Microcircuitry of the songbird basal ganglia nucleus area X

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Abstract

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Understanding learning is one of the most basic human fascinations. As a nation we devote large amounts of resources to discovering therapeutic interventions for and preventing learning disabilities that arise developmentally (ex. mental retardation, autism, attention deficit hyperactivity disorder, dyslexia), due to insults to the nervous system (ex. stroke, traumatic brain injury), or with age (ex. Alzheimer's and Parkinson's diseases). Beyond repairing the brain, these efforts are progressively heading into the ethically gray territory of learning augmentation (Di Pino et al., 2014). Learning is a broad term that can encompass many different sensorimotor, cognitive, emotional and attentional processes on a wide spectrum of timescales. However, at their core all forms of learning occur through some form of interaction with the environment. One common strategy for learning that is used throughout the animal kingdom is trial-and-error. A central question guiding the present work is how the brain accomplishes the "trial" component of trial-and-error learning. In the following pages I will discuss theoretical frameworks that have shaped experimental studies of motor learning, proposed roles of the basal ganglia in regulating voluntary motor behavior and the advantages of neuroethological model systems, with a specific focus on songbirds. Finally, I will focus on the neural mechanisms thought to underlie the exploratory or "trial" component of vocal learning in the songbird, presenting evidence for a novel excitatory basal ganglia circuit component that may serve as one biophysical mechanism for the introduction of exploratory neural variability into motor behavior.

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Huge thanks to Cyril Saint Girons, my husband and best friend, who has been constantly supportive throughout my higher education process and who made me unafraid of Matlab. Thanks for coming to Seattle with me six years ago – I couldn't have done it without you!

Dedication

I dedicate this work to my parents, Elżbieta and Andrzej Budziłło. I am not sure where or whom I would be had they not taken a leap of faith, leaving everyone and everything they knew, and bringing me with them to the overwhelming metropolis of New York City from Poland in 1987.

1. Introduction

Theoretical Frameworks of Learning

Pioneers in diverse fields such as philosophy, psychology, and sociology had been constructing abstract frameworks or models in which to schematize learning processes well before the advent of modern neuroscience. By the middle of the 20th century the burgeoning field of computer science, and especially machine learning, began to play an influential role in guiding experimental approaches to learning research (McCulloch and Pitts, 1943; Turing, 1950). Theoretical models can be valuable tools for understanding biological results. By design, all models distill underlying findings to varying extents, and while the choice of these simplifications may be biased and flawed, ideally models are constructed in a way that inspires testable experimental hypotheses. These in turn can assess the validity of the model and its assumptions, and provide valuable information about the system under investigation.

Supervised and Unsupervised Learning Models

In a very basic sense, actions can be divided into commands (input) and effects (output). In control theory, the processes that govern the matching of inputs to desired outputs are often described in terms of inverse or forward models. A forward model predicts the output that would result from a particular input, while the objective of the inverse model is to find the best input solution to generate a desired output (Wolpert et al., 2011; Giret et al., 2014). These interrelated perspectives can be used collectively to describe different modules of the learning process.

Several types of learning models have been used to describe how this input-output mapping could undergo modification during learning. Such paradigms can be grossly classified into *supervised, unsupervised* and *reinforcement* models. *Supervised* learning is driven by explicitly guided input-output mapping, in practice often through the presentation of training stimuli (Dayan and Abbott, 2001). However in the natural world, learning rarely occurs through direct guidance from an omniscient teacher. Many educators contend that, even in a prototypically didactic setting such as the classroom, learning through explicit instruction may be less effective than learning through activities that promote self-driven exploration and interaction (Bransford et al., 2000). Additionally, by definition the teacher in a *supervised* paradigm already knows the optimal solution to the inverse model. The concept of such a fixed solution is at odds with the continuous flexibility of natural behaviors that may need to be modified and calibrated throughout the lifetime of the animal to account for changes in its internal condition due to growth, injury or aging, or to changes in the statistical properties of its environment (Pascual-Leone et al., 2005). Changes such as these may necessitate different optimal neural solutions at different times. In contrast to *supervised* learning, *unsupervised* learning rewires action-generating circuitry exclusively on the basis of intrinsic network dynamics and inputs with no explicit teacher, often recruiting a Hebbian plasticity mechanism (Hebb, 2005; Dayan and Abbott, 2001). However, basic intuition regarding learning in animals implies that feedback and environmental state both play an important role in guiding behavior, and that natural learning cannot simply function as a closed loop.

Reinforcement Learning Models

An intermediate family of models between *supervised* and *unsupervised* learning that captures these requirements and behavioral observations well is *reinforcement* learning. At their core, *reinforcement* models embody a very

basic intuition formulated by Thorndike: "[actions] which are accompanied or closely followed by satisfaction to the animal will, other things being equal, be more firmly connected with the situation, so that, when it recurs, they will be more likely to recur" (Thorndike, 1911). *Reinforcement* models function by evaluating the outcome of the current commands as compared to the desired output, and generating a signal that can then be used to change future commands. In contrast to *supervised* learning, which is guided by feedback through explicit corrective instruction, *reinforcement* learning uses evaluative feedback in the format of a binary "yes-no" or perhaps continuous "warmer-colder" answer simply to bias future commands with the goal of ultimately narrowing in on a successful behavior. In practice, reinforcement models typically consist of the following elements: an *agent*, which operates on an *environment;* a *policy* that describes how information resulting from those actions is weighted, a *reward function* that assigns values to the immediate desirability of all possible outcomes, and *a value function*, representing a cumulative, long-term evaluation of the animal's state (Sutton and Barto, 1998). One particular formulation of this model that has been implicated in animal learning is the actor-critic architecture (Barto et al., 1983; Barto,1995). We will consider this model in the context of the network of subcortical structures known as the basal ganglia, where it has served as an influential theoretical framework (Houk et al., 1995; Montague et al., 1996).

The Basal Ganglia and Learning

Learning draws on a wide set of sensory, motor, affective and associative processes, and occurs on a broad continuum of timescales. Lowering the dimensionality of this space by concentrating on the study of voluntary *motor* behaviors can provide a window onto this complex suite of mechanisms while staying within a framework

of tractable and intuitively measurable physical outputs. Motor learning is supported by information processing through parallel feedback loops found in extrapyramidal structures such as the basal ganglia and cerebellum (Hikosaka et al., 2002).

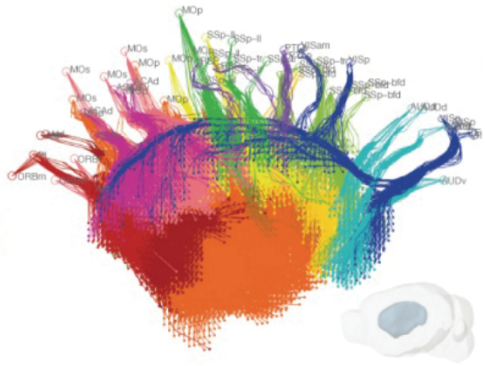

Figure 1. Topography of cortico-striatal projections

Adapted from Oh et al., 2014

3D virtual tractography of striatal projections following anterograde tracer injection into cortical sites marked by open circles

The basal ganglia have long been associated with action selection and voluntary, goal-directed behaviors in humans, as lesions localized to these nuclei cause profound motor and cognitive abnormalities (Rothwell, 2012). Their pivotal role in the brain is underscored by that fact that their structure and function are conserved throughout the vertebrate phylum (Reiner et al., 1998), with evidence of deeply homologous circuitry for action selection reaching as far back up the ancestral ladder as the arthropod complex (Strausfeld and Hirth, 2013). The basal ganglia do not send direct motor commands to the spinal cord, but rather perform a more complicated transformation, adjusting and sequencing actions while integrating information about the animal's attentional and motivational states (Graybiel, 1995). Virtually all cortical regions project to the basal ganglia, and they do so topographically (Kemp and Powell, 1970). Contemporary whole brain connectomics approaches are confirming initial histological and electrophysiological reports of this topographical architecture in an increasingly detailed manner (Fig. 1) (Oh et al., 2014; Hunnicutt et al., 2014). Functionally, this topography allows for information to be

processed in parallel loops, so that output is fed back to the same cortical regions from which input originally arose (Hoover and Strick, 1993).

Organization of the Basal Ganglia

Confusion surrounding the complicated organization of the basal ganglia is foreshadowed by their name, an anatomical misnomer. In modern neuroanatomical terminology the word "ganglion" is typically reserved for a cluster of cells in the peripheral nervous system, while the basal ganglia, a set of interconnected subcortical nuclei, are clearly part of the central nervous system. The relevant structures in this network are the pallidum, the striatum, the substantia nigra and the subthalamic nucleus. The pallidum, named for its pale appearance in unstained tissue, has two parts with distinct functional roles: the medial internal segment of the globus pallidus (GPi) and its more lateral counterpart, the external segment of the globus pallidus (GPe). The neo-striatum (often abbreviated to striatum), an evolutionarily newer structure than the pallidum, has a characteristically striped appearance, due to the bundled arrangement of its emanating output fibers. The striatum is composed of two parts, the caudate and putamen, which are contiguous in the rodent brain, but are largely separated by the internal capsule in humans (Martin et al., 1989). The substantia nigra, named for the ink-like appearance of its melanin granule-containing cell bodies, is composed of two subdivisions – the pars reticulata (SNr), and the pars compacta (SNc). The final member of the basal ganglia is the small, round subthalamic nucleus (STN). Presumably less distinct in its overt appearance, its name reflects its relative location to the overlying thalamus. While the basal ganglia nuclei are highly interconnected with feedback and reciprocal connections existing at each station, Fig. 2 illustrates a simplified description of information flow. In short, cortical, thalamic and midbrain dopaminergic inputs project to the striatum, which in turn projects directly or indirectly to the GPi/SNr.

The indirect pathway first passes through the GPe and STN. Finally, GPi/SNr connect to the thalamus, which can drive activity in cortical regions. The regions listed above are on the sensorimotor end of the behavioral spectrum that the basal ganglia control. There is a more ventrally located loop that preferentially receives input from limbic cortical regions rather than sensorimotor ones, and is primarily involved in the processing of cognitive and affective information. The ventral portion of the striatum is known as the nucleus accumbens, and it is connected with its own complement of distinctly named basal ganglia regions including the ventral pallidum, the nucleus accumbens septi, the olfactory tubercule, the anterior perforated substance, and the ventral tegmental area (VTA). There are many cytochemical and physiological similarities between these ventral and dorsal portions of this system, but also some differences and many unknowns (Voorn et al., 2004).

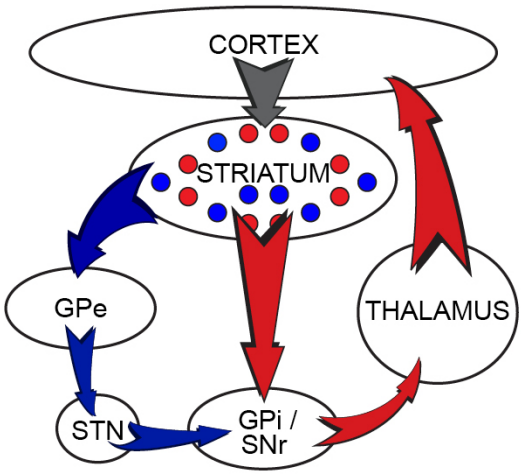

Figure 2. Simplified basal ganglia pathways

Model proposed by Albin et al, 1979 and DeLong, 1980

D1 receptor-expressing MSNs (red) project down the direct pathway, while D2 receptor-expressing MSNs (blue) project down the indirect pathway

Striatal Medium Spiny Neurons give rise to a Direct and Indirect Pathway

What physiological features define the neuronal populations residing in the basal ganglia, and how have these guided our understanding of information processing that occurs within its parallel loops? The striatum is the gateway to the basal ganglia, where the majority of incoming cortical and thalamic projections terminate. Nearly all of the neurons in the striatum are medium spiny neurons (MSNs), and the vast majority of cortical and

thalamic excitatory projections terminate on dendritic appendages known as spines that give these neurons their name (Kemp, 1968). MSNs are estimated to receive on the order of 10,000 inputs through their dendritic spines (Bar-Gad et al., 2003). Intracellular recordings of MSNs show that they are typically hyperpolarized and silent at rest, but at times receive intense cortical drive resulting in a high volume of synaptic activity (Wilson and Kawaguchi, 1996). This bipolar nature of MSNs has been referred to as *up*/*down* state. In the behaving animal, MSNs are characterized by low frequency firing that is time-locked to movement (Hikosaka and Wurtz, 1989).

