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Recurrent inhibitory network among cholinergic interneurons of the striatum

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For my family

and

for Tavaner

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Recurrent inhibitory network among cholinergic interneurons of the striatum

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The striatum is the initial input nuclei of the basal ganglia, and it serves as an integral processing center for action selection and sensorimotor learning. Glutamatergic projections from the cortex and thalamus converge with dense dopaminergic axons from the midbrain to provide the primary inputs to the striatum. Striatal output is then relayed to downstream basal ganglia nuclei by GABAergic medium – sized spiny neurons, which comprise at least 95% of the population of neurons in the striatum. The remaining population of local circuit neurons is dedicated to regulating the activity of spiny projection neurons, and although spiny neurons form a weak lateral inhibitory network among themselves via local axon collaterals, feedforward modulation exerts more powerful control over spiny neuron excitability.

Of the striatal interneurons, only one class is not GABAergic. These neurons are cholinergic and correspond to the tonically active neurons (TANs) recorded *in vivo*, which respond to specific environmental stimuli with a transient depression, or pause, of tonic firing. Striatal cholinergic interneurons account for less than 2 % of the striatal

neuronal population, yet their axons form an extensive and complex network that permeates the entire striatum and significantly shapes striatal output by acting at numerous targets via varied receptor types. Indeed, the persistent level of ambient striatal acetylcholine as well as changes to that basal acetylcholine level underlie the major mechanisms of cholinergic signaling in the striatum, however regulation of this system by the local striatal microcircuitry is not well understood.

This dissertation finds that activation of intrastriatal cholinergic fibers elicits polysynaptic $GABA_A$ inhibitory postsynaptic currents (IPSCs) in cholinergic interneurons recorded in brain slices. Excitation of striatal GABAergic neurons via nicotinic acetylcholine receptors (nAChRs) mediates this polysynaptic inhibition in a manner independent of dopamine. Moreover, activation of a single cholinergic interneuron is capable of eliciting polysynaptic $GABA_A$ IPSCs onto itself and nearby cholinergic interneurons. These findings provide an important insight into the striatal microcircuitry controlling cholinergic neuron excitability.

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Chapter 1. Introduction

1.1 THE BASAL GANGLIA

The basal ganglia is comprised of a group of subcortical, interconnected nuclei involved in the processing of motor, associative, and limbic inputs for the selection, optimization, and execution of voluntary movements. In general, the basal ganglia integrates signals from the cortex, thalamus, and midbrain and then projects to higher cortical areas. The nuclei of the basal ganglia include the striatum, subthalamic nucleus, globus pallidus, substantia nigra, and ventral tegmental area, and although a general input-output pathway exists for signaling through the basal ganglia, reciprocal connections between nuclei and secondary inputs to the basal ganglia add significant complexity to the basal ganglia circuit (Figure 1).

1.1.1 Primary pathway of the basal ganglia circuit

The striatum is the primary input nuclei of the basal ganglia. Indeed, essentially all regions of the cerebral cortex (Parent and Hazrati, 1995) target the striatum and converge with projections from the thalamus (McFarland and Haber, 2000) and the midbrain (Haber et al., 2000). The classical signaling pathway through the basal ganglia begins with striatal projections to the globus pallidus (GP) and substantia nigra (SN). The GP is comprised of an external (GPe) and an internal (GPi) segment while the SN is divided into the pars reticulata (SNr) and pars compacta (SNc). The GPi and SNr are

usually treated as one structure (SNr/ GPi) based on similarities in anatomy and physiology (Francois et al., 1987; Yelnik et al., 1987). Primary striatal output follows two paths. One striatal projection pathway terminates on the SNr/ GPi directly (striatonigral pathway), while a second pathway follows a route to the GPe, STN, and then to the SNr/ GPi (striatopallidal pathway). The SNr/ GPi is the primary output structure of the basal ganglia and sends topographic projections to the thalamus (Sidibe et al., 1997) where they are relayed to the frontal cortex.

1.1.2 Secondary pathway of the basal ganglia circuit

The subthalamic nucleus (STN) also receives significant cortical inputs in a topographic fashion (Nambu et al., 1996). The primary motor cortex, supplementary motor area, and premotor area project to the STN which then projects to the SNR/ GPi and the GPe (Monakow et al., 1978; Nambu et al., 1996; Nambu and Llinas, 1997). Signals from the cortex travel through the STN faster than those through the striatum (Nambu et al., 2000), and signaling through this pathway is thought to prime the SNR/ GPi/ for the arrival of signals through the corticostriatal pathway.

1.1.3 Feedback within the basal ganglia circuit

The complexity of signaling within the basal ganglia network is enhanced by feedback projections from several structures. For example the GPe forms recriprocal connections with the striatum (Bolam et al., 2000), STN (Plenz and Kital, 1999), and the

thalamus (Smith et al., 2004). In a similar respect, the striatum projects back upon the dopaminergic neurons of the SNc (Szabo, 1980; Parent et al., 1983).

1.1.4 Summary

A simplified model of basal ganglia signaling proposes that the basal ganglia provides feedback to the cortex via the thalamus. Cortical inputs are relayed through the basal ganglia via the striatonigral and striatopallidal pathways, and distinct inputs from the thalamus, midbrain, and cortex modulate these signals before they are sent to the thalamus and back to the cortex. Output from the basal ganglia is inhibitory on the thalamus, and enhanced signaling through the striatonigral pathway reduces inhibition of the thalamus by the basal ganglia, while enhanced signaling through the striatopallidal pathway enhances inhibition of the thalamus. It is believed that the imbalance of these pathways underlies symptoms of some movement disorders (Wichmann and DeLong, 1996). For example, a relative increase in activity of the striatopallidal pathway and a reduced activity in the striatonigral pathway results in an enhancement of the inhibition of the thalamus by the basal ganglia. It is possible that this mechanism may contribute to disorders characterized by the reduced ability to initiate movement like Parkinson's disease (Wichmann and DeLong, 2003). The opposite imbalance, enhanced striatonigral and depressed striatopallidal activity, leads to reduced basal ganglia output and disinhibition of the thalamus and cortex. It is possible that this mechanism may contribute to the hyperkinesias typical of hemibalisimus and Huntingon's disease (Pisani et al., 2007).

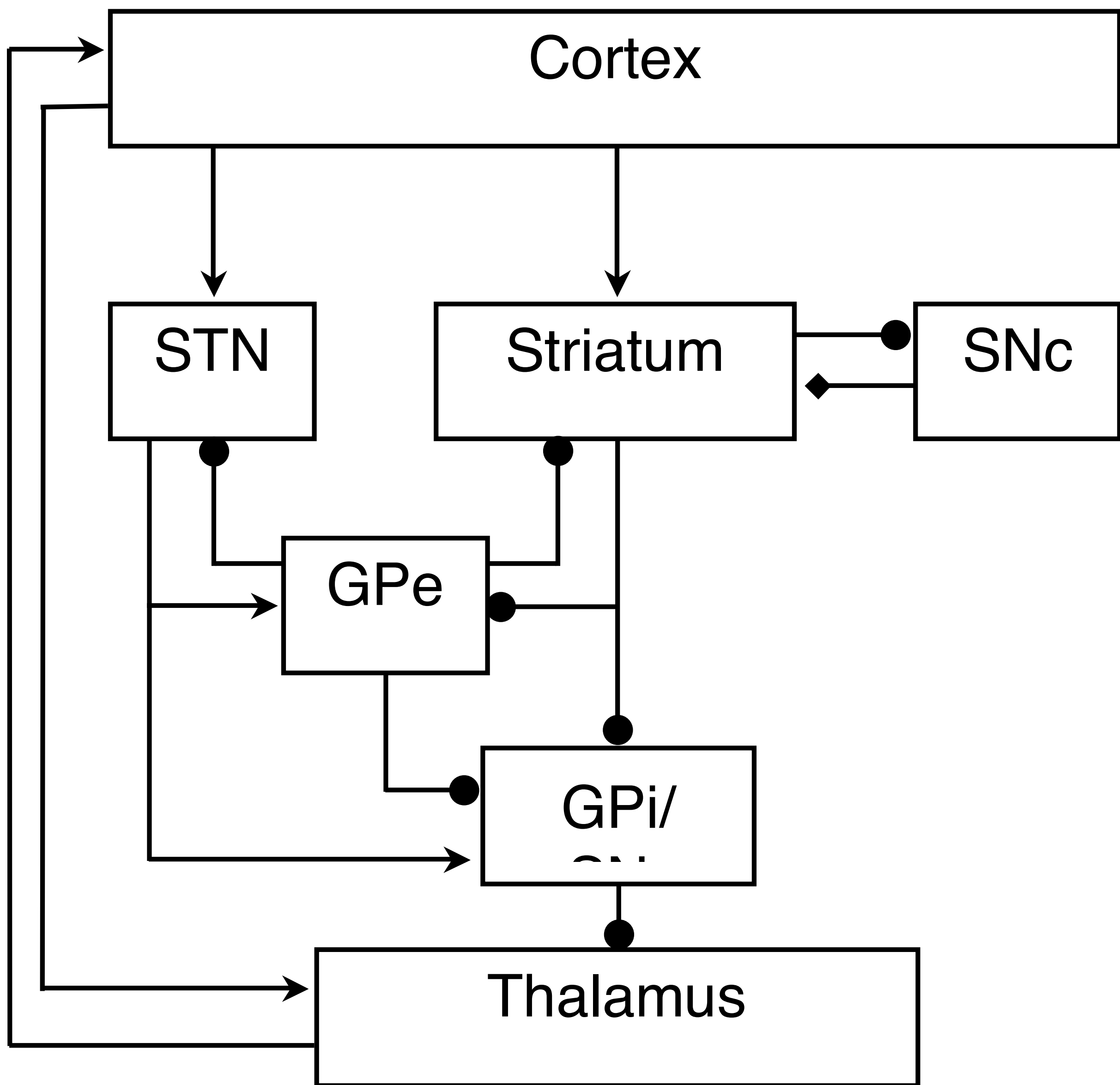


Figure 1. The basal ganglia circuit

Simplified diagram of connectivity within the basal ganglia. The ventral striatum has been excluded for simplicity. Arrowheads signify excitatory connections, circles signify inhibitory connections, and diamonds represent dopamine release sites Subthalamic nucleus (STN), substantia nigra pars compacta (SNc), external segment of the globus pallidus (GPe), internal segment of the globus pallidus (GPi), substantia nigra pars reticulata (SNr).

1.2 THE STRIATUM

The striatum is the primary point of entry for signals directed to the basal ganglia and is divided into two gross compartments containing several nuclei each. The nucleus accumbens and part of the olfactory tubercle make up the ventral striatum, while the caudate nucleus and putamen comprise the dorsal striatum (Packard and Knowlton, 2002). Each nucleus receives differential primary inputs. In a general sense, cortical neurons project to different regions of the striatum and divide the striatum into functional areas that receive sensorimotor, associative, and limbic inputs (Bar-Gad et al., 2003; Tisch et al., 2004). The primary motor cortex, somatosensory cortex, premotor cortex, and supplementary motor area target the dorsolateral striatum (putamen) and comprise the sensorimotor inputs (Takada et al., 1998). The associative inputs target more ventromedial areas of the dorsal striatum (rostral putamen and caudate nucleus) and are made up of projections from the prefrontal and frontal areas as well as temporal, inferior parietal, preoccipital, and parahippocampal areas (Selemon and Goldman-Rakic, 1985). Finally, inputs from the limbic cortex, paralimbic cortex, amygdala, and hippocampus delineate the limbic region located in the most ventral areas of the striatum (ventral putamen, nucleus accumbens, and the olfactory bulb). Thus, all corticostriatal inputs are generally organized topographically (Veening et al., 1980; McGeorge and Faull, 1989).

The striatum also receives projections from the thalamus. The putamen mainly receives input from the centre-median/ parafasicular nucleus complex (CM-PF) of the thalamus (Sadikot et al., 1992; Parent and Hazrati, 1995; McFarland and Haber, 2000), while the caudate nucleus and ventral striatum receive input from the PF and ventral motor nuclei of the thalamus, respectively (Sadikot et al., 1992; Sidibe et al., 1997).

Dopaminergic neurons from the midbrain also target different areas of the striatum. The substantia nigra pars compacta primarily projects to the dorsal striatum, while neurons of the ventral tegmental area predominantly send axons to the ventral striatum (Gerfen et al., 1987).

In addition to ventral and dorsal separation, the striatum can be divided into functional areas defined by the spatial distribution and axonal projections of the primary cell type of the striatum, the medium spiny neuron (MSN) (Packard and Knowlton, 2002). The differential staining of peptides and neurochemicals expressed by MSNs reveals a mosaic of distinct "patches" of similarly labeled neurons separated by an interconnected "matrix" of distinct MSNs. Patches were first described as areas with enriched μ opiate receptor protein (Pert et al., 1976), and it was later shown that acetylcholinesterase was also more prevalent in patches (Graybiel and Ragsdale, 1978; Herkenham and Pert, 1981). In contrast, somatostatin and calbindin-immunoreactive neurons were predominantly found in matrix areas (Gerfen, 1984, 1985; Chesselet and Graybiel, 1986). The inputs to patch and matrix areas differ as well. DA neurons of the VTA mainly project to the matrix areas of the ventral striatum, while SNc neurons project to both the patch and matrix. (Gerfen et al., 1987; Jimenez-Castellanos and Graybiel, 1987). In addition, patch cells preferentially receive input from the basolateral nucleus of the amygdala (Ragsdale and Graybiel, 1988) and prelimbic cortices while matrix cells primarily receive projections from the motor and somatosensory areas of the cortex (Donoghue and Herkenham, 1986). A relationship between the laminar organization of the cortex and the patch-matrix segregation of the striatum has also been shown. Deep cortical layer V and layer VI send axons to the patch compartment, whereas the supragranular layers of cortex target the striatal matrix areas (Gerfen, 1989). However, despite such findings the functional relevance of the mosaic remains poorly

defined due to inconsistent patch and matrix patterns and inadequate localization of markers to specific structures. Therefore, a more practical compartmental organization of the striatum relies upon the differential axonal projections of MSNs.

Although MSNs exist homogenously throughout the striatum, they project to the SNr/ GPi via two distinct routes. MSNs of the "direct pathway" send axons directly to the GPi/ SNr (striatonigral projection), while MSNs of the "indirect pathway" first innervate the GPe (striatopallidal projection). The GPe then projects to the STN and SNr/ GPi (Figure 1).

MSNs are GABAergic and are quiescent until activated by glutamatergic signals from the cortex and thalamus (Wilson, 1994). Striatal targets are GABAergic as well, but are continuously firing spontaneous action potentials (Grace and Bunney, 1984; Cooper and Stanford, 2000; Bevan et al., 2002). Therefore, signaling via the striatonigral pathway inhibits spontaneously active GABAergic projection neurons of the SNr/GPi resulting in disinhibition of the thalamus and transient activation of thalamic targets. In contrast, transient signals from the striatum to the GPe via the striatopallidal pathway inhibit spontaneously active GABAergic projection neurons of the GPe causing disinhibition of the STN. The SNr/GPi receives excitatory glutamatergic signals from the STN. So the relief of tonic inhibition of the STN by signaling from striatopallidal MSNs results in phasic excitation of the SNr/GPi and enhanced inhibition of the thalamus. In general, increased activity of the striatonigral pathway correlates with facilitation of movement, while increased activity of the striatopallidal pathway is associated with inhibition of movement (Bar-Gad et al., 2003).

1.2.1 Primary neurons of the striatum

MSNs account for the majority (≥ 95%) of the neuronal population of the striatum (Chang et al., 1982; Chang and Kitai, 1985; Graveland and DiFiglia, 1985; Rymar et al., 2004). These neurons have a medium sized soma and radiate 25 – 30 dendritic branches that are covered with spines (DiFiglia et al., 1976; Wilson and Groves, 1980). Striatonigral and striatopallidal MSNs are morphologically indistinguishable but vary in their expression of neuropeptides and dopamine receptor subtypes. Striatonigral neurons generally express dynorphin, substance P, and contain dopamine D1 receptors, whereas striatopallidal neurons generally express enkephalin and the D2 dopamine receptor subtype (Surmeier et al., 1996; Wang et al., 2006) .

Labelling of MSNs indicates that while the majority project to downstream brain areas, MSNs also signal within the striatum via extensive local axon collaterals (Bolam et al., 2000). Other MSNs are the primary targets of these intrastriatal spiny neuron collaterals (Pickel et al., 1980; Aronin et al., 1981; DiFiglia et al., 1982; Somogyi et al., 1982; Bolam et al., 1983a; Bouyer et al., 1984; Bolam and Izzo, 1988; Pickel et al., 1992), and all MSNs are GABAergic and inhibitory upon targets inside and outside the striatum (Kawaguchi, 1997).

