CHOLINERGIC MODULATION OF GABAERGIC INHIBITION DYNAMICS IN THE STRIATUM

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Cholinergic Modulation of GABAergic Inhibition Dynamics In The Striatum

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Abstract

The striatum contains medium spiny neurons (MSNs) classified by their axonal projection and expression of dopamine receptors into D1+ and D2+ MSNs; and interneurons including choline acetyltransferase expressing interneurons (ChAT+) that influence MSN activity by triggering GABA release. Here, I identify the potential source of this GABA. In addition, I characterize GABA\(_A\) receptors that mediate the GABA response produced by distinct classes of neurons.

By studying nAChR mediated response in MSNs and in GABAergic interneurons classified as neuropeptide Y (NPY+), parvalbumin (PV+) and tyrosine hydroxylase (TH+), I show direct nAChR mediated response in interneurons but not MSNs, where the response is indirect and mediated through GABA\(_A\) receptors. To identify the neurons that mediate this GABA response, I performed dual recordings from GABAergic interneurons and MSNs. TH+, PV+, and NPY+ NGF interneurons all form synapses on MSNs, but only TH+ and NPY+ NGF interneurons contribute to nAChR mediated GABAergic responses in MSNs.

Through different firing patterns that produce transient and sustained GABA conductance in MSNs, GABAergic interneurons adapt distinct physiological roles. By performing single and dual recordings, I report distinct GABA responses produced by PV+ and NPY+ NGF interneurons. In MSNs, phasic current is mediated by synaptic GABA\(_A\) receptors containing \(\alpha_2, \gamma_2\) and a \(\beta\) subunit; whereas tonic GABA current is mediated by extrasynaptic receptors containing \(\alpha_4, \delta\) (adult mice), and \(\alpha_5\) (young mice) subunit. Using \(\beta_3\) and \(\delta\) subunit selective drugs in mice that lack either subunit, I found that the
β3 subunit is not a component of δ-containing extrasynaptic receptor pool, but is involved in mediating spontaneous slow IPSCs from NGF interneurons.

To further characterize GABA_A receptors, I found that optogenetic activation of D2+, TH+, and PV+ neurons produced a GABA response that exhibited different sensitivity to GABA drugs, demonstrating the expression of various GABA_A receptor subtypes in MSNs. Specifically, D2+ stimulation activated β3 and γ2-containing; TH+ stimulation activated β3-containing; and PV+ stimulation activated α1, β3, and γ2-containing receptors. Taken together, my findings contribute to the understanding of striatal neurons and microcircuitry and set the stage for future studies that will continue to contribute to our understanding of the striatum.
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Chapter 1

Introduction

1.1 Basal ganglia

1.1.1 Structure and projection

The basal ganglia (BG) in the human brain consist of a collection of interconnected subcortical nuclei that mediate a range of functions. In addition to the well-recognized role in motor control, the BG has been shown to mediate a range of non-motor behaviors such as associative learning, reward-related behavior, memory, and emotion. Nonetheless, the physiology and pathophysiology of the motor circuit of the BG is still the best understood. In the classical model, the motor circuit of the BG consists of a direct and an indirect pathway that act in opposing manners to control movement Fig. 1.1. In both pathways, the striatum functions as the input nucleus of the BG and receives excitatory projections from the cortex and thalamus, and modulatory signal from the substantia nigra pars compacta (SNpc). The internal globus pallidus (GPi) and substantia nigra pars reticulate (SNpr) function as the output nuclei of the BG and send output signals to the ventral anterior and ventral lateral thalamus (VA/VL), which then project back to the cortex. In the direct pathway, striatal neurons project directly to the GPi (medial globus pallidus in rodents); however, in the indirect pathway, striatal neurons project to the external globus pallidus (GPe), and then to the GPi either directly or through the subthalamic nucleus (STN). The STN also receives direct input from the cortex through
the so-called hyperdirect pathway, and is considered, like the striatum, an input nucleus of the basal ganglia (Fig. 1.2). How cortical-STN projection incorporates into the classical direct and indirect pathway is unknown, but it is increasingly evident that these two pathways are an oversimplification of BG function. With the exception of the STN, which is glutamatergic, and the SNpc, which is dopaminergic, all other circuitry of the BG is GABAergic (Fig. 1.2). And, because GABAergic neurons of the GPi and SNpr are tonically active, activity of the basal ganglia is mediated through a dis-inhibitory mechanism.

Figure 1.1: Direct- and Indirect-Pathway Basal Ganglia Circuits. Sagittal view of a mouse brain (A), depicting cortex-basal ganglia-thalamus-cortex circuits. Axons from the thalamus and [cortex] form excitatory synapses onto striatonigral/direct-pathway medium spiny neurons (MSNs) (blue) and striatopallidal/indirect-pathway MSNs (red). Direct-pathway MSNs send axons directly to basal ganglia output nuclei (GPm, medial globus pallidus; SNr, substantia nigra pars reticulata), where they form inhibitory synapses. Indirect-pathway MSNs inhibit neurons in the globus pallidus (GP), which in turn make inhibitory connections with the subthalamic nucleus (STN). STN projections target the GPm and SNr, where they form excitatory synapses onto GABAergic basal ganglia output neurons. These inhibitory output neurons send axons to ventroposterior thalamic motor nuclei. Finally, glutamatergic neurons in the thalamus project back to cortex, completing the circuit.

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1.1.2 Model of basal ganglia function

Based on cortical-BG anatomy, the function of the basal ganglia can best be described by the segregation model, in which projections from functionally defined areas of cortex form parallel and closed circuits within the basal ganglia in a topographical manner. Based on this model, five parallel circuits can be identified within the striatum: motor, oculomotor, dorsolateral, lateral orbitofrontal, and anterior cingulate. In addition to cortico-basal ganglia-thalamocortical projections, the basal ganglia also project to the pedunculopontine nucleus (PNN) and the superior colliculus (SC) (Hikosaka et al., 2000) to influence locomotion, and head and eye movement.
1.1.3 Basal ganglia pathology — Parkinson’s disease (PD)

Basal ganglia dysfunction is associated with many neurological disorders and behavioral abnormalities. Parkinson’s disease (PD), one of the first neurological disorders found with a basal ganglia origin, is best known for motor abnormalities that result from the degeneration of SNpc dopaminergic neurons and the consequent depletion of dopamine in the striatum. The lack of dopaminergic modulation within the striatum leads to an imbalance of basal ganglia pathways that involves reduced activity through the direct pathway and enhanced activity through the indirect pathway leading to motor dysfunction. Studies have shown that after dopamine depletion, neurons in the BG and motor neurons in the cortex exhibit synchrony and aberrant oscillation (Costa et al., 2006; Goldberg et al., 2002). MSNs of the striatum also become more synchronous and exhibit an increase in oscillation possibly as a result of changes in cortical influence or a change in striatal microcircuitry (Bracci et al., 2002; Mallet et al., 2006). Therefore, in order for the basal ganglia to function properly, normal microcircuitry of the striatum must be maintained.

1.2 Striatum

1.2.1 Structure and function

The striatum is the largest of the input nuclei of the basal ganglia. Naturally, understanding how the striatum functions is fundamental to understanding basal ganglia function and disorder. Structurally, the striatum is divided by the internal capsule into the caudate and putamen. This structural feature is found only in primates and not in rodents. Functionally, the striatum is divided along the dorsolateral and ventromedial axis so that the dorsolateral striatum is involved in sensorimotor function, whereas the ventromedial striatum is involved in cognitive and limbic function (Voorn et al., 2004). This division matches the three domains of BG function: somatosensory, limbic, and associative/cognitive. In many species, the striatum has a patch/matrix organization. Some neurons are restricted by this organization and keep their dendritic processes within one
compartment or the other, whereas other neurons are less restricted and are able to send	heir dendritic processes across the patch/matrix boundary, exerting a widespread influ-
ence on local striatal circuitry (Crittenden & Graybiel, 2011). In addition, a large number
of molecules are also differentially expressed in these two compartments, suggesting that
this mosaic organization must have important functional implications.

1.2.2 Medium spiny neurons

The striatum has a neuronal population that is roughly 95% medium spiny neurons
(MSNs) and 5% interneurons in rodents (more in primates). MSNs are the primary
input neurons of the striatum, which receive glutamatergic projections from the cortex
and thalamus. They are also the output neurons that send GABAergic projections out of
the striatum to target areas. Based on their expression of dopamine receptors and their
axonal projections, MSNs are categorized into D1 MSNs that form the direct pathway and
project to the GPi/SNpr, and D2 MSNs that form the indirect pathway and project to the
GPe. Activation of D1 MSNs increases thalamic activity and promotes motor activity;
whereas activation of D2 MSNs decreases thalamic activity and suppresses motor activity.
In addition to their differential expression of dopamine receptors, D1 MSNs produce
substance P and dynorphin, whereas D2 MSNs produce enkephalin and adenosine A2A
receptor. Besides projecting outside the striatum, MSNs also form axon collaterals that
synapse on other MSNs within the striatum, producing a feedback inhibition (Plotkin
et al., 2005) (Fig. 1.3). Although studies have shown that a fraction of MSNs are involved
in this feedback inhibition, its function and property is still unclear (Lalchandani et al.,
2013; Taverna et al., 2008). The distinct expression of D1 and D2 receptors leads to
activation of Gαi/oif and Gαi/o, respectively, resulting in distinct intracellular cascades
that account for the opposite effect of striatal dopamine on these two MSNs populations.
The differential expression of dopamine receptors contributes largely to the imbalance
between direct and indirect pathway after dopamine depletion.

Although D1 and D2 MSNs are virtually indistinguishable morphologically, electro-
physiological recording using BAC transgenic mice that identifies D1 and D2 MSNs re-
Figure 1.3: Recent findings in synaptic transmission between spiny projection neurons re-established early suggestions about the importance of a feedback circuitry. For an electrophysiological interpretation of striatal responses through history, see Ref. [58]. Abbreviations: PV, parvalbumin; SS, somatostatin; NPY, neuropeptide Y; NOS, nitric-oxide synthase.

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revealed shared as well as distinct physiological properties between the two populations. All MSNs have medium sized soma, and dense local axon collaterals. Their abundance of spines allows them to receive and integrate diverse input. Both populations exhibit hyperpolarized resting membrane potential, characteristic delayed spiking in response to depolarization, and an outward rectification upon negative current injection (Fig. 1.4). These properties are due to the expression of inward rectifying potassium channel, fast- (Kv4.2) and slow- (Kv1.2) inactivation potassium current, and a persistent potassium conductance (Kv7). A distinguishing feature between the two groups of MSNs is that D2 MSNs are more excitable and thus have a lower rheobase than D1 MSNs (Janssen et al., 2011; Mallet et al., 2006) (Fig. 1.5). The greater excitability of D2 MSNs could be due to their expression of inward rectifying potassium channels that inactivate more readily with depolarization. It’s also been suggested through intrastratal stimulation, that glutamatergic synapses on D2 MSNs are more efficacious and have a higher probability of release (Ding et al., 2008; Kreitzer & Malenka, 2007), thus making D2 MSNs more excitable. Regardless, this feature is thought to serve as a compensation for the relatively poorer arborization of D2 MSNs than D1 MSNs, and has been suggested to
contribute to the selective loss of D2 MSNs following MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment.

Figure 1.4: Characteristic firing of striatal MSN. MSNs exhibit inward rectification in response to hyperpolarizing current injection, and delayed firing in response to depolarizing current injection.

1.2.3 Striatal interneurons

Although interneurons constitute such a minor population of the striatum, they are extremely diverse and form synapses on MSNs. Based on the neurotransmitter released, interneurons are categorized into GABAergic (those that release GABA) and cholinergic (those that release acetylcholine). Despite their low density, striatal interneurons receive input signals from the cortex and thalamus and can transfer these signals into modulatory control over MSN activity (Kemp & Powell, 1971; Koós & Tepper, 1999; Lapper & Bolam, 1992; Parent & Parent, 2006).

1.2.3.1 GABAergic interneurons

GABAergic interneurons can be categorized based on immunohistochemical staining into parvalbumin (PV+), neuropeptide Y (NPY+), and calretinin (CR+). In addition, a population of interneurons has been identified in mice where fluorescent protein was
expressed under the tyrosine hydroxylase (TH) promoter. These neurons have thus been
named TH+ striatal interneurons.

PV+ interneurons are also referred to as fast-spiking (FSs), because, like PV+ in-
terneurons of the hippocampus and cortex, they can fire at a frequency of 200-300 Hz in re-
sponse to positive current injection. PV+ interneurons have aspiny dendrites that branch
modestly, which is in contrast to their extremely dense local axonal plexus (Fig. 1.6). An-
other distinguishing feature of PV+ interneurons is that they form gap junctions, which
enables synchronized activity among connected neurons (Fig. 1.6). PV+ interneurons
synapse mainly onto MSNs, and can inhibit the firing of MSNs (Koós & Tepper, 1999)
in the form of feedforward inhibition. In addition to forming strong synaptic projections
to MSNs, PV+ interneurons receive cortical projections and have faster response latency
than MSNs, which allow them to exert powerful control over MSN activity (Plotkin et al.,
PV+ interneurons are more abundant in the lateral than the medial striatum, so they may play a more prominent role in the somatosensory function of striatum (Bolam & Bennett, 1995). Furthermore, studies have shown that PV+ interneurons preferentially target D1 MSNs.

Figure 1.6: Morphological characteristics of FS GABAergic interneurons and cholinergic interneurons. (a) Reconstruction of FS GABAergic interneuron from an adult brain slice stained with biocytin after whole cell recording in vitro. The soma and dendritic tree are in black and the axon is in red. Note that the dense axonal plexus has close to 5000 varicosities. (b) Electron micrograph of one of the varicosities of the same neuron. The bouton (b) is in symmetrical synaptic contact with the dendritic shaft (d) of an MSN (see inset). The spines (s) emerge from the dendrite.

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NPY+ interneurons can be further categorized based on anatomical and physiological properties into persistent low threshold spiking (NPY+ PLTS) and neurogliaform (NPY+ NGF) (Fig. 1.7). NPY+ interneurons have also been shown to express somatostatin and
nitric oxide synthase; the exact degree of overlap, however, is unclear and controversial as our observation does not agree with that described by English et al. (2011). Striatal NPY+ NGF interneurons are named for their physiological and morphological similarity to neurogliaform neurons found in the cortex and hippocampus. They are of particular interest, because they have been shown to generate a distinct form of phasic GABA response and form strong synapses on MSNs (English et al., 2011). This is in contrast to NPY+ PLTS interneurons, which have been shown to have the least dense axonal arborization (Kawaguchi, 1993) and form sparse synapses on MSNs. In addition, these two NPY+ classes exhibit stark differences in morphology and physiology. NGF exhibit none of the electrophysiological features such as low threshold spike, hyperpolarization rebound spike or plateau potential after the offset of current injection, characteristic of PLTS interneurons (English et al., 2011). Instead, NGF interneurons exhibit inward rectification in response to current injection. They also have more hyperpolarized resting membrane potential, and so do not fire action potential without stimulation (English et al., 2011).