Unlike the projections of almost all other regions of the brain, the basal ganglia nuclei almost exclusively communicate with one another through inhibitory projections, with the notable exception of the STN (Graybiel, 1990). Adhering to this convention, MSNs, which are also the projection neurons out of the striatum, release gamma-aminobutyric acid (GABA). However, MSNs are not a homogeneous population of cell types, instead falling into two classes defined by their projection targets, dopamine receptor subtype and neuropeptide expression (Kreitzer, 2009). If we consider a somewhat oversimplified model of the basal ganglia in which the relevant output of the network is firing rate, these contrasting MSN flavors embody the origins of two ipsilaterally projecting information streams that ultimately yield opposite effects on the generation of movement. *Direct* pathway MSNs express substance P, dynorphin and D1-type dopamine receptors, and project directly to the output nuclei of the basal ganglia, the SNr and GPi. *Indirect* pathway MSNs express enkephalin, neurotensin and D2-type dopamine receptors, and route a detour through the GPe and STN, whose outputs eventually reach the SNr and GPi. Activation of one or the other pathway in this model has opposing behavioral effects due to the inhibitory, sign-reversing synapse inserted at the GPe stopover (Albin et al., 1989; DeLong, 1990). Dopamine release from SNc fibers in the striatum adds an additional layer of control by oppositely affecting excitability of the two MSN classes. Activation of G_s-coupled-D1 receptors increases neuronal excitability through multiple

intracellular signaling mechanisms, including enhancement of L-type calcium channel signaling and inhibition of K^+ channel and Ca^{2+}-dependent (SK) K^+ channel conductances, while G_i-coupled-D2 receptors inhibit neuronal excitability (Gerfen and Surmeier, 2011). Collectively, this results in the promotion of movement through both pathways (Kreitzer and Malenka, 2008).

Basal Ganglia Disorders and the Direct and Indirect Pathways

Basal ganglia disorders, such as Parkinson's Disease (PD), Huntington's Disease (HD) and Tourette's Syndrome, are associated with a spectrum of deficits in the habitual control of action. The framework described above has been instrumental in guiding our understanding of two of these disorders - PD and HD (Albin et al., 1989; DeLong, 1990). PD is characterized by a number of motor and cognitive features including muscle rigidity and jerkiness, difficulty initiating movements and 4-6 Hz involuntary muscle contractions known as resting tremor (although in some patients these oscillations persist during movement) (Rothwell, 2012). At a neural level, PD is characterized by progressive degeneration of dopaminergic neurons, mostly restricted to the SNc, but also in its ventral counterpart, the VTA. Remarkably, the nervous system is able to compensate for the loss of these neurons until there are only approximately 20% of them remaining. Ultimately, however, the loss of dopaminergic input to the striatum is thought to depress movement-promoting signals (Albin et al., 1989; DeLong, 1990). HD has been linked to degeneration of indirect pathway medium spiny neurons, alongside a loss of striatal cholinergic interneurons. The direct/indirect pathway architecture can also be used to explain the hyperkinetic symptoms associated with HD (Albin et al., 1989).

Dopaminergic Modulation of the Striatum

Dopamine modulates excitability of MSNs, but it also controls both presynaptic and postsynaptic expression of long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic inputs onto MSNs (Calabresi et al., 2007; Kreitzer and Malenka, 2008). Midbrain dopaminergic fibers from the SNc and VTA project extensively throughout the striatum, positioning them in a powerful place for modification of synaptic strength (Kreitzer, 2009). Dopamine neurons can release dopamine either tonically, or phasically, as a result of high-frequency bursts of activity (Grace, 1991). Phasic dopamine release increases extracellular synaptic and extrasynaptic dopamine concentration, thus allowing for differential targeting of low-affinity D1 receptors and high-affinity D2 receptors (Venton et al., 2003). It is now clear that dopaminergic fibers in the striatum also co-release glutamate adding another piece to this complicated puzzle (Sulzer et al., 1998; Chuhma et al., 2004; Lavin et al., 2005).

Striatal Interneurons and Ultrastructure

Although much of the focus in the striatum has been on the excitatory corticostriatal inputs onto MSNs and the plasticity of these synapses, these inputs are not the only determinants of MSN activity. There are three other neuron types residing in the striatum, which also receive incoming excitatory inputs and make synapses onto MSNs (Kawaguchi, 1993; Kawaguchi et al., 1995). These are the cholinergic interneurons, the parvalbumin-expressing GABAergic fast-spiking (FS) interneurons, and the GABAergic low-threshold spike interneurons (LTS). Striatal cholinergic interneurons or tonically active neurons (TAN) make up approximately 2% of the cell types in the striatum, are characterized by their slow spontaneous firing *in vivo* (Kimura et al., 1984), and large somata (Zhou et al., 2002). The time-locked nature of their firing and pauses in firing during reinforcement learning has led to the idea that TANs are involved in conveying information about motivation, or perhaps

context, during behavior (Aosaki et al., 1994; Apicella, 2007). On a circuit level TANs are implicated in both MSN plasticity and modulation of dopaminergic activity in the striatum (Mark et al., 2011). Notably, these cholinergic neurons also co-release glutamate, (Schäfer et al., 2002; Gras et al., 2002; Higley et al., 2011) and it is possible that some of these signaling features are in fact mediated by glutamate release (Guzman et al., 2011). The other two interneurons are less well understood - in addition to their low representation in the striatum they do not have easily identifiable characteristic electrophysiological signatures. Although sparsely distributed, FS interneurons are electrically coupled (Koós and Tepper, 1999), allowing them to drive strong and rapid feedforward inhibition onto both classes of MSNs (Tepper et al., 2004). LTS neurons are perhaps the least understood cell type in the striatum. These inhibitory interneurons express nitric oxide synthase, have a depolarized resting membrane potential and long action potential waveforms (Kawaguchi, 1993). Finally, MSNs can affect each other; they make symmetric synaptic contacts onto one another via axon collateral branches, although the strength of inhibition conveyed through this route compared to that coming from the other local inhibitory interneuron populations is likely to be low (Jaeger et al., 1994; Tunstall et al., 2002; Tepper et al., 2004).

Beyond the heterogeneity of cell types within the striatum, there is also puzzling ultrastructure. Early histological studies found what are now called striosomes - patches of striatum with low acetycholinesterase and dense μ-opiate receptor staining (Graybiel and Ragsdale, 1978; Herkenham and Pert, 1981). While most sensorimotor cortical inputs appear to be directed to the surrounding matrix, limbic regions preferentially target striosomes (Groves et al., 1995). Intriguingly, both structural compartments receive mesencephalic dopamine projections, but only striosome neurons send feedback to the SNc.

The Pallidum, Substantia Nigra and Subthalamic Nucleus

Although the striatum has been the core focus in basal ganglia research, appreciation of the other component nuclei has grown, and we are now beginning to understand the neurophysiology of these regions in normal and pathological conditions. Neurons in both the GPe and GPi fire spontaneously at high frequencies (DeLong, 1971) . However, in awake behaving primates, GPe neuron firing patterns exhibit more modulation, with longer pauses in firing and bursts, while GPi neurons fire more irregularly, but without long pauses (DeLong, 1971; DeLong, 1972). Functionally, the SNr plays a similar role to the GPi and the two are typically grouped as a combined basal ganglia output structure. However, there are differences, including diverse developmental origins and the more interconnected relationship between SNr neurons and the nearby dopaminergic neurons of the SNc (Bar-Gad et al., 2003). Finally, although traditionally both of these basal ganglia output structures were thought to be GABAergic, a recent reexamination of SNr outputs to thalamic neurons in mice has led to the discovery of an excitatory component to this projection (Antal et al., 2014). The relevance and function of this surprising finding is yet to be determined.

As the site of the only glutamatergic projection neurons within the basal ganglia, the role of the STN has always been intriguing. The STN is now thought to play an important part in maintaining normal network activity, due to the nature of its pathological activity in Parkinson's disease (Wichmann and DeLong, 1996). STN neurons are tonically active at rest, firing action potentials in bursts during the production of movement (Wichmann et al., 1994; Beurrier et al., 1999). The reciprocal connection between STN and GPe is of particular interest, given that neurons in both of these regions are oscillatory. In PD, STN and GPe neurons are significantly more correlated in their firing, exhibiting bursts of activity in a frequency range that sometimes overlaps with the observed

frequency of tremors (Bevan et al., 2002b). Lesions localized to the STN disrupt these pathological oscillations in the network (Bergman et al., 1990), as does deep brain stimulation (Limousin et al., 1998), alleviating at least some PD symptoms (Limousin et al., 1995; Krack et al., 1998; Rothwell, 2012). GPe and STN both receive cortical input, but removal of these projections *in vitro* does not eliminate oscillatory activity, suggesting that this is a pathological behavior that emerges directly within the network, rather than being inherited from correlated cortical input (Plenz and Kital, 1999). Investigations into the phase relationships that exist between the STN and GPe, as well as the circuit mechanisms responsible for the coupling and decoupling of their firing patterns are likely to inform our understanding of the basal ganglia's activity in normal and pathological states alike (Wilson, 2014; Fujita et al., 2012). Beyond this, characterizing the circuit details of the STN is important, as changes to synaptic input and modulation of synaptic input could alter the dynamics of the network (Bevan et al., 2002a). This is particularly relevant as both of the nuclei are under the influence of dopamine from the SNc, and dopamine levels clearly affect the synchronicity of GPe-STN oscillations (Brown et al., 2001; Cruz et al., 2011).

Beyond the Direct and Indirect Pathways

Early models of basal ganglia function focused on the direct-indirect architecture (Albin et al., 1989; DeLong, 1990). However, this model alone does encapsulate all of the behavioral and neural observations ascribed to normal and pathological basal ganglia activity. For example, synchronization of oscillations between the GPe and STN seem to be involved in aspects of PD that were not easily explained by the direct-indirect pathway architecture (Wichmann and DeLong, 1996). Other themes in basal ganglia processing have emerged outside of this architecture. One that has received particular attention is convergence, which exists at every stage of processing. It is estimated that the number of neurons between cortical projections and striatum decreases 10-fold,

and somewhere between 100 and 1000-fold between the striatum and the GPi/SNr (Bar-Gad et al., 2003). While there is much convergence, neural representations remain sparse, so that a given neuron at each processing layer only has access to a small fraction of the total information present in the input. Some models are focused on describing how sequential convergence of information could operate alongside the functional topography that is apparent throughout the basal ganglia, to determine whether information is processed primarily in parallel or in a funnel-like way (Bar-Gad et al., 2003). Another consistent feature throughout the basal ganglia is the high interconnectivity between its nuclei. Bidirectional flow of information seems to be the rule in most cases. This has particularly interesting implications as many of the basal ganglia nuclei contain spontaneously active oscillatory cell types with the potential to synchronize or desynchronize, affecting the state of the network, and possibly behavior. More recent models of the basal ganglia have focused on its possible role in action selection, the idea here being that rather than simply promoting or inhibiting actions, this circuitry could be involved in selecting the appropriate action from a number of options given the current context (Bar-Gad et al., 2003). Inter-nuclear oscillations have, for example, been suggested as a trigger for action transitions (Fukai, 1999).

