DOPAMINE D2 RECEPTOR ACTIVATION ON STRIATAL INHIBITORY COLLATERALS

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Abstract

The principal neurons of the striatum are GABAergic medium spiny neurons (MSNs), whose collateral synapses onto neighboring neurons play critical roles in striatal function. MSNs can be divided by dopamine receptor expression into D1 and D2 class MSNs, and despite overwhelming evidence for D2 receptors (D2R) in maintaining proper striatal function, it remains unclear how MSN collaterals are specifically altered by D2R activation. This is partly due to the low rate of inhibitory collateral detection between MSNs in conventional ex vivo slice recordings. Furthermore, most studies on MSN collaterals have been conducted either blind or in models in which only one MSN subtype can be distinguished. In this dissertation, I describe a dissociated culture system using striatal and cortical neurons harvested from genetically modified mice. These mice express tdTomato and EGFP downstream of the dopamine D1R and D2R promoters, respectively, allowing for simultaneous distinction between the two major subtypes of MSNs. These neurons develop spines, hyperpolarized resting membrane potentials and exhibit up-and-down states, while also maintaining expression of both fluorophores through time. Paired whole cell patch clamp recordings revealed an enhanced rate of inhibitory functional synapses than previously reported in slice recordings.

Using these cortico-striatal cultures, I report that chronic D2R stimulation, with the D2/3 receptor agonist quinpirole, regulates MSN collaterals in vitro by pre- and post-synaptic
mechanisms. Quinpirole increased the rate and strength of collateral formation onto D2R-containing MSNs (D2 MSNs) as measured by paired whole-cell patch clamp recordings. Additionally, these neurons were more sensitive to low concentrations of GABA and exhibited an increase in gephyrin puncta density, suggesting increased postsynaptic GABA\textsubscript{A} receptors. Lastly, quinpirole treatment increased presynaptic GABA release sites as shown by increased frequency of sIPSCs and mIPSCs, correlating with increased VGAT puncta. Combined with the observation that there were no detectable differences in sensitivity to specific GABA\textsubscript{A} receptor modulators, I provide evidence that D2R activation powerfully transforms MSN collaterals via coordinated pre- and post-synaptic alterations. As the D2 MSNs are highly implicated in Parkinson’s disease and other neurological disorders, these findings may contribute to understanding and treating the changes that occur in these pathological states.
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I. BACKGROUND AND INTRODUCTION

A. Basal ganglia

The basal ganglia are comprised of a group of interconnected subcortical nuclei that are associated with various functions including motor control, addictive behavior and learning (Albin et al., 1989; Bolam et al., 2000). Early speculation on the roles of the basal ganglia was influenced by the clinical descriptions of various basal ganglia disorders. In particular, the progression of Parkinson’s disease, which results from the degeneration of dopamine producing cells of the substantia nigra pars compacta, strongly influenced what was known about basal ganglia processes. Within the past two decades, substantial advances in technology have permitted the blossoming of basal ganglia-related knowledge allowing for a more detailed perspective on basal ganglia anatomy and physiology.

B. Macrocircuits of the basal ganglia

The basal ganglia are often described as being composed of four major nuclei: the striatum, globus pallidus (GP), substantia nigra (SN), and subthalamic nucleus (STN). Upon further examination, the GP can be split into the functionally distinct external (GPe) and internal (GPi) segments. Similarly, the SN can be split into the dopamine producing pars compacta (SNpc) and the basal ganglia output nucleus, the pars reticulata (SNpr).
A simplified version of the principal pathways of information flow in the basal ganglia is illustrated in Figure 1. About 85% of glutamatergic inputs into the basal ganglia are derived from the ipsilateral cortex, contralateral cortex, and intralaminar thalamic nuclei. The topographic projection of the somatosensory and motor cortices onto the striatum is relatively preserved throughout the basal ganglia nuclei. These glutamatergic afferents generally form asymmetric synapses on the spines and shafts of the principal neurons of the striatum (Smith and Kieval, 2000; Doig et al., 2010). Excitatory afferents from the cortex also project to the STN (Monakow et al., 1978). In addition, the striatum receives sparse glutamatergic input from the amygdala and dorsal raphe nucleus. Cortical inputs into the striatum are better understood than thalamic inputs, despite their near equal occurrences (Smith et al., 2004). This is in large part due to the conventional preparation of in vitro coronal striatal brain slices, which sever the majority of thalamo-striatal inputs (Vogt & Vogt, 1941; Cowan & Powell, 1956; Smeal et al., 2007; Smeal et al., 2008). Interestingly, similar thalamic and cortical inputs have been shown onto direct and indirect pathway striatal neurons (Doig et al., 2010), as described below.
Figure 1: Simplified illustration of basal ganglia circuit flow. GPe, external globus pallidus; STN, subthalamic nucleus; GPi, internal globus pallidus; SNpr, substantia nigra pars reticulata; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area.
As the striatum is the main point of entry into the basal ganglia, it is responsible for integrating glutamatergic inputs. The principal neurons of the striatum are GABAergic; thus, outputs from the striatum will be inhibitory. After integrating the inputs via intra-striatal microcircuits, striatal information flows to the rest of the basal ganglia through the direct and indirect pathways to the SN_{pr} (Gerfen et al., 1990). Striatal direct pathway neurons, which also express the dopamine D_{1} receptor, project to the GPi/SN_{pr}, and also send some axon collaterals to the GPe. The indirect pathway neurons, which express the dopamine D_{2} receptor, project only to the GPe. From there, GPe neurons project either to the GPi/SN_{pr}, or first to the glutamatergic STN and then on to the GPi/SN_{pr}. The indirect pathway thus forms a multisynaptic circuit from the striatum to the output nuclei. From the GPi/SN_{pr}, basal ganglia output projects to thalamic nuclei, which in turn project glutamatergic afferents back to the cortex.

Finally, dopamine is a critical neuromodulator of the basal ganglia and is supplied to the striatum, GPe and STN by the dopaminergic cells in the SN_{pc}. Dopamine modulates the flow of information through the basal ganglia, as exemplified by the imbalance observed in Parkinson’s Disease (Albin et al. 1989; DeLong, 1990), and it does so via G-protein coupled dopamine receptors. The role of dopamine in the basal ganglia will be further detailed in Section E.1 of this chapter.

In addition to the major direct and indirect macrocircuits of the basal ganglia, there are also shorter circuits that have been recently suggested to be deregulated in certain pathological states. For example, the GPe and the STN have autonomous pacemaker qualities and are reciprocally connected; therefore, these two nuclei have the ability to tightly regulate each other. There is enhanced functional connectivity detected between GPe and STN in \textit{in vivo}
Parkinsonian states (Mallet at al., 2008a, b; Cruz et al., 2011; Moran et al., 2011). Additionally, there are thalamic afferents that are sent to the STN, and the basal ganglia output nuclei – the GPi/SNpr – have been shown to project to superior colliculus, reticular formation, pedunculopontine nucleus and habenular nuclei.

Because the output nuclei are GABAergic and, like the GPe and STN, have autonomous pacemaker activity, the basal ganglia is a system that is generally inhibited at rest. Inhibition of the thalamus is relieved when glutamatergic input into the striatum creates a loss of the inhibitory tone in the basal ganglia, thus allowing for movement (Albin et al., 1989; Chevalier & Deniau, 1990). Previously it was thought that the direct pathway was responsible for action initiation and the indirect pathways for action suppression. Recent evidence, however, suggests that these systems are far more complex (Cui et al., 2013).

C. Basal ganglia nuclei

The basal ganglia are often divided into dorsal and ventral divisions. The dorsal component is primarily related to motor and associative functions, and is traditionally composed of the striatum, globus pallidus, subthalamic nucleus, and substantia nigra. The ventral component is associated with limbic functions, and is composed of the ventral striatum/nucleus accumbens, ventral pallidum, and the ventral tegmental area. Inputs to the dorsal division are derived mainly from the cortex and thalamus, whereas inputs to the ventral division are derived from the hippocampus, amygdala and limbic cortices (Smith et al., 1998). While both dorsal and ventral subdivisions have distinct inputs and ultimate functions, they are composed of nuclei
containing similar cell types. Therefore, comparable nuclei from the dorsal and ventral divisions of the basal ganglia are presented together here.

1. **Dorsal and ventral striatum**

The striatum is the major input nucleus of the basal ganglia, and is also the largest in size. The striatum is derived from the embryonic telencephalon and is defined by its striated appearance in brain sections, which is a result of alternating gray and white matter. In rodents the striatum is a singular nucleus located prefrontally in either hemisphere, whereas in higher vertebrates the striatum is divided into the caudate nucleus and putamen by the white matter tract known as the internal capsule.

The striatum can also be differentiated into patch and matrix compartments, as determined by neurochemical expression patterns and anatomical connections (Graybiel and Ragsdale, 1978; Graybiel, 1990). The patch compartments receive input from the superficial layers of cortical layer V and analyses reveal that patches also contain high densities of tyrosine-hydroxylase fibers, opiate and dopamine D1 receptors. Matrix compartments receive input from the deeper layers of cortical layer V and layer VI and express acetylcholine esterase, calbindin and somatostatin (Graybiel and Ragsdale, 1978; Gerfen, 1992). Medium spiny neurons (MSNs) in patch and matrix compartments also have distinct efferents: MSNs in patches project to the dopaminergic neurons in the substantia nigra pars compacta, while MSNs in matrices project to the GABAergic neurons in the substantia nigra pars reticulata.
There is a great heterogeneity of cell types within the striatum. With the exception of a small population of cholinergic interneurons, all striatal neurons are GABAergic. The major subtypes of striatal neurons are described here.

i. Principal medium spiny neurons

The principal neurons are the striatal medium spiny neurons and constitute between 90-95% of striatal neurons, dependent on species. MSNs are characterized by their medium sized soma with diameters between 12-20 µm and primary dendrites that are initially smooth and, at 20 µm from the soma, become densely spiny (Wilson, 1994). All MSNs are GABAergic and can be divided into two major subsets based on axonal projections, expression of dopamine receptors, and peptide expression (Figure 2; Gerfen and Young, 1988; Albin et al., 1989). MSNs that express the dopamine D1 receptor (D1 MSNs), substance P and dynorphin generally project directly to the SNpr. MSNs that express the dopamine D2 receptor (D2 MSNs) and enkephalin generally project indirectly to the SNpr via the GPe. In addition, there may be a much smaller subset of striatal neurons that co-express D1 and D2 receptors, or express a D1-D2 receptor heteromer (Perreault et al., 2011), though this remains controversial and is not extensively pursued in this thesis.
Figure 2: MSN properties in ex vivo striatal slices from genetically modified mice. A, Overlying DIC and fluorescence image of BAC drd1 tdTomato mice (top) and characteristic D1-MSN firing properties (bottom). B, Overlying DIC and fluorescence image of BAC drd2 eGFP mice (top) and characteristic D2-MSN firing properties (bottom). C, (top) Slice image of BAC drd1 tdTomato; BAC drd2 eGFP mouse brain displaying simultaneous identification of both D1 and D2 MSNs. C, (bottom) MSN injected with biocytin.

Images and recording traces courtesy of Stefano Vicini, John Partridge, Megan Janssen and Kristen Ade. Panel B is modified with permission from Ade et al., 2008.
Both D₁ and D₂ MSNs have some comparable electrophysiological properties. At rest, MSNs display very hyperpolarized resting membrane potentials (RMP), around -90mV, due to the high expression of the inwardly rectifying Kir2 channels (Wilson 1993; Shen et al., 2007). As observed in ex vivo slice preparations (Figure 3 A), this hyperpolarized state is also achieved with time in vitro (Figure 3 B).

**Figure 3:** Resting membrane potentials of neurons through development. A. RMP values of MSNs in ex vivo slices of mouse striatum at different times points through development, as originally published in Dehorter et al., 2011. B. Data derived from 40 striatal MSNs in vitro dissociated cultures suggest a similar trend (p = 0.0002, unpaired t-test). P, Postnatal day; DIV, days in vitro.

Panel A is from Figure 4 G in Dehorter et al. (2011) Frontiers in Cellular Neuroscience.
The hyperpolarized RMP of MSNs is referred to as the “down-state,” where there is little or no synaptic input. The inward rectifier potassium K\textsubscript{ir}2 channels can also shunt glutamatergic input, minimizing depolarization in response to excitatory activity that lacks spatial or temporal convergence. However, when powerful excitatory input converges onto an MSN, the MSN will depolarize to what is known as the “up-state,” which is at about -50 mV, for hundreds of milliseconds (Figure 4; Wilson and Kawaguchi, 1996; Stern et al., 1998).

![Figure 4: Up-and down states in MSNs in vitro. Whole-cell recordings from two D\textsubscript{2} MSNs at 16 days in vitro with low intracellular chloride levels reveals synchronized up-and down-states when the cells are held in current clamp at their RMP. Dashed horizontal lines indicate the threshold for up-states.](image-url)
Despite exhibiting similar RMPs and gross morphology, D_1 and D_2 MSNs do show some subtle yet important differences. D_1 and D_2 MSNs display slight divergences in morphology: D_1 MSNs have, on average, two more primary dendrites than D_2 MSNs (Figure 5; Gertler et al., 2008). The density of spines on D_1 and D_2 MSN dendrites are comparable; therefore, as D_2 MSNs have fewer dendrites but equal spine densities, D_2 MSNs receive nearly 50% less glutamatergic input than D_1 MSNs. As a result of the divergence in total dendritic area between MSN subtypes, D_2 MSNs have a reduced electronic length and are therefore more excitable than D_1 MSNs. This has been confirmed through studies in which D_2 MSNs were more responsive to intrasomatic current injection than D_1 MSNs (Gertler et al., 2008). D_2 MSNs are also more sensitive to low doses of γ-amino-n-butyric acid (GABA) when compared to D_1 MSNs (Figure 6; Ade et al., 2008).
**Figure 5**: Reconstructions of biocytin-filled D1 and D2 MSNs. **A-C**, Striatal neurons from P35-P45 BAC transgenic mice were biocytin-filled, imaged, and reconstructed in 3D. A GABAergic Interneuron is included for comparison. **D**, Fan-in diagrams displayed no apparent preferred orientation in either the D1 or D2 MSN populations. **E**, Dendrograms displaying in two dimensions the length, number, and connectivity of dendritic segments in sample neurons.

Reprinted with permission from Gertler et al., 2008.
**Figure 6:** Greater sensitivity to exogenously applied GABA in $D_2^+$ than in $D_1^+$ MSNs. Representative traces (A) and summary dose–response data (B) of the whole-cell currents elicited by increasing doses of GABA applied in the presence of TTX (0.5 µM), NBQX (5 µM), and strychnine (0.5 µM) in $D_1^+$ and $D_2^+$ MSNs. The EC$_{50}$ for GABA was 107.3 ± 0.5 and 18.2 ± 1.9 µM and the Hill coefficient was 1.04 ± 0.05 and 1.75 ± 0.18 for $D_1^+$ and $D_2^+$ MSNs, respectively. All recordings were performed with KCl internal solution, and data were derived from at least sixteen cells in each group. Data on whole-cell currents induced by 0.5 µM and 5 µM GABA (C) for $D_1^+$ MSNs (white bar) and $D_2^+$ MSNs (black bar) are illustrated for clarity.

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Aberrant changes in D₂R expression and function in the striatum are closely related to schizophrenia, substance abuse and obesity (Abi-Dargham et al., 2000; Volkow et al., 2001; Asensio et al., 2010; Johnson and Kenny, 2010). The D₂R is also the primary target of action for many antipsychotics (Seeman et al., 1975; Kapur et al., 2000) and for Parkinson’s Disease therapy (Lieberman and Goldstein, 1985). Interestingly, D₂ MSNs of the striatum have been shown to be the first population of neurons affected by DA depletion in Parkinsonism, resulting in alterations of D₂ MSN morphology and function (Albin et al., 1989; Day et al., 2006; Gittis and Kreitzer, 2012; Zold et al., 2011). In addition, it has recently been reported that chronic D₂R upregulation in vivo in the mouse increases MSN excitability and decreases dendritic arborization by the down-regulation of inward rectifying potassium channels (Cazorla et al., 2012).

MSNs have dendritic arbors that span 250-400 μm in diameter (Wilson, 1994) unless obstructed by matrix or striosome compartments, which they do not cross (Wilson and Young, 1988). Their extensive axon collaterals extend a bit beyond the volume of dendritic arbor and primarily target the spine necks and dendrites of other MSNs (Figure 7; Gerfen, 1988), and are therefore referred to as feedback inhibition (detailed further in section F 2 of this chapter). As the striatum is the input nucleus of the striatum, proper integration of information through collaterals provides the striatum with essential computational abilities. In fact, Taverna et al. (2008) found that these feedback inhibitory collaterals are specifically affected in 6-hyrdoxydopamine (6-OHDA) models of dopamine depletion.
**Figure 7:** Neostriatal spiny projection neurons. 

