 42 microns and processed for conventional immunoperoxidase or immunofluorescence (Bubser et al. 2005). BDA was visualized by immunofluorescence using a streptavidin antibody conjugated to the fluorescent protein Cy3 (1:1000, Jackson IR Labs, West Grove, PA) or immunoperoxidase using a streptavidin antibody conjugated to horseradish peroxidase (1:1600, Jackson IR labs, West Grove, PA). FG was

visualized by immunohistochemistry using a rabbit anti-FG antibody (1:3000; Chemicon,Temecula, CA).

Imaging data analysis. Microscopic images captured through a 20x 1.4 plan-Apo objective were acquired using a digital camera coupled to a computer running the cell reconstruction software Neurolucida (Microbrightfield Inc, Williston, VT). BDA-positive fibers and FG-positive cells were charted.

Results

Anterograde tract tracing. The anterograde tracer was deposited into two sites within the M1 motor cortex (see Figure 7) to accurately define the area ablated in the focal cortical lesion experiments described in chapter IV, with anterogradely-labeled fibers seen in the dorsocentral and dorsolateral precommissural striatum (see Figure 7 and 8). We found that the majority of the pyramidal cells in the motor cortex project to the ipsilateral dorsocentral/lateral striatum; a small but significant innervation of the contralateral striatum was also observed. We observed labeling of fiber bundles (pencils of Wilson) as well as thick and thin caliber axons that emanated from these fiber bundles. The cortical innervation of the M1 recipient zone in the dorsocentral/lateral striatum was most dense closest to the corpus callosum and became less dense as a function of distance from the white matter. The extent of anterogradely labeled fibers also gradually decreased as one moves caudally from the injection site.

Interestingly, we observed a somewhat different pattern of corticostriatal innervation contralateral to the BDA deposit compared to the ipsilateral striatum. There was a less extensive zone of innervation in the contralateral striatum that was shifted laterally compared to the ipsilateral striatal innervation seen. Specifically we observed a lateral and a ventral shift in the distribution of corticostriatal fibers in the contralateral striatum (see Figure 7). All analyses of MSNs was performed on the ipsilateral corticostriatal innervation.

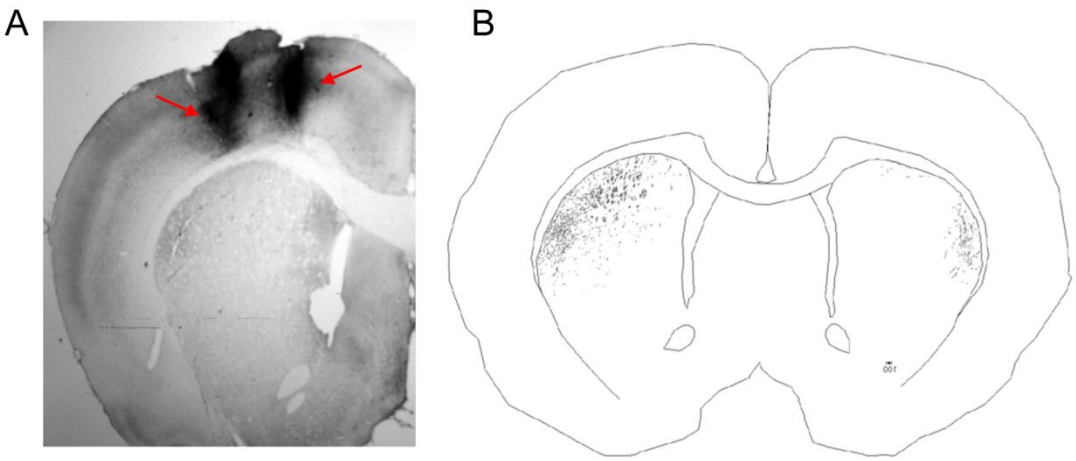

Figure 7. Corticostriatal innervation detected using immunoperoxidase staining of BDA. A.) The anterograde tracer BDA was deposited into the M1 motor cortex; denoted by red arrows (4x magnification). B.) Neurolucida charting illustrates the corticostriatal fiber distribution in dorsolateral striatum.

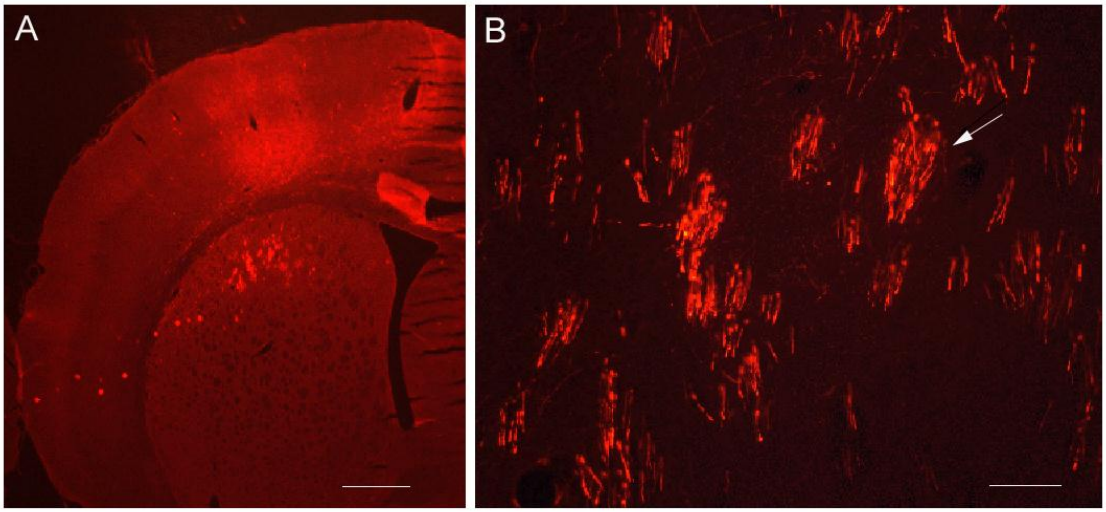

Figure 8. Corticostriatal innervation detected using immunofluorescence staining of BDA. A.) The anterograde tracer BDA was deposited into the M1 motor cortex and corticostriatal fibers are seen in the dorsolateral striatum (4x magnification). B.) A high-powered magnification of the dorsolateral striatum illustrating the anterogradely labeled cortical fibers (20x magnification). White arrow denotes myelinated corticostriatal axon bundle. Scale bars indicates A) 500 µm and B) 100 µm.

Retrograde tract tracing. In addition to the anterograde tracing of BDA-positive fibers in the striatum, we also used retrograde tract tracing to define the cortical origin of the dorsocentral/lateral striatum that was anterogradely labeled from the M1. The retrograde tracer FG was deposited into the striatum and FG-positive cells were charted in the cortex (see Figure 9). FG-positive cells were seen in the sensory-motor cortices both ipsilateral and contralateral to the deposit with the majority being ipsilateral to the deposit (see Figure 9). We observed FG-positive PCs in layers III and V ipsilateral to the iontophoretic deposit. This was slightly different in terms of the contralateral sensorimotor cortical innervation, in which we mainly observed layer V PCs. This is consistent with layers III and V containing IT-type PCs which possess bilateral projections to the dorsolateral striatum.

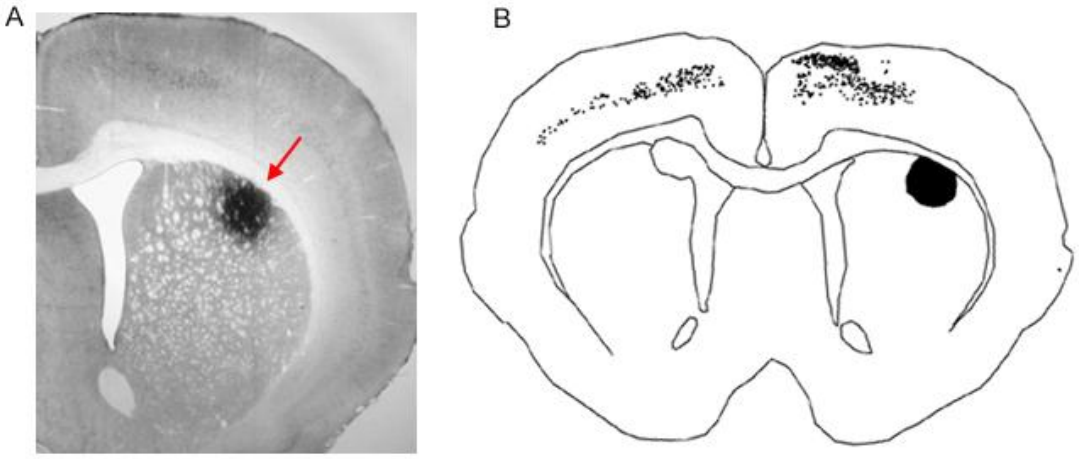

Figure 9. FG-positive cells in the cortex. A.) Photomicrograph illustrating the FG deposit in the striatum. Red arrow indicates the FG deposit site. B.) A Neurolucida charting illustrating the location of cortical cells as revealed using the retrograde tracer FG deposited into the dorsolateral striatum (black circle).

Discussion

The retrograde tracer experiments confirmed the anterograde data we obtained indicating the anatomical organization of a bilateral motor cortex projection to the striatum; these data confirmed previous tract tracing studies (Wilson 1987; McGeorge and Faull 1987, 1989; Wright et al. 2001, and Reiner 2003). Early studies using retrograde tracer deposits into the dorsocentral/lateral striatum reported retrogradely-labeled cells in the M1 and the primary sensory cortex (McGeorge and Faull 1987, 1989), with which our data was in agreement. Our anterograde tracer data were also comparable to previous reports of anterograde labeling from the motor cortex and adjacent cortical territories (Hoffer and Alloway 2001; Ramanathan et al. 2002; Alloway et al. 2006). These data indicated that one can reliably define the location in the striatum that receives inputs from a given zone of cortex, such as the M1 region, although the precise borders of the territory cannot be identified.

CHAPTER III

DOPAMINE DEPLETION-INDUCED DENDRITIC SPINE LOSS

The classic MSN synaptic arrangement described in the previous chapter involves the two major afferents to the striatum: nigral dopamine and cortical glutamatergic neurons terminating on MSN dendritic spines (Bouyer et al. 1984; Freund et al. 1984; Smith et al. 1994). This synaptic architecture suggests a role for dopaminergic modulation of cortical information flow onto MSN dendritic spines. There are a wide variety of structural, biochemical, physiological, and behavioral consequences that result from loss of the dopaminergic innervation of MSNs (Florio et al. 1993; Ingham et al. 1993; Colwell and Levine 1996; Surmeier and Kitai 1997; Meshul et al. 1999; Cepeda et al. 2001; Canales et al. 2002). We will focus on the structural changes that result from striatal DA denervation.

A loss of the modulatory DA influence at the spine neck results in calcium invasion into the dendritic shaft (Carter and Sabatini 2004). The geometries of the spine head and neck determines whether an electrical signal will reside within the spine or invade into the dendritic shaft and affect neuronal physiology (Holmes et al. 1990; Koch and Zador 1993; Svoboda et al. 1996). Dopamine can play a role in dictating both the rate and the duration that calcium accumulates within the spine. The dopamine input onto the spine neck can lead to activation of dopamine receptors that are present on spines, as well as DA heteroreceptors located on corticostriatal terminals, which tonically inhibit glutamate release

(Rubinstein et al. 2001; Bamford et al. 2004b). Thus, the loss of the dopaminergic innervation of MSNs may result in excessive glutamatergic drive and potentially a slow excitotoxic process leading to the eventual retraction of dendritic spines (Nitsch and Riesenberg 1995).

The first documentation of structural alterations in MSNs was from histological analysis of brain tissue from postmortem PD patients, in which the dendritic tree appeared truncated with a noticeable loss of spines (McNeill et al. 1988). Subsequent direct experimental confirmation using a rodent model of parkinsonism suggested that the spine loss observed in PD patients occurred secondary to dopamine denervation of the striatum (Ingham et al. 1989). Dendritic spine loss occurs all along the dendrite (Ingham et al. 1993). The loss of dendritic spines is not immediate, but becomes apparent around 12 days after striatal DA denervation, and persists thereafter (Ingham et al. 1993). The magnitude of the decrease in spine loss ranges from 20-30% when examining all MSNs in rodents (Ingham et al. 1993; Day et al. 2006), while almost 50% spine loss is observed when examining specific subpopulations of MSNs (striatopallidal cells) (Day et al. 2006). Dopamine depletion of the striatum not only causes a loss of spines, but differentially affects subsets of spine types, specifically resulting in a decrease in the percentage of thin spines and an increase in the percentage of stubby spines (Neely et al. 2007).

Dopamine denervation of the striatum results in a significant decrease in MSN dendritic spine density. What is the temporal evolution of MSN spine loss? The experiments discussed below examine this issue.

Methods

Experimental design. Striatal DA depletion was accomplished by means of 6-OHDA injections into the SN and DA denervation was verified via TH immunohistochemistry as well as by western blot assessment of TH immunoreactivity in total homogenates of the dorsolateral striatum. Dendritic spine density and complexity was evaluated in DA-depleted and sham-lesioned animals using Golgi impregnation.

6-OHDA-induced striatal dopamine depletion in rats. Sprague-Dawley male rats (Harlan, Indianapolis, IN) ~90 days of age were group housed with food and water *ad libitum*. Animals were anesthetized with isofluorane. The rats were then unilaterally injected with 6-OHDA (4 ug/uL free base in 0.02% ascorbate) into two sites in the SN (AP: -5.3, L: 1.0 and 2.7, DV: -8.3, relative to bregma), with the lateral site receiving 1.0 µL and the medial site receiving 1.5 µL. Each injection was delivered over a 10 minute period using a peristaltic pump-mounted syringe coupled to a 30 g injection cannula via PE tubing. A sham lesion involved dropping the syringe for 2 minutes into the SN in the control rats. A control group was included in all the time points examined. Groups consisted of six to eight rats per group, and animals were allowed to recover for 5, 12, or 21 days before being sacrificed.

