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CHOLINERGIC-GABAERGIC CIRCUITS IN THE NEOSTRIATUM

BY

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ABSTRACT OF THE DISSERTATION

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The basal ganglia are a system of subcortical nuclei whose functions include regulating the motivation for voluntary actions. One key aspect of this function is to assist in action selection, often referred to popularly as decision making, by keeping track of past results and utilizing this information to produce ideal decisions and by signaling the motivational significance of environmental events. Two types of basal ganglia neurons have been most extensively studied in terms of their role in these functions: the dopaminergic neurons of the substantia nigra pars compacta and the tonically active cholinergic interneurons of the neostriatum. Both have stereotypic responses to motivationally salient stimuli, and it is assumed that this information is used in choosing the most beneficial response.

The focus of my thesis work was to investigate how the responses of neostriatal cholinergic interneurons to motivationally salient stimuli (commonly termed the pause response) are translated into downstream effects in projection neurons. My underlying hypothesis was that if these signals are to participate in the translation of a temporally brief and important environmental event into changes in behavior, that there must be a fast and temporally precise effect on the projection neuron output to other brain structures. Although striatal projection neurons express receptors for acetylcholine, they are of the slow acting muscarinic type, not the fast acting nicotinic type. Therefore, fast cholinergic regulation of projection neurons is assumed to be mediated by changes in synaptic input.

We utilized technologies including optogenetics and transgenic mice to recapitulate the pause response of cholinergic interneurons in acute brain slice preparations, which preserve much of the intrinsic neostriatal circuitry, as well in behaving mice. We obtained recordings of neostriatal neurons using *in vitro* whole-cell and *in vivo* extracellular methods. This revealed a novel cholinergic-GABAergic circuit, involving a recently described GABAergic interneuron similar to cortical neurogliaform interneurons, which translate the pause response into fast and powerful inhibition of projection neurons. In addition to uncovering an unknown and unpredicted mechanism of action of acetylcholine in the neostriatum, these results demonstrate the power of combining traditional electrophysiological recording methods with current optogenetic and transgenic techniques.

PREFACE

The hypothesis guiding my thesis work is that highly conserved firing patterns of cholinergic interneurons in the neostriatum should have a fast effect on ongoing striatal activity. This hypothesis is based upon numerous studies each showing a consistent time-locked response to motivationally salient events, termed the pause response, which varies very little in its temporal structure or in its relationship to stimulus onset. Cholinergic interneurons make up ~1% of the striatum (on average across species) and are sparsely distributed. This makes it nearly impossible to experimentally induce these activity sequences in order to observe directly their downstream effects.

The availability of optogenetic tools, first in the form of plasmids, from which one had to produce your own virus, and finally in the form of readily available and ready to inject live viruses, opened up the possibility of investigating the pause response in more detail than was ever possible. We quickly determined that in acute brain slices, in which cholinergic interneurons expressed an opsin, we could either turn on or off these neurons with optical stimuli. Additionally, the intrinsic physiology of these neurons seemed untouched by transgene expression. Thus, using optogenetics, we were finally able to recapitulate the pause response pattern of cholinergic interneurons and record the effect on other striatal neurons. This led to discovery of a novel circuit in the striatum, which involves a novel interneuron identified in a parallel study in our laboratory. This circuit appeared, at least in brain slices, to translate synchronous excitation of cholinergic interneurons into fast inhibition of all recorded projection neurons. A surprising finding was that the pause seemed to do nothing (in terms of fast effects, we did not look at neuromodulatory effects), rather it was the excitation that mattered, with or without a pause. There was genuinely no expectation of the result that the synchronous excitation would do this, especially in light of the fact that cholinergic interneurons are tonically active. In part due to the unexpected nature of this result, we felt a strong need to attempt to look at this circuitry in vivo. Our first attempts at this, which are not presented here, utilized anesthetized mice using single channel juxtacellular recording. We did manage to record one channelrhodopsin-2 (ChR2) expressing cholinergic interneuron (defined as a tonically active neuron with a wide action potential which was excited by blue light), and a putative spiny projection neuron (pSPN), the latter of which having a complex response to optical stimulation. An essential component of all of our experiments was and is maintaining the strictest standards in terms of the physiological relevance of our optogenetic preparations. A problem with ChR2 in this regard is that it is expressed over nearly the entire plasma membrane surface, including axon terminals, and thus will likely change the biophysics of neurotransmitter release for several reasons, among them its permeability to calcium. The last thing we wanted to do was to generate an artifact with this new tool. For this reason we felt much safer using Halorhodopsin, which can be engineered to avoid axonal and terminal expression and can induce rebound action potentials, following optical hyperpolarization, which are initiated by intrinsic membrane properties. With that in mind, even though I had some data from the ChR2 experiment in vivo, when high-titer Halorhodopsin virus became available I immediately switched preparations. This quickly led to the cessation of anesthetized animal recordings, as it appeared, maybe not surprisingly to some, that the anesthesia changed the activity state of the

cholinergic interneurons. At first, this seemed liked a huge impediment to our progress. However, after going to the International Basal Ganglia Society (IBAGS) meeting in June 2010, I was inspired to initiate a set of experiments combining optogenetic manipulation of cholinergic interneuron activity with singe unit recording in behaving mice. I was extremely lucky to be working at the Center for Molecular and Behavioral Neuroscience (CMBN) at this point. Dr. Gyorgy Buzsaki's laboratory was down the hall, and they are not only expert at *in vivo* recordings but were moving into optogenetics as well. In a few months, I had set up a 16-channel in vivo recording system in our laboratory and was recording, in freely moving mice, Halorhodopsin expressing cholinergic interneurons identified by their immediate and complete inhibition when stimulated with yellow or green light. I quickly identified the parameters necessary to recapitulate the pause response, and then set out to record presumed spiny projection neuron responses to this stimulus. We found that pause-excitation sequences of cholinergic interneurons, in behaving mice, have no measurable effect on projection neurons during the pause phase, but after the pause ends and the cholinergic neurons synchronously fire rebound spikes, projection and other neurons are strongly inhibited. This was the confirmation we needed that the circuit we dissected in vitro was functional in vivo, and was worth all our effort and excitement.

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Additionally I would like to thank my family for supporting me in this academic endeavor, as they always have in the past. Finally, I would like to thank one specific fellow student, Julia C. Basso, who just so happens to be my fiancé.

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Introduction

Overview

The basal ganglia are an interconnected group of subcortical nuclei including the neostriatum, globus pallidus, subthalamic nucleus and substantia nigra. It is involved in multiple behavioral processes including the regulation of the motivation for voluntary movement as well as cognitive processes such as reward learning (Isoda & Hikosaka, 2011; Jankovic & Kurlan, 2011; Shohamy, 2011; Smith *et al.* 2011). Dysfunction of this system underlies the pathophysiology of several neurological disorders including Parkinson's disease (Carlsson, 1972; Surmeier *et al.* 2009), Huntington's disease (Eidelberg & Surmeier, 2011; Vonsattel *et al.* 2011) and Gilles de la Tourette's syndrome (Kalanithi *et al.* 2005; Kataoka *et al.* 2010; Leckman *et al.* 2010), as well as psychiatric disorders such as addiction to drugs of abuse (Fasano & Brambilla, 2002; Graybiel, 2008; Volkow *et al.* 2011).

The neostriatum is the largest nucleus of the basal ganglia (Oorschot, 1996), receives the majority of the cortical and thalamic inputs and is believed to be responsible for the bulk of information processing in the system (Graybiel & Ragsdale, 1979; Albin *et al.* 1989; Graybiel, 1995; Oorschot, 1996; Wise *et al.* 1996). The principal neurons of the striatum, often referred to as medium spiny neurons but more accurately termed spiny projection neurons (SPN), are GABAergic and make up ~95% of the neurons in the structure (Bolam *et al.* 2000). SPNs are divided into direct (striatonigral projecting) and indirect (striatopallidal projecting) pathway types, in reference to their connections with the substantia nigra pars reticulata (SNr) (Penney & Young, 1983; Albin *et al.*

1989; 1995; Parent & Hazrati, 1995). It is important to note that this classification is not perfect, as some neurons of the direct pathway make collateral projections to the globus pallidus (Chang et al. 1981; Kawaguchi et al. 1990). The SNr is the primary output nucleus of the basal ganglia (the others are in primates the internal segment of the globus pallidus and in rodents the entopedunclular nucleus) and makes inhibitory projections primarily to the cortically projecting ventral anterior and ventral lateral thalamic relay nuclei (Graybiel & Ragsdale, 1979; Beckstead, 1983; Finch, 1996; Smith et al. 2009). It is generally considered in the popular two-pathway model of basal ganglia function that activity of direct pathway SPNs results in dis-inhibition of the thalamic relay nuclei due to their inhibition of the inhibitory SNr neurons, and that activity of the indirect pathway has the opposite effect through its inhibition of the globus pallidus which inhibits the subthalamic nucleus which excites SNr neurons (Penney & Young, 1983; Albin et al. 1989; 1995). SPNs are thus considered to control the output of the basal ganglia with activity in each of the two pathways having opponent influence on its output (Penney & Young, 1983; Albin et al. 1989; 1995; Jin & Costa, 2010). The interaction of intrinsic membrane properties of SPNs in combination with synaptic inputs results in clearly identifiable up and down states in electrophysiological recordings (Wilson & Groves, 1981; Wilson & Kawaguchi, 1996), and excitatory inputs are only translated into action potentials when the neuron is in the up state (Wilson, 1986; Stern et al. 1997; Stern et al. 1998). Nearly all cortical areas project to the striatum, and except for the thalamus, the striatum receives inputs from a greater diversity of cortical areas than any other brain structure (Graybiel & Ragsdale, 1979; Ragsdale & Graybiel, 1990; Graybiel, 1991; Reiner *et al.* 2010). The broad cortical excitation drives the down-to-up state transition,

and SPNs over areas of up to 1mm are state-correlated (Stern *et al.* 1998). Individual action potentials, however, are not correlated between pairs of SPNs even when both are in the up state, suggested that additional synaptic inputs contribute to spike timing (Stern *et al.* 1997; Stern *et al.* 1998).

In addition to SPNs, the neostriatum contains GABAergic and cholinergic interneurons, which make synapses on SPNs as well as one another (Tepper & Bolam, 2004). The most studied GABAergic interneuron, termed the fast spiking interneuron, (FSI), expresses the calcium binding protein parvalbumin, is capable of firing at sustained rates of >200 Hz, and strongly inhibits SPNs via GABAA receptors located at proximal dendritic and peri-somatic synapses (Kita et al. 1990; Kawaguchi, 1993; Koos & Tepper, 1999; Koos et al. 2004). More rare GABAergic interneuron types such as those expressing neuropeptide-Y (NPY) (Vuillet et al. 1990; Vuillet et al. 1992; Partridge et al. 2009; Ibanez-Sandoval et al. 2011) or tyrosine-hydroxylase (TH) (Ibanez-Sandoval *et al.* 2010) have also been shown to inhibit SPNs through $GABA_A$ receptors. Cholinergic interneurons are the sole source of acetylcholine to striatal neurons (Graybiel & Ragsdale, 1978; Zhou et al. 2002). Cholinergic influences on SPNs include regulating intrinsic ionic conductances through muscarinic type receptors (Zhou et al. 2003; Lin et al. 2004) (SPNs do not express nicotinic type acetylcholine receptors (Zhou et al. 2002)) and modulation of glutamatergic (Pakhotin & Bracci, 2007; Ding et al. 2010) and dopaminergic (Kudernatsch & Sutor, 1994) inputs through both pre- and post-synaptic receptors. Dopamine plays an essential role in striatal function, acting through DA receptors expressed by SPNs as well as by interneurons and presynaptic terminals of afferent inputs (Young & Penney, 1984; Surmeier et al. 1992; Smith et al.

1994; Nicola *et al.* 2000; Kreitzer & Malenka, 2008; Lerner & Kreitzer, 2011). The roles of ACh and DA have typically been considered neuromodulatory (Kudernatsch & Sutor, 1994; Gerfen *et al.* 1995; Harsing & Zigmond, 1998; Nicola *et al.* 2000; Zhou *et al.* 2003), and thus the GABAergic interneurons have been thought of as playing a more critical role in the timing of individual action potentials in SPNs and thus determining the precise timing of basal ganglia output (Bennett & Bolam, 1994b; Koos & Tepper, 1999; Bolam *et al.* 2000; Koos *et al.* 2004; Gittis *et al.* 2010; Ibanez-Sandoval *et al.* 2010; 2011; Unal *et al.* 2011).

Electrophysiological recordings in behaving animals have identified basal ganglia neurons having activity patterns indicating their direct involvement in learning. Of these, the best known are the dopaminergic (DA) neurons of the midbrain (Bertler & Rosengren, 1959; Barbeau, 1961; 1969; Lanska, 2010), which supply the basal ganglia and frontal cortices with information about motivationally salient environmental events in the form of phasic bursts of dopamine release (Schultz, 1986; 2001; 2007; Hauber, 2010; Schultz, 2010). The cholinergic interneurons (referred to as TANs, for Tonically Active Neuron, in primate extracellular recordings studies and as cholinergic interneurons in intracellular recording studies) respond largely at the same time and to the same stimuli as the DA neurons (Kimura et al. 1984; Apicella et al. 1991b; Morris et al. 2004). This response consists of phasic pauses in ongoing activity, which often include flanking or following excitation above baseline firing rates, the sequence lasting 200-300ms in its entirety (Kimura et al. 1984; Apicella et al. 1991b; Morris et al. 2004; Apicella, 2007; Joshua et al. 2008). Generally, this has been referred to as the pause response, as the pause phase is the most reliably observed. The relationship between the pause response and specific

aspects of learned behaviors has been intensively investigated for nearly three decades and we now have a fairly good understanding of its behavioral correlates. Studies of the precise downstream effects of the pause response on SPNs, in particular those posited to occur at short time scales, have lagged behind and have fallen short of a conclusive functional demonstration. It is unclear how the known modulatory mechanisms of ACh would be capable of affecting SPN spike timing on time-scales short enough for reliable information transfer.

My thesis project aimed to determine what effect the pause response of the TANs/cholinergic interneurons has on the immediate ongoing activity of the projection neurons of the striatum. The hypothesis being tested was that due to the fact that the pause response is so precisely timed, in terms of internal phases and in relation to external stimuli, a similarly temporally contingent change should occur in striatal output. The lack of nAChR expression by SPNs suggests that fast effects of ACh on spike timing in these neurons might arise from the regulation of glutamatergic and/or GABAergic inputs. Both of these inputs are capable of powerfully and precisely timing SPN spikes, although a mechanism by which they could be controlled by the pause response in a temporally precise manner is unknown. The anatomical organization of the cholinergic interneurons precludes experimental induction of the pause response using conventional techniques. We thus used the relatively new approach of optogenetics to enable experimental manipulation of the activity of large numbers of these neurons in mice, and used *in vitro* and *in vivo* electrophysiological methods to monitor the concurrent activity of striatal neurons. Whole cell recordings *in vitro* allow for the monitoring of the synaptic inputs of the recorded neurons as well as the ability to control their activity through current injection or voltage clamp. Extracellular recordings using multi-site probes (tetrodes) *in vivo* allow for the monitoring of action potentials from single neurons in freely moving animals where brain activity is unperturbed.

In the rest of this chapter, I outline the background information relevant to the presented findings. First, I provide a brief review of key studies that investigated the activity of primate TANs in different behaviors and then argue the importance of elucidating the effects of the pause response on striatal function and output. I will then briefly review the behavior-related activities of presumed principal neurons in tasks similar to those in which TANs have been recorded. Following this, I will review the major afferent inputs to the neostriatum. The physiology and anatomy of the spiny projection neurons, GABAergic interneurons and cholinergic interneurons will be reviewed in detail. Lastly, I will review the known mechanisms by which acetylcholine modulates function in the neostriatum, demonstrating a need for further investigation of mechanisms by which the cholinergic interneuron pause response could control spiny projection neuron activity on short time-scales.

Activity patterns of TANs recorded in behaving primates

In primate electrophysiology studies, TANs have consistently been identified as distinct from phasically active neurons (PAN; presumed spiny projection neuron or pSPN) by a set of reliable criteria. TANs fire action potentials in a tonic fashion at rates of 2-10 Hz, have time-locked responses to environmental stimuli and are generally not temporally aligned to motor movement onset or termination (Evarts & Wise, 1984; Kimura *et al.* 1984). PANs fire at rates <1 Hz with long periods (often seconds) of silence interrupted

by bursts of activity, and these activations are often temporally aligned with movement (Apicella *et al.* 1991a). The action potential shapes, firing rates and anatomical distribution of TANs suggest that they are the large aspiny cholinergic interneurons (Aosaki *et al.* 1995). Additional support for this conclusion comes from intracellular (Wilson *et al.* 1990) and juxtacellular (Inokawa *et al.* 2010) recordings in anesthetized rodents, which confirm that neurons with the electrophysiological characteristics of TANs are immunoreactive for choline acetyltransferase. The most recent, and in some ways, the most conclusive evidence comes from experiments where TANs were recorded in awake behaving animals expressing opsins in cholinergic neurons which concluded that they are indeed one and the same (Witten et al. 2011). For the remainder of this thesis, I will refer to cholinergic interneurons as such when discussing *in vitro and in vivo* rodent studies and as TANs when discussing recordings in behaving primates.

The first report of TAN activity related to a specific environmental stimulus demonstrated that a solenoid valve click sound, which predicted the delivery of a juice reward, elicited at a 60 ms latency in 63% of recorded TANs, a pause in tonic firing flanked by excitation above baseline rates, the whole sequence lasting approximately 300ms (Kimura *et al.* 1984). This response existed under conditions of freely dispensed juice as well as that earned by a series of arm movements. Extinction of neuronal responding was achieved through the cessation of juice delivery following the click sound over repeated trials. This was the first reported evidence that brief activity patterns of TANs signal the significance of environmental events. Importantly, as opposed to previous recordings of PANs, TANs did not have activity that was temporally related to movement. An additional important aspect to this finding is that up to eight TANs were

recorded on a single penetration of the electrode and nearly identical responses were observed in all eight. This suggested a high level of synchronization among TANs in relation to the stimulus (Aosaki et al. 1995 calculate that the pause response is synchronized in TANs over a striatal volume of at least 10 mm³). The specifics of the pause response, in particular being closely time-locked to the stimulus and being of short duration, suggested that it might faithfully transfer a signal to other striatal neurons about time-sensitive events. This created much interest in these neurons and many studies followed up on this work to determine the exact behavioral correlates of the pause response. Here I give a brief review of the history of these studies, which focuses entirely on primate species (*m. fuscata, m fascicularis* and *m. mulatta*).

The fact that the DA neurons of the substantia nigra have temporally overlapping responses with the TAN pause response and that the TANs express DA receptors and receive inputs from other DA sensitive striatal neurons suggested the possibility of a causal relationship. Aosaki et al. (1994a) tested this hypothesis by unilaterally eliminating the DA projection to the striatum using the drug MPTP, which selectively kills dopaminergic neurons projecting to the striatum (Aosaki *et al.* 1994a). Before training in a classical conditioning task, 16% of TANs responded to the unlearned CS (approximately this percentage of TANs responding to stimuli of unknown significance has since been replicated many times) while after training this increased to 71% in the caudate and 52% in the putamen (again these approximate figures have been found many times since). The effect of MPTP on the pause response was dramatic, abolishing it with the exception of a greatly diminished initial excitatory component. A similar elimination of the response was produced by the D2-like dopamine receptor antagonist haloperidol,

while the mixed D1/D2-like dopamine receptor agonist apomorphine rescued the response in 50% of TANs in MPTP treated animals. A following study (Watanabe & Kimura, 1998) found similar effects of locally applied DA receptor antagonists in which D1 and D2 antagonists eliminated the response in 2/3 and 1/4 of neurons, respectively. In neither study did the interventions have significant effects on the baseline firing rate of TANs, although their activity patterns were not thoroughly analyzed. These findings demonstrate that the pause response requires an intact nigro-striatal DA projection. The fact that similar effects are seen with chronic DA neuron ablation and short-term striatal application of DA receptor antagonists suggests that the requirement for DA in generation of the pause response reflects activity at DA receptors in the striatum, not a large-scale remodeling of the structure caused by chronic loss of DA.

Although cholinergic interneurons receive cortical glutamatergic inputs, the majority of their glutamatergic input is from the intralaminar thalamic nuclei, whose input to the striatum targets preferentially the cholinergic interneurons. The role of this input in shaping the TAN response to salient stimuli was investigated by Matsumoto et al. (2000). Recordings in the centromedian (Cm) and parafascicular (Pf) intralaminar thalamic nuclei identified neurons having responses to salient events. These responses fell into two categories based upon their response latency and pattern of firing and were referred to as short and long latency facilitation (SLF and LLF, respectively). The shortest occurred at latencies of 50ms, before the TAN pause response begins, and thus could potentially play a role in its generation. Monkeys were trained on a classical task and SLF and LLF thalamic neurons and neostriatal TANs were recorded. They found the typical pause response to stimuli predictive of reward in the TANs and responses in SLF

and LLF neurons to stimuli with or without predictive value. TANs were then recorded while the GABA_A agonist muscimol was injection into Cm/Pf, which did not change baseline firing rates of TANs but eliminated the pause response save for the initial facilitation, which was much reduced. Injection of GABA_A agonists directly into the striatum has a similar effect (Watanabe & Kimura, 1998), and thus these data together suggest that the excitatory input from the thalamus may activate GABAergic interneurons involved in generating the TAN pause response.

The time-course of development of the pause response in TANs in a classical conditioning task was first investigated by Aosaki et al. (1994b). The structure of the pause response was similar to previous reports and it was observed to evolve in as little as 10 minutes of training (Aosaki *et al.* 1994b). This study also demonstrated that the pause response transfers from the original CS to a new earlier CS, supporting the idea that TANs keep track of information relating to the significance of predictive stimuli. Later studies (Apicella et al. 1998, Sardo et al. 2000) investigated the relationship between multiple temporally distinct predictors and the TAN response. As expected, neurons shifted their responding to the earliest cue, although a percentage retained the response to the predictor closest to the outcome. The time between the two cues is critical, as separating them by 4s versus 1s eliminated their interaction in terms of the TAN response, which occurred at the time of the later cue in the 4s delay trials as if the previous had not been given. This suggests a time limit imposed upon the predictive ability of the TAN pause response, a mechanism for which is not known.