**A,** Drawing-tube reconstruction of a neostriatal spiny neuron from an adult rat stained intracellularly with biocytin in vivo. Dendrites are in black; axon collaterals are in red. 

**B,** Electron micrograph of a symmetric axodendritic synapse between two striatal spiny neurons stained intracellularly with horseradish peroxidase. 

Abbreviations: Sp-axon, axon terminal of one intracellularly stained striatal spiny neuron; Sp-dend, dendrite of a second intracellularly stained striatal spiny neuron.

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ii. GABAergic interneurons

There have been at least four types of striatal GABAergic interneurons distinguished by the expression of the following proteins: parvalbumin (PV), calretinin (CR), tyrosine hydroxylase (TH), and neuropeptide-Y (NPY). Depending on the species, GABAergic interneurons together constitute roughly 5-10% of all striatal neurons.

Interneurons that express the calcium binding protein PV are very well characterized and are postulated to play a key role in the regulation of basal ganglia output. PV interneuron somas range between 10-25 µm in diameter and their aspiny dendritic arbors span on average 200-600 µm (Kita et al., 1990; Kawaguchi et al., 1995; Koos and Tepper, 1999). As these neurons fire non-adapting action potentials at 200-300 Hz (Tepper and Bolam, 2004), they are also referred to as fast spiking interneurons, or FSIs. PV-expressing interneurons also express dopamine D₁-class receptors, though their characteristic firing properties allow them to be easily distinguished from direct-pathway D₁ MSNs. These interneurons are also the only striatal neuron known to form gap-junctions with other PV interneurons (Kawaguchi, 1995; Gibson et al., 1999; Koos and Tepper, 1999). PV interneurons mediate feedforward inhibition by forming peri-somatic GABAergic synapses onto striatal MSNs (Parthasarthy and Graybiel, 1997; Tepper and Bolam, 2004), and are thought to contribute greatly to the striatal imbalances exhibited in rat models of Parkinson’s Disease (Mallet et al., 2006).

CR, another calcium-binding protein, is expressed in the most abundant GABAergic interneuron of the striatum in humans (Cicchetti et al., 1998). These neurons have somas that span 12-20 µm in diameter and sparse, aspiny dendrites (Bennet and Bolam, 1993). There are at
least three morphologically distinct types of CR interneurons in the striatum, largely differentiated by size (Prensa et al., 1998; Schlosser et al., 1999; Wu and Parent 2000; Rymar et al., 2004). Although many striatal interneuron subtypes have been studied in detail using transgenic mice, CR-expressing neurons remain relatively unknown as there is no genetic strain available which specifically tags CR transcription. Though immunostaining for the CR protein in mice reveals very few neurons, this is an example of the powerful utility of transgenic mice in the field of neuroscience.

The TH positive striatal neuron has a medium sized soma size, which is on average 15 µm in diameter, and two to four aspiny, varicose dendrites (Tashiro et al., 1989b; Mao et al., 2001; Buscetti et al., 2008). Like CR interneurons, there have been four subtypes of TH neurons identified with varying sizes, dendritic branching, and electrophysiological characteristics (Tepper et al., 2010). The proportion of these neurons has been shown to vary between species, age and potentially dopamine levels (Betarbet et al., 1997; Meredith et al., 1999; Mao et al., 2001; Palfi et al., 2002; Jollivet et al., 2004; Cossette et al., 2005; Mazloom and Smith, 2006; Huot et al., 2007; Darmopil et al., 2008).

Striatal interneurons that express NPY have also been shown to express nitric oxide synthase (NOS) and somatostatin (SS) (Partridge et al., 2009). This class of interneuron has many distinct subtypes, though they can all be characterized by calcium-dependent, low threshold spiking (Tepper and Bolam, 2004). NPY interneurons are responsible for feedforward inhibition in the basal ganglia by sending GABAergic efferents to the soma and distal dendrites of MSNs (Kubota and Kawaguchi, 2000). A distinct subgroup known as neurogliaform (NGF) interneurons are NPY+ but not NOS+ and have recently been suggested to be significantly more
likely to synapse onto a neighboring MSNs than other NPY+ neurons (Ibáñez-Sandoval et al., 2011). Interestingly, these functional synapses between NPY-NGF neurons and MSNs have characteristic slow, GABA_A receptor mediated IPSCs similar to what has been previously observed in NGF neurons of the cortex and hippocampus (Ibáñez-Sandoval et al., 2011; Luo et al., 2013).

iii. **Cholinergic interneurons**

Cholinergic interneurons are the only striatal neuron subtype known that are not GABAergic. They are easily identified by their large soma size, which can span up to 40 µm in diameter, and immunoreactivity to choline acetyltransferase (Bolam et al., 1984). In fact, these neurons were initially mistakenly labeled as striatal projection neurons when they were identified by Ramon y Cajal (1911). Cholinergic interneurons have long, aspiny dendrites and fire slow, repetitive action potentials (Wilson et al., 1990; Bennet and Wilson, 1999). Cholinergic interneurons also express the D_2 class of dopamine receptor; however, given their size, they are distinguished from indirect pathway D_2-receptor expressing MSNs with ease.

The cholinergic interneuron is suspected to play a significant role in activating spontaneous dynamics in striatal cell assemblies (Surmeier et al., 2011). Striatal NPY-NGF interneurons have a high rate of connectivity to MSNs (Ibáñez-Sandoval et al., 2011) and cholinergic interneurons regulate NPY-NGF interneurons (English et al., 2011). This enables cholinergic interneurons to exert rapid inhibitory control of MSNs. Reciprocal connectivity between cholinergic and other GABAergic interneurons form intrinsic oscillators that can
also shape interneuron input to MSNs, giving cholinergic interneurons a central role in striatal function (Carillo-Reid et al., 2008; Surmeier et al., 2011).
**Figure 8:** Immuno-labeling for interneuronal markers in BAC-npy-GFP mice. Overlying fluorescence images with (A) α-NPY, (B) α-Parvalbumin, and (C) α-ChAT show little overlap of PV+ and Cholinergic interneurons with NPY neurons of the striatum.

Images courtesy of Stefano Vicini, John Partridge and Megan Janssen.
2. **Globus pallidus and ventral pallidum**

The globus pallidus is the second largest nucleus of the basal ganglia and also arises from the telencephalon. It includes an internal and external segment (GPi and GPe, respectively), and in rodents the GPi is referred to as the entopeduncular nucleus. The GP is an output nucleus of the striatum and therefore receives GABAergic afferents. The direct and indirect striatal pathways project to the GPi and GPe, respectively, and as the GP is also GABAergic, all efferents from the GP are GABAergic.

The GPe is composed of large, parvalbumin-positive neurons that have local axon collaterals within the GPe. GPe neurons also project to the STN, GPi, and substantia nigra, and nearly one quarter of pallidal neurons give rise to collaterals that project to the striatum (Kita & Kitai, 1994; Bevan et al., 1998). GPe afferents to the striatum have been shown to selectively innervate striatal interneurons (Bevan et al., 1998), specifically the striatal PV+ fast spiking interneurons. This gives the GPe a powerful role in regulating striatal activity, as GABAergic transmission onto PV-interneurons potentially allows for shunting of excitatory inputs (Cobb et al., 1995).

The GPe projects only within the basal ganglia, whereas the GPi, which is often grouped with the SNpr, together function as basal ganglia output nuclei (Nauta and Mehler, 1996).
3. **Subthalamic nucleus**

The subthalamic nucleus (STN) is the only glutamatergic nucleus of the basal ganglia and is derived from the embryonic diencephalon. The STN projects within the basal ganglia, but as it is mostly excitatory, its firing ultimately inhibits thalamo-cortical projections.

Individual neurons from the globus pallidus innervate the STN (Bolam et al., 1993; Smith et al., 1998). The STN also receives glutamatergic input from the motor cortex (Nambu et al., 1996) and the parafascicular thalamic nucleus (Becan et al., 1995). The substantia nigra provides dopaminergic afferents to the STN, and these have also been shown to degenerate in Parkinson’s Disease (Brown et al., 1979; Hassani et al., 1997; Francois et al., 2000; Cragg et al., 2004). The STN projects collaterals to both segments of the GP, as well as back to the substantia nigra. Abnormal activity of the STN has also been reported in a number of other movement disorders, and manipulation of STN activity can improve the symptoms of these disorders (Bergman et al., 1994; Wichmann et al., 1994).

In the absence of GABAergic and glutamatergic inputs, the STN has autonomous pacemaker activity. Driven by voltage-dependent sodium channels, STN neurons display action potentials at a frequency of 5-15 Hz (Bevan and Wilson, 1999). As the STN and GPe are reciprocally coupled, together they are postulated to powerfully regulate basal ganglia output.
4. **Substantia nigra and ventral tegmental area**

The substantia nigra (SN) is the largest nucleus in the midbrain and plays a fundamental role in basal ganglia signaling. The SN is derived from the embryonic mesencephalon and can be divided into two functionally distinct compartments: the SN pars compacta (SN<sub>pc</sub>) and the SN pars reticulata (SN<sub>pr</sub>). While both compartments receive similar inputs, they have distinct neuron types and projections (Gerfen and Wilson, 1996).

i. **SN<sub>pc</sub> and VTA**

The SN<sub>pc</sub> and ventral tegmental area (VTA) are responsible for the dopaminergic tone in the basal ganglia. As the oxidation of dopamine via tyrosinase produces neuromelanin (Simon et al., 2009), the SN<sub>pc</sub> is noticeably darker than its neighboring tissue in primates. The SN<sub>pc</sub> is primarily an input structure to the basal ganglia, supplying the striatum with dopamine, a critical modulator of striatal processing.

ii. **SN<sub>pr</sub>/GPi**

The SN<sub>pr</sub> and GPi are GABAergic nuclei and are the final step of processing within the basal ganglia. The SN<sub>pr</sub> and GPi subsequently project to the thalamus or the SN<sub>pc</sub>, thus controlling both basal ganglia output and dopaminergic input.
D. Ionotropic receptors and transmission in the striatum

1. GABA_A receptors

As 98.86% of all cells in the rat basal ganglia are GABAergic (Oorschot, 1996), proper GABA transmission is critical to basal ganglia function. Besides the STN, which is glutamatergic, and the SNpc, which is dopaminergic, all other nuclei in the basal ganglia are GABAergic. GABA_A and GABA_C are fast-acting ionotropic receptors which bind GABA. Here I will discuss the prominent GABA_A receptors.

i. Structure

GABA_A receptors are pentameric transmembrane pores that are permeable to Cl- and HCO_3- (MacDonald and Olsen, 1994; Farrant and Nusser, 2005; Michels and Moss, 2007). The majority of GABA_A receptors are composed of 2α, 2β and 1γ subunit (Mohler, 2007), although they can also be arranged from any of the following subunits: α1-6, β1-3, γ1-3, δ, ε, θ1-3, π, ρ1-3 (Mohler, 2006; Luscher et al., 2011). The most prominent synaptic GABA_A receptors in the brain incorporate the α1-3, β2-3 and γ2 subunits, whereas extra-synaptic GABA_A receptors often contain the δ subunit in combination with α4, α6, β2, and β3 subunits (Nusser et al., 1998; Jones et al., 1997; Barnard et al., 1998).

Specific subunit combinations confer GABA_A receptors with distinct functional properties, pharmacological sensitivity, and subcellular localization (MacDonald and Olsen,
The α subunit determines GABA potency, and GABA_A receptor decay and desensitization kinetics (Lavoie et al., 1997; McClellan and Twyman, 1999; Mozrymas et al., 2007; Barberis et al., 2007). The β subunit also confers specific pharmacological sensitivity and is a site for post-translational modifications (Wafford et al., 1994; Brandon et al., 2002; Thompson et al., 2004; Mortensen and Smart, 2006; Ade et al., 2008; Janssen et al., 2009; Janssen et al., 2011). The α2 subunit has been shown to be critical in plasticity and synaptic organization (Essrich et al., 1998; Meier & Grantyn, 2004b; Muir et al., 2010), and allows for post-synaptic receptor clustering (Figure 9).
**Figure 9:** GABA<sub>A</sub>R subunits. A, GABA (γ-aminobutyric acid) type A receptors (GABA<sub>A</sub>Rs) are members of the ligand-gated ion-channel superfamily. GABA<sub>A</sub>R subunits consist of four hydrophobic transmembrane domains (TM1–4). TM2 is believed to line the pore of the channel. The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for psychoactive drugs, such as benzodiazepines (BZs). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for various protein interactions as well as for various post-translational modifications that modulate receptor activity. B, Five subunits from seven subunit subfamilies (α, β, γ, δ, ε, θ, π) assemble to form a heteropentameric Cl<sup>-</sup>-permeable channel. Despite the extensive heterogeneity of the GABA<sub>A</sub>R subunits, most GABA<sub>A</sub>Rs expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by δ, ε, θ, or π. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing increased Cl<sup>-</sup>-permeability of the membrane. BZ binding occurs at the interface between the α (1, 2, 3 or 5) and γ subunits and potentiates GABA-induced Cl<sup>-</sup> flux. C, GABA<sub>A</sub>Rs composed of α (1–3) subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas α5, β, γ receptors are located largely at extrasynaptic sites. Both these types of GABA<sub>A</sub>R are BZ sensitive. By contrast, receptors composed of α (4 or 6), β, and δ are BZ insensitive and localized at extrasynaptic sites.

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In the majority of GABA<sub>A</sub> receptors, the GABA binding pocket is located between the α and β subunits. The GABA<sub>A</sub> receptor has additional binding sites that have been harnessed for a variety of pharmacological uses (Figure 10). Benzodiazepines target the interface between the α and γ subunits, and act as allosteric modulators to enhance the frequency of channel opening in the presence of GABA. Similarly, barbiturates enhance the effect of GABA by increasing GABA<sub>A</sub> receptor/channel open time, though they have a different site of action than benzodiazepines. Muscimol, a psychoactive component of hallucinogenic mushrooms, and gaboxadol, a sleeping aid, are agents that bind at the GABA<sub>A</sub> receptor the same site as GABA itself.
**Figure 10:** Binding sites for various drugs and ligands on the GABA$_A$R. A cartoon of the GABA$_A$ receptor showing potential binding sites for various drugs and ligands. The subunit composition of this receptor is typical in the central nervous system. Gephyrin and GABARAP are scaffolding proteins that depend on the presence of the γ subunit to aid in clustering GABA$_A$ receptors at the synapse.

*Figure from Neil Harrison: Navigating Neuronal Pathways.*
Gephyrin is a tubulin-binding scaffolding protein that is found at inhibitory synapses and has been shown to play a critical role in GABA_A receptor clustering (Betz, 1998). Recent work suggests that the α1-3 and β2-3 subunits are responsible for postsynaptic GABA_A receptor stabilization in a gephyrin-dependent fashion (Kirsch et al., 1995; Tretter et al., 2008; Saiepour et al., 2010; Mukherjee et al., 2011; Tretter et al., 2011; Kowalczyk et al., 2013). When gephyrin is removed via siRNA interference or gene targeting, both GABA_A receptor clustering and inhibitory currents are strongly affected. A parallel effect on gephyrin clusters is observed following deletion of the γ2 subunit (Essrich et al., 1998; Kneussel et al., 1999; Li et al., 2005; Yu et al., 2008). The γ2 is also strongly linked to the function of gephyrin as a postsynaptic anchoring protein for GABA_A receptor.

Within the striatum, there is strong expression of the α2, α4, β3 and δ subunits (Laurie et al., 1992; Pirker et al., 2000; Sinkkonen et al., 2000; Heldt and Ressler, 2007). There also seems to be clear expression of α1, α3, α5, β1, β2, γ3, and γ4, and further staining suggests α1 and α5 expression in single dendrites (Korpi et al., 2002). All three of the β subunits are present in the striatum, though there seems to be stronger staining for β1 and β3 than β2 (Pirker et al., 2000; Schwarzer et al., 2001). There have been reports of high θ subunit expression (Bonnert et al., 1999), observed specifically when the θ subunit is combined with the α2, β1, and γ1 subunits. In recombinant form, α2β1γ1θ receptors exhibited a much lower affinity for GABA when compared to receptors composed only of α2β1γ1 (Ranna et al., 2006).
ii. Equilibrium potential

The binding of two molecules of GABA or comparable agonist allows a conformational change to permit bidirectional ion flow into the cell. As the direction of ion movement depends largely on the electrochemical gradient of the ion, GABA\textsubscript{A} receptor channel opening can result in either depolarization or hyperpolarization, depending on the state of the neuron (Figure 11).