Golgi-Cox impregnation of striatal MSNs. Rats were deeply anesthetized with isofluorane, sacrificed, and brains removed from the skull. The tissue was then

sectioned at 150 µm on a vibratome and the sections were immersed in 1% osmium tetroxide in water (EM Sciences, Hatfield, PA) for 30 minutes, followed by incubation in 3.5% potassium dichromate in water for 12 hours at room temperature. After completion of the impregnation step, sections were then "sandwiched" between two glass slides and immersed in 1% silver nitrate solution for 4 hours protected from light. Sections were then washed several times in 0.1 M phosphate buffer (pH 7.4) and mounted on 0.5% gelatin coated microscope slides. Sections were then dehydrated, cleared in xylene and coverslipped.

Dendritic measurements. Randomly selected Golgi-impregnated MSNs in the dorsolateral striatum were reconstructed using the Neurolucida system (Microbrightfield, Inc.), incorporating images obtained at 600x and digitally magnified to 1200.

In a series of preliminary studies we examined Golgi-impregnated MSNs to determine dendritic spine density at different points along the dendritic tree in adult rats with an intact dopaminergic innervation. Although the primary dendrite had a low density of spines, spine density increases sharply and was statistically stable at distances of 60-130 µm distal to the soma (see Figure 11). Spine density was significantly lower at distances greater than 180 µm, although this may reflect difficulties in silver impregnation of the most distal dendrites. In addition, we also performed a Sholl analysis comparing dendritic spine densities in sham-lesioned animals. We observed that spine density did not correlate with

distance from the soma (see Figure 10). We therefore subsequently assessed spine density on 10-20 µm long dendritic segments located at distances 60-130 µm from the soma.

Assessment of striatal DA depletion. Sections through the striatum and SN were processed for tyrosine hydroxylase (TH) immunohistochemistry using a mouse ant-TH antibody (1:3000; ImmunoStar, Inc., Hudson, WI) following our previously described method (Bubser et al. 2005). Only rats with < 3 TH- immunoreactive axon segments per high powered (40x) field of the dorsal striatum were included in the analyses; SN dopamine neurons were almost entirely lost.

Tissue preparation. Samples of dorsolateral striatum were punch dissected from 1.0 mm thick coronal slices with an extra-thin wall stainless steel 19 g punch (~ 0.89 mm OD) for subsequent immunoblot analysis. The small amount of tissue prevented us from preparing synaptosomal preparations without pooling samples, and we therefore performed analyses on whole tissue homogenates. Elapsed time between isofluorane anesthetization of the animal and flash freezing of the dissected samples was <3 minutes. Striatal samples were homogenized in 300 µL 2% SDS with 10 ug/mL leupeptin and 1 ug/mL pepstatin. Protein concentrations were assessed by the Lowry method (Lowry et al. 1951).

Western blot assessment of protein levels. Protein samples from total homogenates (20–40 µg protein per lane) were loaded onto SDS-PAGE gels,

followed by transferred onto nitrocellulose membranes, stained with Ponceau-S (Sigma, St. Louis, MO), and then digitally scanned. After blocking, membranes were probed with mouse anti-tyrosine hydroxylase (Immunostar) and mouse anti-GAPDH (for use as a loading control) (Chemicon). Membranes were then washed and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase-conjugated secondary antibodies were detected with enhanced chemiluminescence (Brown et al. 2005). Specific immunoblot signals were quantified from films exposed in the linear range. The protein band densities in each lane were normalized to GAPDH.

Data analysis. All data were expressed on a per cell basis, after values from 5-9 cells were averaged. A power analysis revealed that in order to achieve 83% power at a Type I error rate of 0.05, the reconstruction of 6 cells per animal was required to detect changes in dendritic spine density.

An unpaired t-test was performed to detect differences in the 6-OHDA lesioned group compared to the respective time point control group. The nonparametric Kruskal Wallis ANOVA was used to assess statistical differences among spine types.

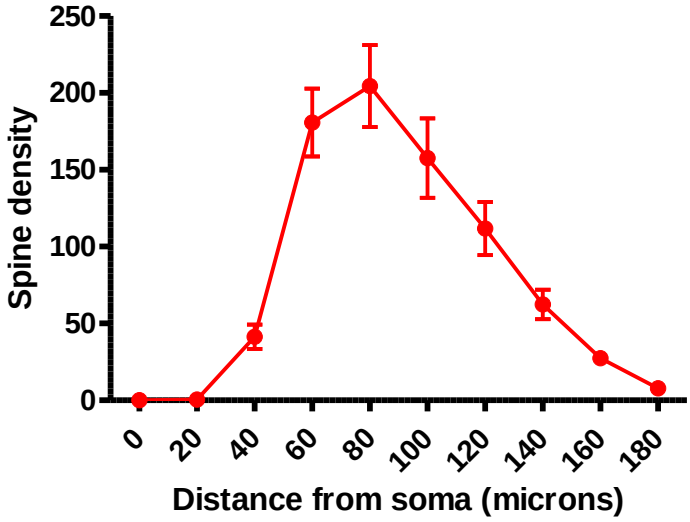

Figure 10. Sholl analysis of spine density on dendrites as a function of distance from the soma. Full reconstructions of MSNs were subjected to a Sholl analysis using Neurolucida explorer.

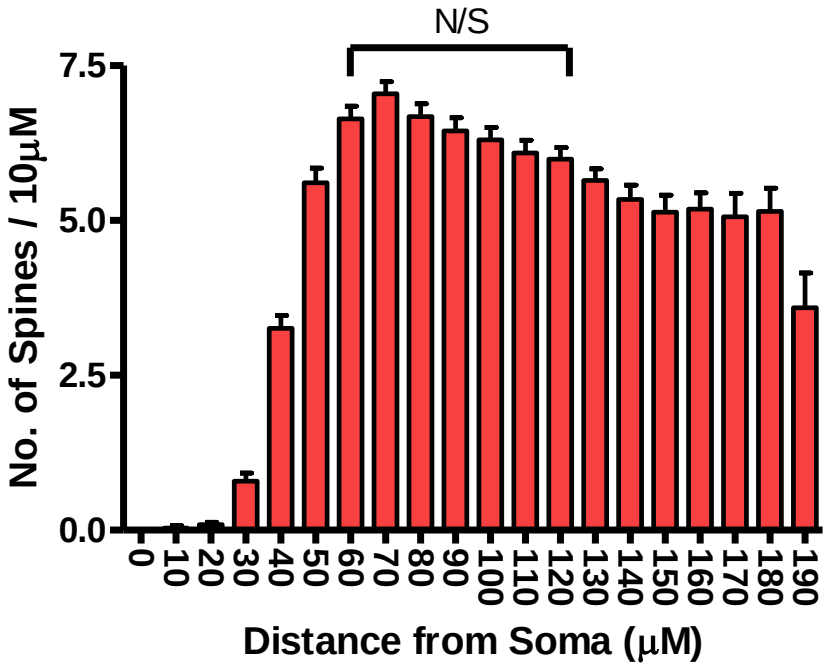

Figure 11. Medium spiny neuron dendritic spine density as a function of distance from soma in the adult rat. In the proximal dendrites spines are relatively sparse. At a distance of 60-120 µm there was no significant difference in spine densities from the previous 10 µm segment of dendrite. Spine densities at distances greater than 180 µm are significant lower because one cannot reliably obtain many longer dendrites due to cutting sections at 150 µm.

Results

Extent of striatal dopamine depletion. To evaluate the extent of the 6-OHDA SN lesions we assessed the amount of the DA biosynthetic enzyme, TH, in both the striatum and the SN by immunohistochemistry. SN lesions produced a near complete elimination of the DA innervation of the striatum. Figure 12 illustrates the amount of striatal (panel A) and SN (panel B) TH-immunoreactivity ipsilateral to the SN lesion and in the control contralateral hemisphere. We also assessed TH-immunoreactivity in the dorsolateral striatum of control and 6-OHDA lesioned animals by western blot. Tyrosine hydroxylase levels were found to be depleted by 91% in animals lesioned with 6-OHDA as compared to control animals and is shown in Figure 13.

Dendritic spine changes. Representative Golgi-impregnated MSNs are depicted in the photomicrographs shown in Figure 14. Visually one can appreciate fewer spines in DA-denervated striatum relative to the control MSN dendrite.

MSN dendritic spine density was not significantly changed 5 days post-operatively (Figure 15), but at 12 days after the lesion a significant but modest 8% decrease in spine density was seen (t_{63}=2.020, p=0.048). We found the spine loss to be progressive and at 21 days post-lesion observed a 16-18% decrease in MSN dendritic spine density compared to the sham-lesioned (t_{215}=8.445, p<0.0001). DA denervation-induced spine loss persists and is progressive, as illustrated by the 28% decrease in MSN dendritic spine density one year following 6-OHDA lesion of the SN (see Figure 16).

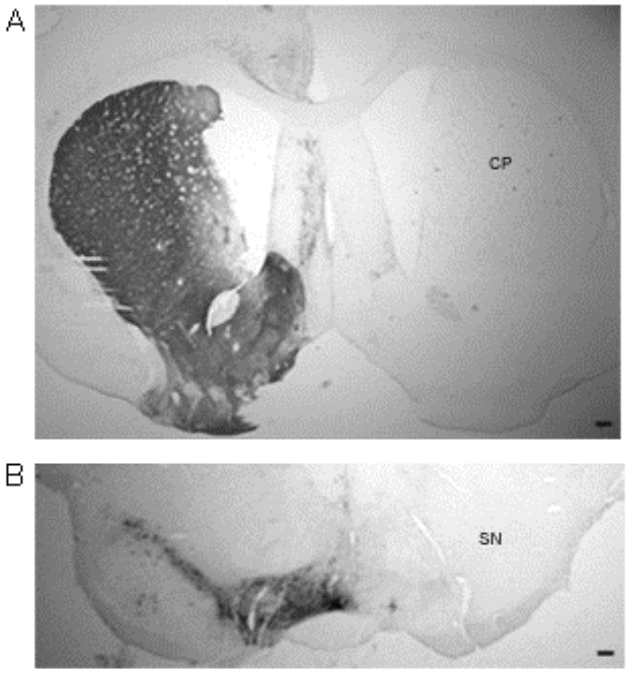

Figure 12. Tyrosine hydroxylase-like immunoreactivity in the striatum (CP; panel A) and substantia nigra (SN; panel B) of an animal with a unilateral 6-OHDA lesion of the right substantia nigra. Scale bars, 100 µm.

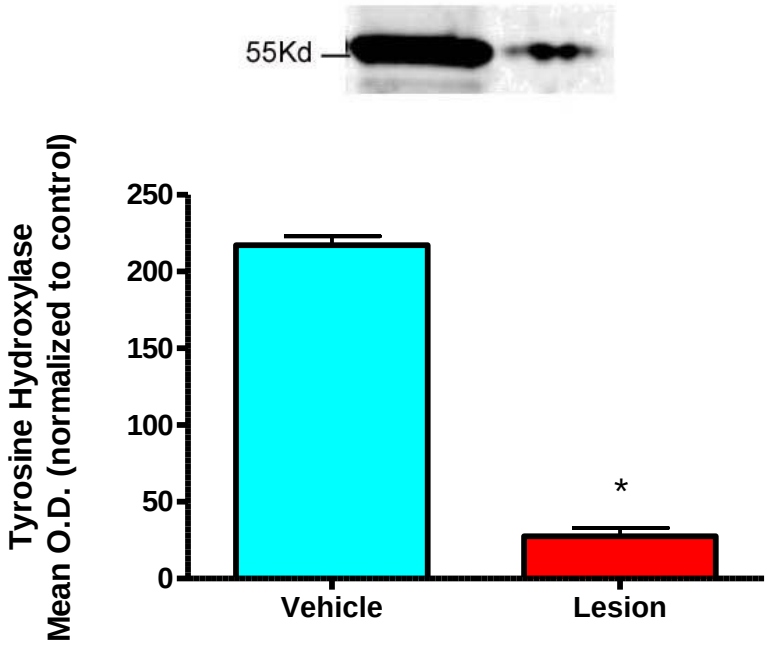

Figure 13. Tyrosine hydroxylase immunoreactivity in the dorsolateral striatum of animals with a unilateral 6-OHDA lesion of substantia nigra or sham lesions (vehicle). A representative immunoblot is illustrated above the quantification. The first band is from animals with a sham lesion and the second lane being from animals receiving 6-OHDA SN lesion. Groups consisted on 4-6 animals. An un-paired t-test was used to compare the lesioned group to its respective age-matched control.
* $p < 0.0001$

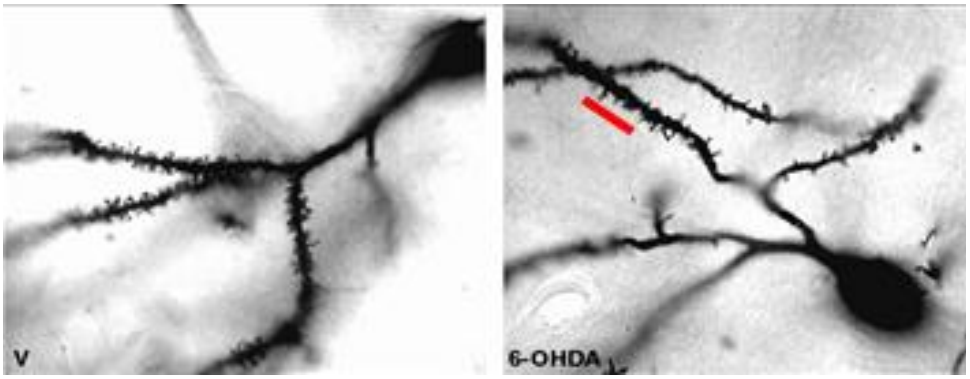

Figure 14. Golgi impregnated MSNs from the intact (left panel, v) and dopamine depleted (right panel, 6-OHDA) striata. One can readily appreciate the difference in spine number on MSNs between lesion and intact animals. The scale bar is 10 µm.