Actor / Critic Reinforcement Learning and the Basal Ganglia

We are now equipped to return to the actor-critic reinforcement learning framework formulated by Sutton and Barto (Fig. 3). The *actor* here is synonymous with an *agent* that acts on the *environment*, and receives feedback based on the results of those actions. The *critic* is a new addition to the network - it also receives feedback, and uses this information to output an evaluation of the relative success of the action that had been taken. A reinforcement signal resulting from this evaluation may then drive an expansion or constriction of stochasticity in future actions. An increase in action variability may lead to the discovery of better suited solutions to the inverse

model (Sutton and Barto, 1998). There is a constant trade-off between such exploration, and exploitation of actions that are already known to lead to positive outcomes or rewards. Importantly, both the *actor* and *critic* in this model receive information about the current context, which can change the immediate objective of a behavior, and consequently its performance evaluation.

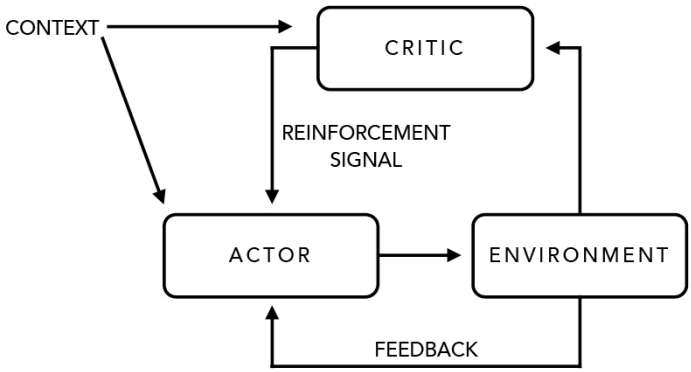

Figure 3. Actor / Critic model
Modified from Barto, 1995

The basal ganglia are proposed to be the biological site of the actor-critic framework (Houk et al., 1995; Montague et al., 1996). A key requirement of any reinforcement learning paradigm is an element that is able to translate some evaluation of feedback into a signal that can then be used to affect future behavioral commands. Remarkably, the activity of midbrain dopamine neurons has been found to match the proposed properties of this integral component of reinforcement learning algorithms. Recordings in VTA and SNc of awake behaving monkeys show that dopamine neurons can dramatically increase their firing rate with a short latency after presentation of an unexpected reward, and cease firing when an expected reward has been omitted (Schultz et al., 1997; Schultz, 1998). In a reinforcement learning paradigm dependent on error prediction, learning should only occur when an outcome is surprising or the error prediction is non-zero. Dopamine neuron activity suggests that

these cells could signal a mismatch between an outcome and the *expectation* of that outcome given the current context, in this way contributing to learning. Dopaminergic neurons can affect MSNs and inputs to MSNs in a myriad of ways described above. One suggestion is that MSNs residing in striosomes, which are reciprocally connected to dopaminergic neurons, could serve as a *critic,* while MSNs residing in the matrix compartment could serve as the *actor* (Houk et al., 1995). An alternative hypothesis is that the dorsal striatum serves as the *actor*, while the ventral striatum is the *critic* (Montague et al., 1996).

Another key element suggested by Sutton and Barto's actor-critic model is a search mechanism for actively injecting trial-to-trial variability into otherwise structured action commands. The introduction of exploratory variability during repetitions of a behavior is essential for presenting the *critic* with opportunities to appropriately reinforce commands that lead to a desired or undesired state. While some forms of variability may reflect limits inherent to the nervous system (Churchland et al., 2006), there is also evidence that variability can actively promote learning (Tumer and Brainard, 2007). Many intrinsic and synaptic factors are likely to control neural variability (Faisal et al., 2008). However, we do not currently understand the precise neural mechanisms underlying the generation of variability in motor learning in mammals.

An Ethological Approach to the Neuroscience of Learning

Although studies in conventional model organisms like rodents and primates have made groundbreaking contributions to the fields of learning and memory, the behaviors studied in these animals in the laboratory are often artificial and lacking in real-world complexity. Some examples of such established paradigms include

simple decision making tasks related to the direction of movement where learning is guided with explicit food rewards (Britten et al., 1992; Cohen and Nicolelis, 2004), the Morris water maze as a means of studying spatial learning and memory (Morris, 1984), and the vestibulo-ocular reflex in primates (Broussard and Kassardjian, 2004). While such laboratory paradigms break down the inherent complexity of behavior into fewer, more manageable and easily observable variables, one is left wondering whether the neural mechanisms underlying such limited laboratory tasks can be abstracted up to more natural and relevant behaviors to the animal. For this reason there is continued value in studying organisms with neuroethologically relevant behaviors despite the richer experimental toolbox and more comprehensive foundation offered by more conventional animal systems (Brenowitz and Zakon, 2015). This approach can be particularly powerful when the behavior is important enough for the organism's survival and reproduction that it has resulted in an expansion of the brain regions involved in its control. Songbirds have emerged as one such valuable ethological model system.

Birdsong as a Model System

Birdsong has captivated the human fascination, inspiring musicians and biologists alike, for centuries. The celebrated classical composer Wolfgang Amadeus Mozart had a beloved pet European starling that he regarded so highly that he held a proper burial ceremony for it. He is reported to have purchased the animal after hearing it mimic a portion of one of his own concertos (Fig. 4) (West and King, 1990). Although early songbird enthusiasts did their best to record bird sounds using musical notation, their data collection was indirect and distorted by the limits of human auditory perception and memory. It was not until the middle of the twentieth century that birdsong truly made its debut on the scientific scene, as audio recording and spectrographic analysis made it possible to conduct careful analysis of the spectral and temporal characteristics of song in a systematic

and unbiased fashion. Groundbreaking behavioral experiments involving isolating nestlings from exposure to their species specific songs, cross-fostering nestlings with distantly related species, and tutoring young birds by cassette tape playback, demonstrated that while there is likely to be some innate wiring that guides preference for species-specific vocalizations, song has a very strong learned component (Thorpe, 1954; Marler and Tamura, 1964). This insight combined with early observations of a discretely organized network of song-specific brain structures (Nottebohm et al., 1976) were two characteristics that propelled the songbird system into the scientific field of learning.

Who Learns to Sing and Why?

Song is a socially complex learned vocal behavior important for survival and reproduction in many bird species. While many animals produce vocalizations, species that partake in vocal *imitation* are surprisingly few in the animal kingdom. This capacity appears to be primarily restricted to humans, marine mammals (Janik, 2000), possibly bats (Boughman, 1998), and many avian species. While some may argue for the inclusion of mice to this list, the evidence that their vocalizations are learned is lacking (Mahrt et al., 2013). Birds throughout the avian family can produce innate vocalizations known as "calls", categorized by the function they serve. Some examples of these include begging calls produced by hungry chicks (Wilkinson, 1980), contact calls amongst a flock (Williams, 1969) and the many different types of alarm calls in response to a predator sighting (Marler, 1955; Klump and Shalter, 1984). Three orders of birds have expanded upon this capacity, evolving the ability to learn complex vocalizations. Among these are the *Apodiformes* (hummingbirds) (Baptista and Schuchmann, 1990), *Psittaciformes* (parrots) (Farabaugh et al., 1994), and the largest with over 4000 member species: the *Passeriformes*, or songbirds (Marler and Slabbekoorn, 2004). This rare capacity coupled with its many parallels to human

communication (Doupe and Kuhl, 1999), have made the songbird a powerful system for investigating the neural bases of speech learning. Studies in the laboratory and in the wild alike confirm that song is a salient and behaviorally relevant signal to birds. Song playback from loudspeakers can be a potent territorial signal (Falls, 1988), and a way of differentiating neighbors from potential intruders (Beecher et al., 1996). Finally, female birds are skilled at hearing even subtle differences in song, and they are sexually responsive to it (McGregor et al., 2013; Woolley and Doupe, 2008).

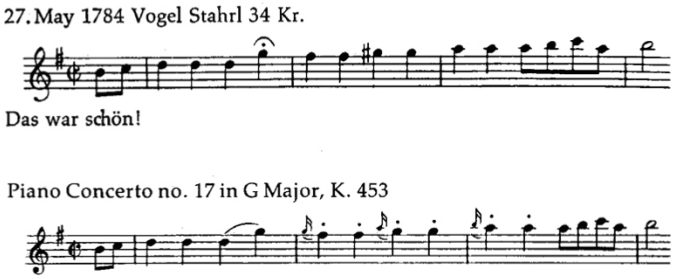

Figure 4. Mozart's starling
From "Mozart's Starling" American Scientist (March - April 1990)

European starling's imitation of an excerpt from a contemporaty concerto composed by Mozart. "Das war schön!" = "That was beautiful!"

Diversity of Birdsong

Song behavior has been adapted in a multitude of ways in different songbird species depending on the nature of their environmental demands (Brenowitz and Beecher, 2005). This natural diversity has been taken advantage of (and perhaps should be taken advantage of more), allowing researchers to match the scientific question being addressed with the most appropriately suited species. A quick internet search shows that the majority of research in songbirds over the past 50 years has been focused on 3 model species – the zebra finch (*Taeniopygia guttata*), (the model organism on which the present work is based), the white crowned sparrow (*Zonotrichia leucophrys*)

and the canary (*Serinus canaria*). Although song function and development in these three example species demonstrates significant variation and each one confers its own research advantages, they represent only a small taste of the incredible diversity present in this bird order. The following is an incomplete list introducing some of this natural variability.

The complexity and structure of song, as well as the volume of song that a bird can produce are all highly variable across species. The full complement of *songs* produced by a given bird is called its *repertoire.* A single *song* can be divided into repeatable units called *motifs*, which are in turn composed of *syllables*, flanked by short-duration gaps in sound production. Finally, *syllables* are composed of one or more spectral components, often called *notes*. Some songbirds, like the brown thrasher, are estimated to have repertoires in excess of 1000 songs (Kroodsma and Parker, 1977; Boughey and Thompson, 1981), while others, like the zebra finch, produce just a single song composed of a handful of syllables in their lifetime. Flexibility is another variable: sedge wrens can invent completely new songs (Kroodsma et al., 1999), but even the more commonplace Bengalese finch performs some improvisation by transitioning between the syllables that it has acquired with different probabilities (Okanoya, 2004). While they can also improvise, nightingales are famed for their ability to learn long concatenated strings of songs, and be able to repeat them back in original order with impressive accuracy (Hultsch and Todt, 1989; Hultsch, 1992).

Only males sing in the vast majority of songbird species, but sexual differences exist on a spectrum. There are particular species and situations in which female birds sing, in some cases even engaging in song duets with males (Langmore, 1998). Adult male canaries have a much more extensive song repertoire than females.

However, female canary song volume increases after systemic administration of synthetic testosterone (Nottebohm, 1980). In zebra finches injection of testosterone alone does not increase song production capacity in females (Wade and Arnold, 2004). Accordingly, the song controlling parts of the brain are largely non-existent, and markedly less pronounced that they are in female canaries (Nottebohm and Arnold, 1976).