Early in development, chloride transporters maintain a high concentration of chloride intracellularly so that when GABA\textsubscript{A} receptors open, chloride will efflux out of the cell and depolarize the membrane (Sung et al., 2000). As mature neurons have lower concentrations of intracellular chloride, activation of GABA\textsubscript{A} receptors in developed neurons generally allow chloride influx, hyperpolarizing the cell. In addition to chloride flux, GABA\textsubscript{A} receptors also permit the passage of bicarbonate ions (HCO\textsubscript{3}^-). The equilibrium potential for a given anion is reached when there is zero net flow through the GABA\textsubscript{A} receptor ion channel.

**Figure 11:** GABA\textsubscript{A} receptor channel opening affects the membrane potential of the cell based on the gradient of chloride. **A.** If the neuron has high intracellular chloride, GABA\textsubscript{A} receptor opening will cause chloride efflux and an inward current. In **B** we see that lowering the intracellular chloride reduces the driving force of the ion. **C** demonstrates low intracellular chloride resulting in chloride influx and an outward current. \(E_{Cl}\), chloride equilibrium potential, \(V_h = -70\) mV.
Due to the strong expression of inwardly rectifying potassium channels, MSNs have very hyperpolarized resting membrane potentials. Therefore, in MSNs, GABA \textsubscript{A} receptor opening generally results in an outward flow of chloride and depolarization of the membrane (Plenz, 2003; Bracci and Panzeri, 2006). The equilibrium potential in striatal neurons has been estimated to be approximately -60 mV (Blackwell et al., 2003). MSNs project weak GABAergic collaterals to neighboring MSNs that have been historically difficult to detect. By altering the intracellular solution to increase chloride concentration, one can increase the driving force of chloride efflux allowing for better detection of smaller currents.

**iii. Phasic transmission**

GABA transmission in the striatum can be phasic or tonic. Phasic transmission is the fast synaptic transmission in response to the release of pre-synaptic GABA vesicles into the synaptic cleft, and subsequent binding to post-synaptic, low affinity GABA\textsubscript{A} receptors to evoke inhibitory post synaptic current (IPSC) (MacDonald and Olsen, 1994; Stell and Mody, 2002). A single vesicle, or quantum, of GABA contains thousands of molecules, allowing for the concentration of GABA in the cleft to reach approximately 1 mM (Koos et al., 2004; Farrant and Nusser, 2005). GABA binds and unbinds rapidly from the GABA\textsubscript{A} receptor to evoke high-amplitude, rapidly decaying responses that are important in regulating spike timing and spike-timing dependent synaptic plasticity.

There are a variety of post-synaptic phasic event types that can be studied. The post-synaptic response to a single vesicle of neurotransmitter can be pharmacologically isolated using action potential blockers and antagonists to the channels that are not of interest. These are
referred to miniature events, and in the case of GABA, these events are known as mIPSCs. The study of mIPSCs can supply information on presynaptic release probability and release sites, and/or postsynaptic receptor composition and number. Similarly, sIPSCs are currents in response to GABA released in spontaneous, action potential-dependent manners. Evoked IPSCs, referred to as eIPSCs in this dissertation, are the result of an action potential from an identified presynaptic neuron simultaneously activating many presynaptic sites to create a large post synaptic current. The study of mIPSCs, sIPSCs, and eIPSCs together can provide detailed information on the functional synapses of a neuron. For example, the average amplitude of the eIPSCs of a cell can be divided by the average mIPSCs to get a rough estimate of either the number of synaptic boutons or how many vesicles are released in a given eIPSC. This is also known as quantal content.

Another method of studying synaptic transmission between two specific neurons is by examining fluctuations in the amplitude of eIPSCs in response to different concentrations of extracellular calcium. Calcium is required for action potential mediated presynaptic vesicle release; therefore, altering the extracellular calcium concentration will change the probability of release. The variance of the peak amplitude can be analyzed to extrapolate information on the average amplitude in response to a vesicle of neurotransmitter, the number or independent release sites, and the probability of presynaptic release. This is referred to as Mean-Variance analysis (Reid and Clements, 1999; Clements and Silver, 2000).

The kinetics of phasic transmission in MSNs is affected by GABA<sub>A</sub> receptor subunit composition. In α2 subunit knock out mice, MSNs in the nucleus accumbens displayed a decrease in mIPSC amplitude and a clear prolongation of mIPSC decay (Dixon et al., 2010).
Similarly, using a α2 knock out mouse, Ade et al. (2008) found prolongation of mIPSC and sIPSC decay, though no changes in amplitude were detected. In a study with β3 subunit knock out mice, MSNs in the striatum had faster mIPSC decay rates compared to control MSNs (Janssen et al., 2011). It is clear from other brain areas that GABA_A receptor composition strongly affects deactivation kinetics (Verdoorn et al., 1990; Macdonald and Olsen, 1994; Gingrich et al., 1995; Tia et al., 1996; Lavoie et al., 1997; Bianchi et al., 2002; Picton and Fisher, 2007; Bright et al., 2011; Eyre et al., 2012). Therefore, as one would expect, differences GABA_A receptor subunit composition also affect MSN IPSC kinetics.

iv. Tonic transmission

High-affinity, extrasynaptic GABA_A receptors mediate tonic GABAergic transmission (Glykys and Mody, 2007) and are important in regulating network excitability (Semyanov et al., 2003; Scimemi et al., 2005). When activated by micromolar levels of GABA, these receptors allow currents that have small amplitudes and longer decay times, with minimal desensitization (Tossman and Ungerstedt, 1986). GABA_A receptors that mediate tonic currents are composed of at least one α and one β subunit, along with a δ or ε subunit (Glykys and Mody, 2007). The α subunit present is usually α 4, 5 or 6 (Ade et al., 2008; Santhakumar et al., 2010). Interestingly, D_2 MSNs display larger tonic currents at younger ages than D_1 MSNs, potentially attributable to α5 subunit containing GABA_A receptors (Ade et al., 2008) and phosphorylation of the β3 subunit (Janssen et al., 2009).
2. **Glutamate receptors**

The basal ganglia receive a large number of glutamatergic afferents into the striatum from the cortex and thalamus, and the STN is the only glutamatergic projecting nuclei within the basal ganglia. Corticostriatal and thalamostriatal afferents can be distinguished by selective expression of vesicular glutamate transporter 1 and 2, respectively (Doig et al., 2010). Although these afferents innervate D₁ and D₂ MSNs in comparable proportions, corticostriatal afferents commonly target MSN spine heads and thalamostriatal afferents target dendritic shafts and spine heads (Smith et al., 2004). These two groups of afferents also have differences in release probability and short-term plasticity, suggesting they code information in temporally distinct ways, ultimately constraining striatal circuitry regulation (Ding et al., 2008).

The two main ionotropic glutamate receptors in the basal ganglia are the AMPA receptor and the NMDA receptor. When glutamate is present, these channels open to allow for bidirectional flow of calcium, potassium and sodium, usually depolarizing the membrane. Permeability through the NMDA receptor pore is voltage-dependent, as Mg²⁺ blocks the channel at rest. Although both D₁ and D₂ MSNs express NMDA and AMPA receptors, D₂ MSNs display larger NMDA:AMPA ratios (Kreitzer and Malenka, 2007), suggesting differences in the preservation of synaptic activity to long-term adaptive responses (Rao and Finkbeiner, 2007).
E. Metabotropic receptors and transmission in the striatum

1. Dopamine

Dopamine (DA) is a major neurotransmitter that acts as a neuromodulator in the central nervous system. The largest producing nucleus of DA is the SNpc. DA acts by binding to G-protein coupled surface receptors that can be divided into the D₁ and D₂ classes of DA receptors. The D₁-class incorporates D₁ and D₅, and the D₂-class includes D₂, D₃ and D₄. Activation of D₁-class of DA receptors, which are Gₛ coupled, increases intracellular cyclic adenosine monophosphate (cAMP) levels by activating adenylate cyclase. On the other hand, D₂-class receptors are Gᵢ/₀ coupled and inhibit adenylate cyclase, reducing intracellular cAMP levels. Dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) is expressed in MSNs and is regulated by DA and NMDA receptor activation. As a net effect, DA receptor activation modulates the excitability of the cell by targeting calcium, sodium and potassium channels. In addition, depending on the G-protein activated, it has been shown that DA can change AMPA/NMDA receptor insertion (Surmeier; 2007). Therefore, DA increases or decreases excitability depending on which receptor class is targeted (Figure 12).
Figure 12: Dopaminergic signaling affecting the integration of glutamatergic signaling in MSNs. A, Schematic representation of a striatopallidal MSN dendrite and spine. DA and muscarinic receptor activation modulates intrinsic excitability by altering the gating of Ca\(^{2+}\), Na\(^{+}\) and K\(^{+}\) channels. B, Signal transduction pathways mediating the effects of D1 receptors in striatonigral MSNs and D2 receptors in striatopallidal MSNs. Abbreviations: ACh, acetylcholine; AMPAR, AMPA receptor; DA, dopamine; DAG, 1,2-diacylglycerol; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; Glu, glutamate; IP3, inositol 1,4,5 trisphosphate; NMDAR, NMDA receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP-2B, protein phosphatase 2B; RCS, regulator of calmodulin signaling.

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D₁ receptors are the most abundant DA receptor type in the central nervous system followed closely by D₂ receptors. The remaining receptors, D₃-₅, are expressed at much lower levels in the brain (Hurley and Jenner, 2006). DA receptors are located on the dendritic shafts of neurons in the striatum, and DAergic terminals are more abundant in the striosomes than the matrix compartments (Bolam and Smith, 1990; Miura et al., 2007). DA has also been shown to control specific MSN axon collaterals (Tecuapetla et al., 2009).

The importance of DA in the brain is most recognized in its absence, as illustrated by Parkinson’s Disease where severe motor deficits are the result of degenerating SN_{pc} dopaminergic cells. Animal models of DA depletion often utilize 6-OHDA lesions of the SN_{pc} to selectively destroy dopaminergic and noradrenergic neurons. MSN excitability is enhanced in these models, perhaps by decreasing the strong role of potassium currents in maintaining MSN membrane properties (Fino et al., 2007). Early studies found that 6-OHDA lesions resulted in a reduced number of asymmetric synapses in striatal MSNs, but no change in symmetric synapses (Ingham et al., 1998). Recent evidence specifically points to the D₂ MSN to be specifically affected in DA-depleted states, demonstrated through a reduction in primary dendrites and a decrease in excitatory spine number (Day et al., 2006; Mallet et al., 2006; Shen et al., 2007; Day et al., 2008). Furthermore, dopamine depletion has been shown to dramatically reduce collaterals between MSNs, specifically D₂ MSNs, and this is a major focus of this thesis (Taverna et al., 2008).
**Figure 13**: TH-positive fibers in the striatum. In BAC D2-eGFP mice stained with nuclear marker DAPI, immuno-labeling for TH-positive fibers demonstrates dense dopaminergic innervation of the striatum.

*Image courtesy of Stefano Vicini and John Partridge.*
**Figure 14:** Dopamine modulates MSN cell excitability. A, Representative current-clamp recording from a D$_2^+$ MSN illustrating the responses to a series of depolarizing current injections (20 pA steps) from −70 mV, recorded with K-gluconate internal in the absence and presence of quinpirole (10 µM) and BMR (25 µM). B, Representative example of a D$_1^+$ MSN in the same conditions as A, but with the D$_1$-like selective agonist, SKF-81297 (10 µM). C, Summary plot showing the averaged rheobase current in D$_2^+$ (n = 5) and D$_1^+$ (n = 7) MSNs with dopamine agonist and BMR application. D, Summary of action potential firing frequency in response to increasing depolarizing current injections recorded with K-gluconate internal solution in D$_2^+$ MSNs (■) in the absence and presence of 10 µM quinpirole (□) and 25 µM BMR (●). Data derive from the same cells in C. E, Summary of action potential firing frequency in D$_1^+$ MSNs (■) in the absence and presence of 10 µM SKF-81297 (□) and 25 µM BMR (●).

*Significance to D$_2^+$ control cells; # significance between D$_2^+$ and D$_1^+$ cells. Calibration: 20 µM, 500 ms.

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2. **GABA\textsubscript{B} receptors**

GABA\textsubscript{B} receptors are metabotropic receptors that can be found in pre- and post-synaptic regions. Activation of presynaptic GABA\textsubscript{B} receptors reduces calcium conductance, which inhibits presynaptic release of neurotransmitters. Postsynaptic GABA\textsubscript{B} receptors are also found in the central nervous system, and are coupled to inwardly rectifying GIRK or Ki3 potassium channels, so their activation increases membrane potassium conductance (Nicoll, 2004). It is important to note that there are no GIRK channels in striatal MSNs.

In 1979 Bowery et al. discovered a population of receptors that were GABA sensitive but were not chloride dependent nor blocked by bicuculline. These GABA\textsubscript{B} receptors were found to be sensitive to baclofen, a GABA analogue that, when bound to GABA\textsubscript{B} receptors, reduces transmitter release and depresses normal neuronal activity (Bowery et al., 1979; Bowery et al., 1983). GABA\textsubscript{B} receptors have been suggested to be involved in the actions of ethanol (Dzitoyeva et al., 2003), GHB (Dimitrijevic et al., 2005), and pain (Manev and Dimitrijevic, 2004), and also potentially play an important role in development (Dzitoyeva et al., 2005).

The presence of GABA\textsubscript{B} receptors in the striatum has been confirmed, and baclofen, an agonist of the GABA\textsubscript{B} receptor, causes a reduction in size of evoked EPSPs in striatal neurons *in vitro* (Calabresi et al., 1990; Calabresi et al., 1991; Nisenbaum et al., 1992). GABA\textsubscript{B} activation also generates slow IPSPs via increased potassium conductance (Calabresi et al., 1991; Nisenbaum et al., 1992; Nicoll, 2004).
F. Striatal GABAergic microcircuits

1. Feedforward inhibition

Feedforward inhibition in the striatum is unique as the outcome of the operation does not influence the inhibiting cell itself. PV+ fast-spiking interneurons are the most well studied interneuron in this microcircuit as they form large IPSPs onto MSNs that are strong enough to delay action potential firing of coupled MSNs, or inhibit firing completely (Plenz and Kitaï, 1998; Koos and Tepper, 1999). These synapses form at a rate of 25% for PV-MSN pairs within a 250 µm range of each other, and have a failure rate of less than 1% (Koos and Tepper, 1999). PV+ interneuron afferents have been shown to target both MSN subtypes, and one PV+ cell can be coupled to anywhere between 135 – 541 MSNs (Koos and Tepper, 1999). PV+ neurons form extensive peri-somatic synapses and axo-dendritic synapses onto MSNs resulting in large and fast IPSPs with low failure rates (Bennet and Bolam, 1994).

The role of NPY+ interneurons in feedforward inhibition is less clear. Some studies have failed to find synaptic connections between NPY+ interneurons and MSNs (Gittis et al., 2010). This, however, may be due to neuron selection as recent studies identify the subclass of NPY-NGF cells to form frequent, long-lasting IPSCs onto MSNs (Ibáñez-Sandoval et al. 2011, English et al. 2011; Luo et al., 2013).
2. Feedback inhibition

Feedback inhibitory microcircuits, where neurons within a structure extend collaterals to neighboring neurons, generally diminish excitation. Feedback inhibition is found in a variety of different brain areas including the cortex and hippocampus. In the hippocampus, pyramidal neurons in the CA3 form excitatory connections onto basket cells, which project inhibitory collaterals which feed back to inhibit the CA3 cells. As D1 and D2 MSNs are the output neurons of the striatum, GABAergic feedback collaterals between MSNs are critical for information processing to produce appropriate signals. MSNs comprise the vast majority of striatal neurons and have extensive dendritic arbors and axons that often extend beyond their dendrites. Therefore, it was expected early on that MSN collaterals provided the majority of striatal inhibition. Until recently, however, the degree of connectivity between MSNs has been reported at minimal rates (Jaeger et al., 1994), resulting in speculation that MSN collaterals were not significant contributors in shaping the basal ganglia circuit.

Feedback inhibition between MSNs, also referred to as lateral connectivity, occurs more commonly at distal dendrites. Therefore, signals may be diminished by the strong shunting effect of the prominent inward rectifying potassium currents. About 88% of MSN-MSN contacts are made onto MSN dendritic shafts and spines, and the remaining 12% of synapses made at the soma (Gerfen and Wilson, 1996). Therefore, the extremely low rate of collateral detection reported in earlier studies may partly be due to the severing of distal synapses with the conventional ex vivo slice preparation. Many other factors can affect the rate and size of MSN-
MSN functional synapses—such as age, *in vitro* system, and chloride driving force—all of which can be optimized to maximize the detection and study of these collaterals.