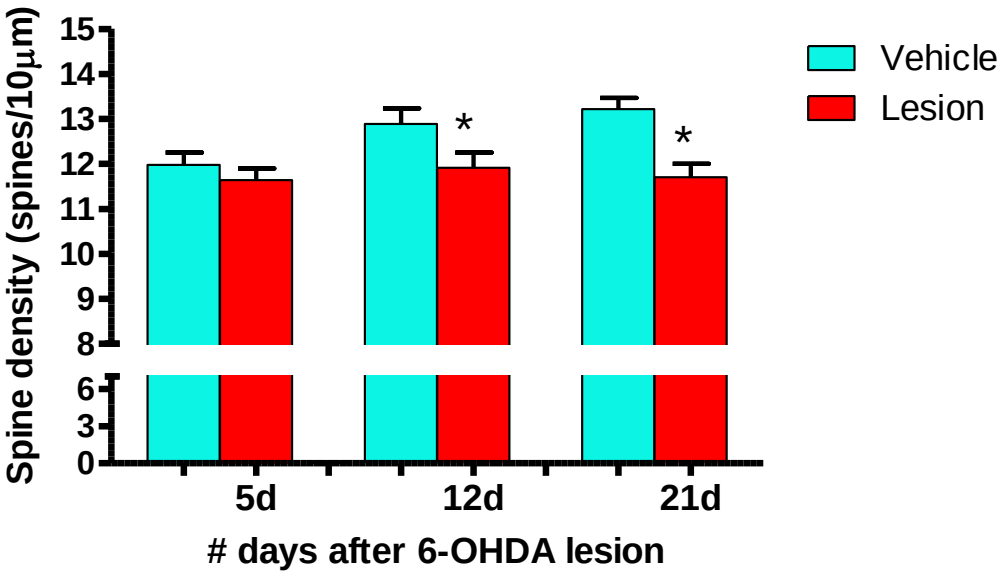

Figure 15. Striatal DA depletion results in decreased MSN dendritic spine density. Lesioned animals were compared to age-matched control animals (vehicle). Significant decreases in spine density were observed at 12 and 21 days post-lesion. Groups consisted on 6-8 animals. An unpaired t-test was used to compare the lesioned group to its respective age-matched control.
* $p < 0.05$ (corrected for multiple comparisons)

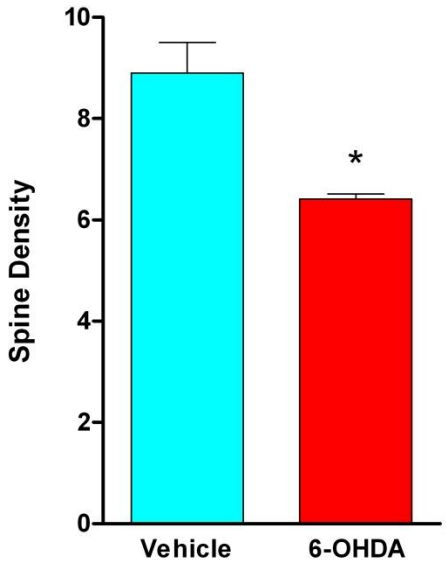

Figure 16. Dendritic spine loss is progressive and persists for at least a year. 3 month old animals received 6-OHDA lesions of the substantia nigra and spine density assessed 12 months later. Note that spine density is significantly lower than that depicted in Figure 11. Groups consisted on 6-8 animals. An un-paired t-test was used to compare the lesioned group to the age-matched control.
* $p < 0.001$

Despite a significant decrease in dendritic spine density seen at 12 and 21 d after the 6-OHDA lesions, there was no significant change in the length of the longest MSN dendrite (see Figure 17).

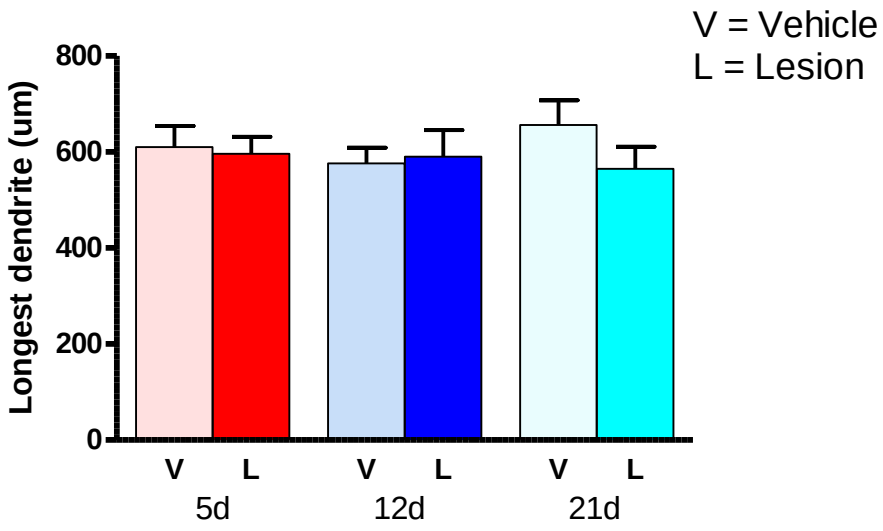

Figure 17. No significant difference observed in the longest dendrite measured at 5, 12 and 21 days post 6-OHDA SN lesions as compared to the control counterparts. Groups consisted on 6-8 animals.

Changes in different morphological types of spines. We observed that ~65% of MSN dendritic spines in adult control animals were of the thin type, followed by stubby spines accounting for 30%, and <5% of MSN spines being mushroom-shaped. Filopodia, defined as spine-like processes >4.5 μm in length, were almost never seen in the adult striatum. MSN dendritic spine types are illustrated in Figure 18.

We uncovered a small but significant shift in the proportions of the three mature spine types present on MSNs in the dopamine-denervated striatum, with significantly fewer thin spines (59%; $H = 13.00$, $p = .0046$) and a somewhat greater percentage of stubby spines (38%; $H = 8.45$, $p = .0376$). The proportion of mushroom-shaped spines did not differ significantly in animals with striatal dopamine denervation relative to sham-lesioned control animals (see Figure 19).

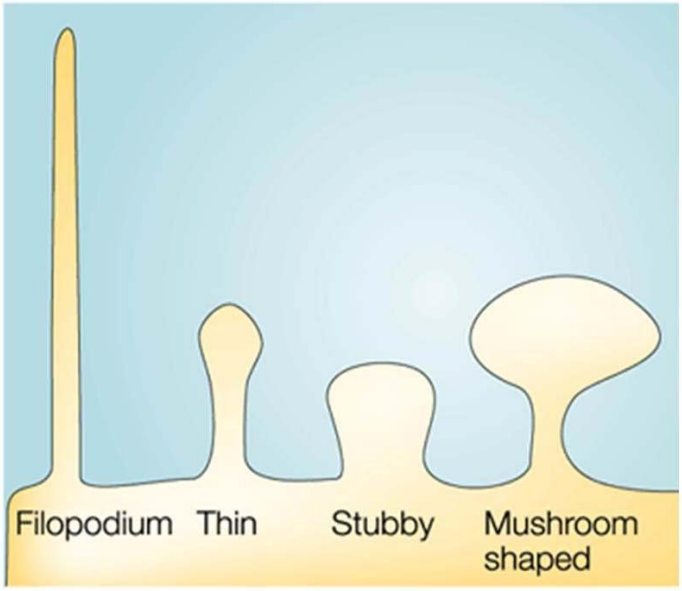

Figure 18. Schematic illustrating the morphologies of dendritic spine types. Filopodia are immature spines that are > 4 µm in length. Based on their shapes, mature dendritic spines can be categorized as thin-, stubby-, or mushroom-shaped. Image from *Hering et al. 2001.*

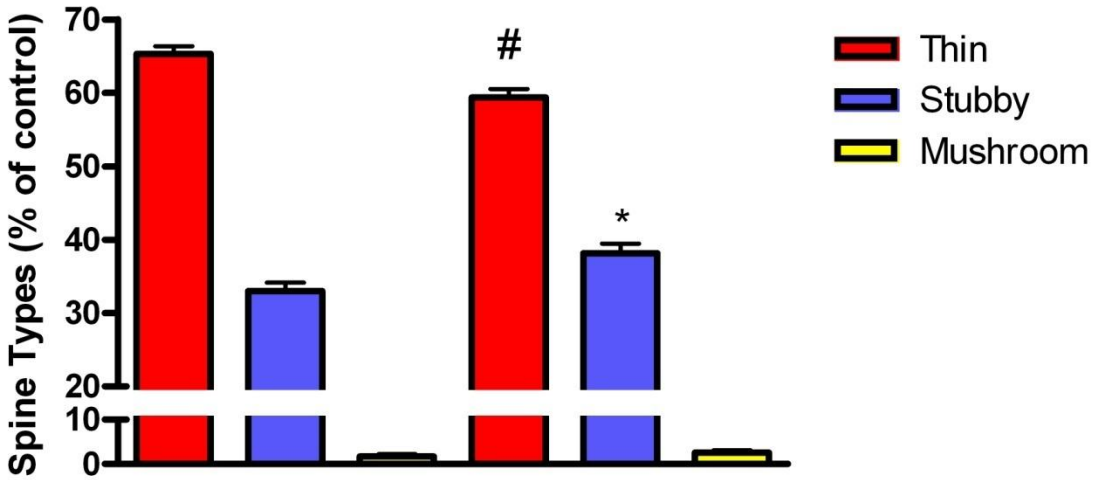

Figure 19. Changes in the proportions of different classes of dendritic spines in animals with cortical lesions and striatal dopamine depletion. Striatal dopamine depletion resulted in a loss of thin spines and an increase in stubby spines. Note that the data are plotted as a percent of total spines, and therefore does not illustrate the changes in spine density.

— — — — — —

Groups consisted on 6-8 animals.

* $p < 0.01$ for dopamine-depleted relative to thin spines in control subjects

* $p < 0.01$ for dopamine-depleted relative to stubby spines in control subjects

Discussion

Our results confirm and extend previous findings that MSN dendritic spine loss has a relatively slow onset after striatal DA depletion. However, once established, the spine changes are progressive and the spine loss is stable for at least up to a year.

Several lines of evidence indicate that the loss of striatal DA is the cause of MSN dendritic spine loss (Ingham et al. 1989, 1993; Meredith et al. 1995; Day et al. 2006; Neely et al. 2007). These include the response to 6-OHDA lesions of the SN (Ingham et al. 1989, 1993; Deutch 2006) and the observation that chronic D_2 receptor antagonists also result in MSN spine loss (Kelley et al. 1997). Moreover reserpine depletion of catecholamine stores decreases the density of dendritic spines (Day et al. 2006). Medium spiny neuron dendritic spine loss occurs in the MPTP mouse models of parkinsonism and in D_2, but not D_1 receptor null mice (Villalba et al. 2009; Deutch 2007). Because the extent of striatal DA depletion in the MPTP model is considerably less than the near total loss seen in the 6-OHDA model, it appears that complete disruption of the striatal DA innervation is not required for spine loss. However, it is not clear what degree of DA denervation is required before spine loss is seen. Studies evaluating the effects of DA denervation on specific subpopulations of MSNs (using transgenic GFP-expressing DA receptor mice) have shown that MSN spine loss is restricted to striatopallidal (D_2-expressing) cells, but not striatonigral (D_1-expressing) cells (Day et al. 2006). However, a recent study in non-human primates found that MPTP treatment resulted in loss of spines on direct pathway MSNs as well as

presumptive indirect pathway MSNs (Villalba et al. 2009). It is not clear if the observation of spine loss occurring on both subsets of MSNs is because of the extended survival of MPTP treated primates (for up to 18 months), or simply reflects a species difference. Interestingly, in a postmortem study of MSNs in idiopathic PD, we found that spine loss was much greater than seen in the animal models of parkinsonism (Zaja-Milatovic et al. 2005). Once again, it is not clear whether the spine loss occurring on both populations of MSNs is due to a species difference or because these patients survived for an extended period of time. Nonetheless, the extent of spine loss in idiopathic PD is so large that it is reasonable to speculate that dendritic spine loss is not restricted to the indirect pathway MSNs (Zaja-Milatovic et al. 2005).

MSN spine loss was seen at 12 days or longer after DA depletion. We found in animals that survived for 12 months after 6-OHDA (i.e., the animals were 15 months old at the time of sacrifice), that sham-lesioned animals suffered a significant loss of spines relative to control animals that were 3 months old. However, the degree of spine loss seen in these older animals referenced to control is the same as that seen in younger animals, suggesting that MSN dendritic spine loss is progressive. Because the spine loss is progressive, following spine loss in animal models of parkinsonism may be useful as a metric for studying progression in PD.

Dendritic spines. Dendritic spines are remarkably plastic structures, changing in number and shape over time scales ranging from seconds to years (Crick 1982;

McKinney 2005; Alvarez et al. 2007; Harms and Dunaevsky 2007; Chen et al. 2009). Long-lasting changes in dendritic spine number have been documented in a number of neuropsychiatric disorders (Ferrante et al. 1991; Kaufmann and Moser 2000; Hill et al. 2006; Kalivas 2009; Tackenberg et al. 2009). Cajal first thought that dendritic spines existed to increase the surface area of neurons. Later dendritic spines were hypothesized to serve as a defense mechanism and likened to the spines found on plants and animals, which play a role in protection. Spines are the sites of synaptic input and offer isolated compartments for the sharp rises in calcium that drive specific signaling cascades that would otherwise be toxic to the cell (Segal 1993, 1995).

Postmortem PD studies have reported that DA depletion not only causes a loss of MSN dendritic spines, but also decreases the length of the dendritic arbor of MSNs (Stephens et al. 2005; Zaja-Milatovic et al. 2005). We did not observe a significant decrease in the length of the longest dendrite examined three weeks following 6-OHDA lesions. However Solis and colleagues (2007) reported a decrease in dendritic length at 4 weeks post-operatively in Wistar rats. Solis et al. (2007) also administered 8 mg/kg methamphetamine two weeks prior to sacrifice in order to assess lesion extent by rotational behavior. Because methamphetamine has been reported to cause a neurotoxic loss of nigrostriatal DA terminals, it is not clear if the reported decrease in dendritic length is due to methamphetamine treatment or DA depletion. Future studies will be required to determine if and when the dendritic length changes in rodent models of parkinsonism.