As noted previously, the glutamatergic fibers from the cortex and thalamus converge within the striatum, and both corticostriatal and thalamostriatal inputs target MSNs. However, several notable differences exist between the two projections indicating differential effects of signaling through these pathways. First, terminals from corticostriatal fibers form synapses primarily with dendritic spines (Bolam et al., 2000), while the thalamic terminals predominantly form asymmetric synapses on dendritic shafts of MSNs (Smith and Bolam, 1990; Sadikot et al., 1992; Smith et al., 1994; Sidibe and Smith, 1996). Although thalamostriatal terminals may form a minority of synapses upon spines as well (Xu et al., 1991). Second, anatomical studies indicate thalamostriatal

fibers selectively target striatonigral MSNs and corticostriatal fibers target striatopallidal MSNs (Sidibe and Smith, 1996; Berretta et al., 1997; Parthasarathy and Graybiel, 1997), however synaptic stimulation of striatal afferents has failed to discriminate between striatonigral and striatopallidal populations (Ding et al., 2008). Finally, paired pulse experiments combined with manipulations of extracellular calcium indicate a high basal probability of quantal transmitter release at thalamostriatal synapses upon MSNs and a low probability of release by corticostriatal terminals (Ding et al., 2008). Differences in probability of transmitter release did not differ between striatonigral and striatopallidal MSNs.

MSNs typically rest at around the K^+ equilibrium potential of -80 mV (downstate) but exhibit plateau – like shifts to a depolarized state near -60 mV (upstate) in response substantial excitatory inputs from the cortex or thalamus (Wilson and Kawaguchi, 1996; Plenz and Kitai, 1998). The membrane potential of MSNs is controlled by the interplay of constitutively active K^+ channels, ionotropic receptors, metabotropic receptor effects, and voltage gated cation channels. Indeed upstate transitions follow AMPA and NMDA receptor activation, and are characterized by the closure or inactivation of several K^+ channels (Kir2, Kv1, and Kv4), reduced SK channel opening (Wickens and Wilson, 1998), and enhanced L-type Ca^{2+} (Cav 1.2 and Cav 1.3) channel currents (Vergara et al., 2003). MSNs typically do not fire action potentials while in the downstate, and it appears that transitions to the upstate potential are necessary for action potential generation (Wilson and Groves, 1981; Wilson and Kawaguchi, 1996; Wickens and Wilson, 1998). Thus, upstates directly modulate basal ganglia output.

1.2.2 Interneurons of the striatum

Striatal interneurons comprise less than 3% of the rat striatum (Rymar et al., 2004) and can either act as feedforward modulators of MSN excitability (Tepper and Bolam, 2004; Tepper et al., 2004) or as independent sources of input to other striatal neurons (Kawaguchi, 1993; Bennett et al., 2000). Four interneuron populations have been identified based on differential morphology, neurochemistry, and physiology (Kawaguchi et al., 1995). Three of the neuron types are GABAergic while the fourth type is cholinergic.

Although axon collaterals from MSNs form an inhibitory network within the striatum, synaptic connections between MSNs are weak and located on distal dendrites (Wilson and Groves, 1980; Bolam et al., 1983b; Jaeger et al., 1994; Tepper et al., 2004). On the other hand, accumulating evidence indicates that striatal interneurons are powerful regulators of MSN activity. For instance, simultaneous electrophysiological recordings between connected neurons indicate that two types of GABAergic interneurons exert strong inhibitory control over MSNs (Koos and Tepper, 2002; Tepper and Bolam, 2004), and the effectiveness of acetylcholine receptor antagonists for the treatment of Parkinson's disease suggests powerful modulation of MSN activity by the striatal cholinergic interneurons (Katzenschlager et al., 2003).

1.2.2.1 Fast spiking interneurons

Fast spiking interneurons are the best characterized of the striatal interneurons (Tepper and Bolam, 2004), and received their name based on their ability to maintain 200 – 300 Hz firing with little or no spike frequency adaptation. The resting membrane potential of FSIs ranges from -70 to -80 mV with input resistance near 100 MΩ (Koos and Tepper, 1999, 2002). Early neurochemical studies identified a small population of

GABAergic neurons within the striatum that displayed more intense staining for glutamic acid decarboxylase (GAD) than did MSNs (Ribak et al., 1979; Bolam et al., 1985; Bennett and Bolam, 1994), and it was later reported that these neurons co-expressed the calcium binding protein parvalbumin (Gerfen, 1984; Kawaguchi, 1993). These neurons account for less than 1% of the neuronal population within the rat striatum (Larsson et al., 2001; Luk and Sadikot, 2001; Rymar et al., 2004), but their complex axonal branches ensure effective connectivity with MSNs and other FSIs (Kawaguchi, 1993; Kita, 1993; Bennett and Bolam, 1994; Koos and Tepper, 1999; Kubota and Kawaguchi, 2000). FSIs also display a dendritic field ranging up to 600 μm that receives input from the cortex, striatal cholinergic interneurons, other FSIs, and from the GPi (Figure 2) (Chang and Kita, 1992; Lapper et al., 1992; Bevan et al., 1998; Ramanathan et al., 2002). Furthermore, FSIs have gap junctions (Kita et al., 1990), and recordings between pairs of FSIs indicate a level of electrotonic coupling between neurons (Koos and Tepper, 1999).

FSIs exert powerful feedforward inhibition upon MSNs. FSI dendrites mostly form perisomatic synapses with MSNs (Kita et al., 1990; Kita, 1993; Bennett and Bolam, 1994), and estimates based on quantal analysis of synaptic potentials suggest that a single FSI forms multiple ($N \geq 7$) synapses with each MSN (Koos et al., 2004; Tepper et al., 2004). Additionally, single action potentials in FSIs rarely fail to evoke a response and produce relatively high conductance (~ 3 nS) IPSCs in connected MSNs (Koos and Tepper, 2002). The number, location, and strength of FSI synapses on MSNs allows single action potentials in FSIs to delay or prevent spiking in MSNs, and the summation of IPSPs resulting from multiple action potentials with short inter - spike intervals enables pronounced inhibition (Koos and Tepper, 1999; Koos et al., 2004). Importantly, FSIs and MSNs receive excitatory glutamatergic input from the same areas of cortex (Lapper et al., 1992).

1.2.2.2 Persistent low threshold spiking interneurons

A second class of striatal interneuron was identified based on selective histochemical staining for NADPH diaphorase/ nitric oxide synthase (NOS), neuropeptide Y, and somatostatin (Vincent et al., 1983; Dawson et al., 1991; Kawaguchi, 1993). Subsequent NADPH labeling of biocytin filled neurons characterized these cells as persistent low threshold spiking (PLTS) interneurons based on the consistent presence of low threshold calcium spikes riding on a persistent depolarization following return from hyperpolarization (Kawaguchi, 1993). Both dual – labeling for GAD67 (Vuillet et al., 1990; Kubota et al., 1993) and attempts to detect GAD mRNA (Chesselet and Robbins, 1989; Catania et al., 1995) failed to conclusively illustrate the GABAergic nature of PLTS interneurons (Kawaguchi et al., 1995), however GABAergic boutons were found on PLTS axons using a colloidal immunogold labeling technique (Kubota and Kawaguchi, 2000). In addition, positive staining of PLTS interneurons for NOS combined with reports that nitric oxide (NO) excites at least one other striatal neuron suggests that PLTS cells also release NO as a neurotransmitter (Kawaguchi, 1993; Centonze et al., 2001). Therefore, PLTS interneurons join FSIs as powerful regulators of striatal activity.

Indeed, despite a population that comprises less that 1% of the total neuronal population of the striatum (West et al., 1996; Rymar et al., 2004), PLTS interneurons extend dense axonal and dendritic processes (up 1 mm in rats) enabling PLTS output to affect a large number of cells over a relatively vast area (Kubota and Kawaguchi, 2000). Ultrastructural evidence indicates that PLTS interneurons receive direct cortical (Vuillet et al., 1989; Thomas et al., 2000) and thalamic (Sidibe and Smith, 1999) innervation and

project to MSNs (Koos and Tepper, 1999) as well as cholinergic interneurons (Vuillet et al., 1992) but not FSIs (Figure 2) (Morello et al., 1997). Whole cell recordings revealed that PLTS interneurons rest close to -60 mV with relatively high input resistances of 500 MΩ - 1.5 GΩ (Kawaguchi, 1993; Kubota and Kawaguchi, 2000; Centonze et al., 2002), allowing for the possibility that small excitatory conductances may evoke action potentials in these cells. Simultaneous recordings between connected PLTS – MSN pairs show that PLTS – evoked postsynaptic responses in MSNs are similar to FSI – evoked responses (Tepper and Bolam, 2004). Like FSIs, summation of short interval inhibitory responses may significantly delay MSN firing. Moreover, although not yet shown, it may be possible for a hyperpolarizing inhibitory input to cause transmitter release via low threshold spiking from PLTS neurons. In this manner, PLTS interneurons may act to facilitate inhibition between neurons. For example, PLTS interneurons may translate typically slower hyperpolarizing metabotropic responses into faster GABAergic inhibitory signals. Therefore, the morphological and electrophysiological properties of PLTS interneurons enable a sparse number of cells to translate weak excitatory or inhibitory synaptic inputs into strong inhibition of a large number of distant neurons.

1.2.2.3 Calretinin – immunoreactive interneurons

The third known type of GABAergic interneuron in the striatum differentially expresses the calcium binding protein calretinin (Jacobowitz and Winsky, 1991; Resibois and Rogers, 1992; Bennett and Bolam, 1993). Early observations identified that catretinin - positive interneurons label positively for GABA and GAD, thus suggesting that these interneurons are GABAergic (Kubota et al., 1993; Kawaguchi et al., 1995). Calretinin – positive interneurons are similar in size to MSNs and FSIs, and like FSIs and

PLTS neurons they comprise less that 1% of the neuronal population of the striatum (Rymar et al 2004). These interneurons are distributed throughout the striatum, including the patch and matrix compartments (Rymar et al., 2004), however a greater density of calretinin – immunoreactive cells is found towards the rostral end of the striatum. Ultrastructural studies suggest that calretinin - positive interneurons form connections with MSNs as a significant number of calretinin - immunoreactive terminals formed asymmetrical synapses on spines, however symmetrical connections were observed as well (Bennett and Bolam, 1993).

The electrophysiological properties of striatal calretinin - immunoreactive interneurons remain unknown, however Koos and Tepper presented evidence of a novel striatal neuronal behavior from an unlabeled cell type. This cell fired low – threshold spikes similar to PLTS interneurons, but failed to maintain a persistent depolarization following membrane hyperpolarization. Simultaneous recordings from this unknown cell type connected to MSNs showed that these neurons can delay or block spiking of MSNs much like PLTS interneurons and FSIs (Koos and Tepper, 1999). Although pure speculation, it is possible that the behavior of the unlabeled cell type described by Koos and Tepper will later be found in caltretinin – immunoreactive interneurons.

1.2.2.4 Cholinergic Interneurons

Cholinergic interneurons make up a small percentage of the striatal neuronal population (< 1% in rats; Larsson et al., 2001; Rymar et al., 2004), yet they have dense, widespread axonal arbors that provide a rich source of ACh within the striatum (Oorschot, 1996). Indeed, striatal acetylcholine release appears to be integral for regular movement control and dysregulation of this system plays a prominent role in Parkinsons

disease (PD; Fahn et al., 1990; Kaneko et al., 2000; Salamone et al., 2001; Zhou et al., 2002). Within the striatum, acetylcholine acts as both a classical neurotransmitter and as a modulator of signaling and cell activity, thereby providing a multifaceted system for control over striatal output.

Cholinergic interneurons fire action potentials spontaneously at 1-10Hz *in vitro* (Kawaguchi, 1993; Bennett and Wilson, 1999). This firing is driven autonomously by intrinsic conductances and is modulated by interactions between these conductances and synaptic inputs to these neurons (Bennett and Wilson, 1998). Cholinergic interneurons receive glutamatergic inputs from the cortex and thalamus (Lapper and Bolam, 1992; Thomas et al., 2000; Reynolds et al., 2004) and dopaminergic inputs from the midbrain (Chang, 1988) in addition to local cholinergic and GABAergic inputs from other striatal interneurons (Tepper and Bolam, 2004). Indeed, clear ultrastructural evidence indicates that PLTS interneurons and MSNs form synapses with cholinergic interneurons (Vuillet et al., 1992), however studies have failed to illustrate connectivity from FSI axons onto cholinergic interneurons (Chang and Kita, 1992). In turn, ultrastructural evidence shows putative cholinergic axon terminals forming synapses with MSNs, FSIs, PLTS interneurons and other cholinergic axons (Figure 2), however these connections are rare as it has been estimated that only 9% of observed cholinergic terminals form synapses (Chang and Kita, 1992; Contant et al., 1996; Descarries et al., 1997). These rare cholinergic synapses are primarily located on distal dendritic shafts, spine necks, and axon boutons (Bolam et al., 1984; Phelps et al., 1985; Descarries et al., 1997).

Cholinergic signaling may occur via direct synaptic transmission but it is believed that a greater number of targets is reached by extrasynaptic or intersynaptic transmission of ACh (Contant et al., 1996; Descarries and Mechawar, 2000; Koos and Tepper, 2002). It is estimated that removal of synaptic ACh by acetylcholinesterase occurs rapidly (~ 1

ms; Dani et al., 2001), however repetitive firing of cholinergic interneurons and the probable diffusion of ACh from synaptic clefts (Barbour and Hausser, 1997) results in a basal ambient ACh concentration in the striatum (Descarries et al., 1997).

The most suggestive evidence concerning the function of cholinergic signaling within the striatum has come from *in vivo* electrophysiological recordings of putative cholinergic interneurons. Microelectrode studies in live animals grouped recorded neurons based on differences in basal action potential firing patterns (Crutcher and DeLong, 1984; Kimura et al., 1984; Alexander and DeLong, 1985b). 'Phasically active neurons' (PANs) exhibited relatively extended silences interrupted by short – duration groups of action potentials, and this group includes MSNs (Kimura et al., 1990; Wilson, 1993). In contrast, a population of 'tonically active neurons' (TANs) were observed to fire continuously at 3 – 12 Hz and are now believed to correspond to the cholinergic interneurons of the striatum (Wilson et al., 1990; Aosaki et al., 1994b; Reynolds et al., 2004).

TANs and PANs exhibit differential activity in response to sensory stimuli, during learning, and during conditioned movement (Apicella, 2007) Specifically, PANs are normally quiescent but fire action potentials preceding or following the execution of a conditioned movement by a trained animal (Alexander and DeLong, 1985a; Zhou et al., 2002). TANs, however, exhibit a transient depression, or pause, of firing in response to the presentation of a cue that an animal has learned will predict a rewarding or aversive stimuli (Kimura et al., 1984; Aosaki et al., 1995; Ravel et al., 1999) and to the stimuli itself (Graybiel et al., 1994; Apicella et al., 1997). In this respect, TANs are thought to be involved in the detection of stimuli that have motivational significance (Apicella, 2007).

However, the link between the TAN pause and learning is not simply a stimulus – induced response. Early studies illustrated that the pause response develops over time as the animal learns (Aosaki et al., 1994a), and that the number of responsive TANs increases as well. In addition, the pause response extinguishes alongside the conditioned behavior in the absence of reward (Aosaki et al., 1994b), and the population behavior of TANs accurately predicts the probability that an animal will initiate movement in response to a conditioned stimulus (Blazquez et al., 2002). These findings support the hypothesis that modulation of TAN firing is integral to processing, not just reporting, information regarding salient environmental stimuli as it relates to motor output.

