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Acute and Chronic Dopaminergic Modulation of Striatal Circuitry
and Function in Health and Disease

by

Philip Ross Lutton Parker

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

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by Philip Ross Lutton Parker

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This work is dedicated to my father, Michael Parker, and my mother, Susan Lutton. My insatiable passion for scientific thought and experiment exists only because of their cultivation of my curiosity and critical thinking during my youth, and their continued support in my adulthood.

Abstract of the Dissertation

The basal ganglia are an evolutionarily conserved set of nuclei that are crucial for context-dependent learning and selection of appropriate behavioral outputs. The striatum is considered the input nucleus of the basal ganglia because it not only receives the bulk of excitatory synaptic drive from sources outside the basal ganglia, but also contains two populations of medium spiny neurons that give rise to the direct and indirect basal ganglia pathways. The activity of direct and indirect pathway medium spiny neurons (dMSNs and iMSNs) is sufficient to promote or suppress behavioral output, respectively. Changes in synaptic strength at excitatory inputs to MSNs are thought to underlie context-dependent learning, whereas the acute modulation of MSN intrinsic excitability affects motivation or vigor of an ongoing behavior. Previous research has revealed that the neuromodulator dopamine appears to be in a unique position to exert both acute and chronic modulatory effects over MSNs and their excitatory inputs. The differential expression of Gs-coupled D1 and Gi-coupled D2 receptors by dMSNs and iMSNs, respectively, results in opposing changes in direct and indirect pathway function with changes in striatal dopamine levels. However, previous technical limitations have restricted the study of dopamine's acute actions in the striatum and the long-term effects of dopamine on MSN subtypes and excitatory inputs of distinct origins. Here we use recently developed optogenetic and chemogenetic techniques combined with behavior and *ex vivo* brain slice electrophysiology to study the mechanisms of acute and chronic dopamine signaling in MSNs and their excitatory inputs. In Chapter One, we review the evidence for the dual-pathway model of basal ganglia function and its modulation by dopamine in the context of controlling behavioral output. In Chapter Two, we perform an electrophysiological comparison of common optogenetic proteins to determine the optimal tool for our experiments. We then use optogenetic control of dopamine release to show specific modulation of dMSN intrinsic excitability and D1-mediated invigoration of behavior in Chapter Three. In Chapter Four, we provide evidence that the chronic loss of dopamine in Parkinson's disease results in a reorganization of the thalamostriatal system that has negative outcomes on motor control. Finally, we discuss the overarching implications of these findings in the context

of basal ganglia function in health and disease in Chapter Five. These findings not only represent a long overdue reevaluation of the assumptions surrounding the role of dopamine in striatal function, but also provide new testable hypotheses addressing the cellular and synaptic bases of an evolutionarily conserved system for the dynamic regulation of ethologically relevant behaviors.

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List of Commonly Used Abbreviations

6OHDA: 6-hydroxydopamine
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ChR2: channelrhodopsin-2; hChR2(H134R)
CM: centromedian nucleus of the thalamus
CNO: clozapine-N-oxide
CS: corticostriatal
dMSN: direct pathway medium spiny neuron
eCB: endocannabinoid-dependent
DBS: deep brain stimulation
EPSC: excitatory postsynaptic current ("evoked" for Figure 2,3)
GABA: gamma-Aminobutyric acid
GPe: globus pallidus pars externa
GPi: globus pallidus pars interna
iMSN: indirect pathway medium spiny neuron
IPSC: inhibitory postsynaptic current
LTD: long-term depression
LTP: long-term potentiation
MSN: medium spiny neuron
NMDA: *N*-Methyl-D-aspartic acid
PD: Parkinson's disease
Pf: parafascicular nucleus
PKA: protein kinase A
QX-314: *N*-Ethylidocaine chloride
RGS4: regulator of G-protein synthesis 4
SEM: standard error of the mean
SNc: substantia nigra pars compacta
SNr: substantia nigra pars reticulata
STN: subthalamic nucleus
Tau: membrane time constant
TEA: tetraethylammonium
TS: thalamostriatal
VGLUT1/2: vesicular glutamate transporter type 1/2

Chapter 1

Introduction: Acute and Chronic Modulation of Basal Ganglia

Circuits by Dopamine

Evolutionary conservation of bidirectional control over behavioral output by dual-pathway basal ganglia circuitry

The brain of a human and that of a mouse could not be more apparently different at face value. Yet every vertebrate brain that has been subject to anatomical and immunohistochemical study shares a common layout. The hindbrain and midbrain are critical structures for the control of basic bodily functions, as well as fundamental behaviors such as approach, avoidance, and prey capture. The forebrain receives sensory information from the outside world that is then transformed into outputs that dynamically regulate midbrain and hindbrain function to control behavior and maximize our individual survival (Grillner and Robertson, 2015). While our species has benefited from a substantial increase in the size and complexity of the neocortex, a forebrain structure that endows us with unprecedented conscious perception of the environment and control over our behavior, we share a common organization in the basic layout of ventral forebrain structures critical for learned context-dependent control over our actions – the basal ganglia (Reiner et al., 1998).

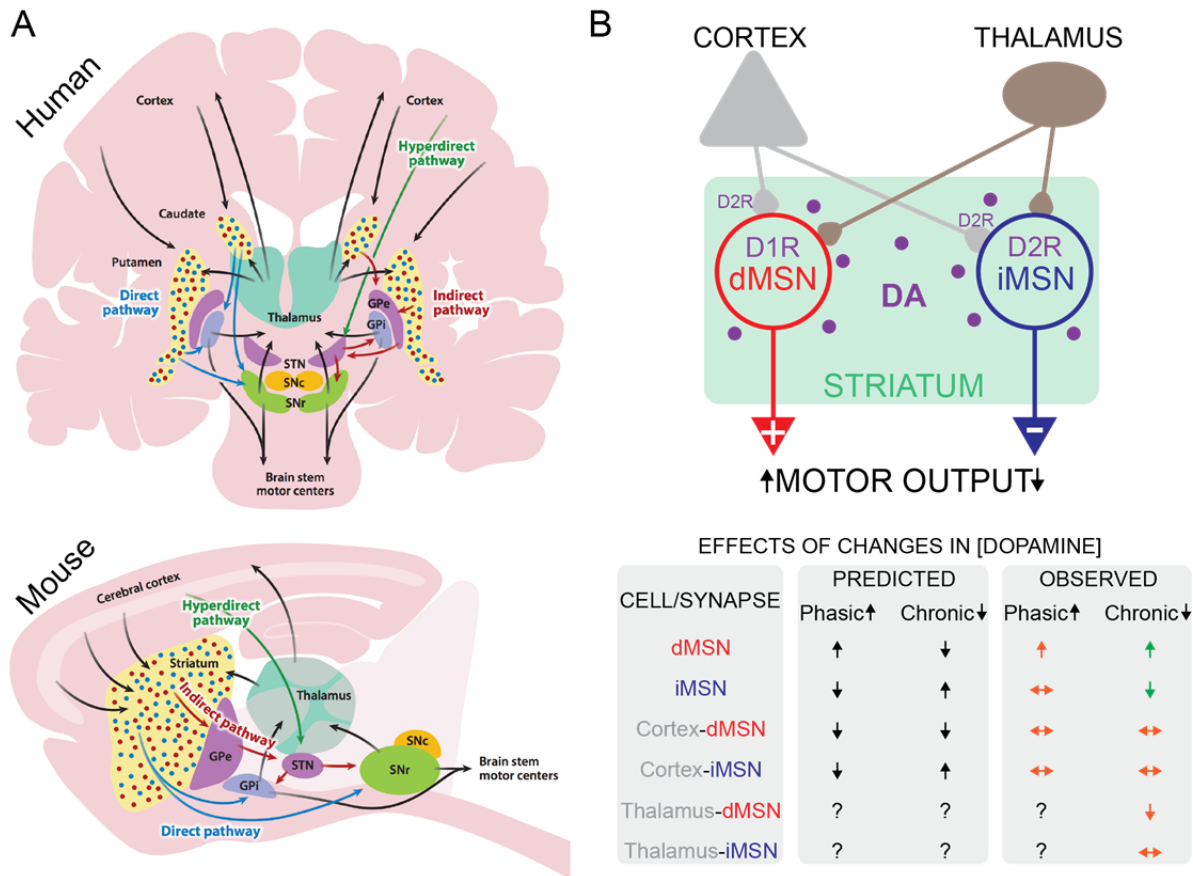


Figure 1: Model of basal ganglia function and modulation by dopamine

A) Schematic of a coronal section through the human brain (top) and a sagittal section through the mouse brain (bottom) showing conservation of basal ganglia structures and circuits across species. The striatum (caudate + putamen) receives inputs from cortex and thalamus, and gives rise to two output pathways that differentially project to downstream basal ganglia structures. The direct pathway projects to basal ganglia output nuclei (substantia nigra pars reticulata, SNr; globus pallidus pars interna, GPi). The indirect pathway projects to the globus pallidus pars externa (GPe), which in turn projects to the subthalamic nucleus (STN), which finally innervates the SNr and GPi. The output nuclei exert tonic inhibitory control over subcortical motor, cognitive, and limbic structures. The substantia nigra pars compacta (SNc) contains dopamine neurons projecting heavily to the striatum. Adapted from (Nelson and Kreitzer, 2014).

B) *Top*: simplified schematic of basal ganglia circuitry and sites of modulation by dopamine. Direct pathway activity enhances motor output, while indirect pathway activity suppresses motor output. Dopamine acts at D1 receptors on direct pathway medium spiny neurons (dMSNs), D2 receptors on indirect pathway medium spiny neurons (iMSNs), and D2 receptors on cortical terminals contacting both MSN subtypes (interneurons not shown for simplicity). *Bottom*: predicted and observed effects of phasic increases and chronic decreases in dopamine concentration. The predicted effects are based on Gs- and Gi-coupling of D1 and D2 receptors, respectively, and represent the expected outcomes based on the classical model of basal ganglia organization and function. Observed changes in orange are addressed in the dissertation, while other groups previously observed the changes in green.

The cell types and connectivity of the basal ganglia nuclei are strikingly conserved across vertebrate evolution, and thus laboratory animals such as mice have become critical models for the study of basal ganglia function (Figure 1A). The basal ganglia include an input nucleus, intermediate nuclei, and output nuclei, which are termed the striatum, the globus pallidus pars externa (GPe) and subthalamic nucleus (STN), and the substantia nigra pars reticulata (SNr) and globus pallidus pars interna (GPi), respectively (Nelson and Kreitzer, 2014). Immunohistochemistry in most vertebrates reveals two populations of neurons within the striatum – one expressing substance P and one expressing enkephalin (Reiner et al., 1998). These two populations correspond to medium-sized spiny neurons (MSNs) that differentially project to downstream basal ganglia nuclei. Direct pathway MSNs (dMSNs) project to the output nuclei, while indirect pathway MSNs (iMSNs) project to the GPe. Activity in dMSNs and iMSNs ultimately has opposing effects on the output nuclei, with dMSN inhibiting and iMSNs disinhibiting basal ganglia output, thus enhancing or suppressing thalamocortical and brainstem activity, respectively (Albin et al., 1989; DeLong, 1990).

Through a series of studies in animal models and humans with basal ganglia-related diseases and injuries, Albin, Young, and Penney (1989) produced a model of basal ganglia function whereby activity within the direct pathway promotes movement, while activity within the indirect pathway suppresses movement (Figure 1A). Despite this relative oversimplification, the predictions of the model have largely held true, as our lab recently confirmed using genetic targeting of the light-gated ion channel

channelrhodopsin-2 (ChR2) to direct and indirect pathway MSNs in mice (Kravitz et al., 2010). Activation of iMSNs was sufficient to grossly inhibit motor output, evidenced by increased freezing and decreased initiation of ambulatory bouts. Conversely, dMSN activation was sufficient to decrease freezing and increase ambulation. These data suggest that the two MSN populations bidirectionally modulate motor actions, and thus are poised to exert powerful control over behavioral output.

Anatomical and intrinsic physiological properties of direct and indirect pathway medium spiny neurons

Anatomically, dMSNs and iMSNs are largely indistinguishable and, unlike laminar structures such as the neocortex and hippocampus, both classes of neurons show apparently random distributions throughout the striatum. Both classes of cells also contain numerous ramifying dendrites dense with spines that receive glutamatergic and dopaminergic input. However, the efferent axonal projections of MSNs are the classical method of differentiation between the two cell types. Axons from iMSNs terminate in the GPe, whereas axons of dMSNs project to the GPi and SNr, occasionally sending a collateral into the GPe (Kreitzer, 2009).

With the exception of slight differences in intrinsic excitability, *ex vivo* patch clamp recordings from dMSNs and iMSNs in brain slices also reveal few distinguishing features between the two cell types. MSNs are notoriously difficult to excite, resting around -80 mV in brain slices and resisting depolarization due to the expression of a constellation of potassium channels that clamp the membrane near the potassium equilibrium potential (Kreitzer, 2009). *In vivo*, MSNs are depolarized closer to firing

threshold due to barrages of excitatory input from multiple sources (Mahon et al., 2006). These inputs are thought to drive state transitions in the dendrites via NMDA receptors and voltage-gated calcium channels, studied mainly in the context of sleep and anesthesia (Plotkin et al., 2011). Recent evidence suggests that, at least at the population level, both dMSNs and iMSNs are excited in preparation of motor output, further underscoring the oversimplifications inherent in the classical dual-pathway rate model of basal ganglia function (Cui et al., 2013; Jin et al., 2014).

Excitatory inputs to medium spiny neurons

Because of their large potassium conductances, MSNs rely heavily on excitatory input to drive spiking. In the dorsal striatum, these inputs arise primarily from the neocortex and the thalamus, while the ventral striatum additionally receives input from the amygdala and hippocampus (Groenewegen, 2003). Cortical and ventral thalamic inputs primarily contain vesicular glutamate transporter type 1 (VGLUT1), while intralaminar thalamic inputs contain type 2 (VGLUT2) (Fremeau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001). This distinction has been used to differentiate between cortical and thalamic inputs in electron microscopy studies, which have revealed a preferential innervation of MSN dendritic shafts by terminals from the parafascicular/centromedian nucleus (Pf), whereas other thalamic VGLUT2+ and cortical VGLUT1+ terminals preferentially innervate spines (Raju et al., 2006; Smith et al., 2014). The implications of this finding include distinct pre- and postsynaptic properties, as well as differential modulation by G-protein coupled receptors that are compartmentally organized within MSN dendrites (Kreitzer, 2009).

While the basic properties of thalamostriatal inputs have only recently begun to be elucidated, the existence long-term plasticity at these synapses has yet to be demonstrated conclusively. Conversely, corticostriatal synapses are well characterized and exhibit both pre- and postsynaptic forms of long-term plasticity in both dMSNs and iMSNs (Kreitzer and Malenka, 2008). Endocannabinoid-mediated long-term depression (eCB-LTD) is a particularly well-studied form of synaptic plasticity, and is mediated by the postsynaptic release of anandamide or 2-arachydonoylglycerol from MSNs acting at presynaptic CB₁ cannabinoid receptors. Critically, these receptors are found only at VGLUT1-expressing terminals in the striatum, and induction of eCB-LTD does not occur at VGLUT2-expressing thalamostriatal synapses (Uchigashima et al., 2007; Wu et al., 2015). This differential expression of plasticity mechanisms indicates that cortical and thalamic inputs to striatum are likely distinct not only in the information they transmit to the striatum, but also in their regulation by neurotransmitters such as dopamine.