The TAN response has been shown to have different qualities in classical versus operant conditioning paradigms. One early study (Apicella et al. 1997) noted that reward-

predictive cues elicit responding in a higher percentage of TANs in an operant task than a classical task, while the reward itself elicited responses in a higher percentage in the classical. Overall, a higher percentage of neurons in both tasks responded to the earliest signal, being the reward predictor and not the reward itself. A key additional finding in this study was that free rewards (the least predictable of the three tested in this study) elicited pause responses in the largest percentage of TANs. Additionally, the initial excitatory phase of the response was greatest in the free reward situation, a fact examined in more detail in later work. These data fit into a larger framework including later studies which lead one to conclude that the more unpredictable a salient event, the greater the number of TANs will respond and the greater the response magnitude will be, especially in terms of excitatory phases.

A significant avenue of investigation in the field has been whether TANs code for events with negative as well as positive valence. An initial study (Ravel et al. 1999), found that 80% of TANs responded to either free rewards or air puffs to the eye (assumed to be aversive). The neurons seemed to be salience detectors, as a neutral sound elicited a response in only 22% of TANs. The response to aversive stimuli was distinct form the reward-triggered response, being compressed to have a pause-excitation lasting a total of 100 ms and often having a second short pause following the excitation (Ravel et al. 1999). Additional studies (Ravel et al. 2003; Joshua et al. 2008), found similar results and in addition identified distinct groups of TANs which are reward or aversive stimulus selective, although most TANs which respond to one respond to both (Ravel et al. 2003). From this data one must conclude that TANs are not exclusively responsive to rewards, and are sensitive to salient stimuli with either positive or negative valence.

TANs were also found to respond differentially depending on the spatial location of reward predictive cues in the visual environment (Shimo et al. 2001, Ravel et al. 2006). In one, study monkeys were trained in a task in which they were required to make saccadic eye movements to four quadrants of their visual field, one of which was rewarded (Shimo et al. 2001). Some TANs were found to have a pause response to cues in visual fields other than that which predicted reward, and additionally to have quadrant specific responsiveness. The spatial and rewarding nature of the responding was not entirely dissociated in this study, as the monkey had to repeat each trial until it was correctly performed in order to advance to the next, so that each stimulus predicted, at the least, moving closer in time to the eventual reward receipt, as the monkey was well trained in this task. An additional study found that when monkeys were required to dissociate stimulus location and arm movement direction, different sub-groups of TANs became active in each task (Ravel et al. 2006). Together, these data demonstrate that TANs include spatial information in their responses although this information is in addition to, and not instead of the salience coding.

The potential coding for omission of expected outcomes and reward probability by TANs were most thoroughly investigated in classical conditioning tasks by Joshua et al. (2008) and Apicella et al. (2009), and in operant tasks by Morris et al. (2004) and Apicella et al. (2011). In the classical conditioning task, both groups found the expected responding to cues predictive of reward or aversive outcomes, although the specifics of the response were different. In Joshua et al. (2008), the cue-evoked response was invariant, whether the cue predicted rewarding or aversive events of several different outcome probabilities. In contrast, Apicella et al. (2009) found that responses to reward predictive cues varied significantly between different blocks of trials in which the cue predicted different outcome probabilities. This difference was mainly in the excitatory phases of the response, in particular the initial excitatory phase, which in Apicella et al. (2009) was greatest for the cue when the outcome probability was 100% and nearly eliminated when the outcome probability dropped to 25%. In both studies, opposite results were observed to outcome delivery, which elicited larger excitation the more unexpected it was. It is unclear what this difference signifies as the monkeys in both studies were of the same species (m. fascicularis) and both tasks were based upon classical conditioning. The key difference may be that the Joshua et al. (2008) study utilized different randomly presented cues each predicting a specific outcome and associated probability, while the Apicella et al. (2009) study used the same cue for all trials but blocks of 40-70 trials were distinct in the predictability of the reward following the cue. The overall predictability of the procedures was thus likely different (although impossible to determine specifically) and the differential responding again points to the idea that the TANs are very sensitive to the predictability of salient events. Though response to the reward delivery was similar in these studies, the response to omission was different. In the Joshua et al. (2008) study the omission was coded for by a very similar though slightly attenuated response as compared to the reward delivery, while in Apicella et al. (2009), they found very small activations or depressions in firing when the outcome was omitted, at different latencies than the pause response. These differences may also be due to the block versus randomized trial organization of the task, the mechanism of which is impossible to determine from the presented data. The conclusion is that in at least some situations, in classical conditioning tasks, the TAN pause responses are

capable of coding for reward probability at cue and outcome and for the omission of expected salient events. Additionally, the Joshua et al. (2008) study demonstrates that the response to both the delivery and omission, but not the predictive cues, of rewarding and aversive stimuli are coded for by different pause responses in that the aversive responses are compressed by approximately 50% as compared to the reward responses, similar to previous reports (Ravel et al. 1999).

The responses of TANs in operant and classical conditioning paradigms are different. Apicella et al. (2011) found that probability coding was much weaker in the operant task, and that there was essentially no initial excitatory phase to the pause response. Morris et al. (2004) found that an initial excitatory phase was the only part of the response that did code for probability. Additionally, outcome omission in their study resulted in a long latency and attenuated pause response in contrast to the findings presented in Joshua et al. (2008). The conclusion is that in at least some instances, TANs code for probability and omission in operant tasks, although the coding for both is weaker than in classical tasks. Significantly, the excitatory phases of the responses are essential to the probability coding but less so to the outcome versus omission coding.

This long and complex literature leaves many questions unanswered, but does provide us with several conclusions about the information coded for by the TAN pause response. The most important conclusion is that TANs are sensitive to surprising or unpredictable events of either positive or negative valence, meaning that they are first and foremost saliency detectors. This conclusion is supported by the fact that the more predictable an event, the less TANs respond. The fact that a particular response component, namely their excitatory phases, are mostly absent when events are highly predictable suggests a differential function for the different phases of the response. TANs can also code for differences between events with positive or negative valence, although this needs further investigation, as does the finding that in some circumstances TANs code for outcome omission. An important factor to be considered is the homogeneity of the neuronal populations being sampled in different studies. All TANs are tacitly considered to be cholinergic interneurons, and although this is likely the case in the majority of recorded neurons, it is likely that other interneuron types are included in some experiments. Part of the reason for the confidence in the classification of TANs as a single class of neurons was due to the fact that they were the only striatal neurons observed to have a tonic firing pattern and that their action potential waveforms were wider than those observed in other neuron types. The description of additional striatal neurons with intrinsically generated tonic firing patterns and more diverse action potential waveforms, including TH+ interneurons and the PLTS type NPY+ interneuron (Ibanez-Sandoval et al. 2010, 2011), suggest that these may have been misclassified as TANs in at least some experiments. Future work using techniques such as optogenetics will allow the investigation of this possibility.

All uncertainties aside, it is likely that the pause response plays an important role in shaping striatal function since it is so reliably evoked in behaviors known to involve the basal ganglia. For the pause response to have an effect on learning and behavior it must be translated into changes in striatal output. The question of what mechanism underlies this translation was the primary motivation and goal of my thesis work.

Activity patterns of spiny projection neurons

In contrast to cholinergic interneurons, other neostriatal cell types do not have reliable response patterns to stimuli (Apicella *et al.* 1991a). An important point to consider is that while the cholinergic interneurons have a relatively unique physiological profile, which lends itself to accurate identification in extracellular recordings, the same is not true of spiny projection neurons or GABAergic interneurons (with the possible exception of the FSI). The result is that recorded populations of presumed projection neurons are more likely to be contaminated with other neuron types. Additionally, there is no way to reliably distinguish between direct and indirect pathway projection neurons (except by antidromic activation which is not feasible in most situations), which are assumed to have very different activities at any given moment. Nevertheless, a brief review of recordings of presumed spiny projection neurons in behaving primates is relevant to the discussion at hand.

This discussion will be limited to studies where the activity of principal neurons was recorded in relation to stimuli such as reward predictive cues or rewards delivered at precise time points, which enable the temporal alignment of neuronal activity to the event. This will obviously ignore an important avenue of research which has been reported in the last decade or so investigating the relationship between striatal interneurons and SPNs and various local field potential oscillations during performance of behavioral tasks. This was decided based on the fact that TANs have not been recorded in these specific studies. The presented studies have examined striatal neurons other than TANs in experiments similar enough to TAN studies to allow direct comparison of the responses. The goal of this thesis is to determine how a specific firing pattern of TANs (the pause response) is translated into striatal output, justifying this restriction of the reviewed literature.

In some studies TANs and presumed SPNs (pSPN) were recorded in the same task or very similar tasks allowing for the direct comparison of their responses to stimuli (Kimura et al. 1990, Apicella et al 1991a, Apicella et al 1991b). In Kimura et al. 1990, TANs and pSPNs were recorded in striatum of monkeys performing an operant conditioning task. Alignment of the TAN activity to movement onset showed no modulation while alignment to the reward delivery demonstrated a pause response to the reward with a strong initial excitatory phase. Conversely, alignment of pSPN activity to reward showed a lack of correlation of neural activity to reward delivery, while alignment to the onset of movement showed significant modulation of neuronal activity preceding and during the movement required to elicit reward presentation. This study was chosen as an example because the pSPNs were identified as projection neurons by antidromic stimulation of the globus pallidus. This is a marked improvement over other studies, which relied on other less reliable physiological criteria to identify neurons as projection cells. These basic findings, that the TANs code for the stimulus tied to reward and the projection neurons code for the actually movement was supported by numerous previous and subsequent findings. These data are not informative about the direct effect of the TAN pause response on the projection neurons, as the recorded projection neurons were silent at the time of reward delivery. In contrast, Apicella et al. (1991a) recorded pSPNs in an operant task, which had significantly different responses than TANs recorded in a similar task in Apicella et al. (1991b). TANs were found to have typical pause responses, and as in other studies these responses were highly conserved across the population of recorded neurons. Presumed projection neurons had different and widely varying responses. Reward delivery elicited no fewer than five distinct response types including

short latency excitations (onset ~200 ms, duration ~300 ms), long latency brief excitation (onset ~800ms, duration ~200ms), long latency long excitation (onset 800ms, duration 1000ms) very long latency very long excitation (onset 1200ms, duration >3s) and oscillatory excitations (onset in the range of 10ms, duration ~200ms, frequency ~1-2 Hz). This result is not surprising when one considers that different groups of projection neurons must be activated and inhibited in turn to correctly engage motor programs to physically attain the reward (in this case licking movements, which were temporally aligned with some but not all of the pSPN responses), and to suppress competing motor programs which would interfere with the achievement of the intended movement. In one study, pSPNs were found to be active immediately before reward receipt and to terminate this activity upon delivery of reward {Takigawa, 2002 #301}.

It is difficult to propose a mechanistic link between the TAN pause and these varied responses. It is assumed that the correct activation sequence of projection neuron ensembles is shaped in part by the activities of TANs. The goal of this thesis is to elucidate possible mechanisms by which this admittedly hypothetical modulation functions.

Anatomy and Physiology of Cortical inputs to the Neostriatum

The striatum receives input from nearly all frontal cortical areas (Graybiel & Ragsdale, 1979; Graybiel, 1991; Reiner *et al.* 2010). These inputs are arranged in a topographical manner, with functionally related cortical areas projecting to nearby areas in the striatum (Yeterian & Van Hoesen, 1978; Ragsdale & Graybiel, 1990). This relationship is shared with the thalamus, as highly interconnected cortical and thalamic

areas input to nearby regions in the striatum (Cheatwood *et al.* 2003; Cheatwood *et al.* 2005; Kamishina *et al.* 2008). The striatum receives inputs from prefrontal, limbic as well as motor, somatosensory and associative areas of cortex (Graybiel, 1995). Thus, although thought of by some as a primarily motor-related structure, the diversity of inputs suggests otherwise. The organization of these inputs on the levels of systems, cell types and synapses is complex. A distillation of the essentials is presented below.

Two types of cortical projection neurons innervate the striatum (Somogyi et al. 1981; Cowan & Wilson, 1994; Reiner et al. 2010). Unilaterally projecting pyramidal tract (PT) neurons, present mainly in deeper areas of cortical layer 5, project to the brainstem, make a collateral projection to the striatum (Reiner et al. 2003). Bilaterally projecting intratelencephalic (IT) neurons reside in superficial areas of layer 5 and in layer 3 of the cortex and do not project to targets outside of the telencephalon (Reiner et al. 2003). Differences in size, axonal conduction speeds, synaptic terminal size and synaptic targets differentiate PT and IT neurons. PT neurons are larger, conduct action potentials faster, have larger presynaptic terminals and preferentially target indirect pathway SPNs, while IT neurons are smaller, conduct slower, have smaller terminals and preferentially target direct pathway SPNs (Cowan & Wilson, 1994; Reiner et al. 2003; Lei et al. 2004). The level of differential targeting of direct and indirect pathway neurons has been the point of some argument in the literature, but the most convincing studies confirm a significant level of segregation (Reiner et al. 2003; Lei et al. 2004). The functional significance of this arrangement is unclear at present, and is not generally considered in the classical two-pathway model of basal ganglia function and dysfunction. Two ideas presented in the literature about the function of the fast PT input to indirect

pathway neurons are that either the inputs directing the current motor movements also input to indirect pathway neurons to inhibit potentially interrupting following movements, or that this input is involved in the timing of the termination of the current motor movement (Mink, 1996; Graybiel, 2005).

Overlaid on this level of cellular organization is the differential targeting of patch and matrix areas of the striatum by different cortical layers and areas (Graybiel & Ragsdale, 1978; 1979). Generally, deeper layers, as well as more prefrontal and limbic related structures, synapse in the patch, while more superficial layers and more motor related areas synapse in the matrix (Graybiel & Ragsdale, 1978; 1979; Ragsdale & Graybiel, 1981; Albin et al. 1989; Ragsdale & Graybiel, 1990) but see (Kincaid & Wilson, 1996). The functional significance of this organization has been considered in light of the fact that the projection neurons in the patch compartments preferentially target the SNr DA neurons projecting back to striatum while the matrix projection neurons target the SNr GABAergic neurons which output to the thalamus (Gerfen, 1984). The idea is that the more cognitive cortical areas (prefrontal, limbic) preferentially gate the DA input to the matrix, which is more strongly connected to the more motor related cortical areas, resulting in cognitive control of movement initiation. The fact that the cholinergic interneurons preferentially avoid the patch compartments (Martone et al. 1993; Martone *et al.* 1994), suggests that they may be functionally integrated into this scheme, although potential connections with patch-matrix border crossing interneurons may play an additional important role.

Overall, the relevance of the topography of the cortico-striatal anatomy to this thesis is limited, as the majority of the input to cholinergic interneurons is from the

thalamus and the pause response is not seen to vary across striatal areas or to have anomalous findings in small areas of recording, the existence of which might suggest modulation by afferent input source or patch-matrix organization.

Anatomy and Physiology of Thalamic inputs to the Neostriatum

The thalamic input to the striatum is known to be critical for generation of the pause response (Matsumoto et al. 2001), and thus this input will be reviewed in detail. This input constitutes along with that from cortex the sole extrinsic excitatory drive to the striatum. The synaptic organization of thalamic inputs, however, contrasts greatly with that of the cortical inputs. These differences have largely been elucidated by anatomists, who have taken advantage of the fact that most thalamo-striatal axon terminals express vesicular glutamate transporter-2 (VGLUT2) while those from cortex express vesicular glutamate transporter-1 (VGLUT1) (Smith et al. 2009). The thalamic nuclei from which the majority of inputs to striatal interneurons arise are the centromedian and parafascicular (Cm, Pf) (Lapper & Bolam, 1992; Sadikot et al. 1992a; Sidibe & Smith, 1999; Matsumoto et al. 2001; Cheatwood et al. 2005). The ventral anterior (VA) and ventral lateral (VL) thalamic relay nuclei primarily serve as an intermediate step between the basal ganglia output nuclei, (the globus pallidus (in primate) and entopedunclular nucleus (in rodents) and substantia nigra pars reticulata in both) and the cerebral cortex, constituting the minority of thalamo-striatal afferents (Deschenes et al. 1995; Parent & Hazrati, 1995; Finch, 1996).

Inputs from the globus pallidus to the VA/VL nuclei differ from those to the Cm/Pf. Individual pallidal afferents terminate in small areas of VA/VL while the input to

Cm/Pf is homogenous and single axons contact the dendrites of multiple neurons across large areas (Sadikot *et al.* 1990; Deschenes *et al.* 1995). The input from the substantia nigra pars reticulata primarily targets the VA/VL, and its innervation of the Cm/Pf is less well characterized (Beckstead, 1983). Inputs from both structures to the thalamus are GABAergic and are considered to inhibit the relay of information from the thalamus to other structures (Parent & Hazrati, 1995). Besides input from the basal ganglia nuclei, the Cm/Pf also receives inputs from motor and sensory related cortical areas as well as from the brainstem reticular formation (Steriade & Glenn, 1982; Parent & Hazrati, 1995). The VA/VL nuclei receive inputs from the basal ganglia output nuclei and cortex (Sawyer *et al.* 1994a; Sawyer *et al.* 1994b; Parent & Hazrati, 1995).

Most Cm/Pf neurons project to the striatum, although there are some which project to cortex or to both cortex and striatum (Sadikot *et al.* 1992a; Sadikot *et al.* 1992b; Parent & Parent, 2005). In the striatum of rodents, the Cm/Pf input makes synapses with principal neurons as well as all interneurons examined (Lapper & Bolam, 1992; Finch, 1996; Rudkin & Sadikot, 1999; Smith *et al.* 2009) (neurochemically identified by expression of somatostatin (SOM), choline acetyltransferase (ChAT), nitric oxide synthase (NOS), neuropeptide-Y (NPY) or parvalbumin (PV)) except those expressing calretinin (PV interneurons get a very sparse input from the Pf (Rudkin & Sadikot, 1999)) while in monkeys this input is much stronger). In the case of cholinergic interneurons this is their main excitatory drive (Lapper & Bolam, 1992; Rudkin & Sadikot, 1999; Sidibe & Smith, 1999; Zackheim & Abercrombie, 2005). Detailed anatomical examination of principal neurons revealed that on the sub-cellular level thalamic inputs are anatomically diverse. Pf inputs primarily target dendritic shafts, while Cm inputs primarily target spines (Lacey et al. 2007), as do cortical inputs. It was initially considered that the spine targeting inputs might be functionally isolated from the effects of DA, as the DA terminals are largely restricted to spines (Smith et al. 1994). A recent report which utilized serial section electron microscopy to reconstruct in three dimensions the distance of TH staining terminals from those staining for VGLUT1 and VGLUT2 (marking dopaminergic, cortical and thalamic terminals, respectively) demonstrated equidistance from a dopaminergic terminal, and thus the current thinking is that thalamic and cortical inputs may be similarly affected by dopamine (Moss & Bolam, 2008). Thalamic synaptic input to principal neurons has been directly observed in brain slices and thalamic inputs are found to have more depressing synapses as compared to cortical facilitating synapses (Ding *et al.* 2008). The preferentially targeting of direct versus indirect pathway SPNs has been examined, and the Cm nuclei have been found to primarily innervate SPNs that project to the internal globus pallidus, which in turn projects back to the intralaminar nuclei (Sidibe *et al.* 1997; Sidibe *et al.* 2002).

In contrast to the projection neurons, cholinergic interneurons receive thalamic inputs targeting proximal dendrites as opposed to the cortical input, which is more distal, and the thalamic input is the main excitatory drive of these neurons (Lapper & Bolam, 1992; Kawaguchi *et al.* 1995). This input is glutamatergic (as are the other inputs from thalamus to other striatal neurons) and has been shown to directly activate cholinergic interneurons and generate burst-pause sequences in a brain slice preparation (Ding *et al.* 2010). These activity sequences differentially regulate the cortical input to direct and indirect pathway SPNs through presynaptic muscarinic receptors (Ding *et al.* 2010). This

study also directly demonstrated that the cortical input is very weak compared to the thalamic input in terms of ability to elicit depolarizations and action potentials in cholinergic interneurons. The role of the thalamic input in generating the pause response in TANs has been described in previous sections (Matsumoto *et al.* 2001). Briefly, it has been demonstrated the activity of Cm/Pf neurons is required for a functional pause response to be generated (Matsumoto *et al.* 2001).

Recordings obtained from non-human primates of neurons in the thalamic Cm/Pf nuclei support a hypothesis whereby cholinergic interneurons are involved in what is referred to as re-bias procedures (Minamimoto et al. 2009). Cm/Pf neurons respond to salient auditory, visual or tactile cues with either of two mutually exclusive responses (Matsumoto *et al.* 2001). Short latency facilitation neurons (SLF), present mainly in the Pf, respond at 5-50 ms latencies, while long latency facilitation neurons (LLF), mainly residing in CM respond at 175-275 ms latencies, with the magnitude of both varying inversely with the predictability of the stimulus (Matsumoto et al. 2001; Minamimoto & Kimura, 2002). During GO-NOGO reward learning trials, unexpected (low probability of occurrence) signals that instruct the animal to switch programs mid-task are accompanied by the strongest activation of both types of Cm/Pf thalamic neurons. When the monkey fails in switching, there is no corresponding activity in the Cm/Pf, suggesting that for proper behavioral switching (re-biasing) the Cm/Pf response to the unexpected stimulus is required. It is suggested that the cholinergic neurons might be part of a mechanism whereby the thalamic input controls the striatal-dependent rebiasing of actions, in particular when an unexpected event occurs and requires a change

in behavioral program (Yamada et al. 2011). A mechanism for this translation is not known.

In conclusion, it has been demonstrated through anatomical and physiological measures that the primary excitatory input to cholinergic interneurons comes from the intralaminar thalamic nuclei, and that neurons in these nuclei participate in behaviors likely requiring the involvement of cholinergic interneurons. The modulation of striatal output by cholinergic interneurons in these situations is not known.