The song learning process itself also demonstrates species specificity. Hearing song is an essential component of song learning, as is the case for speech learning in humans (Doupe and Kuhl, 1999). In fact, deafening birds before singing results in abnormal learning (Konishi, 1965; Brainard and Doupe, 2000a). While song is undoubtedly both a sensory and motor process for all songbirds, when the sensory period of song acquisition occurs, whether it has a critical window, and how much overlap there is between the sensory and motor phases of song learning are all features that vary between species (Brainard and Doupe, 2000a). Zebra finches start vocalizing during their sensory window (Böhner, 1990), while white-crowned sparrows must maintain a memory of their tutor's song for weeks or months before having the opportunity to practice it (Marler, 1970). For canaries, known as open-ended learners due to their continued ability to learn new songs, this critical window may never end (Marler and Waser, 1977; Nottebohm et al., 1986). Mozart's pet, the European starling, is another example of an open-ended learner, but unlike canaries, for which learning persists but is always seasonally dependent, starlings can learn new songs continuously (Böhner, 1990; Chaiken et al., 1994).

Many bird species undergo seasonally dependent changes in their hormonal circulation, morphology, and singing behavior (Tramontin and Brenowitz, 2000). Transitions between breeding and non-breeding seasons are associated with changing levels of neurogenesis; in fact the birth of new neurons in the adult brain was first

reported in songbirds (Goldman and Nottebohm, 1983). Such seasonally dependent species provide a framework for exploring the minimal circuitry requirements for maintaining a motor behavior, as well as how new neurons are incorporated into pre-existing motor circuits. Finally, songbird diversity is continually evolving, often on surprisingly short time scales. In zebra finches, for example, it may also be possible to selectively direct changes in song in the laboratory over multiple generations (Fehér et al., 2009).

Song Learning in the Zebra Finch

Zebra finches first emerged as the standard laboratory songbird species due to several behavioral advantages, but now also due to the momentum gained in understanding their detailed neurophysiological and anatomical makeup (Brenowitz and Zakon, 2015). Native to Australia, these social birds are known as opportunistic breeders for their ability to maintain a state of continuous song production and interest in breeding throughout the year. Zebra finch males learn one simple song in their lifetime. Although somewhat dull-sounding relative to other songbirds, the simplicity of their song is a desirable feature for interpretation of laboratory perturbations. The song consists of a single repeating motif, approximately one second in length, that contains a handful of syllables (Zann, 1996). Only male zebra finches learn to produce song, but birds of both sexes engage in calls, vocalizations that are neither learned nor are dependent on the neural circuitry essential for song (Simpson and Vicario, 1990).

Zebra finches learn their songs in a trial-and-error fashion within the first three months of life (Tchernichovski et al., 2001). The sensitive period for song exposure in zebra finches begins in the nest and typically ends at about 60 days after hatching, although it can be extended artificially in the laboratory by depriving the bird of tutor song exposure until later in development, at approximately 45 days. Initial learning following such restricted access to

tutor song occurs rapidly, making this a useful paradigm for dissociating the effects of development and experience on song learning mechanisms (Tchernichovski et al., 2001; Derégnaucourt et al., 2005). The young zebra finch begins producing early vocalizations, known as subsong, shortly after fledging, at about 25 days. Subsong is characterized by a high degree of spectral and temporal variability, and has been likened to babbling in human infants (Doupe and Kuhl, 1999; Aronov et al., 2008). Over the following two months the bird shapes his vocalizations until he reliably produces a song that is similar in spectral and temporal structure to that of the tutor song. This process occurs via trial-and-error, requiring thousands of song repetitions (Tchernichovski et al., 2001). By approximately 90 days after hatching, the song is "crystallized" or highly stereotyped between renditions. However, residual variability exists even in the mature finch, and can be brought to light by manipulations that perturb auditory feedback either permanently by deafening (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Brainard and Doupe, 2000b), or temporarily by distorting its frequency or duration (Tumer and Brainard, 2007; Sakata and Brainard, 2008; Andalman and Fee, 2009; Ali et al., 2013), or by changes in social context (Sossinka and Böhner, 1980).

Organization of the Songbird Brain

What is the neural basis of song behavior, and does it translate to known motor mechanisms in mammals? Mammals and birds evolved from a common reptilian ancestor; consequently much of the avian brain is homologous to that of mammals, with one potential exception (Jarvis et al., 2005). Homology in the brain is typically inferred from a number of features including comparative location, embryological origin, afferent and efferent connections, and expression of neurotransmitters and developmental transcription factors. In birds, much of the circuitry essential for motor, sensory and cognitive processing resides in the pallium (Reiner et al.,

2005). Although pallium is functionally analogous to mammalian cortex, the two are not equivalent. Cells are organized within nuclei in the pallium, unlike the familiar laminar organization of cortex. And while some of the cell types in the pallium are homologous to those in mammals, there is not a perfect match (Butler et al., 2011). For example, there are no pyramidal cells in the avian pallium. Despite this potential shortcoming, evidence to date suggests that the similarities between avian and mammalian brains far outnumber the differences, and that functional homologies exist even when anatomical ones are not apparent. For clarity from this point on I will refer to avian pallial nuclei as "cortex-like".

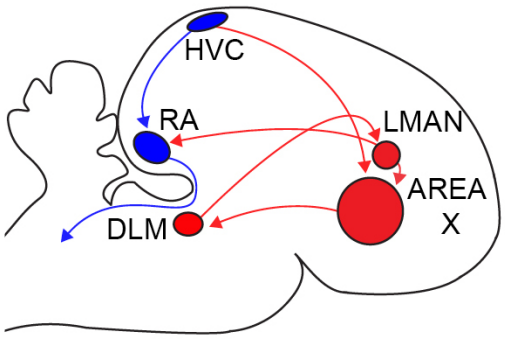

Figure 5. Schematic side view of the songbird brain

Direct Motor Pathway (DMP) in blue
Anterior Forebrain Pathway (AFP) in red

Two neural circuits mediate song production and learning (Nottebohm et al., 1976). These were first described in canaries, and while this network of structures has been corroborated in all songbird species studied to date, the majority of subsequent anatomical and physiological descriptions have been focused on zebra finches. Strikingly, not only do the song nuclei occupy a disproportionately large portion of the songbird brain, but they also stand out in unstained tissue, even to the untrained eye. Both song circuits stem from the premotor cortex-like nucleus HVC, once called the Hyperstriatum Ventrale, pars caudale then the "High Vocal Center" due to its central role

in the uncharted territory of the songbird brain, but now used as a proper name. HVC bridges auditory and motor song information, receiving input from neighboring auditory regions, as well as thalamic nucleus Uvaeformis (Uva) (Nottebohm et al., 1982; Wild, 1994).

Song Production through the Direct Motor Pathway

HVC neurons projecting to the robust nucleus of the arcopallium (RA), a region likened to layer 5 of motor cortex (Karten, 1991), form the first leg of the Direct Motor Pathway (DMP) (Fig. 5, blue), a circuit essential for the production of normal adult song (Nottebohm et al., 1976). RA neurons, in turn, project directly to a series of interconnected brainstem nuclei: the tracheosyringeal branch of the hypoglossal nucleus (nXIIts), which controls muscle contractions in the syrinx (the vocal organ of the bird), and respiratory control nuclei retroambigualis (RAm) and parambigualis (PAm), which are active during breath expiration and inspiration, respectively (Wild, 1993). RA also projects to a midbrain vocal control nucleus, the dorsomedial nucleus of the intercollicular complex (DM), involved in the production of unlearned vocalizations (Vicario and Simpson, 1995) and to the medial portion of the dorsolateral thalamus (DLM) (Wild, 1993; Vates et al., 1997; Goldberg and Fee, 2012). The significance of this latter projection will be addressed at a later time. Notably, RA and its projections to the syringeal muscles themselves are organized topographically in a loosely muscle-oriented fashion (Vicario and Nottebohm, 1988; Vicario, 1991).

How is song encoded in this motor circuit? Behaviorally, lesions of either HVC or RA eliminate the production of normal song (Nottebohm et al., 1976), while electrical stimulation of either causes the production of vocalizations (Vicario and Simpson, 1995). At a neural level, however, HVC and RA show distinct song-related activity. Action

potential bursts in a single HVC neuron appear to be uniquely associated with a precise time in the song (Hahnloser et al., 2002). RA neurons are activated in stereotyped burst patterns during the production of specific notes, or spectral components of the song (Yu and Margoliash, 1996). The prevailing model of this motor production circuit posits that HVC serves as a sort of clock, with specific HVC neurons switching on at each moment in song, coordinating ensembles of RA neurons that then lead to the production of spectral features at that time by activating the appropriate complement of syringeal muscles (Vu et al., 1994; Leonardo and Fee, 2005). In support of this idea, implanting a cooling device under the bird's skull directly over HVC slows the production of song, while cooling RA has no effect on song tempo (Long and Fee, 2008). It has been suggested that HVC neurons activate each other sequentially via a disynaptic inhibitory interneuron mechanism in a "synaptic chain" during the production of song (Kosche et al., 2015) (but see Long et al., 2010). HVC and RA neurons repeat these same firing patterns when the bird is sleeping, with interesting implications for the role of sleep in consolidation of motor memory (Dave and Margoliash, 2000; Hahnloser et al., 2006).

Song Learning through the Anterior Forebrain Pathway

A separate population of cells in HVC gives rise to a distinct neural circuit known as the Anterior Forebrain Pathway (AFP), which converges on RA indirectly by way of the basal ganglia (Fig. 5, red). The first stop in the AFP is area X, a structure homologous to the mammalian striatum (Farries and Perkel, 2002; Carrillo and Doupe, 2004; Farries et al., 2005; Reiner et al., 2004). As expected for a striatal nucleus, area X receives dense projections from midbrain dopaminergic neurons (Lewis et al., 1981; Bottjer, 1993). The next leg of the AFP circuit terminates in the thalamic nucleus DLM. Most neurons in DLM express similar properties to thalamocortical projection neurons in mammals (Luo and Perkel, 2002). Accordingly, DLM projects to the cortex-like lateral magnocellular

nucleus of the anterior nidopallium (LMAN), in this way completing a cortical - basal ganglia – thalamic – cortical loop. LMAN neurons project to RA, merging the AFP back with the DMP. Collateral branches of these axons also send feedback to area X (Vates and Nottebohm, 1995). Topography is preserved in all parts of the AFP loop downstream of HVC (Johnson et al., 1995; Vates and Nottebohm, 1995; Luo et al., 2001), and is not dependent on vocal learning (Iyengar et al., 1999). RA neurons thus receive topographically relevant indirect input from the AFP, with an estimated delay of 40 - 50 ms compared to the direct HVC input (Kimpo et al., 2003).

Many studies over the past three decades have contributed to the unified interpretation that this circuitous connection between HVC and RA is a basal ganglia motor loop that underlies song learning in the juvenile bird and protracted song plasticity in the adult (Doupe et al., 2005). Bilateral lesions of area X, DLM or LMAN in juvenile birds all lead to pronounced song learning deficits (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Goldberg and Fee, 2011). Lesions of LMAN or area X also prevent the degradation of song that normally follows deafening (Brainard and Doupe, 2000a; Brainard and Doupe, 2000b; Nordeen and Nordeen, 2010). Deafening-induced changes to song are thus not simply passive. Instead, erroneous auditory feedback actively drives changes in the motor program, and this process requires the AFP.