Dopaminergic modulation: Acute mechanisms

Probably the most pivotal finding for the study of dopaminergic modulation of basal ganglia circuitry was the discovery that dopamine receptors are differentially expressed by the two MSN subtypes. dMSNs express Gs-coupled D1 receptors, while iMSNs express Gi-coupled D2 receptors, and thus dopamine oppositely regulates the levels of cyclic-AMP and protein kinase A (PKA) activation in MSNs. The result is that ionic conductances in dMSNs are altered by dopamine signaling in a manner consistent with increases in excitability (increased calcium and sodium conductances, decreased potassium conductances), while iMSN conductances are oppositely changed. Thus,

dopamine is hypothesized to excite dMSNs and inhibit iMSNs through the regulation of intrinsic ionic conductances (Figure 1B) (Kreitzer, 2009; Kreitzer and Malenka, 2008; Tritsch and Sabatini, 2012). However, these studies have not addressed the effects of phasic dopamine release on MSN excitability, and instead rely upon reduced preparations (mostly dissociated striatal neurons) and bath application of dopamine receptor agonists and antagonists, which act on the timescale of minutes rather than seconds. The question of how phasic dopamine release occurring at physiological timescales affects MSN excitability remains unanswered.

Dopaminergic modulation of presynaptic glutamate release from cortical terminals has also been proposed in the striatum. Some corticostriatal terminals express D2 receptors, and indirect measures of synaptic release have suggested that D2 receptor antagonism reduces glutamate release from corticostriatal terminals (Bamford et al., 2004; Higley and Sabatini, 2010). Such a mechanism would in theory be independent of the postsynaptic target, and thus cortical drive of both dMSNs and iMSNs would be inhibited by phasic dopamine release (Figure 2B). However, D2 receptor activation in iMSNs can be sufficient to cause eCB release, and given the lack of specificity in eCB spread from MSNs this is a plausible mechanism for decreased corticostriatal transmission (Yin and Lovinger, 2006). D2 receptors are also present on cholinergic interneurons, which directly modulate corticostriatal release (Kreitzer, 2009). Thus evidence for direct modulation of corticostriatal transmission by dopamine is still lacking.

Evidence for modulation of postsynaptic glutamatergic MSN synapses is much stronger, and likely lends insight into long-term plasticity mechanisms. D2 receptor activation does not obviously affect corticostriatal NMDA currents in iMSNs, however it does result in a ~50% reduction in calcium influx through NMDA receptors and R-type calcium channels (Higley and Sabatini, 2010). Thus dopamine can act through D2 receptors to modulate calcium dynamics, and presumably postsynaptic plasticity, in iMSN spines independent of the effects of phasic corticostriatal excitatory drive. Gs-coupled adenosine A2A receptor activation has the opposite effect on calcium influx, and thus Gs/Gi modulation of NMDA receptor and voltage-gated calcium channel currents likely extends to dMSNs as well (Higley and Sabatini, 2010).

Dopaminergic modulation: Chronic mechanisms

In addition to rapid changes to intrinsic excitability, dopamine can modulate striatal circuitry on long timescales by effectively gating the induction of LTD. In iMSNs, the release of endocannabinoids is negatively controlled by the regulator of G-protein synthesis 4 (RGS4) protein. Because the levels of RGS4 activity are determined by PKA activity, D2 receptor-mediated decreases in PKA activity inhibit RGS4, thus permitting the synthesis and release of eCBs from iMSNs and eCB-LTD at cortico-iMSN synapses (Lerner and Kreitzer, 2012). By extension, other G-protein coupled receptors in both iMSNs and dMSNs are hypothesized to similarly gate eCB-LTD, and thus D1 receptor activation in dMSNs is thought to suppress eCB-LTD at cortico-dMSN synapses. Similarly, D1 and D2 receptors have been shown to oppositely regulate

postsynaptic NMDA receptor-mediated long-term potentiation (LTP) and LTD in dMSNs and iMSNs (Shen et al., 2008).

The most extensively studied long-term effects of dopamine modulation are arguably in the context of Parkinson's disease (PD). In humans, PD is characterized by a progressive death of dopamine neurons in the substantia nigra pars compacta (SNc) (Ungerstedt, 1968). The result is a constellation of symptoms that include bradykinesia/akinesia, postural instability, tremor, and rigidity (Albin et al., 1989). Given the massive innervation of striatum by SNc neurons, symptoms in PD are widely thought to stem from changes to striatal circuitry in the absence of dopamine, which are hypothesized to result in hypoactivity of the direct pathway and/or hyperactivity of the indirect pathway due to the loss of dopamine's actions at Gs-coupled D1 and Gi-coupled D2 receptors, respectively (Figure 1B). These changes would then propagate through the basal ganglia circuitry, resulting in widespread pathological activity throughout the motor system (Albin et al., 1989; DeLong, 1990).

Intriguingly, direct evidence for hyperactivity of the indirect pathway and hypoactivity of the direct pathway is seen in recordings from downstream basal ganglia nuclei in patients (Albin et al., 1989). However, in PD patients and animal models alike, changes to MSN intrinsic properties and spine density are opposite the predictions of the classical model (Fieblinger et al., 2014; Suárez et al., 2014). Namely, dMSN excitability is increased rather than decreased, and iMSN excitability is decreased rather than increased. iMSN spine density is decreased rather than increased, and dMSN spine

density appears unchanged. Thus every change associated with MSN intrinsic physiology and anatomy appears either contradictory or compensatory in response to the expected changes predicted by the classical model (Figure 1B).

Goals of the dissertation

The idea of dopamine as a critical modulator of basal ganglia circuit function is not novel, and our basic understanding of its actions is reasonably detailed. However, limitations in our ability to specifically control endogenous dopamine release in time and space have hindered our abilities to test key theories about dopamine function. Further, our inability to identify and control the activity of pre- and postsynaptic neuronal populations has likely caused oversimplifications in our understanding of dopaminergic modulation, and preclude us from making claims about input- and output-specific effects of dopamine on striatal circuitry. To this end, our goal is to utilize a combination of techniques developed in recent years that allow genetically defined neuronal populations to be visualized and their activity controlled in order to test the hypothesis that dopamine exerts rapid and long-term effects in the striatum to promote and suppress activity of the direct and indirect basal ganglia pathways, respectively.

Our first task is to test which optogenetic proteins will prove most effective to address this hypothesis. Since the introduction of the first channelrhodopsin protein, a number of modified versions have appeared that claim to offer improved properties for stimulation of synaptic terminals (Lin et al., 2009; Mattis et al., 2011), and thus we wish to select the opsin that will provide the most efficient and accurate production of data.

Our first major question will be on the topic of striatal circuit modulation by phasic dopamine release. Given the expression of D1 receptors in dMSNs, we expect dopamine to excite direct pathway neurons. Given the expression of D2 receptors in iMSNs and corticostriatal terminals, we expect dopamine to suppress iMSNs firing and release of corticostriatal glutamate onto both MSN subtypes. The implications of these cellular and synaptic findings will be tested in a behavioral context where dopamine will be optogenetically released from terminals in the striatum during a Pavlovian behavior.

Our second major question will be on the role of dopamine in the long-term modulation of striatal circuitry. We will utilize a chronically dopamine depleted mouse model of PD to address whether excitatory inputs from neocortex and thalamus are differentially altered in the absence of dopamine receptor activation. We will then interrogate the role of MSN synaptic inputs in the generation of pathological behavioral using inhibitory chemogenetic techniques.

Together, these two major aims will address two longstanding and untested hypotheses predicted by the classical model of basal ganglia function. These experiments are critical not only for our understanding of how dopamine regulates basal ganglia circuitry, but also the accuracy of our current models describing the synaptic and circuit underpinnings of basal ganglia function.

Chapter 2

Comparison of Optogenetic Tools for Stimulation of Cell Type-Specific Synaptic Inputs

Abstract

A longstanding difficulty for studies of *in vivo* and *ex vivo* electrophysiology and behavior is the activation or suppression of specific cell types and synaptic inputs. Recent advances in optogenetic and chemogenetic technologies offer the potential to overcome these barriers through light- or chemical-mediated modulation of neuronal activity. We aimed to assess the feasibility of stimulating genetically defined synaptic inputs to striatal MSNs using an array of different optogenetic proteins, including hChR2(H134R) (ChR2 for simplicity), ChETA, ChIEF, and the red-shifted opsin C1V1. We first tested the basic properties of each opsin in dMSNs using a Cre-recombinase-dependent AAV vector in a D1-Cre transgenic mouse line. Light stimulation of all tested opsins resulted in substantial whole-cell currents in MSNs, which showed differential properties with respect to desensitization and decay time constants. ChR2 and ChIEF, however, were the only opsins that consistently resulted in reliable synaptic release from cortical terminals. Because of the unavailability of ChIEF-expressing viral constructs and the widespread use and availability of ChR2-expressing viral constructs, we determined that Cre-mediated expression of ChR2 is the most efficient method for stimulation of long-range synaptic inputs to striatal MSNs. Further development of

ChIEF-expressing constructs is proposed as a future tool for more reliable excitation of long-range synaptic connections.

Introduction

Since the beginnings of neurophysiology, the study of synaptic connections between neurons has relied largely on precise control over the strength and timing of presynaptic input activation by electrical stimulation of axonal fibers. While these studies have been pivotal to our understanding of synaptic function throughout the nervous system, the weak specificity of electrical stimulation precludes us from understanding the true nature of many neuronal connections. On the one hand, electrical stimulation can activate multiple populations of axons, as with intrastriatal stimulation that presumably elicits release from multiple glutamatergic (cortical, thalamic, hippocampal, amygdalar, etc.), GABAergic (MSNs, interneurons, GPe, etc.), monoaminergic (dopaminergic, serotonergic, noradrenergic, etc.), and other types of axons (Kreitzer, 2009). On the other hand, cell type-specific expression of opsins such as ChR2 has revealed unexpected release of multiple neurotransmitters from single populations of neurons. For example, dopamine neurons were recently discovered to co-release GABA and glutamate – findings that would have been extremely difficult to reveal with traditional methods (Tritsch et al., 2012).

The power of optogenetics for interrogating cell type-specific synaptic connectivity is unparalleled by any other method, and given the need for such a tool in our studies we aimed to assess the drawbacks and benefits of various opsins developed in recent

years. For our purposes, the especially important factors to consider for optogenetic control of cells and synapses are threefold. First, the opsin should show strong whole cell conductances, and especially strong expression in axons to allow for terminal stimulation in *ex vivo* slices. Second, the opsin should undergo limited desensitization with extended or repeated stimulation. For the most part, electrical stimulation shows effectively zero desensitization at physiological frequencies, however many opsins show reduced current with repeated light stimulation. Finally, the opsin should have fast kinetics as, again, electrical stimulation is limited only by the intrinsic physiological properties of the neurons in which it acts. Thus the onset and offset kinetics should be as close to a square functions as possible.

The opsins of interest here are as follows. ChR2 is the most widely used of the opsins, but is notorious for having relatively poor abilities in following high frequency stimulation (Zhang et al., 2006). ChETA was developed as a mutation of ChR2 for this reason, and is expected to have significantly faster kinetics (Gunaydin et al., 2010). ChIEF was developed independently (Lin et al., 2009) and claims to have faster kinetics and less desensitization than ChR2. Finally, C1V1 is an opsin with a red-shifted excitation wavelength that can theoretically be used in conjunction with ChR2 to achieve dual-wavelength activation of distinct neuronal populations (Erbguth et al., 2012).

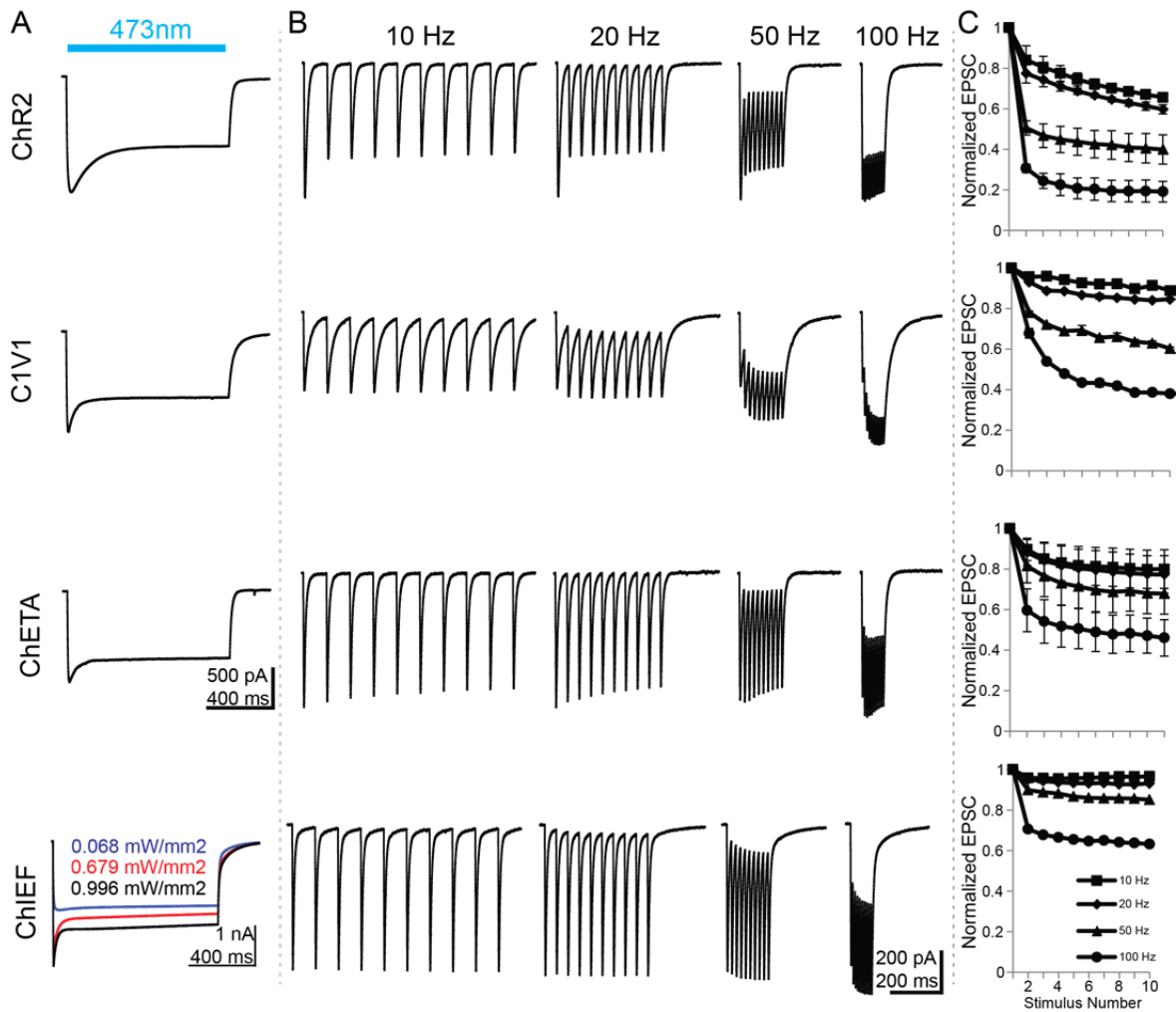


Figure 2: Comparison of whole cell opsin-mediated currents in MSNs

A) Whole cell currents induced by 470nm illumination for 0.5 s reveal distinct onset and offset kinetics, as well as desensitization, of each opsin.

B) Whole cell currents induced by low and high frequency stimulation across a range of 10 to 100 Hz reveal differential abilities of each opsin to follow repetitive stimulation with consistent current magnitudes.

C) Quantification of evoked postsynaptic currents (EPSCs) by light stimulation of MSNs expressing each type of opsin. Repetitive stimulation more strongly attenuated currents in some opsins compared to others.