CR+ interneurons have not been physiologically characterized because no recordings have been done in neurons fluorescently labeled as CR+. For this reason, CR+ interneurons are not studied in this dissertation.

TH+ interneurons are indistinguishable in size from MSNs, but they exhibit distinct physiological properties that were used by Tepper et al. (2010) to categorize the TH+ interneurons into four classes (Ibáñez-Sandoval et al., 2010). Even though these interneurons express tyrosine hydroxylase, the enzyme involved in dopamine synthesis, studies have yet to show that these TH+ striatal interneurons are dopaminergic. Evidence in support of their dopaminergic nature comes from studies that demonstrate an increase in TH+ interneurons in the striatum after SNpc dopaminergic neurons are destroyed, possibly as compensation to local DA depletion (Huot & Parent, 2007; Ibáñez-Sandoval et al., 2010).
Figure 1.7: Photomicrographs of electrophysiologically identified NPY-PLTS and NPY-NGF interneurons stained with biocytin after whole cell recording. A. NPY-NGF interneuron. Note the dense and highly branched and compact dendritic and axonal arborizations. Inset shows part of the highly varicose, dense local collateral arborization at high magnification. B. NPY-PLTS interneuron. Arrowhead points to axon initial segment, arrows to parts of the axon. Note the sparse and poorly branched dendritic and axonal arborizations. Upper inset shows a varicose segment of a secondary dendrite at high magnification. Lower inset shows part of the diffuse axonal arborization with less prominent, more widely and irregularly spaced varicosities than in the NPY-NGF cell in panel A. C. A second NPY-NGF interneuron displaying a rich, branched dendritic tree. D. A second NPY-PLTS interneuron exhibiting the typical sparse and extended dendritic arborization. Arrows point to parts of the sparse axonal arborization. Inset shows sparse spine-like dendritic specializations. Scale marker in B applies to A and B. Scale marker in D applies to B and D.

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1.2.3.2 Cholinergic interneurons

Cholinergic interneurons are large, aspiny, and tonically active with a low firing rate (1-5 Hz); they have extensive axonal projections, which provide the striatum with a high concentration of acetylcholine (Fig. 1.8). Cholinergic interneurons were first identified in vitro due to their expression of choline acetyltransferase (ChAT+), which is the enzyme that synthesizes acetylcholine (Bolam et al., 1984). Subsequently, it’s been shown that
these ChAT+ interneurons correspond to the tonically active neurons (TANs) recorded from primate striatum in vivo, which pause in firing in response to reward-related stimuli (Aosaki et al., 1994; Bennett & Wilson, 2003). Studies have demonstrated that this pause is accompanied by an increased activity of dopaminergic neurons in the SNpc (Fiorillo et al., 2003), suggesting that dopamine may act on D2 autoreceptors to inhibit ChAT+ interneuron activity. Similar to dopamine, the effect of acetylcholine on MSN activity is mainly through modulatory and direct activation of GABAergic interneurons (Koós & Tepper, 2002; Zhou et al., 2002).

Figure 1.8: Morphological and physiological identification of neostriatal cholinergic interneurons. A, Synthetic projection micrograph of a cholinergic neuron prepared from a 300-µm-thick whole mount is shown. The large soma and thick primary dendrites that branch to form fine-diameter secondary and higher order processes are characteristic of cholinergic interneurons. In this particular example, the axonal arborization gives rise to a dense plexus that innervates the area surrounding the soma and dendrites. B, During whole-cell recording, cholinergic cells are readily identified by their response to intracellular current injection. Injection of a negative current pulse produces an initial hyperpolarization followed by an Ih -dependent sag in the membrane potential. Depolarizing current induces regular spiking and results in a long-lasting afterhyperpolarization after cessation of current injection. C, In the absence of applied current, spontaneous regular spiking (rate = 2.87 Hz; CV = 0.157) was observed in this particular neuron. The membrane potential is indicated for the initial point of each trace in B and C, and the recording was made at 35 ± 2°C.

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1.2.4 Interneuron MSN connectivity

MSNs are the only source of output from the striatum. As a result, the only way for interneurons to influence striatal output is by modulating MSN activity through feedforward inhibition. This feedforward inhibition provides gain control and helps to maintain temporal specificity of excitatory glutamatergic signals from the cortex. As mentioned above, PV+ interneurons have the strongest control over MSN activity, followed by NPY+ NGF (English et al., 2011) and TH+ (Ibáñez-Sandoval et al., 2010); NPY+ PLTS interneurons have the weakest (Gittis et al., 2010) control. Despite their weaker connectivity, subtypes of TH+ and possibility all NPY+ PLTS interneurons exhibit spontaneous firing in acute slices, suggesting that they may modulate striatal output by controlling ambient GABA. These findings are investigated further and summarized in this dissertation.

1.3 GABA

GABA is the major inhibitory neurotransmitter in the CNS. It is an amino acid synthesized from glutamate by L-glutamic acid decarboxylase (GAD); therefore, the expression of GAD determines the GABAergic nature of neurons. The striatum, being largely GABAergic, has strong immunoreactivity for GAD65 and GAD67.

1.3.1 GABA$_A$ receptor

1.3.1.1 Structure

GABA$_A$ receptors belong to the Cys-loop ligand-gated ionotropic superfamily that also includes nACh receptors, 5-HT3 receptors and glycine receptors. GABA$_A$ receptors are composed of five subunits out of a repertoire of 19 ($\alpha_{1-6}, \beta_{1-3}, \gamma_{1-3}, \delta, \epsilon, \theta, \rho_{1-3}, \pi$) identified in mammals (Olsen & Sieghart, 2008). The majority of GABA$_A$ receptors are composed of a combination of two $\alpha$, two $\beta$, a $\gamma$ or a $\delta$ subunit (Fig. 1.9). Each subunit has four transmembrane domains, and has both the N and C terminus in the extracellular space (Fig. 1.10). M2 of each of the five subunits contributes to the ion selectivity of the
receptors, which conduct chloride and bicarbonate ions when two molecules of GABA bind to the binding site formed by \( \alpha \) and \( \beta \) subunits. An intracellular loop between M3 and M4 binds and interacts with intracellular proteins to mediate trafficking and subcellular localization of GABA\(_A\) receptors (Chen & Olsen, 2007; Jacob et al., 2008). GABA\(_A\) receptors are ubiquitously expressed in the CNS and, together with glycine receptors, are the major mediators of inhibitory neurotransmission. The main GABA\(_A\) receptor combination found in the CNS is \( \alpha_1\beta_2\gamma_2 \). Other common combinations include \( \alpha_2\beta_3\gamma_2 \) and \( \alpha_3\beta_n\gamma_2 \). Less common ones are \( \alpha_4\beta_n\gamma_1 \), \( \alpha_4\beta_n\delta \), \( \alpha_5\beta_2\gamma_2 \), and \( \alpha_6\beta_2/3\gamma_2 \) (Möhler et al., 2002). Like other receptors of the same family, subunit composition determines the pharmacological and physiological properties, as well as the subcellular and developmental expression of GABA\(_A\) receptors.

Figure 1.9: Phylogenetic tree analysis of the 19 known genes coding for human GABA\(_A\) receptor subunits. The immature amino acid sequences were obtained from the UniProt database (94). The alignment was done with ClustalX (95), and Dendroscope (64) was used for depiction of the dendrogram.

*From Sigel and Steinmann, 2012; J Biol Chem*
Figure 1.10: Schematic representation of the major isoform of GABA<sub>A</sub> receptors, α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>. The GABA<sub>A</sub> receptors are integral membrane proteins. Five subunits are grouped around the central ion pore. A, topology of a single subunit. All subunits share this topology. B, top view of the pentamer. The sidedness of the subunits is symbolized + and -.

*From Sigel and Steinmann, 2012; J Biol Chem*

### 1.3.1.2 GABA<sub>A</sub> receptor pharmacology

GABA<sub>A</sub> receptors are modulated by benzodiazepines (BZD) and classes of other drugs, such as anxiolytics, anticonvulsants, barbiturates, alcohol, general anesthetics and neurosteroids. Benzodiazepines are allosteric modulators of GABA<sub>A</sub> receptors that bind to the benzodiazepine binding site (BZ site) formed at the interface between an alpha subunit (α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, α<sub>5</sub>) and a gamma subunit (typically γ<sub>2</sub>). They act by modulating the affinity and efficacy of GABA, but also by stabilizing GABA<sub>A</sub> receptor at different conformations. Single channel recording revealed that BZs increase the frequency (Vicini et al., 1987) and open time (Twyman et al., 1989) of GABA<sub>A</sub> channels. Clinically, BZs are used for its anxiolytic, sedative, and muscle relaxant properties. Through studies using genet-
ically modified mice, we are beginning to understand which GABA\textsubscript{A} receptor subunits are associated with the various clinical effects of BZs.

Diazepam is a classic non-selective BZ that has been shown to have different clinical effects at different receptor subtypes. Alpha 1 containing receptors are largely responsible for the unwanted sedative and anterograde amnestic effects of diazepam, whereas $\alpha_2$ containing receptors mediate the anxiolytic and myorelaxant effects of diazepam (Crestani et al., 2001; Löw et al., 2000; Rudolph et al., 1999). Because it’s non-selective, diazepam can be used to probe for the presence of $\gamma_2$ containing receptors, which constitute the majority of GABA\textsubscript{A} receptors. Clonazepam is a BZ that varies structurally from classical BZs, but is similar functionally.

Zolpidem (Ambien) is a hypnotic drug prescribed for insomnia. Like classical benzodiazepines, zolpidem binds at the interface between $\alpha$ and $\gamma$ subunits, but it differs in structure and pharmacological profile from classical BZD. Zolpidem binds with high affinity to GABA\textsubscript{A} receptors containing the $\alpha_1$ subunit, and lower affinity to those containing $\alpha_2$, and $\alpha_3$ subunit. In addition, the $\gamma_2$ subunit is required for zolpidem sensitivity. Therefore, zolpidem can be used to probe for the presence of $\alpha_1\beta_n\gamma_2$ GABA\textsubscript{A} receptors, which have the highest affinity for zolpidem. Determining the subunit composition of GABA\textsubscript{A} receptors is key to finding specific drug targets in the CNS.

Etomidate is a clinically used anesthetic selective for $\beta_2/3$ subunits. It has been shown to directly activate $\beta_3$ subunit containing recombinant receptors and enhance GABA induced current at these receptors (Janssen et al., 2009). Critical residues for etomidate function are found in the M2 and M3 of $\beta_2$ and $\beta_3$ subunits (Jurd et al., 2003). Etomidate was used in the studies described in this dissertation to probe for the function and subunit composition of $\beta_3$ containing receptors in D2 MSNs.

THIP (4,5,6,7-tetrahydroisoxazo- zolo[4,5-c]pyridine-3-ol also gaboxadol) was once an experimental sleep aid drug that ended in clinical trials due to its hallucinogenic effects (Wafford & Ebert, 2006). It has super-agonist effect on $\delta$ containing extrasynaptic GABA\textsubscript{A} receptors and low affinity for $\gamma_2$ containing synaptic GABA\textsubscript{A} receptors.
(Mortensen et al., 2010). THIP was used here to probe for the function and subunit composition of δ containing receptors in MSNs.

1.3.1.3 GABA$_A$ receptor subcellular localization

Subunit composition defines subcellular localization and physiological properties of GABA$_A$ receptors (Mody & Pearce, 2004). Traditionally, GABA$_A$ receptors are found at synapses where they mediate fast synaptic responses, which can be measured in whole cell current clamp as sIPSCs (action potential dependent) and mIPSCs (action potential independent). Now, we know that GABA$_A$ receptors are also found at extrasynaptic sites, where they are activated by GABA spillover from synapses to produce a slow tonic response (Farrant & Nusser, 2005). GABA$_A$ receptors at synaptic sites contain the γ$_2$ subunit, whereas those at extrasynaptic sites contain the δ or, less frequently, the α$_5$ subunit (Glykys & Mody, 2006). This association between subunit composition and subcellular localization suggests that intracellular trafficking mechanisms of GABA$_A$ receptors rely on the expression of specific subunits (Luscher et al., 2011). The low affinity of synaptic receptors for GABA, together with diffusion and efficient GABA reuptake system contribute to the phasic nature of synaptic responses. Synaptic GABA$_A$ receptors have low affinity for GABA and exhibit desensitization, whereas extrasynaptic GABA$_A$ receptors have high affinity and desensitize to a lesser extent (Farrant & Nusser, 2005). As a result, synaptic responses require high concentrations of GABA (0.3-1 mM) and are comprised of a fast rise and slow decay component (Nusser et al., 2001), whereas extrasynaptic receptors can be activated by micromolar concentration of GABA to generate a response that has little desensitization (Semyanov et al., 2004).

1.3.1.4 GABA$_A$ receptor subunit composition

Difference in subunit composition contributes directly to differences in intrinsic properties such as channel kinetics, conductance, and GABA sensitivity between synaptic and extrasynaptic GABA$_A$ receptors. For example, α$_2$ containing receptors activate faster and deactivate slower than α$_1$ containing receptors (Lavoie et al., 1997). Furthermore,
the expression of $\alpha_1$ subunit increases with development to confer changes in functional and pharmacological properties. Recently, a form of slow phasic response (referred to as slow IPSC) presumably due to activation of GABA$_A$ receptors located perisynaptically, has been identified in striatal MSNs (Ibáñez-Sandoval et al., 2011). Slow IPSCs exhibit a rise and decay component similar to conventional synaptic IPSCs (referred to as fast IPSC), but with slower kinetics. On average, fast IPSCs have decay time constant of less than 5 ms. Slow IPSCs, on the other hand, have decay time constant up to 100 ms (Ibáñez-Sandoval et al., 2011). Study by English et al. (2011) found that slow IPSCs in the striatum are mediated by the NPY+ NGF interneurons. Here, we expand on this finding and attempt to elucidate the subtypes of GABA$_A$ receptors that mediate slow IPSCs.