Physiology and Anatomy of Neostriatal Spiny Projection Neurons

SPNs represent \approx 95% of the striatal neuron population, receive excitatory inputs from cortex and thalamus, and have strong influence over basal ganglia output (Albin *et al.* 1988; Gerfen, 1988; Albin *et al.* 1995; Bolam *et al.* 2000). *In vivo* intracellular recordings have demonstrated that these neurons have clearly identifiable up and down states governed by the interplay of intrinsic ion mechanisms with glutamatergic input primarily originating in the cerebral cortex (Wilson & Groves, 1981; Nisenbaum *et al.* 1994; Nisenbaum & Wilson, 1995; Wilson & Kawaguchi, 1996). Intrinsic potassium conductances are involved in the state setting behavior and the reliably observed inward and outward rectification properties (Wilson, 1993; Nisenbaum & Wilson, 1995; Wilson & Kawaguchi, 1996). Projection neurons receive input from both ipsilateral and contralateral cortices (Wilson, 1986), and projection neurons of the patch and matrix compartments are nearly identical in terms of excitability and intrinsic membrane properties (Kawaguchi *et al.* 1989). Spike timing in projection neurons, while their excitability

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and the strengths of these synaptic inputs is modulated by neurotransmitters such as DA, ACh, serotonin and histamine (Lavoie & Parent, 1990; Bolam *et al.* 2000; Tepper *et al.* 2004; Ellender *et al.* 2011; Parent *et al.* 2011).

Two functionally distinct SPN types, striatonigral projecting (direct pathway) and striatopallidal projecting (indirect pathway), can be distinguished (Cepeda *et al.* 2008; Day *et al.* 2008) (Gerfen, 1988; Gerfen *et al.* 1995; Bolam *et al.* 2000). These distinctions can be drawn on the basis of physiology, anatomy, gene expression profiles and afferent as well as the already mentioned efferent connectivity. Functionally, the two types exert opponent influence on the initiation of voluntary movement, with activity of direct pathway neurons being facilitatory and indirect pathway neurons being inhibitory (Penney & Young, 1983; Albin *et al.* 1989; 1995; Levy *et al.* 1997).

Electrophysiological experiments comparing the membrane properties of direct and indirect pathway SPNs in acute brain slices using whole cell patch clamp recordings demonstrate many similarities but also some notable differences (Day *et al.* 2006; Cepeda *et al.* 2008; Day *et al.* 2008). Indirect pathway neurons have more hyperpolarized action potential thresholds, the significance of which is not yet understood (Cepeda *et al.* 2008). There is also evidence that indirect pathway neurons receive more active cortical input (at least *in vitro*) and that their dendrites are more excitable and convey back-propagating action potentials farther from the soma than those of direct pathway neurons (Day *et al.* 2008).

The most reliable means of identification for direct pathway neurons is expression of substance P and D1-like dopamine receptors, and for indirect pathway neurons expression of enkephalin and D2-like dopamine receptors (Gerfen *et al.* 1990; Cepeda *et*
al. 2008). Additionally, differential but incompletely restricted expression of muscarinic ACh receptor subtypes exist (Misgeld, 1989; Yan & Surmeier, 1996; Calabresi et al. 2000; Zhou et al. 2003; Cepeda et al. 2008). The above-mentioned genes and their protein products have been used to identify neuron type both in fixed tissue (using single cell RT-PCR and Immunocytochemistry) and in the acute brain slice in vitro (using single cell RT-PCR and/or fluorescence signal in transgenic mice expressing EGFP under the promoters for one of these genes). The differential expression of dopamine receptors is a central aspect of one of the most commonly held theories regarding the symptomatology of Parkinson's disease (Albin et al. 1989; 1995). The two types of DA receptors act generally to make the neurons more or less excitable (D1- and D2-like, respectively) (Gerfen & Keefe, 1994; Gerfen, 2000; Gerfen & Surmeier, 2011), and additionally play a role in differential regulation of lateral inhibition (Tecuapetla et al. 2009) and the plasticity of their glutamatergic inputs (Shen et al. 2008). The dendritic morphology of the neurons is different in that indirect pathway neurons have significantly sparser dendritic fields (Gerfen & Surmeier, 2011).

Physiology and Anatomy of inhibitory GABAergic interneurons

The powerful effect of GABA on SPN activity has been demonstrated by the observation of increased spiking activity in SPNs following GABA_A receptor antagonists (Nisenbaum & Berger, 1992). Blockade of GABA_A receptors *in vivo* in behaving cats results in either hyperactivity or dystonia (following injection of antagonists into the caudate or putamen, respectively) (Yoshida *et al.* 1991). The role of GABAergic interneurons in these effects was demonstrated in a more recent study that found that

selective inhibition of glutamatergic inputs to FSIs (which strongly inhibit SPNS via GABAergic synapses) elicits dystonia in rats (Gittis *et al.* 2011b). The role of other interneurons besides the FSI in shaping striatal function and behavior is less well understood. Below is a brief history of the investigation of neuronal types within the striatum, followed by a description of the current thinking about their functional roles.

The number of different neuron types in the striatum has been a constant question throughout its study. Early reports such as Vogt and Vogt, (1920) suggested the existence of only two cell types, one large and one small, and until relatively recently the less numerous large cells were considered to be the projection cells, influenced by a large number of interneurons. The application of more sophisticated anatomical methods including electron microscopic investigation of Golgi stained tissue and tract tracing allowed neuroanatomists to identify cell types more accurately. By the early 1980's, two importance pieces of information were known. First, a group of small and numerous (>90% of all neurons) highly spiny neurons, not large primarily aspiny neurons, were the projection neurons (Fonnum et al. 1978; Bolam et al. 1981; Somogyi et al. 1981; Mehler, 1982). Second, there likely existed five to seven morphologically distinct cell types in the striatum, several of which were believed to be interneurons (DiFiglia et al. 1976; Pasik et al. 1976; Dimova et al. 1980; Bishop et al. 1982; Braak & Braak, 1982; Chang & Kitai, 1982; Chang et al. 1982; Wilson et al. 1982). Nearly all of these were found to receive input from the cortex as assayed using electrophysiological methods (Bishop et al. 1982) and at least some interneurons were thought to be GABAergic (Bolam et al. 1983a). Neurons containing choline acetyltransferase (Bolam et al. 1984), substance P (Bolam et al. 1983b), enkephalin (DiFiglia et al. 1982; Somogyi et al. 1982), somatostatin (DiFiglia

& Aronin, 1982; Takagi et al. 1983) and parvalbumin (Kita et al. 1990) were identified in the striatum, although at this point the relationship between these neurochemical features and the anatomically identified neuron types was unknown. Projection neurons were shown to receive GABAergic synapses (Aronin et al. 1986), some of which originated from PV+ interneurons (Kita et al. 1990; Bennett & Bolam, 1994b; a). GABAergic synapses upon non-GABA staining neurons were also found, (Pasik et al. 1988) suggesting that neurons besides projections cells received GABAergic inhibition. NPY expressing neurons were also found to be GABAergic by virtue of their GAD staining and also to receive GABAergic synaptic inputs (Vuillet et al. 1990). Cholinergic synapses onto medium sized spiny neurons, some of which were striato-nigral projecting, were identified (Izzo & Bolam, 1988) and reciprocal synaptic connections between cholinergic and NPY expressing neurons were found (Vuillet et al. 1992). Cholinergic synapses were also found to colocalize on single dendrites with dopaminergic afferents, the latter also synapsing on cholinergic neurons themselves (Chang, 1988; Pickel & Chan, 1990), with DA presumably acting at D2 receptors expressed by Cholinergic interneurons (Le Moine *et al.* 1990). Muscarinic acetylcholine receptors were found to be expressed by both substance P and somatostatin staining neurons (Ariano & Kenny, 1989) and later by identified SPNs as well (Kitai & Surmeier, 1993; Hersch et al. 1994; Harsing & Zigmond, 1998; Yan et al. 2001). Intracellular recordings and subsequent morphological assessment of tonic firing neurons in the striatum led to the suggestion that they were the choline acetyltransferase expressing neurons (Wilson et al. 1990), and the matrix localization preference of these neurons as well as intrinsic membrane properties (including long AHP and a sag in the membrane potential during negative current

injection) were described by (Kawaguchi, 1992). Thus by 1992 there was ample evidence that the striatal interneuron network was very complex and consisted of GABAergic interneurons which targeted projection neurons as well as other interneurons, and that at least some of the interneurons were modulated by DA and ACh.

The advent of whole cell recording (Hamill *et al.* 1981) under visualization enabled the enhanced ability of investigators to target different cell types in the striatum, where the vast majority of cells are projection neurons. In a landmark study, whole cell recordings were obtained from projection neurons as well as three types of interneurons identified by their electrophysiological properties as well as unique immunocytochemical staining. They were characterized as the fast spiking parvalbumin expressing interneuron (FSI), the PLTS (*p*lateau potential, *low t*hreshold *s*pike) NADPH expressing interneuron (later shown to express NPY and somatostatin as well) and the large aspiny cholinergic interneuron (Kawaguchi, 1993). Kubota et al. (1993) extended this group to include calretinin, (CR) expressing interneurons (although no recordings were obtained, and have not been to this day) (Kubota *et al.* 1993). This classification scheme of the FS, PLTS, CR and cholinergic interneurons representing the primary interneuron types of the striatum persisted for more than a decade.

The parvalbumin expressing fast spiking interneuron was the first GABAergic interneuron directly demonstrated to make inhibitory synaptic connections with and strongly inhibit the activity of projection neurons (Koos & Tepper, 1999). The idea of feed-forward inhibition, where cortical afferents would excite FSIs, which would then inhibit projection neurons, dominated the field for more than a decade. Only recently have we come to appreciate the diversity of striatal interneurons in terms of their unique

physiology, in particular their connections with each other and with projection neurons.

Some of these findings might not be considered surprising, as GABAergic interneurons throughout the forebrain have a shared developmental origin. Indeed, at least one neostriatal GABAergic interneuron, the fast spiking PV+ interneuron, shares remarkable functional similarity with the corresponding cortical fast spiking PV+ interneuron (Anderson *et al.* 2002; Inta *et al.* 2008). Based on this analogy, the other striatal interneuron types might serve distinct functions due to differences in intrinsic properties, connectivity and neuromodulation (Lawrence, 2008; Woodruff & Yuste, 2008). In fact, recent publications from our laboratory have identified an NPY expressing GABAergic interneuron similar to the cortical neurogliaform interneuron, which will be discussed further in later sections (Ibanez-Sandoval *et al.* 2011).

Recent advances in the understanding of GABAergic interneuron-based inhibition in the neostriatum

Our laboratory was the first to show electrophysiological evidence that PV+ fast spiking interneurons (FSI) are highly connected to and strongly inhibit principal neurons (Plenz & Aertsen, 1996; Koos & Tepper, 1999; Gustafson *et al.* 2006; Taverna *et al.* 2007; Gittis *et al.* 2010; Planert *et al.* 2010). Additional studies have validated these findings *in vivo* (Mallet *et al.* 2005), and much work has gone into elucidating the details of the FSI to SPN connection in terms of direct and indirect pathway differences in both normal states and in models of basal ganglia disorders (Gittis *et al.* 2010; Planert *et al.* 2010; Gittis *et al.* 2011a). The primary reason for the focus on FSIs is that they are relatively easily characterized anatomically and electrophysiologically. It is now know

that many interneurons are not PV+ or ChAT+. These include populations of neurons expressing neuropeptide Y, calretinin, NOS and tyrosine hydroxylase.

In the past, *in vitro* slice recording experiments (let alone *in vivo* experiments) targeting these other GABAergic interneuron types was essentially impossible, and the only way to hope to record and identify them was obtaining whole cell patch clamp recordings of unusual looking neurons and then filling them with Biocytin or another marker, allowing for post-hoc identification using Immunocytochemistry. Little progress was made in the investigation of the less common interneuron types until the advent of transgenic mice expressing EGFP under the promoters for their neurochemically identifying genes. The GENSAT project at Rockefeller University, as well as several other laboratories around the world, has now produced an impressive catalog of mice with EGFP labeling based on single gene expression. Recent studies in the basal ganglia have taken advantage of this tremendous resource, such as those looking at direct versus indirect pathway projection neurons (Cepeda et al. 2008, Tecuapetla et al. 2009), and novel tyrosine hydroxylase and NPY expressing GABAergic interneurons (Ibanez-Sandoval et al. 2010; 2011; Unal et al. 2011). Another variety of transgenic mouse, becoming available in ever-increasing types, are those in which Cre-recombinase is expressed under single gene promoters. Injection of a vector carrying a floxed copy of a fluorescent protein's gene into an area of interest in these mice can allow for visualization of single cell types. Additionally, double transgenic mice created by cross breeding EGFP and Cre-recombinase transgenics are feasible, as we have recently demonstrated with Cholinergic-Cre / NPY-EGFP animals (English et al. 2011). These mice can be injected with vectors carrying genes for fluorescent proteins other than

EGFP allowing for visualization of two distinct cell types during *in vitro* slice recording experiments and in fixed tissue.

Physiology and Anatomy of Neuropeptide-Y expressing GABAergic interneurons

The NPY expressing neuron is a critical element in the experimental results presented in this thesis and thus they will receive a disproportionate amount of attention is this review. Kawaguchi was the first to characterize the electrophysiological properties of an interneuron since termed the PLTS neuron (plateau potential, low threshold spike) which expresses neuropeptide Y (NPY), nitric oxide synthase and somatostatin, and has unique electrophysiological characteristics which distinguish it from SPNs, FSIs and Cholinergic interneurons (Kawaguchi, 1993; Kubota et al. 1993; Kawaguchi et al. 1995). These key features (determined using intracellular recordings in acute brain slices) include a high input resistance, low threshold spiking, spontaneous activity, a relatively depolarized resting membrane potential and a long lasting plateau potential following intracellular current induced depolarization. Recordings from EGFP+ neurons in NPY-EGFP transgenic mice identified a percentage of these neurons as being identical to the PLTS neuron (Ibanez-Sandoval et al. 2011), and will be referred to as NPY-PLTS. The majority (79%) of EGFP+ neurons in these animals are of the PLTS type, however, another neuron with stronger EGFP labeling (and also later found to have stronger NPY immunoreactivity, but no reactivity to NOS or SOM) has been described in a recent paper from our laboratory (Ibanez-Sandoval et al. 2011). These neurons resemble SPNs in terms of their hyperpolarized resting membrane potential, inward rectification, relatively wide APs (as compared to FSI and PLTS) and have a deep AHP (as compared to PLTS and SPN) (Ibanez-Sandoval et al. 2011). Thus, these neurons can be distinguished from all previously identified striatal neurons based upon both their intrinsic electrophysiological properties and neurochemistry. Both NPY+ interneuron types receive glutamatergic input from cortex, with the PLTS responding more strongly to electrical stimulation of the cortex (as measured by analysis of evoked EPSPs), probably due to higher input resistance (Ibanez-Sandoval et al. 2011). Their dendritic and axonal morphology, as well as their inhibitory synaptic input strength to SPNs are very different as well (Ibanez-Sandoval et al. 2011). NPY-PLTS neurons have sparse dendritic fields, and have relatively short and spatially restricted axonal fields. The second type of NPY interneuron, on the other hand, has highly branched dendrites, and a highly ramifying axonal field. Given these differences, it is not surprising that the connection probabilities from the two NPY neurons to SPNs are vastly different. Paired whole cell recordings revealed that 14% of PLTS neurons make inhibitory GABAergic synapses with SPNs compared with 85% of NPY-II neurons (Ibanez-Sandoval et al. 2011). An important point, described in more detail in following chapters, is that the synapse from the second type of NPY+ neuron onto SPNs has an IPSP/C whose decay time constant is considerable slower than that for IPSP/Cs arising from either FSI or PLTS neurons onto SPNs. This is not due to the synaptic location on the cell, as even distal SPN-SPN synapses make shorter IPSP/Cs (Ibanez-Sandoval et al. 2011). This synapse is similar to the synapse in described in the hippocampus and cortex as GABA_Aslow, the biophysics of which are attributed to low and long lasting GABA transients and the involvement of extrasynaptic receptors (Pearce, 1993; Banks et al. 1998; Tamas

et al. 2003). Due to the similar morphological, neurochemical, and synaptic properties of the this second NPY+ neuron to the cortical neurogliaform interneuron, it has been termed NPY-NGF (Ibanez-Sandoval *et al.* 2011), and it will referred to as such from now on.

Physiology and anatomy of Neostriatal Cholinergic Interneurons

Cholinergic interneurons fire spontaneously in vitro and in vivo at rates of 2-10Hz in all species investigated (including mice, rats, non-human primates and humans). In the absence of motivationally salient stimuli their activity is weakly correlated (Kimura et al. 1984; Aosaki et al. 1994a; Aosaki et al. 1995; Morris et al. 2004; Schmitzer-Torbert & Redish, 2008; Christian KE Moll, 2009; Witten et al. 2010). Intracellular recordings obtained in vivo from anesthetized rodents demonstrated that individual action potentials are triggered by small EPSPs (Wilson et al. 1990), while an intrinsic pacemaker mechanism is ultimately responsible for their ongoing activity (Bennett et al. 2000). A specific sequence of intracellular events underlies this mechanism: wide action potentials generate significant calcium entry that results in activation of calcium activated potassium (SK) channels. This results in hyperpolarization of the neuron which then activates I_h, which depolarizes the cell into the range where non-inactivating voltage gated sodium channels begin to open (\approx -65 mV), bringing the membrane potential to spike threshold (Bennett et al. 2000). Two distinct firing patterns, described as either rhythmic or bursting (Wilson et al. 1990; Bennett & Wilson, 1999; Bennett et al. 2000), can be observed in cholinergic interneurons recorded in brain slices in vitro, and vivo in both juxtacellular recordings in anesthetized animals and in tetrode

recordings in freely moving mice (English, 2009; English *et al.* 2011). An additional property of striatal cholinergic interneurons is a voltage gated inward rectifier that produces a regenerative hyperpolarizing current. This current is activated by the offset of a depolarization or a hyperpolarization of sufficient magnitude (Wilson, 2005). It has been suggested that the duration of the pause-response is timed via the serial interaction of the regenerative hyperpolarizing current with the depolarizing I_h , as the duration of the pause in firing generated by these mechanisms nearly matches the time course of the pause in firing observed in response to salient stimuli (Wilson, 2005). Thalamic inputs are hypothesized to engage this mechanism through direct glutamatergic depolarization of the cholinergic interneuron and or indirect hyperpolarization through glutamatergic activation of intermediate GABAergic interneurons. Cholinergic interneurons express D1 and D2 like DA receptors, the latter having been posited to play a role in the generation and/or maintenance of the pause response (Le Moine *et al.* 1990; Zhou *et al.* 2002; Tepper & Bolam, 2004).

Cholinergic interneurons make up only 0.5-2% of the neuron population but form synaptic contacts with all neostriatal neurons (Koos *et al.* 2004; Tepper & Bolam, 2004; Grilli *et al.* 2009), and in addition regulate the striatal circuitry through volume transmission mediated activation of extrasynaptic cholinergic receptors (Wilson *et al.* 1990; Contant *et al.* 1996). The striatum has extremely high levels of acetylcholinesterase, the metabolic enzyme for acetylcholine, which is expected to allow for very rapid termination of cholinergic transients (Zhou *et al.* 2002), although a precise timescale of this process has not been established. As with other striatal neuron types the cholinergic interneuron receives excitatory input from cortex and thalamus, with the intralaminar thalamic nuclei providing the majority of the excitation (Wilson *et al.* 1990; Lapper & Bolam, 1992; Sidibe & Smith, 1999; Reynolds *et al.* 2004). The section on the thalamic input to the striatum provides a more detailed description of these important inputs to cholinergic interneurons. Cholinergic interneurons are sparsely distributed throughout the striatum, have been observed to avoid the mµ-opioid staining compartments referred to as patch or striosome (Aosaki *et al.* 1995)

Cholinergic regulation of striatal circuitry

The two classes of central nervous system acetylcholine receptors, ionotropic nicotinic receptors and metabotropic muscarinic receptors, are present at high levels in the neostriatum and strongly modulate intrinsic excitability and synaptic connections of striatal neurons.

Muscarinic receptors are classified on the basis of their second messenger coupling mechanisms: M1 family receptors (M1, M3 and M5) are coupled to G_q and phospholipase C activation while M2 family receptors (M2 and M4) are coupled to G_{io} and adenylate cyclase inhibition. In the striatum these receptors are located on SPNs, GABAergic and cholinergic interneurons and glutamatergic as well as dopaminergic afferents (Zhou *et al.* 2003). SPN M1 receptor activation enhances NMDA currents, and induces membrane depolarization mediated by the modulation of a constitutively active potassium conductance (M-current; Calabresi *et al.* 2000), resulting in greater input resistance and reduced action potential afterhyperpolarization (Calabresi *et al.* 2000; Figueroa *et al.* 2002; Lin *et al.* 2004). M4 receptors, primarily expressed by SPNs of the striatonigral pathway, reduce high voltage calcium channel currents decreasing spike

AHP (Calabresi et al. 2000; Figueroa et al. 2002). Studies of muscarinic effects on GABAergic interneurons are limited, however it is known that activation of presynaptic muscarinic receptors on the terminals of striatal fast spiking, parvalbumin expressing, interneurons reduces their inhibition of SPNs (Koos & Tepper, 2002). M2 and M4 receptors on cholinergic interneurons act as inhibitory autoreceptors and thus influence the tonic level of ACh in striatum (Calabresi et al. 1998a; Zhou et al. 2003). Cortical afferents express M2 and M3 receptors, which inhibit and facilitate glutamate release, respectively (Zhou et al. 2002), while dopamine release is inhibited by activation of presynaptic M4 receptors (Kudernatsch & Sutor, 1994; Zhou et al. 2003). The potential role of muscarinic regulation of cortical glutamatergic inputs in mediating aspects of the pause response has been investigated in several studies. In one study, decreases in cortico-SPN EPSCs were found to be elicited by Cholinergic interneurons, both at the level of tonic Ach maintained by their constant firing and by additional spikes timed in close proximity to cortical excitation (Pakhotin & Bracci, 2007). A recent study demonstrated that induction of a burst-pause response in cholinergic interneurons via thalamic stimulation in the brain slice preparation modulated cortico-striatal synapses onto principal neurons in a direct/indirect pathway specific manner, having the effect of first presynaptically reducing the activity of both pathways followed by selective facilitation of the indirect pathway (Ding *et al.* 2010). The time-course of this response was relatively fast (sub-second) and is a possible candidate mechanism by which the pause response might be involved in regulating the ongoing activity of striatal principal neurons and additionally in the experience dependent plasticity at cortico-striatal synapses thought to be involved in striatal dependent learning. It is unclear how

precisely this modulation of inputs can regulate SPN spike timing.