Each opsin will be tested first in striatal dMSNs in D1-Cre mice where voltage clamp recordings will be performed to assess channel properties (except for ChIEF which will be delivered to layer II/III cortical neurons via *in utero* electroporation under the control of the synapsin promoter). The opsins will then be expressed in the cortex of Emx1-Cre mice under Cre-dependent control in an EF1a-driven adeno-associated viral construct (see above for ChIEF), and synaptic currents will be measured in MSNs. Light pulses will be delivered through the microscope objective using a TTL-controlled LED.

Results

Whole cell voltage clamp recordings from EYFP⁺ MSNs revealed whole cell opsin-evoked currents for all four opsins tested (Figure 2). ChR2 showed the largest whole cell current, ChIEF showed the fastest rise time, ChETA showed the fastest offset time, and ChIEF showed the least amount of desensitization to constant light (Figure 2A, 3A-D). Brief light pulses at frequencies ranging from 10 to 100 Hz (Figure 2B) mirrored the constant light measurements, with ChIEF being the most reliable after ten pulses (Figure 2C). The dynamic range of ChIEF was greatest, with a fairly linear increase in current amplitude up to 1.5 mW/mm² light power. ChR2 showed the smallest dynamic range, with maximal currents reached at only 0.5 mW/mm² (Figure 3E).

Though all opsins were expressed successfully in cortical neurons, light stimulation of corticostriatal afferents during recordings from MSNs revealed synaptic responses only with ChR2 (Figure 4A,B). Within cortex, synaptic responses in layer V were seen with

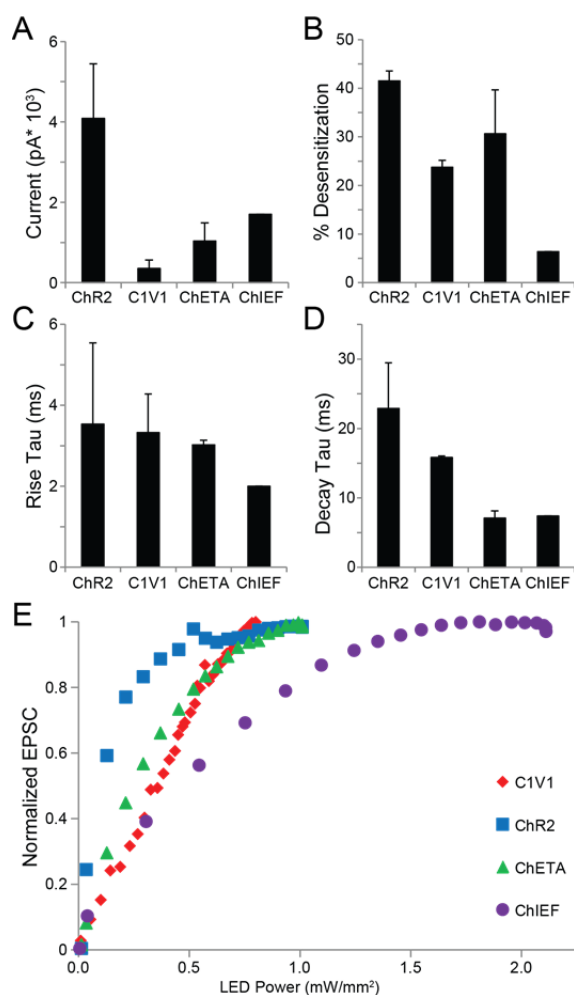


Figure 3: Summary of channel properties of optogenetic proteins in MSNs.

A) Maximum current evoked by $\sim 2 \text{ mW/mm}^2$ 470 nm light. All values are mean $\pm$ SEM.

B) Amount of desensitization of opsin current measured as the steady state current compared to the peak current with 0.5 s light stimulation.

C) Amount of time to reach maximal current from the onset of the light pulse.

D) Decay time constant of the current relative to the offset of the light stimulation.

E) Comparison of the evoked postsynaptic current amplitude at different light powers, normalized to the maximal response for each opsin, revealing different dynamic ranges.

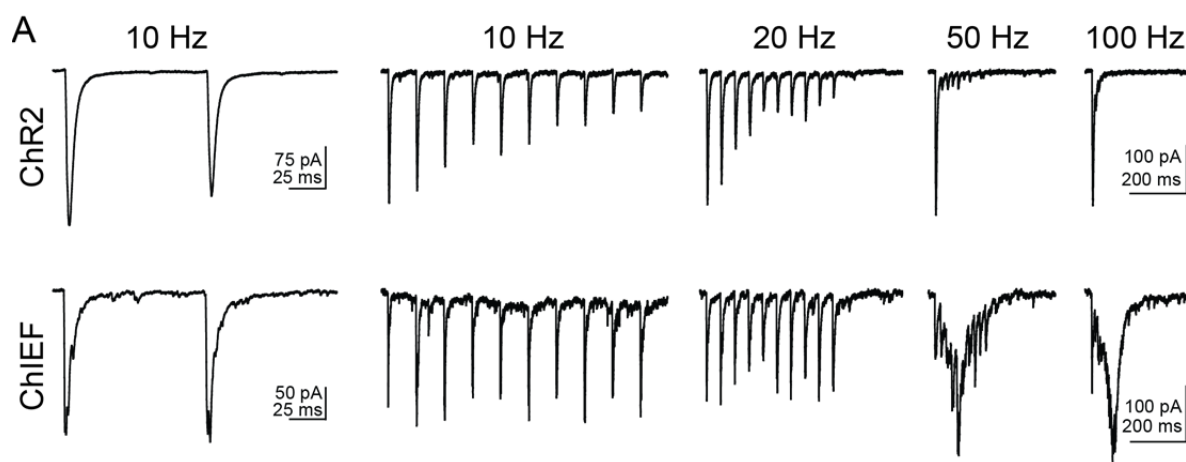


Figure 4: Whole cell synaptic responses evoked by optogenetic terminal stimulation

A) Whole cell excitatory synaptic currents in the presence of $50 \mu\text{M}$ picrotoxin induced by brief 470nm light pulses at various frequencies. Top: ChR2-mediated corticostriatal responses in an MSN. Bottom: ChIEF-mediated LII/III to LV responses.

stimulation of ChIEF+ layer II/III terminals (Figure 4A,B), but ChETA and C1V1 were not able to induce terminal stimulation (data not shown).

Finally, as a control, LED kinetics were measured with a photodiode to ensure that we achieved reliable control of the light stimulation. The amplitudes of light pulses were consistent up to high frequencies, suggesting consistency of light power. The decay constant of the light pulses appeared to be surprisingly slow, akin to the decay constant of whole cell ChETA responses, however this is likely an artifact of slow discharge from the photodiode mechanism (data not shown).

Discussion

Each opsin had strengths and weaknesses in terms of expression, kinetics, and desensitization. In terms of expression, ChR2 was far superior, as every other opsin showed effectively no trafficking to corticostriatal axons even after 6 weeks. ChR2 also induced the largest whole cell current, with the next largest response from ChIEF at less than half the amplitude. ChIEF and ChETA had the fastest kinetics, with ChIEF showing supreme onset and offset time constants. ChIEF also showed the least amount of desensitization, which at low light power was insignificant in magnitude.

In terms of channel function, ChIEF appeared to be superior to all other channels. However, we expressed ChIEF using a difficult method (*in utero* electroporation) because a Cre-dependent virus was not available. Realistically, ChIEF is likely to be difficult to use in practice for this reason. Instead, ChR2 is the most viable option

because of its robust expression, despite the fact that the desensitization and kinetics of the channel are not ideal. Further, because of these properties, ChR2 use will not be viable for experiments to measure endogenous short-term synaptic plasticity. However, it should still be useful to compare between two experimental groups in this context, and thus for the purposes of our experiments it is sufficient.

In future experiments beyond the scope of this dissertation, the use of ChIEF will be especially critical for the characterization of novel synaptic pathways. Further comparison of ChIEF to electrical stimulation of known monosynaptic pathways should also be performed to assess its ability to recapitulate endogenous short-term synaptic dynamics. The modification of ChIEF to enhance axonal trafficking, and the packaging of ChIEF into a vector comparable to that of ChR2 should also be performed, as this will allow for Cre-dependent expression of ChIEF for cell type-specific targeting in long range projections.

Chapter 3

Phasic Dopamine Release Selectively Excites Direct Pathway Medium Spiny Neurons

Abstract

Widespread dopamine receptor expression in every striatal cell type and numerous striatal afferents suggests a potentially complex constellation of roles for phasic dopamine release in the striatum. Here, we tested whether phasic dopamine affects the intrinsic excitability of MSNs or release of transmitter from their glutamatergic cortical inputs. Dopamine release was achieved in slices via viral-mediated transduction of ChR2 in DAT-Cre expressing SNc neurons, and was verified using amperometry and voltammetry. Stimulation of dopamine terminals resulted in fast inhibitory currents in MSNs that were blocked by picrotoxin. Steady-state current-induced firing of iMSNs was unaffected by dopamine release, however dMSNs were rapidly excited by dopamine. This effect was blocked by a D1 receptor antagonist. Dopamine release had no effect on corticostriatal EPSCs in either dMSNs or iMSNs. These results suggest that the phasic release of dopamine preferentially acts on direct pathway neurons, and that the role of dopamine in invigorating behavior is mediated primarily through dMSNs. We tested this hypothesis using a Pavlovian licking paradigm combined with striatal infusion of a D1 or D2 receptor antagonist, or activation of ChR2-expressing striatal dopamine terminals. D1 antagonists decreased anticipatory licking, while dopamine terminal stimulation increased anticipatory licking during the task. D2 receptor antagonists had

no effect on anticipatory licking. These results support our *ex vivo* findings that the main effects of phasic dopamine release are mediated through D1 receptors on dMSNs.

Introduction

The idea of dopamine as a signal for learning has dominated in neuroscience for decades after the discovery that some dopamine neurons appear to encode a reward prediction error (Schultz et al., 1997). Specifically, when an animal receives a reward during a Pavlovian or operant paradigm, dopamine neurons fire if the reward is unexpected or larger than expected, and do not fire if the reward is as expected. If reward is omitted, there is a decrease in dopamine neuron firing rate (Schultz et al., 1997). Thus, some dopamine neurons appear to be updating their downstream targets about reward outcomes. This reward prediction error has been incorporated into learning models and predicts extremely well both the firing of some dopamine neurons and the behavior of animals in operant choice paradigms (Schultz, 2006).

Recently, however, it has become clear that dopamine is involved in other functions besides basic reward outcome updating. First, dopamine neurons have been further categorized based on anatomical and genetic criteria, and it is becoming clear that dopamine neurons projecting to different downstream targets are involved in distinct aspects of behavior (Lammel et al., 2008, 2011, 2012, 2014). Second, dopamine signaling does not always appear as a rapid burst of activity, as recent work has shown a slow ramping of dopamine levels in the striatum that peak as the animal reaches the reward (Howe et al., 2013). For the purposes of our experiments, it is particularly

interesting that, as dopamine neuron responses decrease over trials for expected rewards, the cues that signal the coming rewards begin to elicit burst responses in dopamine neurons (Schultz et al., 1997). These bursts are thought to release a bolus of dopamine into the striatum, which then invigorates responding in the task. Indeed, optogenetic stimulation of dopamine terminals in conjunction with cue onset decreases response latency in the task (Berke JD, personal communication). Furthermore, infusion of a D1 receptor antagonist into the striatum increases the latency to respond to these cues, suggesting a role for D1 receptors and potentially the direct pathway in the invigoration of movement (Nakamura and Hikosaka, 2006).

The effects of dopamine receptor activation on MSN intrinsic properties have been extensively studied in reduced preparations. By dissociating MSNs into single-cell cultures, indirect effects of dopamine on MSNs (e.g., modulation of cholinergic tone via D2 receptors) have been controlled for and the signaling cascades and downstream targets of dopamine receptors are largely understood. D1 and D2 receptors are oppositely coupled to adenylyl cyclase via G-protein signaling, such that D1 receptors stimulate and D2 receptors reduce the production of cyclic AMP and thus activation of protein kinase A (PKA). This modulation of PKA has opposing effects on sodium, potassium, and calcium currents in the two MSN subtypes (Surmeier et al., 2007). However, the time scale of these experiments are very slow compared to the time scale of physiological dopamine release, and thus it is not known whether rapid activation of either dopamine receptor subtype has significant effects on the firing of MSNs.

We sought to address the true effects of phasic dopamine release on MSN firing by recording from identified direct and indirect pathway MSNs in acute brain slices from DAT-Cre mice injected with a Cre-dependent ChR2 virus in the SNc. First we will address the effects of dopamine release on the firing rate of identified dMSNs and iMSNs. Our expectations based upon the literature are increases in dMSN and decreases in iMSN firing with dopamine release. Then we will assess whether corticostriatal inputs to MSNs are altered by phasic dopamine release. Given the reported expression of D2 receptors on cortical terminals, we predict a decrease in the probability of release, and thus the amplitude of excitatory postsynaptic currents (EPSCs) in d- and iMSNs.

Results

Injection of AAV-EF1a-DIO-ChR2-EYFP into the SNc of DAT-cre;D1-tdTomato mice resulted in strong labeling of cell bodies in the midbrain and extensive labeling of axons throughout the striatum and nucleus accumbens. These EYFP⁺ neurons were also labeled by immunostaining against tyrosine hydroxylase, a synthesizing enzyme necessary for the production of dopamine (Figure 5A).

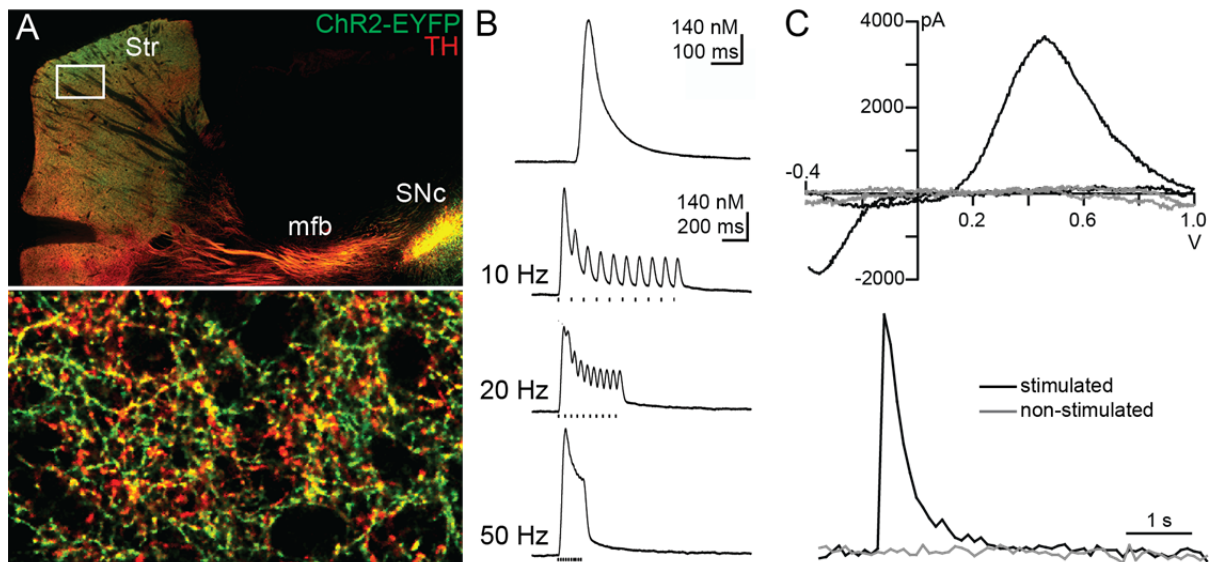


Figure 5: *Ex vivo* optogenetic control of nigrostriatal dopamine release.