Clearly, the AFP is essential for song learning and plasticity – how does neural activity at the different stages of this circuit support these observations? As described above, RA-projecting HVC neurons fire action potentials once during the song motif (Hahnloser et al., 2002). In contrast, area X-projecting HVC neurons demonstrate patterned activity during song, but they are reported to fire a few times during the motif, suggesting that they are not performing the same clock function there (Kozhevnikov and Fee, 2007). Neurons in all of the AFP nuclei in

anaesthetized adult birds are responsive to auditory stimuli, and are particularly tuned to playback of the bird's own song or the tutor's song over the songs of other conspecific individuals (Doupe and Konishi, 1991; Solis and Doupe, 1999). This selectivity is not homogeneous throughout the circuit, as neurons in area X are more broadly tuned than those in LMAN (Doupe and Solis, 1997). While auditory responses in LMAN and area X can be detected in juvenile birds as young as 30 days, selectivity for the bird's own song is refined over the course of development and song learning (Solis and Doupe, 1997). Importantly, LMAN and area X neurons are not only responsive to song, but also exhibit patterned firing during its production. This activity cannot be due entirely to auditory feedback as it persists in deafened birds, and in many cases can be detected prior to vocalization (Hessler and Doupe, 1999). Neurons in DLM, the intermediate nucleus of the circuit, also fire action potentials immediately preceding song production (Goldberg et al., 2012; Goldberg et al., 2013).

Hessler and Doupe also observed that firing patterns in the AFP were not as stereotyped between song renditions as had previously been reported in HVC and RA. In fact, RA neuron activity is initially highly variable in the juvenile bird, with the characteristic sparse bursting described by Yu and Margoliash emerging over the course of development (Ölveczky et al., 2011). Anatomically, the inputs from LMAN arise as early in development as 15 days post hatch, while HVC inputs lag behind for another couple of weeks (Mooney, 1992). These findings led to the overall hypothesis that the AFP drives variable song by injecting noisy LMAN output into RA, muddling the more stereotyped program encoded by the motor pathway. In support of this, pharmacological inactivation of LMAN increases song stereotypy (Ölveczky et al., 2005), while removal of HVC input by surgically transecting the axon tract between HVC and RA leads to juvenile-like firing patterns in RA neurons (Aronov et al., 2008).

How do these contrasting inputs combine at the level of RA? During song learning, projections from the AFP and the DMP synapse on the same RA neurons, but differ in their postsynaptic receptor composition. Synapses made by LMAN terminals are largely blocked by NMDA receptor antagonists (Mooney and Konishi, 1991), while HVC fibers form mixed AMPA/NMDA receptor synapses (Stark and Perkel, 1999). The glutamate receptor specificity at the convergence point of the two pathways lends itself to the possibility that long-lasting changes during song learning occur in RA neurons. As a matter of fact, there is evidence for song experience-dependent synaptic plasticity and reorganization in RA (Sizemore and Perkel, 2011; Garst-Orozco et al., 2014).

In summary, AFP neurons 1) receive a corollary discharge signal that is related to the production of the current song; 2) can discriminate the bird's song (or the song that he is striving to produce) from other auditory stimuli; 3) are more variable in their firing than the DMP; 4) have the potential to cause long lasting changes in RA. Finally, through area X, the circuit is accessible for modification by dopaminergic neurons, which in mammals have been implicated in supplying signals that can reinforce successful behaviors while weakening connections that resulted in the production of undesirable ones (Pignatelli and Bonci, 2015). It is not difficult to see the appeal of this system to neuroscientists and theoreticians studying reinforcement learning.

The Anterior Forebrain Pathway and Reinforcement Learning

Doya and Sejnowski were among the first to draw parallels between the AFP and a reinforcement learning system much like the adaptive critic model formulated by Sutton and Barto. They proposed that LMAN could serve as the *search* element, whose function is to introduce variability into the motor commands, and area X could be the *critic*, able to reinforce trials in a graded fashion (Doya and Sejnowski, 1995). Using this architecture and

drawing on additional anatomical and physiological constraints, they were able to build a simple neural network model that learned to imitate birdsong syllables within several hundred trials. Subsequent models have expanded up on this general reinforcement-based framework, while others have explored other aspects of the song circuit, including motor encoding, synaptic plasticity in RA, auditory encoding of the song, and the physics of the syrinx (Troyer and Doupe, 2000a; Troyer and Doupe, 2000b; Mindlin and Laje, 2006; Fee and Goldberg, 2011; Fiete et al., 2007; Fiete and Seung, 2008; Giret et al., 2014). The reinforcement learning framework continues to guide experimental predictions in the AFP. In particular, there are strong candidate sites for the *search* element in the songbird, with mappable neural and behavioral correlates.

Variability in the Anterior Forebrain Pathway

There is ample evidence in support of the idea that the AFP is the source of a variability signal that could be used by the songbird for the exploratory component of trial and error learning. However, it is not completely evident where this signal originally arises, versus where it may be tuned to guide vocal learning. Neither is it known what intrinsic and circuit level mechanisms underlie the expansion or constriction of variability on a moment-to-moment basis in response to song evaluation or changes in the animal's context. The majority of work has focused on the role of the AFP output nucleus LMAN, but neural activity here could be inherited from further upstream in the pathway. Matters are further complicated by additional projections to and within the AFP, such as feedback from LMAN to area X, direct input from RA to DLM, and other recurrent connections (Wild, 1993; Vates et al., 1997; Goldberg and Fee, 2012; Hamaguchi and Mooney, 2012).

Lesion Studies

While it is clear that bilateral lesions of any component of the AFP in juvenile birds abolish learning, a closer examination of the defects resulting from region-specific lesions is rather puzzling. LMAN lesions in juvenile birds lead to a loss of song variability and crystallization of song in its immature state (Scharff and Nottebohm, 1991). In adult birds, LMAN lesions result in a long-term reduction of both neural and behavioral variability (Ölveczky et al., 2005; Kao and Brainard, 2006; Ali et al., 2013). DLM lesions in juvenile birds similarly decrease song variability (Goldberg and Fee, 2011). In contrast, area X lesions during early song development interfere with the song learning process, but rather than locking the song in prematurely like LMAN lesions they lead to protracted song variability over the lifetime of the animal (Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Goldberg and Fee, 2011). Area X lesions in adults only cause a transient loss of spectral variability in song (lasting a few days) (Kojima et al., 2013; Ali et al., 2013), but do prevent song degradation that normally occurs after deafening, as well as any future feedback-driven changes to song production (Nordeen and Nordeen, 2010; Ali et al., 2013).

The combined effects of AFP lesions seem to paint a picture in which LMAN is essential for the generation of variability in song, while area X guides performance-based changes in the song, playing only an auxiliary role in variability production. One possibility is that area X structures LMAN variability, perhaps by controlling the gain of topographically organized ensembles of LMAN neurons in a temporally precise manner during song production. However, there are potential confounds associated with brain lesions, especially in interconnected circuits like the AFP. Lesions can affect other parts of the circuit, whose activity is dependent on the lesioned area, and they can cause compensatory changes through homeostatic mechanisms. In fact, Kojima and Doupe found

that area X lesions in adults alter activity in LMAN. Specifically, while LMAN neurons continue to fire action potentials preceding song production at a similar frequency, their normal pattern of firing (high frequency bursts), is permanently disrupted by removing area X from the circuit (Kojima et al., 2013). It is possible that residual neural variability in LMAN is artifactual, arising from a now miswired circuit. An alternative hypothesis is thus that area X generates variability in the circuit, but in its absence a compensatory mechanism in LMAN promotes neural variability. Mapping the precise relationship between neural variability in the basal ganglia and behavioral (song) variability will be pivotal for unraveling this complicated story in the future.

Social Context Studies

One additional paradigm that has contributed to teasing apart the details of this circuit in zebra finches takes advantage of the moment-to-moment changes in song that can occur during transitions in social context. Although zebra finch song is fairly stereotyped in its mature form, it does express residual variability that is dampened in the presence of a female bird (Sossinka and Böhner, 1980). Functionally, the exploratory 'undirected' state could allow continuous improvement and adjustment of the song, while the exploitative 'directed' state conservatively replays the current best motor program. This effect is assessed by measuring the variability surrounding the fundamental frequency of a harmonic syllable in the song (Fig. 6) (Kao and Brainard, 2006). Although the absolute change in this variability is small (the coefficient of variation of fundamental frequency roughly halves from 2% to 1%), and imperceptible to the typical human listener, this finding is highly robust across experiments and laboratories. More importantly, it is behaviorally relevant to the birds: not only can female zebra finches discriminate between directed and undirected songs, but they show preference for directed song (Woolley and Doupe, 2008).

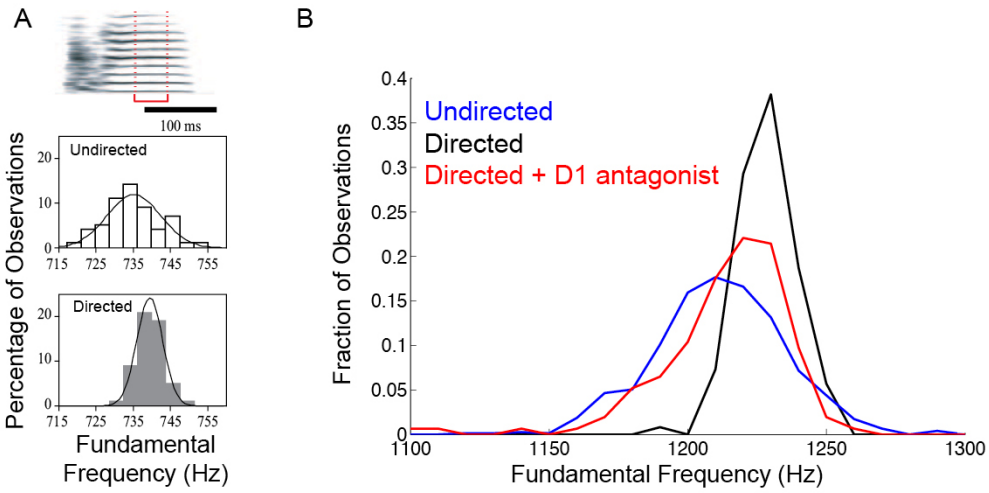

Figure 6. Social context dependent plasticity of song

A. Modified from Kao and Brainard, 2006 - Spectral variability decreases during directed song.
B. Modified from Leblois et al, 2010 - D1 receptor antagonist prevents decrease in song variability.

Social context dependent plasticity requires the AFP. Immediate early gene expression, a proxy for increases in neural activity, is upregulated in area X during the production of undirected song compared to directed song (Jarvis et al., 1998). LMAN neurons switch from bursty to regular firing in directed song (Kao et al., 2008), and both reversible and permanent lesions of LMAN prevent the change in behavioral variability (Kao and Brainard, 2006; Stepanek and Doupe, 2010). While LMAN neurons demonstrate social context-dependent changes, and are essential for ultimately expressing the behavior, dopaminergic modulation in area X appears to be the real trigger. Extracellular dopamine levels in area X are elevated during the production of directed song, and the behavioral transition is dependent on both the dopamine transporter (DAT) and D1 dopamine receptor activity in area X (Sasaki et al., 2006; Leblois et al., 2010). Finally, *in vivo* neural recordings in area X during the production of song demonstrate that the trial-to-trial variability of area X projection neuron firing decreases during directed song

(Woolley et al., 2014). This change in neural variability emerges within area X – it is not acquired from HVC input or from feedback connections with LMAN.

Dopamine in the Song System

Dopamine release in area X may trigger changes in neural activity that ultimately lead to the production of less variable song. Dopamine has also been posited as a key component in reinforcement learning. Could dopamine facilitate song learning in the juvenile bird by structuring the variability of future motor commands based on evaluations of song performance? There is some evidence that dopamine signaling is involved in song learning (Kubikova and Kostál, 2010). Immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, and dopamine turnover rates alike, increase steadily over the course of the sensorimotor phase of song development (Soha et al., 1996; Harding et al., 1998). Additionally, chronic systemic infusion of D1 and D2 receptor antagonists in juvenile birds permanently affects singing and courtship behavior (Harding, 2004). While dopaminergic projections from the midbrain terminate all over the songbird brain, including the cortex-like song nuclei HVC and RA (Appeltants et al., 2000), a large part of these effects is likely to be mediated by the dense projection to area X (Lewis et al., 1981; Bottjer, 1993). The VTA is the source of much of this dopaminergic input, but SNc contributes as well, consistent with song being both a sensorimotor behavior and a salient social communication signal.