Several groups have now shown clear evidence for functional connectivity between MSNs. This dissertation aims to study the properties of these collateral synapses to elucidate a functional role for these connections (Tecuapetla et al., 2009). As D₂ MSN collaterals are specifically regulated by dopamine (Taverna et al., 2008), this dissertation will perform an in-depth investigation on how D₂ receptor activation alters feedback inhibition.

Feedback inhibition is important in processing the numerous inputs MSNs receive from cortical cells and striatal interneurons. Now that evidence points towards an abundance of MSN collaterals, the task is to determine the properties and importance of these synapses.
Figure 15: Schematic summary of the canonical striatal microcircuit. Explaining recurrent and alternating network states in the striatal microcircuit. a) Represents the intrinsic oscillator made up by the cholinergic interneurons and any of several classes of GABAergic interneurons. These interneurons may exhibit pacemaking properties according to context. Dashed circle indicates that the excitatory drive may impinge on many of the neuron pools represented by the symbols. Afferent inputs or a continuous excitatory drive turn on these pools according to intensity and location. b-d) Dashed lines enclose represented pools of SPNs (10-40 neurons) recruited by the oscillator as explained in B. These pools have mixtures of direct and iSPNs (unpublished) which are interconnected according to certain rules (Taverna et al., 2008). The synchronized firing of these neuron pools underlie the networks states (peaks of spontaneous synchronization) that alternate their activity in regular and fixed patterns such as reverberating cycles of activity. That is the three pools represented may alternate their activity in a fixed sequence. They are not anatomically separated as in the scheme, but most probably intermingled (Carrillo-Reid et al., 2008). Alternation of activity among the pools may be explained either by a sequential activation of oscillators, or more likely, to a recovery variable that stops synchronized activity within a pool. This recovery variable is under study but may emerge from intrinsic properties, e.g., repolarizing $K^+$-currents (see above) and inhibitory interconnections among SPNs. In any case the sequence of network states that show a reverberating cycle is orchestrated by GABAergic interneurons, that unlike SPNs, are active during each network state (Carrillo-Reid et al., 2008).

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II. MATERIALS and METHODS

A. Animals

Mice with bacterial artificial chromosome (BAC) D1-tdTomato were crossed with BAC D2- enhanced green fluorescent protein (EGFP) mice (Figure 16; GENSAT; Gong et al., 2003; Shuen et al., 2008). The drd2-EGFP line in our colony was backcrossed onto a C57BL/6 background (Chan et al., 2012) to avoid the abnormalities reported in Kramer et al. (2011). The characteristics of MSNs in the presence and absence of treatments were also confirmed in cultures harvested from two separate mouse strains: drd2-cre;rosa26-tdTomato (Madisen et al., 2010) and npy-EGFP;drd1a-tdTomato (Shuen et al., 2008; Partridge et al., 2009). These mice were derived from FVB/B6/129/Swiss and C57BL/6- B6SJLF1 strains, respectively.

Figure 16: A sagittal section of a BAC drd1a-tdTomato; BAC drd2-EGFP mouse illustrating distinct striatonigral (red) and striatopallidal (green) pathways. As seen here, td-Tomato and GFP expressing fibers and can be traced to the substantia nigra and GPe, respectively. Td-tomato expression was also noted in cortical layers.
B. Primary cortico-striatal cultures

I adapted the procedure originally described in Segal et al. (2003). BAC *drd1a-tdTomato* mice and BAC *drd2-EGFP* mice harbor bacterial artificial chromosome (BAC) transgenes with td-Tomato reporter downstream of D₁ receptor promoter and EGFP reporter downstream of the D₂ receptor promoter, respectively (GENSAT; Gong et al., 2003; Shuen et al., 2008). Crossing these two strains resulted in healthy mouse pups, a subset of which co-expressed tdTomato in D₁ cells and EGFP in D₂ cells. Primary cultures were prepared from the progeny of this cross. Pups of either sex were decapitated at postnatal day 0 (P 0) in accordance with the guidelines of the AMVA Panel on Euthanasia and the Georgetown University Animal Care and Use Committee. A sterile buffer solution containing 0.7% NaCl, 0.04% KCl, 0.02% KH₂PO₄, 0.3% C₆H₁₂O₆, 0.2% NaHCO₃ and 0.001% Phenol Red (all from Fisher Scientific; Hampton, NH) was prepared. Whole brains were removed into the ice-cold buffer solution with the addition of 0.3% BSA (Sigma; St. Louis, MO) and 0.01% MgSO₄ (Fisher Scientific). Meninges were removed and brains were examined for fluorophore expression using Dual Fluorescent Protein Flashlight (NIGHTSEA, Bedford, MA). Striata from brains expressing both tdTomato and EGFP were dissected, using GFP expression as a guide (Figure 18). Cortical tissue from littermates that did not express either fluorophore was separately dissected.

Once collected, cortical and striatal tissues were minced finely and centrifuged at 1000 rpm (rotations per minute) for 2 minutes in separate tubes. The supernatants were removed and the pellets were mixed into the buffer solution containing 0.04% trypsin (Sigma). After 15
minutes in a 37°C water bath shaking at 180 rpm, an equal volume of buffer solution containing 0.001% DNAase, 0.01% trypsin inhibitor and 0.01% MgSO₄ was added to the suspensions and centrifuged at 2000 rpm for 5 minutes. The supernatants were then aspirated and replaced with buffer solution containing 0.01% DNAase, 0.05% trypsin inhibitor and 0.04% MgSO₄. The pellet was tritutated first with normal glass Pasteur pipettes and then with Pasteur pipettes that had been fire-fined to reduce the diameter of the opening. Then, an equal volume of buffer solution containing 0.03% MgSO₄ and 0.001% CaCl₂ was added. Cells were centrifuged at 1000 rpm for 7 minutes. The supernatants were removed and pellets were resuspended in 2 ml of Basal Eagle’s Medium (Invitrogen, Carlsbad, CA). Cell concentrations of the single cell suspensions were determined with a hemocytometer. Cells were combined to achieve a 1:3 ratio of cortical to striatal neurons and diluted to a final concentration of 5x10⁵ cells/ml in Neurobasal Medium, supplemented with 0.25% glutamine, 1% penicillin-streptomycin, 2% B27 (all from Invitrogen), 50 ng/mL BDNF (Alomone, Jerusalem, Israel), and 30 ng/mL GDNF (Sigma). 150 µl of the cell suspension was plated onto poly D-lysine (10 µg/ml, Sigma) coated glass coverslips (1.13 cm²) that had been previously incubated in Basal Eagle’s Medium (Invitrogen, Carlsbad, CA), and cells were incubated at 37°C in 95% O₂/5% CO₂ for 40 minutes. 100 µl was then removed from each coverslip and 200 µl of supplemented Neurobasal Medium was added. At two days in vitro (DIV), 250 µl of supplemented Neurobasal Medium with 50 ng/mL BDNF and 30 ng/mL GDNF was added to each well. 125 µl of supplemented Neurobasal media was exchanged twice weekly, although BDNF and GDNF were not included after DIV 2 (Tian et al., 2010).
C. **Electrophysiology**

Whole-cell voltage-clamp and current-clamp recordings were performed on striatal neurons identified by either tdTomato or EGFP fluorescence. Cells were visualized with a Nikon Eclipse TE2000-S inverted microscope with 4x and 40x phase contrast objectives (Tokyo, Japan), a super high pressure mercury lamp (Nikon), and T-FLC Nikon fluorescence filters. Images were taken with an Infinity 2-1M camera and Infinity Capture software (Lumenera; Ottawa, ON, Canada). All recordings were done at room temperature in extracellular solution (ES) continuously perfused at a rate of 2.5 ml/min with a Masterflex C/L Dual-Channel Variable-Speed Tubing Pump (Cole-Parmer, Vernon Hills, IL). ES was composed of (in mM, all from Fisher Scientific, Pittsburg, PA): NaCl (145), KCl (5), MgCl$_2$ (1), CaCl$_2$ (1), HEPES (5), glucose (5), sucrose (15), phenol red (0.25mg/l), and adjusted to pH 7.4 with NaOH. Recording electrodes were pulled on a two step vertical pipette puller (PP-83, Narishige, Tokyo, Japan) from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) with resistances of 3-5MΩ. To characterize basic electrophysiological properties, electrodes were filled with intracellular solution containing (in mM): K-gluconate (145), HEPES (10), ATP·Mg (5), GTP·Na (0.2), and EGTA (0.5), adjusted to pH 7.2 with KOH. The equilibrium potential ($E_{Cl^-}$) for Cl$^-$ in this condition was -71.4 mV. To study inhibitory synaptic transmission, K-gluconate (145) was replaced with a mixture of K-gluconate (100) + KCl (44). The $E_{Cl^-}$ for this solution was -29.6 mV, allowing GABAergic currents to be recorded as inward at -70 mV while preventing series resistance errors created by extremely large currents. The calculated liquid junction potentials for these conditions were 15.8 mV and 12.2 mV, respectively, and the values
reported have been compensated accordingly. Whole cell voltage-clamp recordings were performed with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and access resistance was monitored throughout the recordings. Currents were filtered at 1 kHz with an 8-pole low-pass Bessel filter and digitized at 5 kHz using an IBM-compatible microcomputer equipped with Digidata 1440B (Molecular Devices) data acquisition board and pCLAMP10 software.

Stock solutions of bicuculline methobromide (BMR), tetrodotoxin (TTX), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (all from Abcam, Cambridge, MA), and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP, Sigma) were prepared in water and diluted in ES to a final concentration of 25 µM, 500 nM, 5 µM and 1 µM, respectively. Stock solutions of etomidate, flumazenil and diazepam (all from Sigma) were prepared in dimethyl sulfoxide (DMSO) and diluted in ES (final DMSO concentration <0.01%). GABA (Sigma) was dissolved in water and diluted in ES to the desired concentrations. Solutions were rapidly exchanged using the Y-tube method (Murase et al., 1989).

Recordings from identified neurons were conducted in voltage clamp at -70 mV and current clamp at the resting membrane potential (RMP) of the cell. Recordings of cells which had leak currents of greater than 100 pA or RMP of less than -50 mV were discarded. Synaptic connections were studied in paired recordings while both neurons were held in voltage-clamp configuration at -70 mV. Cells were alternately stimulated at 3 second intervals with a voltage step to induce an action potential (+100 mV, 4 ms). Neurons were considered coupled if the presynaptic action potential induced an evoked inhibitory post synaptic current (eIPSC) in the postsynaptic cell (e.g., Figure 26). These presynaptic action potentials were also presented at 10
hz (with 5 stimuli) and 20 hz (10 stimuli) (e.g., Figure 27) over 3 seconds, though these data was not extensively pursued. In addition, if two neurons were coupled, action potentials were elicited in current clamp from the presynaptic neuron, while the post-synaptic neuron was held in voltage clamp (e.g., Fig. 28). Preceding all stimulation, a test voltage pulse (-5 mV, 20 ms) was given to monitor series and input resistance and experiments with unstable holding currents were discarded. For variance-mean (V-M) analyses of eIPSCs, changes in extracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_o$) were induced with the Y-tube application of four $[\text{Ca}^{2+}]_o$ concentrations. The resulting variances were studied as in Clements and Silver (2000) by plotting the variance of the peak amplitude against the mean.

IPSC parameters were examined in Clampfit 10.2 (Molecular Devices). eIPSC averages were based on >20 events with failures removed. The decay phase of the averaged eIPSCs was fit to a double-exponential function and $\tau_w$ was calculated as

$$\tau_w = \frac{A_1 \times \tau_1}{A_1 + A_2} + \frac{A_2 \times \tau_2}{A_1 + A_2}$$

where $\tau_1$ and $\tau_2$ are the fast and slow decay time constants, respectively, and $A_1$ and $A_2$ are the contribution of the first and second exponentials to the amplitude (Nusser et al., 2001). Miniature (in TTX and NBQX) and spontaneous events were identified using semi-automated threshold-based software (Mini Analysis, Synaptosoft, Fort Lee, NJ) and visually confirmed (Ade et al., 2008). >100 events were averaged per cell and decay kinetics were determined using a single-exponential equation.

In some experiments, cells were held in current-clamp and given hyperpolarizing and depolarizing steps of 10-20 pA each (e.g., Figure 29). From this, I was able to extract information
regarding the presence of inwardly rectifying potassium channels and A-type potassium channels, along with other membrane properties of the neuron (e.g. Table 1).

D. **Treatments**

Neurons were pharmacologically stimulated between DIV 18-21. To activate dopamine D$_2$ receptors, neurons were treated with the selective D$_{2/3}$ receptor agonist (±)-quinpirole dihydrochloride (10 µM; Sigma) diluted in Neurobasal media (Deyts, et al., 2009). To study non-specific actions of quinpirole, I pretreated a subset of coverslips for 10 minutes with D$_{2/3}$ receptor antagonist sulpiride (10 µM; Sigma). I then chronically treated the cells with 10 µM sulpiride + 10 µM quinpirole for 72 hours.

A smaller subset of coverslips was treated with a low dose of quinpirole (500 nM). Control neurons were given an equal volume of Neurobasal media without the addition of drug. All cells were returned to the incubator for 72 hours before experimentation.

E. **Histochemistry**

**BAC drd1a-tdTomato; BAC drd2-EGFP** mice aged p1, p5 and p10 were killed by decapitation. Whole brains were dissected out and placed into a 4% PFA/4% sucrose PBS solution at room temperature for 24 hours. Brains were then washed 3x with PBS and 150 µm sagittal sections were cut with a Lancer Vibrotome (Series 1000, Sherwood Medical, St. Louis, MO). Slices containing both striatum and substantia nigra were mounted on glass coverslips with
VectaShield and visualized with an inverted Olympus microscope (Shinjuku, Tokyo) and a mercury lamp powered by Ludl Electronic Products, Ltd. Power Supply (Hawthorne, NY). Images were captured using a 10x objective with a Nikon digital camera (DMX 1200) and stitched together using NIS-Elements 2.2 software (Nikon).

Anatomical reconstruction was achieved by including 1% biocytin in the intracellular solution of a subset of neurons (Partridge et al., 2009). To preserve the cell body, the pipette was carefully removed after 5-8 minutes to form an outside-out patch. Coverslips containing recorded, filled neurons were placed in 4% paraformaldehyde/4% sucrose/PBS solution for 15 minutes at room temperature (RT), rinsed 3 times in 1x phosphate buffered saline (PBS) and subsequently permeabilized and stained with fluorescein-avidin dye (Vector Labs, Burlingame, CA) at 2.5 μL/mL for 120 minutes. Coverslips were briefly washed in PBS and mounted on glass slides using Vectashield anti-fade solution (H-1000, Vector Labs).

To better study D$_2$ MSN synapses, mature neurons were fixed and washed as described above. Next, coverslips were permeabilized and blocked as in Tian et al. (2010), and incubated with antibodies to either rabbit α-GFP (1:200, Invitrogen) and mouse α-gephyrin (1:500, Synaptic Systems, Goettingen, Germany), or mouse α-GFP (1:500, Invitrogen) and rabbit α-VGAT (1:1000, Abcam) for two hours at room temperature. Appropriate secondary antibodies conjugated with Alexa-488 or Texas Red were diluted 1:1000. Coverslips were washed three times and mounted with Vectashield for visualization. All histochemistry procedures were conducted at room temperature.
F. Microscopy and image analysis

Images of fixed and stained neurons were acquired using a Nikon FS1 upright microscope equipped with a two channel confocal imaging module (CLS-2HS, ThorLabs Imaging System, Sterling, VA), a multichannel fiber coupled laser source (ThorLabs), and mercury lamp (Intensilight C-HGFI, Nikon, Tokyo, Japan). To study neurite arborization in biocytin injected cells, single confocal images were captured using a 20x objective. Cells were traced with the ImageJ NeuronJ plugin and dendritic arborization was analyzed with Sholl Analysis plugin (National Institutes of Health, Bethesda, Maryland). 3-5 neurons were analyzed per condition in 3 separate cultures and results were plotted as the number of intersections per 10 µm concentric circle from the soma. To study dendrite puncta, confocal images were captured using a 60x objective and puncta measures were quantified with a custom macro for NIH ImageJ software (courtesy of F. Vanevski). Analyses were performed on puncta that were associated with either GFP+ or non-GFP neurons. One representative 70-100 µm dendritic segment starting at the base of the primary dendrite was chosen per each cell. At least 10 neurons from different coverslips and cultures were analyzed for each condition. Puncta density and fluorescence intensity of immunoreactive puncta were calculated by thresholding images at two times background in all conditions. Means from each representative dendrite per neuron were averaged to obtain population means.
G. Statistics

Kolmogorov-Smirnov tests were conducted on all data sets to check for normal distribution and appropriate statistics were chosen dependent upon these results. Statistical tests and graphical plots were carried out in Prism 5 software (GraphPad) and are detailed in Results and Figure Legends. All data values in the text and figures are presented as mean ± SEM.
III. RESULTS

A. MSN subtypes and their projections in BAC drd1a-tdTomato;BAC drd2-EGFP mice through development

To confirm previous findings on the maintenance of dopamine receptor expression through development (Goffin et al., 2010; Thibault et al., 2013), I compared receptor promoter activity in fixed sagittal slices of BAC drd1a-tdTomato;BAC drd2-EGFP mice at postnatal days 1 (P1), 5, and 10 (Figure 17). Both fluorophores were visible at P1 indicating the presence of D₁ and D₂ receptors. However, while striatal D₂ receptor expression appeared stable, striatal D₁ receptor expression seemed to increase from P1 to P10.