A) Injection of AAV-DIO-EF1a-ChR2-EYFP into the substantia nigra pars compacta (SNc) of DAT-Cre mice results in ChR2-EYFP expression in tyrosine hydroxylase (TH) positive neurons of the SNc and their terminals in the striatum. The image below is a magnification of the region boxed in white above.

B) Amperometric measurements of dopamine release in the striatum from acute brain sections in response to brief pulses of 470 nm light. *Top*: dopamine release in response to a single 5 ms pulse of light. *Bottom*: stimulation at higher frequencies reveals individual phasic release events that summate.

C) Voltammetric measurements in the same slices at baseline (grey trace) and in response to a single 5 ms pulse of light. Note the oxidation and reduction peaks in the voltammogram around +0.5 V and -0.4 V, respectively.

D) Voltammetry measurement of dopamine release at the oxidation peak across time, with stimulation occurring immediately prior to the rapid rise in the current. Note the similar time course (~1s) of the dopamine signal between the amperometry and voltammetry measurements.

In voltammetric recordings from striatal slices, brief light pulses (470 nm, 1-5 ms, ~1 mW/mm²) resulted in a large peak and trough in the voltammogram, corresponding to the oxidation and reduction peaks of dopamine. When this oxidation peak was measured across time, there was a clear phasic signal that decayed with the expected kinetics of

dopamine clearance (Figure 5C). Furthermore, amperometry at the peak oxidative voltage revealed reliable dopamine release up to relatively high stimulation frequencies (Figure 5B).

In initial voltage clamp recordings from MSNs, we were surprised to see fast ionotropic currents with a reversal potential around the chloride equilibrium in our conditions, at -40 mV (Figure 6D,E). These currents were blocked by bath application of 50 μ M picrotoxin, suggesting a GABAergic component to the release. While this was at first a concern, we learned that another group had observed a similar phenomenon, and they ultimately published a study showing direct release of GABA from dopaminergic neurons (Tritsch et al., 2012). In the remaining experiments, we included picrotoxin in the bath to block GABAergic responses, as we were primarily interested in the modulatory effects of dopamine. However, these experiments have been performed with intact GABAergic transmission with the same results (data not shown), save for a brief inhibition of MSN firing following dopamine terminal stimulation.

As an important technical side note, we noticed a steady reduction in both the amperometry signal and the GABA-mediated inhibitory postsynaptic current (IPSC) as the experiment progressed (Figure 6D). Thus it became clear that under these experimental conditions, optogenetic release of dopamine from SNc terminals in the striatum undergoes progressive depression, or “run down,” and we accordingly decreased our rate of stimulation to prevent this presumed artifact from affecting our results.

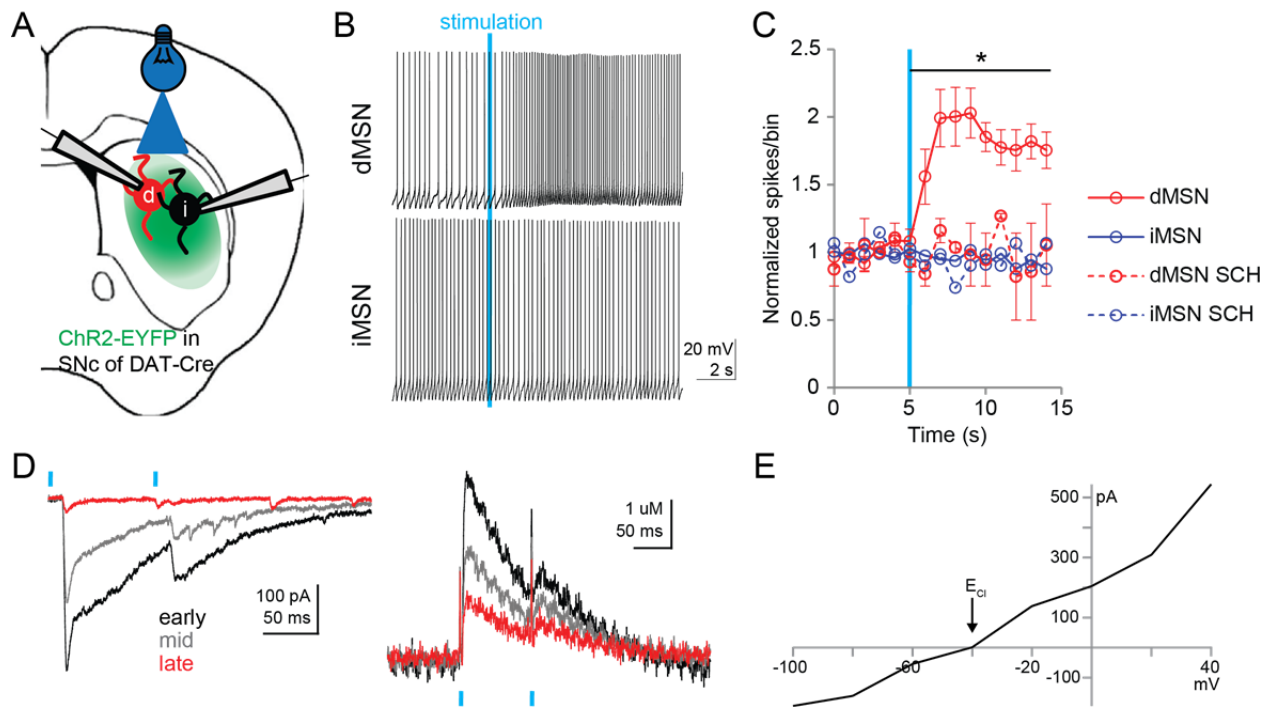


Figure 6: Phasic dopamine release rapidly increases dMSN excitability.

A) Simultaneous paired whole cell current clamp recordings in the presence of 50 μ M picrotoxin were obtained from one dMSN and one iMSN in ChR2-EYFP-expressing DAT-Cre mice. MSNs were injected with steady state positive current to induce low frequency (~5 Hz) firing, then 470 nm light was flashed briefly through the objective to cause phasic dopamine release.

B) Example traces from a dMSN (top) and iMSN (bottom) recorded simultaneously, showing the positive effect of phasic dopamine release on dMSN firing and no effect on iMSN firing.

C) Summary data showing the increase in dMSN firing after dopamine terminal stimulation, which was blocked by 5 μ M SCH23390, a specific D1 receptor antagonist. Phasic dopamine release had no effect on iMSN firing rate.

D) Voltage clamp recordings from an MSN in the absence of picrotoxin show fast ionotropic currents (left) in conjunction with simultaneous amperometric measures of dopamine release (right). Both the dopamine and presumptive GABAergic current depressed over the course of the experiment (black, grey, and red traces are early, mid, and late experiment, respectively), which in this case used 20 s inter-trial intervals. For all other experiments we used 2 min inter-trial intervals for this reason.

E) Current-voltage plot showing the reversal of the fast ionotropic current from D, which reversed around the equilibrium potential for chloride under our recording conditions.

Patch clamp recordings from MSNs were conducted in pairs (dMSN + iMSN) or alone, and MSNs were identified by their tdTomato expression (dMSNs) or lack thereof (iMSNs) (Figure 6A). Interneurons were rarely encountered, as they are less than 5% of the striatal population, and were easily identified by intrinsic properties, spike pattern and waveform. Steady-state current was injected in MSNs to achieve tonic firing around 4-8 Hz, typical for MSNs *in vivo*. Compared to a 5 s baseline spiking

period, activation of dopamine terminals resulted in no change in the firing rate of iMSNs across a 5 s time

period ($98.1 \pm 2.5\%$, $p > 0.05$). However, the dMSN firing rate doubled after phasic dopamine release ($173.3 \pm 11.5\%$, $p < 0.05$), and this increase in spike frequency was sustained until the cell was hyperpolarized back to rest, suggesting the enhancement of a voltage-gated conductance by D1 receptors. Indeed, the D1 receptor antagonist SCH23390 completely blocked this increase in firing rate, implicating D1 receptors in this rapid modulation (Figure 6B,C).

We next tested whether phasic dopamine release acutely modulates MSN excitatory inputs. Voltage clamp recordings from identified MSNs (Figure 7A) revealed no changes in the frequency or amplitude of spontaneous excitatory currents in dMSNs or iMSNs after dopamine terminal stimulation (Figure 7B,C). We then recorded from single identified MSNs in voltage clamp while stimulating the cortex using a bipolar electrode (Figure 7D) in slices previously described to leave corticostriatal transmission intact. 0.5 Hz stimulation of cortical inputs resulted in EPSCs of consistent size (Figure 7E), and optogenetic stimulation of dopamine terminals had no effect on the amplitude of these responses in either d- or iMSNs (Figure 7F). Given the modulation of dMSNs in these very same slices, we conclude that the phasic release of dopamine does not affect the release of glutamate from corticostriatal terminals onto MSNs.

To test the effects of phasic dopamine release on a Pavlovian behavior, we administered liquid sucrose rewards to water-restricted head-fixed mice on a spherical

treadmill in the presence of an auditory cue (Figure 8A). The onset of the reward delivery was variable within the range of 2-3 seconds after cue onset. With repeated exposure, mice displayed regular licking starting shortly after the cue onset in anticipation of reward delivery (Figure 8E). The magnitude of this anticipatory licking was modulated by reward size, and we therefore reasoned that striatal dopamine release in response to the cue could affect the vigor of the response. To test this hypothesis, we stimulated the terminals of SNc neurons in the striatum (Figure 8B,C) in conjunction with cue onset on 20% of trials (50 Hz, 10 ms pulses, 500 ms duration). Anticipatory licking rate was significantly increased on stimulation trials (Figure 8E,F), and the amount of post-cue consummatory licking was unaffected, suggesting invigoration of the learned response. Conversely, infusion of the D1 antagonist SCH23390 into the striatum (Figure 8I) reduced anticipatory licking without affecting consummatory licking (Figure 8J), suggesting a specific role for D1 receptors, presumably on dMSNs, in the vigor of this learned behavioral response. Infusion of a D2 antagonist had no effect on anticipatory or consummatory licking (Figure 8J).

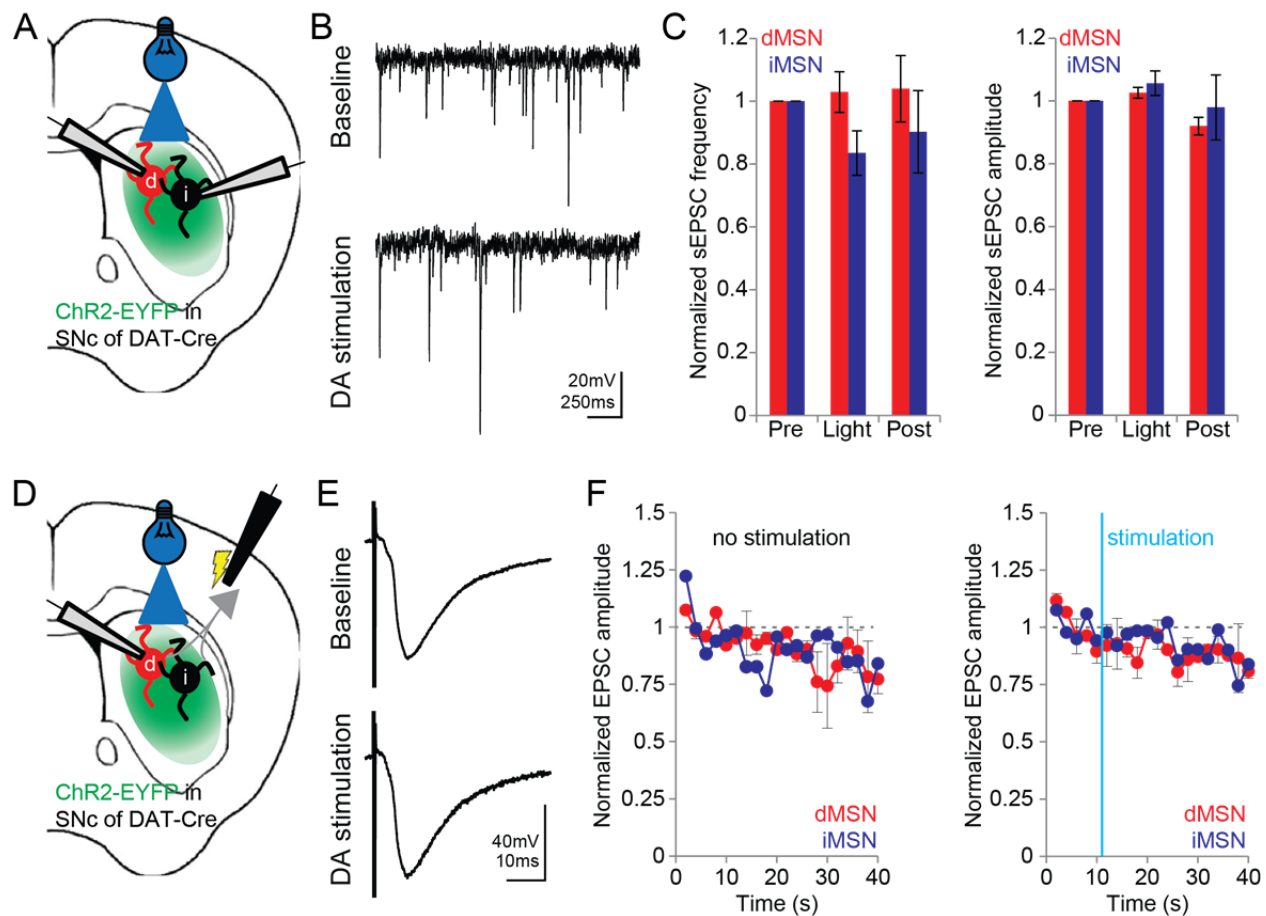


Figure 7: Excitatory inputs to MSNs are not affected by phasic dopamine release.

A) Simultaneous paired whole cell voltage clamp recordings were obtained from one dMSN and one iMSN to record spontaneous excitatory currents before and after dopamine terminal stimulation in ChR2-EYFP-expressing DAT-Cre mice.

B) Example traces from an iMSN before (top) and immediately following 470 nm light stimulation of nigrostriatal dopamine terminals in the striatum.

C) Summary data showing normalized frequency (left) and amplitude (right) of spontaneous excitatory synaptic currents (sEPSCs) in dMSNs (red) and iMSNs (blue). Despite a trend toward decreased frequency in iMSNs with phasic dopamine release not significant changes were seen.

D) Whole cell voltage clamp recordings were obtained from identified MSNs, and corticostriatal inputs were electrically stimulated at 0.5 Hz using a bipolar electrode in cortex. After five baseline EPSCs, ChR2-EYFP-expressing nigrostriatal dopamine terminals were stimulated with 470 nm light.

E) Example EPSCs during the baseline (top) and immediately following dopamine terminal stimulation.

F) *Left:* 0.5 Hz stimulation of corticostriatal inputs causes minor rundown of the synaptic response in dMSNs (red) and iMSNs (blue) over 40 s of stimulation. This control involved no stimulation of dopamine terminals to measure the intrinsic rundown with the stimulation protocol. *Right:* the same corticostriatal stimulation protocol resulted in similar amplitude responses after dopamine terminal stimulation (blue line), revealing no increase or decrease in synaptic release due to dopamine modulation compared to the “no stimulation” condition.