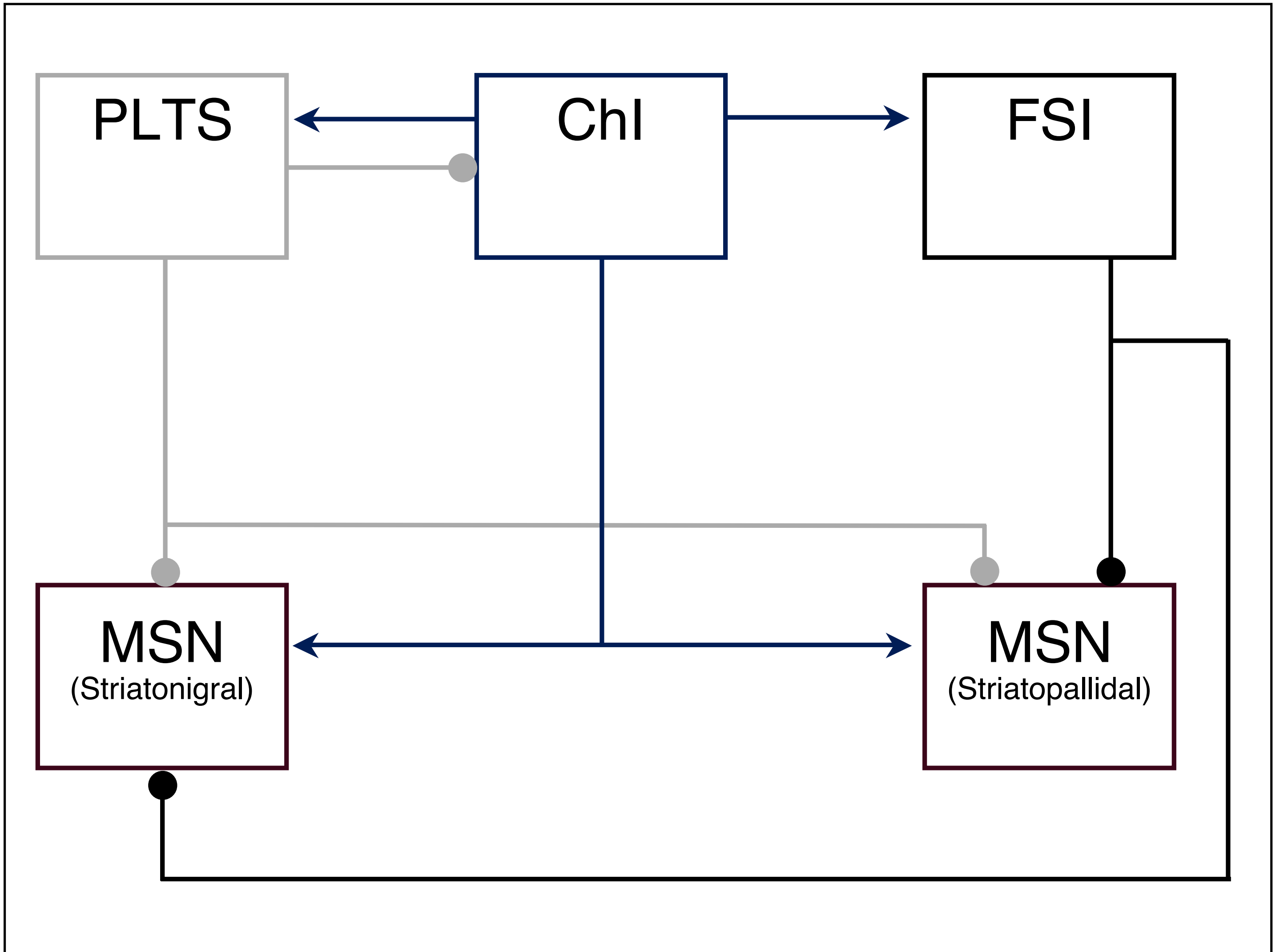


Figure 2. Targets of interneurons within the striatum

Schematic diagram illustrating intrastriatal neuronal connections. Axons of MSNs and axo – axonic connections have been left out for simplicity. Arrows signify an excitatory synapse, and circles signify an inhibitory synapse. Persistent low threshold interneuron (PLTS), cholinergic interneuron (ChI), fast spiking interneuron (FSI), medium spiny neuron (MSN).

1.3 ACETYLCHOLINE IN THE STRIATUM

Within the striatum, the results of cholinergic signaling are primarily mediated by muscarinic receptors, however nicotinic receptors play an important role in the shaping of striatal output as well. Muscarinic acetylcholine receptors (mAChRs) are metabotropic and have seven transmembrane domains. Molecular cloning has identified five distinct receptor isoforms ($M_1 – M_5$) that are grouped into M_1 – like mAChRs (M_1, M_3, M_5) and M_2 – like mAChRs (M_2, M_4) (Wess, 1996; Caulfield and Birdsall, 1998). M_1 – like mAChRs are G_q – coupled and result in the mobilization of intracellular calcium through activation of phospholipases. M_2 – like mAChRs couple to $G_{i/o}$ proteins that inhibit adenylyl cyclase to reduce cyclic – AMP formation and also inhibit calcium channels. In the striatum, expression of M_1 and M_4 is dominant over M_2, M_3, and M_5 mostly because M_1 and M_4 mAChRs are found on MSNs (Weiner et al., 1990; Levey et al., 1991; Gomeza et al., 1999; Pisani et al., 2007).

Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors made up of five subunits that interact to create a central hydrophilic pore (Role and Berg, 1996; Albuquerque et al., 1997; Dani, 2001; Dani et al., 2001). Neuronal nAChRs are comprised either of a combination of α ($\alpha2 – \alpha10$) and β ($\beta2 – \beta4$) subunits or of just α subunits, and the combination of different subunits confers distinct functional and structural properties to the receptor. The majority of nAChRs in the striatum are $\alpha2\beta4$ – containing and $\alpha7$ – containing receptors (Wada et al., 1989; Seguela et al., 1993; Colquhoun and Patrick, 1997) and are mainly located on axonal terminals and modulate transmitter release (Dani and Bertrand, 2007). nAChR channels are permeable only to monovalent and divalent cations, and many important roles of nAChRs are due to Ca^{2+}

entry through these channels (Dani and Bertrand, 2007). Indeed signaling through nAChRs can result in Ca^{2+} - induced Ca^{2+} release (Sharma and Vijayaraghavan, 2003), modulation of neurotransmitter exocytosis (Tredway et al., 1999), and possibly contribute to the induction of synaptic plasticity (Mansvelder and McGehee, 2000).

1.3.1 ACh signaling on medium spiny neurons

MSNs predominantly express M_1 and M_4 mAChRs (Weiner et al., 1990; Levey et al., 1991; Bernard et al., 1992; Ince et al., 1997), and M_4 receptor mRNA is significantly more abundant on striatonigral MSNs compared to striatopallidal MSNs (Yan et al., 2001). While the ultimate effects of M_4 activation upon MSN activity are poorly characterized due to inadequate pharmacological isolation, it is well understood that M_1R activation depolarizes MSNs by suppressing standing K^+ currents (Hsu et al., 1996). Acetylcholine binding to M_1Rs hydrolyzes membrane – bound phosphotidyl inositol (PI) into cytoplasmic diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 then signals to mobilize Ca^{2+} from intracellular stores, and this Ca^+ acts with DAG to activate protein kinase C (PKC). M1 – dependent PI hydrolysis ultimately leads to suppression of K^+ currents through KCNQ (M – channel) and Kir2 channels (Galarraga et al., 1999; Shen et al., 2005). Notably, M_1R activation potently reduces Kir2 currents in striatopallidal MSNs, while only weakly reducing currents through the same channels in striatonigral MSNs (Shen et al., 2007). The result is that tonic muscarinic activation provided by continuous striatal cholinergic interneuron firing persistently enhances the excitability of MSNs by reducing Kir2 channel currents, which are active at resting potentials and tend to hold the MSNs in the down – state near the K^+ equilibrium potential.

M_1R activation also suppresses voltage – activated Ca^{2+} channels on MSNs. It was first shown that muscarine reduces the duration of Ca^{2+} - dependent plateau potentials (Misgeld et al., 1986), and later studies illustrated that specific Ca^{2+} channels were inhibited by M_1R activation of distinct signaling pathways. M_1Rs inhibit N – and P/Q – type Ca^{2+} channels by activating a pertussis toxin – sensitive G protein (Howe and Surmeier, 1995). Ca^{2+} entry through these Ca^{2+} channels is important for Ca^{2+} - dependent K^+ channel activation during action potential after – hyperpolarizations (Vilchis et al., 2000), and inhibition of N – and P/Q – type Ca^{2+} channels by M_1R activation reduces AHP length to shorten the interval between MSN action potentials (Perez-Rosello et al., 2005). Additionally, M_1R activation of a pertussis toxin – insensitive G protein inhibits L – type Ca^{2+} channels (Howe and Surmeier, 1995; Perez-Rosello et al., 2005). Ca^{2+} entry through L - type Ca^{2+} channels occurs during upstates (Carter and Sabatini, 2004) and is required for the induction of corticostriatal LTD on MSNs (Calabresi et al., 1994). Thus M_1R modulation of Ca^{2+} channels on MSNs serves to enhance striatal output by increasing firing frequency and reducing the susceptibility of MSNs towards LTD induction mechanisms.

Acetylcholine also acts through postsynaptic M_1Rs to promote excitatory signaling upon MSNs by enhancing membrane depolarization resulting from NMDA receptor activation (Calabresi et al., 1998a). It is believed that M_1R activation results in phosphorlyation of NMDARs via inositol triphosphate – dependent Ca^{2+} release from intracellular stores and subsequent activation of phosphokinase C by diacylglycerol and Ca^{2+}. Phosphorlyation of NMDARs enhances cation entry through activated receptors and may play a role in the induction of postsynaptic long-term potentiation (LTP) of corticostriatal synapses (Carter and Sabatini, 2004).

Finally, tonic activation of M_1Rs on MSNs is involved in the suppression of inhibitory signaling onto these neurons. When paired with mAChR activation, Ca^{2+} influx facilitates the release of endocannabinoids, and the subsequent retrograde signaling inhibits release of GABA upon MSNs (Narushima et al., 2007). This study showed that the coincidence of a single action potential from ChIs with MSN depolarization was capable of suppressing IPSCs on MSNs. Therefore continuous M_1R activation by tonic ChI firing should prime MSNs to release endocannabinoids in response to Ca^{2+} influx.

In summary, tonic M_1R activation by ChI firing generally enhances the excitability of MSNs. mAChRs suppress the persistent inhibitory influence of Kir2 channels, promote upstate transitions by enhancing currents through NMDARs, augment MSN output by increasing spike rate, reduce the efficacy of inhibitory signaling onto MSNs, and prevent LTD induction.

1.3.2 ACh signaling on fast spiking interneurons

Immunolabeling first illustrated the presence of cholinergic synapses upon parvalbumin positive neurons (Chang and Kita, 1992), and functional studies later found that ACh acts through both nicotinic and muscarinic receptors to influence FSI output. Postsynaptic nicotinic responses of FSIs isolated by focal pressure application of ACh failed to desensitize, thereby suggesting a tonic depolarizing influence of nicotinic receptors on FSIs (Koos and Tepper, 2002). This finding is supported by previous reports that nonselective cholinergic agonists increase striatal GABA levels in a nAChR – dependent manner (Limberger et al., 1986; Koos and Tepper, 2002). In contrast, simultaneous recordings of connected neurons illustrate that muscarine reduces the

amplitude of FSI input onto MSNs (Koos and Tepper, 2002). Thus, nAChRs and mAChRs provide oppossing mechanisms for regulating FSI output (Table 2).

1.3.3 ACh signaling on cholinergic interneurons

Striatal cholinergic interneurons express M_1, M_2 and M_4 mAChRs (Bernard et al., 1992; Yan and Surmeier, 1996; Alcantara et al., 2001; Bonsi et al., 2008), and activation of these autoreceptors serves to reduce basal striatal acetylcholine levels (James and Cubeddu, 1987). M_2 and M_4 mAChRs are found on somato – dendritic regions of cholinergic interneurons in addition to axon terminals (Alcantara et al., 2001; Zhang et al., 2002b), and activation of these receptors results in both the opening of Kir3 K^+ channels and inhibition of N- and P- type Ca^{2+} channels (Yan and Surmeier, 1996; Calabresi et al., 1998c). K^+ channel activation by mAChRs induces membrane hyperpolarization of ChIs and provides a mechanism for feedback regulation of ChI action potential frequency and overall excitability (Bonsi et al., 2008). In addition, N- and P- type Ca^{2+} channel inhibition by mAChR activity reduces Ca^{2+} entry into ChIs by acting through $G_{i/o}$ proteins (Yan and Surmeier, 1996). N- and P- type Ca^{2+} channels are commonly found at axonal terminals of other cell types, and inhibition of these channels in ChIs presumably acts to reduce transmitter release (Dunlap et al., 1995). In mice lacking either M_4 or M_2 and M_4 mAChRs, standard stimulation protocols failed to induce corticostriatal LTD onto MSNs confirming the necessity of these receptors for this form of plasticity (Bonsi et al., 2008). Muscarinic autoreceptors clearly provide an important brake for the striatal cholinergic system, and the dysregulation of these receptors may contribute to Parkinson’s disease symptoms (Table 2; Pisani et al., 2007).

1.3.4 ACh signaling on striatal afferent axon terminals

Glutamatergic and dopaminergic axon terminals within the striatum express ACh receptors (Hersch et al., 1994; Jones et al., 2001), and cholinergic control of glutamate and dopamine release represents a powerful method of shaping striatal output (Table 3). By recording from MSNs in striatal slices, it was shown that M_2 and M_3 mAChR agonists reduced the amplitude of evoked EPSPs, and it is believed that this occurs through the reduction of Q – type Ca^{2+} channel activity and subsequent decrease of transmitter release from glutamatergic terminals (Calabresi et al., 1998b; Barral et al., 1999). In line with this finding, it was recently shown that individual action potentials from cholinergic interneurons control glutamate release upon MSNs and other cholinergic interneurons by acting through presynaptic mAChRs (Pakhotin and Bracci, 2007). Likewise, muscarinic receptor activity modulates dopamine release from terminals of axons arising from midbrain nuclei, yet studies of muscarinic control of DA release contradict and the different findings may result from variable expression of mAChR subtypes on DAergic terminals. For example, *in vivo* microdialysis studies report that M_1 – like receptor activation increases while M_2 – like receptor activation decreases striatal DA levels (Xu et al., 1989; Smolders et al., 1997), however knockout of M_1 and M_2 receptors failed to alter dopamine release evoked by a general muscarinic agonist (oxotremorine; Zhang et al., 2002a).

In contrast, nAChRs clearly, and powerfully, regulate striatal DA release by acting through β2 – containing nAChRs on dopaminergic axon terminals (Jones et al., 2001; Rice and Cragg, 2004). Specifically, voltammetric measurements of DA in striatal slices show that the β2 – containing nAChR antagonist, DHβE, decreases action potential – dependent DA release, indicating that nAChR activity enhances striatal DA release

from single action potentials (Zhou et al., 2001). Moreover, this nAChR – dependent enhancement contributes to a use – dependent short – term depression of DA release during high frequency DA neuron activity by significantly reducing the number of available DA – carrying vesicles to be released during later spikes (Cragg, 2003). Thus nAChR activity enhances release of DA from single DA neuron action potentials but limits DA release during high – frequency periods of DA neuron firing.

1.4 Dopamine and the striatum

Axons originating from dopaminergic neurons in the midbrain branch extensively within the striatum to form a broad network (Prensa and Parent, 2001), and labeling studies paired with ultrastructural evidence indicate that cholinergic and dopaminergic axons physically intertwine within the striatum (Contant et al., 1996; Descarries and Mechawar, 2000; Zhou et al., 2001). Like striatal ChIs, midbrain DA neurons are tonically active both *in vivo* and *in vitro*, and changes in their activity are known to encode information regarding reward – relevant events (Schultz et al., 1997). MSNs and striatal interneurons express DA receptors and are targets of dopaminergic axons (Centonze et al., 2003; Maurice et al., 2004; Surmeier et al., 2007), however DA, like ACh, is thought to influence these targets mainly through volume transmission (Grace, 1991; Barbour and Hausser, 1997). The convergence and similarity of cholinergic and dopaminergic systems within the striatum suggests a close functional interaction between these two neurotransmitter systems.

1.4.1 Dopamine signaling on medium spiny neurons

As mentioned previously, MSNs are divided into two populations based largely on their axonal projections. Each population differentially expresses either D1 or D2 receptors. Striatonigral MSNs project to the SNr and GPi and express D1 receptors, while the striatopallidal MSNs project to the GPe and express D2 receptors. Classically, activation of D1 receptors excites direct pathway MSNs whereas D2 receptor activation reduces indirect pathway MSN excitability, however these generalizations are not completely accurate. The effects of DA signaling are more complicated for at least two reasons. First, DA release occurs on two timescales. DA neurons are tonically active and fire intrinsically – driven action potentials at 1 – 10 Hz *in vivo*, but these neurons also fire brief groups of action potentials at a higher frequency, or 'bursts', in response to specific environmental stimuli. Second, MSNs transition between two states, and DA signaling affects each state differently (Table 1).

1.4.1.1 Modulation of striatonigral MSN function by D1 receptors

D1 receptors on MSNs couple to $G_{olf\alpha}$ to drive phosphorylation by PKA through an adenylyl cyclase/ cAMP – dependent cascade (Herve et al., 1995; Zhuang et al., 2000), and the results of D1 receptor activation differ depending on the state of the MSN. For MSNs in the downstate, activation of this pathway modulates voltage – gated and ionotropic receptor channels to promote upstate transitions. The dominating features of MSN upstate transitions are the closure or inactivation of several K^+ channels (Kir2, Kv1, and Kv4) and reduced SK channel opening (Wickens and Wilson, 1998), and D1 receptor activation works to promotes these changes. Additionally, phosphorylation resulting

from D1 receptor signaling increases L-type Ca^{2+} (Cav 1.2 and Cav 1.3) channel openings, which has been shown to promote upstates in slices (Vergara et al., 2003), and also reduces SK channel activity by reducing Ca^{2+} entry through Cav2 Ca^{2+} channels (Vilchis et al., 2000). The result of these changes is to promote the excitability of direct pathway MSNs and enhance the probability that glutamatergic input will evoke transition to the upstate and action potential firing.