D₁ receptor containing MSNs (D₁ MSNs) project directly to the substantia nigra pars reticulata (SN_ṛ), and D₂ receptor containing MSNs (D₂ MSNs) project indirectly to the SN_ṛ via the external segment of the globus pallidus (Gong et al., 2003; Shuen et al., 2008). In BAC drd1a-tdTomato;BAC drd2-EGFP mice, D₁-MSN fibers from the striatum of these mice visibly projected to the substantia nigra at P1 (Figure 17 A), and by P5, D₂-MSN fibers projected to the external globus pallidus (Figure 17 B). In line with previous findings of D₁ receptor expression in the cortex (Gong et al., 2003), I also observed consistent D₁ receptor expressing neurons in the cortex through development in these mice.
Figure 17: Distribution of striatonigral and striatopallidal neurons and their projections in BAC drd1a-tdTomato; BAC drd2-EGFP mice through early development. Photomicrographs of fixed sagittal slices (150 μm) from mice at P1 (A), P5 (B), P10 (C). Slices in the left column are further magnified in the middle and right columns. The insets in the white dashed boxes are magnified below (merged on left, channels separated in middle and right), illustrating a lack of colocalization between red and green cells through early development. Calibration bars are from left to right: 0.5mm, 0.25mm 0.45mm, and 0.15mm.
B. MSNs in co-culture develop considerably between first and second weeks in vitro

To perform targeted studies of D₁ and D₂ MSNs, I took advantage of BAC *drd1a*-tdTomato;BAC *drd2*-EGFP mice to further develop the model originally described by Segal et al. (2003) and extended by Tian et al. (2010). Brains were harvested for cultures at P0-1, and exact dissection was possible due to the strong expression of the D₂ receptor in the striatum (Figure 18, left). As D₁ and D₂ receptors are also expressed in the cortex at P1 (Figure 17 A), I co-cultured striata that co-expressed EGFP and tdTomato, and cortices were selected from littermates that expressed neither fluorophore.

![Figure 18: Cortico-striatal co-cultures from BAC drd1a-tdTomato; BAC drd2-EGFP mice. (Left) Dissected brain at P0, arrowheads indicate EGFP-positive striata in both hemispheres allowing for precise dissection. (Right) Schematic of the culture preparation in which cells are dissociated and combined at a 1:3 ratio of cortical (non-fluorescing) to striatal (tdTomato and EGFP fluorescing) neurons.](image)
The inclusion of 50 ng/mL BDNF and 30 ng/mL GDNF in the feeding media as previously reported in Gertler et al. (2008) and Tian et al. (2010) drastically increased the diversity and complexity of the cell types observed in the cultures (Figure 19).

**Figure 19:** Growth factors in culture. Phase contrast micrographs of DIV 7 (A₁, B₁), 14 (A₂, B₂), and 24 (A₃, B₃) neurons plated without (A₁₋₃) and with (B₁₋₃) BDNF and GDNF, illustrating that cortico-striatal cultures plated in the presence of 50 ng/mL BDNF and 30 ng/mL GDNF develop more diverse phenotypes and retain greater neuron density.
Density of MSNs with fluorophore expression did not significantly change between the first and second week in vitro: D₁ MSN density decreased from 66±10 to 48±6 cells/mm² and D₂ MSN density increased from 31±15 to 43±5 cells/mm², respectively. Colocalization of both fluorophores was detected in 5-10% of the neurons and their density also remained relatively unchanged through development: 12±5 cells/mm² and 14±3 cells/mm² overlapped at the first and second week in vitro, respectively. MSNs retained expression of both fluorescent proteins through the third week in vitro, at which point green, red and non-fluorescent cells were observed at densities of 43±4, 49±4, and 46±6 cells/mm², respectively (p>0.05; Friedman test). Again, 11% of neurons (17±3 cells/mm²) co-expressed both fluorescent markers (p<0.0001 compared to all other cell types; Friedman test). Neurons that expressed both fluorophores were not extensively pursued and thus not included in this dissertation (data obtained in 113 fields from 4 distinct culture preparations).

Pure striatal cultures were attempted to verify the findings from Segal et al. (2003), which describe a crucial role for excitatory input onto MSNs for spine development and glutamatergic transmission. In my hands, however, these cultures did not survive for more than one week in vitro. In addition, when recorded during the first week, these MSNs displayed infrequent NMDA receptor mediated currents.
Figure 20: Fluorophore expression through development in vitro. Examples of cocultures of cortical and striatal neurons through development in vitro at DIV 7 (A) and DIV 15 (B) in a field with red neurons (top left), green neurons (top right), phase contrast (bottom left) and all images are superimposed (bottom right) (40x magnification). Red neurons are D₁, green neurons are D₂, and yellow neurons indicate colocalization. (C) An example of a biocytin injected MSN that illustrates axonal and dendritic arborization. The phase contrast image overlayed to green (top left) and to the red (top right). The middle two panels illustrate the injected neuron (left) with inset in white dashed box magnified to the right. Dendrites are further magnified on the bottom, displaying branched, spiny dendrites.
Neurons in culture were easily identifiable at an early time point using a light microscope equipped with phase contrast and blue and green excitation fluorescence filters (see Methods). Fluorophore expression allowed for targeted recordings from distinct MSNs (Figure 20 A, Figure 21). To study membrane properties of neurons in culture at early and late time points, whole cell patch clamp recordings were conducted from MSNs during the first (DIV 7-10) and second (DIV 13-16) week in vitro with potassium gluconate solution (K gluconate). Though many properties were comparable between MSN subtypes, there was an effect of time in vitro on resting membrane potential (RMP) in D₁ MSNs, capacitance in D₂ MSNs, and input resistance in both subtypes (Table 1).

Figure 21: Drd1a-tdTomato;BAC drd2-EGFP neurons in cultures retain fluorophore expression. A, Examples of co-cultures of cortical and striatal neurons in vitro. Red neurons are D₁ MSNs, green neurons are D₂ MSNs. The two images are shown superimposed to the DIC image. B, Confocal laser scanning microscopy image of a D₂ MSN from Drd1a-tdTomato/Drd2-EGFP culture at DIV 21.
Table 1: Membrane properties of D\textsubscript{1} and D\textsubscript{2} MSNs in primary cultures through development. Whole cell recordings from identified MSNs suggest that, while there are changes observed between one (DIV 7-10) and two (DIV 13-16) weeks in vitro, there is no difference between the D\textsubscript{1} and D\textsubscript{2} MSNs at either time point. Resting membrane potential (RMP) values are corrected for liquid junction potential. Inward rectification index was calculated from a standard current-voltage plot. Comparing MSN subtypes between the first and second week in vitro: \textasciitilde p < 0.05 using an unpaired t-test for normally distributed data, and *p < 0.05 using a Mann-Whitney test for data that was not normally distributed.

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<td>D\textsubscript{1}+</td>
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<td><strong>RMP (mV)</strong></td>
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<td>-70.8±2.9 (n=11)</td>
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<tr>
<td><strong>Capacitance (pF)</strong></td>
<td>32±8 (n=5)</td>
<td>35±5 (n=7)</td>
<td>51±13 (n=4)</td>
<td>50±4 (n=16)</td>
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<tr>
<td><strong>Input Resistance (mΩ)</strong></td>
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<td>891±331 (n=12)</td>
<td>362±46 (n=15)</td>
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<td><strong>Inward Rectification Index</strong></td>
<td>1.1±0.1 (n=3)</td>
<td>1.3±0.5 (n=4)</td>
<td>2.2±0.7 (n=4)</td>
<td>2.2±0.3 (n=18)</td>
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Time in vitro also altered MSN morphology: MSNs at DIV 14 displayed more extensive dendritic complexity and more mature spines in comparison to DIV 7 (Figure 22 A, D). Active membrane properties also matured between the first and second week, as seen with differences in action potential firing patterns (Figure 22 B, E) and up-and-down states (Figure 22 C, F) (Randall, et al., 2011). These spontaneous membrane potential fluctuations that lead to bursts of action potentials have been previously reported in organotypic slices (Plenz and Kitai, 1998) and dissociated cultures (Randall et al., 2011) (Figure 23).
Figure 22: Intrinsic MSN properties at the first and second week in vitro. Images of D2 neurons at DIV 7 (A) and DIV 14 (B). Insets of dendritic segments are shown on the right and illustrate the typical shift from filopodia to spines with development (calibration bars are 100 µm left, 25 µm right). Voltage traces from patch clamp recordings from cultured MSNs at DIV 7 (B) and DIV 14 (E), with K-glucuronate based intracellular solution, illustrate responses to current injections of increasing amplitude. Spontaneous membrane potential fluctuations illustrate the occurrence of putative “up” and “down-states” in MSNs recorded in current clamp at DIV 7 (C) and DIV 15 (F).
Up and down states, paired with the occurrence of spines, suggest the successful innervation of MSNs by excitatory cortical neurons (Segal et al., 2003; Tian et al., 2010). The characteristics of MSNs observed in cultures from drd1a-tdTomato; drd2-EGFP remained consistent across three additional mouse strains and genotypes (drd2-EGFP, drd2-cre;rosa26-tdTomato, and npy-EGFP;drd1a-tdTomato, data not shown).

**Figure 23:** Synchronized activity in MSNs. A, Synchronized action potentials from two MSNs at DIV 16 recorded in cell attached mode with K-gluconate internal solution demonstrate the occurrence of up states. B, Dual whole-cell recordings from D2 neurons at DIV 14 in current clamp at RMP show synchronous up states that are prolonged upon removal of Mg$^{2+}$ (purple region).
C. Co-cultures express functional GABA<sub>A</sub> receptors

As MSNs are GABAergic, I next assessed sensitivity to GABA by locally applying GABA in cultures at two weeks in vitro at three doses. Twenty-second applications revealed two phases of response—a peak and a plateau—to low GABA concentrations (Figure 24). Both D<sub>1</sub> and D<sub>2</sub> MSNs displayed similar sensitivities to GABA.

Figure 24: Cocultures exhibit sensitivity to GABA. Whole cell currents in a representative D<sub>2</sub> MSN demonstrate responses to two GABA concentrations at DIV 15 (top). Summary plots of peak responses to GABA illustrate the presence of GABA<sub>A</sub> receptors on both MSN subtypes at DIV 14-16 (bottom). KCl-based intracellular solution described in Materials and Methods was used.
As the GABA_A receptor is a pentameric structure with differential subunits conferring different channel properties, subunit selective pharmacology was also applied to dissect out potential differences between MSN subtypes in culture. To target the extrasynaptic δ subunit containing GABA_A receptor, I locally applied the δ-preferring agonist THIP (Brown et al., 2002). Interestingly, currents in response to THIP were larger in D_2-MSNs, suggesting a greater number of δ-containing GABA_A receptors in D_2-MSNs. In addition, I tested the GABA_A receptor β 2/3 subunit selective agonist etomidate on whole cell currents, which again revealed an increase in β2/3 expression in D_2-MSNs in vitro. These data are summarized in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>0.3 µM GABA</th>
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<th>3 µM GABA</th>
<th>1 µM THIP</th>
<th>5 µM Etom</th>
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<td><strong>Peak (pA)</strong></td>
<td></td>
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<td>D1</td>
<td>20±12</td>
<td>97±42</td>
<td>228±72</td>
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<td>(n=14)</td>
<td>(n=8)</td>
<td>(n=14)</td>
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</tr>
<tr>
<td>D2</td>
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<td>88±22</td>
<td>477±96</td>
<td>18±3**</td>
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<td>(n=19)</td>
<td>(n=21)</td>
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<tr>
<td><strong>Steady State (pA)</strong></td>
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<td>86±24</td>
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<td>(n=14)</td>
<td>(n=8)</td>
<td>(n=14)</td>
<td>(n=15)</td>
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<tr>
<td>D2</td>
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<td>142±35</td>
<td>14±2***</td>
<td>81±10</td>
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<td>(n=19)</td>
<td>(n=21)</td>
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**Table 2:** GABA currents in D_1 and D_2 MSNs in primary cultures at DIV 14-16. Local application of three concentrations of GABA, of δ subunit preferring agonist THIP and of β2/3 selective agonist etomidate on whole cell currents of identified MSNs. The peak current values are the maximum amplitude of the response, and steady state values are measured at 6-8 seconds after application. Current density can be extrapolated by normalizing peak current data to cell capacitance, which is similar between D_1 and D_2 MSNs (Table 1). *p < 0.05 **p < 0.01 and *** p < 0.001, comparing MSN subtypes using a Mann-Whitney test.
To study single GABAergic synapses of MSNs in vitro, I isolated miniature inhibitory postsynaptic currents (mIPSCs) with local application of 5 μM NBQX and 0.5 μM TTX. Although mIPSCs may be derived from presynaptic terminals from cortical GABAergic neurons, striatal interneurons, or MSNs, the occurrence of mIPSCs indicates successful innervation and the presence of postsynaptic GABA_A receptors. mIPSCs were studied in both D_1 and D_2 MSNs at two weeks in vitro, and the kinetics and frequency of these events were comparable between D_1 and D_2-MSNs (Figure 25).

**Figure 25:** Co-cultures express functional GABA_A receptors and GABAergic synapses. mIPSCs recorded in a D_2 MSN at DIV 15 (top). Summary scatter plots show no differences in mIPSC amplitude, decay, and frequency (bottom; n=26 D_1 MSNs, n=22 D_2 MSNs). A KCl-based intracellular solution was used.
D. Ease in study of MSN inhibitory collaterals

Previous studies have detected an increased rate of coupling between neurons *in vitro* when compared to *ex vivo* slice (Tunstall et al., 2002; Czubayko and Plenz, 2002). Synapses made by neuron collaterals can be studied by performing dual patch-clamp recordings and eliciting an action potential with a single depolarizing step (Figure 26 A, B). As MSN synapses are GABAergic, pipettes were filled with an enhanced [Cl]\textsubscript{-} intracellular solution to achieve an \( E_{\text{Cl}} \) at -30 mV to allow evoked inhibitory postsynaptic currents (eIPSCs) to be detected as inward. Cells were recorded in voltage-clamp at -70 mV and action potentials were artificially elicited in each cell to determine the presence of synaptic coupling.

Out of 104 recorded pairs, evoked inhibitory post synaptic currents (eIPSCs) were detected in 13% of pairs at DIV 7-8 (n=8), and 42% of recorded pairs at DIV 13-16 (n=96; 5 culture preparations). eIPSCs detected at one week *in vitro* were exclusively between D\textsubscript{1} MSNs. In addition to synaptic currents, there were frequent occurrences of what appeared to be autaptic currents immediately following stimulation in both D\textsubscript{1} and D\textsubscript{2} MSNs. These autaptic currents were found in 50% of MSNs at DIV 7-8 (n=8), and 57% of MSNs at DIV 13-16 (n=96). Local application of BMR eliminated synaptic and autaptic eIPSCs and most other spontaneous activity, indicating that the synapses between MSNs *in vitro* are GABAergic (Figure 26 B).
Figure 26: Paired recordings reveal large evoked IPSCs between MSN subtypes. A, Superimposed eGFP fluorescence and DIC images illustrating an example of a dual patch-clamp recording from a pair of D2 MSNs (calibration bar, 25 µm). B, Example traces of evoked synaptic currents in a D2 neuron at DIV 15. A 100 mV test pulse is given to the presynaptic neuron (top), creating an action current (middle) and an evoked current in the postsynaptic neuron (bottom), showing amplitude fluctuation. Local application of BMR (blue) eliminates evoked currents. C, Inhibitory collaterals can be studied by averaging the eIPSC to look at amplitude and rise time (pink), decay (green) and charge transfer (blue) of the eIPSC. eIPSCs were studied with high chloride intracellular solution.
In the first week *in vitro*, eIPSCs between D₁ MSNs were detected though sIPSCs occurred at low frequency in both D₁ and D₂ MSNs. By the second week *in vitro*, synapses had formed between all possible MSN pair combinations (Table 3). Directly stimulating targeted neurons typically resulted in a fast latency to peak (5.2 ±0.5 ms, n=33; 4 culture preparations), single-peaked response, indicating that they were due to the activation of a specific presynaptic MSN rather than to polysynaptic activity. Taking advantage of this configuration, I studied the properties of these identified collaterals (Figure 26, Table 3). As the rate of synapse formation at the first week in vitro was low, the analysis of eIPSCs was limited to DIV 14 and above.
| Table 3: Properties of evoked IPSCs in D1 and D2 MSNs in primary cultures at DIV 13-16. Four combinations of MSN pair recordings were conducted and eIPSCs were elicited in voltage clamp (see Methods). In the first row, **p < 0.01, comparing D1-D1 pairs to D2-D2 pairs using a $\chi^2$ test. In all subsequent rows, statistics were conducted using Kruskal-Wallis test with Dunns post-test to compare MSN pairs. |
The study of these synapses was not limited to single action currents; trains of stimulation were also delivered to study how the eIPSCs were affected by repeated activation (Figure 27). I observed that eIPSC peak would vary per stimulus at low-frequency trains, whereas higher frequency trains (20 hz) would attenuate the size of the eIPSC over the course of the protocol.