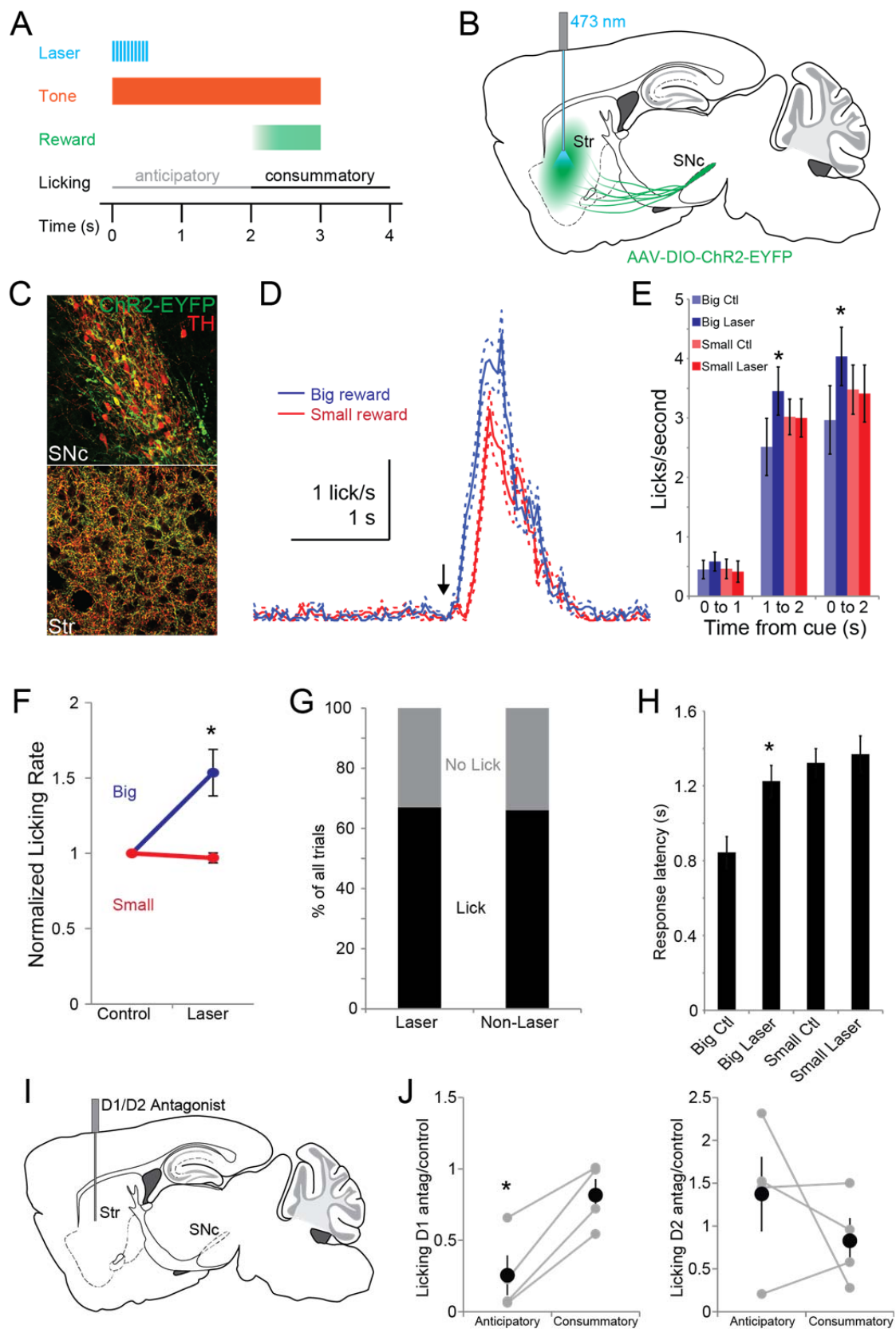


Figure 8: Phasic striatal dopamine release invigorates behavioral output.
See following page for legend.

Figure 8: Phasic striatal dopamine release invigorates behavioral output.

A) Schematic of behavioral paradigm. 2-3 s after the onset of an auditory tone a sucrose reward is delivered to the animal. With training, animals display two main epochs of licking: anticipatory (after the tone but before the possibility of reward delivery) and consummatory (after the possibility of reward delivery). On 20% of trials, 470nm light is delivered through an optical fiber implanted in the ventral lateral striatum (see B).

B) ChR2-EYFP was expressed in the SNc of DAT-Cre mice, and a 200 μ m optical fiber was implanted in the ventral lateral striatum. 10 ms pulses of 50 Hz 473 nm laser light was delivered for 500 ms at the start of the cue on 20% of trials.

C) Confocal images showing expression of ChR2-EYFP (green) and tyrosine hydroxylase (TH, red) in the SNc (top) and striatum (bottom).

D) Average licking rate for an example animal aligned to the cue (arrow), showing that the response to the big reward (blue) is faster and larger than the response to the small reward (red). Dotted lines indicate SEM.

E) Average licking rate for small and big rewards during non-laser (Ctl) and stimulation (Laser) trials. There was a selective increase in anticipatory licking for stimulation in big reward trials.

F) The same data in F over the full anticipatory licking period, normalized to baseline licking.

G) There was no difference in the probability of licking for trials where the laser was on versus off.

H) Latency to the first lick was shorter for big reward trials, and was increased on big reward trials with phasic dopamine terminal stimulation.

I) A separate group of animals was bilaterally implanted with 33-gauge cannulas in the ventral lateral striatum, and infused with a control medium or dopamine receptor antagonist (saline versus SCH23390 to block D1 receptors; DMSO versus sulpiride to block D2 receptors).

J) Anticipatory licking in the presence of a D1 (left) or D2 (right) antagonist compared to control infusions. There was a selective decrease in anticipatory licking after D1, but not D2, receptor antagonist infusion.

Discussion

These experiments have addressed four major predictions of the classical model (Figure 1B, bottom). One prediction was robustly confirmed, the increased excitability of dMSNs to phasic dopamine release, while three others were not supported by the evidence: modulation of iMSN intrinsic excitability, and suppression of transmission at dMSN and iMSN cortical inputs. These findings are not only surprising, but require reshaping of the classical model of dopaminergic modulation of basal ganglia circuits (Surmeier et al., 2007). Certainly dopamine must have some effects on iMSNs and corticostriatal inputs, however these effects may not occur under circumstances of rapid, phasic dopamine release. Instead, perhaps decreases in dopamine tone or slow changes in dopamine concentration have a greater affect on these other targets.

Indeed, recent unpublished work in our lab has implicated iMSNs in the specific encoding of value-related information across slower time periods. Thus dopamine may modulate iMSNs on a slower time course through changes in tonic firing rate in order to update the striatum about the overall value of engaging in a particular task.

The rapid increase in dMSN excitability by dopamine is also in line with recent unpublished work in our lab. dMSNs appear to encode information related to the direction and vigor of movement, and thus cue-induced dopamine release in the striatum would be expected to rapidly modulate an animal's behavioral output given the identity of the cue. Indeed, stimulation of dopamine terminals in the striatum significantly enhanced anticipatory licking in our Pavlovian task, while D1 receptor antagonism reduced the anticipatory licking response. It is interesting that this effect was specific to the big reward trials, and suggests that the mechanisms behind dopamine's modulation of dMSN gain may not be linear. Indeed, previous work has shown increased L-type calcium channel function with D1 receptor activation at depolarized, but not hyperpolarized, membrane potentials (Hernández-López et al., 1997). This suggests that dMSN firing will be most robustly enhanced if the cell is very depolarized or already firing, as in our experiments. Because the anticipatory responses in the small reward condition are much weaker than in the large condition, dMSNs may not be sufficiently depolarized to permit significant L-type channel modulation by D1 receptors. This hypothesis will be most rigorously tested with *in vivo* single unit recordings of identified MSNs during the Pavlovian paradigm with optogenetic activation of striatal dopamine terminals. Indeed, current methods of identifying neurons with *in vivo* extracellular

recordings requires ChR2 (Lima et al., 2009), thus a second opsin such as C1V1 would be required to specifically stimulate dopamine neurons. Given the current unavailability of robust red-shifted opsins (see Chapter 2), this experiment may have to await advances in optogenetic protein development.

Another group has recently found similar effects of dopamine terminal stimulation in the striatum on the latency of operant response execution during an operant paradigm. Stimulation of dopamine terminals during reward consumption instead affected the animal's choice behavior on the next trial, and not the vigor of the response (Berke JD, personal communication). Thus dMSNs are poised to tightly control motor responses to environmental cues, and their rapid excitation by phasic dopamine release and activation of D1 receptors appears to strongly invigorate behavioral responses.

Given the recent observations that dopamine levels in the striatum ramp slowly over the course of behavioral task trials (Howe et al., 2013), further study of dopaminergic modulation of striatal circuits should utilize *in vivo* techniques or patterns of dopamine release seen *in vivo*. Our experiments use brief pulses of stimulation to induce burst-like releases of dopamine from terminals, akin to activity seen in response to the cue during operant behaviors (Schultz et al., 1997). However, it is not clear whether this type of dopamine transmission is typical for ethologically relevant behaviors outside of the laboratory. The reduced preparation utilized here is critical for understanding the basic properties and mechanisms of dopaminergic modulation of dMSN firing. However, in order to understand the computational effects of dopamine in this context, intact preparations will be necessary. For example, the modulation of dMSN firing is clearly

dependent upon membrane potential. But is this truly a bimodal effect (Hernández-López et al., 1997), or are there non-linear relationships inherent between the gain increases and either the membrane potential, dopamine concentration, or both? These questions will require simultaneous *in vivo* measurements of dMSN membrane potential, striatal dopamine concentration, and behavioral output.

Similarly, a major question not addressed by these experiments is the role of acute changes in dopamine concentration that are not phasic in nature. For example, dopamine concentration varies across behavioral tasks depending upon the rate of reward, and these changes are associated with different levels of invigoration in responding (Niv et al., 2007). Given the recent work in our lab showing iMSN tracking of reward state, these slower changes in dopamine may preferentially effect iMSN excitability and corticostriatal transmission. Thus, while phasic dopamine may act specifically at dMSNs to invigorate behavior, slower changes in dopamine levels that are not “chronic” are likely important for the overall level of motivation and engagement. This hypothesis can be tested using the block structure of the present behavioral paradigm, combined with measurements of MSN activity and dopamine neurons as described above.

Chapter 4

Pathway-Specific Remodeling of Thalamostriatal Inputs in Parkinsonian Mice

Abstract

Suppression of movement in Parkinson's disease (PD) is thought to arise from increased efficacy of the indirect pathway basal ganglia circuit, relative to the direct pathway. However, the underlying pathophysiological mechanisms remain elusive. To examine whether changes in the strength of synaptic inputs to these circuits contribute to this imbalance, we obtained paired whole-cell recordings from striatal direct- and indirect-pathway medium spiny neurons (dMSNs and iMSNs) and optically stimulated inputs from sensorimotor cortex or intralaminar thalamus in brain slices from control and dopamine-depleted mice. We found that dopamine depletion selectively decreased synaptic strength at thalamic inputs to dMSNs, suggesting that thalamus drives asymmetric activation of basal ganglia circuitry underlying parkinsonian motor impairments. Consistent with this hypothesis, *in vivo* chemogenetic inhibition of intralaminar thalamic neurons reversed motor deficits in dopamine-depleted mice. These results implicate thalamostriatal projections in the pathophysiology of PD and support interventions targeting thalamus as a potential therapeutic strategy.

Introduction

The underlying pathophysiology of Parkinson's disease (PD) remains poorly understood despite decades of research. The pathological hallmark of PD is a progressive loss of dopamine neurons in the substantia nigra pars compacta, which extensively innervates the dorsal striatum (Ehringer and Hornykiewicz, 1960). Loss of striatal dopamine is proposed to increase efficacy of the indirect pathway basal ganglia circuit (thought to normally suppress movement) and/or decrease efficacy of the direct pathway circuit (thought to normally facilitate movement) (Albin et al., 1989; DeLong, 1990). Consistent with this model, activation of the direct pathway in parkinsonian animals restores normal motor function (Kravitz et al., 2010). A number of studies revealed PD-associated intrinsic and synaptic changes in the basal ganglia pathway cells of origin, the direct and indirect pathway striatal medium spiny neurons (dMSNs and iMSNs) (Day et al., 2006; Fieblinger et al., 2014; Suárez et al., 2014). However, anatomical observations provide only an indirect measure of synaptic strength, and physiological readouts have not addressed changes to excitatory striatal inputs arising from distinct brain regions, which may be differentially affected in PD.

Firing of striatal MSNs requires significant excitatory synaptic drive (Wickens and Wilson, 1998), which they receive primarily from the cortex and thalamus (Kemp and Powell, 1971). Corticostriatal afferents have received the most attention in PD research, and dysregulation of synaptic plasticity at these inputs is thought to contribute to imbalanced basal ganglia circuit function (Kreitzer and Malenka, 2008; Surmeier et al., 2009). In contrast, fewer studies have examined the thalamostriatal system in PD (Smith et al., 2014). VGLUT2+ neurons in the intralaminar thalamus provide a major

source of excitatory input to the striatum (Freneau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001). Intriguingly, intralaminar neurons in the centromedian/parafascicular nucleus (Pf) show increased activity and pathogenic oscillations in PD animal models (Jouve et al., 2010; Orioux et al., 2000; Parr-Brownlie et al., 2009; Yan et al., 2008), as well as tremor-locked activity in human patients (Magnin et al., 2000). Deep brain stimulation (DBS) targeting Pf shows significant promise for reducing PD symptoms such as tremor, gait, and sensorimotor neglect (Caparros-Lefebvre et al., 1999; Jouve et al., 2010; Mazzone et al., 2006; Peppe et al., 2008; Stefani et al., 2009).

To examine input strength from the two major dorsal striatal afferents to MSN subtypes, we optically stimulated ChR2-expressing axons arising from either sensorimotor cortex or intralaminar thalamus, while performing simultaneous whole-cell recordings from dMSN/iMSN pairs. Following dopamine depletion, we found that the relative strength of thalamic inputs to dMSNs and iMSNs is reversed, biasing excitatory drive to the indirect pathway. Consistent with this observation, chemogenetic inhibition of intralaminar thalamus markedly improved parkinsonian motor deficits.

Results

Reduced thalamostriatal drive to dMSNs in parkinsonian animals

To directly assay the relative excitatory input strength onto dMSNs versus iMSNs, we performed simultaneous voltage-clamp recordings from identified d/iMSN pairs in acute sections containing the dorsolateral striatum (DLS) from mice expressing Cre-dependent ChR2-EYFP in thalamostriatal (VGLUT2-Cre) or corticostriatal (Emx1-Cre) projections. One to two weeks before recordings, 6-hydroxydopamine (6OHDA) or saline was injected into the medial forebrain bundle to produce parkinsonian and control animals, respectively (Figure 9D). In VGLUT2-Cre animals, strong EYFP expression was centered in Pf (Figure 9A), and dense axonal expression was observed in the DLS (Figure 9B). Brief light pulses through the microscope objective elicited fast excitatory postsynaptic currents (EPSCs) in both MSN subtypes (Figure 9F). On average, dMSNs in control animals displayed larger EPSC amplitudes than iMSNs (dMSN, 408.2 ± 86.0 ; iMSN, 311.1 ± 58.7 pA; $n = 10$; Figure 9G), and the average pairwise ratio revealed stronger dMSN synaptic drive (d:i ratio, 1.5 ± 0.3 ; Figure 9H). However, one to two weeks following 6OHDA-mediated ablation of striatal dopamine, EPSC amplitudes in iMSNs were significantly larger than those in dMSNs (dMSN, 180.0 ± 36.2 ; iMSN, 406.8 ± 80.9 pA; $n = 16$; Figure 9G), and the average pairwise ratio revealed a shift to preferential iMSN synaptic drive (d:i ratio, 0.70 ± 0.2 ; $p = 0.016$ vs. control; Figure 9H).