Nicotinic receptors are composed of five transmembrane subunits surrounding a cation conducting ligand gated pore, and as ligand gated channels open on submillisecond to millisecond timescales (Albuquerque *et al.* 1997). They are traditionally divided into two primary classes, heteromeric alpha and beta subunit containing and homomeric alpha-7 subunit containing receptors (Albuquerque *et al.* 1997; Albuquerque *et al.* 2009). From a functional point of view, the receptors can be classified into at least 3 types based on their pharmacological profile and affinity of acetylcholine for activation and for inactivation (Albuquerque *et al.* 2009). Type I receptors correspond to alpha-7 subunit containing homomeric channels, are rapidly desensitizing, have low affinity for acetylcholine and are primarily involved in direct nicotinic receptor mediated fast synaptic excitation, while Type II receptors, most often composed of alpha4/beta2 subunits, have high affinity for acetylcholine, are desensitized more slowly by low agonist concentrations and are involved primarily in presynaptic facilitation of GABA release. Type III receptors exhibit mixed or intermediate characteristics.

Nicotinic receptors are highly expressed in the striatum, and are located on GABAergic interneurons as well as on the presynaptic terminals of glutamatergic and dopaminergic afferents but are not expressed by SPNs (Koos & Tepper, 2002; Liu *et al.* 2007; Pisani *et al.* 2007; Sullivan *et al.* 2008). Activation of nicotinic receptors located on GABAergic interneurons has been shown to increase inhibitory input to projection cells (de Rover *et al.* 2002), and to be critical in a mechanism by which cholinergic interneurons engage recurrent inhibitory circuits that appear to avoid SPNs or other interneurons, selectively inhibiting the cholinergic interneurons (Sullivan *et al.* 2008).

Additionally, electrophysiological recordings of fast spiking GABAergic neurons demonstrate that these neurons can be directly depolarized by application of nicotinic agonists acting through a very slowly or non- desensitizing nicotinic receptor (Koos & Tepper, 2002). Nicotinic EPSPs have never been observed in FSIs (Koos & Tepper, 2002; Tepper *et al.* 2004). Thus it seems that the nicotinic receptors on FSIs are extrasynaptic, and thus may be tuned to the overall ACh tone. An intriguing possibility is that nAChRs, especially those containing the β 2 subunit, serve not only has ionotropic receptors but have the ability to activate intracellular signaling cascades at ligand concentrations below that required for pore opening (Kabbani *et al.* 2007). Thus at least some nAChRs present on FSIs, and possibly other striatal neurons, may act through this mechanism.

Glutamate and dopamine release from afferent terminals is enhanced by activation of presynaptic nicotinic receptors, which include both heteromeric and homomeric subtypes (McGehee *et al.* 1995; Zhou *et al.* 2002; Cragg, 2006; Pakhotin & Bracci, 2007; Wonnacott, 2008). It has recently been shown that optogenetic activation of Cholinergic interneurons *in vivo* can directly induce DA release in the nucleus accumbens core, demonstrating the high level of control of DA release by ACh (Irving, 2011). One likely effect of the pause response is presynaptic modulation of DA release. Cholinergic modulation of glutamate co-released from DA terminals has not been investigated.

Cholinergic interneurons do not express nicotinic receptors. As mentioned, SPNs do not express nicotinic acetylcholine receptors, indicating that nicotinic effects on the output of the striatum are mediated via regulation of their inputs.

Goal of the thesis

The pause response and related activity sequences of cholinergic interneurons are postulated to influence neostriatal output on short time-scales. This idea is supported by the strict temporal relationship between the responses and the varied stimuli to which they are sensitive. The known mechanisms of action of acetylcholine in the striatum are not believed to be capable of translating the activity of cholinergic interneurons into precise changes in spike timing of projection neurons. Part of the reason for this lack of information is the fact that it was not previously feasible to precisely control the activity of cholinergic interneurons. We used optogenetic techniques to solve this problem and were thus able for the first time to control large numbers of cholinergic interneurons. The goal of this thesis is to elucidate mechanisms capable of translating the pause response of cholinergic interneuron populations into fast changes in principal neuron activity.

Experimental Methods

Molecular biological techniques applied to systems neuroscience

In the last decade two major advances in molecular biology have been applied in neuroscience. The first is the genetically programmed expression of ectopic fluorescent reporter genes in specific populations of neuronal cells in transgenic mice. The second is the advent of optogenetics, which allows one to bi-directionally control the activity of specific neuron populations with light. Both of these techniques were absolutely essential to the experiments we performed and thus their methods are described in detail in this chapter.

Transgenic mice

All transgenic mice used in our experiments were housed in the animal colony at Rutgers-Newark which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

ChAT-Cre transgenic mice, (*B6;129S6-Cholinergic<tme1(cre)Lowl>/J*, (Jackson Laboratory)) were bred in the animal colony at Rutgers-Newark. The founders were homozygous for the transgene and the line was maintained as such. In some circumstances homozygous animals were crossed with the background strain C57BL6. This strain of mice has an expression cassette consisting of an internal ribosome entry

sequence (IRES) followed by the gene for Cre-recombinase inserted directly downstream of the endogenous stop codon of the choline-acetyltransferase (ChAT) gene. Cre expression is thus regulated at the level of transcription by the endogenous regulatory sequences of the ChAT gene. In these mice Cre expression in the striatum is restricted solely to cholinergic interneurons.

NPY-EGFP transgenic mice (*B6.FVB-Tg(NPY-hrGFP)1Lowl/J* strain (Jackson Laboratory)) express enhanced green fluorescent protein under the promoter for neuropeptide-Y and in the striatum enable the visualization of multiple interneuron types (Ibanez-Sandoval *et al.* 2011).

ChAT-EGFP transgenic mice (*B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J* (Jackson Laboratory)) express enhanced green fluorescent protein under the promoter for the choline acetyl-transferase gene and in the neostriatum this expression is restricted to Cholinergic interneurons (English *et al.* 2011).

NPY-EGFP and ChAT-EGFP animals were bred in the animal colony at Rutgers-Newark. Both strains were acquired as hemizygotic pairs, necessitating the genotyping of offspring for use in experiments. Genotyping procedures included extraction of DNA from tail samples obtained under isoflurane anesthesia followed by polymerase chain reaction experiments to determine the presence of absence of the transgene as well as a control reaction. Primer sequences (presented as 5'-3') were as follows: NPY-EGFP, common: TATGTGGACGGGGGCAGAAGATCCAGG, mutant: CCCAGCTCACATATTTAT CTAGAG, wild type: GGTGCGGTTGCCGTACTGGA; ChAT-EGFP, forward: AGT AAGGCTATGGGATTCATTC, reverse: AGTTCACCTTGATGCCGTTC, internal positive control forward:

CAAATGTTGCTTGTCTGGTG, internal positive control reverse: GTCAGTCGAGTGCACAGTTT.

Optogenetic control of cholinergic interneuron activity

To enable the experimental manipulation of the activity of populations of cholinergic interneurons we utilized two different optogenetic methods capable of exciting or inhibiting cholinergic interneurons. Channelrhodpsin-2, originally found in the unicellular algae Chlamydomonas reinhardtii, is a non-specific cation channel gated by blue light (peak activation at 470 nm), being closed in the absence of light (Nagel et al. 2003). Channelrhodopsin-2-h134 is an engineered mutant of the wild type channelrhodopsin, which has been modified to have an increased steady-state photocurrent. The h134 variant will be referred to simply as ChR2 from here on, as it is the only version in common use. In neurons expressing ChR2, application of blue light opens the ChR2 conductance to cations resulting in depolarization of the neuron and under some conditions action potential initiation. Halorhodopsin, originally found in the halobacterium *Natronomonas pharaonis*, is an inward chloride ion pump driven by the energy from yellow light (peak activation is at 590 nm) (Mukohata & Kaji, 1981; Sugiyama & Mukohata, 1984; Han & Boyden, 2007; Zhang et al. 2007). We utilized modified versions of the wild-type Halorhodopsin protein, termed eNpHR and NphR3.0/Halo3, which have the benefits of decreased cellular toxicity and increase photocurrent, achieved through increased ER export by addition of ER export signal from K_{ir} 2.1 and increased plasma membrane (PM) insertion by addition of a PM insertion sequence from nAChR (Gradinaru et al. 2007; Zhang et al. 2007; Gradinaru et *al.* 2008). In neurons expressing Halorhodopsin, application of yellow light induces the pumping of Cl⁻ ions into the cell, resulting in a hyperpolarization which under certain conditions inhibits the firing of action potentials. When expressed in cholinergic interneuron of the neostriatum, ChR2-induced depolarization is sufficient to drive action potential firing, while Halorhodopsin induced hyperpolarization is sufficient to stop ongoing tonic firing.

We made intrastriatal injections in ChAT-Cre animals of viral vectors where transgene expression is dependent upon Cre for transcription, resulting in cholinergic interneuron specific expression (Figure 2, (Johnson *et al.* 1996; English *et al.* 2011)).

In order to express ChR2 and Halorhodopsin in neostriatal cholinergic neurons we utilized two main classes of viral vectors. A schematic of their transgene is shown in figure 1. For ChR2 expression we used adeno-associated virus serotype 2 or serotype 5 coding for the ubiquitous promoter elongation factor-1 α (EF1 α) followed by a fusion gene of ChR2h134 and enhanced yellow fluorescent protein coded in the inverse open reading frame orientation flanked by opposing pairs of recombination incompatible loxp and loxp2722 sites (AAV2/5-DIO-ChR2-EYFP) (Schneider *et al.* 2008; Minamimoto *et al.* 2009). For eNpHR we used a replication and chromosomal integration deficient recombinant VSVG pseudotyped HIV-1 virus coding for EF1 α followed by a fusion gene of NpHR and a red fluorescent protein (mCherry, (Price, 1986)) flanked by opposing pairs of recombination incompatible loxp and loxp2722 sites (Atavo *et al.* 2008). For NpHR3.0/Halo3 we used adeno-associated virus serotype 2 or serotype 5 coding for EF1 α followed by a fusion gene of Halo3 and enhanced yellow fluorescent protein coded in the inverse open reading frame orientation protein coded in the inverse open reading pairs of recombination incompatible loxp and loxp2722 sites (plenti-DIO-NpHR-mCherry) (Atasoy *et al.* 2008). For NpHR3.0/Halo3 we used adeno-associated virus serotype 2 or serotype 5 coding for EF1 α followed by a fusion gene of Halo3 and enhanced yellow fluorescent protein coded in the inverse open reading frame orientation

flanked by opposing pairs of recombination incompatible loxp and loxp2722 sites (AAV2/5-DIO-Halo3-EYFP) (Gradinaru *et al.* 2008; Minamimoto *et al.* 2009). In all vectors reaction of the transgenes with Cre-recombinase restores the correct open reading frame allowing for productive transcription.



Figure 1. Reaction of Cre-recombinase with virus transgene turns on transcription of the opsinfluorescent protein fusion gene. Live recombinant virus contained a transgene similar to that picture. The basic layout of the transgene (top), is a ubiquitously active promoter sequence, followed by an opsin-fluorescent protein fusion gene configured in the inverse open reading frame, the latter of which is flanked by incompatible pairs of loxp sites (orange and red sites are incompatible). The result of the reaction of the initial sequence (top) with Cre-recombinase is a permanent transcriptionally productive alignment of the opsin-fluorescent protein fusion gene with the promoter sequence (bottom).

Virus Production

Adeno-associated virus serotype 2 (AAV-2) was used for the expression of ChR2-YFP and serotype 5 (AAV-5) virus for eNpHR3.0-YFP and ChR2-mCherry. The AAV-2 vector was produced at Vector Biolabs. The AAV-5 vectors were produced by the vector core of the University of North Carolina. The transfer vector plasmids and the transgene constructs were designed by Karl Deisseroth at Stanford University. (http://www.stanford.edu/group/dlab/optogenetics/sequence_info.html).

Lentivirus mediated, Cre/loxP controlled expression of eNpHR1.0-mCherry was

carried out with integration deficient lentiviral (IDL) particles to prevent chromosomal rearrangements that may occur across multiple proviral loxP or lox2227 recombination sites when integrating virus is employed. IDL particles were produced in 293FT cells (Invitrogen) grown to 95–100% confluence in DMEM (+10% fetal bovine serum (vol/vol) and 1% L-glutamine (vol/vol)) using TransIT-293 (virus) transfection agent as described previously47. Briefly, confluent 293FT cells in each of six 175-cm² flasks (Falcon) were co-transfected with 22 µg of the lentiviral transfer vector DNA (pLenti:EF1:DOI:eNpHR1.0-mCherry:WPRE) and the second generation packaging plasmids pCMV-dR8.74-D64V (15 µg), and pMD2.G 5 µg; (Addgene, 12259) supplemented with a plasmid carrying a suppressor of a dsRNA inhibitor (pAdvantage, Promega, 2 µg). The *pCMV-dR8.74-D64V* plasmid encodes the lentiviral integrase carrying a D64V point mutation that completely blocks proviral integration 48 and was a gift from R. Yanez-Munoz (Royal Holloway-University of London). The medium was changed to a viral production medium (Ultraculture, Lonza, +1% pen-strep (vol/vol), 1% sodium pyruvate (vol/vol), and 5 mM sodium butyrate) 24 h after transfection, and the virus-containing supernatant was collected and concentrated with ultracentrifugation 48 h post-transfection. The titer of the concentrated IDL particles was not directly determined, but comparison with lentivirus stocks of known titer injected in mouse brains indicated that it approached 10^9 IU ml^{-1} .

The *eNpHR1.0-mCherry* transgene was produced by adding the endoplasmic reticulum export and plasma membrane localization signals described previously31 in two rounds of extension PCR using a high-fidelity DNA polymerase (Accuprime Pfx, Invitrogen) to the coding sequence of *NpHR-mCherry* produced by K.D.

(http://www.stanford.edu/group/dlab/optogenetics/sequence info.html). The primer for first and second PCR rounds were, respectively, sequences the 5'-GTCGTCTCTGTTCTCTGCTTCAGGACACAGAGACCCTGCCTCCCGTGAC CGAGAGT-3' 5'and TTACACCTCGTTCTCGTAGCAGAACTTGTACAGCTCGTCCATGC-3', 5'and GGCCTGCGCTAGCGCCACCATGAGGGGTACGCCCCTGCTCCTCGTCGTCTCTC TGTTCTCTCTGCTTCAG-3' 5and 'CGGACCCATATGGGCGCGCCTTACACCTCGTTCTCGT-3'. The PCR product was subcloned in an inverted orientation between the loxP/lox2722 flanking recombination sites, replacing the ChR2-YFP coding sequence in an AAV:EF1:DOI:ChR2-YFP:WPRE plasmid from which the entire expression cassette (EF1:DOI:eNpHR1.0*mCherry:WPRE*) was subsequently cloned into a third generation (Tat independent) selfinactivating lentiviral expression vector. Detailed map is available on request.

Intracerebral virus injection

All *in vivo* and *in vitro* surgical procedures were performed in accordance with the US National Institutes of Health *Guide to the Care and Use of Laboratory Animals* and with the approval of the Rutgers University Institutional Animal Care and Use Committee. The virus injection surgeries were performed in a custom-built surgical setup inside an isolation cabinet under Biosafety Level-2 (BL-2) confinement. Mice were anesthetized with isoflurane and the skull was exposed under antiseptic conditions using local anesthesia with bupivacaine. A small burr hole was drilled at coordinates 0.5-1.0 mm anterior to Bregma, 1.5-2.2 mm lateral. We injected 0.5-1.5 µl of concentrated virus

stock solution using a Nanoject-2 pressure injection apparatus and glass pipettes over 10– 40 min at a depth of 2.4–2.7 mm from the surface of the brain. Animals were housed in a BL-2 safety cabinet for at least 6 d. Experiments were conducted 7–30 d following injection.

Immunocytochemistry

Fixation was performed after establishing anesthesia with ketamine (400 mg per kg of body weight, intraperitoneal) with transcardial perfusion using 10 ml of ice-cold oxygenated Ringer solution followed by 75–100 ml of 4% paraformaldehyde (wt/vol) and 15% saturated picric acid (vol/vol) in 0.15 M phosphate buffer. Brains were kept in the same fixative overnight. We cut 60-µm sections on a Vibratome. The immunocytochemical labeling of ChAT included pre-incubation in 10% methanol (vol/vol) and 3% hydrogen peroxide (vol/vol) in phosphate-buffered saline (PBS), blocking of nonspecific binding with 10% normal donkey serum (vol/vol), 3% bovine serum albumin (vol/vol) in a 0.5% Triton X-100 solution (vol/vol) in PBS, followed by incubation in the blocking solution containing goat antibody to ChAT (1:200, cat. #AB144P, Millipore) for 48 h at 20–25 °C. After wash, sections were incubated in donkey antibody to goat IgG conjugated to Alexa-594 (1:100) in PBS at 20–25 °C overnight. Sections were mounted in Vectashield medium.

In vitro voltage and current clamp recordings of striatal neurons

All *in vitro* recordings were obtained from acute para-horizontal striatal slices at 7-21 days post virus injection. All experiments are performed in adult animals, 49-309

days of age at the time of recording. Striatal slices were made as follows: animals were given an intraperitoneal injection of ketamine/xylazine then perfused with ice cold artificial cerebrospinal fluid (aCSF) containing (in mM): 242.0 sucrose, 2.5 KCl, 0.0 CaCl₂, 7.0 MgCl₂, 28.0 NaHCO₃, 1.25 NaH₂PO₄, 10.0 glucose, and 15.0 sodium pyruvate, bubbled with a 95% oxygen / 5% carbon dioxide gas mixture. The brain was then removed from the skull, blocked, and mounted in a vibratome in the same solution with electronic cooling to maintain near freezing temperature. 300µm slices were made and stored in solution which was bubbled continuously with a carbon-dioxide and oxygen mixture and kept at 34 degrees Celsius for at least 30 minutes prior to recording. All recordings were made in the same storage aCSF or a modified aCSF containing 1mM Ca2 and 3.5mM K. Solution was kept at 30-32 degrees Celsius throughout recording. Electrode solution for current clamp and some voltage clamp recordings consisted of a standard KMeSO4 based solution, while a subset of voltage clamp recordings were made using CsCl⁻ based solution or aCSF. In some experiments internal solution also contained Alexa 594 (25 nm) to enable post-hoc identification of recorded neurons.

A combination of double and triple, cell attached and whole cell current and voltage patch clamp recordings were made using three Axon instruments Multiclamp 700B amplifiers run through an Instrutech digitizer and controlled by an Apple G4 Powermac desktop computer running Axograph data acquisition software. This recording system enabled the simultaneously recording of up to three neurons in voltage or current clamp configurations. Cell attached recordings, using aCSF as electrode internal solution, allowed us to record the *in vitro* firing of neurons without disrupting

the local extracellular and intracellular ionic concentrations or osmolarities. These factors were essential in our verification that transgene expression does not change the spontaneous activity of cholinergic interneurons, a key physiological characteristic and part of their regulatory mechanism, which is disrupted by whole cell recordings and could be affected by increased local potassium concentrations. Whole cell voltage and current clamp recordings of opsin expressing Cholinergic interneurons and GABAergic interneurons enabled us to record their intrinsic electrophysiological properties in detail. Similar recordings of SPNs enabled us to observe their response to stimulation of Cholinergic or GABAergic interneurons.

In vitro optical stimulation

ChR2-YFP was activated using a 750-mW blue LED (http://www.cree.com/). Light was either projected onto the slice through the condenser of the microscope with the bottom differential interference contrast polarizer removed or projected onto the slice from above (approximately 2 cm). The intensity and duration of the illumination were controlled through a digital to analog converter output of a ITC-18 digitizer and a Mightex SLA LED driver. Halo3-EYFP and eNpHR1.0-mCherry were activated with alternating pulses of 200–300-ms green (514 \pm 20 nm) and blue (470 \pm 20 nm) light delivered through the epifluorescence illumination pathway using Chroma Technologies filter cubes under temporal control with a Uniblitz shutter (Vincent Associates). Blue light was delivered to facilitate recovery from photodesensitization. Optical stimuli were delivered at 20–60-s intervals to allow recovery to baseline.

In vivo optical stimulation

We chronically implanted 125-µm multi-mode optic fibers (part #AFS105/125Y, Thor Labs) as part of the optrode. To minimize tissue damage and increase the lateral distribution of light, we etched optical fibers by immersing ~200 µm of the tip of the fiber in hydrofluoric acid (Sigma-Aldrich) overlaid with mineral oil and then slowly lifting the fiber tip into the protective oil layer (over ~30–60 min), resulting in a smooth, gradual taper and a tip diameter of <50 µm. Implanted fibers were coupled to a 594-nm DPSS laser (LaserGlow Technologies) via modified light-coupling connectors (part # 86024-5500, Thor Labs) and ceramic attachments encasing the external end of the fiber. Light intensity at the fiber tip was measured before implantation as 10–30 mW. Illumination duration was controlled via a TTL-gated shutter with a transition time of less than 0.5 ms (Uniblitz LS2; Vincent Associates). Stimulation timing was controlled via Spike2 software running a CED micro MKII Digitizer (software and hardware were from Cambridge Electronic Design).

Chronic in vivo extracellular recording

Optrodes were composed of four independently movable tetrodes mounted in a five cannula array surrounding a central optic fiber with lateral distances between the five elements set at 200 μ m. Tetrode wires were gold-plated to impedances of <400 kOhm measured at 1 kHz, no more than 1 h before implantation. Coordinates targeting dorsal striatum were +0.5–1.0 mm anterior, 1.6–2.0 mm lateral and –2.4–2.7 mm ventral (relative to Bregma). Animals were implanted with optrodes >7 d post virus injection. Wires were advanced slowly until units were encountered. The recorded extracellular

potential was pre-amplified $20 \times$ using a headstage pre-amplifier (Plexon) and further amplified $100 \times$ and band-pass filtered (0.1–10,000 Hz) using an analog amplifier (Grass Technologies), digitized at 25 kHz (micro MKII Digitizer, Cambridge Electronic Design) and recorded for off-line analysis using Spike2 software (Cambridge Electronic Design).