In contrast, D1 receptor activation limits action potential firing during the upstate of MSNs by reducing voltage – dependent Na^{+} channel function. D1 receptor - dependent PKA phosphorylation of the pore – forming Na^{+} channel subunit leads to a transition into a non-conducting state that reverses only upon hyperpolarization associated with return to a downstate (Surmeier et al., 1992; Carr et al., 2003). It is believed that limited action potential generation by Na^{+} channel phosphorylation serves to either prevent weaker glutamatergic input to evoke MSN firing or to act as a brake upon firing and encourage transition to the downstate.

D1 receptor signaling on MSNs induces longer lasting changes as well. Notably, surface expression of AMPA and NMDA receptors is increased by D1 receptor – dependent PKA activity (Snyder et al., 2000; Hallett et al., 2006). The trafficking pathway remains poorly defined, but it is known to require FYN kinase and the striatal enriched protein phosphatase (STEP) (Braithwaite et al., 2006). In addition, studies *in vitro* have demonstrated LTP after high frequency stimulation of corticostriatal afferents (Kerr and Wickens, 2001; Centonze et al., 2003). This LTP required co-activation of D1 and NMDA receptors but did not depend upon Ca^{2+} entry from L-type Ca^{2+} channels. This evidence suggests that coincident dopaminergic and glutamatergic signaling results in the strengthening of synapses. As a result, the cumulative effect of D1 signaling upon striatonigral MSNs appears to enhance the excitability of these neurons by promoting

upstate transitions and strengthening of synapses, however D1 receptor activation also reduces the firing of these neurons.

1.4.1.2 Modulation of striatopallidal MSN function by D2 receptors

D2 receptors are highly expressed on striatopallidal MSNs, and like the activation of D1 receptors on striatonigral MSNs, these subunits modulate ionotropic and voltage gated currents to influence the downstate and upstate responses of MSNs to glutamatergic input.

D2 receptor activation stabilizes the downstate of striatopallidal MSNs by affecting L – type Ca^{2+} channels, Kir K^{+} channels, and Nav 1.1 channels via the D2 – coupled $G_{i/o}$ protein cascade. $G_{\beta\gamma}$ activates phospholipase C (PLC) to induce IP3 – dependent Ca^{2+} release from intracellular stores. This Ca^{2+} can be taken up by calmodulin which ultimately reduces L – type Ca^{2+} (Ca_V 1.3) channel activity by acting on protein phosphatase 2B (Hernandez-Lopez et al., 2000). Unlike other L – type Ca^{2+} channels which are high – voltage activated, Ca_V 1.3 channels activate in response to modest depolarization (Koschak et al., 2001; Olson et al., 2005) and their inhibition by D2 receptor activation results in a reduction in spiking (Hernandez-Lopez et al., 2000; Olson et al., 2005). Additionally, $G_{\beta\gamma}$ releases diacylgylcerol (DAG) to stimulate protein kinase C (PKC) and reduce Nav 1.1 Na^{+} channel opening (Surmeier et al., 1992). This mechanism likely works by augmenting slow inactivation of Na^{+} channels (see above), and functionally limits MSN spiking. Finally, an enhancement in Kir channel opening stabilizes the downstate of MSNs thereby increasing the threshold that must be overcome by excitatory input to induce an upstate transition (Freedman and Weight, 1989).

D2 receptor activation also reduces the excitability of MSNs by reducing the number of functional AMPA receptors available in the postsynaptic membrane. Studies in slices have suggest that D2 receptor activation of $G_{\alpha i}$ subunits produces dephosphorylation of GluR1 at S845 (Hakansson et al., 2006). Previous studies have shown that phosphorylation of S845 produces trafficking of GluR1 subunits to the membrane and increases AMPA channel conductance (Roche et al., 1996; Banke et al., 2000), thus dephosphorylation should promote trafficking of GluR1 away from the membrane. Indeed, D2 receptor activation reduced AMPA receptor currents in dissociated MSNs (Hernandez-Echeagaray et al., 2004).

In contrast to the depressing actions of $G_{\beta\gamma}$ during striatopallidal MSN downstates, D2 activation of this protein enhances firing of striatopallidal MSNs during upstates. Cav2 Ca^{2+} channel inhibition by $G_{\beta\gamma}$ reduces Ca^{2+} - dependent K^{+} entry (SK channel) during action potential AHPs and this reduces intervals between spikes (Salgado et al., 2005). Thus like D1 receptor signaling on striatonigral MSNs, D2 signaling on striatopallidal MSNs elicits variable responses during downstates and upstates. D2 signaling enhances the stability of the downstate, but once in the upstate D2 signaling acts to shorten the time between action potentials to presumably increase the efficacy of MSN signaling.

D1 effects Striatonigral MSNs		D2 effects Striatopallidal MSNs	
Downstate	Upstate	Downstate	Upstate
↓ K Channel function (Kir2, Kv1, Kv4, SK)	↓ Voltage gated Na channel function	↓ L - type Ca channel function (CaV 1.3)	↑ Cav 2 Ca channel inhibition (reduces SK current)
↑ L - type Ca channel function (CaV 1.2 and 1.3)	↑ AMPA and NMDA surface expression	↓ Nav1 channel function	
↑ N and P/Q type Ca channel function (CaV 2.2 and 2.1)	D1 receptors are required for LTP	↓ AMPA receptor surface expression	

Table 1. Effects of dopamine signaling upon medium spiny neurons

1.4.2 Long term depression of corticostriatal synapses upon MSNs

Long term depression (LTD) of glutamatergic neurostransmission can be induced by paring high frequency stimulation with postsynaptic depolarization and has been observed in both direct and indirect pathway MSNs (Calabresi et al., 1992a; Calabresi et al., 1992b; Lovinger et al., 1993; Wang et al., 2006). This LTD is most likely induced postsynaptically via calcium entry from L – type Ca^{2+} (Cav 1.3) channels combined with signaling through mGluRs, and it is well established that the induction of this LTD is dependent upon D2 receptor activation (Calabresi et al., 1992a; Calabresi et al., 1992b; Wang et al., 2006). The differential expression of dopamine receptors by the two populations of MSNs suggests that the location of the D2 receptor involved in corticostriatal LTD may not be located on MSNs. Indeed, recent evidence indicates that corticostriatal LTD depends upon D2 receptor – dependent inhibition of ChI firing and subsequent disinhibition of Cav 1.3 Ca^{2+} channels on MSN terminals by reduced M1 muscarinic receptor activity. The resulting increase of Ca^{2+} within the spine produces endocannabinoid release into the synaptic cleft and CB_1 – dependent depression of glutamate release from corticostriatal terminals (Wang et al., 2006).

LTD of glutamatergic synapses upon MSNs is also induced by dopamine – dependent release of nitric oxide. As mentioned earlier, NOS staining selectively identifies PLTS interneurons within the striatum, and burst firing by midbrain dopamine neurons results in NOS activation *in vivo* (Sammut et al., 2006). Dopamine – dependent NOS activity in these cells requires D1 – family DA receptor activation, and pharmacological stimulation of these receptors on PLTS interneurons produces NO release and LTD induction (Centonze et al., 2003).

1.4.3 Dopamine signaling on fast spiking interneurons and PLTS interneurons

As previously described, phasic DA neuron signaling elicits striatal NOS activity presumably from PLTS interneurons. Thus, as striatal interneurons act as feedforward modulators of MSN output, DA – dependent regulation of striatal interneurons adds to the complexity of dopaminergic modulation of striatal output (Table 2). Both FSIs and PLTS interneurons express DA receptors and receive dopaminergic innervation (Kubota et al., 1988; Kawaguchi et al., 1995), and *in vitro* studies report that DA application depolarizes the membranes and increases the input resistance of both cell types (Bracci et al., 2002; Centonze et al., 2002). In both interneuron populations, DA acts postsynaptically through D1 – like receptors (Centonze et al., 2003) while D2 receptor activation fails to alter resting membrane conductances. However, DA – dependent depolarization of FSIs augmented IPSCs on MSNs in only a subset of recorded neuron pairs suggesting that some FSIs may be more responsive to DA than others (Tecuapetla et al., 2007). Regardless, it stands that dopaminergic enhancement of striatal GABAergic interneuron excitability extends the inhibitory power of DA. Not only can DA inhibit MSNs directly, but DA – dependent enhancement of GABAergic signaling upon MSNs also provides a powerful mechanism to limit striatal output.

1.4.4 Dopamine signaling on cholinergic interneurons

Dopaminergic regulation of ChI output is mediated by D2 and D1 – like DA receptors (Table 2). Specifically, D1 – like receptor activation increases striatal

acetylcholine release *in vivo*, while spontaneous acetylcholine release is reduced by D2 receptor activation (Damsma et al., 1990; DeBoer and Abercrombie, 1996). DA depolarizes ChIs through D1 receptor activation of an adenylyl cyclase – cAMP dependent pathway that closes a resting K^+ conductance and opens a nonselective cation conductance (Aosaki et al., 1998). Membrane depolarization results in an increase in the spontaneous firing frequency of ChIs that likely raises the ambient level of striatal ACh. Paradoxically, D1 – like receptor activation also enhances $GABA_A$ currents in ChIs (Yan and Surmeier, 1997). This study reported that the only D1 family receptor expressed by ChIs is the D5 receptor, and that D5 receptor activation triggers a cascade involving PKA and PP1 to boost $GABA_A$ currents. Centonze et al confirmed that D5 receptors are primarily responsible for the D1 – like DA effect upon ChIs by using D1 knockout mice (Centonze et al., 2003). Moreover, D5 receptors are required for the induction of LTP of glutamatergic input to ChIs (Suzuki et al., 2001). Thus it appears that D5 receptors perform a dual regulatory function upon striatal ACh by both enhancing and reducing the excitability of ChIs.

Dopamine D2 receptors are located on somatodendritic areas and on the axons of cholinergic inteneurons (Alcantara et al., 2003). Activation of D2 receptors on ChIs reduces striatal ACh levels by reducing autonomous spiking and by reducing transmitter release (DeBoer and Abercrombie, 1996; Yan and Surmeier, 1997; Deng et al., 2007). D2 signaling reduces ChI firing by stabilizing Na^+ channels in a slow – inactivating state (Maurice et al., 2004) and by increasing currents through HCN channels (I_h ;Deng et al., 2007). Functionally, these effects decrease the frequency of spontaneous action potentials in cholinergic interneurons and reduce the ambient concentration of striatal ACh. Additionally, D2 receptors suppress N-type Ca^{2+} channels to presumably reduce

striatal ACh efflux from ChI terminals (Yan and Surmeier, 1997). The combined result of D2 activation upon ChIs is a robust decrease in ambient ACh levels.

1.4.5 Dopamine signaling on striatal afferent axon terminals

D2 receptors, but not D1 receptors, are located on corticostriatal terminals as well as dopaminergic terminals within the striatum (Sesack et al., 1994; Wang and Pickel, 2002), and signaling through these terminal D2 receptors provides yet another powerful control point for DA in the striatum (Table 3). D2 receptor activation on corticostriatal terminals inhibits glutamate release in a manner that limits glutamate release from only "weaker" terminals (Bamford et al., 2004a; Bamford et al., 2004b). That is, D2 activation provides greater inhibition to terminals with a low probability of release but leaves terminals with a high probability of release relatively unaffected. Moreover, D2 inhibition of corticostriatal glutamate release was substantially greater when firing was evoked at higher frequencies (10 – 20 Hz), suggesting that DA signaling on corticostriatal terminals functions as a low – pass filter for weak terminals. The end result is that DA limits the efficacy of signaling through weak glutamatergic terminals but allows signaling through strong terminals (Horvitz, 2002).

D2 receptors located on dopaminergic dendrites and terminals within the striatum also inhibit neurotransmitter release (Kennedy et al., 1992; Cragg and Greenfield, 1997). However, D2 – dependent inhibition of DA release may depend upon the location of the terminals within the striatum (Cragg et al., 1997; Cragg and Greenfield, 1997). The SNc and VTA project to different areas of the striatum, and significantly less D2 DA receptor mRNA was found in SNc neurons compared to those in the VTA (Hurd et al., 1994; Haber et al., 1995). The mechanism of inhibition of dopaminergic terminals by D2

receptors is not completely understood but may work through reducing N and P/Q – type Ca^{2+} channel currents at terminals (Cardozo and Bean, 1995). Autoreceptor – dependent inhibition of DA release provides a mechanism for feedback inhibition of DA neuron activity within the striatum, presumably to maintain homeostatic levels of ambient DA in this brain area.

	Dopaminergic		Cholinergic	
	D1	**D2**	**nACh**	**mACh**
ChI	Membrane depolarization ↑ $GABA_A$ current	↓ ACh release ↓Na channel function ↑ HCN current	unknown	↓ ACh release membrane hyperpolarization
FSI	Membrane depolarization	↓ GABA release	membrane depolarization	no effect
PLTS	Membrane depolarization	unknown	membrane depolarization	no effect
Calretinin positive	unknown	unknown	unknown	unknown

Table 2. Dopaminergic and cholinergic modulation of striatal interneurons

	Dopaminergic		Cholinergic	
	D1	**D2**	**nACh**	**mACh**
Glutamatergic terminal	no effect	↓ release at weak terminal	unknown	↓ release
Dopaminergic terminal	no effect	↓ release of DA	↑ release by single AP	↓ release

Table 3. Dopaminergic and cholinergic modulation of striatal afferent terminals

1.5 Dopaminergic and cholinergic modulation of striatal output

Early clinical studies demonstrating the effectiveness of ACh antagonists and DA agonists for the treatment of Parkinson's disease led to the proposal that ACh and DA exert opposing actions in the striatum (Barbeau, 1962). Support for the balance hypothesis is contributed by observations of each system during associative learning tasks. For instance, *in vivo* studies illustrate that salient reward – relevant stimuli induce coincident but opposing changes in the tonic firing patterns of both midbrain DA neurons and striatal TANs (Aosaki et al., 1994b; Schultz, 1998; Morris et al., 2004). The firing rate of midbrain DA neurons increases in response to a primary reward or a conditioned cue predicting a reward while TAN firing is transiently suppressed (Schultz, 1986; Aosaki et al., 1994b).

Additionally, several lines of physiological evidence support the hypothesis that ACh and DA balance the other's effects within the striatum. These include the spatial overlap of the cholinergic and dopaminergic systems in the striatum (Zhou et al., 2002), dopaminergic signaling on most targets including MSNs and glutamatergic terminals, and cholinergic signaling on MSNs and DA terminals.

One theory regarding the antagonistic interaction between ACh and DA views the dopaminergic system as a mechanism that limits the effectiveness of weak glutamatergic inputs to elicit striatal output while enhancing the effectiveness of strong glutamatergic signals to result in striatal output (Nicola et al., 2000; Horvitz, 2002). Here, combined pre- and postsynaptic actions of DA reduce the ability of glutamate to evoke MSN output such that only strong, converging cortical input effectively induces MSNs to fire action potentials. Moreover, when glutamatergic inputs are strong enough to overcome the

suppressing effect of DA, DA signaling then facilitates MSN output. The persistent DA receptor activation resulting from tonic DA neuron firing therefore, sets a threshold that glutamatergic input must overcome to elicit striatal output and then augments MSN output when the threshold has been surpassed. In contrast, when strong glutamatergic signaling occurs in coincidence with the phasic DA signal, MSN output is enhanced over the level provided by tonic DA. Yet when the phasic DA signal occurs independently of strong glutamatergic signaling, MSN output is further suppressed. The interaction of dopaminergic signaling upon striatal afferent terminals, interneurons, and MSNs presumably underlies the mechanism for this hypothesis (Table 4).

On the other hand, the DA – ACh balance hypothesis predicts that ACh opposes the threshold set by the tonic DA signal. Indeed, persistent mAChR activation on MSNs by tonic ChI firing antagonizes the effects of tonic DA by increasing postsynaptic excitability via membrane depolarization (Calabresi et al., 2000). In this regard, the balance of the tonic levels of ACh and DA helps to set a threshold that glutamatergic input must overcome to elicit striatal output. However, the functions of the striatal cholinergic system extend beyond the role of ACh to balance DA. For example, cholinerigic signaling reduces glutamate release, enhances DA release, and increases the excitability of FSIs (Table 4). Each of these effects contrast with the idea that ACh acts to enhance the excitability of the striatum, and clearly point to other roles for ACh signaling within the striatum.