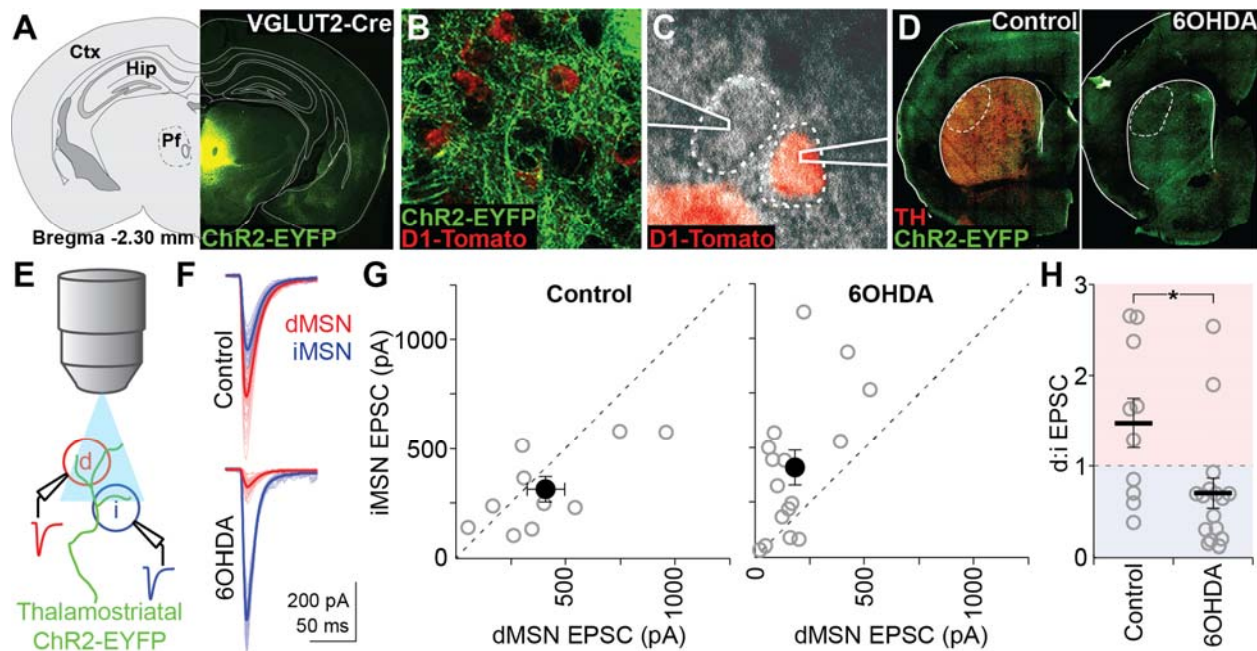


Figure 9: Dopamine depletion selectively reduces thalamic input to dMSNs.

A) AAV-DIO-ChR2-EYFP expression targeting intralaminar thalamus in VGLUT2-cre x D1-Tomato mice. Ctx, cortex; Hip, hippocampus; Pf, parafascicular nucleus.

B) ChR2-EYFP+ axons in the DLS surrounding D1-Tomato+ dMSNs.

C) DIC image showing simultaneous patch-clamp recordings from one D1-Tomato+ and one D1-Tomato- MSN.

D) Epifluorescence image of ChR2-EYFP and tyrosine hydroxylase (TH) expression in coronal sections containing the DLS of saline- (control) and 6OHDA-injected animals. Recordings were obtained within the dotted region.

E) Schematic of recording setup: whole-cell responses to thalamostriatal stimulation evoked by 470nm LED pulses through the objective were recorded simultaneously from one dMSN (red) and one iMSN (blue).

F) Example traces from a pair of MSNs in a control and 6OHDA-lesioned animal. Thick traces are averages and thin traces are individual sweeps.

G) Individual (grey) and mean (black) values for dMSN and iMSN EPSC amplitudes for all recorded pairs in control (left, n = 10 pairs) and 6OHDA-lesioned (right, n = 16 pairs) animals.

H) Pairwise ratios of dMSN and iMSN EPSC amplitudes in control and 6OHDA-lesioned animals. Points above 1 (red shading) represent dMSN-favored pairs and points below 1 (blue shading) represent iMSN-favored pairs. All summary data are presented as mean $\pm$ SEM. Asterisk is $p = 0.016$.

No relative change in corticostriatal synaptic strength in parkinsonian animals

ChR2-EYFP injections into Emx1-Cre animals resulted in extensive labeling of sensorimotor cortical neurons across layers (Figure 10A) and intense axonal labeling in the DLS (Figure 10B). As with thalamostriatal recordings, brief light pulses through the

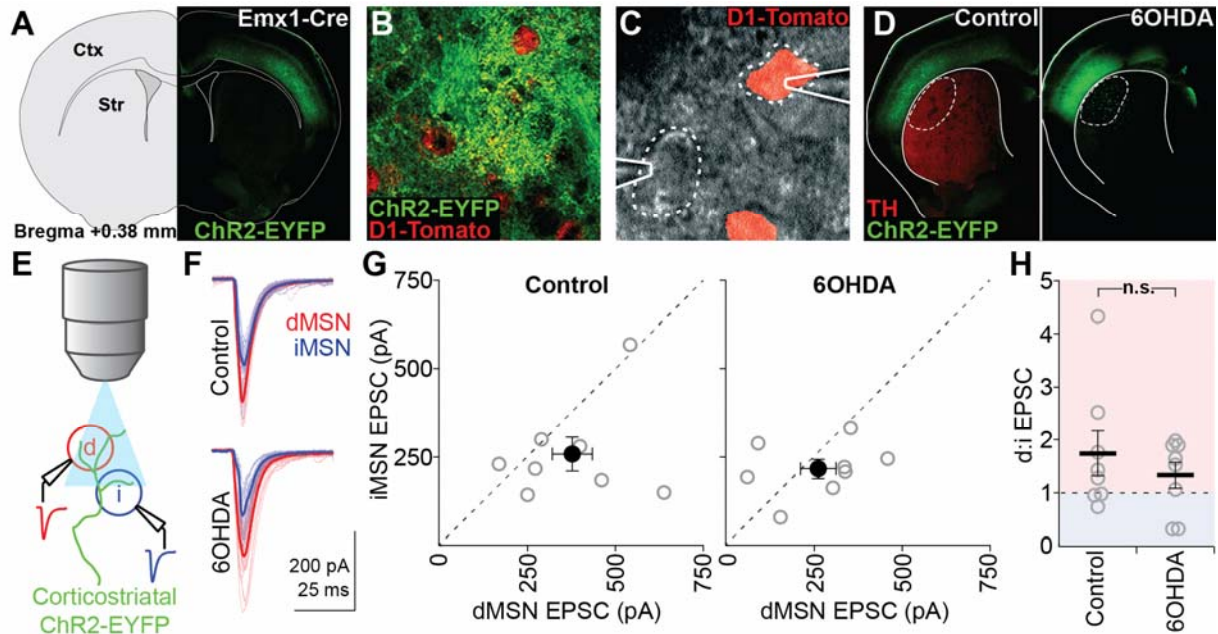


Figure 10: Maintenance of relative corticostriatal drive after dopamine depletion.

A) AAV-DIO-ChR2-EYFP expression targeting sensorimotor cortex in Emx1-cre x D1-Tomato mice. Ctx, cortex; Str, striatum.

B) ChR2-EYFP+ axons in the DLS surrounding D1-Tomato+ dMSNs.

C) DIC image showing simultaneous patch-clamp recordings from one D1-Tomato+ and one D1-Tomato- MSN.

D) Epifluorescence image of ChR2-EYFP and tyrosine hydroxylase (TH) expression in coronal sections containing the DLS of saline- (control) and 6OHDA-injected animals. Recordings were obtained within the dotted region.

E) Schematic of recording setup: whole-cell responses to corticostriatal stimulation evoked by 470nm LED pulses through the objective were recorded simultaneously from one dMSN (red) and one iMSNs (blue).

F) Example traces from a pair of MSNs in a control and a 6OHDA-lesioned animal. Thick traces are averages and thin traces are individual sweeps.

G) Individual (grey) and mean (black) values for dMSN and iMSN EPSC amplitudes for all recorded pairs in control (left, n = 16 pairs) and 6OHDA-lesioned (right, n = 12 pairs) animals.

H) Pairwise ratios of dMSN and iMSN EPSC amplitudes in control and 6OHDA-lesioned animals. Points above 1 (red shading) represent dMSN-favored pairs and points below 1 (blue shading) represent iMSN-favored pairs; n.s. is not significant. All summary data are presented as mean $\pm$ SEM.

microscope objective (Figure 10E) elicited short-latency EPSCs in both MSN

subtypes (Figure 10F). Paired recordings revealed larger EPSCs in dMSNs than

iMSNs in control animals (dMSN, 403.6 ± 40.9 ; iMSN, 323.7 ± 45.2 pA; d:i ratio, $1.5 \pm$

0.2 ; n = 16; Figure 10G), as seen with our thalamostriatal stimulation and in previous

investigations of relative cortical drive to d/iMSN pairs (Kress et al., 2013).

Recordings from 6OHDA-lesioned animals revealed no significant change in the relative

corticostriatal drive to dMSNs and iMSNs compared to control animals (dMSN, 316.3 ± 43.3 ; iMSN, 240.8 ± 23.1 pA; d:i ratio, 1.4 ± 0.2 ; $n = 12$; $p = 0.741$ vs. control; Figure 10G,H).

Postsynaptic decrease in AMPAR-mediated responses at thalamo-dMSN synapses

To test whether thalamostriatal connectivity changes in parkinsonian animals were associated with alterations in pre- or postsynaptic function, we measured AMPA- and NMDA-EPSCs (Figure 11A) and paired pulse ratios (PPR; Figure 11D). Thalamostriatal inputs showed a specific reduction in dMSN:iMSN AMPA ratio, but not in dMSN:iMSN NMDA ratio (control vs. 6OHDA; d:i AMPA, 1.2 ± 0.2 vs. 0.7 ± 0.1 , $p = 0.026$; d:i NMDA, 1.1 ± 0.2 vs. 1.2 ± 0.3 , $p = 0.628$; $n = 18$ vs. 15 ; Figure 11B). In theory, the decreased thalamostriatal d:iMSN AMPA ratio in parkinsonian mice could arise from a postsynaptic decrease in dMSN and/or an increase in iMSN EPSC amplitude. However, the AMPA:NMDA ratio was selectively decreased at thalamo-dMSN, but not thalamo-iMSN, synapses (control vs. 6OHDA AMPA:NMDA; dMSN, 4.3 ± 0.7 vs. 2.2 ± 0.3 , $n = 18$ vs. 16 , $p = 0.015$; iMSN, 4.3 ± 0.8 vs. 3.5 ± 0.5 , $n = 19$ vs. 16 , $p = 0.389$; Figure 11C), indicating a decrease in dMSN EPSC amplitude. Cortico-dMSN synapses showed a slight increase in AMPA:NMDA ratio (control vs. 6OHDA AMPA:NMDA; dMSN, 3.3 ± 0.3 vs. 4.5 ± 0.5 , $n = 16$ vs. 13 , $p = 0.028$; iMSN, 3.4 ± 0.5 vs. 3.8 ± 0.4 , $n = 12$ vs. 12 , $p = 0.568$; Figure 11C) but no significant change in dMSN:iMSN AMPA and NMDA currents

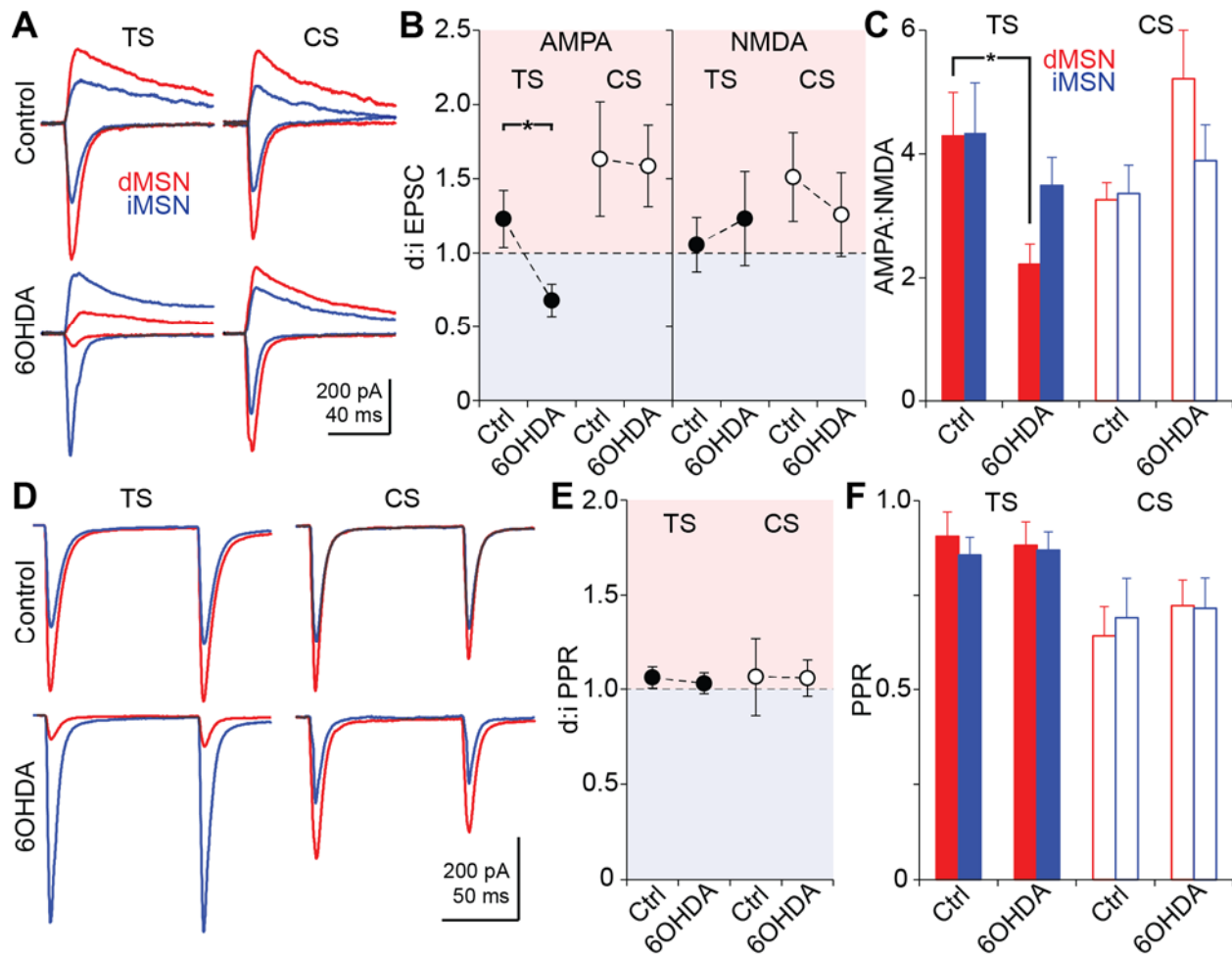


Figure 11: Selective reduction in postsynaptic AMPAR-mediated currents at thalamo-dMSN synapses in parkinsonian animals.

(A) Averaged traces of four individual dMSN (red) and iMSN (blue) pairs showing thalamostriatal (TS) and corticostriatal (CS) responses in control and 6OHDA-lesioned animals. For each pair, lower traces were recorded at -80 mV and upper traces were recorded at +40 mV.

(B) dMSN:iMSN ratio of AMPA (left) and NMDA (right) currents at TS (filled circles) and CS (open circles) synapses. Points above 1 (red shading) represent dMSN-favored pairs and points below 1 (blue shading) represent iMSN-favored pairs. TS control $n = 18$, TS 6OHDA $n = 15$, CS control $n = 10$, CS 6OHDA $n = 10$. Asterisk is $p = 0.026$.