Analysis of in vitro data

Analysis was performed in Axograph2.0 (J. Clements) or with routines written in IgorPro (WaveMetrics). Rise times were defined as the time difference between the data points at which the amplitude of the response was 10% and 90% of peak. For the analysis of the correlation of fIPSC and sIPSC amplitudes, individual response amplitudes were defined as the mean in a 1-ms (fIPSC) or 15–35-ms (sIPSC) window (Fig. 1f). The wide window averaging was carried out to eliminate the contribution of the uncorrelated stochastic channel noise associated with the sIPSC. An exponential function was then fitted to the fIPSC and sIPSC amplitudes of subsequent compound responses expressed as functions of recording time, which revealed that both amplitudes decayed over repeated stimulations. The exponentially fitted trend of amplitude decay was then subtracted from the individual amplitudes and the de-trended amplitudes were expressed relative to the respective average fIPSCs and sIPSCs amplitudes, thus defining Δ fIPSC and Δ sIPSC (Fig. 1f). This procedure removes a source of correlated variance of unknown origin, but the uncorrelated nature of the residual variance excludes in itself the possibility of shared receptor mechanisms or neurotransmitter pools underlying the two response components.

Analysis of in vivo data

Spike2 software was used for spike detection and sorting. Signals were band-pass filtered (300-6,000 Hz, digital two-pole Butterworth filter) and an appropriate spike trigger threshold was set by the experimenter (approximately 3–5 times the s.d. of the noise). Wavemarks defined as 0.5-ms pre and 1.0-ms post peak threshold crossing were extracted from each channel when at least one channel was triggered. After detection, the mean of the peak amplitudes (negative going) on the four channels was measured and this data was combined with the relative ratios of the peaks on the four channels, yielding five variables, from which three principal components were extracted using a principal component analysis routine of Spike2. The events were then projected in a thus defined three-dimensional space and were automatically over-clustered using the K-mean statistics (10–20 clusters are initially cut for data actually having less than five units). Clusters that were manually classified as noise on the basis of waveform shapes and inter-stimulus interval (ISI) histograms were discarded. The remaining potential units were then recombined and reclassified the same way a second time, with the effect of reducing the bias introduced in the first iteration by the noise and improving the extraction of principal components most discriminative among extracellular spikes. The identified clusters were then subjected to principal component analysis based on multidimensional data defined by all amplitude values in the spike waveforms. K-means were again used to automatically over-cluster the data, and the clustering information from waveforms and relative amplitude ratios was reconciled manually. Auto and crosscorrelation histograms were constructed and units were classified as putative single units if there was a clear refractory period (>3 ms) and if, in the ISI histogram, 10% or less of the spikes in the first 50 ms occurred in the first 5 ms (ref. 49). Unit clusters that had

classifiable waveforms similar to single units, but did not meet these criteria were classified as multiple unit recordings.

Differences in waveform shape and firing pattern as well as optical responses were used to classify cell types. In accordance with previous reports, putative SPN single units had firing rates <2 Hz (mean = 0.74 Hz, s.d. = 0.62) and band-pass filtered (300–6,000 Hz) waveform valley widths >0.35 ms (mean = 0.51 ms, s.d. = 0.09)50. ChAT units were identified based on zero latency optical inhibition. Notably, the waveforms of ChAT and SPN units were similar, the most reliable difference being an initial positive phase present in most ChAT units (Fig. 6). Spikes of ChAT units fired tonically whereas SPNs tended to fire single spikes or bursts interspersed with long (>1 s) periods of silence. Units classified as 'other neurons' had firing rates similar to ChAT interneurons, but had significantly shorter waveforms than all other unit classes and their firing rate was not directly modulated by illumination.

To examine the relationship between optical stimulation and changes in the firing rate of SPNs, PSTHs were constructed using 50-ms binning and the mean and the s.d. of the spike number per bin were calculated for the 10–20 s preceding the stimulus (20–40 bins). A statistically significant change in firing rate change was defined as two consecutive bins outside mean ± 2 s.d. defining a significance level of P = 0.0019.

Statistical methods

Given the small number of observations in most cases, the nonparametric Wilcoxon ranksum test was used to compare the means of populations. These calculations and linear regression analysis were performed in IgorPro or StatView. Population measurements are reported as mean \pm s.d. unless otherwise indicated. The statistical significance of firing rate changes *in vivo* were determined as described above.

*The majority of the methods text has been reproduced from English et al. 2011.

Results

Expression of excitatory and inhibitory opsins in neostriatal cholinergic interneurons enables optical bi-directional control of their activity

Many of the reported TAN pause responses include an initial excitatory phase (Kimura et al. 1984; Morris et al. 2004), thus we used channelrhodopsin expression in these neurons to replicate this synchronous excitation. AAV2/2-DIO-ChR2-EYFP (Sohal et al. 2009), was injected into the dorsal striatum of transgenic mice expressing Crerecombinase under the regulatory sequences for choline acetyltransferase (ChAT-Cre mice, described in detail in the methods section). This vector contains a ubiquitously active promoter followed by a fusion gene consisting of Channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (EYFP), coded in the inverse open reading frame orientation and flanked by pairs of opposing, recombination incompatible, loxp sites (loxp and loxp2722). Reaction of this sequence with Cre recombinase restores the correct ORF and through the high transcriptional activity of the EF1 α promoter yields high expression of the ChR2-EYFP fusion protein. Expression is restricted to cholinergic interneurons in our preparation (fig 2.), and Immunocytochemistry for ChAT protein demonstrates near perfect targeting, with 81/82 EYFP+ neurons colocalizing ChAT (98.7%, 81/82). It should be noted that every neuron targeted for recording in acute brain slices from these animals



Figure 2. Verification of opsin targeting and visualization of brain slice recording area A. Confocal image series demonstrating Colocalization of the ChR2-EYFP fusion protein with the Alexa-594 antibody fluorophore-tagged for choline acetyltransferase. B. 20X image of the same brain slice as in A, showing multiple EYFP+/ChAT+ neurons and dense axon expression of ChR2-EYFP. C. ChR2-EYFP expressing cholinergic interneuron imagined in a live slice demonstrates the feasibility targeting method for patch clamp recording of fluorescent cells. D. Low magnification image of the live brain slice under near IR DIC imaging, with the corresponding EYFP signal shown in E. Note the white line is dental floss used to hole the slice in place. Reproduced from English et al. 2011.

based on EYFP signal matched the electrophysiological characteristics of Cholinergic interneurons (n>50), and that the one neuron in the Immunocytochemistry count may have simply failed to immunoreact.

Acute neostriatal slices were prepared from these animals and the area of infection was localized using epifluorescence imaging 4X at magnification. Cholinergic identified interneurons were by fluorescent reporter (EYFP) expression and were targeted for cell attached and whole cell recording using near IR-DIC imaging at 80X magnification. Cell attached recordings of ChR2-EYFP+ neurons demonstrate that pulses of 5ms, 470nm light result in single action potentials (fig 3. B, C).

Additionally, ramp pulses induced action potentials with reliable trial-to-trial jigger. Whole cell recording of these neurons (fig. 3A), in current clamp mode demonstrates that optically induced action potentials appear physiological, being characteristically wide and

having a long AHP.

The most frequently observed response of cholinergic interneurons to motivationally salient stimuli is a populationwide pause in ongoing activity lasting 200-300 ms followed by a brief period of synchronized excitation (Pisani et al. 2007). The effect of this activity sequence on SPNs is currently unknown. from Our data experiments previous demonstrate that synchronized cholinergic interneuron excitation leads to rapid and strong inhibition of SPNs via



Figure 3. Basic physiology and optical responses of channelrhodopsin-2 expressing cholinergic interneurons. A. Current-voltage series from a cholinergic interneuron expressing channelrhodopsin-2 targeted for recording by its EYFP signal. Note the prominent sag in response to hyperpolarizing current injection, sustained low firing in response to positive current injection and rebound action potentials in response to the offset of hyperpolarizing current pulses. B. Optically elicited action potential in a EYFP+ cholinergic interneuron. Note the characteristically wide action potential is elicited by the optical stimulus at short latency. Blue bar is 5 ms pulse. C. Cell attached recording in voltage clamp mode of unclamped spikes in a EYFP+ cholinergic interneuron. Note the tonic background firing and the precise experimental timing of spikes at 5 Hz by pulses of blue light. Blue bard is 5 ms pulse. D. Ramp optical stimulus reliably elicits spikes, with latencies ranging from 1 to 10 ms over six trials. 40 µm. Reproduced from English et al. 2011.

novel cholinergic-GABAergic circuits. We suspected that the post-pause excitation might activate the same mechanism, however it remained possible that the pause would prevent this activation. To test this we recorded SPNs in acute brain slices with expression of Halorhodopsin-3 (Halo3), an engineered version of NpHR, exclusively in cholinergic interneurons.

Adeno-associated virus (AAV2/2-DIO-Halo3-EYFP) coding for Halo3-EYFP (in





the same configuration as for the ChR2 virus described above) was injected into dorsal striatum of ChAT-Cre transgenic mice resulting in expression of the transgenes exclusively in cholinergic interneurons (fig 4. EYFP+ Cholinergic inset). interneurons were visualized in slices acute brain with epifluorescence imaging and subsequently targeted for recording under near IR-DIC imaging. Whole cell current clamp recordings using normal electrode internal solution were obtained from these neurons. Somatically injected current pulses elicited voltage responses characteristic of cholinergic interneurons, including reliably timed rebound spikes at the offset of hyperpolarizing pulses, tonic firing, and a prominent I_h. Rebound

firing could also be elicited by an optically induced hyperpolarization (80 ms, 570 nm; fig 4.). Cell attached recordings obtained in voltage clamp configuration (using aCSF as electrode internal solution to avoid changing the local ionic and osmotic environment) of unclamped action potentials demonstrated spontaneous firing of Halo3-EYFP expressing Cholinergic interneurons and that the effect of optical stimulation (80 ms, 570 nm; fig 4.) is a pause in tonic firing followed by a short-latency rebound single spike. Thus we are able to elicit pause-excitation responses in cholinergic interneuron populations *in vitro* in acute brain slices.

Synchronized activation of Cholinergic+ interneurons induces cholinergic-GABAergic inhibition of SPNs in vitro

Previous studies in non-human primates have demonstrated that under some experimental conditions, particularly those involving unexpected stimuli or outcomes, the pause response of

TANs (presumed

Cholinergic

interneurons) has an initial excitatory phase. The effect of this synchronized activity of a large proportion of the cholinergic interneuron population on SPNs is currently



Figure 5. Optical stimulation of ChR2 expressing cholinergic interneurons elicits an IPSC/P in spiny projection neurons which reliably inhibits action potential firing. A. Current-voltage series recorded from a spiny projection neuron in an acute neostriatal slice with expression of ChR2-EYFP in cholinergic interneurons. Repeated optical stimulation (blue bar is 5 ms pulse, applied at 0.05 Hz) elicits an IPSP which reverses near the reversal potential for chloride ions, suggesting that it is meditated by chloride permeable GABA_A receptors. Note the corresponding IPSC recorded in voltage clamp at -80 mV. B. Repeated trials of optical stimulation under the same conditions as in A demonstrate the reliability of the inhibition (seven of seven trials shown). Reproduced from English et al. 2011.

unknown. To enable the recording of the effect of this event on SPNs we recapitulated this response *in vitro* in acute brain slices by expressing the blue-light activated non-specific cation channel channelrhodopsin-2 (ChR2) selectively in cholinergic interneurons

Whole cell current and voltage clamp recordings of SPNs were obtained in acute neostriatal slices prepared from animals expressing ChR2-EYFP in cholinergic interneurons. Recordings were targeted to the area of infection in dorsal striatum by fluorescent imaging at 4-80X magnifications, and SPNs were targeted by characteristic morphology and patched under near IR-DIC imaging. Their identity confirmed was bv determining the current-voltage relationship by injecting current somatically, which revealed the characteristic inward rectification and ramp depolarization leading to



Figure 6. The optically induced IPSP/C in SPNs persists in the presence of AMPA receptor inhibition Top: Responses of an SPN to optical stimulation during hyperpolarizing and depolarizing current injection. Vrest = -88 mV. Arrow first depolarizing indicates IPSP. Bottom: IPSC recorded in a different SPN using CsCl internal solution. The neuron is voltage clamped at -90 mV. 20 µM CNQX is present in the aCSF in both recordings. Blue bar represents 5 ms blue light optical stimulus. Both experiments were performed in slices from animals expressing ChR2-EYFP in Cholinergic interneurons. Reproduced from English et al. 2011.

spiking. Subsequent filling with the fluorescent dye Alexa 594 enabled the visualization of dendrites, which confirmed their highly spiny nature. Pulses of blue light (5ms, 470nm) applied at 0.05 Hz produced in SPNs recorded under current clamp an IPSP which reversed at the Cl⁻ reversal potential (~60 mV) and inhibited action potential firing induced by somatic current injection (fig 5.). It was not possible to determine if naturally occurring action potentials are inhibited by optical stimulation due to the fact that SPNs are not spontaneously active in acute brain slice preparations. The power of spiking
inhibition was observed to be most effective at low SPN firing rates (~10-30Hz, fig 7.). Application of either the GABA_A receptor antagonist bicuculline or the type-II nAChR receptor antagonist DHβE blocked the IPSC in SPNs recorded in voltage clamp (fig. 8, n=10 for each). Blockade of glutamate receptors did not effect the IPSP or spike inhibition (fig. 6, n=10), ruling out the possibility that glutamate co-released from Cholinergic interneurons was responsible for the induction of GABA release. These results suggest that synchronized firing of cholinergic interneurons activates GABA release (most likely through action potential firing of one or more GABAergic interneurons), via nAChRs, the effect of which is a powerful inhibition of SPNs. It should be noted that all (94/94) SPNs responded to stimulation in this preparation with an IPSP or IPSC, suggesting that this circuit has very high connectivity, targets SPNs of both direct and indirect pathways and likely does not obey patch-matrix boundaries.

Further analysis of the SPN GABAergic IPSC (recorded in the presence of CNQX

GABAergic

to

isolate

currents) revealed that it is composed of three components with different time constants, from now on termed the fast $(\tau=5.2+/-1.8 \text{ ms}),$ slow $(\tau=96+/-11.7 \text{ ms})$



Figure 7. The magnitude of inhibition of SPNs depends on postsynaptic firing rate Left. Decrease in momentary firing rate for 5 ranges of initial rate (one-way ANOVA, p<.05, Tukey post-hoc test, **p<.01). Right. Representative examples of inhibition at low and high rates of firing. Blue bars represent 5 ms optical stimulus. Reproduced from English et al. 2011.

ms) and late (τ =906+/-106 ms) responses (n=6 for τ calculations) (fig. 8). These components were observed to vary independently (fig. 8). Decreasing the rate of GABA reuptake through application of the GABA reuptake transporter (GAT-1) inhibitor NO711 changed the time course of the slow component only (fig. 8, 322% increase, n=4, Wilcoxon test, P=0.02, n=4). Together, these data suggest that there are multiple distinct sources of GABA release activated by synchronous activation of cholinergic interneurons. We did not investigate the late component further because it accounts for such a small proportion of the total current.

In an attempt to determine whether the GABA release induced by stimulation of cholinergic interneurons was mediated by action potential firing of a GABAergic neuron or direct facilitation of GABA release by nicotinic depolarization of the axon terminals we applied TTX (1 μ M), which completely blocked the SPN IPSP/C and then applied the potassium channel blocker 4-AP (n=2), as this protocol can under some circumstances demonstrated reveal direct presynaptic facilitation of neurotransmitter release (Petreanu *et al.* 2009; Cruikshank *et al.* 2010). We found that upon application of 4-AP, the response



Figure 8. Pharmacological and temporal characteristics of the IPSC elicited in SPNs by optical stimulation of ChR2 expressing cholinergic interneurons. A. Optogenetically elicited IPSCs in two SPNs (blue traces) were blocked by bicuculline (Bic, left) or DH β E (right, red traces). B. Kinetic components of the compound IPSC. Left, three distinct components of the IPSC exhibiting different rdecay values. Right, non-monotonic transition between the fIPSC and the sIPSC. Note the negative inflection following the transition (red arrowhead). Inset, decomposition of the compound IPSC (black trace) into a fIPSC (blue trace) and sIPSC (red trace). C. Independent trial-to-trial amplitude variance of the fIPSC and the sIPSC. Left, overlay of four responses exhibiting identical sIPSC, but different fIPSC, components (colored arrows point to fIPSC peaks). Shaded areas are averaging windows. Middle, variable sIPSC components. Right, relative sIPSC amplitudes plotted against corresponding relative peak fIPSC amplitudes (n = 5). Red line is linear regression (n.s. not significant, P > 0.2). D. Inhibition of GAT-1 selectively prolonged the sIPSC. Note that the fIPSC was unaffected (arrow and inset). Red arrow, transition point of the response components. In all figures, the blue bars represent optical stimuli. Reproduced from English et al. 2011.

was transiently rescued while the drug washed in, then disappearing again. Successful permanent rescue would be evidence for an action potential independent mechanism (as axon potentials are blocked by TTX). The obtained result is not conclusive. The fact that TTX block of sodium channels is use dependent is a possible explanation for the transient recovery. It is possible that if a GABAergic interneuron mediating the response was not active, that it would still fire a few action potentials in the presence of TTX before TTX blocked the sodium channels and thus axon potentials in that neuron.

These data represent the first report of a neostriatal nicotinic-GABAergic circuit which targets SPNs. Additionally, the Cholinergic interneuron activity that elicits this response closely resembles activity sequences of these neurons which signal the occurrence of motivationally salient events recorded in non-human primates, and thus may play a role in the translation of these sequences into changes in striatal output.

SPN inhibition is distinct from previously described cholinergic-GABAergic recurrent inhibitory circuits of cholinergic interneurons

It has recently been reported that cholinergic interneurons activate a recurrent GABAergic inhibitory circuit through nAChRs (Sullivan et al. 2008). Due to the similarity with the circuit we are investigating we compared the recurrent inhibition of cholinergic interneurons and the feed-forward inhibition of SPNs. Both responses were reliably evoked by optical stimulation of ChR2 expressing cholinergic interneurons in neostriatal brain

slices. Both are blocked by the GABA_A receptor antagonist bicuculline (fig 8,10), and the type II nAChR antagonist DHBE, but not by antagonists of glutamate receptors. However, the feed-forward inhibition was found to be different from the recurrent inhibition in several characteristics. First, the onset latency of the IPSC elicited in Cholinergic interneurons via the recurrent pathway is significantly shorter than simultaneously recorded feed-forward IPSCs in SPNs (fig 10; note the responses elicited in the cholinergic neuron occur before spiking in this neuron due to holding at hyperpolarized potential. Other ChR2 expressing cholinergic interneurons are assumed to fire sooner and elicit the response in the recorded neuron). This effect is independent of stimulus intensity and is thus not likely to be an artifact of the preparation. Second, over a large range of stimulation intensities, the optical stimulation power necessary to activate the recurrent circuit was significantly less than that required to activate the feed-forward circuit. This was demonstrated by the fact that minimal stimulation values evoked recurrent inhibitory responses in cholinergic interneurons without a corresponding response in simultaneously recorded SPNs, while maximal stimulation of the same pair of neurons evoked a response in both (fig. 10). Additionally the recurrent circuit elicits trains of IPSCs in recorded cholinergic interneurons while the feed-forward circuit always elicits a unitary (though multi-component) response in SPNs (fig. 10).

These data support the idea that the response of SPNs to synchronized firing of cholinergic interneurons represents a novel feed-forward GABAergic circuit distinct from the recurrent circuit targeting cholinergic interneurons.



Figure 10. The feed-forward inhibition of SPNs and recurrent inhibition of cholinergic interneurons are mediated by distinct mechanisms. A. Top: Recurrent IPSCs (arrows) elicited by optogenetic stimulation (blue bar) are mediated by GABA_A receptors (top; red trace shows the ChR2 current after blocking the IPSCs). Bottom: Single spikes evoked in voltage clamp in a cholinergic interneuron elicit a train of recurrent IPSCs (arrows point to onsets of the IPSCs). Note that over successive trials the onset latencies remain approximately constant (arrows) while the IPSC amplitudes vary significantly. This observation strongly suggests that the recurrent IPSCs originate from stereotypical burst firing of one or more interneurons that is driven by a regenerative intrinsic ionic mechanism triggered by nicotinic depolarization (see also a for another example). B. Feed-forward inhibition in an SPN and recurrent inhibition in a simultaneously recorded cholinergic interneuron exhibit distinct temporal patterns. Note the IPSC train in the cholinergic interneuron (top traces, arrows) that is absent in the SPN (bottom). C. Simultaneously recorded recurrent IPSCs and feed-forward IPSCs exhibit different onset latencies at 3 different optical stimulus intensities. Note that the onset latencies in the SPNs at low stimulus intensities are significantly longer then the latency at maximum intensity reported in the main text. D. Cumulative comparison of the onset latency difference in 3 Cholinergic-SPN pairs at 9 different stimulus intensities. The latencies are statistically significantly different (paired t-test, p<.0001). Note that the average difference in onset latency (4.8+1.7 ms), far exceeds the typical total conduction delay among local circuit neurons which strongly suggest that the responses cannot originate from the same source. E. Triple (left) and paired recording (right) examples in which the recurrent inhibition in Cholinergic interneurons could be activated within a specific range of stimulus intensities (green, blue and black traces) without triggering a response in the simultaneously recorded nearby SPNs. Note that sub-maximal, but higher stimulation intensities (pink traces) can elicit IPSCs in the SPNs demonstrating that the feed-forward circuit to SPNs is not compromised. IPSC is shown for one SPN in the left panel (pink trace). Similar data was obtained in 16 pairs and 2 triples, representing a total of 20 instances in which recurrent inhibition could be independently triggered. Reproduced from English et al. 2011.