We did not evaluate the structural differences in striatopallidal versus striatonigral MSNs. Future experiments examining specific populations of MSNs in response to DA deafferentation will be critical.

Post-synaptic glutamatergic signaling. Although the primary cause of dendritic remodeling is the loss of DA signaling through the D_2 receptor (Day et al. 2006; Deutch et al. 2007), it appears likely that changes in cortically-derived glutamate contributes to the changes in MSN spines. Dopamine replacement therapy (L-DOPA) in PD patients or in animals with striatal dopamine depletion does not restore spine loss (Stephens et al. 2005; Zaja-Milatovic et al. 2005; Deutch et al. 2007), suggesting that after a period of time the dopamine receptor is uncoupled from its intracellular effectors. This led us to hypothesize that directly manipulating corticostriatal glutamate release might reverse the MSN spine loss seen in the dopamine-denervated striatum.

Glutamatergic mechanisms are critically involved in determining both dendritic spine development and maintenance (Korkortian and Segal 2000; Passafaro et al. 2003; Lippman and Dunaevsky 2005; McKinney et al. 2005; Bloodgood and Sabatini 2007). For example, glutamatergic signaling through NMDA receptors increases intra-spinous calcium levels, which determines spine morphology (Segal 2003). These considerations suggest that corticostriatal neurons play a central role in determining the structure of MSN dendrites. The increase in glutamatergic drive onto MSNs in the DA-denervated striatum results in changes in expression of glutamate receptors that are localized to the

MSN spine, as well as scaffolding proteins associated with the receptors (Weihmuller et al. 1992; Fitzgerald et al. 1995; Dunah et al. 2000; Brown et al. 2005; Hallett et al. 2005; Bayer et al. 2006). For example a decrease in NMDA receptor NR1 and NR2B subunits occurs in response to striatal DA denervation (Dunah et al. 2000; Brown et al. 2005). The decrease in NR2B is accompanied by an increase in tyrosine-phosphorylated NR2B, apparently as a compensatory mechanism to inhibit the binding of CAMKII and allow for a slow dissociation of preformed CAMKII-NR2B complexes (Chase and Oh 2000). Striatal DA depletion is also accompanied by changes in phosphorylation of glutamate receptors, including phospho-Ser^{831}-GluR1 suggesting changes in trafficking (Brown et al. 2005).

Clinical relevance. Several studies have reported that relatively late in the course of PD, a decreased responsiveness of motor symptoms to DA replacement therapy occurs (Marsden and Parkes 1977; Rinne 1981; Clissold et al. 2006). In newly-diagnosed patients with PD, including those who have been treated with levodopa or DA agonists, there is an increase in striatal D_2 binding potential (Rinne 1981; Kaasinen et al. 2000), consistent with the extensive (~70%) loss of striatal DA before motor symptoms appear (Hornykiewics and Kish 1987). However, in advanced PD somewhat lower D_2 binding densities are seen than in newly diagnosed patients. This may be due to levodopa treatment or may be secondary to the loss of dendritic spines, on which DA receptors reside. Dendritic spine loss once established by striatal DA denervation, does

not respond to DA replacement therapy (Stephens et al. 2005; Zaja-Milatovic et al. 2005), suggesting that to effectively treat late stage PD some intervention in addition to DA replacement is needed.

CHAPTER IV

DECORTICATION ATTENUATES DENDRITIC SPINE LOSS

Introduction

A recent *in vitro* study that examined the role of corticostriatal projections in dendritic remodeling in the dopamine-denervated striatum reported that complete decortication prevents the development of spine loss on striatal MSNs in organotypic slice co-cultures (Neely et al. 2007). However, the ability of decortication to *reverse* spine loss that has already been established, which may be more relevant to treatment of PD, has not been examined in these cultures nor *in vivo*. We therefore determined if decortication can reverse or prevent MSN dendritic spine loss *in vivo*.

Methods

Animals. Adult male Sprague-Dawley rats (Harlan; Indianapolis, IN), were group housed on a 12:12 light dark schedule with food and water freely available. All studies were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and under the oversight of the Vanderbilt University Animal Care and Use Committee.

Experimental design. We first determined if focal lesions of the motor cortex *in vivo* could *reverse* the spine loss that occurred in response to striatal DA

denervation. In the next experiment we determined if cortical lesions could *prevent* the development of MSN spine loss.

In the *reversal* experiment 6-OHDA lesions of nigrostriatal DA neurons were made, and then four weeks later, when spine loss was present, the motor cortex was lesioned with ibotenic acid (IA). Animals survived for four weeks after the cortical lesions before being sacrificed.

In the *prevention* experiment striatal DA depletion was also accomplished by means of 6-OHDA lesions, but during the same surgery IA was used to lesion the motor cortex. Animals were sacrificed four weeks later.

Surgical manipulations. Animals were anesthetized with isofluorane and lesions of the motor cortex were made by injecting 1.0 µL of 45 nM ibotenic acid (Tocris; Ellisville, MO) into the M1 cortex (AP: +0.7; ML: +2.0, +3.6; DV: -2.0, -2.3) at a rate of 200 nL/min. Control (sham) cortical lesions involved incision of the skin and placement of a burr hole.

Striatal DA denervation in the same animals was accomplished by injecting 6-OHDA HBr (4.0 µg/µL free base; Sigma-Aldrich; St. Louis, MO) into two sites in the SN (AP: -5.4, ML: +1.0, +2.4, DV: -8.4) in a volume of 1.5 µL (lateral injection site) and 1.0 µl (medial injection site) at a rate of 100 nl/min.

Golgi impregnation. Animals were transcardially perfused with 0.1 M phosphate buffer followed by an ice cold solution of 2.5% glutaraldehyde (EM Sciences; Hatfield, PA) and 2% paraformaldehyde (VWR; West Chester, PA) in 0.1M

phosphate buffer (pH 7.45). Brains were removed and the forebrains postfixed for 3 hours. Coronal sections (150 μm) were cut on a vibrating microtome. The sections were then incubated in 1% osmium tetroxide (EM Sciences) for 40 min, after which sections were transferred to 3.5% potassium dichromate (Sigma-Aldrich) for 16 hours in a humid chamber. The sections were then "sandwiched" between glass slides and incubated in the dark in 1% silver nitrate (Sigma-Aldrich) for 4-6 hours. Sections were washed in water, mounted on 0.5% gelatin-coated slides, dehydrated, cleared, and coverslipped with DPX (Sigma-Aldrich).

Dendritic analyses of Golgi-labeled MSNs. Microscopic images were acquired by a digital camera coupled to a computer running the cell reconstruction software Neurolucida (Microbrightfield Inc, Williston, VT), using a 60x 1.4 plan Apo objective with a 10x ocular. The image was digitally magnified by a factor of 2 to yield a final magnification of 1200x.

Data from animals with cortical lesions that impinged on the corpus callosum were excluded from subsequent analyses, as were data from animals in which the lesions did not involve layer V, where the majority of cells that innervate the striatum are located.

Golgi-impregnated MSNs in dorsolateral striatum were reconstructed by a person unaware of the treatment conditions of the animals. Neurons were randomly selected from the M1 recipient zone of dorsolateral striatum, provided that the cells had a soma diameter of 12-17 μm and through a 10x objective appeared to be well impregnated. Dendritic spine density was measured on

dendritic segments 10-20 μm in length that were located 60-120 μm distal to the MSN soma.

Immunohistochemistry. Animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Forebrain and midbrain blocks of tissue were postfixed overnight and then cryoprotected in 30% sucrose for 2-4 days. Coronal sections (42 μm) were cut on a freezing microtome.

To assess the extent of cortical IA or SN 6-OHDA lesions, free-floating brain sections were processed as described previously (see Bubser et al. 2005), using mouse anti-NeuN (1:1000; Chemicon, Temecula, CA) to reveal the borders of the cortical lesions, and mouse anti-TH (1:3000; ImmunoStar, Inc.) to stain dopaminergic neurons.

FluoroJade C staining. In order to determine if the cortical lesions resulted in any overt transsynaptic cell loss in the striatum, animals received IA injections of the motor cortex, and were sacrificed at various times between 2 and 28 days after the cortical lesions. The brains were processed to stain degenerating neurons using Fluorojade C (FJC; Schmued et al. 2005). Animals were perfused with 4% paraformaldehyde and sections cut through the forebrain, mounted on 0.5% gelatin-coated slides, and dried overnight. Slides were then incubated in a basic ethanol solution (1% NaOH in 80% ethanol), dehydrated for two minutes in 70% ethanol, and then incubated in 0.06% potassium permanganate for 10 minutes. Finally slides were incubated in 0.001% FJC (Chemicon) for 10 minutes, rinsed

three times in water, dried overnight, and dehydrated and cleared in xylene before being coverslipped with DPX.

Anterograde tract tracing. Collateral sprouting from remaining cortical cells could mask an effect of the cortical lesion on MSN spine loss *in vivo*. In order to determine if there is significant sprouting of cortical cells in response to M1 cortex lesions, we subjected rats to M1 lesions and four weeks later iontophoretically deposited the anterograde tracer BDA into the non-lesioned (contralateral) M1 cortex. Methods are as previously described in chapter II.

Data analysis. Dendritic spine densities were determined on branches of four primary dendrites from each reconstructed neuron, with at least five MSNs assessed in each animal (an average of 7.7 cells analyzed for each group in the prevention study, and an average of 10.0 MSNs for each group analyzed in the reversal experiment). We analyzed MSNs located in the striatal zone that receives inputs from the lesioned motor cortex, as well in a second region located ventromedial to the M1-innervated sector that does not receive significant M1 inputs (see Figure 20).

Average spine densities for each cell were collapsed to yield a mean MSN spine density. In turn, these mean “per cell” spine densities were collapsed to generate a mean MSN value for each animal. This latter “per animal” MSN spine density value was used for subsequent statistical analyses by means of two-way ANOVAs and subsequent Bonferroni t-tests if warranted by significant main

effects or a significant interaction. The degree of MSN spine loss in the non-M1-recipient zone of the striatum was analyzed relative to the 6-OHDA plus decortication group separately.

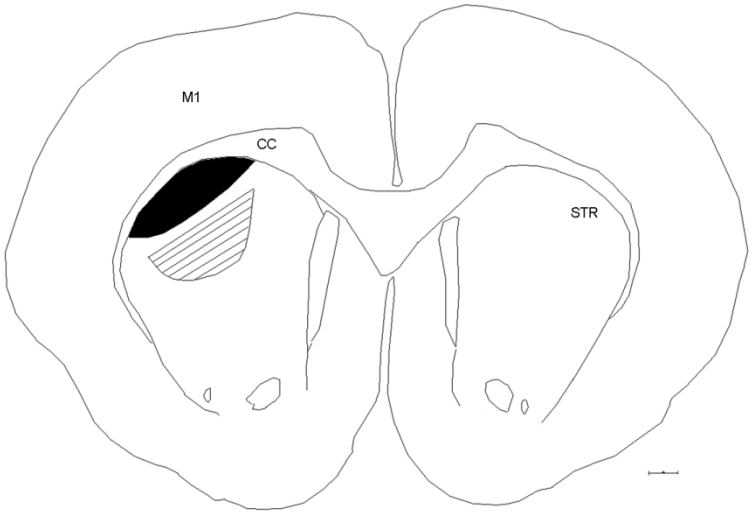

Figure 20. MSNs were analyzed in the striatal region that receives afferents from the M1 cortex (black). In addition we also analyzed MSNs in a region that does not receive a significant input from the M1 cortex as an internal control site (hatched). CC, corpus callosum, STR, striatum. Scale bar, 500 μm.

Results

Characterization of M1 motor cortex lesions. Ibotenic acid injections lesioned the M1 motor cortex (see Figure 18); the lesions impinged medially on the M2 area, with some degree of lateral invasion into the forelimb region of the primary somatosensory cortex (see Figure 21). Although the lesions sometimes involved areas adjacent to M1, for simplicity sake we refer to the lesioned region as the M1 area. The IA injections did not result in cavitation, and NeuN staining revealed intact underlying tissue (see Figure 21). In most cases the lesion involved layers I-VI, although loss of cells in layer VI was variable.

FluoroJade C staining. We also determined if IA lesions of the motor cortex led to any overt degeneration of striatal MSNs, using FJC. Unilateral IA lesions of the M1 cortex were placed and animals sacrificed 3, 7, 14, or 21 days later. We observed that ibotenic acid injections caused extensive neuronal loss in the vicinity of the IA injection, as reflected by a dense aggregation of cortical FJC-positive cells seen at two and four days postoperatively (see Figure 22). FJC accumulation in the myelinated bundles of corticostriatal axons was seen ipsilateral to the lesion, but very few degenerating axons were seen in the contralateral striatum (see Figure 22). At no time point up to 28 days after the ibotenic acid injection did we observe any FJC-positive cells in the striatum of animals with M1 lesions, consistent with a lack of transsynaptic degeneration of striatal neurons.