(C) Unpaired data showing average values of AMPA:NMDA ratios at TS (filled bars) and CS (open bars) synapses for dMSNs and iMSNs in control (Ctrl) and 6OHDA-lesioned animals. dMSNs and iMSNs: TS control $n = 18$ and 19 , TS 6OHDA $n = 16$ and 16 , CS control $n = 16$ and 12 , CS 6OHDA $n = 13$ and 12 . TS asterisk is $p = 0.015$, CS asterisk is $p = 0.028$.

(D) Averaged traces of four individual dMSN (red) and iMSN (blue) pairs showing thalamostriatal (TS) and corticostriatal (CS) responses in control and 6OHDA-lesioned animals. For each pair, the paired pulse ratio (PPR) was calculated as EPSC2/EPSC1.

(E) dMSN:iMSN ratio of PPR at TS (filled circles) and CS (open circles) synapses. Points above 1 (red shading) represent dMSN-favored pairs and points below 1 (blue shading) represent iMSN-favored pairs. TS control $n = 10$, TS 6OHDA $n = 16$, CS control $n = 8$, CS 6OHDA $n = 8$.

(F) Unpaired data showing average PPR at TS (filled bars) and CS (open bars) synapses for dMSNs and iMSNs in control (Ctrl) and 6OHDA-lesioned animals. All summary data are presented as mean $\pm$ SEM. TS control $n = 10$, TS 6OHDA $n = 16$, CS control $n = 16$, CS 6OHDA $n = 12$.

(control vs. 6OHDA; d:i AMPA, 1.4 ± 0.2 vs. 1.6 ± 0.2 , $p = 0.365$; d:i NMDA, 1.3 ± 0.1 vs. 1.3 ± 0.2 , $p = 0.900$; $n = 10$ vs. 10 ; Figure 11B).

The PD-associated thalamostriatal reorganization could also be produced by changes in presynaptic glutamate release, which should be reflected in PPR measurements (Figure 11D). By experimentally manipulating the external calcium concentration, we confirmed the sensitivity of optogenetically-evoked PPR measurements to changes in presynaptic release probability (Figure 12). The dMSN:iMSN PPR was unchanged at both corticostriatal (control vs. 6OHDA; 1.0 ± 0.1 vs. 1.0 ± 0.1 , $p = 0.899$; $n = 16$ vs. 12) and thalamostriatal (1.1 ± 0.1 vs. 1.0 ± 0.1 , $p = 0.755$; $n = 10$ vs. 16 ; Figure 11E) synapses, and raw PPRs for all connection types showed no significant changes after dopamine depletion (Figure 11F), suggesting no significant change in presynaptic release probability at thalamostriatal and corticostriatal synapses in parkinsonian animals.

Inhibition of VGLUT2-expressing thalamic neurons rescues parkinsonian motor behavior

Given the reported increase in firing of thalamic Pf neurons in PD models (Jouve et al., 2010; Magnin et al., 2000; Orioux et al., 2000; Parr-Brownlie et al., 2009; Yan et al., 2008), and the decrease in thalamic connectivity with dMSNs after chronic dopamine depletion, we hypothesized that thalamostriatal inputs might be actively contributing to decreased spontaneous motor activity in parkinsonian mice through selective drive of the indirect pathway, relative to the direct pathway. We reasoned that disconnection of

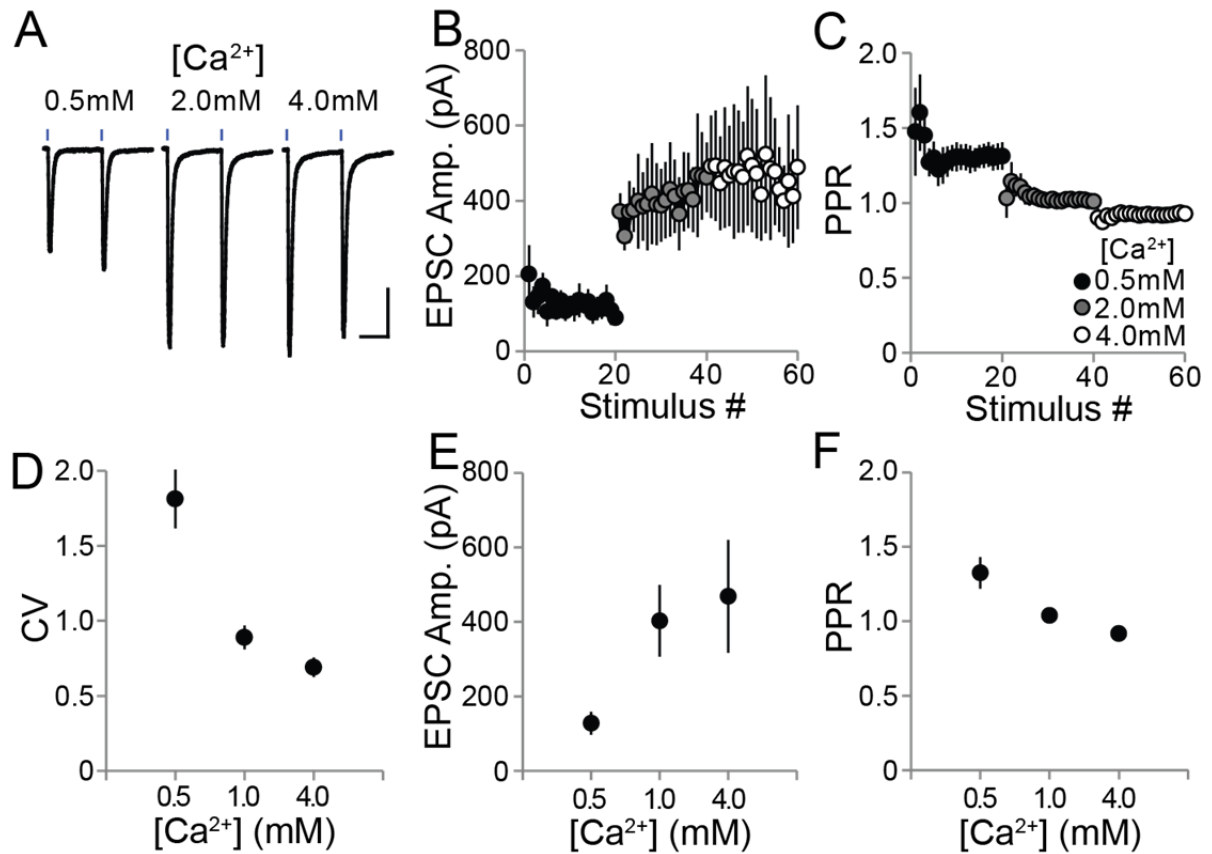


Figure 12: Dependence of paired pulse ratio of optogenetically-evoked EPSCs on presynaptic release probability.

(A) Example responses in a MSN to thalamostriatal stimulation in a VGLUT2-Cre mouse expressing Chr2-EYFP at varying external calcium concentrations.

(B) EPSC amplitudes plotted as a function of stimulus number. Circle color represents different calcium concentrations: black 0.5 mM, grey 2.0 mM, white 4.0 mM.

(C) Paired pulse ratio plotted as a function of stimulus number across different calcium concentrations.

(D) Average coefficient of variation for each calcium concentration.

(E) Average EPSC amplitude for each calcium concentration.

this pathway could rescue motor behavior. To test this hypothesis, we targeted Pf in VGLUT2-Cre mice with a virus encoding Cre-dependent hM4D-mCherry, a Gi-coupled receptor activated exclusively by clozapine N-oxide (CNO) (Armbruster et al., 2007). In mice co-injected with Cre-dependent Chr2-EYFP virus, optogenetically-evoked EPSCs in both dMSNs and iMSNs were strongly suppressed by bath application of CNO (baseline, 308.1 ± 97.0 ; CNO, 141.8 ± 65.7 pA; $p = 0.025$; $n = 6$; Figure 13A-C),

confirming hM4D-mediated suppression of glutamate release. Intraperitoneal injection of CNO into parkinsonian mice significantly increased the amount of time spent ambulating (saline vs. CNO, $37.3 \pm 5.0\%$ vs. $56.1 \pm 5.2\%$, $p = 0.003$; $n = 16$), and decreased the amount of time spent freezing ($19.8 \pm 4.5\%$ vs. $8.3 \pm 2.1\%$, $p = 0.012$) relative to saline injection (Figure 13D,E). CNO injection also increased average center-point velocity (saline vs. CNO, 2.0 ± 0.3 vs. 3.4 ± 0.5 cm/s, $p = 0.009$; Figure 13F). Activation of thalamic hM4D receptors in control animals had no effect on spontaneous motor behavior (saline vs. CNO; ambulation, $52.7 \pm 3.5\%$ vs. $53.5 \pm 3.2\%$, $p = 0.888$; freezing, $4.1 \pm 1.0\%$ vs. $4.3 \pm 1.4\%$, $p = 0.917$; velocity, 2.7 ± 0.2 vs. 2.8 ± 0.2 cm/s, $p = 0.660$; $n = 17$; Figure 13E,F), consistent with a detrimental gain-of-function of thalamostriatal inputs in parkinsonian animals.

Discussion

We performed simultaneous paired voltage-clamp recordings to directly compare the strength of excitatory inputs from cortex or thalamus onto direct- and indirect-pathway MSNs in the dorsolateral striatum. While chronic dopamine depletion did not change the relative strength of cortical inputs to the two MSN subtypes, thalamostriatal synaptic strength was decreased onto dMSNs relative to iMSNs, resulting in asymmetric drive of the indirect pathway by thalamostriatal afferents. This change could be explained by a decrease in postsynaptic AMPAR-mediated EPSCs specifically at thalamo-dMSN synapses. Gi-DREADD-mediated inhibition of VGLUT2+ intralaminar thalamic neurons

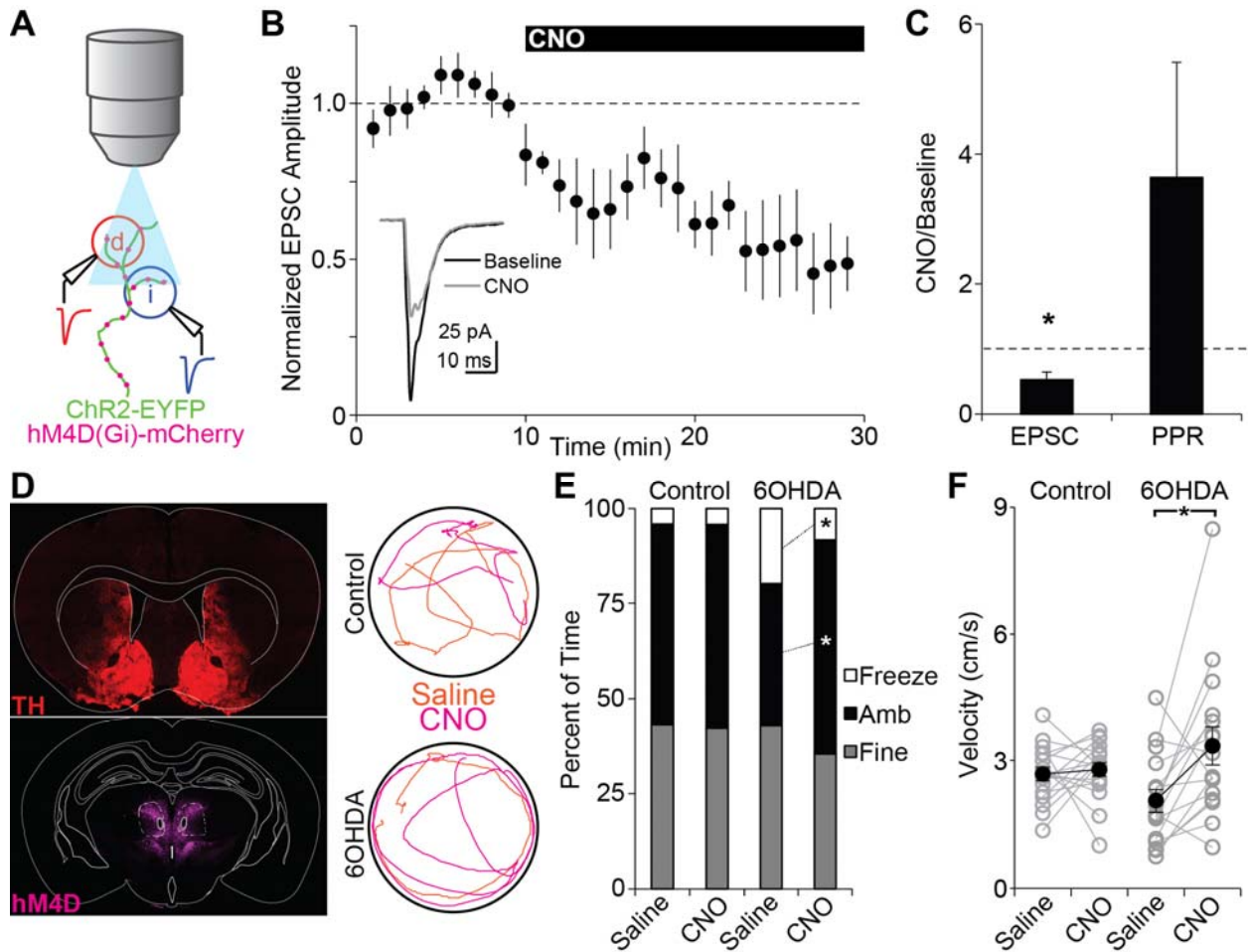


Figure 13: hM4D-mediated inhibition of VGLUT2+ intralaminar thalamic neurons rescues parkinsonian motor behavior.

(A) Schematic of recording setup. Intralaminar thalamic neurons were transduced with ChR2- and hM4D-expressing viruses. EPSCs from thalamostriatal stimulation evoked by 470nm LED pulses through the objective were recorded simultaneously from one dMSN (red) and one iMSNs (blue). hM4D receptors in thalamostriatal axons were activated by bath application of clozapine N-oxide (CNO).

(B) Averaged data from MSNs ($n = 6$) showing reduction in thalamostriatal EPSC amplitude after bath application of CNO. Inset: traces from an iMSN showing synaptic responses to ChR2-mediated thalamostriatal stimulation before (baseline) and after (CNO) bath application of CNO. CNO versus baseline, $p = 0.025$.

(C) Bilateral dorsolateral striatal 6OHDA lesions in a VGLUT2-Cre animal (top) and hM4D-mCherry expression in intralaminar thalamus (bottom; Pf = parafascicular nucleus).

(D) Example trajectories over 30 s of a control (top) and a 6OHDA-lesioned mouse (bottom) injected with saline (grey) or CNO (magenta).

(E) Average percent of time spent freezing, ambulating, or engaged in fine movement across a 15 minute open field session for control and 6OHDA-lesioned animals injected with saline or CNO. Control $n = 17$, 6OHDA $n = 16$. Control versus 6OHDA saline asterisk is $p = 0.001$ freezing, $p = 0.015$ ambulation. 6OHDA saline versus CNO asterisks are $p = 0.0124$ freezing, $p = 0.003$ ambulation.

(F) Individual (grey) and average (black) center point velocities for control and 6OHDA-lesioned animals injected with saline or CNO. Control versus 6OHDA saline asterisk is $p = 0.048$. 6OHDA saline versus CNO asterisk is $p = 0.009$.

ameliorated the parkinsonian phenotype, indicating that thalamus contributes significantly to the parkinsonian phenotype.