Dopaminergic neurons are well placed to affect song behavior. Songbird VTA is homologous to mammals, containing the same cell types (Gale and Perkel, 2006). The dynamics of dopamine release from midbrain fibers and its reuptake in area X resemble those of the mammalian basal ganglia as well, although there is evidence for

unexpected monoamine transporter cross-talk (Gale and Perkel, 2005). VTA receives feedback from the striatum in addition to its outgoing projections there. Axon collaterals of area X's output projections to DLM synapse in the ventral pallidum, which in turn projects to the VTA. This connection forms an anatomical loop between VTA and the song system, and confers selective auditory responses to playback of the bird's own song in VTA neurons (Gale et al., 2008; Gale and Perkel, 2010). VTA neurons also receive input from a cortex-like region (Aiv), and it is hypothesized that this projection may involved in the evaluation of auditory feedback for reinforcement learning (Person et al., 2008; Mandelblat-Cerf et al., 2014). Dopaminergic neurons thus receive song-specific auditory information and are in a position to alter AFP output. However, while dopamine can be correlated with certain aspects of song learning, its involvement in social context-dependent learning is more straightforward – the decrease in song variability occurring with directed song can be eliminated by infusion of a D1 receptor antagonist into area X (Leblois et al., 2010).

Relationship Between Variability in Social Context and Juvenile Learning

Importantly, directed song can also be elicited in juveniles, revealing surprisingly improved song copies from immature birds (Kojima and Doupe, 2011). The modulation of variability observed during this state transition is thus not simply a peculiarity of adult birds, but may represent a window onto hidden variability-generating mechanisms that are used throughout the learning process, and persist into adulthood. Determining the cellular and circuit level effects that dopaminergic modulation has on the basal ganglia during social context-dependent plasticity is thus likely to inform our understanding of dopamine's role in juvenile learning.

The Area X Files: Unraveling a Mysterious Basal Ganglia Nucleus

In order to understand how dopamine modulates the songbird basal ganglia, we must take a step back and describe the cytochemical landscape on which it is exerting its action. There are many similarities between the basal ganglia system in mammalian and non-mammalian vertebrates (Smeets et al., 2000). Area X is a component of the larger basal ganglia circuitry present in all birds (Karten and Dubbeldam, 1973). The broader avian basal ganglia include a lateral striatum, medial striatum and pallidum (Reiner et al., 2004), as well as an STN homolog (Jiao et al., 2000), although in songbirds the STN is not reciprocally connected with the pallidum, nor is there evidence for a projection to area X (Person et al., 2008).

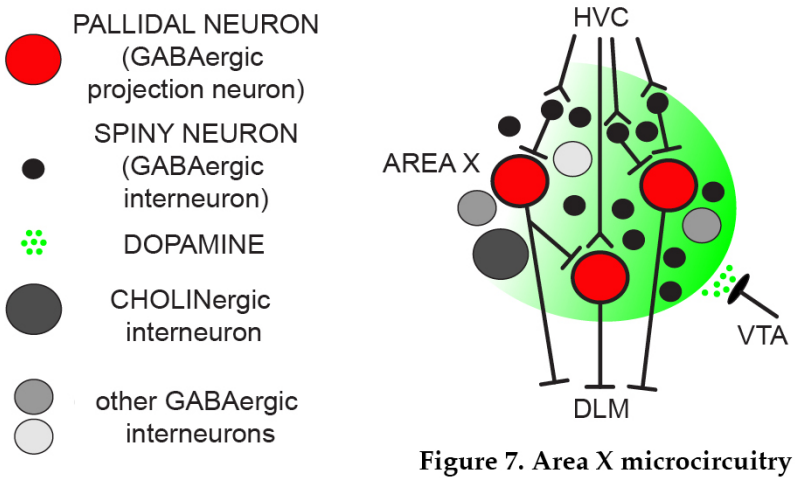

Figure 7. Area X microcircuitry

Area X is Striatal

Area X is homologous to mammalian striatum and is largely composed of the same complex assortment of cell types (Fig. 7) (Farries and Perkel, 2002; Reiner et al., 2004; Carrillo and Doupe, 2004; Person et al., 2008; Goldberg

et al., 2010; Goldberg and Fee, 2010). The vast majority of these are small with spiny dendritic arbors, and express GABA along with the neuropeptides enkephalin or substance P.

The intrinsic features of area X spiny neurons include very negative resting potentials, lack of spontaneous firing, ramping to depolarizing pulses, and Cs+ dependent inward rectification in response to hyperpolarization, all of which are consistent with the mammalian literature regarding MSNs (Farries and Perkel, 2002). Spiny neurons receive excitatory synaptic input from the cortex-like regions HVC and LMAN, and inhibitory input from local interneurons (Farries et al., 2005). However, unlike in the mammalian striatum, spiny neurons make only local connections in area X (Reiner et al., 2004; Person et al., 2008). During song production spiny neurons fire action potentials sparsely (Goldberg and Fee, 2010). Dopamine has modulatory effects on the excitability and plasticity of these neurons akin to those reported in mammals: D1 receptor activation increases their excitability, and vice versa for D2 receptor activation (Ding and Perkel, 2002). Dopamine also mediates presynaptic depression of HVC inputs, and long-term potentiation at spiny neuron synapses (Ding et al., 2003; Ding and Perkel, 2004).

A second, sparse population of large neurons expresses choline acetyltransferase (ChAT) (Zuschratter and Scheich, 1990). These neurons do not project out of the nucleus (Carrillo and Doupe, 2004). In the *in vitro* preparation these cells fire action potentials at very low rates, on the order of 1 Hz, and exhibit long-lasting action potential after-hyperpolarizations (Farries and Perkel, 2002). Their activity *in vivo* resembles that of the striatal tonically active cholinergic interneuron of the mammalian striatum (Goldberg and Fee, 2010). Finally, two other classes of GABAergic interneurons have been identified in area X, displaying electrophysiological and anatomical characteristics that are a good match with fast-spiking cell (FS) and the low-threshold spiking (LTS). FS neurons are characterized by their narrow spike waveforms, and while they show no spontaneous firing *in*

vitro, burst at high frequencies during song production. LTS cells have broad action potential waveforms and remain relatively depolarized at rest, which can result in spontaneous bursts of firing *in vitro.* During song production these bursts are long, with reported frequencies in excess of 1 kHz (Goldberg and Fee, 2010). It is likely that FS and LTS neurons express parvalbumin and nitric oxide synthase, respectively, as both markers can be detected in area X (Wallhäusser-Franke et al., 1995; Reiner et al., 2004). The synaptic inputs to these latter three interneuron types have not been explicitly studied, but the safest assumption is that they are all highly interconnected and receive inputs from HVC and LMAN. It is also not known whether these cells express dopamine receptors, or how dopamine could affect their firing properties directly or indirectly.

Area X is Pallidal

Electrophysiological characterization of area X neurons *in vitro* also revealed the presence of a cell type with no counterpart in the mammalian striatum. These neurons fire highly regular action potentials spontaneously at approximately 20 Hz in room temperature recordings (and closer to 40 Hz at 30°C, unpublished data), and can sustain firing at frequencies over 100 Hz in response to depolarizing current injections (Farries and Perkel, 2002). Their firing properties and other electrophysiological features, namely hyperpolarization-induced sag and rebound activity, resemble those of globus pallidus neurons more so than those of any mammalian striatal cell type. These pallidal-like cells were positively identified as the only projection neurons from area X to the thalamus (Carrillo and Doupe, 2004; Reiner et al., 2004; Farries et al., 2005; Person et al., 2008). They express glutamic acid decarboxylase (GAD), an essential enzyme in the GABA synthesis pathway, and form strong, monosynaptic inhibitory synapses onto DLM neurons (Luo and Perkel, 1999a; Luo and Perkel, 1999b). Oddly, many of them also express the neuropeptide enkephalin (Carrillo and Doupe, 2004). In mammals it is neurons

from the globus pallidus that form inhibitory synapses in the thalamus, suggesting that area X in fact contained *both* striatal and pallidal components (Carrillo and Doupe, 2004; Reiner et al., 2004; Person et al., 2008). In further support of this, area X projection neuron firing *in vivo* resembles that seen in the primate GPi (Goldberg et al., 2010).

Variability of Area X Pallidal Neuron Firing Can Affect Downstream Signaling

Pallidal neuron firing statistics are an important determinant of DLM activity. Pallidal terminals form large, calyx-like synapses in DLM (Luo and Perkel, 2002), and stimulation of these afferents to DLM causes strong, reliable inhibitory postsynaptic events (Luo and Perkel, 1999a). Silencing area X with GABA increases DLM spontaneous firing rate (Kojima and Doupe, 2009), while glutamate receptor blockade directly in DLM decreases firing rate (Leblois et al., 2009). How does pallidal neuron activity contribute to signal transmission in DLM? There are at least two non-mutually exclusive ways in which DLM neurons are thought to fire action potentials (Kojima and Doupe, 2009; Goldberg et al., 2013). DLM neurons demonstrate *both* inhibitory and excitatory postsynaptic responses (Luo and Perkel, 2002). In fact, in addition to its primary outputs to the brainstem, RA sends a projection to DLM (Wild, 1993; Vates et al., 1997), and there is some evidence that this input can drive DLM neurons during singing (Goldberg and Fee, 2012). One proposition is that pauses in pallidal neuron firing disinhibit DLM neurons, allowing them to fire to other inputs, such as those from RA (Goldberg et al., 2012). However, there is also evidence that DLM neurons can fire action potentials without excitatory input, via a post-inhibitory rebound mechanism that is highly dependent on variability and pauses in pallidal firing (Person and Perkel, 2005). DLM neurons exhibit such post-inhibitory rebound firing with an approximate delay of 50 ms (Luo and Perkel, 1999a).

Some Pallidal Neurons Make Local Connections

While most recorded pallidal neurons make *direct* projections to DLM, a subset make local connections within area X in what appears to be a circuit analogous to the mammalian *indirect* pathway, despite lacking the intermediary STN (Carrillo and Doupe, 2004; Farries et al., 2005; Goldberg et al., 2010; Pidoux et al., 2015). These two classes of pallidal neurons exhibit different activity profiles during the production of song in the awake, behaving bird. Specifically, GPe-like neurons fire in bursts, while GPi-like neurons, which are confirmed projection neurons, fire continuously (Goldberg et al., 2010). Although there is some evidence that these classes differentially express the neuropeptide enkephalin and the pallidal postembryonic marker Nkx2.1 (Carrillo and Doupe, 2004), at this time there is no surefire way of distinguishing pallidal interneurons in the *in vitro* preparation, and so it is not clear how they could be affecting area X network dynamics. It is also unknown what role dopamine may be playing in this putative direct/indirect analog. This single-nucleus packaging of area X presents a significant deviation from typical basal ganglia organization. Nonetheless, there is much evidence in support of the idea that the computations inherent to this striato-pallidal nucleus are analogous to those in mammalian basal ganglia (Farries et al., 2005; Goldberg et al., 2010; Goldberg and Fee, 2010).