Pause-excitation sequences of Cholinergic+ interneurons induce cholinergic-GABAergic inhibition in SPNs in vitro

We performed whole cell current and voltage clamp recordings of SPNs simultaneous with whole cell and cell attached recording of Halo3-EYFP expressing cholinergic interneurons (fig 11). To better visualize distal synaptic inputs, in a subset of voltage clamp recordings of SPNs electrode internal solution contained cesium (to decrease the electrotonic length of the neuron) alone or cesium and high [Cl⁻] (to decrease the electrotonic length and to shift the Cl⁻ reversal potential to close to 0 mV, amplifying the magnitude of GABA_A mediated currents recorded at membrane potentials of ~90mV). SPNs recorded in current clamp mode responded to optically elicited rebound firing of cholinergic interneurons with an IPSP capable of inhibiting action potential firing induced by somatic current injection through the patch pipette (fig. 11). No effect in the SPN was observed during the pause period of the optically induced sequence. In SPNs recorded in voltage clamp, the corresponding IPSC appeared similar to the IPSC resulting from optical stimulation of ChR2 expressing Cholinergic interneurons, including its inhibition by either GABA_A or type II nAChR antagonists (n=5), similar onset latency and slow time course (relative to spontaneous IPSCs and those from FSI-SPN synapses) as well as the existence of multiple components. It is important to note that in both ChR2 and Halo3 based preparations, the elicited responses in a subset of SPNs lacked the initial fast component of the GABAergic IPSP/C. Additionally, in some Halo3 experiments two periods of SPN action potential inhibition were apparent, both requiring nicotinic receptors, and corresponding temporally to the first and second spikes occurring after the optically induced hyperpolarization and pause in action potential firing (fig. 11, n=2).

In summary, synchronized firing of cholinergic interneurons, with or without a preceding cessation of tonic firing, engages powerful feed-forward GABA_A receptor mediated inhibition of SPNs through nAChR activation of GABA release. Importantly, rebound spikes driven by ionic mechanisms intrinsic to the cholinergic interneurons are able to engage this inhibitory circuitry. This provides evidence in support of the idea that the inhibitory responses elicited in SPNs by optically induced action potentials in ChR2 expressing Cholinergic interneurons are not an artifact of the likely supraphysiological release of ACh caused by overly synchronous firing across multiple neurons or by expression of ChR2 in presynaptic terminals, an effect which has been documented for other neuron types (Zhang and Oertner, 2006). This is the first data reporting fast effects of pause-rebound excitation sequences of cholinergic interneurons on SPNs.



Figure 11: Optogenetically reproduced pause-excitation population response of Cholinergic interneurons elicits powerful inhibition in SPNs in vitro. A. Synaptic responses of a SPN to a pause-excitation population response of cholinergic interneurons. 1, rebound excitation of the interneurons triggered coincident large IPSPs that efficiently blocked action potential generation. 2. Spike trains of cholinergic interneurons recorded using cell-attached (large spikes with red asterisks) and extracellular (small spikes with blue asterisks) recording. 3. Another cholinergic interneuron recorded in current clamp. Inset, eNpHR3.0-YFP–expressing cholinergic interneurons and cell-attached recording pipettes (red). 4. PSTH of Cholinergic interneurons demonstrating pronounced pause-excitation activity. B. Simultaneous current recordings (blue and red) from two SPNs showing IPSCs elicited by the rebound activation of cholinergic interneurons (bottom) induced using eNpHR1.0-mCherry. Simultaneous voltage recording from a third SPN showed optical inhibition and rebound firing. C. Top, short and long latency inhibition (black trace) in a SPN elicited by cholinergic interneurons were blocked by DHβE (red traces). Bottom, cell-attached

In vitro Cholinergic-SPN paired recordings demonstrate cholinergic-

GABAergic circuits targeting SPNs exist in non-transgenic animals with no opsin expression

Due to concern that ChR2 and Halo3 expression in and optical stimulation of Cholinergic neurons could be creating a non-physiological preparation, we performed paired recordings between Cholinergic interneurons and SPNs in wild type animals with no expression of ChR2 or Halo3, and in transgenic animals expressing EGFP under the endogenous regulatory sequences for choline acetyl-cholinesterase (Cholinergic-EGFP). Individual spontaneous actions potentials in cholinergic interneurons do not trigger postsynaptic responses in SPNs. We recorded Cholinergic neurons in whole cell current clamp configuration and injected negative current (50-100 pA) to suppress tonic firing, then elicited single action potentials by injecting positive current through the patch pipette (300 pA, 5 ms, 0.05 Hz). This stimulation protocol evoked an inward current in SPNs voltage clamped at -90 mV, similar to the ChR2 and Halo3 induced currents (approximately 50% of pairs, n=21). Recurrent inhibition in cholinergic interneurons was also evoked in this experiment, and as in previous experiments using opsins it was distinct from the SPN response (fig. 11). The IPSC evoked in SPNS by single cholinergic interneuron spikes was found to reverse at the Cl⁻ reversal potential and to be sensitive to the GABA_A receptor antagonist bicuculline (10 µM, n=4) and the nAChR antagonist DHbE (100 nM, n=3) (fig 11), similar to the results in our other preparations.

These date demonstrate that the cholinergic-GABAergic circuit targeting SPNs is not an artifact of our opsin preparations, rather it is a genuine and novel functional



Figure 12. *GABAergic IPSCs are elicited in SPNs by activation of single cholinergic interneurons.* **a.** Photomicrograph of an EGFP expressing cholinergic interneuron (*left*, arrow) recorded and labeled with Alexa-594 (*middle*, arrow). A simultaneously recorded SPN is also labeled (*right* arrowhead). Overlay is on the right. **b.** Whole cell current clamp recording of the EGFP positive neuron demonstrates characteristic responses to current injection (same as in *a*). Photomicrograph taken during recording (inset). **c.** IPSCs elicited in the SPN (*top*, same as in **a**) and the cholinergic interneuron (*bottom*, arrows, same as in **a** and in Supplementary Fig. 4a) to single spikes of the cholinergic interneuron elicited in voltage clamp (middle trace, black). Double exponential fit to the IPSC in the SPN is shown. **d.** Another paired recording demonstrates that the IPSC in the SPN (black) is blocked by bicuculline (red, block; green, partial recovery), and by DHbE (yellow). Inset shows the membrane potential responses of the presynaptic interneuron to injected current. **e.** Scatter plot of individual IPSC amplitudes during pharmacological challenge. *Reproduced from English et al.* 2011.

Parvalbumin expressing fast spiking interneurons and NPY-PLTS

interneurons do not participate

in the cholinergic-GABAergic circuit targeting SPNs

We hypothesized that the cholinergic-GABAergic inhibition was mediated by action potential firing of GABAergic interneurons, known to express nAChRs and in the case of the parvalbumin expressing FSI, to be depolarized by cholinergic agonists acting at nAChRs expressed by these neurons (Koos & Tepper, 2002). We thus chose to evaluate the PV+ FSI first



Figure 13: FSIs do not mediate the inhibition of SPNs by Cholinergic interneurons. (a) Paired recording from a synaptically connected FSI and SPN. Intracellularly injected current pulses in the interneuron elicited voltage responses that identified it as an FSI (bottom). Note the typical large-amplitude IPSCs elicited in the SPN (arrows). (b) Optical stimulus (blue bar) elicited a compound IPSC, including a large fIPSC in the SPN (blue trace, top), but failed to trigger action potentials or large depolarizing potentials in the FSI (red trace, bottom), as compared with the responses elicited in NPY-NGF neurons. Reproduced from English et al. 2011.

thus chose to evaluate the PV+ FSI first. Whole cell voltage and current clamp recordings were obtained of FSIs in slices with ChR2 expression in cholinergic interneurons (as described in previous sections). Paired recordings of FSIs and SPNs demonstrated a high probability of inhibitory synaptic connections, as has been previously demonstrated (Koos & Tepper, 1999); fig. 13). Optical stimulation induced IPSC/Ps in all recorded SPNs. FSIs had no response to the optical stimulus in the majority of cases and in some cases a small GABAergic IPSP was observed (fig. 13, n=8; data not shown). These experiments suggest that FSIs are not involved in the cholinergic-GABAergic circuit under investigation.

PLTS

NPY type expressing interneurons were evaluated next, although their inhibition of SPNs has previously been shown to be weak and were thus not particularly likely to be the neuron we were looking



Figure 14. NPY-PLTS interneurons do not mediate the inhibition in SPNs. a. Photomicrograph of a brain slice from a double transgenic Cholinergic-Cre/NPY-EGFP mouse expressing Cre-recombinase in Cholinergic interneurons and EGFP in NPY expressing neurons. Intrastriatal injection of AAV2-DIO-ChR2-mCherry allowed distinct visualization and targeted whole cell patch clamp recording of cholinergic interneurons (red arrows) and NPY expressing neurons (green, arrows). **b.** Simultaneous whole-cell recording from an NPY-PLTS interneuron and a SPN demonstrates that optogenetically elicited IPSCs in the SPNs are not accompanied by inward currents in the NPY-PLTS neurons (bottom). NPY expressing neurons are identified as NPY-PLTS interneurons based on their membrane potential responses to injected current (top, compare to Supplementary Fig. 4e). Note the rebound LTS. c. A spontaneously active NPY-PLTS interneuron (top traces) exhibits long-lasting inhibition in response to optogenetic stimulation of cholinergic interneurons (blue bar). Note the IPSC in the SPN (bottom, red trace). Also note that the optical stimulus does not trigger spikes or elicit EPSPs in the NPY-PLTS interneuron. Also note the lack of monosynaptic responses in the SPN in response to spiking in the NPY-PLTS neuron (double arrows). Reproduced from English et al. 2011.

for since the circuit under investigation is so robust in its inhibition of SPNs (Gittis *et al.* 2010). Cholinergic-Cre and NPY-EGFP transgenic mice were crossed to produce Cholinergic-Cre / NPY-EGFP double transgenic mice. Such mice were injected with the AAV vector carrying floxed ChR2-EYFP as described previously. PLTS neurons were targeted by EGFP signal visualized with epifluorescence microscopy and patched under near IR-DIC imaging (fig. 14). Whole cell current and voltage clamp recordings of EGFP+ PLTS interneurons demonstrated the typical current-voltage relationship of these neurons, low threshold spikes and plateau potential (fig 14). Optical stimulation of cholinergic interneurons in this preparation reliably induced IPSCs in SPNs (fig. 14),

with either no response in the PLTS interneuron (fig. 14) or inhibition of spiking (fig 14). This suggests that PLTS type NPY expressing interneurons are not involved in the cholinergic-GABAergic circuit under investigation.

These data show that neither parvalbumin expressing FSIs nor PLTS type NPY expressing interneurons participate in the cholinergic-GABAergic feed-forward or recurrent inhibitory circuits. There remains a possibility that direct facilitation of GABA release from the presynaptic terminals of either or both of these interneurons, which we would be blind to due to the restrictions of somatic recording, make a contribution to the observed responses in SPNs and Cholinergic interneurons. However, the most likely conclusion to be drawn from these experiments is that a yet to be described GABAergic interneuron or interneurons mediate these circuits.

NPY-NGF interneurons participate in feed-forward cholinergic-GABAergic circuits which inhibit SPNs

A novel GABAergic interneuron has recently been identified in NPY-EGFP transgenic mice, which expresses NPY but is distinct from the NPY-PLTS interneuron (Ibanez-Sandoval et al. 2011.). Details of this neuron are described in previous sections and in a recent paper from our laboratory and will not be repeated here (Ibanez-Sandoval *et al.* 2011). Whole cell current clamp recordings of these neurons visualized as EGFP+ using epifluorescent imaging and targeted for recording with near IR-DIC imaging, were obtained in acute neostriatal brain slices and their responses to Cholinergic interneuron stimulation were recorded. Spikes in cholinergic interneurons induced by somatic current injection elicited depolarizing postsynaptic potentials in these neurons which were

insensitive to either GABA or glutamate receptor antagonists (n=4 for bicuculline, n=3for CNQX), but were blocked by inhibitors of nAChRs (fig. 15, n=3, response amplitude was 0.96 ± 0.7 mV (range, 0.28-2.27 mV), had a rise time 4.7 ± 5.3 ms (range, 9.0-24.7ms), a decay time constant of 75.6 ± 40.2 ms (range, 27.8-147 ms), an onset latency of 3.6 ± 1.6 ms and exhibited no transmission failures). This is the first evidence that the NPY-NGF interneurons are involved in the cholinergic-GABAergic circuits under investigation. Stimulation of multiple cholinergic interneurons via ChR2 optical stimulation elicits not only nicotinic EPSPs in these neurons but in some neurons action potentials as well (2/7 tested). Triple NPY-NGF, Cholinergic and SPN recordings were obtained, demonstrating ChR2 mediated spiking in Cholinergic interneurons, ChR2 and nAChR mediated spiking in NPY-NGF interneurons (2/7), and GABAergic IPSPs in SPNs elicited by ChR2 stimulation or by action potential firing of the recorded NPY-NGF interneuron induced by direct somatic current injection (3/3). We thus demonstrated the functionality of the entire disynaptic cholinergic-GABAergic circuit intact in the acute brain slice preparation. An additional finding, possibly of great importance to further understanding of the striatal microcircuitry, is that the NPY-NGF interneurons are gap-junction coupled (fig. 18 C). This supports the idea that the cholinergic interneurons engage widespread inhibition involving many NPY-NGF interneurons.

It is important to note that the NPY-NGF interneuron IPSP/C recorded in the SPN matches only one of the components of the response evoked by the cholinergic-GABAergic circuit, namely the slow component. The fast component was never observed in SPNs when NPY-NGF neurons were directly excited by somatic current injection. Thus it appears that multiple GABA sources participate in the response, as predicted by

our initial analysis of the independent variability of the different components and experiments blocking GABA reuptake. Our current experiments have yet to elucidate the



Figure 15. Synaptic interactions of Cholinergic and NPY-NGF interneurons and SPNs. (a) Top, characteristic passive and active properties of Cholinergic and NPY-NGF interneurons. Bottom, synaptic circuitry of a Cholinergic and a NPY-NGF interneuron and a SPN recorded simultaneously. GABAergic (blue) and nicotinic (red) interactions are indicated. Circular arrow represents recurrent inhibition. (b) Action potentials of the Cholinergic interneuron elicited in voltage clamp (bottom) induced nEPSPs in the NPY-NGF interneuron (top; red, average) that were blocked by DHBE (blue). (c) Recurrent GABAergic IPSCs in the same cholinergic interneuron (spike subtracted). Note the short τ decay of the IPSC and block by bicuculline (blue trace). (d) Train stimulation of the cholinergic interneuron (middle) elicited nEPSPs in the NPY-NGF neuron (top traces, arrows). The partial depression of the nests contrasted with the complete failure of the recurrent IPSC after the first stimulus (bottom traces, arrowheads). (e) Spiking in the NPY-NGF interneuron (middle) elicited IPSCs in the SPN (bottom: blue, average) and the Cholinergic interneuron (top). Note the slow time course of the IPSCs and the absence of a fIPSC in the SPN. Bicuculline block, green. (f) Top, inhibition of GAT-1 increased τ decay of the IPSC elicited in an SPN by an NPY-NGF interneuron (different neurons than in a-e). Bottom, overlay of the same IPSCs (green traces) with optogenetic IPSCs recorded under the same pharmacological conditions (blue, same conditions as in Fig. 1). Unitary responses were scaled in amplitude. Note the similarity of rise times and τ decay. *Reproduced* from English et al. 2011.

other source(s) of GABA.



Figure 16. Optogenetic activation of cholinergic interneurons elicits nEPSPs and GABAergic IPSPs and triggers action potential firing in NPY-NGF interneurons. (a) Confocal image of cholinergic interneurons expressing ChR2-mCherry (arrows) and two EGFP-expressing NPY-NGF neurons (arrowheads) in a Cholinergic-cre; Npy-GFP mouse. (b) Top, confocal images of a NPY-NGF interneuron (arrowhead) and a simultaneously recorded nearby SPN intracellularly labeled with Alexa 594 (arrow). Bottom, photomicrographs of the neurons during recording. Inset, high magnification showing SPN dendritic spines (arrows, same neurons as shown in c). (c) Top, optical stimulation of cholinergic interneurons elicited large amplitude depolarizations and action potentials in a NPY-NGF interneuron that were blocked by DHBE (blue trace). Simultaneously elicited optogenetic compound IPSC in the SPN (bottom green trace). Bottom, single action potential in the NPY-NGF neuron (black trace) elicited a slow IPSC in the SPN (green trace). (d) EPSP-IPSC sequence elicited with optical stimulation of cholinergic interneurons in an NPY-NGF neuron (different neuron than those shown in **b**, c and e). Left, reversal of the IPSP (arrow). Right, the IPSP (green trace), the compound response (black trace) and the isolated nEPSP (blue trace) were recorded at \sim -45 mV. Note the large amplitude and slow time course of the isolated IPSP. (e) The compound optogenetic response of another NPY-NGF interneuron (black trace) was gradually increased in amplitude by application of bicuculline (red trace), leading to action potential firing (top inset). The isolated nEPSP (red trace) was blocked by ~95% by 200 nM DH β E (blue trace). The residual response (blue trace, bottom inset) was not sensitive to 6,7-dinitroquinoxaline-2,3-dione (DNQX, green trace). Reproduced from English et al. 2011.



Figure 17. Electrophysiological and morphological properties of NPY-PLTS and NPY-2 interneurons. **a.** Photomicrographs of an NPY-PLTS interneuron targeted in NPY-EGFP mice (*left*) intracellularly labeled with Alexa-594 (*right*). **b-e.** Electrophysiological characteristics of NPY-PLTS neurons. Characteristic high maximal firing rate of an NPY-PLTS interneuron in response to current injection pulses (**b**, **e**), and typical *in vitro* spontaneous activity of the same interneuron (whole cell recording, **b**; cell attached recording, **c**). Note that spontaneous activity is seen only in some NPY-PLTS neurons. **d.** A hyperpolarizing current pulse elicits rebound firing driven by an LTS followed by a long lasting plateau depolarization (arrow) illustrating defining characteristics of these neurons (different neuron from that in *a*). **f.** Photomicrograph (*top*) and membrane potential responses (*bottom*) of a typical NPY-2 interneuron. Note the striking electrophysiological and morphological differences from NPY-PLTS interneurons. *Reproduced* from English et al. 2011.



Figure 18. Synaptic connections of and gap junction coupling between NPY-2 interneurons.a. Triple recording from 2 NPY-2 interneurons (top panels, responses of the neurons to current pulses) and an SPN (bottom). Spikes elicited in the NPY-2 interneurons (middle, red traces) elicit IPSCs in the SPN (bottom, arrows; individual traces, gray; average, pink). Note that one of the NPY-2 interneurons is stimulated in voltage clamp (middle). b. Overlay of the average IPSCs elicited in the SPN (top) by the 2 interneurons (bottom panel: peak-scaled IPSCs). Note the characteristic slow time course of the IPSCs (blue and red) compared to a regular, fast spontaneous IPSC (black). c. Electrotonic coupling of the same 2 NPY-2 interneurons shown by membrane potential deflections in one neuron (top traces) induced by hyperpolarizing (black) and depolarizing potentials in the other neuron (bottom traces). Reproduced from English et al. 2011.



Figure 19. Stimulation of Cholinergic interneurons in current and voltage clamp. Left: Overlay of nEPSPs elicited in a NPY-2 interneuron (top, average in pink) by single action potentials in a Cholinergic interneuron evoked in current clamp (bottom). Note the similarity of this response to the nEPSPs shown in Fig. 2. k. Right: An unclamped action potential elicited in a Cholinergic interneuron in voltage clamp with a large 70-100 mV command potential is seen as a fast inward current (a.p. arrow). The response triggers a recurrent ("IPSC", IPSC arrow). The corresponding nEPSP elicited in an NPY-2 interneuron is shown in Fig. 2. Reproduced from English et al. 2011.

Pause-excitation sequences of Cholinergic+ interneurons inhibit SPNs in vivo

We obtained chronic *in vivo* tetrode recordings of neostriatal neurons from animals expressing Halo3 in cholinergic interneurons to test the hypothesis that optical induction of a pause-rebound sequence *in vivo* would elicit inhibition of the ongoing activity of SPNs, as predicted by our *in vitro* data. We chose to test the pause-rebound sequence over the excitation-pause-rebound sequence for three reasons. First, the pause-excitation sequence is by far the most commonly observed. Second, in order to illuminate large areas of the striatum *in vivo* it is necessary to use an illumination intensity which is likely to result in overly synchronous action potential firing across multiple Cholinergic interneurons. Third, the experiments involving ChR2 have the potential to generate artifactual results due to the fact that ChR2 is expressed in axon terminals and thus the direct depolarization of these terminals might lead to supraphysiological levels of ACh release.

We injected Cholinergic-Cre mice with AAV2/5-Halo3-EYFP to selectively express Halo3 in Cholinergic+ interneurons. Recordings made using independently were moveable wire tetrodes (2- 4 per animal, unilaterally implanted in the dorsal medial and dorsal lateral striatum) which were part of an optrode array. Details of the optrode are expanded upon in the materials and methods section. Briefly, an optic fiber was implanted at fixed depth, а corresponding to the beginning of the area of infection of AAV2/5-Halo3-EYFP. Tetrodes were implanted parallel to the optic fiber at lateral distances of 200-300µm, and were advanced slowly (<50 µm per day) until units were encountered. Spike sorting was performed offline and is explained in detail in the methods



Figure 20: Characteristics of three recorded unit types and optically induced pause in cholinergic interneurons (a) Left, waveforms of distinct types of units included in analysis. Average unit waveform is shown in gray, population averages are in color. Overlay demonstrates feature differences between unit types. Right, firing rates (mean \pm s.e.m.) of the three types of units. SPNs exhibited significantly lower firing rates than other unit types (t test, P < 0.01). (b) Examples of spike trains of putative SPNs and Cholinergic interneurons. Note that a bursting episode was selected for the SPN. (c) Characteristics of the population response of cholinergic interneurons elicited with optogenetic inhibition. Note the instantaneous inhibition of firing and the excitation phase that is similar to the responses of putative cholinergic interneurons in primates (bins, 30 ms). Reproduced from English et al. 2011.

section. Recordings were obtained while the animal freely explored a circular environment,

empty except for bedding or in some cases a small running wheel. Analysis was not restricted to specific behavioral states, however, since the recordings were made in the light during the animals normal inactive/sleep period, the majority of the recordings were from immobile states, and possibly contained episodes of sleep. Observed units include presumed SPNs (pSPN), presumed parvalbumin expressing fast spiking interneurons (pFSI) and Cholinergic interneurons as well as a previously undescribed neuron with a very short action potential and intermediate firing rate which we refer to as "other". Neuron types were identified based upon waveform width, average firing rate and firing pattern, with the exception of the cholinergic interneuron, which was identified by zero latency inhibition to optical stimulation. The exact parameters defining each neuron class can be found in the methods section of the attached manuscript (English et al. 2011). Sampling of Cholinergic interneurons was positively biased, as the ratio of ChAT+ to pSPN units was on the order of 1:10 (6 cholinergic interneurons and 9 single, 4 multi unit pSPNs, while anatomical evidence has previously demonstrated this ratio to be closer to 1:100. This is likely due to several factors, most importantly being the far greater size and more linear shape of the Cholinergic interneurons (resulting in a greater extracellular dipole to sample from with the electrode) as well as their higher and more regular rates of action potential firing.