Remodeling of striatal afferents after dopamine depletion

Spine measurements are typically used to assay striatal synaptic changes after dopamine depletion, however these do not take into account the identity of the synaptic input, and thus it is not clear how changes (or lack thereof) in spine density reflect alterations of striatal inputs of distinct origins. Historically, cortical and thalamic terminals have been differentiated by their expression of VGLUT1 and VGLUT2, respectively (Freneau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001). Because VGLUT2+ Pf terminals primarily target MSN shafts (Raju et al., 2006), spine changes are thought to largely reflect changes in cortical innervation and may not be sensitive to alterations in these thalamic inputs. Recent evidence suggests there is in fact a selective loss of striatal VGLUT2+ terminals in monkey MPTP models (Villalba et al., 2013). Our data are the first to suggest a pathway-specific loss of postsynaptic AMPARs at thalamostriatal synapses in parkinsonian animals. This change could be mediated by long-term depression (LTD) at thalamo-dMSN synapses – a process that has not yet been described in the literature. We have conducted initial experiments that suggest NMDAR-mediated LTD akin to that seen in the hippocampus or cortex exists at thalamo-dMSN synapses, and that this LTD is occluded in parkinsonian mice (data not shown). This experiment, in addition to input-specific measurements of strontium-mediated synaptic events to measure quantal size, will give critical support to our hypothesis that thalamic inputs to dMSNs are depressed in parkinsonian animals.

The lack of change in the relative d/iMSN cortical synaptic drive in our study is quite surprising given the rich literature surrounding corticostriatal synaptic reorganization in PD. However, even with significant alterations to dMSN and iMSN morphology and physiology, broad activation of Thy1-ChR2 afferents in striatum reveals minor changes in overall EPSC amplitudes (Fieblinger et al., 2014). Our data suggest that, despite the many changes seen to the presumptive corticostriatal anatomy in PD models, the ultimate outcome of these changes is the maintenance of relative drive to the direct and indirect pathways. Certainly *in vivo* measurements of synaptic input to these two pathways will be critical. However, recent work suggests that these data largely agree with those seen *in vitro* (Pala and Petersen, 2015).

Amelioration of parkinsonian deficits with thalamic inactivation

Neurons in the intralaminar thalamus have been implicated in a number of important behaviors. Experiments in monkeys have connected the activity of CM/Pf neurons to sensory stimuli signaling the requirement for a low-reward action when a high-value reward is anticipated, thus implicating the intralaminar thalamus in biasing action choice (Minamimoto et al., 2005, 2014). Pf neurons in PD patients and animal models show higher firing rates and increased oscillatory activity (Jouve et al., 2010; Orioux et al., 2000; Parr-Brownlie et al., 2009; Yan et al., 2008), which is time-locked to tremor in patients (Magnin et al., 2000). In parkinsonian rats, Pf lesions decrease response latency on a motivational task (Henderson et al., 2005), and Pf DBS completely rescues sensorimotor neglect (Jouve et al., 2010). This latter finding is associated with the

reversal of PD-associated hyperactivity in iMSNs and neurons in the globus pallidus, subthalamic nucleus, entopeduncular nucleus and substantia nigra pars reticulata. In PD patients, Pf DBS effectively reduces tremor and, as seen in rodent models, reduces levodopa-induced dyskinesia (Caparros-Lefebvre et al., 1999; Mazzone et al., 2006; Peppe et al., 2008; Stefani et al., 2009). The combination of asymmetric intralaminar drive of the indirect pathway in our study with these observations of increased intralaminar activity and oscillations supports the notion that a parkinsonian gain-of-function of Pf contributes significantly to basal ganglia pathophysiology in PD (Chen et al., 2014).

If indeed thalamic inputs to dMSNs are depressed through NMDAR-mediated LTD, an alternative method for improving the parkinsonian phenotype could be the *in vivo* optogenetic induction of LTP at thalamo-dMSN synapses, similar to the recent optogenetic induction of LTD at cortical inputs to dMSNs to reduce cocaine sensitization (Creed et al., 2015). Semaphorin-3E/Plexin-D1 signaling is also known to enhance thalamo-dMSN synapses in development, and thus overexpression of Semaphorin-3E in thalamostriatal neurons or Plexin-D1 in dMSNs could increase transmission at thalamo-dMSN synapses in parkinsonian animals. These approaches would lend insight into whether restoration of transmission at this synapse, which we have shown to be specifically suppressed in parkinsonian animals, could be a useful target for intervention in patients with PD.

Combining input-specific targeting of ChR2 and cell-identified pairwise recording techniques, our data implicate a pathway-specific synaptic remodeling resulting from the loss of dopamine in a PD model that contributes significantly to the disease pathophysiology. These results highlight the need for a better understanding of the molecular mechanisms behind thalamostriatal plasticity in normal and disease states, and closer consideration of the thalamus as a major player in the pathophysiology of Parkinson's disease.

Chapter 5

Significance of the Dissertation and Remaining Questions

The most evolutionarily ancient vertebrates benefited from a complex, modular nervous system that allowed them to dynamically control their behavior in response to current and previous environmental factors. Complex vertebrates, such as mammals, have greatly expanded on that system, and our level of intentional control over our motor behavior is unprecedented. The basal ganglia and their rich connectivity with structures throughout the brain, such as the brainstem, midbrain, and thalamus, exert widespread control over our behavior via neural computations that initiate and suspend specific behavioral outputs (Grillner and Robertson, 2015; Reiner et al., 1998). In this context, striatal dopamine transmission is undoubtedly a critical component in both the learning and performance of motor behavior. However, the mechanisms by which dopamine acts and its effects on neural computations are still unclear despite decades of research. As the development of tools capable of manipulating genetically defined components of brain circuitry has flourished in recent years, our understanding improves, and the findings of this dissertation make two major contributions to this understanding.

The first important advance of the dissertation is the observation that phasic dopamine transmission, thought to widely affect striatal circuitry and its inputs, acts specifically at direct pathway neurons to increase the gain of inputs, thereby enhancing the vigor of ongoing behavior. While phasic dopamine likely has other acute effects on basal

ganglia circuit function, these have not yet been shown to occur *in vivo* during behavior. For example, dopamine's actions through D2 receptors on striatal cholinergic interneurons are thought to be necessary for one component of the burst-pause-burst activity of cholinergic neurons in response to rewarding stimuli (Ding et al., 2010). However, a role for this specific action of dopamine in learning or modulating ongoing behavior has yet to be illustrated. Combining cell type-specific targeting of optogenetic and chemogenetic manipulations with *ex vivo* recordings and *in vivo* behavior as we have done here, along with *in vivo* recording and imaging, will be of great benefit to addressing the many existing hypotheses on the role of phasic dopamine release in basal ganglia-dependent behaviors.

The dissertation's second finding of significant impact is that the chronic loss of dopamine in Parkinson's disease does not act to disrupt the balance of corticostriatal drive to the two basal ganglia pathways, but rather reorganizes striatal inputs from thalamus to remove excitatory drive from direct pathway neurons, thus suppressing movement. The corticostriatal system has remained the central focus of most PD research, and understandably so. The cortex provides the bulk of the excitatory input to the striatum, specifically targeting dopamine receptor-rich MSN spines, and animals with less expanded cortices do not seem to endogenously develop PD. However, as a relatively restricted population of neurons that widely broadcast excitatory signals to the striatum, thalamic neurons are in a unique position to robustly alter ongoing patterns of striatal activity. The thalamostriatal system is ripe for study, and thus far has received little attention within PD research or in basal ganglia studies in general. Given the large

number of basal ganglia related disorders, it seems likely that the thalamostriatal system, or thalamic inputs to other basal ganglia nuclei, will be ultimately be implicated in pathophysiology leading to obsessive compulsive disorder, Tourette's syndrome, and others. In the very least, the dissertation provides incentive for others to further explore the nature of the thalamostriatal system and its importance in health and disease.

We would also like to underscore the technical strengths of the experiments described here, as these are truly the reason we were able to address such longstanding hypotheses regarding dopaminergic modulation of striatal circuits. First, critical to many of these experiments is the use of simultaneous paired whole cell recordings. There simply is no substitute for this technique in the comparison of neuromodulation or synaptic input to two populations of neurons. The lack of iMSN modulation by phasic dopamine release would be difficult to convincingly show were it not that a neighboring dMSN is simultaneously excited rapidly. The decrease in thalamo-dMSN transmission in PD mice would be similarly difficult to show convincingly without a neighboring iMSN that has intact transmission. These experiments provide an internal control that simply does not exist in single cell patch clamp recordings, or in "sequential paired recordings" that are becoming increasingly popular. Furthermore, performing these experiments in animals that express ChR2 in a genetically defined population of presynaptic neurons with postsynaptic neurons identifiable by fluorophores expression allows for extremely precise control and identification of the neuronal populations in question. Many hypotheses remain regarding the role of dopamine in basal ganglia circuit function, and

these hypotheses have recently become testable due to these powerful advances in relevant techniques.

We addressed eight of the twelve predictions about acute and chronic dopamine modulation introduced in Figure 1B, and together these findings suggest that, despite the diverse potential array of dopamine's actions within the striatum, its presence and absence have an especially profound influence over behavior that seems to be dominated mechanistically by direct pathway neurons and their inputs. Phasic dopamine release enhanced the gain of dMSNs to invigorate learned behavior, and chronic dopamine depletion depressed thalamo-dMSN inputs to create a state of akinesia/bradykinesia in parkinsonian mice. As we move beyond the basic rate model of basal ganglia function and gain a better understanding of the complex computations conducted within the striatum and the basal ganglia circuit as a whole, the mechanistic changes we have described here will be put into a more useful light. In this regard, we hope to not only come to an understanding of how the brain works, but also to put that understanding to good use by improving the lives of those who suffer in its dysfunction.

Materials and Methods

Animals

All experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the University of California, San Francisco. VGLUT2-Cre, Emx1-Cre, and D1-Tomato mice were purchased from Jackson Laboratories (stock numbers 016963, 005628, and 016204 respectively), and DAT-Cre mice were obtained from R. Edwards at UCSF (MGI: 3689433). The three Cre lines were crossed to the D1-Tomato line and bred in-house.

Surgeries

Six- to ten-week-old mice were anesthetized (isoflurane 2%) and placed in a stereotaxic frame. Using bregma as a reference, a mounted drill was used to create holes in the skull, and a 33-gauge needle (Plastics One) was inserted for viral injections into cortex (AP +0.5/1.5, ML +/-2.0, DV -1.0 from dura), thalamus (AP -2.3, ML +/-0.5, DV -3.25 from dura), striatum (AP +0.8, ML +/-1.5, DV -2.5), or SNc (AP -3.45, ML +/-1.5, DV -4.3). A volume of 0.75 μ l/site of virus (AAV5-EF1a-DIO-ChR2(H134R)-EYFP, AAV8-EF1a-DIO-hM4D-mCherry, AAV5-EF1a-DIO-ChETA-EYFP, AAV5-EF1a-DIO-C1V1, titer $\sim 10^{12}$, UNC Vector Core) was injected at a rate of 0.2 μ l/min. The needle was left in place for 5 min before being withdrawn and the scalp was sutured. For PD experiments, two weeks later, the scalp was reopened and 1 μ l of 5 μ g/ μ l 6-hydroxydopamine (6OHDA; Sigma-Aldrich) or saline was injected unilaterally into the medial forebrain bundle (MFB; AP -1.0, ML -1.0, DV -4.9 from dura) or bilaterally into the DLS (AP +0.8, ML +/- 2.2, DV -2.5 from dura).

For Pavlovian experiments, Vetbond (3M) was applied to the skull, and a custom metal headbar was attached to the skull with dental acrylic (Jet). A 200 μm optical fiber attached to a ceramic ferrule or a 33 gauge cannula (Plastics 1) was slowly inserted into the ventral lateral striatum (AP +1.0, ML +/- 2.2, DV -4.0).

In utero electroporation

A pCAGGs plasmid containing the ChIEF coding region (Lin et al., 2009) was introduced into the developing cortex and striatum *in vivo* by intraventricular injection and electroporation. Intraventricular injections were carried out in E14 timed-pregnant mice, where the morning of the plug is designated embryonic day 1 (E1). Electroporations were performed using an Electro Square Porator ECM830 (Genetronics) (5 pulses, 50V, 100 ms, 1 s interval). DNA was prepared in endotoxin-free conditions and 1 μl was injected per brain.

Electrophysiology

One to two weeks after 6-OHDA/saline injection, acute slices (300 μm) were cut through the DLS in sucrose-based artificial cerebrospinal fluid (ACSF) using a vibratome (Leica VT1200S). Slices recovered NaCl-based ACSF for 30 min at 32 deg C and then were held at room temperature until being transferred into the recording chamber at 32 deg C. ACSF [consisting of (in mM): NaCl 125, NaHCO_3 26, NaH_2PO_4 1.25, KCl 2.5, MgCl_2 1, CaCl_2 2, glucose 12.5, and 50 μM picrotoxin to suppress GABAergic responses] was perfused over the slices at ~2 ml/min while simultaneous voltage clamp recordings were made from one D1-Tomato+ and one D1-Tomato- MSN using 2-4 M Ω patch pipettes

pulled from borosilicate glass on a Sutter P-97 and filled with internal solution [consisting of (in mM): CsMeSO₃ 120, CsCl 15, NaCl 8, EGTA 0.5, HEPES 10, QX-314 5, TEA-Cl 10, Mg-ATP 2, and Na-GTP 0.3]. Light pulses (470 nm, 0-2 mW/mm², 0.5-5 ms duration) were delivered through a 40x immersion objective at 0.15 Hz using a high-intensity LED (Thorlabs LED4C driven by a Prizmatix BLCC-2). MSNs were held at -80 mV and depolarized to +40 mV to measure NMDAR-mediated currents (measured at 50 ms after stimulus onset when AMPA-mediated currents were negligible). For hM4D experiments, clozapine-N-oxide (CNO, 10 μ M; Sigma-Aldrich) was added to the bath solution after a ten-minute baseline recording.

Open Field Behavior

One to two weeks after 6-OHDA/saline injection, activity in the open field was tracked using ETHOVISION 9 software (Noldus) on two separate days. On the first day, half of each group (control, 6OHDA) received intraperitoneal (i.p.) saline injections while the other half received i.p. CNO injections (0.1 mg/kg CNO in saline) 30 min before open field measurements. Two days later, the experiment was repeated and the saline/CNO cohorts were switched. Ambulation was defined as movement of the mouse center-point >1.75 cm/s. Fine movement was defined as movement of the mouse center point <1.75 cm/s with >2% change in image pixels. Freezing was defined as movement of the mouse center-point <1.75 cm/s with <2% change in image pixels. Statistical significance was evaluated using a two-way ANOVA and post-hoc Bonferroni-corrected paired student's t-test.

Pavlovian behavior

One week after headbar surgery, animals were slowly habituated to the spherical treadmill. A 3 s 2 kHz tone was played every 20-40 seconds, 20% sucrose in water was delivered 2-3 seconds after the tone onset. Licking was measured with a custom IR diode lickometer system. Data were acquired and analyzed in Matlab 2007b using custom software. 5 mW laser light (OEM Lasers) was used to stimulate dopamine terminals using 10 ms pulses at 50 Hz for 500 ms at cue onset. For antagonist experiments, 0.5 μ l of SCH23390 (5 μ M) or sulpiride (10 μ M) were infused into the striatum at a rate of 0.1 μ l/min.

Histology

Mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA), brains were rapidly dissected out and placed into 4% PFA overnight. The next day they were rinsed in PBS and switched to 30% sucrose-containing PBS for 48 h, after which slices (30 μ m) were cut on a sliding microtome (Leica SM2010R). After blocking with 10% donkey serum and permeabilization with 0.1% Triton-X-100 for 30 min at room temperature, slices were incubated in primary antibody (rabbit anti-tyrosine hydroxylase 1:500, Pel-Freeze; mouse anti-RFP 1:500, Rockland Immunochemicals) overnight at 4°C. Slices were then incubated in secondary antibody (donkey anti-mouse Alexa568, donkey anti-rabbit Alexa647, 1:1000, Invitrogen) for 1 h at room temperature and mounted onto slides for imaging with a Nikon 6D epifluorescence microscope.

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