There are at least four additional roles for the cholinergic system of the striatum. 1) Muscarinic autoreceptors on cholinergic axon terminals underlie feedback regulation of ACh release. 2) nAChRs enhance release of DA during tonic DA neuron firing, but reduce DA release during high – frequency DA neuron firing. Therefore, DA release is enhanced when DA bursts coincide with pauses in ChI firing. 3) mAChRs suppress

glutamate release from glutamatergic terminals such that a pause in ChI firing again functionally enhances glutamate release. 4) nAChR signaling depolarizes FSIs to enhance GABAergic inhbition within the striatum. Notably, the combined effects of these cholinergic mechanisms can be reconciled with the hypothesis that the phasic signal of the cholinergic system, like the phasic dopaminergic signal, establishes conditions in the striatum that favor the ability of coincident glutamatergic signaling to elicit striatal output. According to this idea, a pause in ChI firing enhances DA release, reduces inhibition of glutamate release, and decreases inhibition of MSNs by FSIs. Each of these effects occurs in the area of the striatum targeted by the topographic glutamatergic inputs. Additionally, outside the target area, a pause in ChI firing results in decreased MSN excitability and increased GABA release. In this respect, the cholinergic system promotes signaling through the targeted area of the striatum and reduces the possibility of signaling outside the target area.

Tonic ACh effects		Phasic ACh effects (Pause in tonic firing)	
muscarinic	nicotinic	muscarinic	nicotinic
↓ DA release	↑ DA release	relieve inhibition of DA terminals	↑↑ DA release
↓ ACh release	↑ FSI excitability	relieve inhibition of ACh terminals	relieve FSI excitability
↓ GABA release from FSIs		relieve inhibition of FSI terminals	
↓ Glutamate release		relieve inhibition of glutamate terminals	
↑ MSN excitability		relieve excitability of MSNs	

Table 4. Tonic versus phasic effects of dopamine signaling in the striatum

1.6 Hypothesis and specific aims

ChIs are thought to correspond to the tonically active neurons (TANs) recorded *in vivo*, which respond to sensory stimuli with a transient depression, or pause, of tonic firing during sensorimotor conditioning (Wilson et al., 1990; Aosaki et al., 1994b). These neurons are spread throughout the striatum and give rise to extensive axonal arbors forming a dense plexus of cholinergic fibers (Zhou et al., 2002). Notably, despite the topographical arrangement of striatal inputs and evidence suggesting that the TAN pause results from thalamostriatal signaling, TANs respond as a population during sensorimotor conditioning (Apicella, 2007). Indeed, the percentage of TANs that respond to a stimulus directly correlates with the probability of the stimulus to induce a motor response (Blazquez et al., 2002), suggesting that striatal output can be dependent upon TAN activity.

Accumulating evidence indicates an important role of the local microcircuitry in regulating the activity of striatal projection neurons, the MSNs. Spiny neurons form a weak lateral inhibitory network among themselves via local axon collaterals, yet feedforward signaling by striatal interneurons exerts more powerful control over MSN excitability. For example, single action potentials from one FSI can inhibit MSN firing (Koos and Tepper, 1999), and as described previously, at least two types of GABAergic interneurons exist in the striatum along with FSIs. These are the PLTS interneuron and the calretinin – positive interneuron. PLTS interneurons and MSNs, but not FSIs, make synaptic contacts with ChIs. Local electrical stimulation has revealed that GABAergic input can delay action potential firing of ChIs (Bennett and Wilson, 1998), and electrical stimulation of cortico- and thalamostriatal glutamatergic fibers produces monosynaptic

EPSPs followed by polysynaptic $GABA_A$ IPSPs in cholinergic interneurons (Suzuki et al., 2001). However, the regulation of ChIs by the striatal GABAergic network is not completely understood, and these studies suggest a role for the feedforward modulation of ChI function by GABAergic neurons within the striatum. The working hypothesis of this dissertation is that striatal GABAergic neurons form an inhibitory network among cholinergic interneurons. The existence of such a network may reconcile evidence illustrating that TANs respond to specific sensory cues as a population spread throughout the striatum with evidence that the striatal inputs likely to transmit the relevant sensory cues to TANs target the striatum topographically.

The first specific aim will test the hypothesis that polysynaptic GABAergic inhibition transiently suppresses the tonic firing of striatal cholinergic interneurons. For this aim, GABAergic responses evoked by intra – striatal synaptic stimulation will be pharmacologically isolated in ChIs under whole cell voltage and current clamp. GABAergic IPSCs will be characterized based on physiological and pharmacological tests, and then the effect of evoked polysynaptic IPSCs on ChI firing will be evaluated.

The second specific aim will test the hypothesis that dopamine receptor activation modulates polysynaptic inhibition upon cholinergic interneurons of the striatum. As explained above, strong evidence exists linking the striatal cholinergic network with the axonal network formed by midbrain DA neurons projecting to the striatum. Therefore, this aim will monitor changes in polysynaptic inhibitory responses in ChIs in response to pharmacological manipulation of DA receptors and striatal DA concentration in order to identify the type and location of DA receptor located in the polysynaptic circuit.

Chapter 2: Materials and methods

2.1 Slices and Solutions

All animal procedures were performed in accordance with the NIH guideline and approved by the Institutional Animal Care and Use Committee at UT Austin. Oblique horizontal slices (30-45°, 230 μm) containing the dorsal striatum were prepared from male Sprague-Dawley rats (21-28 day). Slices were cut using a vibratome (VT1000S; Leica Microsystems, Bannockburn, IL) in ice-cold saline containing (in mM): 2.5 KCl, 1.25 NaH_2PO_4, 7.5 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, 205 sucrose, 25 $NaHCO_3$ (saturated with 95% O_2 and 5% CO_2), and then incubated at 35°C for >1 hr in physiological saline containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, 21.4 $NaHCO_3$, 5 kynurenic acid (saturated with 95% O_2 and 5% CO_2, ~295 mOsm/kg). Recordings were made at 35°C in the same saline (without kynurenic acid) perfused at 2-3 ml/min.

2.2 Electrophysiological Recordings

Cells were visualized using an upright microscope (BX51WI; Olympus America, Center Valley, PA) with IR/DIC or oblique illumination optics. Whole-cell recordings were made with borosilicate glass pipettes (1.6-2.2 MΩ) filled with internal solution containing (in mM): 115 K-methylsulfate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na_2-GTP, and 10 Na_2 phosphocreatine (pH 7.25-7.3, ~280 mOsm/kg).

Data were acquired using a Multiclamp 700A or 700B amplifier (Molecular Devices, Union City, CA), filtered at 2-5 kHz, digitized at 5-20 kHz, and collected using Axograph X (Axograph Scientific, Sydney, Australia). The membrane potential was corrected for a liquid junction potential of 7 mV.

2.3 Dopamine Depletion

Rats were injected intraperitoneally with the vesicular monoamine transporter inhibitor reserpine (5 mg/kg, 24 hr prior to dissection) and the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine (AMPT; 300 mg/kg, 4 hr before dissection, plus 200 mg/kg, 2 hr before dissection). Slices were pre-incubated in reserpine (1 μM), AMPT (30 μM), and D-amphetamine (1 μM), and recordings were done in the continuous presence of reserpine (1 μM) and AMPT (30 μM) to ensure complete depletion of dopamine.

2.4 Drugs

6,7-dinitroquinoxaline-2,3-dione (DNQX), 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid), D-(-)-2-amino-5-phosphonopentanoic acid (APV), 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (5R,10S)-(-)-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5-imine maleate (MK-801), dihydro-β-erythroidine hydrobromide (DHβE), and (R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390) were obtained from Tocris

Bioscience (Ellisville, MO). All other chemicals were obtained from Sigma/RBI (St. Louis, MO).

2.5 Data analysis

Data are expressed as means ± SEM. Statistical significance was determined with Student's t test. The difference was considered significant at $p < 0.05$. The onset of suppression in tonic firing was defined as the first of three consecutive bins (bin width = 20 ms) that deviated significantly from the baseline firing frequency before the stimulus, whereas the offset was defined as the first of three consecutive bins that returned to the baseline level (Aosaki et al 1995).

Chapter 3: Results

3.1 Aim 1: Polysynaptic GABAergic inhibition transiently suppresses the tonic firing of striatal cholinergic interneurons

3.1.1 Polysynaptic $GABA_A$ IPSCs in cholinergic interneurons

Cholinergic interneurons were identified by their large soma (25-50 μm) and unique electrophysiological properties (Fig. 3A,B), such as spontaneous action potential firing, a long-duration afterhyperpolarization, and a depolarizing sag in response to hyperpolarizing current injection (Wilson, 2005; Sullivan et al., 2008). In order to investigate local synaptic inputs onto these neurons, we made whole-cell voltage clamp recordings at a holding potential of -87 mV and measured synaptic currents evoked every 20 s by intrastriatal stimulation using a bipolar electrode (100-200 μm tip separation) placed 100-300 μm from the recorded cell. This causes stimulation of both intrinsic fibers from striatal neurons and extrinsic inputs from other structures. Recordings were done in the presence of an AMPA/kainate receptor antagonist DNQX (10 μM) to eliminate responses produced by stimulation of glutamatergic fibers from the cortex and thalamus (Lapper and Bolam, 1992). A single stimulus induced compound postsynaptic currents (PSCs) containing multiple peaks in 7 cells tested with various stimulus intensities (50-800 μA, 0.2 ms) (Fig. 3C). At threshold intensity (50-150 μA), a single peak (150 ± 29 pA) was elicited with a long latency (14.2 ± 0.6 ms) after the stimulus. The PSC increased in amplitude, duration, and number of peaks with an increase in stimulus intensity. At the same time, the onset latency became shorter up to a certain

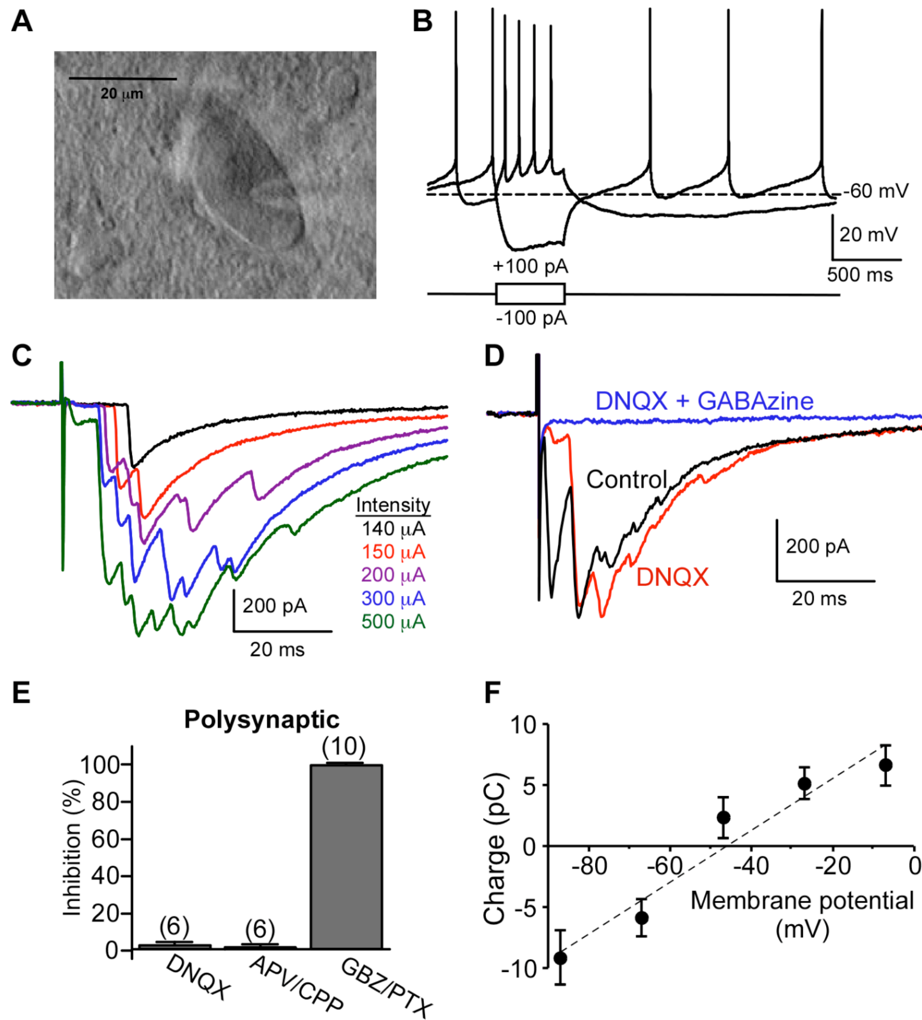
level in each cell, ranging from 6.6 to 10.8 ms (8.2 ± 0.7 ms), consistent with the idea that it is evoked polysynaptically. Higher intensity stimulation produced an additional component (30-200 pA) that occurred with a much shorter latency (1.2-3.6 ms, 2.1 ± 0.3 ms). The latency to this early component was not changed by further increase in stimulus intensity, implying that it is a monosynaptic response. The range of the onset latency of the two components had no overlap. Furthermore, the late component was selectively suppressed with an increase in extracellular concentrations of divalent cations (4 mM Ca^{2+} and 4 mM Mg^{2+}, n = 4) (Fig. 4), a treatment commonly used to block polysynaptic responses by elevating action potential threshold (Cruikshank et al., 2002; Liao and Walters, 2002). Therefore, the early and late components represent monosynaptic and polysynaptic PSCs, respectively.

GABAergic transmission makes a major contribution to the striatal microcircuitry (Tepper and Bolam, 2004; Tepper et al., 2004). Indeed, both monosynaptic and polysynaptic PSCs were completely blocked by bath application of $GABA_A$ receptor antagonists, GABAzine (10 μM) or picrotoxin (100 μM) (Fig. 3D,E). Furthermore, the charge transfer mediated by the polysynaptic component reversed at -45 ± 3 mV (n = 12) (Fig. 3F), close to the estimated equilibrium potential for Cl^- (E_{Cl} = -47 mV) under our recording conditions, as would be expected for Cl^--permeable $GABA_A$ conductance. Together, these results demonstrate that intrastriatal stimulation produces rapid monosynaptic and delayed polysynaptic $GABA_A$ IPSCs in cholinergic interneurons under AMPA/kainate receptor blockade. It should be noted that polysynaptic $GABA_A$ IPSCs were consistently elicited in all cholinergic interneurons tested for intrastriatal stimulation in this study. In contrast, only monosynaptic IPSCs, without any polysynaptic IPSCs, were observed in spiny projection neurons (n = 8, data not shown). These monosynaptic IPSCs in spiny neurons and cholinergic interneurons are most likely caused by

stimulation of intrinsic GABAergic fibers from striatal GABAergic neurons, although extrinsic GABAergic inputs from the globus pallidus may also be involved (Kita, 2007).

It has been shown that electrical stimulation of the subcortical white matter, which contains cortico- and thalamostriatal glutamatergic fibers, produces polysynaptic $GABA_A$ IPSCs in cholinergic interneurons via AMPA receptor-dependent excitation of striatal GABAergic neurons (Suzuki et al., 2001). To test if the intrastriatal stimulation described above can invoke this mechanism, we also recorded PSCs in the absence of DNQX. Intrastriatal stimulation (200-800 μA) produced a large monosynaptic component (50-600 pA) with a short latency (2.6 ± 0.1 ms, $n = 10$) followed by a delayed polysynaptic component (Fig. 3D). The early component was largely reduced by DNQX (10 μM) and abolished by the combination of DNQX and GABAzine (10 μM) or picrotoxin (100 μM), indicating that it represents a mixture of monosynaptic AMPA EPSCs and $GABA_A$ IPSCs. In contrast, DNQX had no effect on the late component, which was abolished by subsequent application of GABAzine or picrotoxin, as described above (Fig. 3D,E). Furthermore, NMDA receptor antagonists, APV (100 μM) or CPP (100 μM), failed to affect either the early or late component (Fig. 3E). Therefore, activation of glutamatergic fibers does not contribute to the generation of polysynaptic $GABA_A$ IPSCs by intrastriatal stimulation. All of the experiments hereafter were performed in the presence of DNQX to eliminate monosynaptic AMPA EPSCs.

Figure 3.



Intrastriatal stimulation elicits monosynaptic and polysynaptic $GABA_A$ IPSCs in cholinergic interneurons.

(A) Photomicrograph of a recorded cholinergic interneuron in a striatal slice. (B) Representative traces of a spontaneously firing cholinergic interneuron depicting its response to positive and negative current injections. (C) A series of PSC traces evoked by intrastriatal stimulation of various intensities. The experiment was done in the presence of DNQX (10 μM). (D) Representative traces of PSCs in control (black), in DNQX (red), and in DNQX plus GABAzine (10 μM; blue). (E) Summary bar graphs showing the effects of DNQX (10 μM), APV (100 μM) or CPP (100 μM), and GABAzine (GBZ, 10 μM) or picrotoxin (PTX, 100 μM) on early monosynaptic (left) and late polysynaptic (right) components of PSCs. (F) Summary graph plotting the charge transfer of the polysynaptic component versus the holding potential (n = 12). The dotted line represents linear fit to the data.