Pallidal Neuron Firing Variability is Modulated by Social Context and Dopamine

Spiny neurons are thought to receive much of the cortex-like input to area X, but ultimately it is activity in the pallidal neurons that can enact downstream changes in AFP signaling. Pallidal projection neurons receive direct excitatory input from HVC and LMAN fibers, and polysynaptic inhibitory inputs through the spiny neurons and possibly other interneuron types (Farries et al., 2005; Leblois et al., 2010). *In vivo* their firing rate increases in response to playback of the bird's own song (Leblois et al., 2010). Dopamine inhibits the strength of this neural

response while decreasing the variability in neural firing (Leblois et al., 2010). D1 receptors are also implicated in mediating the social context-dependent constriction of song variability (Leblois et al., 2010). Accordingly, i*n vitro* recordings show that D1 receptor activation decreases the variability of pallidal neuron firing, simultaneously increasing their excitability (Leblois et al., 2009; Leblois et al., 2010).

The noisiness that is normally present in pallidal neurons during undirected song may be an important determinant of DLM neuron firing mechanisms (Person and Perkel, 2005). Dynamic changes in pallidal neuron variability state could therefore profoundly affect downstream AFP signaling. What is the mechanism behind neural variability here, and how is it modified by D1 receptors? Woolley and Doupe (2014) recently showed that trial-to-trial neural variability in neither HVC inputs nor spiny neurons changes during directed song, a high-dopamine state. However, variability of pallidal spike timing and variability in the duration of pauses in pallidal firing alike decreased significantly. A mechanism underlying social context-dependent changes in neural variability (which may also guide exploration during juvenile learning) can thus be pinned down somewhere between the input (spiny) and output (pallidal) neuron layers in area X. The following chapters focus on *in vitro* electrophysiology, microscopy and theoretical approaches that we employed with the goal of understanding 1) what inputs pallidal neurons receive; 2) how these inputs collectively impact the intrinsic oscillatory activity of pallidal neurons, and 3) what circuit mechanisms could be contributing to dopamine-mediated changes in neural variability.

2. Dynamic Modulation of Basal Ganglia Output By a Novel Excitatory Cell Type

This work will be submitted for publication, with the following contributing authors:

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Abstract

Vocal learning and social context-dependent plasticity in songbirds depend on a forebrain circuit through the basal ganglia nucleus area X. While area X contains many cell types, its output neurons are the initial site of dopamine-mediated changes to trial-to-trial neural variability that occur when the bird engages in courtship song. Here we present evidence for a novel, tonically active, excitatory interneuron in area X that makes strong synaptic connections onto output neurons, often linked in time with inhibition. Dopamine receptor activity dynamically modulates the coupling of these excitatory and inhibitory events *in vitro*, as well as the trial to trial variability of a modeled of area X output neuron receiving excitatory and inhibitory inputs. The excitatory interneuron thus serves as one biophysical mechanism for the introduction of neural variability in this predominantly inhibitory nucleus.

Introduction

Trial-and-error learning is a common strategy for the acquisition of complex motor behaviors, and for their maintenance through changes such as growth, injury or aging. Theoretical models of motor learning include three stages: generation of random variations in the motor signal, a mechanism for evaluating the quality of the outcome, and a means to integrate more successful variations back into the original motor routine (Sutton and Barto, 1998). Strong candidate neural mechanisms exist for two of these theoretical components, namely the involvement of the dopaminergic system in reward prediction error (Schultz et al., 1997; Schultz, 1998), and the potential of synaptic plasticity mechanisms to modify circuits that generate motor output (Wickens et al., 2003). However, the cellular and circuit mechanisms that generate and modulate variability in motor commands remain unknown.

Birdsong, used for territory defense and mate selection, is learned in a trial-and-error fashion, and provides an invaluable model for elucidating how variability is generated and used to improve motor behavior (Woolley and Kao, 2014). Early song learning involves highly variable vocal "babbling" (Doupe and Kuhl, 1999; Aronov et al., 2008). This variability decreases over the course of learning, but residual variability persists in adults and can be altered rapidly by social context (Sossinka and Böhner, 1980). This ongoing variability could support song maintenance or adult learning (Tumer and Brainard, 2007; Sakata and Brainard, 2008; Andalman and Fee, 2009; Ali et al., 2013). Songbirds possess discrete forebrain circuitry in which the approximate functional roles of brain nuclei in song learning and production have been established (Fig. 8*A*) (Nottebohm et al., 1976). Thus, this

system provides a unique opportunity for determining the biophysical mechanisms underlying the acquisition and performance of a complex natural behavior.

The Anterior Forebrain Pathway (AFP) is a basal ganglia loop essential for song learning as well as for generating and regulating song variability in both juveniles and adults (Fig. 8*A*) (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Doupe et al., 2005; Kao et al., 2005; Aronov et al., 2008; Kojima and Doupe, 2011). Variability generated in the AFP is delivered to the motor pathway through its output area, the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN) (Olveczky et al., 2005; Kao and Brainard, 2006), but could be inherited from further upstream in the circuit. During courtship of a female, elevated dopamine within area X, a striato-pallidal nucleus at the start of the AFP (Reiner et al., 2004a; Carrillo and Doupe, 2004) reduces song variability (Sasaki et al., 2006). Area X transforms highly stereotyped inputs from premotor, cortex-like nucleus HVC (used as a proper name) (Reiner et al., 2004b) into variable firing of its output neurons in a fashion independent of feedback from LMAN (Woolley et al., 2014). While the production of variability is likely distributed throughout the AFP loop, area X appears to play a vital role.

How could variable firing arise in area X and how could dopamine modulate this variability? Area X spiny neurons faithfully report the stereotyped, time-locked inputs from HVC via inhibitory connections with pallidal neurons (Reiner et al., 2004a; Person et al., 2008; Woolley et al., 2014). These carry the output of area X to thalamic nucleus DLM (Carrillo and Doupe, 2004; Reiner et al., 2004a; Farries et al., 2005; Person et al., 2008), which in turn drives LMAN (Fig. 8*A*) (Boettiger and Doupe, 1998; Kojima and Doupe, 2009). There are additional inhibitory and cholinergic interneuron classes whose roles are unclear (Farries and Perkel, 2002; Goldberg and Fee, 2010).

Area X also receives dense dopaminergic inputs from the midbrain (Lewis et al., 1981; Bottjer, 1993), homologous to the mammalian pathway that conveys reward prediction error (Schultz et al., 1997; Schultz, 1998; Gerfen and Surmeier, 2011).

To examine the biophysical and circuit properties of area X, we used brain slices to dissect the area X microcircuitry, focusing on synaptic inputs to the pallidal output neurons. We report a novel, local source of glutamate, which shifts the tone of this basal ganglia nucleus from strictly inhibitory to mixed inhibitory-excitatory. This excitatory component of area X is not only well placed to contribute to variability in pallidal neuron firing, but also provides one mechanism for dopaminergic modulation. Finally, a simple, biologically-constrained model of local synaptic circuitry in area X can account for generation and modulation of variable activity in pallidal output neurons. Together our results suggest a plausible biophysical mechanism for the generation of trial-to-trial behavioral variability.

Results

Spontaneous synaptic events in area X pallidal neurons are both inhibitory and excitatory

We made whole-cell recordings from pallidal neurons in adult zebra finch brain slices in a preparation of area X isolated from its afferent nuclei in the pallium and midbrain (Lewis et al., 1981; Reiner et al., 2004a; Person et al., 2008). Spontaneous interspike interval (ISI) averaged 18.92 ± 10.16 ms and mean coefficient of variation (C.V.) of ISI was 0.22 ± 0.20 (n = 19, ± *SD*), similar to results with cell-attached recordings ($P \geq 0.35$, df = 130, Fig. 8*B-D*). Frequent disruptions in membrane potential between action potentials and skewed ISI histograms (Fig. 8*B-C*)

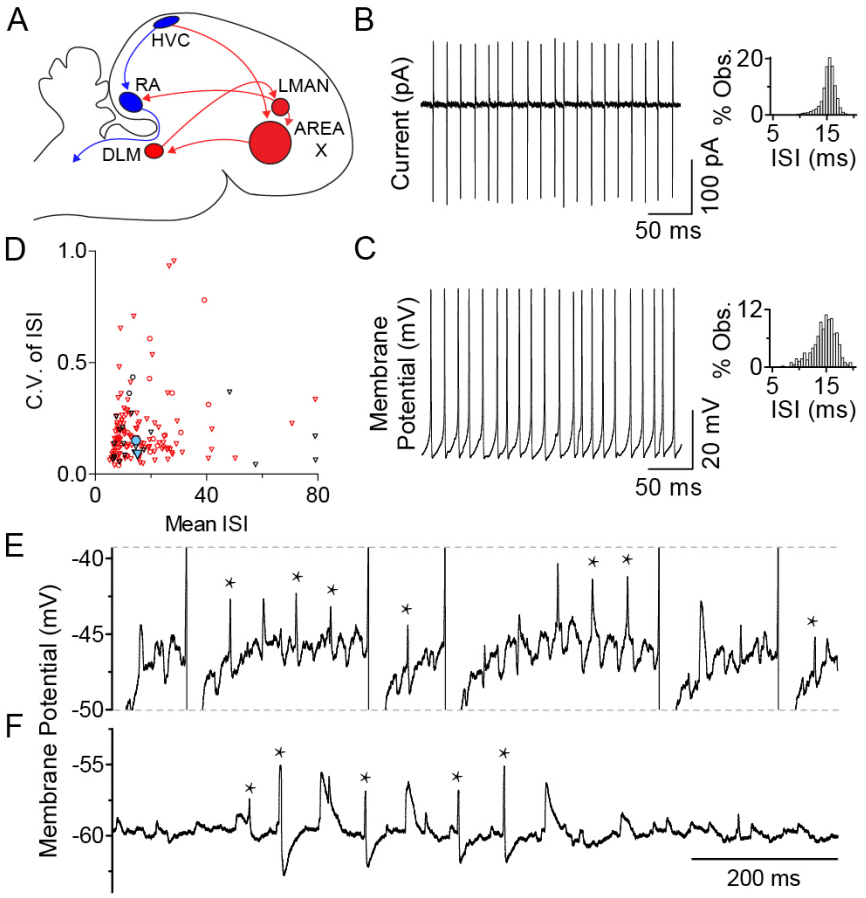

Figure 8. Pallidal neurons are spontaneously active and receive synaptic inputs

A. Parasagittal schematic diagram of the songbird brain. *B.* Example pallidal neuron recording in cell-attached configuration. Right, interspike interval (ISI) distribution. *C.* Example recording from a second pallidal neuron in current-clamp configuration with no current injection. Note sub-threshold membrane potential activity. Right, ISI distribution. *D.* ISI mean and C.V. for all pallidal neurons recorded; n = 113 cell-attached isolated (red triangles), 19 cell-attached intact (black triangles), 19 whole-cell isolated (red circles), 2 whole-cell intact (black circles), blue triangle = example from *B.* blue circle = example from *C. E, F.* Example recordings from two pallidal neurons in current-clamp configuration receiving continuous current injections of -200 pA (*E*) and -90 pA (*F*) to silence spontaneous firing. Spikes in *E* are truncated to emphasize subthreshold activity. Asterisks indicate coupled excitatory-inhibitory synaptic potentials.

hinted that synaptic potentials could distort the regularity of intrinsic pacemaking in pallidal neurons. We injected hyperpolarizing current to slow or stop spontaneous firing, revealing substantial spontaneous synaptic

activity (Fig. 8*E-F*). In all cases we saw both inhibitory and excitatory postsynaptic potentials (IPSPS/EPSPs), despite the absence in the preparation of cell bodies from regions known to send excitatory projections to area X. A large proportion of EPSPs were closely followed by IPSPs (Fig. 8*E-F*, asterisks). We shifted to voltage-clamp recordings to isolate the conductances underlying synaptic events.