The pause phase of the Cholinergic interneuron pause-response recorded in nonhuman primates is between 200-300 ms, thus we determined the optical stimulus parameters necessary for induction of both 200 and 300ms pauses in Halo3 expressing Cholinergic interneurons *in vivo*. A square pulse of yellow light delivered from a laser, gated by a fast transition time (<500µs) shutter was found to inhibit action potentials in Cholinergic interneurons for the duration of the illumination, with near zero latency to the onset of inhibition. Importantly, rebound action potentials occurred in nearly every trial with a variable 20-50 ms delay. We thus demonstrated that optical stimulation of Halo3 expressing cholinergic interneurons elicits responses in these neurons, which closely mimic both the responses we elicited in these interneurons in our in vitro experiments and the responses of cholinergic interneurons recorded in non-human primates to salient stimuli. In a small subset of experiments we used 1000 ms light pulses, which resulted in complete during-illumination inhibition and reliable rebound action potential firing. Cholinergic neurons have never been observed to pause for this long *in vivo*. This protocol was essential, however, to determine whether changes in SPN firing rate were timed to the pause phase or the rebound excitation phase of the optically elicited cholinergic interneuron activity sequence.

SPNs were recorded while optical stimulation was applied at 20 or 30second intervals (0.05 or 0.033Hz). We chose this infrequent stimulation to reduce the possibility of additive effects or long synchronization term of cholinergic interneuron

populations, as well as because our *in vitro* data suggested that the feed-forward cholinergic-GABAergic circuits begin to fail if

stimulated

repeatedly



Figure 21: Pause-excitation sequences of cholinergic interneurons inhibit SPNs in vivo in freely moving mice. Inhibition of firing of SPNs (bottom three PSTHs) by pause-excitation activity pattern of cholinergic interneurons (top). Lower three PSTHs show (respectively, from top to bottom) cumulative response of all SPNs, SPN responses following 200-ms optical inhibition and responses following 1,000-ms inhibition. The population mean and 2 s.d. below the mean firing rates are indicated by blue and red lines, respectively. Note that the end of the optical stimulus was closely followed by strong inhibition of firing in SPNs. Consecutive bins with firing rates more than 2 s.d. below the mean are indicated by bins colored in blue. Note that the optical inhibition itself did not elicit an observable firing rate change in the SPNs. Horizontal bars denote periods of illumination (bins, 50 ms). Reproduced from English et al. 2011.

at shorter intervals. As previously reported, we found that pSPN firing rates *in vivo* are low, averaging less than 1 Hz. We thus needed long recording sessions (2-4 hours) to observe

statistically significant effects of cholinergic interneuron pause-rebound sequences on SPN firing. Peri-stimulus time histograms of SPN activity were constructed around the optical stimulus. Pauses in ongoing cholinergic interneuron activity of either 200, 300 or 1000ms had no significant effect on SPN firing rate. In contrast to this, a significant effect on SPN firing rate was observed following the offset of the optical stimulus, at the time that ChAT+ interneurons fired rebound action potentials. Inhibition was defined as having at least two consecutive 50 ms bins falling below two standard deviations of the mean within 200 ms of the termination of the optical stimulus. Inhibition meeting these criteria was observed in 9 single unit and 3 multi unit pSPNs. Importantly, each neuron met the criteria for significant inhibition when tested in isolation. These 12 neurons were not the only SPNs recorded. However, due to the nature of the experiment, we were not able to identify our exact recording location in relation to virus infection area for each recording session. Thus we only included in our analysis those sessions were an optically elicited effect was observed in either Cholinergic interneurons or SPNs on at least one tetrode, which confirmed our electrode location as being inside the infection area. In all cases where optical effects were observed on a given tetrode, all units recorded on that tetrode responded (in one case three single unit SPNs on a single tetrode). Thus, although our experiments were not capable of determining the exact percentage of neurons affected by our stimulation protocol, this number appears to be quite high based upon the available data.

Recordings of pFSIs did not show any effect of optical stimulation on firing rate. However, two units, which did not meet the classification criteria for any of the above mentioned groups (termed "other") were inhibited at the same time as the SPNs in these experiments. These neurons had intermediate firing rates, which were slower and less regular than FSIs, but had extremely narrow action potentials, and thus may be a type of interneuron other than FSI or Cholinergic. This suggests the intriguing possibility that the pause-rebound sequences of Cholinergic interneurons function to regulate on fast timescales not only SPN activity but in addition have the ability to coordinate large groups of interneurons through precisely timed inhibition

These data demonstrate that optogenetic methods can be used to identify Cholinergic units in vivo, that pause-rebound excitation sequences of Cholinergic interneurons can be elicited in freely moving mice using optogenetic techniques, and that these events trigger powerful inhibition of both pSPNs and asof-yet unidentified presumed interneurons. This is the first known report of optogenetic recapitulation of this activity in behaving animals and represents a major step forward in our ability to interrogate



Figure 22. Optogenetically elicited pauseexcitation sequence of Cholinergic interneurons inhibits putative neostriatal interneurons in vivo. Top: 200 ms optical stimulus (yellow bar) elicits pause-excitation sequences in cholinergic interneurons (same as in Fig. 6). Bottom: Short waveform, fast firing units classified as "Other" neurons (distinct from SPN and Cholinergic units, see Methods) are silenced by the excitation phase of the cholinergic population activity.\ Orange bar indicates the end of stimulus. 50 ms bins. Horizontal lines in bottom trace are average (red) and 2 SD (orange) of the firing rate. Bins in blue are > 2SD below the mean.

brain circuits in detail in freely moving animals.

Auditory and visual stimuli elicit short latency responses in positively identified cholinergic interneurons

Of the six cholinergic interneurons recorded *in vivo* which were identified positively by their optical responses, all were tested for responding to either visual or auditory stimuli. Sound (50 dB, 3 kHz, 100 ms) or light (field illumination shift from 20-100 lumens, 100 ms) stimuli were applied either at semi-random 1-20s or regular 5s intervals for



Figure 23. Optogenetically identified cholinergic interneurons respond to salient visual and auditory stimuli in mice. Top: 200 ms optical stimulus (green bar) inhibits action potential firing in this unit identifying it as a cholinergic interneuron. *Bottom:* Response of the same cholinergic interneuron to auditory stimulus (red bar). Note the apparent lack of desensitization in the response. 10 ms bins.

periods of up to one hour. All optogenetically identified cholinergic interneurons responded to these stimuli with short latency (50-100ms) excitation lasting 20-50ms. This response persisted for up to 100 trials (the most tested) over the course of up to one hour (the longest time tested). Thus it appears that similar to TANs recorded in primates, cholinergic interneurons in mice respond to salient stimuli. The stimulus was not paired

with a reward or any other outcome, however it is likely that both a loud noise or sudden bright light would be at least mildly aversive to, or at least garner the attention of the animal. The high percentage of cholinergic neurons responding to salient but otherwise non-information carrying stimuli observed in mice as compared to the approximately one-fifth of TANs observed to respond to similar stimuli in primates suggests that murine cholinergic interneurons and/or the thalamic nuclei which innervate them may be sensitive to a broader range of stimuli than those in primates.

Discussion

Physiologically realistic optogenetic recapitulation of activity sequences of striatal cholinergic interneurons

The central hypothesis guiding my thesis work is that highly conserved firing patterns of cholinergic interneurons in the neostriatum should have effect the ongoing activity of striatal projection neurons on short timescales, faster than the typical neuromodulatory effects of acetylcholine. This hypothesis is based upon numerous studies of cholinergic interneurons in non-human primates, which reliably report consistent time-locked responses to salient events. This activity, termed the pause response (although it often includes excitatory phases), varies very little in its temporal structure (one exception is the "contracted" response observed in response to learned predictors of stimuli with negative valence (Ravel *et al.* 1999; Joshua *et al.* 2008)).

Cholinergic interneurons make up only 1% of the striatum (Apicella, 2007), on average, and are sparsely distributed with little spatial organization (the exception is their avoidance of the mµ-opioid staining striosome compartments (Aosaki *et al.* 1995)). The ongoing activity of these neurons is typically only weakly correlated (Morris *et al.* 2004), except for the brief periods of synchronization following salient events (Kimura *et al.* 1984; Morris *et al.* 2004). Thalamic inputs are assumed to initiate these events, taking advantage of intrinsic membrane properties (Matsumoto *et al.* 2001), which can generate complex pause responses (Wilson, 2005), and possibly through the activation of GABAergic neurons as well. In the time since these responses were first recorded in 1984 (Kimura *et al.* 1984), basal ganglia neurophysiologists have been postulating quite

liberally about what they actually do. Almost all studies of cholinergic function in the striatum, ranging from behavioral pharmacology to intracellular recordings in brain slices, have focused on the neuromodulatory roles of ACh, of which there are many (i.e. modulating the activity of GABAergic interneurons (Koos & Tepper, 2002) and glutamatergic inputs from cortex (Pakhotin & Bracci, 2007; Ding et al. 2010)). Although the pause response is likely to affect neuromodulatory pathways, most if not all of them are relatively slow, and would therefore be poor candidates for the job of translating these fast sequences into temporally precise changes in striatal activity and ultimately basal ganglia output. This is not to say that the pause does nothing in terms of neuromodulation, just that the pause-response likely has several functional effects, at least one of which should be fast acting. Mechanisms by which the pause-response likely acts on neuromodulatory systems include regulation of ongoing activity (Misgeld, 1989; Kitai & Surmeier, 1993; Calabresi et al. 1998b; Figueroa et al. 2002; Koos & Tepper, 2002), and synaptic plasticity at cortico-striatal and thalamo-striatal glutamatergic terminals (Pakhotin & Bracci, 2007; Ding et al. 2010), and regulation of dopamine release (Exley & Cragg, 2008). We chose to focus not on these potential effects but to search for fast-acting mechanisms.

It is impossible to control the activity of numbers of cholinergic interneurons using standard electrophysiological or pharmacological methods, much less to reliably produce sequences consisting of both inhibition and excitation. Thus there was no way to experimentally recapitulate the pause response and finally determine what effect it has on striatal neuron activity using conventional techniques. To overcome this obstacle we used the relatively new technologies of optogenetics, which enable optical depolarization or hyperpolarization of neurons through the ectopic expression of optically activated proteins. Channelrhodopsin-2 membrane is an engineered variant of the channelrhodopsin protein found in the unicellular green algae Chlamydomonas reinhardtii, which opens a non-specific cation conductance when exposed to blue light (maximum peak is at 470 nm) (Zhang et al. 2006). Halo3 is an engineered variant of the Halorhodopsin protein found in Natroamonis pharaonis which pumps chloride ions into the cell using the energy from yellow light (maximum peak is at 590 nm) (Mukohata & Kaji, 1981; Kamo et al. 1985; Gradinaru et al. 2008). Selective expression of either protein in a given cell type of interest can be achieved by the use of Cre-LoxP systems which regulate expression at the level of gene transcription. In our experiments we took advantage of a recently engineered viral vector system in which genes of interest, coded in the reverse ORF, are flanked by opposing pairs of incompatible LoxP sites. When this sequence reacts with Cre-recombinase the correct ORF is permanently restored. When these vectors are injected into animals expressing Cre recombinase in single cell types, the virus enters all cells but the inserted gene is only expressed in Cre expressing cells, while the rest make nonsense transcripts and no functional proteins.

We utilized this strategy to selectively express either ChR2 or Halo3 in Cholinergic+ neurons. One of several lentiviral or adeno-associated viral vectors carrying either ChR2, eNphR or Halo3 linked to a fluorescent protein, oriented as described above and in previous sections, were injected into the striatum of Cholinergic-Cre transgenic mice. Expression of Cre-recombinase in this strain is controlled by the endogenous regulatory sequences for choline acetyl-transferase. This means that the Cre gene is integrated into the mouse chromosome directly following the coding region for Cholinergic. This type of transgenic animal typically yields the most specific expression of the ectopic gene. We used both homozygous and heterozygous transgenic animals, as the quantity of Cre protein necessary to react with any LoxP sites present is produced even with only one copy of the Cre gene and no differences were observed between the two groups of mice in our studies or by others (English et al. 2011). We verified the selectivity of opsin expression in two ways. First, we performed immunocytochemistry for the cholinergic protein, and observed nearly perfect overlap of cholinergic staining and opsin-fluorescent protein expression. Second, we patched a large number (>50) of fluorescent neurons in acute neostriatal brain slices from these animals and obtained recordings in whole cell current clamp mode to observe the intrinsic electrophysiology of these opsin-expressing neurons. All such neurons matched the well established characteristics of cholinergic interneurons in the striatum, which include tonic action potential firing between 2-10Hz, prominent Ih, wide action potentials and rebound spiking elicited by the offset of hyperpolarization induced by somatic current injection. In some neurons recorded in whole cell configuration tonic firing ceased after a period, which is a known phenomenon, caused by dialysis of the neuron with the electrode internal solution. To ascertain whether opsin expressing Cholinergic interneurons maintained tonic firing, as do healthy non-opsin expressing neurons, we obtained cellattached recordings in a subset of neurons (~20) in order to leave the intracellular milieu unchanged. The same aCSF used as external slice perfusion solution was used as electrode internal solution in these recordings in order to preserve the local ionic and osmotic environment as we patched the cell. Normal electrode internal solution has high potassium concentrations (matching intracellular concentrations) as compared to the

aCSF and possibly differences in osmolarity (although we did attempt to match this parameter), which tends to evoke depolarization of the neuron as it is approached with the electrode, introducing the possibility that neurons could be switched into an active up-state by this procedure. Nevertheless, the vast majority of neurons recorded in both configurations were tonically active.

We recapitulated the initial excitatory phase of the pause-response in brain slices by illuminating cholinergic interneurons expressing ChR2 with carefully designed pulses of blue light. Whole slice illumination was delivered through a high output light emitting diode (HO-LED), at first mounted below and focused thru the microscope condenser. The HO-LED was controlled by Axoclamp software, enabling us to control the intensity of the LED in a precise (sub-millisecond precision) fashion. Several potential problems with this preparation were immediately apparent. ChR2 appears to be expressed everywhere on the cell membrane of Cholinergic interneurons, and although we did not directly observe or attempt to measure expression at axon terminals, its presence had to be assumed. The time-course of the ChR2 current is longer than the normal depolarized state of an axon terminal elicited by an action potential, and thus optical activation of terminal ChR2 might lead to supraphysiological levels of ACh release as is observed in other neurons (Zhang et al. 2008). Additionally, ChR2 is calcium permeable, and the direct influx of calcium through an exogenous channel is likely to alter neurotransmitter release. Lastly, ectopic expression of high levels of a membrane protein in axon terminals also has the possibility to interfere with normal protein distribution in the membrane. The eventuality of these potential problems could not be determined in this preparation, thus we were forced to rely on other experiments to control for these

possibilities, namely the generation of rebound action potentials in large numbers of cholinergic neurons through hyperpolarization with Halo3.

The initial excitatory phase of the cholinergic interneuron response recorded in primates has a trial-to-trial jitter of ~20ms (as measured in single neurons) and thus we aimed to achieve this level of near-synchrony across the population of neurons expressing ChR2. We found that the most effective way of generating reliable jitter was to depolarize the neuron optically but let the endogenous pacemaker induce the spike, in essence limiting the latency until the next spike. In fact, using a low level of light combined with a ramped onset we could reliably induce action potentials occurring after the light had turned off – we gave just enough light to push the membrane potential to a level where the pacemaker took over and induced a spike. The caveat of this experiment, of course, is that we are recording only one to three neurons at a time, and so we were unable to definitively say that other neurons were firing more synchronously. This was especially concerning since we record from the top of the slice and were initially illuminating from the bottom. We thus set up a top mounted LED, and found the same results, that we could produce semi-synchronous activity using low intensity light pulses. Not all experiments used this stimulation, and in fact many experiments were performed with high intensity light pulses, which result in almost no jitter in spiking, and also generate larger postsynaptic responses. Critically, all phenomena were observed with both stimulation parameters.

To recapitulate the pause-excitation sequence, the response most often observed in primates, we selectively expressed different versions of Halorhodopsin in Cholinergic interneurons. The first version available, referred to as NpHR, had a significant
limitation in that it tended to remain sequestered in the endoplasmic reticulum (Gradinaru et al. 2008). This posed two problems, namely possible cellular toxicity and limited membrane localization and thus limited current. A second version of the protein, enhanced NpHR (eNpHR), solved these problems by adding two elements fused to the protein, one from a nAChR and one from a K_{iR} channel, which direct the protein out of the ER and into the plasma membrane (Gradinaru *et al.* 2008). This second version had a significant benefit as well in the fact that its placement on the cell membrane was limited to the soma and dendrites, and not in axons or axon terminals. The current produced by eNpHR was still rather low, on the order of ~100pA. A third version of halorhodopsin, termed NpHR3.0 or Halo3, was developed which generates almost 1 nA of current, and it is expressed throughout the membrane, including axons and terminals. In our experiments we used both eNpHR and Halo3, and did not observe any qualitative differences in terms of downstream effects on SPNs. It should be noted that in vivo experiments exclusively used the more efficient Halo3 as we aimed to affect large numbers of cholinergic interneurons over large areas of striatum using as little light as possible to minimize the possibility of heating the tissue.

The pause length in most primate recordings is ~200-300ms, and thus we aimed to reproduce this both in our *in vitro* and *in vivo* experiments. *In vitro*, we used a similar set-up as with the previously described ChR2 experiments, using a below or above slice-chamber mounted HO-LED. Cholinergic interneurons patched in cell-attached configuration allowed for the recording of unclamped spikes, and application of green or yellow light inhibited ongoing activity with near zero latency (as best as was measurable). This effect persisted using pulses ranging from 10–1000 ms. Additionally,

regardless of the inhibition length rebound spikes with a 5-100 ms jitter were reliably elicited. This jitter is desirable as the primate responses have approximately this level of synchrony. *In vivo*, we used 200, 300 or 1000ms pulses from a yellow laser, delivered through an optic fiber, to recapitulate these patterns. Like the Cholinergic neurons recorded in the slice, presumed cholinergic interneurons (definitively identified as such by their zero-time lag inhibition induced by yellow light application) responded with inhibition and rebound firing with a substantial jitter, ranging from 20-150 ms.

Thus we succeeded in reproducing physiologically realistic activity sequences of cholinergic interneurons, both *in vitro* and *in vivo*, using cell type specific expression of excitatory and inhibitory opsins. It should be noted that this was also the first time that positively identified dorsal striatal cholinergic interneurons were recorded in behaving animals. As expected, the physiology of these neurons matched that of presumed cholinergic interneurons or TANs in primates.

Determination of the effect of cholinergic interneuron activity sequences on SPNs

To test our hypothesis that the highly preserved activity sequences of cholinergic interneurons should have a temporally precise effect on SPN activity we obtained whole cell recordings of SPNs in brain slices where we could recapitulate these patterns experimentally, as is described above. Semi-synchronous firing of ChR2 expressing Cholinergic interneurons elicited in SPNs, recorded in whole cell current clamp configuration, a GABAergic IPSP which occurs secondary to activation of type-II nAChRs. Current injection induced spiking of SPNs was blocked by the IPSP in a firing rate dependent manner. This result was not at all expected, as there have been no reports of cholinergic-GABAergic circuits innervating SPNs. Detailed analysis of the corresponding IPSC revealed that it consists of multiple components, which vary independently and have different time-courses. The fastest response is similar in temporal structure to IPSCs at FSI-SPN synapses (Koos & Tepper, 1999; Gittis *et al.* 2010; Planert *et al.* 2010), while a slower component appears unlike anything previously observed in the striatum, and in fact appears similar to the GABA_A "slow" described in the hippocampus and neocortex (Pearce, 1993; Banks *et al.* 1998; Tamas *et al.* 2003; Szabadics *et al.* 2007). A very slow (termed late) component rounds out the three, and due to its proportionally small contribution was not investigated further. These data show that the interneuron network in the striatum is much more complex than has previously been considered, and that an intrinsic excitatory drive exists which selectively targets interneurons. This forces a new consideration of the role of ACh and cholinergic interneurons in the striatum and possibly in other brain structures as well.

A circuit which appears pharmacologically similar to the feed-forward cholinergic-GABAergic circuit linking Cholinergic interneurons and SPNs has been described previously in which Cholinergic interneurons activate a nAChR and GABA_A receptor mediated recurrent inhibition (Sullivan *et al.* 2008). We aimed to determine whether or not there was overlap between the two, and found that all of the evidence was in support of the existence of distinct circuits. The most visible difference in the responses is that the recurrent inhibition displays trains of synaptic inputs, whereas the feed-forward inhibition never manifests as a train. Additionally, the latency to the onset of the recurrent inhibition is significantly shorter than that for the feed-forward pathway.

Separation is also demonstrated by experiments where multiple levels of optical stimulation were used, which revealed that the threshold stimulation level for eliciting a response was lower for the recurrent pathway than for the feed-forward. Although not quantified, the recurrent inhibition was in general, more reliably encountered, and especially in the non-opsin based experiments. A final piece of evidence is that the inputs to NPY-NGF interneurons (which appear to mediate the slow component of the feed-forward response) from Cholinergic interneurons undergo less desensitization than the recurrent inhibition, as there exist stimulation frequencies where NPY-NGF interneurons are depolarized by multiple stimuli in a train while recurrent inhibition fails after the first stimulus. This also suggests that the desensitization of the disynaptic feedforward pathway largely occurs at the GABAergic and not the nicotinic synapse, at least when considering the slow component. We were unable to resolve any differences in receptor pharmacology, most likely due to the relative non-specificity of the available antagonists of nicotinic receptors, a problem exacerbated by the incomplete knowledge of the subunit assembly patterns in the central nervous system.