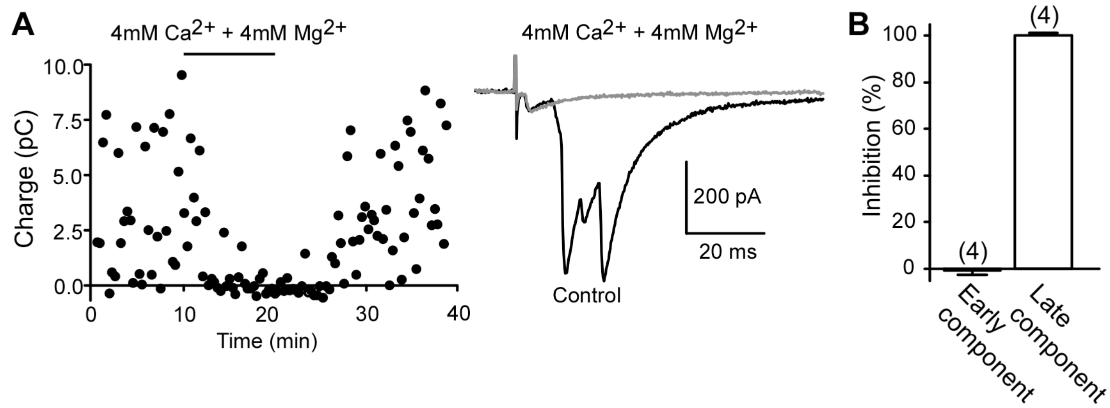


Figure 4. High divalent cation solution selectively blocks the late component of PSCs.

(A) Time graph illustrating the effect of high divalent cation solution containing 4 mM Ca2+ and 4 mM Mg2+ on PSCs. Representative traces of PSCs from the same experiment in control and in high divalent cation solution are shown on the right. (B) Summary bar graph showing the selective blockade of the late component of PSCs by high divalent cation solution.

3.1.2 β2-containing nAChRs mediate polysynaptic $GABA_A$ IPSCs

It is possible that polysynaptic $GABA_A$ IPSCs are mediated by stimulation of local cholinergic fibers, causing excitation of striatal GABAergic neurons via nAChRs. To test this possibility, we examined the effects of different nAChR antagonists. Bath application of general nAChR antagonists, hexamethonium (10-50 μM) or mecamylamine (1-10 μM), selectively abolished the polysynaptic component of $GABA_A$ IPSCs without affecting the early monosynaptic component (Fig. 5A,B). MK-801, a commonly used NMDA receptor antagonist, is known to potently block nAChRs as well (Amador and Dani, 1991; Yamakura et al., 2000). Indeed, MK-801 (20-100 μM) also inhibited the polysynaptic component by 92 ± 5% (n = 8) (Fig. 5B). Furthermore, DHβE (100 nM), a selective antagonist of nAChRs containing β2 subunits, completely suppressed the polysynaptic component (Fig. 5A,B). β2-containing nAChRs are readily desensitized by relatively low concentrations of nicotine (<1 μM) (Giniatullin et al., 2005). Consistent with the involvement of β2-containing nAChRs, the polysynaptic component of $GABA_A$ IPSCs was eliminated by bath application of nicotine (500 nM) for ~5 min (Fig. 5A-C). Recovery from this desensitizing effect of nicotine required tens of minutes, in agreement with the recovery time course of the effects of nicotine on β2-containing nAChR-mediated responses in previous studies using brain slices (Zhou et al., 2001; Mansvelder et al., 2002). During this slow recovery, the number of peaks in IPSCs gradually increased (Fig. 5A, bottom trace), suggesting a gradual increase in the number of spikes in GABAergic neurons mediating polysynaptic transmission. Scopolamine (1 μM), a muscarinic ACh receptor antagonist, had no measurable effect on either component of $GABA_A$ IPSCs (n = 3, data not shown). These results demonstrate that

stimulation of intrastriatal cholinergic fibers excites GABAergic neurons via activation of β2-containing nAChRs, inducing polysynaptic $GABA_A$ IPSCs in cholinergic interneurons.

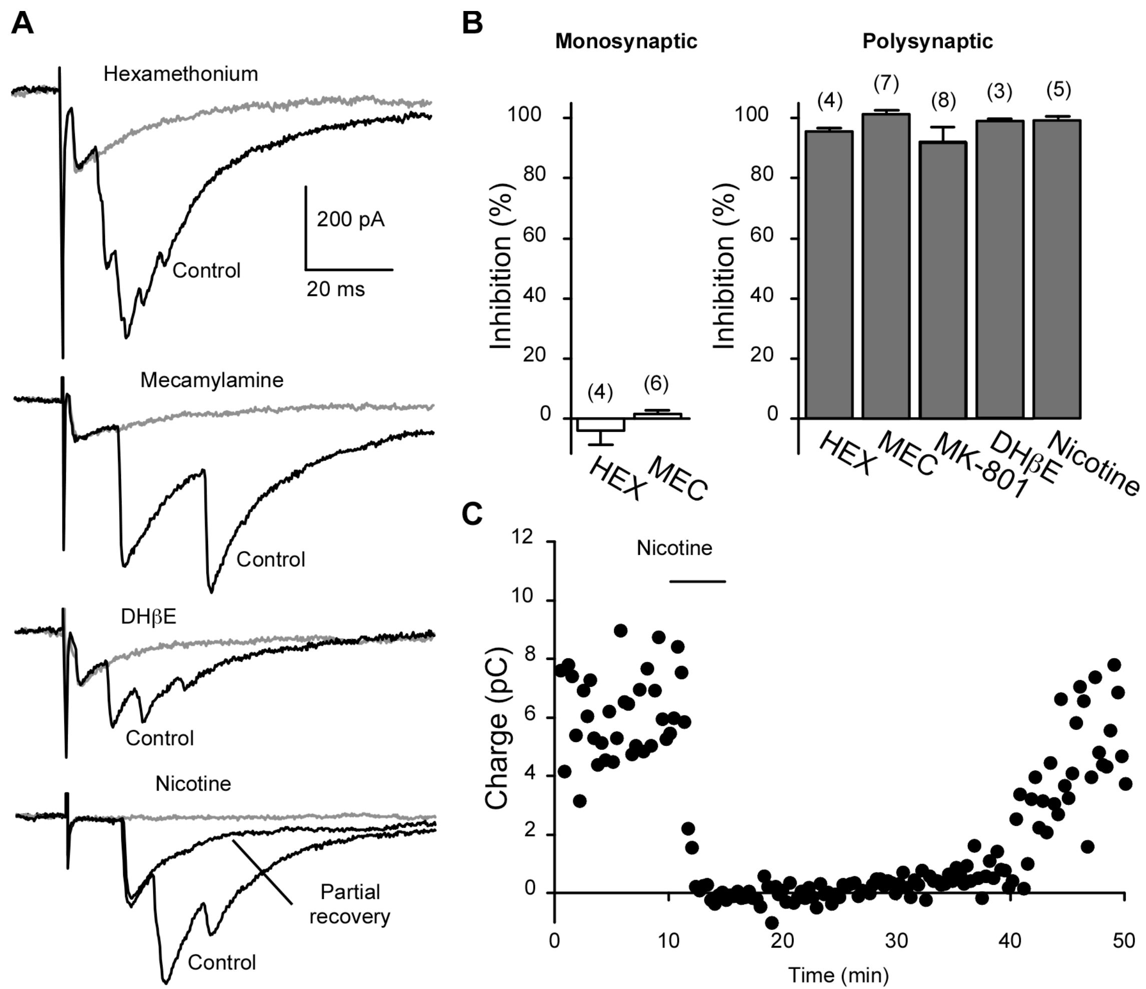


Figure 5. β2-containing nAChRs mediate polysynaptic $GABA_A$ IPSCs.

(A) Representative traces of $GABA_A$ IPSCs illustrating the effects of hexamethonium (50 μM), mecamylamine (10 μM), DHβE (100 nM), and nicotine (500 nM). All of these drugs selectively blocked the late polysynaptic component. (B) Bar graphs summarizing the effects of hexamethonium (HEX, 10-50 μM), mecamylamine (MEC, 1-10 μM), DHβE (100 nM), and nicotine (500 nM) on monosynaptic (left) and polysynaptic (right) components of IPSCs. (C) Time graph of a representative experiment depicting the effect of nicotine (500 nM) on the charge carried by polysynaptic $GABA_A$ IPSCs. Note the slow time course of recovery.

3.1.3 Activation of a single cholinergic interneuron elicits polysynaptic $GABA_A$ IPSCs

We next asked if activation of a single cholinergic interneuron can drive polysynaptic inhibition. To test this, an unclamped action potential was evoked every 20 s by a 2-ms depolarizing pulse in cholinergic interneurons voltage clamped at -87 mV. These experiments were done in the presence of a muscarinic ACh receptor antagonist scopolamine (1 μM). The generation of an action potential was confirmed by a large action current during the voltage step (Cui et al 2007). This resulted in a "feedback" PSC in the same neuron in 9 out of 428 neurons tested (197 ± 35 pA, 16.0 ± 1.2 ms latency from the peak of action current) (Fig. 6). We further performed dual recordings from pairs of cholinergic interneurons (20-150 μm apart). An unclamped action potential evoked in one neuron produced a "feedforward" PSC in the other neuron in 7 out of 199 pairs (136 ± 50 pA, 13.8 ± 1.1 ms latency). The mean success rate to cause a PSC with each action potential was 48 ± 5% in these 16 cases. Interestingly, both feedback and feedforward PSCs were observed in 3 pairs. In 2 of these 3 pairs, activation of one of the two neurons evoked PSCs in itself and the other neuron (an example is illustrated in Fig. 6A-C), whereas in the other pair, PSCs were induced only in one of the two neurons by activation of either neuron. In the former case, the induction of feedback PSCs and that of feedforward PSCs succeeded or failed together with each action potential, suggesting that both neurons in the pair were contacted by the same GABAergic neuron(s) (Fig. 6B,C). Reciprocal feedforward PSCs were not observed in 199 pairs tested. These PSCs elicited by an action potential in a single presynaptic neuron routinely had only one peak, although 2-3 peaks were occasionally detected in 2 out of the 16 cases (Fig. 6D),

suggesting multiple spikes in a GABAergic neuron connecting two cholinergic interneurons in the pair and/or recruitment of multiple GABAergic neurons.

Feedback (n = 5) and feedforward (n = 5) PSCs were completely abolished by both DHβE (100 nM) and GABAzine (10 μM) (Fig. 6B,E), consistent with the idea that they are polysynaptic $GABA_A$ IPSCs mediated by activation of GABAergic neurons via β2-containing nAChRs. In line with this, PSCs reversed at -49 ± 3 mV (n = 3), close to the estimated E_{Cl} of -47 mV (Fig. 6F). These results show that action potential firing in a single cholinergic interneuron can induce polysynaptic IPSCs in both itself and nearby cholinergic interneurons.

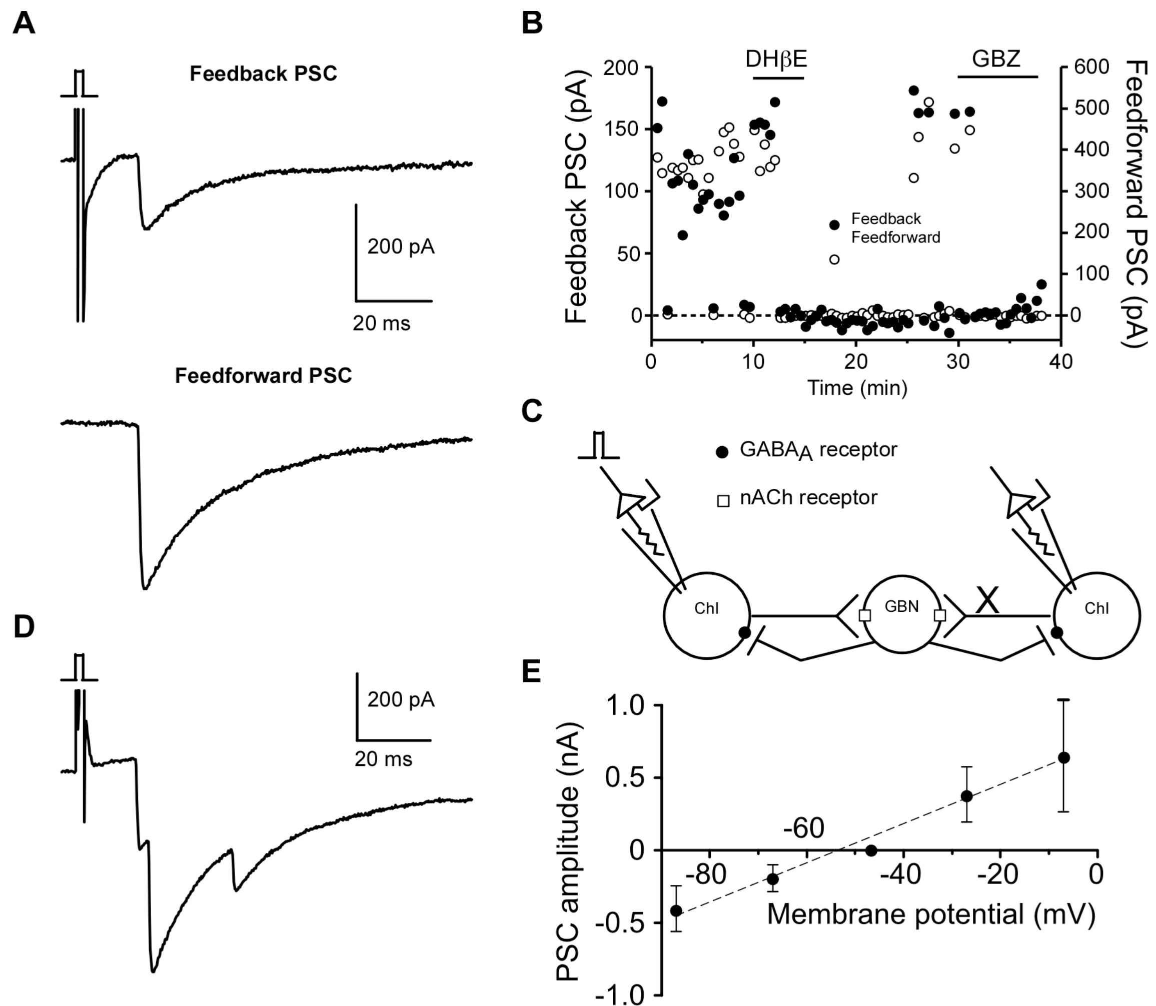


Figure 6. Recurrent polysynaptic inhibition between single cholinergic interneurons.

(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 of 80 mV from a holding potential of -87 mV. (B) Representative time graph showing the effects of DHβE (100 nM) and GABAzine (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). An intermediate GABAergic neuron (GBN) 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. (D) An example trace of a feedback PSC exhibiting three distinct peaks. (E) DHβE and GABAzine completely eliminated PSCs produced by activation of single cholinergic

interneurons in 10 experiments performed as in (B). (F) Summary graph showing I-V relationship of PSCs evoked by stimulation of single cholinergic interneurons ($n = 3$). The dotted line represents linear fit to the data.

3.1.4 Polysynaptic $GABA_A$ IPSCs are depressed by repetitive firing

Cholinergic interneurons fire tonically at ~1-8 Hz both *in vitro* and *in vivo* (Wilson et al., 1990; Bennett et al., 2000). If action potentials in single cholinergic interneurons are capable of eliciting polysynaptic IPSCs, one would expect to observe many spontaneous polysynaptic IPSCs. However, we rarely detected large spontaneous IPSCs (>50 pA) resembling the polysynaptic IPSC triggered by an action potential elicited in voltage-clamped cholinergic interneurons. Thus, it is possible that polysynaptic IPSCs are depressed during repetitive firing of cholinergic interneurons. To test this, we evoked a train of action potentials (5 at 2 Hz) in cholinergic interneurons that produced either feedback (n = 4) or feedforward (n = 1) IPSCs, and then elicited a single action potential with different intervals (1-10 s) after the train (Fig. 7A). These recordings were done in scopolamine (1 μM). The average success rate of a presynaptic action potential to evoke a polysynaptic IPSC was 59 ± 8 % for the first spike of the train (n = 5) (Fig. 7B,C). However, subsequent spikes in the train completely failed to produce polysynaptic IPSCs, suggesting a failure of firing in GABAergic neurons mediating polysynaptic transmission. The success rate gradually recovered over seconds and reached 60-90% of the initial value after 10 s. There was no detectable change in the amplitude of polysynaptic IPSCs during the recovery period, implying that GABAergic synapses on the postsynaptic cholinergic interneurons were not significantly depressed.