Figure 27: Paired voltage-clamp recordings allow for the study of MSN collaterals in a variety of configurations. In a pair of DIV 13 D₂ MSNs where cell 1 is strongly coupled to cell 2 and cell 2 is weakly coupled to cell 1, action currents are delivered at 10 hertz (A) and 20 hertz (B). I₁, I₂, current traces from cell 1 and cell 2, respectively V₁, V₂, voltage traces from cell 1 and 2, respectively. Note the presence of autapses in both neurons.
It was also useful to observe the pre-synaptic cell in current-clamp, so that the pre-synaptic cell could be artificially injected with current to depolarize the cell. In this configuration the cell was induced to fire action potentials while the post-synaptic cell was in voltage clamp (Figure 28). Though I primarily utilized the voltage clamp protocol in which a single action current is injected (Figure 26), these other stimulus protocols (Figure 27, 28) may be useful in further characterizing synapses in a system such as this in which connections occur at high frequencies.

![Figure 28: Paired current- and voltage-clamp recordings allow action potentials to elicit post-synaptic currents. Same MSNs as Figure 27.](image)

<table>
<thead>
<tr>
<th>V1</th>
<th>I1</th>
<th>I2</th>
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<tbody>
<tr>
<td><img src="trace1.png" alt="trace" /></td>
<td>![trace2.png]</td>
<td>![trace3.png]</td>
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| 10 mV | 250 ms | 40 pA |
E. **MSN inhibitory collaterals shape MSN firing**

High frequency MSN collaterals have the ability to shape firing patterns of the neurons they are coupled with. Figure 29 is an example of two MSNs that are unidirectionally coupled and in current clamp configuration. MSN#1 is coupled to MSN#2, as determined by the stimulation protocol described in Figure 5. While MSN#1 is at rest, MSN#2 fires action potentials in response to current injections of increasing intensity (Figure 29 A). When MSN#1 is also induced to fire, the firing rate in response to the same number of current steps in MSN#2 is considerably attenuated (Figure 29 B). Upon closer examination, with every action potential in MSN#1 there is an inhibitory postsynaptic potential (IPSP) in MSN#2. If BMR is locally applied while both MSN #1 and #2 are induced to fire these IPSPs disappear, demonstrating GABAergic coupling between MSNs. In addition, BMR application reduces the firing rate in both cells despite following the same current step protocol as in Figure 29B, similarly to what previously reported in D2-MSNs in Ade et al. (2008). This may be due to the difference between the RMP and $E_{Cl}$ of the cell.
Figure 29: Lateral (feedback) inhibition shapes firing patterns. Paired recording from two DIV 27 D2 MSNs with the K-glutamate based internal solution: MSN#1 (top) is unidirectionally coupled to MSN#2 (bottom). In panel A, MSN #1 is at rest while MSN#2 is injected with increasing 10 pA current steps, eliciting action potential firing. B, When MSN #1 is also induced to fire action potentials the firing rate in MSN#2 is attenuated despite receiving the same current steps as in panel A. Note that IPSPs are apparent in MSN#2. C, In the presence of 25 µM BMR both MSNs fire at a lower rate than what is observed in panel B, and IPSPs are eliminated. Traces in bold are voltage responses to a 250 pA current injection, and all panels receive a maximum of 270 pA.
MSN pairs can also be studied while in continuous current clamp. In a pair of neurons that are not coupled, up-states occur synchronously in both cells (green bars, Figure 30 A), but action potentials in one cell do not induce firing in the other neuron of the pair. On the other hand, when an action potential is induced in a pre-synaptic neuron that is unidirectionally coupled (dashed lines, Figure 30 B) an up-state is provoked in the receiving neuron. These recordings were conducted with a higher internal chloride concentration (\(E_{\text{Cl}} = -29.6 \text{ mV}\)) than in Figure 29 (\(E_{\text{Cl}} = -71.4 \text{ mV}\)), illustrating drastic changes in excitability occurring with differences in chloride concentration.
Figure 30: Coupling shapes action potential firing between MSNs during state transitions. Paired recording from uncoupled (A) and unidirectionally coupled (B) D2 MSNs at DIV 27. When MSN#1 on top is coupled to MSN #2 on bottom (B), current injections to elicit action potentials in the first MSN (top trace) induce up states in the second MSN (bottom trace) as indicated by dotted lines. Note the occurrence of spontaneous up and down states that elicit action potentials (green bars). The internal solution is composed of KCl.
As considerable differences between cultures at one and two weeks in vitro were observed, I next studied MSNs at three weeks in vitro (DIV 21-24). To ensure that MSNs maintained expression of characteristic electrophysiological properties, whole-cell patch clamp recordings were performed as previously described, first using K-gluconate as the internal solution. Both MSN subtypes at this time point continued to exhibit membrane properties reminiscent of those reported in brain slices (Dehorter et al., 2011), including action potential firing patterns, inward rectifying potassium currents and the delay to first action potential suggestive of A-type potassium channel expression (Table 4).
<table>
<thead>
<tr>
<th></th>
<th>Nontreated</th>
<th>Treated</th>
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<tr>
<td></td>
<td>D&lt;sub&gt;1&lt;/sub&gt; MSN</td>
<td>D&lt;sub&gt;2&lt;/sub&gt; MSN</td>
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<tr>
<td><strong>RMP (mV)</strong></td>
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<td>-75.5 ± 1.5 (n=43)</td>
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<tr>
<td><strong>Capacitance (pF)</strong></td>
<td>54 ± 5 (n=16)</td>
<td>43 ± 4 (n=23)</td>
</tr>
<tr>
<td><strong>Input Resistance (mΩ)</strong></td>
<td>265 ± 18 (n=19)</td>
<td>215 ± 23 (n=42)</td>
</tr>
<tr>
<td><strong>Inward Rectification Index</strong></td>
<td>1.7 ± 0.1 (n=8)</td>
<td>1.7 ± 0.1 (n=22)</td>
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</table>

**Table 4:** Membrane properties of mature D<sub>1</sub> and D<sub>2</sub> MSNs in primary cultures in nontreated and quinpirole treated conditions. Whole cell recordings from identified MSNs showed that chronic D<sub>2</sub>R activation increased the capacitance and inward rectification index in only D<sub>2</sub> MSNs. D<sub>1</sub> MSNs remained unchanged despite the treatment, serving as an internal control. (Data derived from at least 4 culture preparations). **p < 0.001, comparing same cell type between treatment conditions using an unpaired t-test, † p < 0.05 comparing different cell types within a treatment condition using an unpaired t-test.**
G. **Paired recordings from MSN subtypes in mature neurons**

Recurrent axon collaterals provide the striatal network with the capacity to integrate and store complex signals (Churchill and Sejnowski, 1992; Plenz, 2003). Thus, I again investigated the occurrence of synaptic coupling between mature MSNs in this culture model. Concurrent whole-cell recordings were established from identified MSN pairs within the same visual field (Figure 31 A). As seen in the example recording in Figure 31B, eIPSCs could be studied in this configuration. Though autaptic currents were often detected in both recorded MSNs, this issue was not extensively pursued. In addition to being blocked by 25 µM BMR (Figure 31 B, blue trace), eIPSCs were also blocked by perfusion with 0.5 µM TTX and were unaffected by 5 µM NBQX (data not shown). Figure 31C shows a schematic diagram of the potential synaptic arrangement between MSNs, revealing that a maximum of four synapses can be simultaneously studied in this configuration.
Figure 31: A maximum of four synapses can be directly studied between mature MSNs. **A**, Superimposed GFP, RFP and phase contrast image of a D1-D2 MSN pair (scale bar, 30 µm). **B**, Example voltage clamp traces from reciprocally coupled MSNs at DIV 24. Yellow arrows mark the stimulation pulse, dashed purple lines highlight both autaptic (1 and 4) and synaptic (2 and 3) responses. Local perfusion of 25 µM BMR (blue trace, superimposed) abolished all evoked and most spontaneous responses. **C**, Schematic diagram illustrating that a maximum of 4 synapses can be studied in a paired recording, as labeled, if the pair are both reciprocally connected and have autapses.
To determine whether eIPSCs were a result of direct monosynaptic or indirect polysynaptic connections, I examined eIPSC latency to peak and failure rate. MSNs consistently displayed rapid and homogenous latency to peak (4.3±0.2 ms), and low rates of failures (8.9±2%), suggesting that eIPSCs were a result of direct monosynaptic connections (n=50 cells from 6 culture preparations). Although clear evidence of multi-peaked, long lasting eIPSCs suggestive of polysynaptic activity was occasionally observed, I found this in less than 5% of the MSN pairs examined and these neurons were excluded from the study. A summary of the relative occurrence of the synaptic and autaptic currents detected between the two MSN subtypes is shown in Figure 33A and B, respectively (open bars).

H. **Chronic D₂R activation increases coupling onto D₂ MSNs**

It has been shown that MSN collaterals are negatively regulated by dopamine depletion (Taverna et al., 2008), but it remains unknown which dopamine receptor these changes can be attributed to. To investigate if D₂R activation affects GABAergic coupling between MSN subtypes, neurons were examined in an environment where DA levels could be tightly regulated. As cortico-striatal cultures are devoid of dopaminergic input, I treated mature cultures with the D₂R class agonist quinpirole (10 µM) for 72 hours. Pilot experiments determined this treatment regimen as optimal (Figure 32). Cultures treated with the D₁ class receptor agonist SKF (1 µM) and quinpirole (1 µM) at DIV 2 showed no difference in connectivity between D₂ MSNs at DIV 14 (n=10 per condition, p=0.606; χ²-test). Although cultures treated with quinpirole (10 µM) at
DIV 10 exhibited increased connectivity between D2 MSNs by DIV 13 (n=8 per condition, p=0.021; $\chi^2$-test), cultures DIV > 18 were used for experimentation to avoid confounds that could be ascribed to development in vitro. To address potential non-specific effects of 10 µM quinpirole, I conducted additional paired recordings from coverslips co-treated with 10 µM quinpirole and 10 µM D2R selective antagonist sulpiride. In this condition, D2 MSNs were detected as coupled in 40% of 58 synaptic pairs, from two culture preparations (p=0.025 when compared to nontreated D2 MSN pairs; $\chi^2$-test). Coverslips treated in parallel with 500 nM quinpirole exhibited 75% coupling in 28 pairs (p=0.156 when compared to nontreated D2 MSN pairs; $\chi^2$-test).

**Figure 32:** GABAergic connectivity between D2 MSNs with various D2R agonists and antagonists. * p < 0.05, *** p < 0.001; $\chi^2$ test (total number of cells per pair tested in parentheses).
I then examined if chronic D$_2$R activation with 10 µM quinpirole altered the rate of functional synapse formation between MSN pairs. Postsynaptic D$_2$ MSNs were more likely to receive coupling in the treated condition, regardless of presynaptic partner (Figure 33 A). Interestingly, the rate of autapse formation also increased with quinpirole treatment, again in exclusively D$_2$ MSNs (Figure 33 B). In support of this observed increase in connectivity, the likelihood in which two D$_2$ neurons were reciprocally coupled was also significantly increased: reciprocal connections were found in 26 of 67 (39%) cells analyzed in nontreated conditions, and increased to 60 of 97 (62%) after chronic D$_2$R activation (p=0.004; $\chi^2$ test).

![Graph A: Synaptic Connectivity](image)

![Graph B: Autaptic Connectivity](image)

**Figure 33:** GABAergic synaptic connectivity between MSNs is sensitive to D$_2$R activation. A, B, Rates of connectivity between specific mature MSN pairs (DIV 21-24) at (A) synapses and (B) autapses exhibit an increased rate of synapse formation with 72 hour 10 µM quinpirole treatment, exclusively when the postsynaptic neuron is a D$_2$ MSN. * p < 0.05, *** p < 0.001; $\chi^2$ test (total number of cells per pair tested in parentheses).
To study changes in synapse strength following quinpirole treatment, the size and kinetics of eIPSCs between the two conditions were compared. Charge transfer and time to decay ($\tau_w$) increased in treated D$_2$ MSNs, regardless of presynaptic partner (Figure 34 A, B). Similar results were seen for peak amplitude in D$_2$ MSNs (-89±22, n= 35 cells to -191±25, n=54 cells, p=0.0002; Mann-Whitney test) but not D$_1$ MSNs (-74±20, n= 32 cells to -96±24, n=31 cells, p=0.213; Mann-Whitney test). These results correlated with a decrease in failure rate (Figure 34 B), as well as a decrease in coefficient of variation for postsynaptic D$_2$ MSNs, (0.50±0.05, n=45 cells to 0.34±0.02, n=55 cells, p= 0.007; Mann-Whitney test) but not D$_1$ MSNs (0.42±0.04%, n=30 cells to 0.39±0.02, n=27 cells, p= 0.867; Mann-Whitney test). Taken together, paired recordings from identified MSNs suggest that activation of the D$_2$R increases the rate of collateral formation onto D$_2$ MSNs and these collaterals form larger, longer, and more reliable eIPSCs when compared to nontreated controls.
Figure 34: Chronic D2R activation increases the strength of D2 eIPSCs. A, eIPSC traces with superimposed averages from representative pairs of MSN combinations in nontreated and treated conditions. B, Summary plots compare charge transfer, fitted decay time constant, $\tau_w$, and rate of failure, separated by experimental condition ($n=13$ and 15 for D1-D1 nontreated and treated MSNs, respectively; $n=19$ and 16 for D2-D1 nontreated and treated MSNs, respectively; $n=16$ and 21 for D1-D2 nontreated and treated MSNs, respectively; $n=22$ and 33 for D2-D2 nontreated and treated MSNs, respectively.) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney test).
I. \textbf{D}_2\textbf{R} activation influences \textbf{D}_2 \textbf{MSN} morphology and synapse formation

It has been previously reported that \textbf{D}_2 \textbf{MSN} morphology is regulated by the presence of dopamine (Gertler et al., 2008, Day et al., 2008). I therefore examined if quinpirole treatment altered \textbf{D}_2 \textbf{MSN} morphology in a subset of MSNs (n=11-12/condition from 3 cultures). Biocytin injections allowed for morphological reconstruction and subsequent analysis of the dendritic arbor (Figure 35 A and B). To do so, Sholl analyses were employed by studying the number of neurites intersecting each 10 µm concentric circle, starting at the neuron soma. These values were plotted (Figure 36 A), and a greater number of intersections indicated a neuron with a more complex arbor.

\textbf{Figure 35:} Sholl analysis of \textbf{D}_2 \textbf{MSNs}. \textbf{A, B}, Biocytin injected (left) and traced (right) nontreated (\textbf{A}) and \textbf{D}_2 agonist treated (\textbf{B}) \textbf{D}_2 \textbf{MSNs} (scale bar, 50 µm). Green concentric circles start at the soma and increase by 10 µm increments. The numbers of dendrites that encounter these circles give an indication of dendritic complexity of the neuron.
The plot of dendritic complexity (Figure 36 A) illustrates slight but significant increases in dendritic arborization with the treatment. Analysis of phase contrast images confirm an increase in primary dendrite number (Figure 36 B) and report a reduction in soma diameter (Figure 36 C) in treated D2 MSNs whereas D1 MSNs remain unchanged (n>50/condition).

**Figure 36**: D2R activation increases dendritic complexity. 

**A**, Analysis of sholl crossings and dendrite lengths reveals an increase in primary dendrites and enhanced number of crossings with no change in dendritic length (inset). There was a significant main effect of the treatment (p < 0.0001) and of distance from the soma (p < 0.0001). Sholl crossings were analyzed with two-way ANOVA and Bonferroni’s post-test, and individual points and dendritic length were analyzed with unpaired t-tests (n=11-12/condition from 3 cultures). 