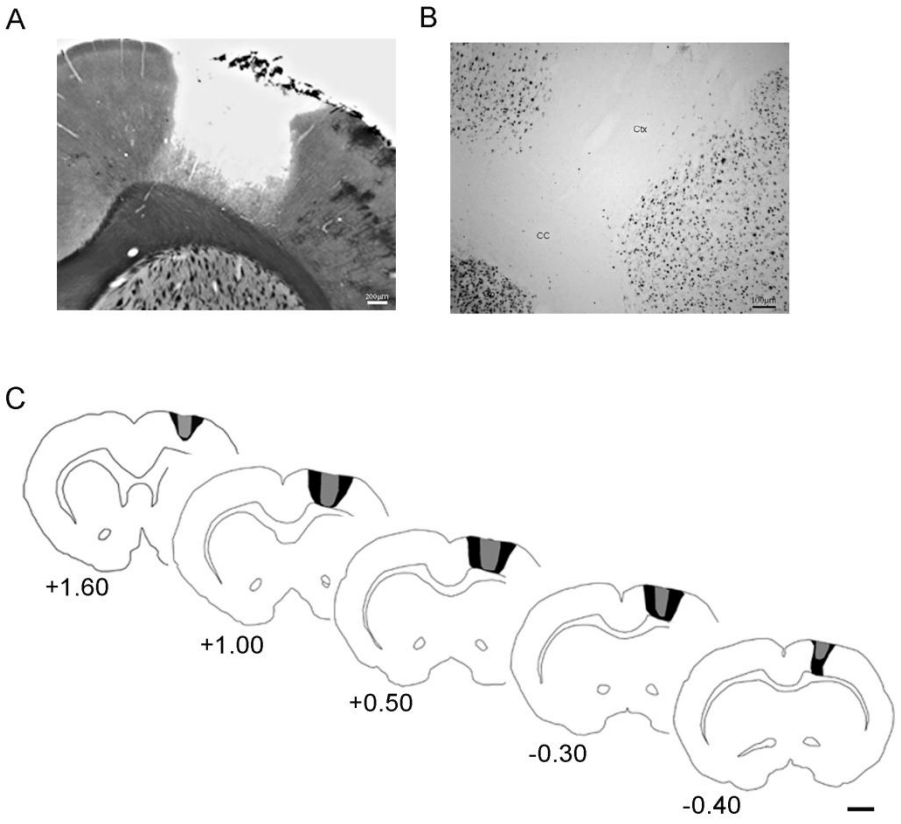

Figure 21. Characterization of focal cortical lesions. (A) A representative 150 µm coronal section illustrating the cortical lesion, which in this case spanned layers I-V. (B) The absence of NeuN-positive cells illustrates the loss of cortical neurons in the lesioned area and shows that the underlying tissue is intact. Reconstructions of the largest (black) and smallest (gray) cortical lesions as assessed by the loss of NeuN-like immunoreactive neurons is shown in panel C. Numbers refer to distance from the bregma skull suture (Paxinos and Watson 2007). Scale bars in panel A; 200 µm, panel B; 100 µm, and panel C; 500 µm.

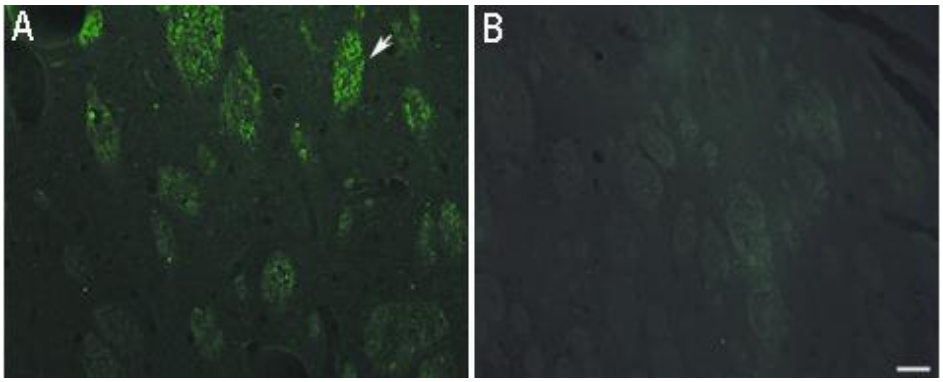

Figure 22. No Fluorojade C-positive (degenerating) MSNs were seen in the striatum after cortical ibotenic acid lesion. Arrow denotes FJC-positive fiber bundle. Although we did not see any degenerating (FJC-positive) MSNs, we did observe FJC staining of fascicles of corticostriatal axons in the striatum, consistent with degeneration of the corticostriatal innervation. This staining was observed predominantly on the side of the cortical lesion (panel A), with few stained fascicles seen contralaterally (panel B), and was most intense in animals sacrificed four days after IA injections. Groups consisted of 3-4 animals. Scale bar, 50 μm.

Cortical lesions reverse dopamine depletion-induced spine loss. In this experiment we assessed if decortication could reverse MSN spine loss that was caused by 6-OHDA lesions performed four weeks earlier. Cortical lesions significantly attenuated but did not totally reverse established MSN spine loss in the M1 recipient zone (omnibus ANOVA $F_{3,21} = 7.98$, $p = .001$), with a significant main effect of dopamine depletion ($F_{1,21} = 20.2$, $p = .0002$) but no significant interaction uncovered. Post-hoc analyses revealed that striatal dopamine depletion resulted in a significant decrease in spine density (17.9%) compared to that seen in sham-lesioned control animals ($p = .007$); cortical lesions alone did not change MSN spine density relative to control (sham-lesioned) animals (Figure 23). However, M1 lesions placed four weeks after striatal dopamine denervation significantly attenuated the degree of MSN spine loss compared to the 6-OHDA-lesioned group alone ($p = .008$), with a 9.4% decrease in spine density relative to sham-lesioned rats. Thus, animals with striatal dopamine depletion suffered a loss of dendritic spines that was almost 50% less than that seen in animals without cortical lesions. The cortical lesions significantly attenuated MSN spine loss only in the M1-recipient zone of the striatum, and not in the ventromedially adjacent striatal sector that does not receive significant M1 inputs (see Figure 23). Representative dendritic segments are illustrated in the photomicrographs shown in Figure 24.

Effects of concurrent cortical and 6-OHDA lesions on MSN spine density. The omnibus ANOVA for the analysis of the effects of cortical lesions performed at

the same time as 6-OHDA lesions was significant ($F_{3,27} = 9.37$, $p = .0002$), with a significant main effect of dopamine depletion ($F_{1,27} = 23.88$, $p < .0001$) but no significant cortical lesion x 6-OHDA lesion interaction. Post-hoc tests revealed that striatal dopamine depletion in animals with an intact cortex significantly decreased MSN spine density in the M1-recipient zone of animals relative to control rats (18.6%, $p = .002$). In contrast, spine loss in animals with both cortical and 6-OHDA lesions averaged 10.3%. Although spine loss was attenuated in the combined cortical + 6-OHDA lesions relative to the 6-OHDA-lesioned group alone, this effect did not reach statistical significance ($p = .06$) (see Figure 25). In the non-M1 recipient zone of the striatum there was no attenuation of spine loss (see Figure 25).

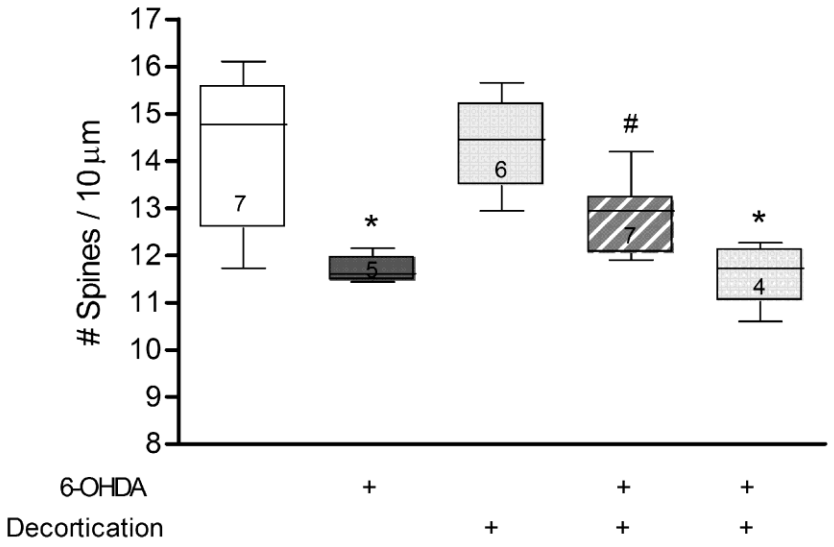

Figure 23. Cortical lesions significantly reverse dopamine depletion-induced MSN spine loss. Cortical lesions attenuated spine loss only in the M1 recipient zone (hatched bar) and not an adjacent territory (stippled bar). Numbers inside each bar indicate the number of animals/group. A two-way ANOVA was used to assess differences amongst the groups.

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* $p < 0.01$ relative to control animals

# $p < 0.001$ relative to 6-OHDA-lesioned animals with intact cortex

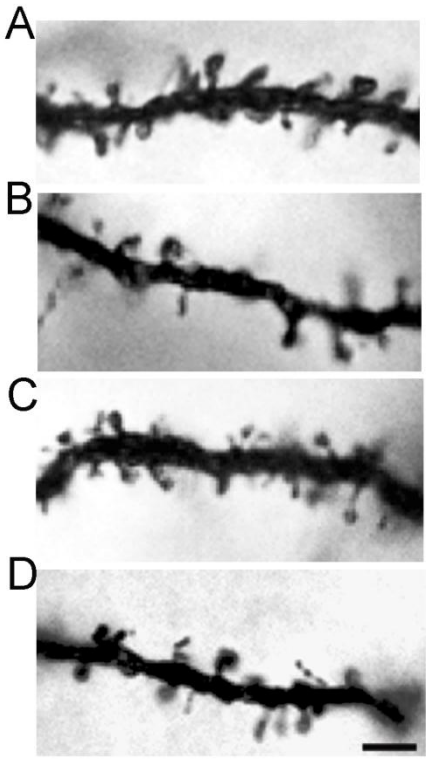

Figure 24. Photomicrographs of representative Golgi-impregnated MSN dendritic segments are shown for A) control; B) 6-OHDA-lesioned; C) cortically-lesioned; and D) 6-OHDA- plus cortically-lesioned animals. Scale bar, 4 μm.

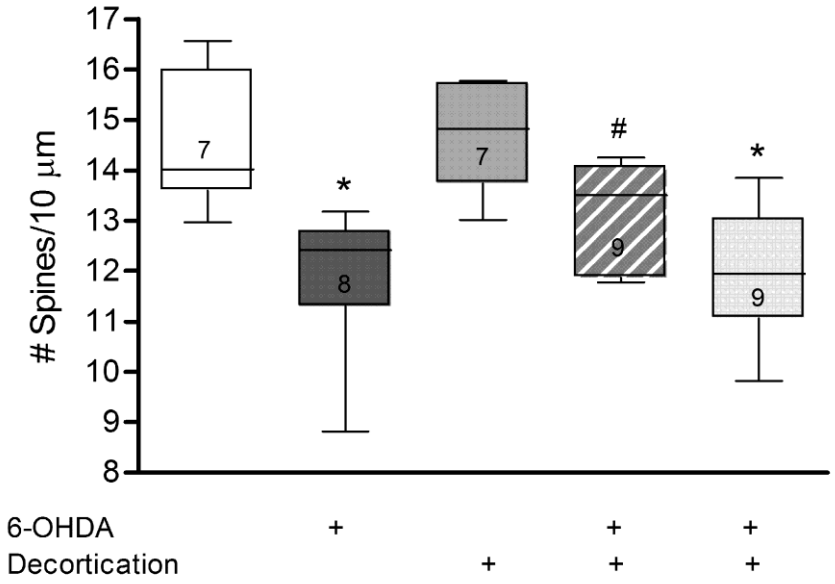

Figure 25. Cortical lesions performed at the same time as 6-OHDA lesions of nigrostriatal dopamine neurons attenuate MSN spine loss. Cortical lesions attenuated dopamine depletion-induced spine loss only in the M1 recipient zone (hatched bar) and not in an adjacent region of the striatum that does not receive inputs from the motor cortex (stippled bar). Numbers inside each bar indicate the number of animals/group. A two-way ANOVA was used to assess differences amongst the groups.

— — — — — — — — —

* $p < 0.01$ relative to control animals
# $p = 0.06$ relative to 6-OHDA-lesioned animals with intact cortex

Compensatory corticostriatal sprouting. BDA deposited into the M1 cortex revealed corticostriatal fibers in the dorsolateral striatum, predominantly ipsilateral to the deposit in control animals. However in addition we observed a small but significant innervation of the contralateral striatum (see Figure 26). Animals receiving M1 cortical lesions revealed a similar ipsilateral striatal innervation, but there appeared to be an expansion of the contralateral corticostriatal projection (Figure 26).

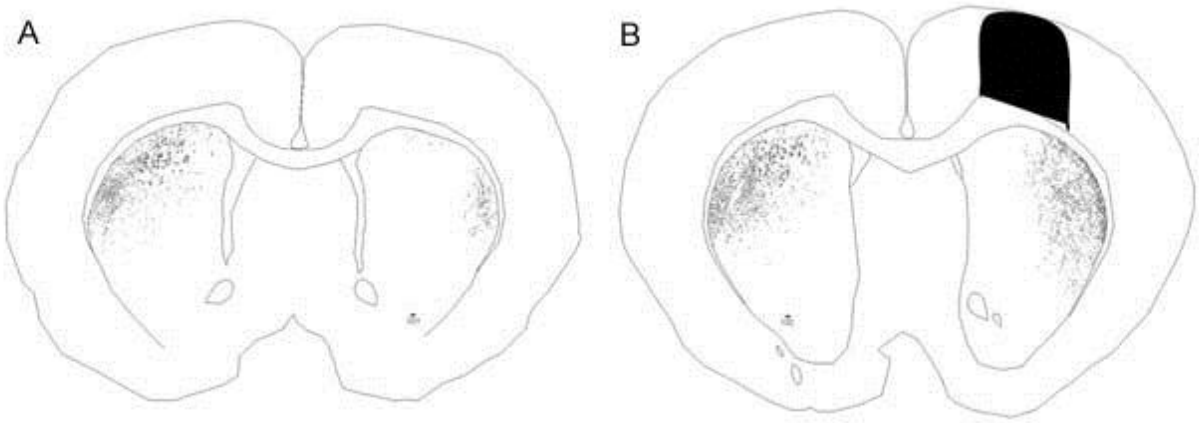

Figure 26. Corticostriatal sprouting following M1 focal lesions. BDA was deposited into the M1 cortex (unlesioned contralateral cortex) and BDA-positive fibers were charted in the striatum of A.) sham-lesioned animal and B.) an animal who received a focal cortical lesion (illustrated in black).