We further investigated this short-term depression by applying a train of 5 intrastriatal stimuli at 2 Hz. These experiments were done in the presence of DNQX (10 μM), scopolamine (1 μM), and sulpiride (10 μM). In 4 cells tested at threshold intensity (100-200 μA), the first stimulus in the train evoked single-peak IPSCs averaging 210 ±

52 pA with a latency of 13.3 ± 0.5 ms. The mean success rate for the first stimulus to trigger a polysynaptic IPSC was 64 ± 9%. The amplitude, latency, and success rate were comparable to the values for the IPSCs produced by an action potential elicited in a single cholinergic interneuron voltage clamped at -87 mV, suggesting that a single cholinergic fiber, likely severed from the tonically firing soma in a slice preparation, is stimulated at threshold intensity. In line with this idea, the second through fifth stimuli in the train were completely ineffective at triggering IPSCs (Fig. 7D), similar to the spike train evoked in a single cholinergic interneuron. However, when higher stimulus intensity (500-600 μA) was used to evoke polysynaptic IPSCs with multiple peaks, the fifth stimulus in the 2-Hz train still produced robust polysynaptic IPSCs: the charge carried by polysynaptic IPSCs evoked by the fifth stimulus was 40 ± 9% of the charge of those evoked by the first stimulus (n = 5) (Fig. 7D,E). The onset latency of polysynaptic IPSCs was prolonged from 8.9 ± 0.5 ms for the first stimulus to 13.6 ± 0.7 ms for the fifth stimulus ($p < 0.01$), suggesting that nAChR-mediated excitation of GABAergic neurons was reduced during the train. Although monosynaptic IPSCs also displayed some depression during the 2-Hz train, the magnitude of depression was much smaller than that for polysynaptic IPSCs: the amplitude of monosynaptic IPSCs elicited by the fifth stimulus was 77 ± 7% of the amplitude of those caused by the first stimulus (n = 4).

Taken together, these results demonstrate that repetitive firing of cholinergic interneurons leads to depression of polysynaptic $GABA_A$ IPSCs mostly due to a reduction in cholinergic excitation of GABAergic neurons without much depression of GABAergic synapses onto the postsynaptic cholinergic interneurons. However, simultaneous activation of multiple cholinergic fibers can partially overcome this depression and produce sufficient nAChR-dependent depolarizations in GABAergic neurons, thus reliably triggering polysynaptic IPSCs in cholinergic interneurons.

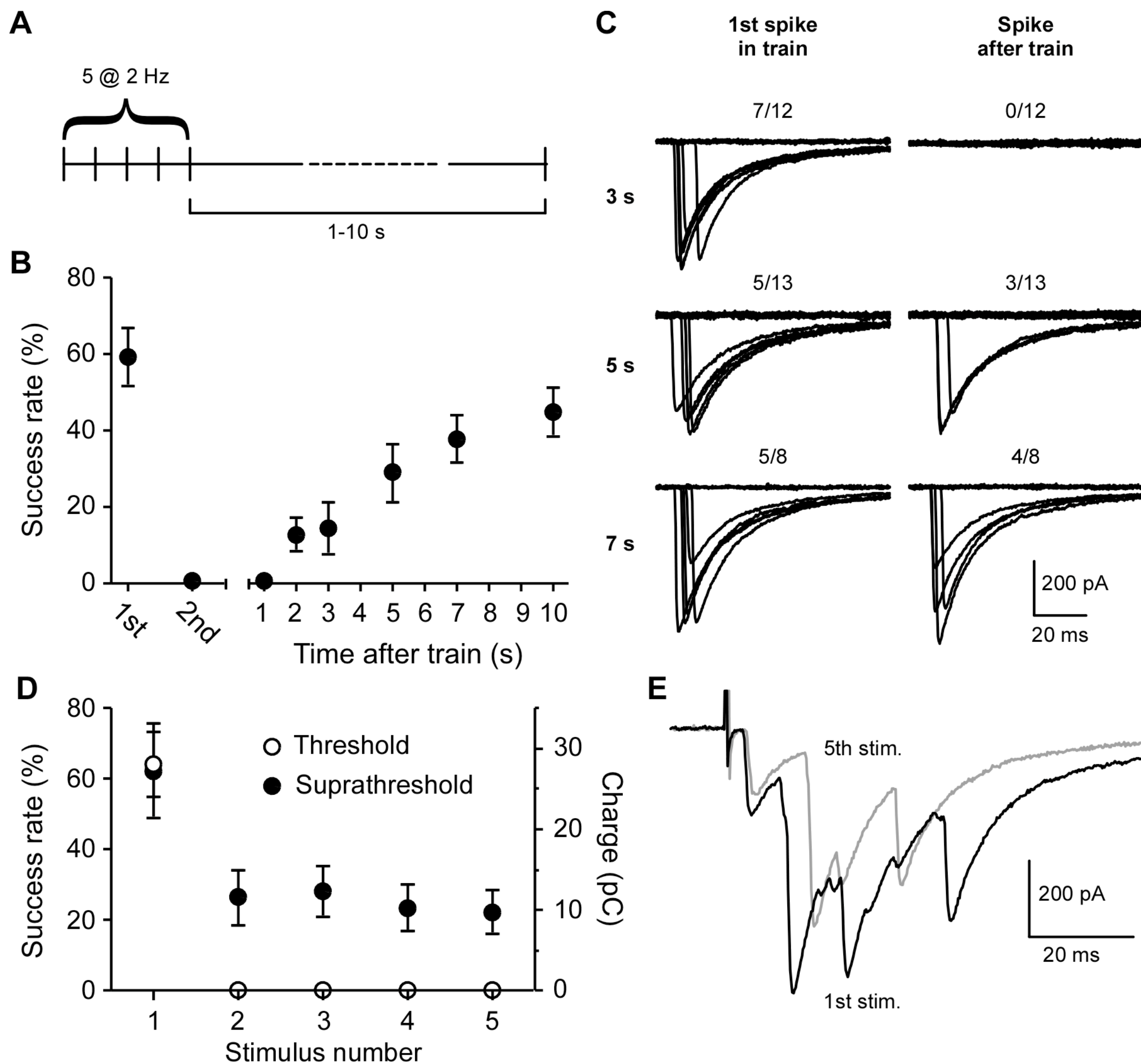


Figure 7. Short-term depression of polysynaptic IPSCs.

(A) Illustration of the protocol used to evoke a train of action potentials in presynaptic cholinergic interneurons. Depolarizing pulses were applied in a train of 5 at 2 Hz, followed by a single pulse applied at various intervals after the train. (B) Summary data from 5 cells tested with the protocol depicted in (A). The success rate for a presynaptic action potential to evoke an IPSC is plotted for the first and second spikes in the train and for the single spikes evoked at different intervals (1-10 s) after the train. Data for the third through fifth spikes in the train, which always failed to evoke IPSCs, are not shown for simplicity. (C) Traces of IPSCs from an experiment using the protocol in (A). Feedforward PSCs were evoked in this particular experiment. In each row, traces

of IPSCs are shown superimposed for the first spike in the train (left column) and for the single spike evoked at the indicated interval after the train (right column). The number of successes out of the number of trials is indicated above each group of traces. (D) Summary graph illustrating short-term depression of polysynaptic IPSCs evoked by a train of 5 intrastriatal stimuli at 2 Hz. The success rate for each stimulus to elicit a polysynaptic IPSC is plotted for experiments where IPSCs were evoked using threshold stimulus intensity (n = 4), while the charge carried by polysynaptic IPSCs is plotted for experiments using suprathreshold stimulus intensity (n = 5). (E) Representative traces of IPSCs induced by the first and fifth stimuli in the 2-Hz train using suprathreshold intensity.

3.1.5 Polysynaptic inhibition leads to suppression of tonic cholinergic interneuron firing

Striatal cholinergic interneurons (i.e., TANs) display a pause of tonic firing in response to sensory stimuli during associative learning *in vivo* (Aosaki et al., 1994b; Apicella, 2007). To examine how polysynaptic inhibition affects cholinergic neuron activity, we tested the effect of intrastriatal stimulation on the spontaneous firing of cholinergic interneurons recorded using cell-attached configuration. Firing recordings were done in DNQX (10 μM) to prevent time-locked action potentials triggered by monosynaptic EPSPs and also in eticlopride (100-200 nM) to eliminate the potential contribution of D_2 receptor-dependent inhibition of Na^+ channels in cholinergic interneurons resulting from stimulation of dopaminergic fibers (Maurice et al., 2004). Under these conditions, intrastriatal stimulation (400-600 μA) produced a pronounced suppression of tonic firing that started ~20 ms after the stimulus and lasted for ~250 ms (Fig. 8). Furthermore, $GABA_A$ antagonists, GABAzine (10 μM) or picrotoxin (100 μM), and a general nAChR antagonist hexamethonium (50 μM) blocked this suppression of tonic firing. None of these drugs significantly changed the baseline firing frequency. These results demonstrate that nAChR-mediated polysynaptic GABAergic inhibition produces a transient suppression of cholinergic interneuron firing.

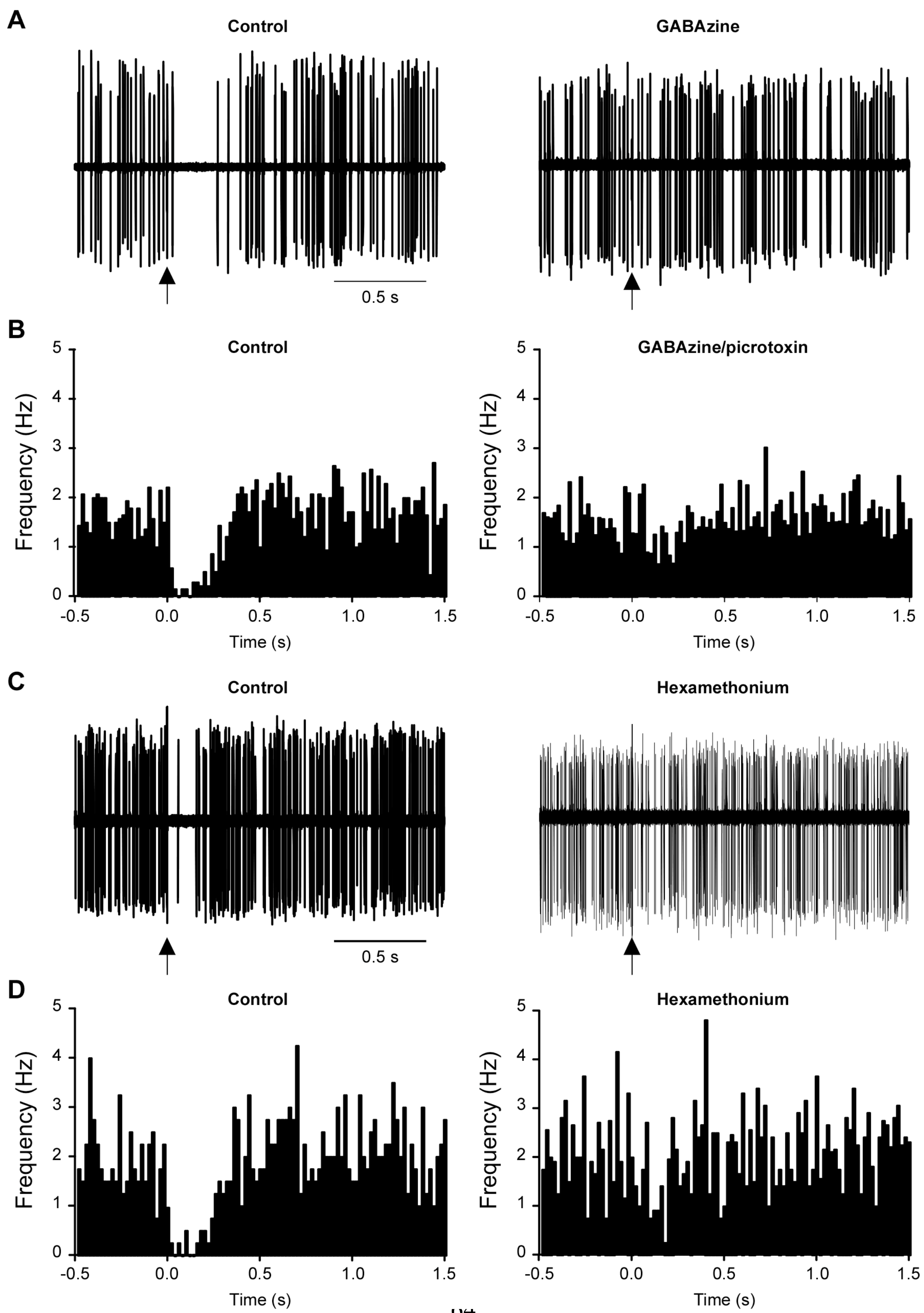

A
Control
GABAzine
0.5 s
B
Control
GABAzine/picrotoxin
Frequency (Hz)
Time (s)
C
Control
Hexamethonium
0.5 s
D
Control
Hexamethonium
Frequency (Hz)
Time (s)

Figure 8. Polysynaptic $GABA_A$ IPSCs cause transient suppression of spontaneous cholinergic interneuron firing.

(A) Representative traces of the firing activity of a cholinergic interneuron in control and in GABAzine (10 μM). Fifty traces are overlaid in each condition. Intrastriatal stimulation was applied at the time indicated by the arrow. The baseline firing frequency was 1.1 Hz and 1.2 Hz in control and in GABAzine, respectively. (B) Peristimulus time histograms (bin width = 20 ms) of cholinergic interneuron firing in control and in GABAzine (10 μM) or picrotoxin (100 μM). Intrastriatal stimulus was applied at time 0. The data were averaged from 7 cells. The duration of the firing suppression in control was 260 ms in these 7 cells. (C) Overlay of 50 traces of the firing of a cholinergic interneuron in control and in hexamethonium (50 μM). The baseline firing frequency was 3.0 Hz and 3.3 Hz in control and in hexamethonium, respectively. (D) Averaged histograms in control and in hexamethonium (50 μM) from 4 cells. The duration of the firing suppression was 220 ms in these 4 cells.

3.2 AIM 2: DOPAMINE RECEPTOR ACTIVATION MODULATES POLYSYNAPTIC INHIBITION UPON CHOLINERGIC INTERNEURONS OF THE STRIATUM

3.2.1 Dopamine receptor activation is not required for polysynaptic IPSCs in cholinergic interneurons

Recent evidence indicates that phasic activation of β2-containing nAChRs expressed on dopaminergic terminals facilitates dopamine release in the striatum (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004). To examine the involvement of dopamine in the polysynaptic transmission, we next tested dopamine receptor antagonists. Combined application of a D_1-like receptor antagonist SCH 23390 (1 μM) and a D_2-like receptor antagonist sulpiride (10 μM) had no significant effect on polysynaptic IPSCs (n = 4) (Fig. 9A). Moreover, polysynaptic IPSCs were easily evoked in slices prepared from dopamine-depleted rats (n = 8) (Fig. 9B). We further confirmed that these polysynaptic IPSCs evoked in dopamine-depleted slices were blocked by GABAzine (10 μM, n = 5), hexamethonium (50 μM, n = 5), or DHβE (100 nM, n = 2) (Fig. 9B,C). From these results, we conclude that dopamine is not directly involved in the generation of nAChR-mediated polysynaptic $GABA_A$ IPSCs.

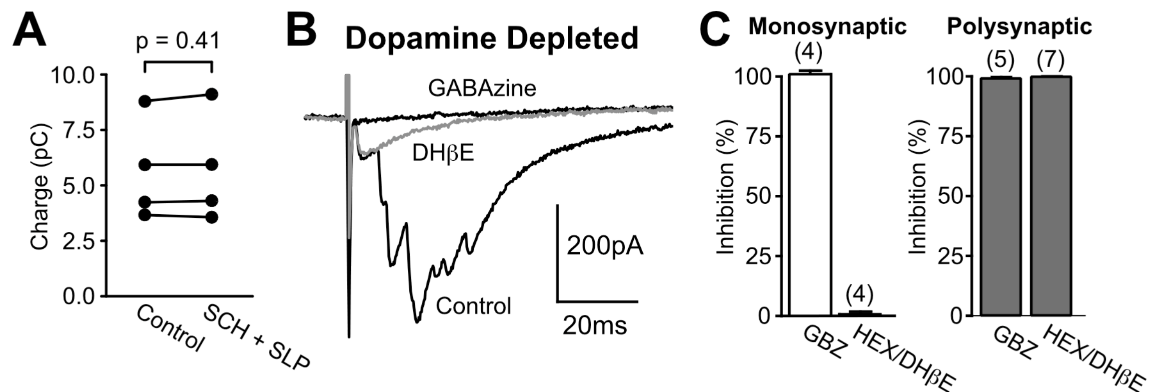


Figure 9. Dopamine does not mediate polysynaptic IPSCs.

(A) The charge carried by polysynaptic IPSCs before and after coapplication (7-10 min) of SCH 23390 (SCH; 1 μM) and sulpiride (SLP; 10 μM) is plotted for individual cells (n = 4). (B) An example trace of an IPSC having both monosynaptic and polysynaptic components in a cholinergic interneuron from a dopamine-depleted rat. A trace (gray) in DHβE (100 nM) and that in GABAzine (10 μM) are also shown. (C) Summary bar graphs illustrating the effect of GABAzine (GBZ, 10 μM) and that of hexamethonium (HEX, 50 μM) or DHβE (100 nM) on monosynaptic (left) and polysynaptic (right) components of IPSCs in cholinergic interneurons from dopamine-depleted rats.