1.3.1.5 Changes in GABA$_A$ receptor during development

The composition of GABA$_A$ receptors changes with development (Fritschy & Brüning, 2003). In the striatum of young mice, tonic current is mediated by $\alpha_5$ containing receptors (Farrant & Nusser, 2005). In adult mice, $\delta$, $\alpha_4$ and $\alpha_6$ subunits begin to play a more prominent role (Nusser & Mody, 2002). D2 MSNs have larger tonic current than D1 MSNs in young mice (Janssen et al., 2009). This difference is reversed in adult mice as the expression of $\alpha_5$ subunit decreases in D2 MSNs, and the expression of $\delta$ subunit increases in D1 MSNs (Farrant & Nusser, 2005). Alpha5 subunits usually combine with other $\alpha$, $\beta$ and $\gamma_2$ subunits to form extrasynaptic GABA$_A$ receptors such as $\alpha_5\beta_3\gamma_2$. Previous studies in our lab have shown that $\alpha_5$ and $\beta_3$ subunits form extrasynaptic receptors that mediate endogenous tonic current in young D2 MSNs and that $\delta$ subunits do not (Ade et al., 2008; Janssen et al., 2009). This observation can be explained by two possibilities. First, the expression of extrasynaptic receptors containing the $\delta$ subunit is too low to mediate tonic current in young D2 MSNs. Second, $\beta_3$ subunit containing extrasynaptic receptors are activated by the concentration of GABA present endogenously in acute brain slices, whereas $\delta$ subunit containing extrasynaptic receptors are not activated in
young D2 MSNs. We investigate these two possibilities by using $\delta$ and $\beta_3$ subunit selective GABA$_A$ receptor agonists.

### 1.3.2 Chloride reversal potential

Because GABA$_A$ receptors are highly permeable to Cl$^-$ ions, whether a cell depolarizes or hyperpolarizes in response to GABA$_A$ receptor activation depends on the chloride reversal potential or the intracellular chloride concentration [Cl$^-$]$i$. [Cl$^-$]$i$ undergoes a developmental shift thought to be determined by the expression of two cation-chloride cotransporters: K$^+$.Cl$^-$ (KCC2) and Na$^+$.K$^+$.2Cl$^-$ (NKCC1)(Fig. 1.11). In young brains, NKCC1 maintains an elevated [Cl$^-$]$i$ such that chloride flows out of the cell when conductance increases, making activation of GABA$_A$ receptors depolarizing. As the brain matures, the expression of NKCC1 decreases while that of KCC2 increases. This lowers the [Cl$^-$]$i$ so that the reversal potential of Cl$^-$ ($E_{Cl^-}$) rests 15-20 mV negative to the resting membrane potential. As a result, GABA$_A$ receptor activation in the mature brain leads to chloride influx, which favors inhibition. A recent study presents strong evidence, which suggest that cytoplasmic impermeant anions and polyanionic extracellular glycoproteins are the primary determinants of neuronal Cl$^-$ concentration, and that cation-chloride cotransporters serve only as conduit of chloride flux to maintain Cl$^-$ concentration (Glykys et al., 2014). They also deduct that the developmental decrease in [Cl$^-$]$i$ results from increases in both cytoplasmic and extracellular anions (Glykys et al., 2014). Given their finding, a re-evaluation of the determinants of neuronal [Cl$^-$] is needed. Simply thinking of GABA$_A$ receptors as either excitatory or inhibitory, however, is an incomplete representation of the role of GABA$_A$ receptor mediated current in neuronal signaling.

### 1.3.3 GABA$_B$ and GABA$_C$ receptors

GABA$_B$ receptors are heterodimeric Gi/Go-protein coupled receptors that are involved in certain presynaptic modulations of striatal activity. They activate potassium channels and inhibit calcium channels (Ulrich & Bettler, 2007). GABA$_C$ receptors are now consid-
Figure 1.11: Early expression of NKCC1 and late expression of KCC2 determines developmental changes in [Cl\textsuperscript{-}]i. Schematic diagram depicting the Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{-} co-transporter NKCC1, the K\textsuperscript{+}Cl\textsuperscript{-} co-transporter KCC2 and voltage-gated calcium currents, as well as the gradients of chloride ions. (a) NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride ([Cl\textsuperscript{-}]i) is relatively high. (b) KCC2 expression predominates in mature neurons. Note that the activation of GABA (gamma-aminobutyric acid) type A receptors generates an efflux of chloride and an excitation of immature neurons, and an influx of chloride and an inhibition of adult neurons. ClC2, voltage-gated chloride channel 2; E\textsubscript{Cl}, chloride reversal potential; RMP, resting membrane potential (V\textsubscript{rest}); VDCC, voltage-dependent calcium channel. Adapted, with permission, from Ref. 42 ©1998 The American Physiological Society.

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1.3.4 Tonic vs. phasic GABA current

Tonic GABA current, which results from the opening of δ or α5 containing GABA\textsubscript{A} receptors, can strongly influence the excitability of a network through inhibitory hyper-
polarization or shunting inhibition (Brickley et al., 2001). Inhibitory hyperpolarization occurs in cells where the reversal potential of GABA is more hyperpolarized than the resting membrane potential. Shunting inhibition, on the other hand, occurs as a result of an increase in membrane conductance associated with GABA tonic current. Under this condition, synaptic excitatory inputs fail to alter the membrane potential sufficiently to generate action potentials. Shunting inhibition through GABA tonic current is thought to occur throughout the CNS. In whole cell voltage clamp, phasic GABA currents, mediated by activation of synaptic GABA<sub>A</sub> receptors, can directly inhibit firing activity in the postsynaptic neurons. Tonic GABA current is usually unmasked by the application of a GABA<sub>A</sub> receptor antagonist such as bicuculline or GABAzine, and is represented by an upward shift in holding current (Ade et al., 2008; Janssen et al., 2009).

The balance between excitation and inhibition is crucial to generating desired striatal output signals. One of the mechanisms that helps keep this balance in check is shunting through GABA<sub>A</sub> receptor mediated tonic current. We and others have demonstrated the occurrence of GABA tonic current in D2 MSNs in the striatum of acutely isolated mouse brain slices (Fig. 1.12). In addition, previous studies have shown that in this preparation, GABA tonic current is action potential dependent, suggesting contribution from active neurons (Ade et al., 2008). Several classes of striatal interneurons fire action potential spontaneously in the absence of excitatory input. Of those, the cholinergic interneurons are of particular interest. Dual recordings of two cholinergic interneurons showed that an action potential elicited in one leads to GABA mediated feedforward response in the other; and also GABA mediated feedback response in the stimulated neuron (Sullivan et al., 2008). Given their large soma and extensive processes, cholinergic interneurons are well-suited to influence striatal network activity, and may do so through GABA tonic current. Here, we investigate the modulatory effect of the cholinergic system on striatal output by activating nAChRs in the striatum.
Figure 1.12: D2+ MSNs demonstrate larger GABA$_A$ receptor-mediated tonic currents than D1+ MSNs. A, Representative traces from a D1+ MSN (left) and a D2+ MSN (right) demonstrate that BIC (25 µM) blocked sIPSCs in both cell types but only revealed an endogenous GABA-mediated tonic current in the D2+ MSN. The remaining sEPSCs in both cell types were blocked by NBQX (5 µM) with no effects on the tonic current. B, The mean of the baseline current during BIC application from the representative traces in A were adjusted to 0, and the amplitude distributions were drawn from segments immediately preceding BIC application. The non-skewed sides of the amplitude histograms were fit with a Gaussian, the peak of which was used to determine the absolute magnitude of tonic current blocked by BIC. C, Summary of tonic currents blocked by BIC (25 µM) with KCl internal (black bars; n = 23 and 44) and with CsCl internal (white bars; n = 7 and 9) in D1+ and D2+ MSNs, respectively.

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1.3.5 GABA$_A$ receptor subtype in the striatum

The expression of GABA$_A$ receptor subunits in the rodent striatum has been investigated extensively and has been shown to be a diverse mixture. $\alpha_{1-4}$, $\beta_{2/3}$, $\gamma_{1-3}$, and $\delta$ subunits have been shown to be expressed in the striatum by immunoreactivity (Pirker et al., 2000). Immunoreactivity for the $\alpha_6$ subunit was not found in the striatum (Schwarzer et al., 2001). This correlates with in situ hybridization studies showing similar findings (Herb et al., 1992; Wisden et al., 1991, 1992). The diversity of GABA$_A$ subunits found in the striatum stem largely from the diversity in its neuronal population (Tepper & Bolam, 2004). Striatal MSNs and interneurons are believed to express different subsets
of receptors that shape their physiological characteristics and function. In MSNs, $\alpha_2$, $\alpha_4$, $\beta_3$, and $\delta$ subunits are more prevalent, whereas in interneurons, $\alpha_1$, $\beta_2$, and $\gamma_2$ subunits are preferentially found (Schwarzer et al., 2001). Genetic and pharmacological methods have shown that $\alpha_5$, $\beta_3$ and $\gamma_2$ containing GABA$_A$ receptors contribute to the generation of tonic current in D2 MSNs, while $\alpha_1$ and $\delta$ subunit containing receptors do not (Ade et al., 2008; Janssen et al., 2009). As assembled receptors, $\alpha_1\beta_2\gamma_2$, the most abundant subtype in the brain, is expressed by neurons of the striatum. In addition, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_2\gamma_2$, and $\alpha_4\beta_3\delta$ (and $\alpha_4\beta_3\delta$) receptors are expected to be expressed by different cell types within the striatum (Fujiyama et al., 1999). Whether these receptors are expressed exclusively by certain cell types or are co-expressed by the same cell type is unclear. Using optogenetics and pharmacology, we can begin to identify receptor subtypes that mediate response evoked by specific neuronal populations in the striatum.

1.4 Dopamine

Dopamine has widespread effect on striatal neurons and is crucial for striatal function and understanding basal ganglia disorders. Striatal dopamine originates from dopaminergic neurons in the SNpc, which send extensive axonal projections to the striatum. The effect of DA depends on the expression of dopamine receptors. However, accumulating evidence suggest that DA neurons and terminals do more than release DA to modulate striatal activity.

1.4.1 Dopamine receptors

Dopamine receptors are G protein coupled receptors that mediate a variety of intracellular signaling after binding and activation by dopamine. Two subtypes of dopamine receptors have been identified: D1-like, which couple to $G_s$ and $G_{olf}$ proteins (D1 and D5) and activates adenylyl cyclase, and D2-like, which couple to $G_i$ and $G_o$ proteins (D2, D3 and D4) and inhibits adenylyl cyclase. All dopamine receptors are expressed in the striatum; however, D1 and D2 receptors are particularly abundant because of their high
Although dopamine has opposing actions on D1 and D2 MSNs, the overall outcome is the same: to facilitate movement. Besides MSNs, striatal interneurons also express dopamine receptors, and thus, can be influenced by activity of dopaminergic neurons in the SNpc. Specifically, GABAergic interneurons express D2 receptors; cholinergic interneurons express both D2 and D5 receptors.

### 1.4.2 Dopamine modulates MSN activity

Directly, DA activates direct pathway D1 MSNs while inhibiting indirect pathway D2 MSNs. By activating D1 receptors, DA increases depolarizing Cav1 L-type Ca\(^{2+}\) channel currents, decreases hyperpolarizing somatic K\(^{+}\) currents and activation of small-conductance K\(^{+}\) channels, which, together, increase firing of D1 MSNs (Gerfen & Surmeier, 2011). DA has the opposite direct effect on D2 MSNs by reducing current through Cav1 L-type Ca\(^{2+}\) channels, and increasing hyperpolarizing current through K\(^{+}\) channels (Gerfen & Surmeier, 2011). The outcome of both is the facilitation of movement. Indirectly, DA can also modulate MSN activity and striatal output by releasing glutamate (Tecuapetla et al., 2010) and GABA (Tritsch et al., 2012). More interestingly, striatal cholinergic interneurons can also drive GABA release from dopaminergic terminals by activating nicotinic receptors. Taken together, DA terminals can be modulated to release DA, glutamate, and GABA through nicotinic activation independent of SNpc activity (Surmeier & Graybiel, 2012). These findings add more complexity to the effect of DA neurons on striatal output.

### 1.5 Achetylcholine

Acetylcholine provides the other modulatory branch of striatal activity. Cholinergic interneurons, which constitute only a few percent of striatal neurons, provide the striatum with one of the highest concentrations of acetylcholine found in the brain (Goldberg et al., 2012). Both muscarinic and nicotinic receptors are expressed in the striatum. Although the expression of nicotinic receptors is more restricted in comparison (Goldberg et al.,
2012). Muscarinic receptors are expressed by all striatal neurons and on axonal terminals that project to the striatum. For instance, activation of muscarinic receptors on terminals of PV+ interneurons diminishes GABA release and frequency of IPSC in MSNs (Koós & Tepper, 2002). Because this dissertation focuses on nicotinic receptor mediated cholinergic response in the striatum, muscarinic receptors will not be discussed further.

1.5.1 Nicotinic acetylcholine receptors

Neuronal nicotinic acetylcholine receptors (nAChR) belong to the same superfamily of Cys-loop ligand gated ionotropic receptors as GABA_A receptors. They are composed of five subunits out of a repertoire of 12 (α2–10, β2–4). The five subunits assemble to form a nonselective cation channel that opens with the binding of two molecules of ACh at the α/β interface. There are two classes of neuronal nAChR. One is formed by a combination of α and β subunits; the other is a homomeric composed of α7–α9 subunits, although only homopentamer composed of the α7 subunit is found in the CNS (Zhou et al., 2002). Despite all the possible subunit combinations, only a few are actually found. Various studies have shown that, in the rodent striatum, two major subtypes of nAChRs are found: one contains the α4 and β2 subunits, and the other contains the α6 and β2 subunit (Gotti & Clementi, 2004). Alpha7 homopentamers are excluded from the striatum (Zhou et al., 2002). Pharmacological and neurochemical evidence for the expression of subtypes by specific neurons are mixed. However, electrophysiological data support the expression of nAChRs by GABAergic interneurons and not MSNs in the striatum (de Rover et al., 2002; Koós & Tepper, 2002).

1.5.2 Cholinergic modulation of striatal activity

Similar to nigrostriatal dopaminergic innervation, cholinergic interneurons have wide and extensive axonal arborization, and serve to modulate striatal activity and output. The rich cholinergic innervation in the striatum is supported by the abundant expression of choline acetyltransferase as well as acetylcholinesterase, both of which are essential in cholinergic neurotransmission (Zhou et al., 2002). Precisely how the cholinergic system is
involved in modulating striatal function is still unclear, but studies suggest a direct effect on GABAergic interneurons, which would lead to an indirect effect on striatal projecting neurons (Sullivan et al., 2008).