Discussion

Lesions of the motor cortex reversed the loss of MSN spines that occur after striatal dopamine denervation. This observation provides the first evidence that MSN spine loss, once established, can be reversed.

Effect of cortical lesions on MSN spines in the intact striatum. We did not observe any effect of the cortical lesions on MSN spine density in animals with an intact striatal dopamine innervation. In contrast, some previous studies have reported that cortical lesions decrease MSN spine density (Kemp and Powell 1971; Cheng et al. 1997). However, these earlier studies examined the effects of cortical aspiration lesions, which can easily damage the underlying striatal tissue, either by direct extension or secondary to edema. Moreover, Cheng and colleagues (1997) noted that the decrease in MSN spine density was transient, being maximal at 10 days postoperatively and returning to baseline levels by 20 days after the lesion, consistent with resolution of edematous changes. Because we examined animals at four weeks after the cortical lesion, we cannot exclude the possibility that there was a transient decrease in spine density in animals with IA cortical lesions that resolved by 28 days post-operatively. However, because dopamine denervation-induced MSN spine loss persists for at least one year (Ingham et al. 1989), the reversal of spine loss that we observed after cortical lesions cannot be due to recovery from the dopamine depletion.

To minimize the possibility of striatal damage by cortical lesions, we used IA to lesion the cortex. Although excitotoxic lesions can cause distant neuronal

loss, we did not observe FJC-positive MSNs at any time up to 28 days post-lesion. Moreover, we found that spine density was unchanged in animals subjected to cortical IA lesions four weeks previously, suggesting that the cortical lesion did not compromise the structural integrity of MSNs.

Effect of cortical lesions on dendritic spines in the dopamine-denervated striatum**.** A previous study in organotypic slice co-cultures found that complete ablation of the cortex performed at the same time as MPP^+ treatment completely prevents MSN spine loss (Neely et al. 2007). In our *in vivo* prevention experiment we observed a strong trend that did not reach statistical significance ($p = .06$). Because the *in vitro* study of Neely and colleagues (2007) indicated that total decortication completely prevented the development of dendritic spine loss in the dopamine-denervated striatum, and the fact that cortical lesions reverse existing spine loss, it is likely that the non-significant trend we observed in animals with focal cortical lesions represents a type II (false negative) error.

We therefore conducted both *in vivo* and *in vitro* studies, with the former examining the effects of a cortical lesion that left intact glutamatergic projections from the thalamus and the contralateral cortex, both of which synapse onto dendritic spines (Freund et al. 1984; Smith et al. 1994; Lacey et al. 2007). Despite not eliminating all glutamatergic inputs, we found that focal cortical lesions *in vivo* significantly reversed dopamine denervation-induced MSN spine loss and tended to prevent the development of spine loss. We suspect that we observed a significant but incomplete reversal of spine loss because of the

presence of some of the remaining glutamatergic inputs after the cortical lesion (corticostriatal sprouting) (Napieralaski et al. 1996; Hughes-Davis et al. 2005).

The vesicular glutamate transporter 2 (VGluT2) is abundantly expressed by thalamostriatal but not corticostriatal neurons (Kaneko and Fujiyama 2002). Raju et al. (2008) found no change in striatal VGluT2 in dopamine-denervated MPTP-treated primates, consistent with intact striatal glutamate inputs from the thalamus. Thus it is unlikely that the attenuation and not complete reversal was due to the thalamus.

Although we performed focal lesions of the M1 cortex there may be residual cells adjacent to the lesion site or in the contralateral cortex that provide glutamatergic inputs to MSNs. These considerations suggest that our studies with unilateral cortical lesions represent a conservative test of the hypothesis that cortical denervation prevents and reverses spine loss *in vivo*. The attenuation, but not complete reversal, of spine loss may be secondary to the presence of surviving ipsilateral corticostriatal neurons that innervate regions immediately adjacent to the denervated sector, or because of sprouting of neurons from the contralateral cortex as we have demonstrated.

Because there are no clearly defined boundaries that distinguish the M1-recipient zone from adjacent striatal tissue that does not receive M1 inputs, we restricted our analysis to a fairly conservative definition of the striatal M1-recipient zone, based on our anterograde tracer studies as well as published data on the striatal projections of the motor and adjacent cortices (Alloway et al. 2006). The cortical lesion attenuated the loss of dendritic spines only in this M1-

recipient zone of the striatum. Spine densities of MSNs located in an area of the striatum ventromedial to the M1-innervated region did not differ significantly from those seen in animals with 6-OHDA lesions but an intact motor cortex. These observations argue that we were able to define accurately the striatal territory receiving inputs from the lesioned cortex.

Methodological limitations of focal cortical lesions. By performing cortical lesions, we partially removed not only the glutamatergic innervation of striatum but also other molecules present in cortical neurons that might play a role in regulating dendritic spine density, such as neurotrophic factors. In order to delineate the effects of cortical factors versus a decrease in corticostriatal glutamatergic neurotransmission, we next examined the effects of mGluR 2/3 agonists, which dampen presynaptic glutamate release, on the effects of DA depletion-induced spine loss.

CHAPTER V

MODULATION OF CORTICAL GLUTAMATE PREVENTS SPINE LOSS

Metabotropic glutamate receptor subtypes. Metabotropic glutamate receptors (mGluR) are seven transmembrane G-protein coupled receptors. The mGluR subtypes are classified into three groups based upon sequence homology, coupling to second messenger systems, and their pharmacological properties (Conn and Pin 1997). Group I metabotropic glutamate receptors include mGluR 1/5 subtypes and are coupled to phosphoinositide hydrolysis. Group II mGluRs contain mGluR 2/3 receptors and are negatively coupled to adenylate cyclase. These group II mGluRs are thought to act as autoreceptors regulating the amount of glutamate released (Lovinger and McCool 1995; Shigemoto et al. 1997; Cartmell and Schoepp 2000). In addition group II mGluRs also inhibit glial glutamate release (Winder and Conn 1996). Finally mGluR 4,6,7, and 8 comprise group III mGluRs, which are also negatively coupled to adenylate cyclase and differ in their sequence homology from group II and their affinities for various mGluR ligands .

Metabotropic glutamate receptor distribution. Immunohistochemical studies have revealed dense mGluR1 staining in the globus pallidus, SN, hippocampus, and cerebellum; lower levels are detected in the cortex and striatum (Berger et al. 2001). Intense immunoreactivity for mGluR5 has been observed in the olfactory

bulb, cortex, striatum, lateral septum, and hippocampus (Romano et al. 1995; Gubellini et al. 2004).

Immunohistochemical studies have detected mGluR2 in the olfactory bulb, cerebral cortex, striatum, hippocampus, and the thalamus (Ohishi et al. 1998). The group II mGluR 3 mRNA is also widely expressed in rodent brain, with prominent expression observed in the cerebral cortex, dentate gyrus, olfactory nucleus, reticular thalamic nucleus, and a few hypothalamic and brainstem nuclei (Ohishi et al. 1993). mGluR 3 is localized to both neurons as well as astrocytes (Ohishi et al. 1993). There is moderate expression of mGluR 3 mRNA in the basal ganglia (Ohishi et al. 1993). *In situ* hybridization and northern blot analysis studies in humans are largely consistent with the rodent studies (Tanabe et al. 1993; Fotuhi et al. 1994; Testa et al. 1994; Makoff et al. 1996; Simonyi et al. 2005).

The group III mGluR (mGluR4-li) immunoreactivity has been observed in the cerebral cortex, striatum, globus pallidus, hippocampus, substantia nigra, and the cerebellum (Berger et al. 2001). mGluR 4 mRNA is detected in the olfactory bulb, cerebral cortex, striatum, limbic forebrain, hippocampus, amygdala, midbrain and the cerebellum; while mGluR 7 mRNA is most densely expressed in the hippocampus, with lesser amounts in the olfactory bulb, cerebral cortex, striatum, and midbrain (Bradley et al. 2006). This is a macroscopic overview of mGluR distribution and now we will discuss the cellular expression of mGluRs in the striatum.

Metabotropic glutamate receptor expression in the striatum. All three mGluR receptor groups have been reported to be expressed by striatal cells. Group I mGluRs are mainly found on MSNs, where they are localized to dendritic spines (Shigemoto et al. 1993), while groups II and III are mainly on presynaptic elements to MSNs (Testa et al. 1995; Kosinski et al. 1999; Smith et al. 2000; Corti et al. 2002; Pisani et al. 2002). In addition, group II mGluRs are expressed on striatal interneurons and astrocytes.

Striatal cholinergic interneurons express mGluRs 1,2, and 5 (Testa et al. 1995; Pisani et al. 2002). Stimulation of group I mGluRs results in a decrease in striatal acetylcholine (ACh) release (Marti et al. 2001). A similar effect has also been observed with mGluR 2/3 agonists (Bonsi et al. 2007). Astrocytes express mGluR 3 but not detectable levels of mGluR 2 (Testa et al. 1995).

Metabotropic glutamate receptors in animal models of parkinsonism. There have been surprisingly few studies of group II agonists in animal models of PD. Of these few reports, group II metabotropic agonists have been suggested to reduce motor deficits in animal models of parkinsonism. Treatment with mGluR 2/3 agonists decreases reserpine-induced akinesia and haloperidol-induced rigidity (Konieczny et al. 1998; Dawson et al. 2000; Murray et al. 2002). The mGluR 2/3 agonist LY379268 has also been suggested to be neuroprotective as reflected by fewer nigrostriatal cells dying in response to MPTP treatment (Battaglia et al. 2003). In addition an enhanced potency of group II mGluR agonists has been reported in DA depletion-induced animals (Picconi et al. 2002).

Blockade of the mGluR 5 receptor has been reported to alleviate akinesia in 6-OHDA-lesioned animals and rats treated with haloperidol (Spooren et al. 2001; Ossowska et al. 2001; Breysse et al. 2002). In addition, the mGluR 5 knockout mouse has been reported to be somewhat resistant to MPTP toxicity, although the mechanism is unclear (Battaglia et al. 2004).

Metabotropic glutamate receptors in idiopathic Parkinson's Disease. Enhanced mGluR 5 binding has been reported in MPTP-treated non-human primates as measured with the PET tracer, [^{11}C]MPEPY, in the striatum (Sanchez-Pernaute et al. 2008). Recently the specific binding of mGluR 2/3 has been reported to be decreased in the caudate nucleus of parkinsonian patients (Samadi et al. 2009). The authors suggest that this decrease in mGluR 2/3 levels may be the result of compensatory changes as a result of increased glutamatergic neurotransmission.

In order to determine if the loss of MSN dendritic spines requires corticostriatal glutamate release, and hence if cortical lesions may mitigate spine loss by suppressing glutamate release, we used organotypic slice co-cultures comprised of cortex, striatum, and ventrolateral mesencephalon (including the SN) to assess the effects of a group II metabotropic (mGluR 2/3) receptor agonist and a mGluR 5 antagonist. Activation of mGluR 2/3 receptors that are located on corticostriatal terminals (Testa et al. 1998) dampens glutamate release (Lovinger 1991; Calabresi et al. 1992; Lovinger and McCool 1995). mGluR 5 receptors are located on the spines of MSNs and couple to Gq, suggesting that they modulate intracellular stores of calcium. Thus mGluR 5 antagonists should

dampen the intracellular calcium seen in MSNs after DA depletion (Taylor et al. 1991; Drissi et al. 1998; Kumar et al. 2008).

In order to examine if corticostriatal regulation of MSN dendritic spines is mediated by cortical glutamate, we determined if suppression of glutamate transmission would also attenuate DA depletion-induced spine loss. We first assessed if stimulation of mGluR 2/3 receptors would prevent MSN spine loss, and then determined if blockade of mGluR 5 receptors would also prevent DA denervation-induced spine loss.

Methods

Organotypic slice cultures. Triple slice cultures consisting of cortex, striatum, and ventrolateral mesencephalon (SN) were prepared from the brains of P1-P2 Sprague-Dawley rats (Harlan) following our previously described method (Neely et al. 2007). A representative organotypic triple slice culture stained with toluidine blue is depicted in Figure 27. Two cultures were plated in each well. At 14 days *in vitro* (DIV), by which time the MSNs achieve a mature dendritic morphology (see Neely et al. 2007), the dopamine innervation of the striatum was denervated by treatment of the cultures with 15 μM 1-methyl-4-phenylpyridinium (MPP^+) (Sigma-Aldrich). This concentration of MPP^+ causes a selective loss of DA but not other neurons in the cultures as assessed by propidium iodide accumulation (Neely et al. 2007). MPP^+ was removed 24 hours later and treatment with the mGluR 2/3 agonist LY379268 (1.0 μM; Tocris) started, with the agonist being added to the cultures at the time of media

changes (every other day for 14 days). Culture media was collected at 14 DIV (just before MPP^+ treatment) and again at 17 DIV, and stored at -80^o C until subsequently assayed for the dopamine metabolite homovanillic (HVA) acid as an index of dopamine denervation. The cultures were harvested at 28-30 DIV for analysis of dendritic spine density.

We determined if co-administration of an mGluR 2/3 antagonist to cultures would block the ability of the agonist LY379268 to prevent spine loss in dopamine-denervated cultures. At 14-16 DIV the cultures were treated with MPP^+, which was removed 24 hours later, at which time either the mGluR 2/3 agonist LY379268 (1.0 μM), the antagonist LY341495 (0.2 μM), or both the mGluR 2/3 agonist and antagonist were added to the culture media. The concentration of LY379268, which has at least an 80 fold higher affinity for group II mGluRs than other metabotropic glutamate receptors (Marek et al. 2000; Schoepp et al. 1999), was based on *in vitro* slice data from Marek et al. (2000) and Picconi et al (2002). The concentration of the antagonist LY341495 (Monn et al. 1999; Schoepp et al. 1999) was also based on the data of Marek et al. (2000). After 14 days, the cultures were harvested and ballistically labeled.