Chapter 4: Discussion

The main finding of the present study is that striatal cholinergic interneurons communicate with each other via GABAergic neurons. This recurrent polysynaptic connection among cholinergic interneurons is dependent on the activation of β2-containing nAChRs. Although nAChR-mediated excitation of GABAergic neurons is depressed during repetitive cholinergic interneuron firing, simultaneous activation of many cholinergic fibers by intrastriatal stimulation reliably evokes polysynaptic $GABA_A$ IPSCs in cholinergic interneurons, resulting in a transient suppression of tonic firing. These findings illustrate a novel microcircuit within the striatum that exerts powerful control over the firing activity of cholinergic interneurons.

4.1 The mechanism of polysynaptic transmission

Polysynaptic $GABA_A$ IPSCs described here have not been observed in previous studies recording synaptic responses of cholinergic interneurons in striatal slices (Bennett and Wilson, 1998; Calabresi et al., 1998c; Momiyama and Koga, 2001; Centonze et al., 2003; Pakhotin and Bracci, 2007). Although the exact reason for the discrepancy is unclear, it may be due to the difference in experimental conditions, such as temperature, location and type of the stimulating electrode, slice plane, and use of MK-801 as an NMDA receptor antagonist, which actually blocks nAChRs as well (Amador and Dani, 1991; Yamakura et al., 2000). Of particular interest is the previous report demonstrating that stimulation of the subcortical white matter containing both cortico- and thalamostriatal glutamatergic fibers results in polysynaptic GABAergic inhibition

through activation of AMPA receptors (Suzuki et al., 2001). This polysynaptic GABAergic inhibition was observed in only ~10% of the recorded cholinergic interneurons, whereas polysynaptic IPSCs were invariably evoked in our case using intrastriatal stimulation. It is possible that our results have characterized part of the pathway underlying the polysynaptic IPSCs described in this previous study. In this scenario, coincident glutamatergic excitation presumably evokes synchronous firing of a population of cholinergic interneurons via AMPA receptors, leading to nAChR-dependent excitation of GABAergic neurons and subsequent inhibition of cholinergic interneurons.

Dopamine has been shown to play an important role in the pause response of tonically active cholinergic interneurons in behaving animals (Aosaki et al., 1994a; Watanabe and Kimura, 1998). However, phasic dopamine release in the striatum may not directly trigger the pause, since it can be observed in the absence of phasic dopamine response under certain conditions during sensorimotor conditioning (Morris et al., 2004). Moreover, systemic administration of a general dopamine receptor agonist apomorphine can restore the pause response suppressed by infusion of dopaminergic neurotoxin into the striatum (Aosaki et al., 1994a), suggesting a permissive role of tonic dopamine in enabling pause generation.

Dopamine receptor antagonists or dopamine depletion did not affect polysynaptic GABAergic inhibition. This result rules out the scheme in which stimulation of cholinergic fibers indirectly excites striatal GABAergic neurons via phasic dopamine release triggered by activation of β2-containing nAChRs on dopaminergic terminals (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004). Therefore, it is most likely that cholinergic fibers make direct synaptic contacts onto GABAergic neurons that in turn innervate cholinergic interneurons, as illustrated in Fig. 6C.

Evoking an action potential in a single cholinergic interneuron produced polysynaptic IPSCs in itself and nearby cholinergic interneurons. It has been reported recently that firing of a single cholinergic interneuron leads to suppression of glutamatergic and GABAergic transmission onto neighboring spiny projection neurons and cholinergic interneurons via activation of muscarinic ACh receptors (Narushima et al., 2007; Pakhotin and Bracci, 2007). Our results demonstrate that activation of a single cholinergic interneuron can also drive excitation of surrounding GABAergic neurons through activation of nAChRs. The latency of polysynaptic IPSCs triggered by single cholinergic neuron activation, as well as those evoked by threshold intrastriatal stimulation, was fairly long (~14-16 ms), which presumably reflects the time for relatively small nicotinic EPSPs to elicit a spike in GABAergic neurons. Although polysynaptic connectivity between single cholinergic interneurons was sparse (~3%), stimulation of intrastriatal cholinergic fibers was capable of eliciting polysynaptic IPSCs in all cholinergic interneurons tested in this study. Hence, each cholinergic interneuron invariably receives input from those GABAergic neurons that are activated by local cholinergic input. In other words, each cholinergic interneuron can be polysynaptically influenced by the activity of other cholinergic interneurons, likely reflecting extensive axonal arborizations of GABAergic neurons as well as cholinergic interneurons.

Our results further showed that repetitive firing of cholinergic interneurons at 2 Hz induces depression of nAChR-mediated transmission onto GABAergic neurons. This ensures that polysynaptic inhibition of cholinergic interneurons is not constantly triggered by the spontaneous activity of individual cholinergic interneurons. Therefore, synchronous activation of multiple cholinergic interneurons is necessary to overcome this depression and mediate efficient inhibition of cholinergic neuron excitability. It remains to be determined whether this short-term depression is due to presynaptic depression of

ACh release, postsynaptic desensitization of nAChRs, or both. Activity dependence of cholinergic transmission in the CNS is not well known, mainly because of the difficulty in evoking cholinergic synaptic responses in brain slices (Dani and Bertrand, 2007). β2-containing nAChRs are readily desensitized by a small but sustained elevation of ACh levels (Giniatullin et al., 2005). However, removal of ACh by acetylcholinesterase at synapses is thought to be very rapid (~1 ms), thereby precluding significant nAChR desensitization by synaptically released ACh (Dani et al., 2001). In line with this, β2-containing nAChRs on dopaminergic terminals remain in a non-desensitized, functional state in the striatum despite the constant release of ACh resulting from the tonic activity of cholinergic interneurons (Zhou et al., 2001). Thus, presynaptic depression may play a more dominant role in the short-term depression of cholinergic transmission onto GABAergic neurons. Indeed, repetitive stimulation at 2-20 Hz has been shown to cause short-term depression of nicotinic EPSPs via a presynaptic mechanism in cervical sympathetic ganglion neurons (Birks and Isacoff, 1988).

The present study strongly suggests the presence of GABAergic neurons expressing β2-containing nAChRs in the striatum. In addition to GABAergic projection neurons, a small percentage (2-3%) of striatal neuronal population is comprised of different types of GABAergic interneurons (Tepper and Bolam, 2004). Detailed histochemical evidence indicates that nAChR β2-subunit immunoreactivity cannot be detected in spiny projection neurons (Hill et al., 1993; Jones et al., 2001). nAChR-mediated depolarization has been recently shown in fast-spiking (FS) interneurons, however pharmacological examination has ruled out the involvement of β2-containing nAChRs (Koos and Tepper, 2002). Furthermore, it has been reported that synaptic connectivity between cholinergic and FS interneurons is unidirectional, in that cholinergic terminals make synaptic contacts onto parvalbumin-positive FS interneurons

but not vice versa (Chang and Kita, 1992). On the other hand, reciprocal synaptic contacts between cholinergic interneurons and neuropeptide Y-containing neurons, which corresponds to persistent and low-threshold spike (PLTS) interneurons, has been detected (Vuillet et al., 1992). It should also be noted that PLTS interneurons have depolarized resting membrane potential (-50 to -60 mV) close to action potential threshold and very high input resistance (>500 MΩ) (Kawaguchi, 1993; Tepper and Bolam, 2004). These intrinsic membrane properties, together with their elongated axonal arbors (up to 1 mm) (Kawaguchi, 1993; Kubota and Kawaguchi, 2000), would make PLTS interneurons suited for transforming small excitatory cholinergic input into extensive inhibition of downstream neurons.

4.2 FUNCTIONAL SIGNIFICANCE OF RECURRENT INHIBITION AMONG CHOLINERGIC INTERNEURONS

It is well established that the pause response of cholinergic interneurons, or TANs, to sensory cues develops during reward-based conditioning (Aosaki et al., 1994b; Morris et al., 2004), and the population response of these neurons has been linked to the behavioral output (Blazquez et al., 2002; Apicella, 2007). Previous studies *in vivo* have indicated the important roles of glutamatergic and dopaminergic inputs in the generation of the pause (Aosaki et al., 1994a; Watanabe and Kimura, 1998; Matsumoto et al., 2001; Reynolds et al., 2004). Accordingly, a number of studies *in vitro* have addressed the potential mechanisms underlying the pause based on the premise that it results from the interplay among glutamatergic and dopaminergic inputs, local GABAergic transmission, and intrinsic cholinergic neuron properties (Yan and Surmeier, 1997; Suzuki et al., 2001; Maurice et al., 2004; Wilson, 2005; Deng et al., 2008). However, a pause mimicking the

in vivo pause has not been readily induced by synaptic stimulation in brain slices. In this study, under AMPA receptor blockade to prevent time-locked action potentials and subsequent prolonged afterhyperpolarizations (Bennett et al 2000), intrastriatal stimulation of cholinergic fibers reliably produced a transient suppression of tonic cholinergic interneuron firing for ~250 ms, which is comparable to the duration of the pause response in behaving animals (Aosaki et al., 1995; Morris et al., 2004). The pause response *in vivo* is frequently preceded by a small, brief (<50 ms) increase in firing frequency (Apicella, 2007), which represents roughly synchronized firing of a population of cholinergic interneurons within a short time window. Successive small nicotinic EPSPs during this time window may well summate to produce sizable depolarizations in GABAergic interneurons, thereby driving their firing. Therefore, the polysynaptic inhibitory mechanism described in this study may contribute, at least partially, to the generation of pause by transforming activation of a population of cholinergic interneurons into recurrent inhibition of a larger population of these neurons.

Chapter 5: Conclusions

The network of ChIs within the striatum plays a pivotal role in regulating striatal activity by affecting varied targets and operating over several timescales. In a simplified view, the tonic activity of ChIs establishes a basal level of excitability within the striatum, while the phasic cholinergic signal functions to limit excitability in areas of the striatum not receiving coincident glutamatergic and dopaminergic inputs. This model requires that ChIs (TANs) can pause simultaneously; either by receiving simultaneous input from striatal afferents or via connections within the striatum.

The primary findings of this dissertation indicate that the population of striatal ChIs is connected via a network of GABAergic neurons (Figure 10), and that signaling through this inhibitory network can suppress the firing of ChIs. It is possible that signaling through the described GABAergic network among ChIs contributes to the pause response observed during learning. This hypothesis relies upon evidence that glutamatergic axons from the thalamus provide the primary excitatory input to ChIs in the striatum, and that these axons target areas of the striatum topographically. Accordingly, the GABAergic network among striatal ChIs acts to reverse the polarity of the thalamic signal while also distributing the inhibition among distant ChIs. In this manner, excitatory signals become inhibitory, and area – specific signals become generalized. As a result, MSNs that are near the target ChI and which receive coincident glutamatergic and dopaminergic input while ACh is reduced, transition to an upstate to fire downstream. In contrast, MSNs located away from the topographically specific glutamatergic and dopaminergic inputs are less excitable due to diminished mAChR activity and reduced inhibition of GABA release from FSI terminals. So a pause mediated by a recurrent inhibitory network among ChIs promotes signaling through the targeted area of the striatum and reduces the possibility of signaling outside the target area.

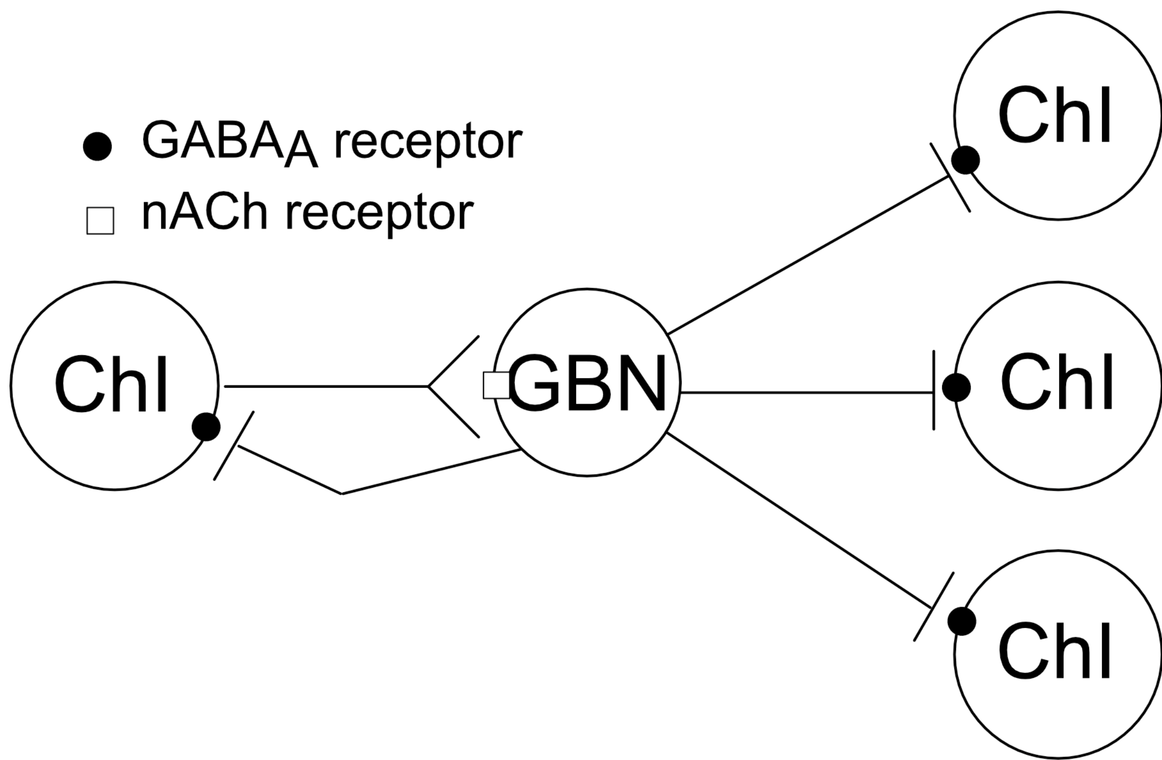


Figure 10. Recurrent inhibitory network among cholinergic interneurons

Diagram of the working hypothesis and findings of this dissertation. The population of cholinergic interneurons (ChI) within the striatum are connected by a network of GABAergic neurons (GBN).

Additionally, the network of GABAergic neurons connecting ChIs provides a location for long – term plastic changes in ChI firing. nAChR activation is required for activation of the GABAergic neurons in the inhibitory network among ChIs, and Ca^{2+} entry through nAChRs provides a possible source of inducing plasticity within the circuit. The mechanism underlying the increase in the number of TANs that pause as an animal learns is unclear, and plasticity dependent upon Ca^{2+} entry through nAChRs may underlie this development. A striking benefit derived from locating the source of plasticity on the GABA neuron within the circuit is that the induction of plasticity will depend upon other striatal inputs as well. For example, the primary source of glutamatergic input to the GABA neuron may arrive from a cortical area, while it is known that the majority of glutamatergic inputs to ChIs are thalamic, and plasticity may be dependent upon coincident nAChR – dependent Ca^{2+} entry and cortex – driven depolarization of the GABA neuron. It is also possible that both thalamic and cortical inputs arrive upon the GABA neuron, as is the case for FSIs. These advantages provided by the recurrent inhibitory network need to be elucidated through experimentation.

Subsequent work is also needed to elucidate the possibility that DA modulates inhibitory signaling between ChIs via the network presented here. As discussed previously, it is possible that GABAergic IPSCs participate in the pause of TAN firing observed *in vivo* during associative learning, yet DA signaling is required for the expression of the pause *in vivo* (Aosaki et al., 1994a) and not necessary for the polysynaptic IPSCs between ChIs presented by this dissertation. Our findings indicate that polysynaptic inhibition between ChIs persists in the presence of dopamine receptor antagonists and in dopamine depleted slices, and this suggests that the GABAergic IPSCs studied in this project may not underlie the pause observed *in vivo*. However, it is likely that the conditions for our experiments and those of Aosaki et al. fail to accurately mimic

the conditions found *in vivo*. Specifically, the role of DA as a modulator of ionotropic signaling is well established, and like other striatal responses, tonic DA helps to establish resting conditions while phasic DA enables the response without actually causing the response. Our synaptic stimulation and Aosaki's use of systemic apomorphine probably overcame the modulatory capabilities of the DA system. Presumably, the synaptic stimulation used in this study recruited so many cholinergic fibers at such a high intensity that any probable signal modulation by DA was obsolete. Likewise, systemic apomorphine probably activated DA receptors in the striatum to levels above the maximum reached by phasic DA neuron signaling *in vivo*. It would be interesting to observe the occurrence of the pause during local injection of selective DA receptor agonists *in vivo.*

To conclude, this dissertation presents evidence for a novel inhibitory network connecting ChIs of the striatum. The reported findings are the first to mimic the learning – related pause of TAN firing in a brain slice preparation. More importantly, signaling through this network requires activation of nAChRs, and provides a mechanism to transfer the simultaneous excitation of a small number of ChIs into the inhibition of a widespread population of striatal ChIs.

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Vita

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