Cholinergic signaling is well recognized to play an important role in modulating striatal output through its antagonistic action with dopamine. Studies have shown that acetylcholine can induce and enhance GABA activity in the striatum by activating GABAergic interneurons such as PV+ interneurons (Koós & Tepper, 2002; Sullivan et al., 2008) (Fig. 1.13). Using channelrhodopsin to selectively activate a population of ChAT+ interneurons, English et al. (2011) showed that light activation of ChAT+ interneurons induced GABA release by NPY+ NGF interneurons, which in turn, produced IPSCs in MSNs. These evidence suggest that GABAergic tonic current in D2 MSNs may in fact depend on the control of GABAergic interneurons by acetylcholine, and propelled our studies where we investigated the cholinergic control of GABAergic interneurons and how it contributes to the occurrence of tonic GABA current in MSNs. GABA response produced by cholinergic interneurons is recurrent, as both intracellular (Sullivan et al., 2008) and light stimulation (English et al., 2011) of cholinergic interneurons produced inhibitory current in the stimulated neuron in addition to other neurons. Evidence suggest that this recurrent current is produced by distinct GABAergic neurons as those involved in feedforward inhibition of MSNs (English et al., 2011).

1.6 Acetylcholine and dopamine in striatum

Acetylcholine and dopamine are important to striatal function. Striatal dopamine is provided by dopaminergic neurons of the SNpc. Striatal acetylcholine is provided by cholinergic interneurons within the striatum. These neurons share several physiological characteristics, such as large soma, extensive axonal branching and spontaneous activity, allowing them to strongly influence striatal output. In addition, both classes of neurons exhibit hyperpolarization-activated cation current (Ih), which appears in response to hyperpolarizing current injection and is believed to contribute to their spontaneous
Figure 1.13: Recurrent polysynaptic inhibition between single cholinergic interneurons. A, Evoking an action potential in one cholinergic interneuron produced a feedback PSC in the same neuron as well as a feedforward PSC in the other neuron recorded simultaneously. An unclamped action potential was elicited by a 2 ms depolarizing pulse. B, Representative time graph showing the effects of DHβE (100 nm) and GABAzine (GBZ; 10 µm) on feedback and feedforward PSCs. The recording was from the same pair of cholinergic interneurons as in A. C, Schematic diagram illustrating synaptic connections between the pair of cholinergic interneurons (ChIs) shown in A and B. A GABAergic neuron (GBN) connecting two cholinergic interneurons is also depicted. In this particular pair, stimulation of one cholinergic interneuron (left) produced GABA A IPSCs in both neurons, whereas stimulation of the other neuron (right) failed to elicit IPSCs in either neuron.

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activity. Indeed, Ih has been implicated in the generation of rhythmic activity of not only neurons, but also cardiac cells (DiFrancesco, 1993; Pape, 1996). The reciprocity of DA and ACh stems from two observations. First, cholinergic antagonists were used to treat PD in which DA levels plummet. Second, in response to reward-related cues, DA neurons burst while ACh neurons fall silent. Not surprisingly, DA and ACh terminals are found in close proximity within the striatum, allowing direct interaction between the two systems (Descarries et al., 1997). Recent advances in optogenetics have helped paint a clearer picture of how these two systems interact. It’s been known for some time that DA can inhibit the spontaneous activity of cholinergic neurons and reduce ACh release by activating D2 autoreceptors on cholinergic terminals (Gerfen & Surmeier, 2011). Whether ACh has a direct antagonistic effect on DA release is less clear. In fact, recent findings suggest the
opposite. Channelrhodopsin induced simultaneous activation of cholinergic interneurons increased striatal DA release. Moreover, the pulse in cholinergic firing is dependent on nicotinic receptor activation, which likely triggers the DA release that subsequently inhibits cholinergic firing. By modulating acetylcholine release, DA can modulate activity of MSNs indirectly.

1.7 Channelrhodopsin

1.7.1 Structure and function

Channelrhodopsin-2 (ChR2) is a light activated ion channel natively expressed by the unicellular alga Chlamydomonas reinhardtii to mediate phototactic behavior (Nagel et al., 2003). When expressed in cell culture and living animals, however, ChR2 has diverse neurobiological applications that allow fast and non-invasive light activation of neurons (Boyden et al., 2005; Li et al., 2005; Nagel et al., 2005). ChR2 belongs to the family of microbial rhodopsins that have a characteristic seven transmembrane domain, which mediates the channel function of the ChR2 (Nagel et al., 2003) (Fig. 1.14). Often, ChR2 is tagged with a red or yellow fluorescent protein to monitor cellular expression and localization. When illuminated, ChR2 undergoes a structural change to increase their conductance for monovalent (Na\(^+\)) and divalent (Ca\(^{2+}\)) cations, leading to membrane depolarization and neuronal activation. Because ChR2 allows fast, reliable, precise and non-invasive activation of specific populations of neurons, it has found wide application in the study of neuronal activity in various brain areas (Fenno et al., 2011).

1.7.2 Advantages

The advantage offered by ChR2 for studying network activity is that it allows the simultaneous activation of multiple cells of the same class. This is a desirable alternative to other methods of neuronal activation such as stimulating electrode, intracellular electrode or drug application, which lack either specificity, temporal precision or the ability to synchronously activate multiple cells. Recent advances in our understanding of the
Figure 1.14: Channelrhodopsin-2 (ChR2) is a light-gated cation channel native to the green alga C. reinhardtii. It consists of seven transmembrane proteins and absorbs light through its interaction with retinal. Here, scientists induce channelrhodopsin coupled to enhanced yellow fluorescent protein (eYFP) into undifferentiated human embryonic stem cells via a lentiviral vector and differentiate these cells into cardiomyocytes. (Credit: Stanford University).

striatal network draw largely from studies that express ChR2 in different classes of striatal neurons (Chuhma et al., 2011; English et al., 2011; Ibáñez-Sandoval et al., 2010; Kozorovitskiy et al., 2012; Ledri et al., 2014; Szydlowski et al., 2013) Cell-type specific expression of ChR2 can be driven in double transgenic mice by breeding Cre recombinase mice with mice that express a loxP flanked stop cassette upstream of ChR2-YFP fusion protein. With the expression of Cre recombinase, the stop cassette will be deleted resulting in the expression of ChR2-YFP fusion protein in Cre expressing tissue (Witten et al., 2011).

In order to elucidate striatal connectivity and to study the properties of different GABAergic synapses between various striatal neurons, we performed single and dual recordings using transgenic mice with Cre dependent expression of ChR2 in striatal D2 MSNs, TH+, PV+ and SST+ interneurons.
Chapter 2

Materials and Methods

2.1 Animals

The use of several strains of transgenic mice examined in this study to genetically identify rare striatal interneurons and have been described previously (Luo et al., 2013). These include the BAC-npy-eGFP and parv-Cre;rosa26-tdTom. Conditional and neuron specific GABA$_A$ $\beta_3$ subunit knockout (KO) mice and appropriate control mice were produced as reported in Janssen et al. (2011), by crossing floxed $\beta_3$ mice ($\beta_3^{f/f}$) (Jackson Labs # 008310; Ferguson et al. (2007)) to transgenic mice that express Cre recombinase under the drd2 promoter (GENSAT, ER44; Gong et al. (2007)). These Cre-dependent $\beta_3$ subunit KO mice were subsequently crossed to "floxed-stop" rosa26-tdTom mice (Jackson Labs # 007914; Madisen et al. (2010)); a 2nd Cre-dependent line to allow visual identification of neurons lacking the $\beta_3$ receptor subunit restricted to D2+ neurons. We designate this line as drd2-Cre;$\beta_3^{f/f}$;Rosa$^{tdTom}$. Control mice used in these studies included drd2-Cre;$\beta_3^{f/+}$;Rosa$^{tdTom}$ and Cre-negative $\beta_3^{f/f}$;Rosa$^{tdTom}$. We did not detect any statistical differences between these two genotypes and combined them for control experiments. We employed commercially available genotyping of tail biopsies of littermates via Transnetyx, Inc. (Cordova, TN) before conducting experiments. We also used global GABA$_A$ $\delta$ -receptor subunit knockout mice previously described (Janssen et al., 2009; Mihalek et al., 1999).
2.2 Slice preparation

Brain slices were prepared from young male and female mice (p15-21). The following mice were used: bacteria artificial chromosome (BAC) drd2-EGFP; drd1a-tdTomato (GENSAT; Gong et al. (2003); Shuen et al. (2008)), BAC-NPY (npy promoter attached to a humanized Renilla GFP Stock 006417, Jackson Laboratory, Bar Harbor, ME), PV-Cre;ROSA-tdTomato (Madisen et al., 2010; Murray et al., 2011), and TH-EGFP (Ibáñez-Sandoval et al., 2010) (Tg(Th-EGFP) stock obtained from MMRC strain #292). Mice were sacrificed by decapitation in agreement with the guidelines of the AMVA Panel on Euthanasia and the Georgetown University animal care and use committee. The whole brain was removed and placed in an ice-cold slicing solution containing (in mM): NaCl (85), KCl (3), CaCl2 (0.5), MgSO4 (7), NaH2PO4 (1.25), NaHCO3 (25), glucose (25), sucrose (75) (all from Sigma, St Louis, MO). Corticostriatal coronal slices (250 µm) were prepared using a Vibratome 3000 Plus Sectioning System (Vibratome, St Louis, MO) in ice-cold slicing solution. They were incubated in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (124), KCl (4.5), Na2HPO4 (1.2), NaHCO3 (26), CaCl2 (2.0), MgCl2 (1), and dextrose (10.0) at 305 mOsm at 32°C for 30 minutes. The slices then recovered for an additional 30 minutes in ACSF at room temperature, 23-25°C. All solutions were maintained at pH 7.4 by continuous bubbling with 95% O2, 5% CO2. During experiments, slices were completely submerged and continuously perfused (2-3 ml/min) with ACSF at room temperature.

2.3 Whole-cell recordings

Hemislices containing the cortex and the anterior striatum were visualized under upright microscopes (E600FN or FN1 Nikon, Japan) equipped with Nomarski optics and an electrically insulated 60x water immersion objective with a long working distance (2 mm) and high numerical aperture (1.0). In drd2-EGFP; drd1a-tdTomato mice, MSNs were classified as being either striatopallidal dopamine D2 receptor positive (D2+) or striatonigral dopamine D1 receptor positive (D1+) based on their expression of EGFP or tdTomato,
respectively. In all other transgenic mice, MSNs were not tagged with fluorescent proteins and the sample population likely contains both D1+ and D2+ MSNs. Neurones were visualized with green or red fluorescent protein, based on animal genotype, and often confirmed with firing patterns. Identification of fluorescent protein-expressing neurones was performed by epifluorescent excitation of the tissue with a mercury-based lamp and standard filter sets.

Recording pipettes 4-6 MΩ were pulled on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II; Drummond, Broomall, PA) and filled with either KCl or potassium gluconate-based internal solutions. The KCl-based internal solution contained (in mM): KCl (145), HEPES (10), ATP.Mg (5), GTP.Na (0.2), EGTA (5), adjusted to pH 7.2 with KOH. In K-gluconate-based internal solutions (labeled as "Kgluc" in some Figures), KCl was replaced with equimolar K-gluconate and pH was adjusted with KOH.

Voltage-clamp recordings were performed using the whole-cell configuration of the patch-clamp technique at a holding voltage of -70 mV using the Multiclamp 700B amplifier (Molecular Device Co., Sunnyvale CA, USA). When Kgluc internal solutions were used, the baseline membrane potential for current-clamp recordings was set at -70 mV before each series of current step injection protocols. In a small subset of cells, we examined evoked excitatory postsynaptic currents via whole-cell voltage-clamp recordings (Partridge et al., 2009). Evoked responses were obtained using a twisted bipolar stimulating electrode placed near the white matter separating the cortex from striatum. For these recordings, we used an internal solution containing (in mM): 145 potassium gluconate, 10 HEPES, 10 BAPTA, 0.2 Na-GTP, 5 Mg-ATP, and supplemented with 5 lidocaine N-ethyl bromide (QX-314), pH adjusted to 7.4 with KOH.

Stock solutions of bicuculline methobromide (BMR), carbachol, atropine, dihydro-β-erythroidine (DHβE), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate (NBQX, Abcam Biochemicals Cambridge, MA), cytisine (Abcam Biochemicals Cambridge, MA), mecamylamine (MEC Abcam Biochemicals Cambridge, MA), methyllycaconitine (MLA, Tocris Bioscience, Minneapolis, MN), tetrodotoxin (TTX; Alomone Labs, Israel), quinpirole, and SKF-81297 (Sigma) were
prepared in water. Stock solutions of zolpidem, etomidate, and diazepam (Sigma) were dissolved in dimethylsulfoxide (<0.01% final concentration). All stock solutions were diluted to the desired concentration in aCSF and applied locally through a "Y tubing" (Murase et al., 1989) modified for optimal solution exchange in brain slices (Hevers & Lüddens, 2002). In most cases, multiple experiments were not performed on the same cell to avoid receptor desensitization.

Currents were filtered at 2 kHz with a low-pass Bessel filter and digitized at 5-10 kHz using a personal computer equipped with Digidata 1322A data acquisition board and pCLAMP10 software (both from Molecular Devices). Off-line data analysis, curve fitting, and Fig. preparation were performed with Clampfit10 software (Molecular Devices). Spontaneous inhibitory postsynaptic currents (sIPSCs) were identified using a semi-automated threshold based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ) and were visually confirmed. Event detection threshold was set at 5 times the RMS (root mean square) level of baseline noise. NBQX was not included in sIPSC measurements to not perturb the network activity. AMPA-mediated spontaneous EPSCs could easily be identified by the rapid decay kinetics (≤ 8 milliseconds) and were excluded from the IPSC analysis, as we have previously described (Forcelli et al., 2012; Ortinski et al., 2004). All detected events were used for event frequency analysis, but superimposing events were eliminated for the amplitude and decay kinetic analysis. AChR mediated current was primarily measured with an all-points histogram that measured the mean holding current 10 seconds before and during drug application. Holding current changes during high frequency stimulation of synaptically connected pairs was assessed similarly after low pass filtering (2-5 Hz).

2.4 Excised patch recordings

Recordings of single channel activity were performed in excised outside-out patches held at -60 mV. Because it was rare to find patches with single channels, kinetic analyses were performed on stretches with few superimposed channel openings. Events for single-channel
dwell time analyses were collected using half-amplitude threshold detection method built into pClamp 10 software and >500 events were collected for all conditions. Superimposed openings were excluded. Mean unitary current was determined from the Gaussian fits of amplitude distributions of openings to the main conductance level lasting more than 0.5 ms.

2.5 Channelrhodopsin stimulation

ChR2 was activated using a blue Lumen 120LED (200 mW) with light projecting onto the slice through the objective lens of the microscope. The intensity and duration of the illumination were controlled by a digital to analogue converter output of Digidata and controller.