This set of experiments demonstrated the functionality and pharmacology of a novel circuit, which was so unexpected that we initially had reservations about its authenticity and were concerned that it might be an artifact, especially since we were using new and relatively untested tools. Ultimately, we concluded that the results were indeed real, in part based on experiments using wild-type animals with no opsin expression, which are described below.

We performed paired recordings of cholinergic interneurons and SPNs in transgenic and non-transgenic animals, neither injected with virus. We found that in a percentage of recorded SPNs (~50%), single spikes of single cholinergic interneurons elicited in SPNs GABAergic IPSC/Ps that required intermediate activation of nAChRs. This was extremely reassuring, as it suggested that the response we saw in the ChR2 experiments was quantitatively and not qualitatively different from an effect observed in normal animals.

The validity of the circuit, both as a biophysical mechanism and as a downstream effect of realistic firing patterns of cholinergic interneurons, was most strongly demonstrated in our experiments where we elicited pause-rebound sequences in cholinergic interneurons expressing Halorhodopsin. We found that rebound action potentials of tonically active cholinergic interneurons, in acute slices, produced a quantitatively and qualitatively similar response in SPNs as that observed in the ChR2 experiments. Current injection induced spiking of SPNs was not affected during the pause, only after the neurons rebounded, and in some instances responded not only to the first but also the second volley of cholinergic interneuron rebound spikes. At this point in time, several hundred milliseconds after any opsin activity, it is extremely unlikely that any artifactual event was in play, and thus this observation provided us with the most definitive piece of evidence that an authentic brain circuit produces the SPN response.

Identification of the intermediate GABA source

The natural next question was to locate the source of GABA producing the inhibition in SPNs. The fast component of the response, in temporal structure, is similar to FSI inputs to SPNs, and FSIs express cholinergic receptors and are excited by

cholinergic agonists. We were thus surprised by our results that FSIs were not activated in preparations in which cholinergic interneuron stimulation elicited inhibition of SPNs. There is a small possibility that the presynaptic terminals of FSIs are directly depolarized by ACh and elicit the fast response. This seems unlikely as single FSI inputs to SPNs are of larger magnitude than the responses observed through cholinergic interneuron activation. A second commonly recorded striatal GABAergic interneuron, the NPY-PLTS neuron, was also found not to be involved. We considered the possibility that the circuit we observed did not require action potential firing of interneurons, rather that nAChRs located on GABA releasing terminals were directly eliciting release. This was impossible to evaluate fully, but the one available experiment suggested that action potentials were required. Around this time a post-doc in the lab identified a new type of NPY expressing interneuron, which was termed the NPY-NGF interneuron due to its similarity with cortical neurogliaform (NGF) interneurons (Ibanez-Sandoval et al. 2011). In paired recordings this interneuron elicited a slow IPSC in SPNs, which was similar to the slow component of the cholinergic-GABAergic response both in time-course and in its sensitivity to GAT-1 blockade. NPY-NGF interneurons were found to receive a nicotinic EPSP when single nearby Cholinergic interneurons were stimulated, and fired action potentials when multiple ChR2 expressing Cholinergic interneurons fired action potentials together evoked by optical stimulation. Triple recordings demonstrated the functionality of this Cholinergic/NPY-NGF/SPN disynaptic circuit in the slice, positively identifying a functional role for the NPY-NGF neuron in this novel circuit. We were unable to determine the source of the fast component of the response, and it is likely that further work involving EGFP labeled interneurons of other types will yield

the answer. It is important to note that while the NPY-NGF neuron does make GABAergic synapses with cholinergic interneurons, it is not the source of the recurrent inhibition previously described (Ibanez-Sandoval *et al.* 2011). The IPSC has a different time course than the train of IPSCs seen in the recurrent inhibition, as well as the fact that spiking in NPY-NGF neurons was never observed following stimulation of single cholinergic interneurons, a procedure which reliably elicits recurrent inhibition.

In vivo investigation of the effects of pause-excitation sequences of cholinergic interneurons on SPN activity

At the completion of our *in vitro* investigation we were convinced that we had identified a novel circuit in the striatum. We also were certain that we were not creating an artifactual preparation. This was very exciting, as the entire idea of this circuit was novel, it involved a novel interneuron, and appeared to act independently of cortically driven FSI inhibition of SPNs. The natural next step was to test if this circuit actually functioned *in vivo*. Acute slice recordings are perfect for the level of circuit dissection at which we worked, however there are several factors beyond the control of the experimenter which become critically important when considering the concerted action of multiple neurons in eliciting a response. The most important in this case was the state of the interneurons in the slice versus *in vivo*. Additionally, a peculiar aspect of the circuit is that it only works if one stimulates cholinergic interneurons at low rates, which is why we used a 20s interval in our experiments. This is particularly curious as the Cholinergic interneurons are tonically active, and so there is always ACh being released. There was some concern that in the slice a proportion of Cholinergic interneurons were

not active, creating an unrealistically low ACh tone, which could alter the state of nAChRs. There was no way to determine this accurately in the slice.

In order to address the issue of *in vivo* functionality we performed single and multi-unit recordings of cholinergic interneurons and SPNs in behaving mice expressing Halo3 in Cholinergic interneurons. We built custom optrodes carrying multiple moveably tetrodes and a fixed optic fiber. This allowed us to illuminate a large area from which we could record multiple neurons at four independent sites. Initially were not counting on being able to record Cholinergic interneurons, however, we were pleasantly surprised at the relative ease at which they were found. This is likely due to the relatively large size and relatively polarized shape of these neurons as compared to other striatal neurons, both of which lead to a larger dipole from which to sample spikes with the extracellular electrode. In vivo recordings of Halorhodopsin expressing cholinergic interneurons demonstrated that pulses of yellow light immediately inhibited action potential firing during the pulse. At the end of the pulse, following a short delay, rebound action potentials were reliably elicited. We recorded presumed SPNs during this stimulation and found that only the rebound activity, not the pause (even at lengths of up to 1 second) affected spontaneous action potentials in SPNs. The effect was strong inhibition, which developed quickly and collapsed with a bi-exponential time-course. This suggests that the circuit identified *in vitro* is functional *in vivo*.

A recent study, which in some respects is similar to ours, has recently been reported (Witten *et al.* 2010). The authors investigated the role of cholinergic interneurons in the nucleus accumbens (NA) in regulating the activity of projection neurons and in conditioned place-preference for cocaine. Their results are strikingly

different from ours and suggest a divergence in interneuron function between the two structures. They did not find fast circuits activated by synchronized cholinergic interneuron activation or inhibition, rather they found very strong cholinergic modulation of synaptic inputs to SPNs, recorded *in vitro*, the timescale of which suggests a neuromodulatory mechanism. Their *in vivo* experiments demonstrated that inhibition of cholinergic interneurons in the NA causes principal neuron firing rate changes during the pause only, in contrast to our results demonstrating changes only during post-pause excitation. Additionally, they found that changes in firing rate were bi-directional in different SPNs, while our experiments showed inhibition in all units. These differences suggest that the nucleus accumbens and the dorsal striatum (neostriatum) differ not only in their afferent input but in their intrinsic interneuron circuitry as well.

Responses of cholinergic interneurons to salient stimuli

We found that all cholinergic interneurons recorded *in vivo* respond to salient stimuli. The stimuli were not predictive of any other event. The stimuli themselves likely were of negative valence to a mouse, being a loud noise or a bright light. Approximately one fifth of TANs in primates respond to similar stimuli. The difference in the percentage of responding neurons could be for many reasons. First, we sampled relatively few neurons compared to the primate studies. Second, these types of stimuli are likely more aversive to mice than to monkeys, considering their natural environments. Lastly, the neostriatal cholinergic system of the mouse may be hypersensitive as compared to the primate, possibly reflecting a greater need for striatal based, as opposed cortically based, fast decision making in response to stimuli. Evidence for a basic difference in the system comes from the fact that the response in the mouse starts out with a burst when this is the least commonly observed response pattern in primates. Further investigation of the behavioral correlates of cholinergic interneuron firing patterns in mice will all for a more complete reconciliation with the findings from primates.

Cholinergic-GABAergic circuits may be involved in thalamic gating of behavioral program switching in the striatum

Recordings in non-human primates of thalamo-striatal neurons to salient events suggest that there should be a mechanism by which these inputs change the activity of SPNs in a fast and reliable manner. The fact that these thalamic inputs preferentially target interneurons suggests this as a possible mechanism of translation (Lapper & Bolam, 1992; Ding *et al.* 2010). The circuit that we identified, likely to be activated by thalamic input may be a part of this proposed mechanism. The effect of the circuit activity is to eliminate ongoing activity and output of the striatum, and may allow for a new set of actions to be put into play by the following set of activity patterns (re-biasing) (Minamimoto & Kimura, 2002; Smith *et al.* 2011; Yamada *et al.* 2011). This inhibition is not thought to be direct or indirect pathway SPN specific, or to respect patch-matrix boundaries (Smith *et al.* 2011; Yamada *et al.* 2011).

These data supports the idea of a segregation of externally driven interneuron networks, for example cortically driven FSIs and thalamic driven Cholinergic-GABA

inhibitory pathways. It also fits in with the idea of Kimura and colleagues of re-biasing of striatal processing being driven by thalamic inputs (Matsumoto *et al.* 2001; Minamimoto & Kimura, 2002; Kimura *et al.* 2003; Kimura *et al.* 2004). Future experiments aimed at recording large-scale ensembles of projection neurons active during a pre-biasing through re-biasing procedure, coincident with manipulation of cholinergic interneurons, would be able to directly test this hypothesis.

Possible effects of DA on the pause response and its fast effects on SPNs

DA modulates cholinergic interneurons by changing membrane properties through activation of DA receptors expressed in these neurons and through modulating synaptic input from DA-sensitive presynaptic neurons. Both DA pathways are important to this thesis, as the interaction of intrinsic membrane with excitatory and inhibitory synaptic inputs are thought to generate the pause responses (see Introduction section).

D2-type DA receptors are expressed directly by cholinergic interneurons and their activation in *in vitro* preparations results in a reduction in the rate of autonomous firing (in the presence of synaptic input blockade) through inactivation of sodium currents (Maurice et al. 2004). The fact that we observed the cholinergic-GABAergic circuit to undergo significant use-dependent suppression, and that cessation of tonic firing in single cholinergic interneurons facilitated their activation of cholinergic-GABAergic inhibition of SPNs, suggests that the magnitude of the effects of the pause response on SPNs are inversely correlated with the tonic firing rate of cholinergic interneurons and the corresponding background level of ACh. It would be expected then that the effect of direct activation of D2 receptors in cholinergic interneurons, at least in the *in vitro*

preparation, would be an enhancement of the cholinergic-GABAergic circuit's inhibition of SPNs.

Although chronic DA depletion caused by lesion of the nigro-striatal DA pathway does not affect tonic ACh levels as measured by microdialysis, the modulation of striatal ACh levels by pharmacological dis-inhibition of excitatory inputs from the Pf nucleus of the thalamus is switched from a net reduction to net increase following chronic DA depletion (Zackheim and Abercrombie, 2005). The explanation for the effect of increased excitatory drive from the Pf leading to decreased ACh levels (without DA depletion), even though cholinergic interneurons receive excitatory synapses from this area, is that there is a presumed intermediate GABAergic interneuron, which also receives this excitatory input, and that the balance is net inhibition (Zackheim and Abercrombie, 2005). This is supported by the fact that local application of bicuculline in striatum reverses the effect of Pf dis-inhibition, resulting in a net increase in ACh, presumably caused by the now-dominant direct excitation of the cholinergic interneurons by the Pf. DA depletion thus mimics the effect of blocking GABAergic input from local interneurons to cholinergic interneurons, suggesting that the Pf to GABAergic interneuron to cholinergic interneuron circuit is regulated by DA. This is in line with experiments in primates, which demonstrate that either inhibition of the Cm/Pf nuclei (Matsumoto et al. 2000) or lesion of the DA projection to striatum (Aosaki et al. 1994a) nearly completely eliminate the pause responses of TANs. The fact that local application of DA antagonists mimics the effect of chronic DA lesions (in terms of its effects on the pause responses; Watanabe and Kimura, 2004), suggests that the presumed GABAergic interneuron linking the Pf and cholinergic interneurons is sensitive to both

chronic and short-term changes in DA signaling.

Relevance to human disease: Introduction

Addiction, Parkinson's disease, Huntington's disease and Gilles de la Tourette syndrome are undoubtedly the most well known disorders of the basal ganglia. The relevance of my thesis work is limited primarily to Tourette's syndrome. Parkinson's disease and Huntington's disease, although obviously arising from basal ganglia dysfunction, and perhaps treatable to some extent with pharmacotherapy targeting cholinergic systems, will not be discussed in detail. There is no connection between the cause of these diseases or their symptoms to my investigation of fast cholinergic signaling. This is not to say that in either of these diseases this circuit might not function correctly, indeed it is known that in dopamine depleted animals the pause responses of cholinergic neurons are abnormal. However, the relevance of this circuit's malfunction to either disorder is unknown, and I have *no a priori* assumptions in this regard.

Addiction

Addiction is a disease whereby people are unable to make proper decisions regarding their well being due to the learned excessive positive salience of a drug or activity. The normal decision-making process breaks down, and a maladaptive motivational shift takes place inducing seeking and consummation of the substance of reward at significant cost to other activities. The rewards can include pharmacological agents such as alcohol, tobacco, opiates and stimulants as well as behaviors such as sex or exercise. The key feature of any addiction is an inability to make decisions whose outcomes are not in line with attaining the reward to which the person is addicted. The brain circuits underlying this behavior are complex and anatomically distributed throughout the brain. Malfunction of the basal ganglia, prefrontal cortex, amygdala and hippocampus are all involved. The result is a condition that is extremely difficult to treat by pharmacological, psychological or other interventions.

The neostriatum and nucleus accumbens are involved in different aspects of decision-making, largely determined by the structures from which they get afferent information. The striatum primarily gets input from different sensorimotor related cortical areas and the thalamus, while the nucleus accumbens/ventral striatum gets input primarily from limbic related structures such as the amygdala and hippocampus, as well as the prefrontal cortex. Due to this, the information processed in the two structures is different. Dysfunction in the nucleus accumbens is related more to emotional disorders such as addiction and depression and dysfunction of the dorsal striatum to disorders involving initiation of and motivation for voluntary action. The physiology and anatomy of the structures are different, as the concentrations of different interneuron subtypes (categorized largely by expression of neurochemical identifiers) differs between the structures, and the circuit function and gene expression of at least once interneuron, the cholinergic interneuron, appears to be different. P11, a protein related to motivational abilities and which may be related to depression, is only expressed in the nucleus accumbens cholinergic interneurons (Svenningsson et al. 2006; Alexander et al. 2010; Egeland et al. 2011). In the accumbens, synchronized activity of cholinergic interneurons does not engage a GABAergic interneuron network as we have demonstrated in the neostriatum (Witten et al. 2010). My thesis work focused solely on

the circuit function of cholinergic interneurons in the neostriatum and thus does not specifically address the neural basis of addiction.

Gilles de Tourette's syndrome

Gilles de la Tourette syndrome (TS) is characterized by involuntary, nonrhythmic motor movements and vocalizations referred to as tics (State et al. 2001). The severity of the disorder is highly variable and affects as much as 1% of the human population to some degree. In comparison to other common neuropsychiatric disorders TS has received relatively little attention in terms of both basic research and therapeutics. Given the role of the basal ganglia in the regulation of voluntary movement initiation and action selection, much TS research is largely focused on basal ganglia function and dysfunction. Recent anatomical investigation of human TS patient brains post-mortem revealed loss of specific cell types in the neostriatum, specifically interneurons expressing parvalbumin or choline acetyltransferase (Kalanithi et al. 2005; Kataoka et al. 2010). Additionally, fMRI studies have revealed activation patterns in basal ganglia nuclei related temporally to tic generation or the voluntary inhibition of tics (Mazzone et al. 2010). Importantly, TS stands in contrast to other basal ganglia related disorders in that there does not appear to be a pathology directly involving DA neurons and that pathological oscillations in basal ganglia nuclei have not been implicated (State et al. 2001; Bloch et al. 2011). As with most neuropsychiatric disorders, a major focus has been on illuminating potential genetic factors in the disorder, mutations which affect neuronal development. Relatively little progress has been made in terms of defining a genotype predictive of TS in large populations, however rare copy number variants (in

the gene SLITRK1) (Abelson *et al.* 2005; Speed *et al.* 2008) and de novo mutations (in the gene HDC) (Ercan-Sencicek *et al.* 2010) have revealed genetic causes of TS in small populations which can inform us about potential etiologies underlying the disorder in general.

The fact that parvalbumin interneurons are depleted in TS patients suggests that a failure of cortically driven feed-forward inhibition may be involved in the etiology of the disease, as normal functions of this pathway include inhibition of striatal output, and pharmacological disfacilitation of this pathway produces dystonia. The potential mechanistic role of the loss of cholinergic neurons was thought to involve reduction in a tonic down regulation of projection neuron activity by acetylcholine. The findings presented in this thesis suggest an alternative or additional mechanism, in which loss of cholinergic neurons would deprive projection neurons of a potent source of inhibition from a GABAergic interneurons expressing NPY. The fact that this circuit is likely engaged by thalamic inputs whose purpose may be to cause motor program switching potentially explains the fact that TS patients get stuck in repetitive motor programs which are involuntary. Thus the failure of the cholinergic-GABAergic circuit might have a direct symptomotological correlate in TS patients. Whether or not this specific conjecture is correct, the dual loss of PV and Cholinergic interneurons in TS patients surely results in the projection cells being over-active due to the loss of two fast GABAergic inhibitory mechanisms, which likely contributes to the symptoms of the disorder.

Conclusion

The data presented here can be broken down into several pieces, which when put together illuminate a novel and probably critically important circuit in the striatum. It is now undeniable that acetylcholine acts in roles outside of neuromodulation the striatumspecifically it acts as a fast excitatory neurotransmitter in a similar capacity to glutamate. The combined evidence from our three *in vitro* preparations (ChR2, Halorhodopsin, and paired recordings) and our *in vivo* experiments demonstrate that fast cholinergic signaling can engage local inhibitory interneurons that shut down the ongoing activity of large groups of SPNs. The fact that all SPNs get the same inhibition suggests that this effect may function to reset the ongoing activity of the striatum, biasing it towards reflecting the next set of cortical inputs. A picture now emerges in which the cholinergic pause response may function to enable switching between the activation of different principle neuron ensembles, each underlying a specific motor program or action. This makes sense in light of the work on thalamic inputs to the striatum (which are thought to drive the pause response) and their behavioral correlates. In fact, this circuit might be the missing link postulated to exist between thalamic activity in response to alerting stimuli and changes in the ongoing evaluation of motivation for voluntary movement, reflected as changes in striatal activity and output. The fact that presumed principal neurons recorded in primates do not reflect activity of this circuit at the time the pause response is engaged is not all that surprising. In addition to the fact that some of the neurons presumed to be projection cells may be interneurons, it is not expected that all projection neurons should respond in the same way or have the same activity patterns simultaneously. Our results show that when projection neurons are very active that they may be less affected by this inhibition, which may function as a contrast detector

between competing projection neuron ensembles. Additionally, some projection neurons in particular primate experiments have been observed to be active then cease their activity at the precise time of the excitation of cholinergic interneurons, suggesting that the pause response might have had the effect predicted by this circuit under those precise experimental circumstances. Large-scale recordings of principle neurons in welldesigned behavioral tasks are needed to investigate further the effects of the cholinergic pause response and the cholinergic-GABAergic circuit described in this thesis.

Our results underscore recent ideas which have been gaining traction in the basal ganglia field including the idea that there are many interneuron types in the striatum with distinct functional roles. It was believed for decades that the two main inhibitory influences over striatal output were the feed-forward inhibition from PV+ interneurons and collateral inhibition between projection neurons. As compared to brain areas such as the hippocampus and neocortex, this system appeared relatively simple, as the latter has at least 10-20 distinct interneuron types with very specific functional roles. The recent findings from out lab and others, which have identified four new GABAergic interneurons expressing tyrosine hydroxylase, as well as the novel NPY-NGF neuron involved in the cholinergic-GABAergic circuit, suggests that the striatum may be more similar to these other brain areas than was previously appreciated. Investigation of the inputs to principal neurons from GABAergic interneurons as well as the inputs between GABAergic interneurons now need to be examined in more detail on a cellular and subcellular level. This will allow the determination of their precise roles in shaping principal neuron activity, and allow us to see if they are as finely tuned as their counterparts in other brain areas.

The relatively recent field of optogenetics is still undergoing many developments. We initiated this study at a time when the potential caveats and problems with channelrhodopsin, and especially Halorhodopsin, were relatively unknown and the problems that were known were serious. We thus included many control experiments, in fact using three distinct *in vitro* preparations to test the same circuit, in order to make sure we were not generating artifacts with optogenetics. Importantly, both for our work in the basal ganglia and for the field of optogenetics, these results provide ample evidence that optogenetic techniques can be used to interrogate neural circuits while maintaining strict physiological relevance.

This work is relevant in a general way to all basal ganglia disorders, including Parkinson's disease, Huntington's disease, addiction and Gilles de Tourette syndrome. Of these, TS is the only one where our data suggest a possible direct mechanistic role in the etiology of the symptoms. The meaning of the loss of interneurons, particularly cholinergic interneurons, in TS patients, is unknown. Our data suggest that a source of inhibition of striatal projection neurons may be deficient in these patients, which could contribute to their symptoms, especially failure to control or stop tics. This work thus highlights the importance of the study of brain circuits, even those containing rare neurons, on a basic level, as even though the players in this circuit together constitute less than 5% of the neurons in the entire structure, they may be invaluable in understanding the mechanism of a common neurological disorder.

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Publications

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