We also tested the effects of mGluR 5 blockade on MSN spine density, using the antagonist MTEP (10 μM). MTEP is a highly selective and potent mGluR 5 antagonist that has been found to have virtually no effects on the other mGluR subtypes.

Ballistic labeling of cultures. Cultures were fixed in 1.5% paraformaldehyde in 0.1M phosphate-buffered saline for 25 minutes and then ballistically labeled with the carbocyanine dye CM-DiI (Invitrogen; Carlsbad, CA), following the general protocol of Gan et al. (2000), as modified by Neely et al. (2009). Cultures were then mounted with Prolong Antifade (Invitrogen).

Assessment of striatal dopamine depletion. In order to determine the extent of MPP^+-induced striatal DA depletion in the cultures, we measured the concentration of the DA metabolite HVA in the culture media both immediately before and 48 hours after MPP^+ treatment. The media was analyzed by HPLC-EC, following our previously described procedure (Deutch and Cameron, 1992). Briefly, 500 µL of media was added to 125 µL of a 1M perchloric acid solution containing 0.2g/l $NA_2S_2O_5$ and 0.05 g/l Na_2-EDTA. The samples were centrifuged at 23,000Xg for 5 min, injected on a C18 column (Alltech, Deerfield, IL) and HVA measured using an ESA 501A Coulochem detector (Chelmsford, MA). HVA levels were expressed as pmol/ml medium. Because DA is rapidly oxidized in the culture media and therefore below detection thresholds in control cultures, we focused on the DA metabolite HVA, which is stable. Spine density in MSNs of cultures in which HVA levels were depleted by <70% were not analyzed.

Dendritic spine analyses in diOlistically-labeled cultures. A confocal laser scanning microscope with a 60x 1.4 NA objective (with a 2x digital zoom to yield

a final magnification of 120x) was used to obtain z-stacks of MSN segments at 0.5 µm intervals. MSN spines were assessed on third and fourth order dendritic segments, of which 3-4 dendrites per neuron were analyzed.

Data analysis. Mean MSN spine density values per neuron were determined, after which these values were collapsed to yield a single “per culture” value. These “per culture” data were then analyzed by two-way ANOVAs.

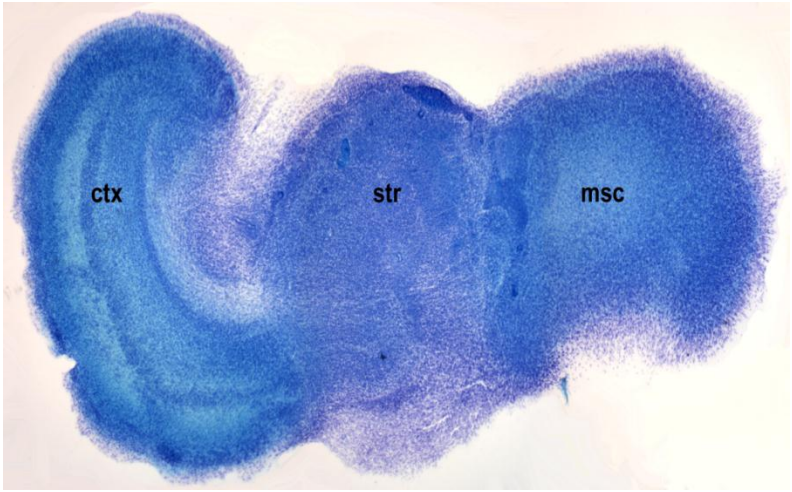

Figure 27. Organotypic triple co-culture stained with toluidine blue. Cultures consisting of the cortex (ctx), striatum (str) and ventral mesencephalon (msc) were prepared from P1-2 Sprague dawley rats. By 14 DIV cultures reestablish appropriate connections while maintaining cellular composition and MSN dendritic spines are mature.

Results

Metabotropic glutamate receptors regulate spines. The treatment of cultures with MPP^+ caused a marked (89.4%) decrease in the concentration of the dopamine metabolite HVA in media ($t_{32} = 17.73$, $p < .0001$), consistent with extensive striatal DA denervation. A two-factor (dopamine x drug treatment) ANOVA was used to assess the effect of the mGluR 2/3 agonist on MPP^+-induced spine loss, yielding an overall significant effect ($F_{3,40} = 6.86$; $p < 0.001$). Main effects of both dopamine innervation ($F_{1,40} = 5.33$, $p = .0262$) and of drug treatment ($F_{3,40} = 9.83$, $p = .003$) were uncovered, with a strong trend toward an interaction ($F_{1,40} = 4.08$, $p = .0502$). Post-hoc analyses revealed a significant decrease (26%) in MSN spine density compared to vehicle-treated cultures ($p = .003$; see Figure. 28). While treatment with the mGluR 2/3 agonist LY379268 had no effect on spine density in MSNs from cultures with an intact striatal dopamine innervation, the mGluR 2/3 agonist completely prevented MSN spine loss in MPP^+-treated cultures, such that spine density did not differ significantly from control (intact dopamine innervation + vehicle-treated) cultures (see Figure 28). Representative diOlistically-labeled MSN dendritic segments are shown in Figure 29.

mGluR 2/3 antagonist blocks ability of LY379268 to attenuate spine loss. In order to determine the specificity of the mGluR 2/3 agonist LY379268, we assessed if treatment with the mGluR 2/3 antagonist LY341495 would block the

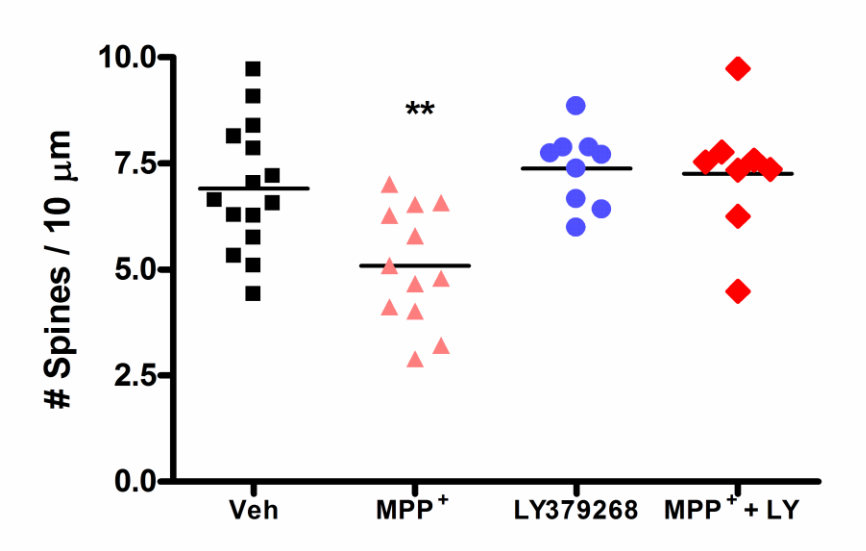

Figure 28. Treatment of slice cultures with the mGluR 2/3 agonist LY379268 completely prevented dopamine depletion induced spine loss. Each symbol represents the mean spine density in a single culture. The data were analyzed by a two-way ANOVA.

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** $p < 0.005$ relative to control cultures

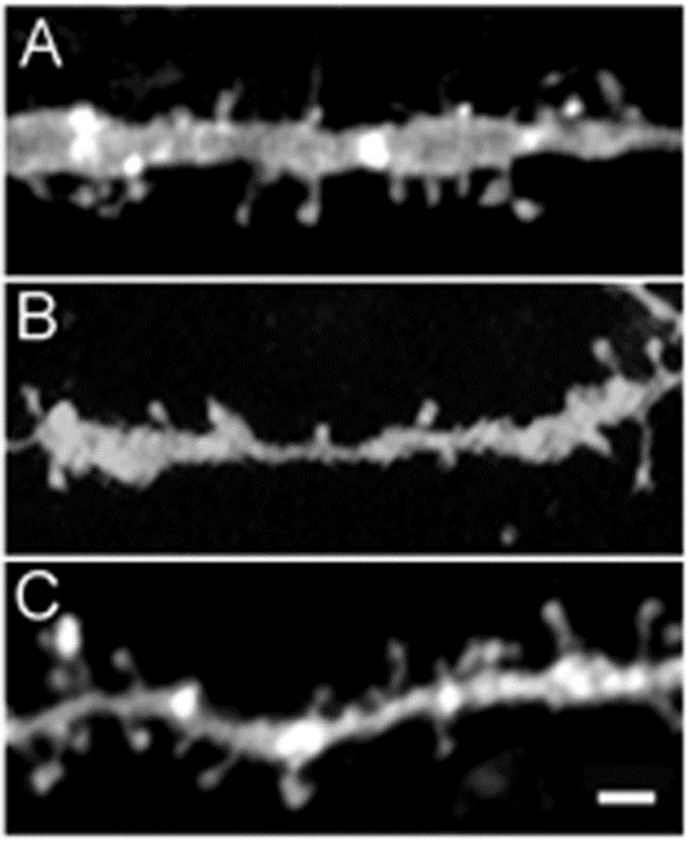

Figure 29. Photomicrographs of ballistically-labeled MSN dendrites. A) dendritic segment of MSN from a control culture, B) MPP^+-treated dendrite, and C) LY379268- plus MPP^+ -treated dendrite. Scale bar, 2 µm.

actions of the group II metabotropic glutamate receptor agonist. As expected, post-hoc analyses revealed that MPP^+ treatment significantly decreased spine density ($p = .01$). The antagonist alone also caused a significant decrease in spine density ($p = .037$), while the combined treatment of the antagonist plus agonist in dopamine-depleted cultures did not significantly differ from the MPP^+-treated cultures alone, and thus completely reversed the protective effects of the mGluR 2/3 agonist (see Figure 30).

Group I mGluR antagonist fails to attenuate DA depletion-induced spine loss.

Treatment of cultures with an intact DA innervation with the mGluR 5 antagonist MTEP had no significant effect on MSN dendritic spine density (Figure 31). As expected, we observed a significant effect of MPP+, which decreased spine density ($p<0.01$). However, treatment of DA-depleted cultures with MTEP did not significantly attenuate the MSN dendritic spine loss seen in response to DA deafferentation (Figure 31).

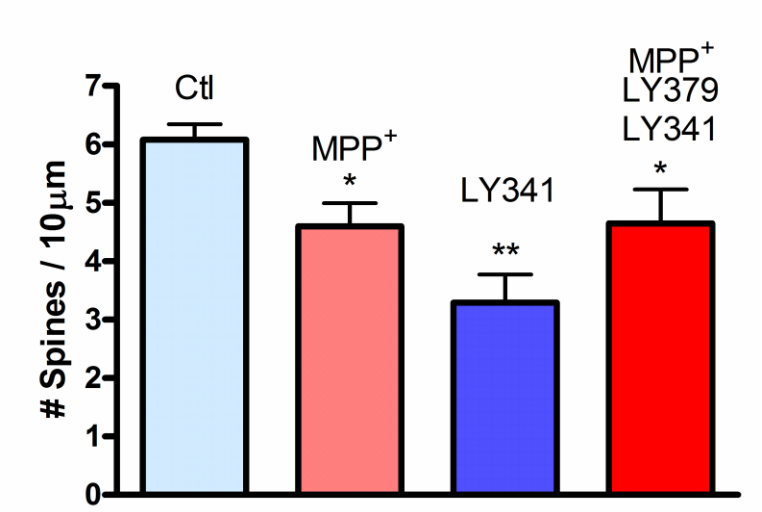

Figure 30. Treatment of slice cultures with the mGluR 2/3 antagonist LY341495 blocks the effects of the mGluR 2/3 agonist LY379268. LY341495 significantly decreased MSN spine density in cultures with an intact dopamine innervation.

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* $p < 0.05$ compared to control cultures
** $p < 0.05$ compared to control cultures

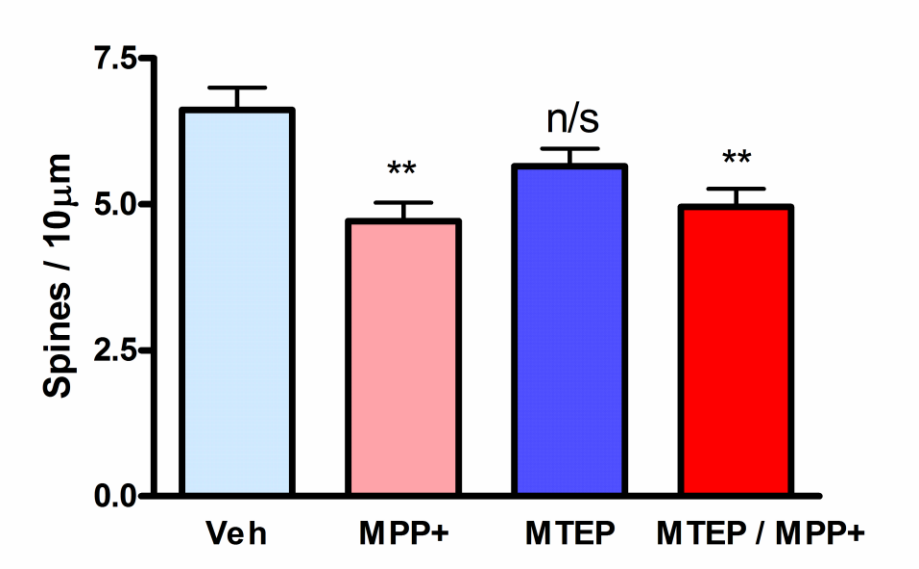

Figure 31. Treatment of slice cultures with the mGluR 1/5 antagonist MTEP fails to prevent dopamine depletion induced spine loss. Cultures were maintained for 14 DIV in which MPP+ treatment began. MTEP treatment began at 15 DIV and was added to the culture media every other day until 29 DIV.