2.6 Statistics

To sample a representative population, each variable examined in this study was derived from at least three different animals from at least two or more breeding pairs. Statistical significance was determined using the paired two-tailed Student’s t-test to compare pre-drug conditions with recordings made under drug conditions of the same cell population. One-way ANOVA for repeated measures followed by Tukey post hoc test was used for comparisons across cell groups. All values in text and figures are expressed as mean ± SEM. In all summary graphs, the number of cells studied is in parenthesis.
Chapter 3

Results

3.1 Characteristics of striatal neurons

In order to record selectively from striatal neurons, we used transgenic mice that express fluorescent proteins under the control of neuronal specific promoters (Fig. 3.1A). Once whole cell recording was achieved, the identity of individual neurons was further confirmed by their response to hyperpolarizing and depolarizing current injections (Fig. 3.1B). D1 and D2 MSNs identified in drd2-EGFP; drd1a-tdTomato mice had medium-sized cell bodies and were characterized by the presence of inward rectification and delayed firing. Persistent low-threshold spiking NPY+ (NPY+ PLTS) interneurons were identified in NPY-EGFP mice as moderately fluorescent bipolar cells that emit two to three primary dendrites with minimal branching. The physiological response of NPY+ PLTS interneurons to current injection is heterogeneous. In the majority of these interneurons, we observed continuous firing in response to positive current injection, and a rebound firing following the offset of hyperpolarizing current injection (Fig. 3.1B). In NPY-EFGP mice, we also observed a minor population of brightly fluorescent round cells with multiple primary dendrites similar to the NPY+ NGF interneurons identified by Ibáñez-Sandoval et al. (2011). Current injections confirmed them as NPY+ NGF interneurons and revealed that these neurons exhibit more distinct and homogeneous physiological properties than PLTS neurons. Regular firing to depolarizing current injection, and inward rectifica-
tion to both hyperpolarizing and depolarizing currents were always observed in NPY+ NGF interneurons (Fig. 3.1B). In PV-Cre/Rosa-tdTomato mice, PV+ interneurons were identified by their large soma and extensive dendritic branching. They exhibited characteristic physiological properties including rapid firing, short duration action potential and short after-hyperpolarization (Fig. 3.1B). In some instances, stuttering was observed in response to positive current injection before maximum firing frequency was reached. Interneurons identified in TH-EGFP mice were small and had heterogeneous physiological properties that often but not always matched those reported by Ibáñez-Sandoval et al. (2010). Attempts were not made to classify these neurons. Based on the morphology of interneurons in these transgenic mice, NPY+ NGF and PV+ interneurons seem to have the most extensive synaptic connectivity with neighboring neurons because of their rich branching. In addition to the differences observed in response to current injection, striatal neurons also exhibit physiological differences apparent in the absence of stimulation. In cell attached and current clamp recordings, without active current injection, the majority of NPY+ PLTS and TH+ interneurons were spontaneously active (Fig. 3.2). Spontaneous firing was never detected in MSNs (not shown), NPY+ NGF or PV+ interneurons, which had hyperpolarized resting membrane potential. Electrophysiological properties always correlated with cell types identified by the expression of fluorescent markers.

3.2 Nicotinic receptor mediated response in striatal neurons

To test the effect of acetylcholine on striatal neurons, we locally applied 100 µM carbachol along with 1 µM atropine to striatal slices to isolate nAChR-mediated effects. To facilitate the detection of GABAergic activity, we used high [Cl−] internal solution. A shown in Fig. 3.3A, carbachol and atropine (CCh+Atr) increased both the holding current and the occurrence of sIPSCs in 72 of 94 MSNs (76%). When BMR (25 µM), the GABA A receptor antagonist, was applied, the effect of CCh+Atr was abolished, suggesting that
Figure 3.1: Identification of MSNs and interneurones. (A) Fluorescent micrographs identifying populations of MSNs, NPY+, PV+ and TH+ interneurones in coronal striatal sections of different mice. Scale bar is 100 µm except for the first panel (70 µm). (B) Electrophysiological characteristics of different striatal neurones during hyperpolarizing and depolarizing current injections.

GABA is released through activation of nAChRs on GABAergic neurons (Fig. 3.3B). In 8 MSNs identified as D2+, CCh+Atr significantly increased sIPSC frequency (247 ± 50%) and amplitude (124 ± 11%) (p<0.05). In 4 of the 8 D2+ MSNs, BMR revealed an underlying tonic current as previously reported (Ade et al., 2008). To eliminate possible contribution from muscarinic ACh receptors, we applied atropine, a muscarinic receptor antagonist, alone before applying carbachol. Atropine at 1 µM had no effect on the holding current or sIPSC frequency in all MSNs tested (n=24). To determine whether the effect of CCh+Atr was action potential dependent, we applied the voltage-gated sodium channel blocker, TTX. In 4 out of 5 MSNs TTX (0.5 µM) blocked the effect of CCh+Atr, suggesting that carbachol evoked GABAergic neurons to fire action potentials, leading to GABA release. Fig. 3.3C shows a summary plot of CCh+Atr evoked current comparing D1+, D2+ and unidentified MSNs. The change in holding current induced by carbachol was not significantly different among these three groups. To test for the presence of endogenous cholinergic tone acting on nicotinic receptors, we locally perfused
Figure 3.2: Membrane potential activity of striatal interneurons in acute slice. Whole cell current clamp recording of striatal GABAergic interneurons. TH and NPY+PLTS interneurons have depolarized membrane potential and fire action potential spontaneously. PV+ and NPY+NGF interneurons are more hyperpolarized and are quiescent in the absence of stimulation.

physostigmine (5 \( \mu \text{M} \)), an inhibitor of acetylcholinesterase (AChE). In 5 D1+ and 6 D2+ MSNs tested, physostigmine had no effect on either holding current or frequency of sIPSCs. Phystostigmine inhibited evoked AMPA-EPSCs in 3 MSNs demonstrating its efficacy on cholinergic tone through muscarinic receptors. This effect was completely reversed upon co-application of 1 \( \mu \text{M} \) atropine.

To determine which nAChR subtype is involved in mediating the carbachol induced current in MSNs, we used the \( \alpha_4 \beta_2 \) selective antagonist, dihydro-beta-erythroidine (DH\( \beta \)E). DH\( \beta \)E (10 \( \mu \text{M} \)) had variable effects on CCh+ Atr induced current in 19 MSNs (Fig. 3.3C). In 14 MSNs, it blocked the response completely. In the 5 remaining MSNs, we observed either a partial or no blockade of CCh+ Atr induced currents. In 12 MSNs identified as either D1+ or D2+, DH\( \beta \)E completely blocked CCh+ Atr induced currents in 5/5 D2+ and 5/7 D1+ MSNs. To further investigate the nAChR subtype, we tested the non-selective nAChR antagonist mecamylamine (MEC, 60 \( \mu \text{M} \)) and the \( \alpha_7 \) subunit-selective antagonist methyllycaconitine (MLA, 50 nM). MEC did not alter
Figure 3.3: Cholinergic modulation of tonic and phasic GABA current in striatal neurons. Representative traces illustrating the effects of carbachol (100 µM) and 1 µM atropine (CCh+Atr, A and B) on D2+ MSNs with KCl internal solution. (A) Pre-application of 1 µM Atr failed to alter and did not prevent CCh+Atr induced changes in holding current. (B) Application of 25 µM BMR unmasked GABAergic tonic current following CCh+Atr induced changes in holding current. (C) Bar graph summarizing the changes in holding current induced by CCh+Atr and CCh+Atr+DHβE in unidentified MSNs (* p<0.005). For comparison, changes in holding currents are also shown for D1+ and D2+ MSNs. (D) Changes in GABA-induced holding current was absent when CCh+Atr was applied in BMR with Kgluc internal solution.

holding current, but did block CCh+ Atr induced currents (n=4 MSNs). MLA had no effect on either (n=5 MSNs). These findings demonstrate that carbachol induces action potentials in GABAergic neurons by activating β₂-containing nAChRs, which then leads to activation of synaptic and extrasynaptic GABA_A receptors on MSNs.

To eliminate the possibility that carbachol may activate MSNs directly, we recorded using K-gluconate (Kgluc) based internal solution and applied CCh+Atr in the presence BMR to block GABA current, which could mask nAChR mediated current (Fig. 3.3D). In 14 MSNs, we did not detect any change in holding current or occurrence of spontaneous synaptic events, demonstrating a lack of nAChR on MSNs. This allows us to conclude
that GABAergic interneurons, and not MSNs, are the main contributor of GABA in response to CCh+Atr induced nicotinic activation.

Figure 3.4: Cholinergic modulation of tonic and phasic current in interneurons. (A) Representative traces showing the effect of CCh+Atr in the absence and presence of BMR on NPY+PLTS and PV+ interneurones while recording with high [Cl\(^-\)] solution. (B) Bar graph showing the summary of average sustained current in response to CCh+Atr in the presence and the absences of BMR in NPY+PLTS and PV+ interneurones.

Recording from GABAergic interneurons using high [Cl\(^-\)] internal solution revealed that CCh+Atr increased both the holding current and the occurrence of sIPSCs in NPY+ PLTS and PV+ interneurons (Fig. 3.4A&B); these responses were significantly reduced by co-application of BMR. This suggests that activation of presynaptic GABAergic interneurons increases GABA activity in MSNs, NPY+ PLTS, and PV+ interneurons.

3.2.1 Direct nicotinic mediated response in GABAergic interneurons

Having concluded that MSNs are not activated directly by CCh+Atr, we went on to investigate which classes of GABAergic interneurons are activated by CCh+Atr and to characterize the nAChR expressed. CCh+Atr induced inward currents in the majority of
GABAergic interneurons recorded with Kgluc internal solution in the presence of BMR (Fig. 3.5A). Because drug application via y-tubing reach neurons in slices at variable rate, a comparison of CCh+ Atr response kinetics could not be carried out. Regardless, these responses were always characterized by a remarkable increase in current noise in all responding neurons (Fig. 3.5). Greater than 60% of interneurons in each class responded to CCh+Atr (53/72 NPY+ PLTS, 32/33 NPY+ NGF, 19/26 PV+ and 60/99 TH+).

Fig. 3.5A (left) illustrates the average current recorded in responding interneurons from each group. Larger CCh+Atr responses were observed in NPY+ NGF than in TH+, NPY+ PLTS or PV+ interneurons (average values). BMR and the voltage-gated Na+ channel blocker TTX did not prevent the action of cholinergic drugs in any interneuron group tested (Fig. 3.5B, middle). Similar results were obtained in the presence of the AMPA receptor antagonist NBQX (5 µM, not shown). Our results suggest that subtypes of nAChR are expressed in most striatal GABAergic interneurons and are potential contributors of GABA current observed in MSNs, NPY+ PLTS, and PV interneurons.

![Figure 3.5: Characteristics of cholinergic response in GABAergic interneurons.](image)

(A) Representative currents elicited by CCh+Atr co-applied with BMR in NPY+PLTS, NPY+NGF, PV+ and TH+ interneurons. (B) Summary of current responses recorded in striatal interneurons in the absence and presence of BMR, TTX or DHβE as measured by the lower white dashed line in A. * significance to NPY+PLTS, + significance to NPY+NGF, ∧ significance to TH+.
3.3 Nicotinic receptor subtypes in GABAergic interneurons

To examine the subtype of nAChRs, we tested the action of nAChR antagonists (Fig. 3.6A&B). When CCh + Atr were co-applied with the DHβE (10 µM), current responses were abolished in all subtypes of interneurons, with the exception of TH+. CCh + Atr induced responses in TH+ interneurons are not mediated by the α7 subtype either, as MLA (50 nM) did not block the response in 8 TH+ interneurons tested. However, mecamylamine (60 µM), a use dependent nAChR blocker, was able to block CCh + Atr mediated currents (<5% of control) in all interneuron groups, including the TH+. This result suggests that α4β2 nAChRs mediate CCh + Atr induced response in NPY+ PLTS, NPY+ NGF, and PV+ interneurons, but not TH+ interneurons.

Figure 3.6: Antagonism of CCh + Atr responses by DHβE compared between an NPY+NGF (A) and a TH+ interneurone (B). Note that in (B) DHβE failed to block the response in TH+ interneurone that was subsequently blocked by mecamylamine. (C) CCh + Atr elicited channel currents in a patch excised from a TH+ interneurone (left). Co-application with mecamylamine also elicited channel currents (right) in a patch excised from another TH+ interneurone.
To directly demonstrate expression of nAChRs in striatal interneurons, we recorded activation of single nAChRs in response to CCh+Atr using outside-out recording. In 8 TH+ neurons we successfully detected channel currents (Fig. 3.6C). Channel current openings were primarily at a main conductance level that averaged 44 ± pS. The mean open time was quite variable between patches (2 to 6 ms) and averaged 3.5 ± 0.7 ms. The open duration decreased significantly when CCh +Atr was co-applied with mecamylamine (Fig. 3.6C, right). Run-down of channel currents in excised patches prevented proper pharmacological analysis. These results are comparable to previous reports characterizing functional properties of neuronal nAChR channels in interneurons and transfected cells (Ragozzino et al., 1997; Shao & Yakel, 2000), and suggest that both β2 and β4 subunit containing nAChRs can be found in striatal TH+ interneurons. To further investigate this possibility we studied the action of cytisine, a partial agonist of β2 and full agonist of β4 subunit containing nAChRs (Zoli et al., 1998). Using 10 µM cytisine, we detected responses in 3/5 NPY+ PLTS neurons (3.3 ± 0.9 pA) and in 2/4 PV+ neurons (13.2 ± 8.4 pA). Cytisine evoked current was significantly larger in 7/7 TH+ neurons (19.4 ± 6.9 pA) than in NPY+ PLTS or PV+ neurons. We also studied 10 µM cytisine induced currents in 4 D2+ MSN. Cytisine increased sIPSC frequency (216 ± 38%) and amplitude (129 ± 4%) and induced a sustained current of 11.0 ± 1.4 pA. Taken together our results strongly suggest the presence of nAChR in striatal interneurons, but not MSNs.

### 3.4 Effect of nicotinic activation on interneuron activity

In order to examine cholinergic control of striatal interneuron activity, we studied the effect of CCh+Atr using cell-attached recordings, which allows detection of firing activity while preserving intracellular ionic composition. Spontaneously active interneurons such as TH+ and NPY+ PLTS are more likely involved in generating ambient GABA that activates extrasynaptic GABA_A receptors to produce a tonic current. In 5/6 TH+ and 4/9 NPY+ PLTS interneurons, CCh+Atr increased action potential firing in cell
attached. Interestingly, CCh+Atr application decreased firing in 3 NPY+ PLTS and failed to have an effect on one TH+ and one NPY+ PLTS neuron. This variability is likely due to the fact that some interneurons do not respond to CCh+Atr. In these non-responding neurons, spontaneous firing is likely suppressed by GABAergic activation evoked by CCh+Atr application. This suggests that cholinergic activation of GABAergic interneurons will depend on a balance between the presence of nAChR and the extent and strength of GABAergic innervations from other interneurons.