**B**, Dendrite counts from phase contrast images confirm a significant increase in number of primary dendrites in treated D2 MSNs (Mann-Whitney test). 

**C**, Soma diameter of D2, but not D1, MSNs decreases slightly in the treated condition (n > 28 cells/group, *p < 0.05; unpaired t-test).
As increased dendritic arborization may improve the likelihood of synapse formation, I studied possible alterations in presynaptic terminals using an α-VGAT antibody, a marker for vesicular GABA transporter. D₂ MSNs showed an increase in VGAT puncta density after D₂R activation specifically in GFP labeled dendrites (Figure 37 A, B).

![image](image-url)

**Figure 37:** D₂R activation increases presynaptic GABAergic function I. A, Confocal images of nontreated (left) and D₂ agonist-treated (right) D₂ MSN segments, stained against GFP (green) and VGAT (red) (scale bar, 5 µm). B, VGAT puncta density (left) and puncta intensity (right) in identified GFP+ and GFP- dendrites as quantified in ImageJ (*p < 0.05; Mann-Whitney U test). A correlated increase in the frequency of spontaneous (Figure 38 A, C) and miniature (Figure 38 B, C) IPSC events was also observed. D₁ MSNs displayed no change in mIPSC frequency (2.3±1.5, n=8 cells to 2.15±0.5, n=8 cells, p=0.869; unpaired t-test). As no change in peak amplitude of mIPSCs was noted in D₂ MSNs between treatments, the smaller average sIPSC peak and increased mIPSC frequency are possibly the result of a preferential increase in
small events in the treated condition (Figure 38 D). $\tau_w$ was not significantly affected by D$_2$R activation (Figure 38 E). It is important to keep in mind that mIPSCs and sIPSCs need not originate exclusively from MSNs; striatal or cortical interneurons could contribute as well. Quantal content, determined by dividing the average amplitude of eIPSCs by the average mIPSC per cell, was also significantly affected in D$_2$ MSNs (6.9±1.8, n=14 cells to 16.4±2.7, n=32 cells, p=0.030; unpaired t-test) but not D$_1$ MSNs (8.2±1.6, n=10 cells to 7.4±5.1, n=5 cells, p=0.853; unpaired t-test). The increase in quantal content further supports the decrease in failure rate of D$_2$ MSN eIPSCs, suggesting either (a) a potential increase in the number of release sites or (b) in release probability following quinpirole treatment.
Figure 38: D2R activation increases presynaptic GABAergic function II. A, B, Representative sIPSC (A) and mIPSC (B) traces from D2 MSNs recorded from nontreated (left) and treated (middle) D2 MSNs. Plots of cumulative inter-event intervals in D2 MSNs (right) show a reduction in time between events in treated D2 neurons. C-E, Summary plots compare frequency (C), peak amplitude (D) and $\tau_w$ (E) of sIPSCs and mIPSCs in D2 MSNs in both experimental conditions ($n=38$ and 55 nontreated and treated cells/group). D1 MSNs similarly exhibited no change in kinetics or amplitude (data not shown). * $p < 0.05$ when comparing mIPSCs or sIPSCs between treatment condition (Mann-Whitney U test). $++$ $p < 0.01$ when comparing mIPSCs and sIPSCs within treatment group (Wilcoxon signed rank test).
To evaluate these two possibilities, I employed a variance-mean (V-M) analysis of eIPSC amplitudes as described by Clements and Silver (2000). The relationship between amplitude variation and amplitude mean with changes in external calcium concentrations ([Ca\(^{2+}\)]_o) can be plotted to determine quantal size (Q), number of release sites (N), and the average probability of vesicular transmitter release (P_r). As expected, changing [Ca\(^{2+}\)]_o from 0 mM to 0.5 mM to 1 mM led to increases in eIPSC amplitude variance and amplitude mean in D_2 neurons from both treated and nontreated conditions. When [Ca\(^{2+}\)]_o was increased to 2mM [Ca\(^{2+}\)]_o, nontreated D_2 MSNs again displayed an increase in eIPSC amplitude variance and amplitude mean (Figure 39 A). D_2 MSNs in the treated condition, on the other hand, exhibited an increase in eIPSC amplitude mean but a reduction of amplitude variance (Figure 39 B). As illustrated in Figure 39C, the V-M plot of D_2 MSNs in the nontreated condition was relatively linear, indicating increased variance at higher [Ca\(^{2+}\)]_o concentrations. On the other hand, the V-M plot of treated D_2 MSNs formed a distinct parabola (Figure 39 D). These data support the hypothesis that D_2R activation via quinpirole increases the number of GABA release sites (N) or vesicular GABA release probability (P_r). However, the lack of parabolic mean-variance relationship in the nontreated condition prevents us from truly distinguishing between these two possibilities.
Figure 39: Variance-mean (V-M) analysis of eIPSC amplitudes. A, B, V-M analyses of eIPSCs from a representative nontreated (A), and treated (B) D2 MSNs. (1) Superimposed eIPSCs illustrate amplitude fluctuations with changes in external calcium ([Ca$^{2+}$]$_o$) concentration. (2) The time course of individual responses is plotted with four [Ca$^{2+}$]$_o$ concentrations as indicated, and mean peak values are overlayed per concentration (3). (4) V-M plots generated per cell highlight differences in release probabilities between conditions. C, D, Summary data show contrasting V-M trends between nontreated (C) and treated (D) conditions, suggesting higher release probability in D2 MSNs treated with quinpirole. V-M is normalized to the mean value of 2mM Ca$^{2+}$ in each cell (n=13 and 10 cells/group from 4 culture preparations).
As the effects of the treatment on eIPSCs were observed only when the postsynaptic target was a D₂ MSN, I hypothesized that D₂R activation preferentially affects postsynaptic D₂ MSNs, independent of the presynaptic partner. The increased frequency of sIPSC and mIPSC events in quinpirole treated D₂ MSNs, along with the reduction in failures of eIPSCs suggest an increase in the number of MSN collaterals and a potential increase in clusters of postsynaptic GABA_A receptors.

J. **Chronic D₂R activation increases GABA sensitivity of D₂ MSNs**

I therefore examined postsynaptic GABA_A receptor clusters with an α-gephyrin antibody. Gephyrin is a synaptic anchoring protein which colocalizes with postsynaptic GABA_A and glycine receptors (Sassoe-Pognetto et al., 2000). Immunohistochemical analysis of gephyrin clusters in D₂ MSN dendrites revealed a significant increase of gephyrin puncta density after quinpirole treatment in exclusively D₂ MSN dendrites (Figure 40 A, B) supporting a postsynaptic increase of GABA_A receptors with chronic D₂R activation.
To directly assess changes in functional GABA_A receptor expression, I investigated the action of several GABA concentrations (0.3, 1, 3, and 10 µM) on whole cell currents in D_1 and D_2 MSNs. In the nontreated condition, D_1 and D_2 MSNs exhibited similar sensitivity to all concentrations of GABA. With chronic quinpirole treatment, however, D_2 MSNs displayed a higher sensitivity at 0.3 and 1 µM GABA (Figure 41 A-C). Similarly, at 3 µM GABA, currents were 94±33 pA, n =12 and 250±65 pA, n=11 in nontreated and treated D_2 MSNs, respectively (p=0.032; Mann-Whitney test). At 10 µM, currents were 201±39 pA, n=23 and 313±46 pA, n=29 in nontreated and treated D_2 MSNs, respectively (p=0.074; Mann-Whitney test). The larger currents in response to GABA in treated D_2 MSNs point strongly to a postsynaptic mechanism.

**Figure 40:** Increased GABA_A_R clusters in D_2 MSNs following D_2R activation. A, Confocal images of D_2 MSN segments with GFP (green) and gephyrin (red) staining in nontreated (left) and quinpirole treated (right) conditions (scale bar, 5 µm). B, Summary plots of gephyrin puncta density (left) and puncta intensity (right) of identified GFP+ and GFP- dendrites (**p < 0.01; Mann-Whitney U test).
for dopamine, but whether this is due to an increase in GABA$_A$ receptor number, as suggested by increased gephyrin clusters, or a change in GABA$_A$ receptor subtype required further elucidation.

Different GABA$_A$ receptor subunits influence receptor conductance, open probability, and ligand affinity (Macdonald and Olsen, 1994). The delta subunit-containing extrasynaptic GABA$_A$ receptor has been shown to be more sensitive to GABA (Farrant and Nusser, 2005). As D$_2$ MSNs displayed increased GABA sensitivity with the quinpirole treatment, I hypothesized that this may be a result of increased expression of the delta subunit containing GABA$_A$ receptor. THIP, a preferring agonist for delta subunit containing GABA$_A$ receptor, was locally perfused while MSNs were in whole cell configuration. Response to 1 µM THIP did not significantly change with the quinpirole treatment in either MSN subtype (Figure 41 A, B). Interestingly, D$_2$ MSNs exhibited larger currents in response to THIP compared to D$_1$ MSNs, suggesting a greater expression of extrasynaptic GABA$_A$ receptors in D$_2$ MSNs basally *in vitro* (Figure 41 C).

Synaptic GABA$_A$ receptors in the striatum contain the β3 subunit (Janssen et al., 2011). Etomidate has been shown to consistently modulate the β2/3 subunit containing GABA$_A$ receptor and was therefore used to assess any potential changes in synaptic GABA$_A$ receptors. As with THIP, there were no detectable differences in peak current responses to etomidate within MSN subtype when compared between conditions (Figure 41 A, B).
**Figure 41:** Enhanced GABA$_A$ sensitivity in D$_2$ MSNs following D$_2$R activation. A, B, Representative currents in response to direct Y-tube application of GABA, THIP (1 µM) and etomidate (5 µM) in nontreated (A) and treated (B) D$_2$ MSNs. C, Summary of direct drug effects in D$_1$ and D$_2$ MSNs in each experimental condition (n=16-24 cells/group) (Kruskal-Wallis test with Dunn’s post test; *p < 0.05, **p < 0.01, ***p < 0.001 comparing the same cell type between conditions. "p < 0.05, ""p < 0.01 comparing different cell types within conditions).
These experiments suggest that the increased sensitivity to GABA observed in quinpirole-treated D₂ MSNs is not related to changes in β2/3 or δ subunit incorporation into functional GABA_A receptors. Thus, taken together, the increase in mIPSC frequency and GABA sensitivity point to an increase in the number of postsynaptic GABA_A receptors with treatment. As I did not observe a correlated increase in mIPSC amplitude, the data point to an increase in the number of inhibitory synaptic sites with chronic D₂R stimulation. Although a preferential increase in other GABA_A receptor subtypes or postsynaptic modifications of existing receptors cannot be excluded, the most plausible explanation at this point is that quinpirole treatment can increase the number and/or density of GABA_A receptor clusters.

K. Pharmacology of inhibitory synaptic connections between MSNs

To further investigate changes in GABA_A receptor subtypes, I utilized benzodiazepine pharmacology. Benzodiazepine sensitivity relies on the presence of specific α and γ2 subunits to increase frequency of opening of the targeted GABA_A receptor subtypes (Macdonald and Olsen, 1994). Therefore, if the targeted subunit is present, an enhancement of IPSC decay time (τ_w) is expected.

Flumazenil, a potentiator of α4, β3 and γ2 subunit containing GABA_A receptors (Ramerstorfer et al, 2010), failed to prolong IPSC decay in either condition, excluding a role for the α4 subunit (data not shown). Diazepam acts as a positive allosteric modulator at the benzodiazepine site of α1-3, or α5-containing GABA_A receptors. Local perfusion of 5 µM diazepam revealed a prolongation of eIPSCs in both nontreated and treated D₂ MSNs, indicating
that D₂ MSNs in both conditions express these subunits (in nontreated D₂ MSNs, \( \tau_w \) increased from 38±3 ms to 48±3 ms, \( n=5 \), \( p=0.025 \); paired t-test. In treated D₂ MSNs, \( \tau_w \) increased from 56±5 ms to 79±3 ms, \( n=8 \), \( p=0.035 \); paired t-test). Interestingly, the increase in \( \tau_w \) of eIPSCs from baseline was larger in quinpirole treated (41%) versus nontreated (23%) D₂ MSNs (\( p<0.0001 \); unpaired t-test). Amplitude of eIPSCs was not affected by the presence of the benzodiazepine (data not shown).

To investigate if quinpirole treatment induced a greater expression of benzodiazepine sensitive GABA\( _A \) receptors over other GABA\( _A \) receptor subtypes, I studied the effect of diazepam on mIPSCs. As an mIPSC reflects the response to a single quantum of neurotransmitter, the similarities between the benzodiazepine-induced prolongation of mIPSC \( \tau_w \) in the nontreated and treated conditions suggest similar expression of diazepam-sensitive synaptic GABA\( _A \) receptors between the two groups. I observed that diazepam significantly prolonged mIPSC decay in both conditions: in nontreated D₂ MSNs, \( \tau_w \) increased from 25±2 ms to 33±1 ms, \( n=12 \) (\( p<0.0001 \); paired t-test); in treated D₂ MSNs, \( \tau_w \) increased from 27±3 ms to 39±3 ms, \( n=8 \) (\( p<0.0001 \); paired t-test). However, unlike eIPSCs, the diazepam-induced change in mIPSC \( \tau_w \) did not differ between conditions (32% nontreated and 44% quinpirole treated, \( p=0.08 \); unpaired t-test). The possibility that mIPSCs and eIPSCs may be derived from different pre-synaptic terminals should be considered. However, the slower decay of eIPSCs compared to mIPSCs suggests they reflect asynchronous summation of multiple synaptic currents produced at distinct sites (Mody et al., 1994). Thus, the enhanced effect of diazepam on eIPSCs compared to
mIPSCs in treated D$_2$ MSNs may be due to increased number of synaptic connections on D$_2$ MSNs with chronic quinpirole treatment.

**Figure 42:** Pharmacological regulation of D$_2$ MSNs IPSCs by benzodiazepines. **A, B,** Superimposed averaged and normalized eIPSC example from a DIV 24 D$_2$ MSN displaying the effect of vehicle (black trace), 10 µM flumazenil (blue trace) and 10 µM diazepam (orange trace). **B, C,** Average fitted decay time constants ($\tau_w$) of IPSCs in (B) nontreated, and (C) treated conditions. $^p < 0.05$, $^{++} p < 0.01$, $^{+++} p < 0.001$ versus vehicle (paired t-test, corrected for multiple comparisons). $^* < 0.05$, $^{***} < 0.001$ between conditions (unpaired t-test).
IV. DISCUSSION

A. Summary

GABAergic MSNs mediate the integration of information within the striatum, ultimately controlling proper motor movement and behavior (Graybiel et al., 1994). Nearly 95% of all striatal neurons are GABAergic MSNs, which can be divided by their differential dopamine receptor expression into D\textsubscript{1} or D\textsubscript{2} MSNs (Gerfen and Young, 1988; Bolam et al., 2000; Kreitzer and Malenka, 2008). As MSN axons branch extensively onto nearby neurons, MSNs have the capacity to tightly regulate striatal activity and output (Wilson, 1994; Plenz, 2003). This feedback inhibition between MSNs is critical in integrating and regulating information and is strongly affected by changes in dopamine levels (Taverna et al., 2008). Previous studies have confirmed that MSNs form GABAergic synapses at dendritic necks and shafts of neighboring MSNs (Gerfen and Young, 1988), however, the study of these collaterals have proven to be challenging as the rate of connectivity detected between MSNs is fairly low (Plenz, 2003).

For this thesis, I have established a dissociated primary cell culture model of the mouse striatum that allows for increased detection of MSN collaterals. Previous studies have shown an increased rate of synapse formation in \textit{in vitro} models compared to conventional \textit{ex vivo} slice preparations (Czubayko and Plenz, 2002). I utilized BAC transgenic mice that express td-Tomato downstream of the D\textsubscript{1} receptor promoter and EGFP downstream of the D\textsubscript{2} receptor promoter to concurrently distinguish between the two MSN subtypes. With this method, cultures retain
expression of their transgene, develop complex dendritic arbors, and exhibit MSN-like firing properties \textit{in vitro}. In addition, the rate of synapse formation is nearly double that reported in previous studies allowing for a more extensive study of MSN collaterals. This model opens the door for targeted studies of MSN collateral synapses that have previously remained elusive.

Despite the overwhelming role for D$_2$Rs in maintaining proper striatal function, it remains unclear how MSN collaterals are specifically altered by D$_2$R activation. The D$_2$R is a G-protein coupled receptor that is critical in maintaining proper function in the central nervous system (Girault and Greengard, 2004). To investigate the role of chronic D$_2$R activation on GABAergic transmission, I took advantage of the cortico-striatal culture model in which MSN subtypes can be distinguished. An \textit{in vitro} model allows for direct pharmacological control of DA receptor activation while maintaining neuronal integrity (Deyts et al., 2009). This provided the unique opportunity to specifically compare the effects of D$_2$R activation on the two major subtypes of striatal neurons.