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** $p < 0.01$ relative to control cultures

Discussion

Mechanism of action of cortical lesions. A variety of *in vivo* and *in vitro* data suggest that striatal dopamine depletion increases glutamate release from corticostriatal terminals (Florio et al. 1993; Meshul et al. 1999; Cepeda et al. 2001; Bamford et al. 2004a,b; Day et al. 2006). Dendritic spine formation and maintenance are determined largely by changes in intracellular calcium levels, including NMDA receptor-driven increases in calcium levels. Accordingly, we hypothesized that one mechanism that contributes to dopamine depletion-induced MSN spine loss is the loss of tonic dopaminergic inhibition of the D_2 heteroceptor on corticostriatal terminals, leading to increased glutamate release (Bamford et al. 2004a), which in turn increases intraspinous calcium and culminates in spine loss. Thus, we anticipated that cortical lesions would reduce excess glutamate release from cortical axons and thereby attenuate MSN spine loss.

Group II metabotropic glutamate receptors that are located presynaptically on corticostriatal terminals (Testa et al. 1998) are release-modulating autoreceptors. We therefore treated organotypic slice co-cultures with an mGluR 2/3 receptor agonist to dampen glutamate release from disinhibited corticostriatal axons. In mature cultures, there are both extensive cortical and nigral projections to the striatum (Neely et al. 2007; Snyder-Keller et al. 2008). MSNs in these cultures have both up and down states, and accordingly one sees both spontaneous and cortically-evoked activity of MSNs (Plenz and Kitai 1998; Snyder-Keller et al. 2008). This is consistent with a

functional cortical regulation of striatal MSNs. The use of these cultures allowed us to determine specifically if the loss of cortical glutamate, as opposed to glutamatergic afferents from other areas such as the thalamus, was responsible for preventing spine loss.

Treatment of dopamine-denervated cultures with the mGluR 2/3 agonist LY379268 completely prevented the spine loss. It appears likely that the ability of LY379268 to block MSN spine loss is due to actions at mGluR 2/3 sites. LY379268 is a preferential agonist at mGluR 2/3 receptors (Schoepp et al. 1999; Imre 2007). The agonist inhibits forskolin-stimulated cAMP accumulation in cells expressing mGluR 2/3 receptors (EC50 $\leq$ 6 nM) but has very weak actions at mGluR4 and mGluR8 receptors (EC50 > 2 μM) and no significant actions at other mGluRs (Monn et al. 1999; Imre 2007). In addition, we found that the mGluR 2/3 antagonist LY341495 completely reversed the protective effects of the agonist on MSN spines in the dopamine-depleted striatum. Moreover, treatment of cultures containing an intact nigrostriatal dopamine system with the antagonist was sufficient to cause MSN spine loss, supporting the hypothesis that MSN spine loss results from excessive glutamate release from corticostriatal axons.

Recently, Seeman and colleagues (2008) have suggested that mGluR 2/3 agonists, including LY379268, are partial agonists at D_2 receptors. Because dopamine denervation-induced MSN dendritic spine loss is not reversed by levodopa treatment of rodents (Deutch et al. 2007), and because MSN spine loss is seen in postmortem striatal samples from PD patients who had received

chronic treatment with levodopa and/or dopamine agonists (Stephens et al. 2005; Zaja-Milatevic et al. 2005), the attenuation of spine loss in animals treated with the mGluR 2/3 agonist is probably not due to any actions of LY379268 at dopamine receptors. This is consistent with the recent report of Fell et al. (2009) who did not observe any interaction between group II ligands and the D_2 receptor.

Group II metabotropic receptor agonists have been reported to afford protection against MPTP-induced striatal dopamine loss (Battaglia et al. 2003). However, we started treatment with LY379268 *after* treatment with MPP^+, rather than administering the mGluR 2/3 agonist prior to or together with MPP^+. Moreover, we observed extensive striatal dopamine depletion in cultures treated with LY379268, as reflected by an almost 90% decrease in media concentrations of HVA, indicative of striatal dopamine denervation.

Taken together, our data strongly suggest that the ability of LY379268 to attenuate MSN spine loss is due to actions at mGluR 2/3 receptors and not an off-target action of the drug. Moreover, the observation that treatment of the cultures with an mGluR 2/3 antagonist in cultures with an intact striatal dopamine innervation elicited spine loss is consistent with the hypothesis that cortical lesions protect against dopamine depletion-induced MSN spine loss by reducing glutamate release from corticostriatal terminals.

It is likely that there are sources of glutamate in addition to corticostriatal axons that contribute to MSN spine loss. Extracellular glutamate levels involve not only synaptically-released glutamate but also glutamate release from

astrocytes through the cystine-glutamate exchanger as well as glutamate uptake through high-affinity glutamate transporters, including the astrocytic transporter GLT-1 (EAAT2) (see Kalivas 2009). Because group II mGluRs are expressed by astrocytes (Testa et al. 1994), it is possible that the effects of the mGluR 2/3 agonist are mediated in part by astrocytic glutamate transporters. In addition, factors extrinsic to MSNs (such as certain trophic factors) and intrinsic to striatal cells (such as L-type voltage-gated calcium channels) may also play important roles.

mGluR5 antagonist fails to prevent spine loss. Group I mGluRs couples to Gq proteins which leads to the activation of PLC and increases in intracellular stores of calcium via the IP_3 receptor. Antagonizing this pathway should decrease release of calcium from intracellular stores in dendritic spines. If intracellular stores of calcium play a role in dendritic spine loss then we should observe an attenuation of the DA depletion-induced spine loss. However, we saw no such MTEP-induced protection.

There could be several explanations as to why we did not observe a protective effect of MTEP in the DA denervated cultures. One possibility is that treating cultures with MTEP every other day was insufficient; however the mGluR 2/3 agonist completely blocked spine loss with every other day administration, suggesting that this explanation probably does not account for the lack of MTEP effects. More likely is the possibility that mGluR 5-mediated decreases in intracellular calcium are either not involved or that mGluR 5 antagonists-elicited

suppression of intracellular calcium stores is insufficient to afford spine protection.

Methodological limitations of MSN spine density analysis. In contrast to studies with group I mGluR antagonists, recent data suggest that mGluR 2/3 agonists do not attenuate levodopa-induced dyskinesias (Rylander et al. 2009). Several studies have pointed to D_1-expressing (direct pathway) MSNs as being critical to the development and maintenance of dyskinesias (Bordet et al. 2000; Carta et al. 2008; Berthet et al. 2009; Darmopil et al. 2009). In contrast, rodent studies indicate that MSN dendritic spine loss in the dopamine depleted striatum occurs in D_2-expressing MSNs (Rodriguez and Pickel 1999; Day et al. 2007; Deutch et al. 2007), although a recent study in the primate suggests that spines are also lost on D_1-expressing cells (Villalba et al. 2009). Because the animals in our study were not treated with levodopa or other dopamine agonists, the attenuation of spine loss that we observed is not related directly to dyskinesias.

Because Golgi impregnation of neurons precludes the determination of the type of MSN, we cannot ascertain if the changes we observed occur in indirect or direct pathway MSNs. The spine loss we observed secondary to DA denervation presumably occurs only in striatopallidal MSNs, with the prevention/reversal of spine loss also occurring on these indirect pathway cells. However, we cannot eliminate the possibility that the striatonigral MSNs are being altered by the manipulations decreasing corticostriatal glutamate levels. Future studies using

different methods that permit evaluation of spine changes in specific types of MSNs will be required to untangle changes in direct and indirect pathway MSNs.

Methodological limitations of slice co-culture LY379268 study. Treatment of the slice co-cultures with LY379268 completely prevented DA depletion-induced loss of spines. The cultures provide a unique reduced system in which to study the mechanism of DA depletion-induced spine loss. The cultures are spontaneously active, reestablish normal innervation patterns of the constituent parts, and possess up and down states. As noted above a caveat to the use of these slice cultures was the fact that we did not replace the mGluR agonist or antagonist more frequently.

Despite our every other day treatment regimen, we found that LY379268 completely prevented DA denervation-induced spine loss. Because the cultures lack key mechanisms for eliminating the active drug, *in vivo* studies of the effects of LY379268 will need to use more frequent dosing of the drug.

Developmental regulation of mGluR 2/3. Ly379268 does not distinguish between mGluR 2 and mGluR 3. Levels of both class II mGluRs are developmentally regulated. mGluR 2 mRNA is low at PND 0 and gradually increases and levels off by PND 21. Unlike mGluR 2, mGluR 3 mRNA is elevated at PND 0 and gradually declines with aging (Jokel et al. 2001). However, we do

not know the relative abundance of mGluR 2 and mGluR 3 receptors at 14-16 DIV, the time point at which LY379268 was added to the culture media. Future studies evaluating specific mGluR 2 and 3 agonists will help clarify which receptor sub serves the prevention of spine loss. In addition to corticostriatal terminals expressing mGluR 2/3 receptors, cholinergic interneurons express mGluR 2 (Bell et al. 2002) and astrocytes express mGluR 3 (Testa et al. 1995). Parsing out the individual roles of corticostriatal as well as interneuron, and astrocytic mGluR 2/3 receptors will be an important topic for future studies.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

To the best of our knowledge, this is the first demonstration of the ability of any treatment to *reverse* DA depletion-induced loss of striatal MSN spines once the spine loss is established. Our *in vivo* data indicate that cortical lesions reverse the structural changes in MSNs, although it is not clear if this is a growth of "new" spines or a rescue of spines that had retracted into dendritic shaft. Consistent with the second possibility are ultrastructural data indicating that the spine apparatus, an electron dense substance normally present only in dendritic spines, can be seen in the dendritic shaft after DA denervation (Nitsch and Riesenberg 1995). The major protein constituent of the spine apparatus is synaptopodin, and thus it will be important to determine relative intraspinous as well as intradendritic levels of synaptopodin in the DA denervated striatum.

Our data suggests that treatments that target corticostriatal glutamatergic projections may be a useful intervention in PD. However, clinical trials of ionotropic glutamate receptor antagonists in Parkinson's disease have been disappointing. These trials focused on symptom reduction and did not assess disease (or symptom) progression. Our data suggest that modulation of glutamatergic transmission through metabotropic glutamate receptors,

specifically mGluR 2/3 agonists, may be warranted in studies aimed at slowing progression in PD by halting the ongoing loss of MSN dendritic spines.

Relation to motor deficits in parkinsonism. Dopamine replacement with levodopa or direct DA agonists does not reverse MSN spine loss in either animal models of parkinsonism or in PD patients (Stephen et al. 2005; Zaja-Milatovic et al. 2005; Deutch 2007). Our data on the effects of mGluR 2/3 agonists point to derangements in corticostriatal glutamatergic systems as contributing to the dendritic remodeling seen in the dopamine-denervated striatum. This has significant implications for the treatment of Parkinson's Disease.

Surprisingly few studies have examined the effects of mGluR 2/3 agonists on parkinsonian-like motor deficits (Murray et al. 2002; Feeley Kearney and Albin 2004). Murray et al. (2002) found that intraventricular administration of the mGluR 2/3 agonist LY379268 dose-dependently reversed reserpine-induced akinesia. In contrast, Ossawska et al. (2007) did not observe any benefit of intrastriatal injections of a different mGluR 2/3 agonist, 2R,4R-APDC on haloperidol-induced motor deficits; it is not clear if this is because of the acute nature of the dopamine blockade achieved with haloperidol. Clearly additional behavioral studies on the effects of group II mGluR agonists in animal models of parkinsonism is needed. Our data suggest that metabotropic mGluR 2/3 agonists may be beneficial in PD by either decreasing glutamatergic neurotransmission or perhaps by attenuating MSN dendritic spine loss.

Dendritic spine loss is progressive and keeps pace with the age-related decline in MSN spine loss. Spine loss compromises cortical input to the striatum and corticofugal circuitry. The activity of corticostriatal neurons may also be decreased as a function of aging, and is associated with a decrease in spines on cortical PCs (Kabaso et al. 2009). Although there have been discussions of potential roles of MSN spine loss in parkinsonism, there are no empirical data that address this issue. This is because we have not previously had a means of reversing spine loss. Our finding of the reversibility of spine loss *in vivo* opens the opportunity to begin to address the functional significance of spine loss in parkinsonism by treating animals and examining them in the spine loss condition, then inducing recovery and reexamining the animals.

Our data indicate that decortication reverses spine loss. In our *in vitro* studies we showed that spine loss can be prevented by mGluR 2/3 agonists. However, we did not determine if the reversal elicited by the cortical lesion is attributable to suppression of glutamate release in the cultures because such studies require that the cultures be maintained for 6-7 weeks, a time point at which our cultures begin to degenerate. However, we are now examining the effects of the mGluR 2/3 agonist on the DA denervated striatum *in vivo*. If these data parallel the finding that decortication blocks spine loss, then mGluR 2/3 agonists should provide an easy means with which to study the effects of DA depletion-induced spine loss.

In addition to understanding the mechanism of spine loss, it will be important to address the status of the presynaptic partners of spines that are lost.

We have now shown that spine loss can be reversed, but have not examined the issue of whether the “new” spines have presynaptic partners, i.e., have established functional synaptic contacts. Moreover it is not known if any contacts of presynaptic elements onto new spines would route appropriately or if there would be ectopic localization of afferents to the spine or dendritic shaft. It is intriguing to note that cortical inputs to the striatum express BDNF, although MSNs do not. Some form of trophic support may be necessary for appropriate rewiring of the striatum to its normal state.

In addition to the utility of the therapeutic strategy to suppress corticostriatal drive to benefit the motor symptoms, it may be possible that the same approach may offer promise for treatment of non-motor symptoms. Patil and colleagues (2007) reported that mGluR 2/3 agonists are effective for treating the positive and negative symptoms of schizophrenia with minimal side effect liability. The deficits in cognitive function in PD are in the executive realm and resemble the executive dysfunction seen in schizophrenia. Because the early study of Patil et al. (2007) suggest that mGluR 2/3 agonists may target to some degree cognitive deficits as well as positive and negative symptoms, it is possible that the mGluR 2/3 agonists may be useful for treating these non-motor (cognitive) as well as the motor symptoms of PD.

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