3.4.1 Nicotinic activation promote GABAergic interneuron firing

To better control these variables, we recorded in current clamp with physiological intracellular chloride (Kgluc) solution. These experiments were performed in interneurons at their resting membrane potential (RMP). CCh+Atr application depolarized the membrane potential of NPY+ PLTS and TH+ interneurons and increased their firing frequency (Fig. 3.7A). NPY+ NGF interneurons, normally quiescent due to their more hyperpolarized resting membrane potential, were depolarized to firing threshold by CCh+Atr application (Fig. 3.7A). CCh+Atr promoted action potential firing in most interneurons (Fig. 3.7B), although the extent of depolarization varied (Fig. 3.7C). CCh+Atr application neither depolarized the membrane potential significantly nor induced action firing in PV+ interneurons, which are quiescent under normal conditions. When high [Cl⁻]i solution was used, however, CCh+Atr depolarized PV+ interneurons sufficiently to induce firing (Fig. 3.7A, bottom right). This activity was blocked by BMR (Fig. 3.7A, bottom right and Fig. 3.7B), suggesting that PV+ interneurons receive strong GABAergic input from other interneurons that are activated by CCh+Atr application. This GABAergic input effectively counterbalances any direct excitatory effect CCh+Atr has on PV+ interneuron membrane potential and prevents action potential.
3.5 GABAergic interneurons generate persistent GABA conductance in MSNs

To further investigate the role of GABAergic interneurons in generating persistent GABAergic conductance in MSNs, we recorded from pairs of interneurons and MSNs. (44
MSN-TH+, 9 MSN-PV+, 50 MSN-NPY+ NGF and 15 MSN-NPY+ PLTS) (Fig. 3.8). In all cases, the presynaptic interneuron was recorded with Kgluc internal solution, whereas the postsynaptic MSN was recorded with high [Cl\(^-\)] internal solution. When the presynaptic interneuron was briefly depolarized to 0 mV from a holding potential of -70 mV to evoke neurotransmitter release, IPSCs were recorded in the synaptically connected MSN. Connectivity was observed in all interneuron groups tested (Fig. 3.8B), except NPY+ PLTS interneurons that have been previously reported to have low connectivity with MSNs (Gittis et al., 2010). PV+ interneurons have the highest synaptic connectivity with MSNs and low failure rate, confirming their prominent role in mediating feed-forward inhibition. Both TH+ and PV+ interneurons produced typical fast synaptic IPSCs in MSNs, whereas NPY+ NGF interneurons produced IPSCs characterized by slow rise and slow decay (Fig. 3.8A, top, English et al. (2011); Ibáñez-Sandoval et al. (2011). Fig. 3.8C summarizes the average peak IPSC amplitude measured in interneuron-MSN pairs. Large variability in IPSC size was seen between pairs, and the average values were not significantly different between interneuron-MSN groups. Train stimulation (20 Hz) of presynaptic interneurons produced robust synaptic activity shown as summating IPSCs and increased baseline holding current in MSNs. Fig. 3.8D summarizes the size of the baseline current elicited by stimulation at 20 Hz. Baseline current shift was significantly larger in MSNs that are postsynaptic to NPY+ NGF interneurons because IPSCs exhibit greater summation due to their slow kinetics.

### 3.6 GABAergic interneurons produce variable response in MSNs

When presynaptic interneurons were made to fire a train of action potential in current clamp by injecting positive current, summating IPSCs and increased baseline holding current were observed in synaptically connected MSNs (Fig. 3.9). Notably, at low presynaptic firing rates (<5 Hz), only NPY+ NGF interneurons were able to reliably produce baseline current shift in MSNs (21.6 ± 4.5 pA, n=5). Baseline current shift was
Figure 3.8: Characterization of GABA current in synaptically connected pairs of interneurons and MSNs. (A) (top) Representative traces from synaptically connected pairs of a presynaptic TH+, NPY+ NGF or PV+ interneurone and a postsynaptic MSN. A depolarization step to +40 mV (4 ms in duration) in either a TH+ or a PV+ interneurone induced a typical rapidly decaying IPSC in the MSNs, but in a NPY+ NGF interneurone, it induced an IPSC with slow rise and decay kinetics (bottom). When the same presynaptic interneurones was given a train of 10 depolarizations (4 ms to +40 mV) at 20 Hz, the MSN innervated by an NPY+ NGF interneurone responded with a steady change in current. (B) Bar graph summarizing the percent synaptic connectivity of attempted dual recordings between presynaptic interneurones and post-synaptic MSNs. The peak response from single IPSCs (C) and baseline current changes (D) are summarized.

Occasionally observed in MSNs that are synaptically connected to TH+ interneurons, however, it depended strongly on the strength of connectivity. Under this recording configuration, where the presynaptic interneurons are in current clamp and the postsynaptic MSNs are in voltage clamp, we applied CCh+Atr and compared the currents induced in MSNs. MSNs synaptically coupled to NPY+ NGF or TH+ interneurons (Fig. 3.10, top) displayed increased sIPSCs frequency and holding current upon CCh+Atr application. However, these changes in conductance always occurred before action potential firing.
in the presynaptic interneuron in all pairs tested, regardless of synaptic connectivity, suggesting contributions from other nearby presynaptic interneurons. This is evident in the action of CCh+Atr on a non-connected NPY+ PLTS-MSN pair (Fig. 3.10, bottom), where interneurons other than the NPY+ PLTS were contributing to the GABAergic activity in the MSNs when CCh+Atr was applied. When cells were synaptically connected, a considerable increase in holding current in the MSN in response to CCh+Atr correlated to presynaptic action potential firing in the interneuron (Fig. 3.11A). This is highlighted by the reduction in holding current in MSNs when presynaptic interneurons (NPY+ NGF, Fig. 3.11A and TH+, Fig. 3.11B) were hyperpolarized to terminate firing during the application of CCh+Atr. Taken together, these results show that cholinergic activation of GABAergic interneurons generates a cloud of GABA that evokes sustained current and regulates the excitability of MSNs. However, for an interneuron that is synaptically connected to MSNs, cholinergic activation allows it to directly influence the firing pattern of MSNs.

Figure 3.9: Representative traces of pair recordings between MSNs and distinct interneurons. Presynaptic interneurone action potentials in response to depolarizing current injections elicited GABAergic currents only in synaptically coupled postsynaptic MSNs. Action potential frequency was < 5 Hz in all examples.
Figure 3.10: CCh+Atr elicit currents in MSNs by activating various interneurons. A comparison of GABAergic currents following perfusion of CCh+ Atr in a NPY+ PLTS/MSN (top) and a TH+/MSN pair (bottom). Note the NPY+ PLTS/MSN pair was not synaptically coupled while the TH+/MSN pair was. CCh+Atr elicited currents in postsynaptic MSNs in both situations before presynaptic action potential firing.

3.7 GABAergic dynamics generated by NPY+NGF and PV+ interneurons

Based on our paired recording studies, NPY+ NGF and PV+ interneurons have the highest and strongest synaptic connectivity with MSNs. To further characterize GABA_A receptor mediated response at the synapses between these two classes of interneurons and MSNs, we performed dual whole cell recordings in mice that express fluorescent markers identifying specific class of interneurons (Luo et al., 2013). Action potential in presynaptic interneuron produced IPSCs in synaptically connected MSNs (Fig. 3.12A). To quantitatively characterize the IPSCs generated by both interneurons, we constructed a scatter plot in which the rise of each IPSC was plotted against the decay. This resulted in a significant clustering of the responses generated by each interneuron class (Fig. 3.12B). To quantitatively characterize the change in postsynaptic current in MSNs in response to
Figure 3.11: Action potential firing in presynaptic interneurone contribute to sustained GABA current in MSNs. Presynaptic hyperpolarization cessation of CCh+Atr induced action potential firing decrease postsynaptic response in both a NPY+NGF/MSN (A) and a TH+/MSN pair (B).

Based on various frequency of action potential in presynaptic interneurons (Fig. 3.13A), we plotted the change in holding current ($\delta I_{\text{hold}}$) against increasing frequencies of presynaptic action potential. Although both interneuron classes produce robust IPSCs in MSNs, at the same firing rate, NPY+ NGF interneurons generate a greater shift in $I_{\text{hold}}$ as a direct result of the slower rise and decay kinetics of IPSCs than those produced by PV+ interneurons (Fig. 3.13B).
Figure 3.12: GABAergic dynamics at interneuron-MSN synapses. (A) A depolarization step to +40 mV (4 ms in duration) in a NGF-NPY and FS-PV interneuron induced slow and fast IPSCs, respectively, in synaptically connected MSNs. (B) Scatterplot in log-log scale of the 10-90% rise time versus the weighted decay time for IPSCs in MSNs in response to action potentials in NGF-NPY and FS-PV interneuron. Each point represents one MSN, with average shown as + in red. IPSCs by FS-PV have faster rise and decay kinetics than NGF-NPY interneurons. *p<0.05 unpaired two-tailed Students t test.

3.8 GABA_A receptor subunit at synaptic and extrasynaptic sites

What exactly accounts for the slow kinetics produced by NPY+ NGF interneurons is still unclear. One postulation is the expression of synaptic GABA_A receptors at perisynaptic or extrasynaptic sites that mediate this slow phasic response. In adult mice, tonic GABAergic currents in MSNs are mediated by extrasynaptic receptors containing the α_4 and δ subunit (Santhakumar et al., 2010), whereas in younger mice, this current is mediated by receptors that contain the α_5 and β_3 subunit (Ade et al., 2008; Janssen et al., 2009). These studies have shown that deletion of the δ subunit removes GABAergic tonic current from adult MSNs while deletion of the β_3 subunit removes tonic current from young MSNs. Although α_4 and δ subunit mediated tonic current has been found in younger MSNs, it does not account for the difference in tonic current observed between D1+ and D2+ MSNs Janssen et al. (2009); rather, it is the differential expression of the β_3 subunit that has been suggested to account for the difference.
Figure 3.13: NGF-NPY and FS-PV interneurons produce different GABA response in MSNs. (A) Depolarization steps elicit action potentials in NGF-NPY and FS-PV interneurons that evoke GABAergic current in synaptically connected MSNs. High frequency action potential firing in NGF-NPY interneuron induced a large shift in holding current, while similar frequency in FS-PV interneuron induced only a moderate shift. Holding current (Ihold) as indicated by white dashed lines was assessed after low pass filtering (2-5 Hz). Note that individual IPSCs in the FS-PV paired MSN persist under high frequency. (B) Summary of the average shift in holding current for presynaptic firing frequencies of <10, <20, <30 and >30 Hz.

In D2+ MSNs that have the $\beta_3$ subunit conditionally removed, tonic current is reduced, the remaining of which is likely mediated by $\alpha_4$ and $\delta$ subunit containing receptors. To investigate if deletion of the $\beta_3$ subunit also affects tonic currents mediated by $\alpha_4$ and $\delta$ subunit, we used THIP (gaboxadol), a $\delta$ subunit-containing GABA$_A$ receptor superagonist (Brown et al., 2002). As shown in examples in Fig. 3.14A and the summary data in Fig. 3.14B, response to low doses of gaboxadol (2 $\mu$M) were abolished in $\delta$ but not $\beta_3$ subunit knock out mice. This suggests that functional $\delta$ subunit containing extrasynaptic receptors are formed in the absence of the $\beta_3$ subunit, and that, in contrast to what was previously shown for tonic current in young mice (Janssen et al., 2009), the $\beta_3$ subunit is not an obligatory component of $\delta$ subunit-containing extrasynaptic receptor pool in adult mice (Santhakumar et al., 2010).

Removing the $\beta_3$ subunit of GABA$_A$ receptors from D2+ MSNs reduced whole cell current mediated by the general anesthetic etomidate, a $\beta_3$ subunit preferring modulator
Figure 3.14: The $\beta_3$ subunit does not associate with the $\delta$ subunit in extrasynaptic GABA$\_A$ receptors. (A) Example traces illustrating 2 $\mu$M THIP induced current in control MSNs and D2+ MSNs from $\beta_3^{f/f}$;Rosa$^{tdTom}$ mice and the lack of response in MSN from $\delta^{-/-}$ mice. Summary of the data obtained is shown in (B) together with the summary of currents (I$_{hold}$) elicited by 10 $\mu$M THIP compared between MSN from control and $\delta^{-/-}$ mice. Note that at 10 $\mu$M THIP begins to activate non $\delta$-containing receptors. *$p<0.05$ One-way ANOVA for repeated measures followed by Dunns post hoc test for the 2 $\mu$M THIP cell groups while the Mann-Whitney test was used for the 10 $\mu$M THIP groups.

(Janssen et al., 2011). Here we extend these results by investigating the action of this general anesthetic on sIPSCs recorded from fluorescently identified D2+ MSNs, which have the $\beta_3$ subunit conditionally deleted. We used a concentration of etomidate (0.5 $\mu$M) that does not produce a sustained tonic current (Fig. 3.15A). As shown in the example traces in Fig. 3.15A and the summary data in Fig. 3.15B, etomidate (0.5 $\mu$M) doubled the decay time of sIPSCs in control D2+ MSNs but not in D2+ MSNs, where the $\beta_3$ subunit was removed. In addition, sIPSCs from MSNs in $\delta$ subunit +/- mice were significantly prolonged by etomidate. This suggests that, in young mice, the $\beta_3$ subunit is found in receptors that mediate both synaptic and extrasynaptic receptors.
Figure 3.15: GABA<sub>A</sub> receptor subunits affect response to etomidate. (A) Example traces at a slow time scale (left) illustrating the lack of effect of 0.5 µM etomidate on holding current in D2+ MSNs deriving from control and β<sub>3</sub><sup>fl/fl</sup>;Rosa<sup>tdTom</sup> compared to unidentified MSNs from δ<sup>-/-</sup> mice. On the right are shown superimposed the average normalized sIPSCs with exponential fits and weighted decay time constant in the absence and the presence of etomidate. (B) Summary graph comparing the weighted decay time constant of average sIPSCs measured in MSNs from the three genotypes in the absence and the presence of 0.5 µM etomidate. *p<0.05 paired two-tailed Students t test (control group) or Wilcoxon matched pairs test. The average increase in Tw in δ<sup>-/-</sup> MSNs is less compared to control but the difference is not significant.

3.9 β<sub>3</sub> subunit contributes to slower IPSC decay

Our previous results revealed that in mice that have β<sub>3</sub> subunit conditionally removed in D2+ MSNs, the decay of mIPSCs recorded in MSNs (D1+ and D2+) were significantly faster than in control littermates (Janssen et al., 2011). Although, the identity of D1+ and D2+ MSNs were undetermined, the fast decaying mIPSCs were most likely from D2+
MSNs, which lack the β₃ subunit. To prove this definitively, we recorded from MSNs in newly bred mice where the β₃ subunit is conditionally deleted in D2+ MSNs that also express red fluorescence (drd2-Cre;β₃∕∕;Rosa<sup>tdTom</sup>). Individual mIPSCs recorded in voltage clamp from control and β₃ subunit KO D2+ MSNs (Fig. 3.16A) were averaged and fitted with double exponential decay to show the difference in decay kinetics (Fig. 3.16B). As seen in the summary results (Fig. 3.16C), in D2+ MSNs where the β₃ subunit is removed, mIPSCs decay faster compared to MSNs in control mice and D1+ MSNs in KO mice.