With this dissertation, I present direct evidence for the regulation of synaptic frequency and efficacy at MSN collateral synapses by chronic D$_2$R activation \textit{in vitro}. My data suggest that D$_2$R activation causes distinctive changes in MSN morphology with coordinated increases in the number of synaptic sites and density of post-synaptic GABA$_A$ receptor clusters. Taken together, my results demonstrate that GABAergic transmission between MSNs is significantly regulated by D$_2$R activation, and this occurs via coordinated pre- and post-synaptic modifications at specific MSN collaterals.
B. Cortico-striatal cultures

Primary culturing techniques have been utilized for many years and recent advances suggest eliminating cytosine arabinofuranoside (Martin et al., 1990), reducing or removing serum (Ahmed et al., 1993), and including low concentrations of growth factors (Gertler et al., 2008). My protocol has amalgamated these past advances, and further demonstrates that the presence of growth factors plays a fundamental role in proper neuronal maturation.

An *in vitro* model that allows for simultaneous identification of both MSN subtypes is useful for the targeted investigation of the role of D2Rs in striatal networks. I report that cortico-striatal cultures from *drd1a-tdTomato; drd2-EGFP* mice retain their fluorescent properties through time *in vitro*. Neurons in these co-cultures have profiles comparable to MSN development observed *in vivo*, including action potential firing patterns, up-and-down states, developed dendritic morphology and spine formation. MSNs in this primary culture concurrently display properties that are not typically seen *ex vivo*, such as autaptic currents and colocalization of D2 and D1 MSNs markers. The absence of natural targets for MSNs in culture may explain the high rate of autapse formation, as is exemplified with *in vitro* microislands (Bekkers, 2005). The considerable extent of neurons that co-express both D1 and D2 markers in primary culture may be a consequence of harvesting MSNs at an early developmental stage (Goffin et al., 2010).

Studies of striatal MSNs in primary culture or organotypic slice models (Plenz and Aertsen, 1996; Segal et al., 2003; Gertler et al., 2008; Tian et al., 2010; Kaufmann et al., 2012) offer advantages and disadvantages compared to acute slice preparations. Primary cultures enable manipulation of the extracellular environment and consequently allow close comparisons
between experimental conditions. In addition, *in vitro* models are useful in distinguishing between the contribution of cell autonomous and environmental factors (Gertler et al., 2008). These benefits come with inherent limitations: dissociated culture models disrupt the natural development of cytoarchitecture, potentially altering cellular and network phenotypes. However, while *in vitro* culture models report enhanced rates of functional connectivity, *ex vivo* slice preparations unavoidably reduce the estimated extent of coupling due to slice thickness and depth of neurons (Chuhma et al., 2011). Therefore, *in vitro* preparations are useful for dissecting out changes in MSN collaterals.

**C. MSN collaterals**

The striatum holds a critical role in information processing within the basal ganglia and is therefore a subject of high interest for the understanding of movement control and movement pathologies. Neuron collaterals endow the striatum with the ability to process and consolidate multiple inputs. This information is relayed mostly to the SNpr, resulting in executive action. Improper striatal function results in a slew of pathological states including Parkinson’s disease, and striatal collaterals have been largely implicated with changes in dopamine levels (Taverna et al., 2008; Lalchandani et al., 2013). Given the infrequent detection of MSN collaterals in the traditional slice preparation, I have developed a dissociated culture model in which collaterals are more frequent, and in which MSNs are identifiable by fluorophore expression.

Previous studies have reported a range of connectivity rates between D1 and D2 MSNs, with variations in methodology potentially leading to diverse results (Jaeger et al., 1994,
Czubayko and Plenz, 2002; Tunstall et al., 2002; Koos et al., 2004; Venance et al., 2004; Gustafson et al., 2006; Tecuapel et al., 2007; Taverna et al., 2008). In these studies, functional coupling was reported at higher rates in culture models compared to ex vivo slices. Here I report the same trend: at two weeks in vitro I detect connections between 41.7% of MSNs. And although dissociated culture models have inherent limitations, such as removing structures from their native environment and bathing neurons in predetermined concentrations of growth factors, conventional ex vivo slices also come with certain constraints. Given that MSN arbors extend beyond the typical 100-300 µm slice preparation (Wilson, 1994), differences in slice thickness and depth of neurons selected can easily affect the percentage of coupling detected. And while dissociated primary cultures are grown in an artificial environment, neurites are not severed after plating, allowing for the study of intact neuron collaterals. Ultimately, it is important to keep in mind the limitations of each preparation when interpreting results.

D. MSN inhibitory collaterals at the first two weeks in vitro

Neurons in these cultures displayed substantial development between the first and second weeks in vitro. In particular, the rates of connectivity observed between MSNs at the first week in vitro were less than 15%, and restricted to D1 MSNs collaterals. This potentially correlates with the finding that D2 MSN projections to the globus pallidus take longer to develop than D1 MSN projections to the substantia nigra in the intact brain. MSNs in the cultures also expressed GABA\(_A\) receptors, as determined by whole cell currents in response to low doses of GABA. While there was no difference in GABA sensitivity between MSN subtypes, there appeared to be
differential subunit expression—D₂ MSNs displayed larger responses to β2/3 and δ subunit specific agonists. The larger β2/3-subunit mediated currents support data previously collected in our laboratory from ex vivo mice striatal slices suggesting that D₂ MSNs have a higher expression of β2/3 subunits. However, the larger currents in response to the δ subunit specific agonist THIP is in contrast to what our laboratory has reported for D₂ MSNs in young mouse brain slices (Ade et al., 2008). This may be indicative of specific culturing conditions causing up-regulation of receptor subtypes responsible for δ-subunit mediated tonic currents, or of an accelerated rate of maturity in vitro, as mature mouse slices have been reported to have larger δ-subunit mediated tonic currents (Santhakumar et al., 2010).

As neural circuits are defined by their connections, MSN collaterals have the ability to integrate multiple inputs to create a defined output to the SNpr. The inwardly rectifying potassium channel is the predominant channel open in resting MSNs; therefore, MSNs generally display resting membrane potentials in the -80 to -90 mV range (Jiang and North, 1991). Timing of collateral firing makes a difference in the message that is relayed as collateral firing can either depolarize or hyperpolarize the neuron. As long-term potentiation and depression are dependent on both glutamatergic input onto MSNs and spike-timing of the MSNs themselves (Shen et al., 2008), MSN collaterals are at the fulcrum of striatal activity.

E. Dopamine and MSN collaterals

Lack of dopamine in vivo has been found not to affect the development of MSNs or proper neural circuits in a DA-deficient mouse model (Zhou and Palmiter, 1995). Dopamine has
been previously implicated in modulating collateral synapses between MSNs (Taverna et al., 2008; Tecuapetla et al., 2009), but these collaterals produce currents that are small in amplitude and occur at very low frequencies. As the primary culture model described does not contain dopaminergic cells of the substantia nigra pars compacta but should nevertheless permit normal MSN and circuit development, these co-cultures provide for the unique opportunity to study how D₂R activation alters synaptic function. This dissertation further optimizes the study of these collaterals by 1) using a system where synapses occur at a higher rate and 2) by loading the recorded neurons with high chloride to increase the driving force and subsequent detection of chloride through the GABA_A receptor. In addition, the use of transgenic mice that allow for live identification of two populations of neurons provides an additional, straightforward internal control.

F. D₂R activation increases collateral frequency and strength

The rate of functional synaptic connectivity in the co-culture model at three weeks in vitro was consistently higher in D₂-D₂ MSN pairs than any other configuration. Despite the difference in frequency of functional synapses in basal conditions, eIPSC size and kinetics remained consistent between MSN pairs. Chronic D₂R activation with 72 hours of 10 μM quinpirole, however, increased both the rate and size of eIPSCs specifically in D₁-D₂ and D₂-D₂ pairs, suggestive of a post-synaptic mechanism of D₂R activation. This increase was not seen in pairs that had been treated with a combination of quinpirole and D₂/₃ antagonist sulpiride, demonstrating that 10 μM quinpirole is effectively targeting D₂ MSNs.
Sholl analyses revealed that chronic D<sub>2</sub>R activation increased both the number of primary dendrites and the dendritic arborization of D<sub>2</sub> MSNs. While this correlates with the increased number of functional synapses onto treated D<sub>2</sub> MSNs, it has recently been reported that chronic D<sub>2</sub>R upregulation in vivo in the mouse increases MSN excitability and decreases dendritic arborization by the down-regulation of inward rectifying potassium channels (Cazorla et al., 2012). There are several reasons that may underlie the divergence of the studies presented here from the results of Cazorla et al. It is likely that there are distinct intracellular pathways and compensatory mechanisms activated by D<sub>2</sub>R upregulation compared to chronic D<sub>2</sub>R activation. In addition, there are inherent differences in the in vivo versus in vitro experimental settings. In fact, the number of primary dendrites of MSNs in dissociated cultures (4-6) is consistent with what has been previously reported, and is lower than what has been observed in slice (6-8) (Gertler et al., 2008). Day et al. (2006) provides evidence that dopamine depletion reduces primary dendrite number selectively in D<sub>2</sub> MSNs from 6 to 4. In combination with the reported reduction in primary dendrites of cortico-striatal cultures that inherently lack DA input (Gertler et al., 2008) these two studies further support the idea that DA activation of D<sub>2</sub>Rs regulates D<sub>2</sub> MSN morphology.

G. Coordinated pre- and post-synaptic mechanisms

Consistent with an increase in functional synapses onto D<sub>2</sub> MSNs, I observed an increase in pre-synaptic GABAergic terminals and post-synaptic gephyrin clusters along D<sub>2</sub> MSN dendrites with quinpirole treatment. These results parallel the enhanced sensitivity to low
concentrations of GABA in treated D$_2$ MSNs. However, the possibility of differential GABA$_A$
receptor subtype expression should also be considered.

The pharmacological dissection of native GABA$_A$ receptor subtypes is challenging
because of the specificity of available modulators and heterogeneity of endogenous subunit
expression. Based on reported expression studies by Pirker et al. (2000), striatal GABA$_A$
receptors should be predominantly composed of $\alpha2$, $\beta1/3$, and $\gamma2$ subunits. $\alpha1$, $\alpha4$, $\alpha5$ and $\delta$
subunits have also been shown to be expressed. Previous studies in our group have suggested
that the presence of specific DA receptors in MSNs affect synaptic and extrasynaptic GABA$_A$
receptor function (Ade et al., 2008, Janssen et al., 2009), with a key role for $\beta3$ subunits (Janssen
et al., 2011). The present results, however, show that the sensitivity to etomidate, a $\beta2/3$ subunit
containing agonist, does not change with chronic D$_2$R activation. Furthermore, currents in
response to THIP, a superagonist of the extrasynaptic $\delta$ subunit containing receptor (Brown et al.,
2002), are unaltered by the treatment, suggesting the increase in GABA sensitivity is specific for
synaptic receptors.

Diazepam appears to prolong the synaptic decay of GABA IPSCs in both nontreated and
treated MSNs, but the actions on $\tau_w$ were enhanced in quinpirole treated D$_2$ MSNs. This effect
was observed to a greater degree on eIPSCs and not on mIPSCs; therefore, I conclude that this is
a result of increased asynchronous summation of synaptic release from multiple sites. While the
potential for changes in juxtasynaptic receptors cannot be excluded, the similarities in response
to THIP point towards similar extrasynaptic GABA$_A$ receptor composition in both conditions.

The possibility that mIPSCs and eIPSCs represented distinct inputs should be kept in
mind when interpreting these results. It remains to be determined whether the increase in
synaptic sites in treated D₂ MSNs is due to a morphological change in dendritic branching (Day et al., 2006), or if it is due to a facilitation of GABA<sub>A</sub> receptor insertion at newly formed inhibitory synapses. Changes in dendritic morphology (Figure 36) and increases sensitivity of treated D₂ MSNs to low doses of GABA with the treatment parallel the increases in gephyrin clusters (Figure 40, 41) and support the hypothesis of a coordinated dopaminergic control of GABA<sub>A</sub> receptor clusters and dendritic morphology in D₂ MSNs. The effect of quinpirole treatment is selective for postsynaptic D₂ MSNs and independent from the presynaptic cell being D₁ or D₂ MSN. This supports the hypothesis that D₂R activation in post-synaptic D₂ MSNs has its effect through one of the multiple intracellular cascades that regulates GABA<sub>A</sub> receptor trafficking (Vithlani et al., 2011) and dendritic morphology (Day et al., 2008).

The role of D₂R is central in neuropsychiatric disorders. The results presented here begin to elucidate the powerful action of biochemical cascades following activation of these receptors in contributing to synapse formation and functionality (Girault and Greengard, 2004). I provide a model for future studies that may define the relative role of distinct components of this cascade, and compare the role of D₁ receptor activation. One must consider when comparing results of DA receptor stimulation that, while DA is released in a context dependent manner in vivo, selective agonists are always present in culture. Prolonged agonist application may alter receptor signaling paradigms, potentially producing different effects in comparison to phasic discontinuous stimulation.

Previous studies have shown that DA regulates the efficacy of MSN collaterals (Taverna et al., 2008). These results extend these findings and provide an experimental assay to mechanistically analyze this effect. Taken together, this dissertation suggests that dopamine D₂
receptor activation facilitates inhibitory synapse formation via coordinated pre- and post-synaptic changes.
V. LIMITATIONS AND FUTURE DIRECTIONS

One of the difficulties in research is focusing on a specific topic-- in doing so we build inherent limitations into our experimental direction. By focusing on the D$_2$ receptor, I neglected the effects of D$_1$ agonist on MSN collaterals, something that is potentially very interesting and relatively unexplored in the field. By conducting my experiments in vitro, dissociated culture, I focused my efforts into a model in which physiological striatal afferents and efferents were missing. And by studying chronic D$_2$ receptor activation on neurons in culture, I removed the normally phasic presence of dopamine and flooded the cells with constant quinpirole, causing undetected intracellular cascades and potentially receptor desensitization.

Therefore, future directions include the study of a D$_1$ agonist on MSN collaterals and how it compares to the outcomes of the current work. To bring physiological relevance to this dissertation, it is important to conduct similar experiments in 6-OHDA lesioned mice that are given a D$_2$ agonist via peristaltic pump. Future experiments can also use a variety of different protocols to assess GABA$_A$ receptor number, such as GABA$_A$ receptor radioligand binding in tissue homogenate.
VI. CONCLUSIONS

- In vivo, D₂ MSNs are more abundant in the striatum than D₁ MSNs early in development, though D₁ MSNs reach their target nuclei before D₂ MSNs do.
- Corticostriatal cultures from Drd1a-tdTomato/Drd2-EGFP mice retain their fluorescent properties through age in vitro, allowing for distinct subtype selection.
- Markers of D₁ and D₂ MSNs overlap to a greater extent in culture than in vivo.
- Action potential firing, presence of up states, synapse formation, and morphology of cultured MSNs are comparable to those found in vivo.
- Up-states occur synchronously in MSN pairs and are sensitive to the presence of magnesium, suggesting a component of NMDA receptor involvement.
- Synaptic connectivity resulting from extensive MSN axon collaterals can be detected easily in culture, and occurs in greater abundance in vitro than in vivo.
- As MSNs are GABAergic, synaptic collaterals shape MSN signaling and subsequent striatal output.
- Stimulating dopamine D₂R for 72 hours with the specific D₂ class agonist quinpirole preferentially increases the strength and occurrence of functional GABAergic synapses onto D₂ MSNs (and not D₁ MSNs).
- Stimulated D₂ MSNs display increases in primary dendrite number and dendritic arborization. This correlates with an increased frequency of sIPSCs and mIPSCs in D₂ MSNs when compared to nontreated neurons. The number of α-VGAT puncta also increases with the treatment.
• Enhanced response to low concentrations of GABA and pharmacological targeting of specific GABA<sub>A</sub> receptors subtypes reveal an increased sensitivity to GABA with no apparent differences in subunit composition between conditions.

• The effect of flumazenil and diazepam on synaptic decay, combined with the increase in frequency of miniature IPSCs and decreased coefficient of variation of eIPSCs, support an increase in synaptic sites in D<sub>2</sub> MSNs when chronically treated with quinpirole.

• D<sub>2</sub> receptor stimulation plays a critical role in modulating feedback GABAergic connectivity in the striatum <i>in vitro</i> potentially due to increased GABA<sub>A</sub> receptor insertion in postsynaptic D<sub>2</sub> MSNs and increases in presynaptic sites.

• This model may facilitate the targeted study of functional and morphological changes observed in other pathological states, potentially providing experimental conditions relevant for Parkinson’s and Huntington’s Disease.
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VII. REFERENCES


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