Figure 3.16: Synaptic decay is altered specifically in β₃ -/- MSNs. (A) Representative raw traces of mIPSCs from a control D2+ MSN and a β₃<i>ff</i>;Rosa<sup>tdTom</sup> D2+ MSNs. (B) Average normalized mIPSCs with double exponential fitting illustrating difference in Tw between the two cells shown in A. (C) Box plot summarizing the weighted decay time constant of average mIPSCs measured in MSNs from the three genotypes as indicated. *p<0.05 One-way ANOVA for repeated measures followed by Tukeys post hoc test.
3.10 β3 containing receptors mediate slow IPSC from NPY+NGF interneurons

Hentschke et al. (2009) showed that deletion of the β3 subunit in hippocampal CA1 pyramidal neurons removes slow sIPSCs produced by NPY+ NGF interneurons. Therefore, we speculate that another contributing factor to the fast mIPSC kinetics in D2+ MSNs with deletion of the β3 subunit could be the lack of slow IPSCs mediated by presynaptic NGF+ NPY interneurons, as shown in the examples in Fig. 3.17A. To investigate this, we looked for the occurrence of slow sIPSCs in several D2+ MSNs in control and in β3 subunit deleted mice. Spontaneous slow IPSCs occurred very infrequently (<1 event/5 min), in 12% (6/51) of D2+ MSNs from 10 control mice and 13% (4/31) of MSNs from 5 δ subunit -/- mice, but they were not observed in 58 D2+ MSNs from 13 mice with the β3 subunit deletion. The differences observed were statistically significant (drd2-Cre;β3f/f vs. control p=0.009; drd2-Cre;β3f/f vs. δ-/- 0.013 p=0.013; Fisher’s Exact Test). This is supported by pair recordings from putative NPY+ NGF interneurons and D2+ MSNs that either express (Fig. 3.17B, left) or lack the β3 subunit (Fig. 3.17B, right). NPY+ NGF interneurons were identified in drd2-Cre;β3f/f; Rosa<sup>tdTom</sup> mice by soma size and characteristic firing pattern (fig31B, inset; Ibáñez-Sandoval et al. (2011); Luo et al. (2013)). As illustrated, a burst of action potentials in NPY+ NGF interneurons elicited a large slow IPSC in D2+ MSNs in control mice, but not in D2+ MSN that lack the β3 subunit. Lack of slow IPSC was observed in 4 additional pairs of NPY+ NGF interneuron and D2+ MSN in β3 subunit conditional deletion mice. Since synaptic connectivity between NPY+ NGF interneurons and MSNs is considerably robust in recordings from control mice, the lack of slow IPSCs strongly suggests that β3 subunit is required to mediate slow IPSCs from NPY+ NGF interneurons.

To further investigate this we recorded from D2+ MSNs during local application of extracellular solution containing 12 mM K⁺ or 50 µM 4-aminopyridine to increase synaptic activity. Under these conditions, we observed occasional bursts of action potentials in NPY+ NGF interneurons and large slow IPSCs in D2+ MSNs. Slow IPSCs occurred
in 60% (9/15) of D2+ MSNs from three control mice, 75% (3/4) of MSNs from one δ subunit -/- mouse, but they were observed in only one out of ten D2+ MSNs from two mice with the β3 subunit deletion (p=0.018 & 0.041; Fisher’s Exact Test). Taken together these data suggest that the role of β3 subunits in mediating synaptic input from NGF neurons as seen in the hippocampus is likely to hold for the striatum as well. Given the rare occurrence of slow IPSCs, their presence should not cause significant changes in the average mIPSC kinetics. Thus, other factors may be responsible for the faster mIPSCs in MSNs from mice with β3 subunit conditional deletion.

3.11 Optogenetic activation of striatal neurons

In order to study the properties of GABA current triggered by different striatal neurons, we used optogenetics to activate large populations of specific neurons. ChR2 expression was introduced into D2+, TH+, and PV+ neurons in a Cre recombinase dependent manner and can be detected by fluorescence. In all ChR2 expressing neurons tested, blue
light induced action potential in cell-attached recording, indication that light exposure can activate populations of these neurons simultaneously and noninvasively (Fig. 3.18A). The identity of ChR2 expressing neurons was confirmed by firing pattern in whole cell current clamp (Fig. 3.18B). Both D2+ and TH+ neurons fired for the duration of light exposure. PV+ neurons, however, fired a single or double action potential only at the onset of light exposure and remained silent afterwards. This observation was independent of the field or intensity of light stimulation. We can only speculate as to the cause of this difference in ChR2 mediated activation.

Figure 3.18: Optogenetic activation of striatal neurons. (A) Light stimulation of TH+, D2+ and PV+ neurons induced action potential in cell-attached recording. (B) Response to hyperpolarizing and depolarizing current injection confirmed neurons identified by ChR2-YFP expression.

3.12 Properties of optogenetically induced GABA current in striatal MSNs

After establishing that light exposure evoked action potential in specific ChR2 expressing neuronal populations, we recorded light triggered GABA current in MSNs, because they are the most abundant striatal neurons and receive synaptic input from almost all other classes of striatal neurons. High chloride intracellular solution was used to facilitate
the detection of GABA current by bringing the GABA reversal potential closer to 0 mV. Under this condition, light activation of D2+, TH+ and PV+ neurons all produced inward currents sensitive to the GABA$_A$ receptor antagonist bicuculline (25 µM), confirming their GABA nature (Fig. 3.19). To better characterize the physiological properties of these currents, we gave a short pulse stimulation of 2-5 ms followed by a long stimulation of 1 s. Short pulse stimulation of D2+, TH+ and PV+ neurons evoked, in MSNs, a rapid inward current that decayed rapidly at the offset of light exposure (Fig. 3.20). Long stimulation of D2+ and TH+ neurons evoked sustained GABA current in MSNs for the duration of light exposure (Fig. 3.20). This was not the case with long stimulation of PV+ neurons, where, similar to the short pulse stimulation, it produced a rapid inward current that decayed rapidly regardless of continued light exposure. This brevity in GABA activity correlated well with the firing pattern of PV+ neurons in response to prolonged light exposure (Fig. 3.18A). In this section of the dissertation, phasic current will refer to the current induced by short pulse stimulation of D2+, TH+ and all stimulation of PV+ neurons, whereas sustained current will refer to the current induced by long stimulation.

![Image](image.png)

Figure 3.19: Light stimulation of ChR2+ neurons induces GABA response in MSNs. Pulse stimulation of TH+ neurons expressing ChR2 induced phasic in MSN that was subsequently blocked by BMR (25 µM).

By investigating the pharmacological properties of light evoked GABA current, we can begin to characterize the identity of GABA$_A$ receptor mediated current associated with the activity of different neuronal populations. We used clonazepam (selective for α$_3$), diazepam (selective for γ$_2$), zolpidem (selective for α$_1$), and etomidate (selective for β$_{2/3}$). Application of clonazepam (200 nM), diazepam (5 µM), zolpidem (1 µM), and etomidate (0.5 µM) significantly (n=13, 6, 14, 10 MSNs; p=0.006, 0.031, 0.012, 0.012)
Figure 3.20: Differential GABA response to light exposure. Short pulse stimulation of TH+, D2+ and PV+ neurons induced a phasic response in MSNs. Long stimulation of TH+ and D2+ neurons produced a response that decays but is sustained for the duration of light exposure. Long stimulation of PV+ neuron produced phasic response similar to short pulse stimulation.

prolonged the decay, while diazepam (n=5; p=0.025) also increased the area of phasic current triggered by short pulse stimulation of D2+ neurons, demonstrating that α1-, α3-, β3- and γ2-GABA<sub>A</sub> receptors are activated by D2+ neurons Fig. 3.21. Etomidate significantly prolonged the decay of phasic current (n=3; p=0.007) triggered by short pulse stimulation of TH+ neurons, while no significant changes were observed with the application of all other drugs. This strongly implicates β3-GABA<sub>A</sub> receptors being activated by TH+ neurons. Zolpidem, etomidate, and diazepam (n=6, 3, 6; p=0.027, 0.013, 0.027) all prolonged the decay of phasic current triggered by short pulse stimulation of PV+ neurons, demonstrating expression of postsynaptic α1β3γ2 receptors.
Figure 3.21: Effect of benzodiazepines on GABA current induced by light stimulation of D2+, TH+, and PV+ neurons. Clonazepam, diazepam, zolpidem, and etomidate prolonged the decay of IPSC induced by short pulse stimulation of D2+ neurons. Diazepam also increased the IPSC area. Etomidate increased the decay of IPSC induced by short pulse stimulation of TH+ neurons. Diazepam, zolpidem, and etomidate prolonged the decay of IPSC induced by short pulse stimulation of PV+ neurons. *p < 0.05; one sample t-test.
Chapter 4

Discussion

4.1 Summary

The striatum is a complex network that consists of a majority of projecting MSNs and a minority of GABAergic and cholinergic interneurons. Together, these neurons orchestrate fundamentally important neurological functions by integrating information received through excitatory glutamatergic input and modulatory dopaminergic input to produce desired output. Understanding how striatal neurons function is central to understanding how the striatum functions as a nucleus within the basal ganglia and how basal ganglia dysfunction manifests as neurological disorders. This dissertation details the findings of three separate but related studies that aimed to further our understanding of striatal function.

The antagonistic influence of dopamine and acetylcholine systems within the striatum, evidenced by the use of muscarinic cholinergic antagonists to treat symptoms of PD, has been known for decades. However, the exact mechanisms of how each of these system functions is still unclear. Here, we focused on striatal cholinergic system, which has been shown to influence MSN activity by triggering GABA release. Our results confirm and expand relevant findings by others. We show that out of the four classes of striatal GABAergic interneurons (TH+, PV+, NPY+ NGF, NPY+ PLTS) shown to express nAChRs, TH+ and NPY+ NGF interneurons can be activated by a nicotinic agonist.
to generate action potentials and release GABA that produce strong currents in MSNs. Through paired recordings of synaptically connected interneurons and MSNs, we confirm that, in addition to PV+ interneurons, which form strong synapses on MSNs, NPY+ NGF interneurons also form synapses of comparable strength on MSNs. Of particular interest is the kinetics of IPSCs generated by NPY+ NGF interneurons in MSNs. English et al. (2011) made the initial observation, and here, we shown the same finding that these IPSCs have extremely slow rise and decay kinetics compared to conventional fast IPSCs recorded in MSNs. This finding strongly implicates the expression of different GABA\textsubscript{A} receptor subtypes in MSNs, because the response produced by GABA depends on the subunit composition of its receptors. The two studies which followed focused on deciphering the expression of receptor subunits in MSNs using knockout mice, pharmacology and optogenetics.

Pharmacology and transgene can be used to directly probe for the expression of functional receptors in neurons. Here, we used two transgenic mice: one has the $\beta$3 subunit removed in D2+ neurons, the other has the $\delta$ subunit removed in all neurons, to probe for the expression of $\beta$3 and $\delta$ subunits in synaptic and extrasynaptic receptors in MSNs. We show that $\beta$3 and $\delta$ subunits do not exclusively associate to form extrasynaptic GABA\textsubscript{A} receptors. Furthermore, our findings suggest that $\beta$3 receptors may mediate the slow IPSCs produced by NPY+ NGF interneurons.

Increasingly, optogenetics is being used to study neuronal network due to its various advantages over other methods of activating neurons. Specifically, optogenetics allows simultaneous and noninvasive activation of multiple neurons. Using light stimulation, we began to characterize and identify GABA\textsubscript{A} receptors and the response they produce at different interneuron-MSN synapses. We recorded from striatal neurons while light activating D2+, TH+, and PV+ neurons, which express ChR2. We found that activation of these three populations produced GABA response in MSNs that exhibit different sensitivity to GABA drugs, demonstrating the expression of a combination of GABA\textsubscript{A} receptor subtypes in MSNs that mediate input from different striatal neurons. As a sub-aim of
the main study, optogenetics also allowed us to look at whether these ChR expressing neurons form recurrent GABA project onto themselves.

As described in this dissertation, our findings contribute to the understanding of striatal microcircuit, GABA response, and receptor subtype expression. Although the exact mechanism of each is far from clear, these findings have, however, set a stage for future studies that will continue to contribute to our understanding of the GABAergic regulation of the striatal circuitry.

4.2 Acetylcholine mediate MSN activity through TH+ and NPY+ NGF interneurons

Our study demonstrates that TH+ and NPY+ NGF interneurons are directly activated by nicotinic agonists and are involved in feedforward inhibition of GABAergic interneurons and MSNs. Although PV+ and NPY+ PLTS interneurons are also directly activated by nicotinic agonists, their activity does not lead to increased GABAergic activity in MSNs.

When MSNs are in whole cell voltage clamp, nicotinic agonists generated a downward shift in holding current (sustained current) accompanied by increased amplitude and frequency of sIPSCs. This response was suppressed by both BMR and TTX, indicating it was GABA- and action potential-mediated. Nicotinic receptor stimulation has been reported to elicit TTX-insensitive GABAergic synaptic currents in rat MSNs (Liu et al., 2007). More recently, Nelson et al. (2014) published a study showing that activation of cholinergic interneurons can robustly trigger release of GABA from dopaminergic terminals to induce IPSCs in MSNs in an action potential independent manner. The reason we failed to detect action potential independent responses in our study could be due to the difference in the method of nicotinic receptor stimulation. Optogenetic activation of cholinergic interneurons allows phasic and synchronous release of acetylcholine and subsequent activation of nicotinic receptors that minimizes the desensitization of nicotinic receptors on dopaminergic terminals (Nelson et al., 2014). Such a condition would not likely be achieved by the application of nicotinic agonists through y-tubing. Further
studies should investigate this and provide alternative explanations for the discrepancy between studies.

Our finding that MSNs do not respond directly to nicotinic agonists preclude contribution from MSN collaterals and identify GABAergic interneurons as the primary candidate source of GABA during nicotinic activation. The expression of functional nicotinic ACh receptors is a feature common to interneurons in many brain areas such as the hippocampus in rats (Jones & Yakel, 1997; Lee et al., 2010). Indeed, all four major classes of striatal GABAergic interneurons express nAChRs and are activated directly by nicotinic agonists. However, the degree and consistency of activation varies. In our acute slice preparation, NPY+ NGF interneurons are activated the most, in terms of current flow in voltage clamp, in response to nicotinic agonist. This finding correlates well with a recent report by English et al. (2011) showing that NPY+ NGF interneurons receive synaptic input that triggered action potential following optogenetic activation of cholinergic interneurons. TH+ interneurons are also strongly activated by nicotinic agonists and contribute to the source of GABA during nicotinic activation. Although PV+ interneurons express nAChRs and exert strong synaptic influence over MSN activity, (Koós & Tepper, 2002), our results and those of others (English et al., 2011; Nelson et al., 2014) suggest that they are not readily activated by cholinergic agonists and thus are not involved in cholinergic induced GABA response in MSNs. Because NPY+ PLTS interneurons do not make detectable synaptic connections with MSNs, they are unlikely to be a source of GABA. Taken together, our study supports the role of striatal NPY+ NGF and TH+ interneurons as the primary source of GABA that mediate the inhibitory response in MSNs during nicotinic activation.

4.3 Endogenous tonic current and cholinergic induced sustained current have distinct sources

Tonic GABA current mediated by extrasynaptic receptors was first discovered in embryonic neurons where synapses are absent. This form of communication is different from
synaptic transmission by producing a persistent conductance that can strongly influence the excitability of neurons. Previous studies reported the existence of an endogenous tonic GABA current in MSNs in acute striatal slices (Ade et al., 2008; Janssen et al., 2011; Santhakumar et al., 2010). As endogenous tonic current was significantly decreased and, in some cases, completely blocked by TTX, it is likely generated by spontaneously firing GABAergic interneurons. This is similar to the sustained GABAergic current induced by cholinergic activation in our study, suggesting that the two currents may be mediated by the same set of extrasynaptic GABA$_A$ receptors. However, three pieces of evidence suggest against this possibility. First, both acetylcholine esterase inhibitor and non-specific blocker of nAChR did not alter endogenous tonic current, indicating that either endogenous nicotinic cholinergic activity is not robust in our slices, or endogenous tonic current is not triggered by nicotinic activity. Second, our results show that interneuron subtypes most sensitive to nAChR agonists do not display spontaneous firing in the absence of stimulation, and therefore, are likely not sources of endogenous tonic GABA. Lastly, unlike endogenous tonic current, which is greater in D2 MSNs (due to greater sensitivity to GABA) than in D1 MSNs, CCh+Atr induced current did not differ between the two MSN populations. Thus endogenous tonic and cholinergic agonist induced GABA currents must have different origins and are mediated by different sets of extrasynaptic GABA$_A$ receptors. Specifically, endogenous tonic current must derive from striatal interneurons that exhibit spontaneous activity including subtypes of TH+ and NPY+ PLTS neurons, but are not necessarily involved in cholinergic regulation of striatal output. Finally, we could not eliminate the possibility that the persistent current response we saw during the application of nicotinic agonist likely represent the combined activation of extrasynaptic and synaptic receptors. It’s possible that phasic response from multiple synapses are integrated into a sustained response when they arrive at the soma where the electrical signals are recorded. One should consider, however, our findings that nAChRs powerfully regulate interneuron firing rates to produce additional GABAergic conductance, which may fulfill diverse roles in striatal control of movements.
4.4 **Cholinergic regulation of MSN activity depends on different nAChR subtypes**

Based on our pharmacological investigation, different nAChR subtypes mediate currents induced by carbachol in distinct interneurons. For instance, sensitivity of currents to DHβE in NPY+ and PV+ interneurons implicates the expression of α₄β₂ nAChRs, the predominant subtype in the CNS. In contrast, currents in TH+ interneurons were largely insensitive to DHβE and MLA, but blocked by mecamylamine. This suggests a possible role of the α₃β₄ receptor in these neurons as supported by the sensitivity to cytisine, a preferred agonist at these receptors (Zoli et al., 1998). Importantly, when evaluating the action of nAChR antagonists on cholinergic induced GABA responses in MSNs, we observed considerable heterogeneity. This suggests that MSNs receive GABAergic innervation from different interneuron populations, which may be competing to mediate nicotinic regulation of MSN output. Further experiments are needed to address receptor subtypes using more specific pharmacological tools (when available) and transgenic mice with deletions of specific subunits. Our results do, however, begin to delineate the complexity in striatal interneuron regulation in which various nicotinic receptor subtypes are expressed to modulate striatal network activity.

4.5 **NPY+ NGF and TH+ interneurons fire action potential after nicotinic activation**

Pair recordings of synaptically connected interneurons and MSNs strengthened the role of NPY+ NGF and TH+ interneurons in transmitting cholinergic effect to influence MSN and striatal output activity. Recordings of connected NPY+ NGF-MSN pairs confirmed the observation in English et al. (2011) that NPY+ NGF interneurons form synapses with and generate IPSCs in MSNs with slow rise and decay kinetics. The slow kinetics of these IPSCs facilitate summation allowing NPY+ NGF interneurons firing at low frequencies (<5 Hz) to generate a persistent GABA conductance in MSNs. Such unique
electrophysiological properties enable NPY+ NGF interneurons to exert powerful control over MSN and striatal output activity in response to cholinergic modulation. Recordings of connected TH+-MSN pairs demonstrate that subtypes of TH+ interneurons produce persistent GABA conductance in MSNs, although to a lesser extent than NPY+ NGF interneurons firing at comparable frequencies. Given that TH+ interneurons are directly activated by nicotinic agonist and produce GABAergic response in MSNs, they could potentially, along with dopaminergic terminals, mediate the fast IPSCs described by English et al. (2011). PV+ interneurons, on the other hand, require higher presynaptic firing rate to produce a persistent conductance.

Because GABAergic response to nicotinic activation of presynaptic GABAergic interneurons is similar between D1+ and D2+ MSNs, specific interneurons may preferentially target striatopallidal and striatonigral MSNs (Gittis et al., 2010). Deciphering this preferential targeting may uncover evidence linking the theory of imbalance between dopamine and acetylcholine to that between the direct and indirect pathway.

Optogenetic control of the activity of cholinergic interneuron in vivo and in vitro regulates MSN firing through GABAergic mechanisms (English et al., 2011; Witten et al., 2010). Our results confirm and extend the evidence of nAChR expression to four subtypes of striatal GABAergic interneurons. Nicotinic activation of these interneurons generates tonic and phasic GABAergic conductance in MSNs, which regulate striatal output. Additional optogenetic studies also show that synchronous cholinergic neuron activation can directly gate the release of dopamine and also GABA by activating nAChR on dopaminergic terminals (Cachope et al., 2012; Threlfell et al., 2012). The theory of acetylcholine dopamine imbalance is widely regarded as a fundamental cause in various basal ganglia disorders (Surmeier & Graybiel, 2012). Dehorter et al. (2009) showed that chronic dopamine depletion induced increased occurrence of large amplitude IPSC bursts in MSNs. The Dehorter et al. study suggests that striatal interneurons other than PV+ mediated this effect. It will be important to establish the changes that occur in cholinergic regulation of striatal function with dopamine depletion in animal models of basal ganglia disorders. Studies that have investigated this show that after dopaminergic neurons
are destroyed by toxins, the number of TH+ neurons in the striatum increases (English et al., 2011) and release of GABA from dopaminergic terminals triggered by cholinergic activation is disrupted (Nelson et al., 2014). Taken together, the critical role of ChAT+ interneurons in modulating striatal output reinforces the reciprocal interaction between acetylcholine and dopamine, which may underlie the imbalance theory between the direct and indirect pathways in basal ganglia disorders.

4.6 GABA$_A$ subtype mediates dynamics of GABAergic inhibition

Synaptic, perisynaptic and extrasynaptic GABA$_A$ receptors have distinct roles in mediating key aspects of neuronal microcircuitry that has been extensively characterized in the cerebellum (Farrant & Nusser, 2005) and the thalamus (Jia et al., 2007). These roles include mediating fast feedforward and feedback inhibition, dendritic shunting, regulation of excitability (Semyanov et al., 2004) and generation of oscillatory activity (Cobb et al., 1995; Leresche et al., 2012). Many of these roles mediated by GABA$_A$ receptors have been shown to occur in the striatum, which is largely GABAergic. MSNs, the GABAergic projection neurons form axon collaterals and send output to target areas (Gittis & Kreitzer, 2012). GABAergic interneurons make extensive synaptic connections within the striatum and influence MSN activity through phasic and tonic GABA conductance. Yet, precisely how GABAergic inhibition function within the striatum remain unclear. Our results extend recent important findings on the dynamics of GABAergic inhibition and GABA$_A$ receptors in striatal neurons.

4.7 Subunit expression contributes to variable GABA response

In recent years, the use of transgenic mice to identify subtypes of GABAergic interneurons using fluorescence has lead to a rapid expansion in our understanding of striatal
GABAergic circuitry (Gittis & Kreitzer, 2012; Tepper et al., 2010). Specifically, targeted paired recording between interneuron and MSN has revealed that PV+ and NPY+ NGF interneurons form strong synaptic connections with MSNs (Luo et al., 2013). After quantifying the rise and decay kinetics of IPSCs in MSNs, it’s apparent that PV+ and NPY+ NGF interneurons produce physiologically distinct responses. While both synapse onto MSNs with almost no transmission failure, PV+ interneurons produce typical fast IPSCs, whereas NPY+ NGF interneurons produce slow IPSCs. Both forms of phasic transmission are sufficient to inhibit MSN firing activity (English et al., 2011; Tepper et al., 2004). In order to better understand how different patterns of presynaptic firing effect MSN activity, we quantified GABA current produced in MSNs by PV+ and NPY+ NGF interneurons firing at various frequencies. NPY+ NGF interneurons, but not PV+ interneurons, firing at high frequencies are able to produce massive GABAergic conductance. This difference in IPSC kinetics and integration between PV+ and NPY+ NGF interneurons could be explained by several possibilities. One possibility is the expression of different GABA_A receptor subtypes. Subunit composition determines both the physiological properties and subcellular localization of GABA_A receptors. Our results show that the δ but not the β_3 subunit is required for the effect of extrasynaptic receptor selective agonist. In contrast, the β_3 but not the δ subunit is required for the prolongation of synaptic decay by the general anesthetic etomidate. To study which GABA_A subunit is associated with different GABAergic interneurons, mice that have fluorescently labeled interneuron and MSNs with GABA_A receptor subunit specific deletions are required.

4.8 Presynaptic and postsynaptic factors mediate distinct signaling

Taken together these data allow us to partially model the subunit composition of synaptic and extrasynaptic GABA_A receptors in striatal MSNs of young mice. Specifically, synaptic and not extrasynaptic GABA_A receptor must contain the β_3 subunit. In contrast, the δ subunit is restricted to extrasynaptic GABA_A receptors as expected from current
evidence in other brain areas. Whether $\beta_3$ or $\delta$ subunits are found in perisynaptic receptors is unknown. Current models propose that the subcellular localization of perisynaptic receptors make them ideal mediators of slow phasic response and are thus central to the synaptic action of neurogliaform neurons in the hippocampus (Capogna & Pearce, 2011). Clearly more studies are needed to extend these findings to other brain regions. The data presented here suggest that, as is the case in the hippocampus, the $\beta_3$ subunit is required to mediate the synaptic action of NGF+ interneurons in the striatum, as removal of the subunit significantly reduced the occurrence of spontaneous slow IPSCs (Fisher’s Exact Test). The infrequent finding of MSNs with slow IPSCs suggests that the faster decay of mIPSCs associated with $\beta_3$ subunit deletion in MSNs is not due to diminished activity at NPY+ NGF and MSN synapses, but rather to enhanced expression of $\alpha_1$ subunit-containing synaptic receptors as we previously proposed (Janssen et al., 2011).

In summary, this work emphasizes that in order to better understand GABAergic control of striatal microcircuitry, we need to consider the activation mode and heterogeneity of presynaptic interneurons together with the subtypes of postsynaptic GABA$_A$ receptors.

4.9 Optogenetics shed light on subunit identity

GABA$_A$ subunit expression in the striatum has been probed using a variety of methods, here we report the use of optogenetics to study GABA$_A$ receptors activated by select classes of neuronal populations. Our findings show that $\beta_3$ and $\gamma_2$-containing receptors are activated by D2 neurons; $\beta_3$-containing receptors are activated by TH neurons; $\alpha_1$, $\beta_3$, and $\gamma_2$-containing receptors are activated by PV+ neurons. Taken together, our results suggest the expression of $\alpha_{1/2/3}\beta_3\gamma_2$ and $\alpha_5\beta_3$ receptors on MSNs. $\alpha_{1/2/3}\beta_3\gamma_2$ receptors likely constitute the majority of low affinity receptors found at synaptic sites, whereas $\alpha_5\beta_3$ may be the subtype mediating tonic current or slow phasic GABA current. More studies using subunit specific drugs are needed to further elucidate the GABA$_A$ receptor subtypes expressed in the striatum.
Chapter 5

Conclusion

5.1 Conclusion

1. Pharmacological activation of nicotinic acetylcholine receptors (nAChRs) excites striatal interneurones and induces a GABA mediated current in medium spiny projecting neurones (MSNs) via feedforward inhibition.

2. Striatal interneurones identified as NPY+, PV+ and TH+ have somatic expression of nAChRs.

3. The neurogliaform (NGF) subtype of NPY+ interneurones exhibit the most robust nicotinic response and have high synaptic connectivity with MSNs.

4. Antagonism of nAChR responses suggests the expression of distinct receptor subtypes between interneurone classes.

5. NPY+ NGF and TH+ interneurones mediate cholinergic control of MSNs and thus striatal output via GABA_\text{A} receptors.

6. Pharmacological activation of nAChRs excites GABAergic interneurons and induces a GABA current in MSNs via feedforward inhibition.

7. NPY+, PV+ and TH+ striatal interneurons have somatic expression of nAChRs.
8. The neurogliaform (NGF) subtype of NPY+ interneurons exhibit the most robust nicotinic response and have high synaptic connectivity with MSNs

9. Antagonism of nAChR responses suggests the expression of distinct receptor subtypes among interneuron classes

10. NPY+ NGF and TH+ interneurons mediate cholinergic control of MSNs and thus striatal output via GABA<sub>A</sub> receptors

11. PV+ and NPY+ NGF interneurons form strong synapses on MSNs, but produce response with significantly different kinetics

12. GABA<sub>A</sub> receptor β3 subunit does not associate with δ subunit in extrasynaptic receptors

13. GABA<sub>A</sub> receptor β3 subunit contributes to slow synaptic decay and may form receptors that mediate slow IPSC from NPY+ NGF interneurons

14. Optogenetic stimulation reveal that D2+, TH+ and PV+ neurons all form axon collaterals

15. D2+, TH+ and PV+ neurons activate different GABA<sub>A</sub> receptors after light stimulation
Appendix A

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