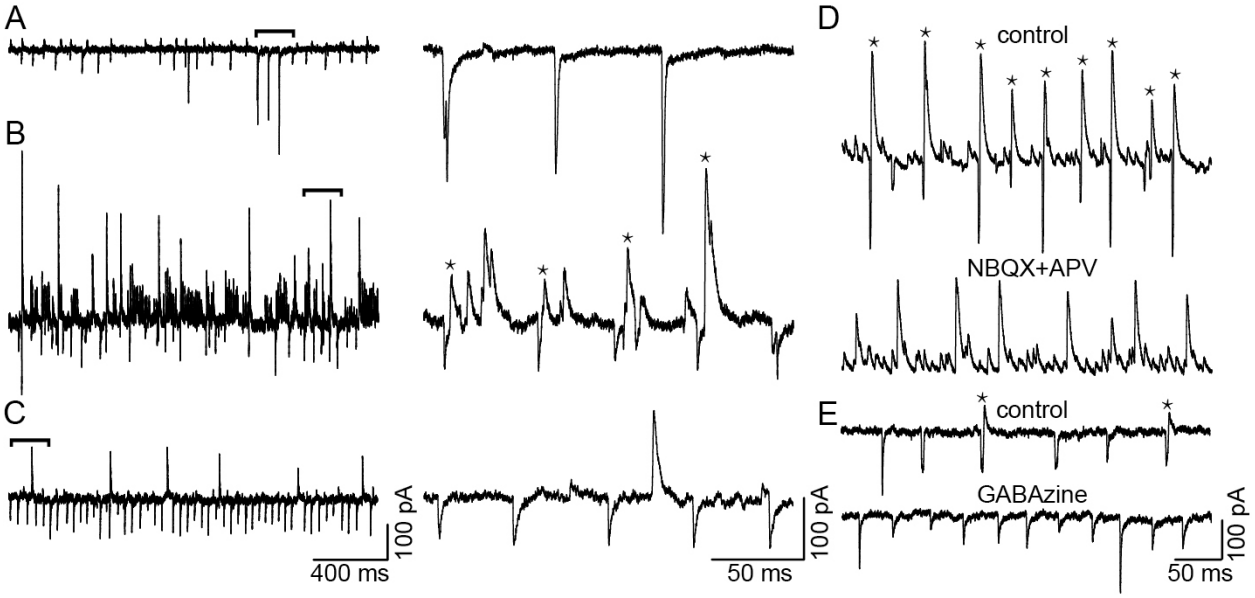

Figure 9. Spontaneous synaptic currents are glutamatergic and GABAergic

A–C. Three example pallidal neurons in voltage-clamp configuration at holding potentials of -60 mV (*A*), -50 mV (*B*) and -60 mV (*C*). Left, 2-s segments showing different temporal structure of spontaneous synaptic currents. Area marked by bracket is magnified in center column. *D*. Pallidal neuron recorded with Cs^+ internal solution in voltage-clamp configuration at a holding potential of -45 mV. Top, synaptic activity during control period; Bottom, after co-application of 20 μM NBQX and 100 μM APV. *E.* Pallidal neuron in voltage-clamp configuration at a holding potential of -60 mV. Top, control period; bottom, after application of 10 μM GABAzine. Asterisks indicate coupled excitatory-inhibitory synaptic currents.

In voltage-clamp, pallidal neurons continued to show steady spontaneous synaptic events, although specific synaptic activity profiles differed across individual neurons (Fig. 9*A-C*). As in current-clamp mode, we often observed coupled EPSC-IPSCs (Fig. 9*B-E,* asterisks*)*. The consistent presence of EPSCs across all recordings in our data suggested that these events did not arise from severed axon terminals in area X originating from HVC or

LMAN but rather from a neuron type whose soma is located within area X. While the vast majority of area X neurons are thought to be GABAergic, the nucleus also contains a small number of cholinergic interneurons. We observed no change in EPSCs in the presence of nicotinic acetylcholine receptor antagonist mecamylamine (data not shown). However, EPSC frequency was strongly reduced with application of glutamate receptor blockers NBQX and APV (Fig. 9*D*, 21.89 ± 4.67 Hz vs. 2.58 ± 0.75 Hz, P = 0.01, n = 5, ± *SE*). We also verified that outward synaptic events are mediated by $GABA_A$ receptors (Fig. 9*E*).

Glutamatergic Currents are Generated within Area X

Glutamatergic EPSCs in pallidal neurons were unexpected given the lack of a known glutamatergic neuron type (Farries and Perkel, 2002). Our preparation did not contain cell bodies of HVC or LMAN neurons, nor any other cortex-like regions (Fig. 10*A*). The regularity of the EPSCs suggested that they represented the activity of one or more presynaptic neurons firing action potentials. Consistent with this hypothesis, the voltage-gated sodium channel blocker tetrodotoxin suppressed EPSC frequency (Fig. 10*B*, P = 0.01, n = 3, 7.31 ± 0.58 Hz vs. 1.14 ± 0.19 Hz, ± *SE*). In TTX residual EPSCs were of dramatically reduced amplitude (Fig. 10*B*, 18 ± 2.39 pA vs. 63.9 ± 8.55 pA, n = 3, ± *SE*). Most forebrain neurons express $GABA_A$ receptors on their soma (Fritschy and Panzanelli, 2014), and while presynaptic $GABA_A$ receptors have been reported in the forebrain, their activation is associated with synaptic bouton depolarization, resulting in a widening of action potential waveform and synaptic transmission facilitation (Kullmann et al., 2005; Ruiz et al., 2010). Thus we reasoned that if EPSCs arise from a soma within area X, application of the $GABA_A$ receptor agonist muscimol should decrease their frequency. Indeed, muscimol reversibly suppressed mean EPSC frequency (Fig. 10*C-D*, P = 0.001, n = 11, 14.27 ± 2.67 Hz vs. 1.66 ± 1.01 Hz, ± *SE*), in addition to IPSC frequency. Finally, EPSCs were highly regular, consistent with spontaneous firing in either a

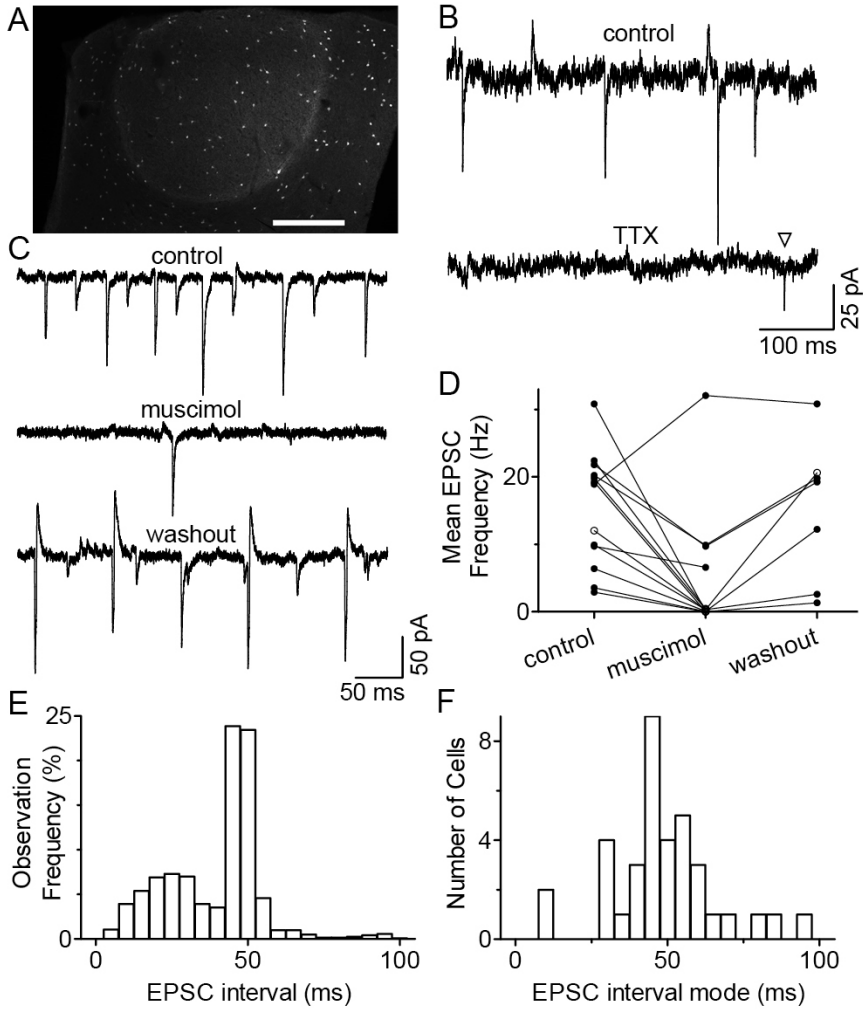

Figure 10. Spontaneous glutamatergic currents arise locally within area X

A. Isolated area X section immunolabeled with anti-ChAT, illustrating cholinergic neuron somata and area X borders. Scale bar = 100 μm. *B.* Pallidal neuron in voltage-clamp configuration at a holding potential of -60 mV. Top, control period; bottom, 1 μM TTX eliminated EPSCs. Inverted triangle highlights a putative miniature EPSC. *C.* Pallidal neuron in voltage-clamp configuration at a holding potential of -65 mV. 10 μM muscimol reversibly decreased EPSC frequency. *D.* Muscimol (1-10 μM) significantly decreased mean EPSC frequency (n = 13 neurons for muscimol application, n = 7 for washout). Example in *C* indicated by open circles. *E.* Example pallidal EPSC interval histogram. *F.* EPSC interval modes cluster around 45 ms (n = 36 pallidal neurons).

small number of presynaptic inputs, or alternatively a synchronized population of presynaptic neurons, and inconsistent with spontaneous vesicular release (Fig. 10*E*). Although the exact shape of EPSC interval distributions varied, in two thirds of all neurons the mode of EPSC interval fell between 40 and 60 ms (Fig. 10*F*). Together, these results provide strong evidence of a spontaneously active glutamatergic neuron located within area X that makes a synaptic connection onto pallidal neurons.

Source of Glutamate in Area X

While *in situ* hybridization has revealed a sparse population of somata in area X that contain VGluT2 mRNA (ZEBrA database), we found numerous VGluT2 protein immunoreactive terminals, but did not observe labeled cell bodies. Given the dense glutamatergic input from song nuclei HVC and LMAN, and the fact that these cortex-like regions in birds express VGluT2, the vast majority of VGluT2+ terminals presumably arise from afferent projections. Nonetheless, a fraction of VGluT2+ terminals could arise from a local glutamatergic cell population.

In mammals, striatal cholinergic interneurons co-release glutamate with acetylcholine (Higley et al., 2011; Guzman et al., 2011). To determine whether area X cholinergic interneurons could release glutamate, we tested for colocalization of VGluT2 and ChAT immunoreactivity in synaptic terminals. We found no significant colocalization of VGluT2+ and ChAT+ terminals, strongly suggesting that cholinergic interneurons are not the source of glutamate in area X (Fig. 11*A-D*, n = 7 birds). Glutamate can also be co-released from GABA neurons in the developing nervous system (Boulland et al., 2004; Gillespie et al., 2005) and in the adult brain (Fattorini et al., 2009; Boulland et al., 2009; Zander et al., 2010; Shabel et al., 2014; Root et al., 2014). To address the hypothesis that

glutamate is released from one of the previously described GABA interneurons in area X: spiny neurons, fast-spiking interneurons, low-threshold spike interneurons, or the subpopulation of pallidal neurons that do not project to the thalamus (Farries and Perkel, 2002; Farries et al., 2005) we double-immunolabeled for VGluT2 and vesicular GABA transporter (VGaT). We found no significant colocalization of VGluT2+ and VGaT+ terminals (Fig. 11*E-H*).

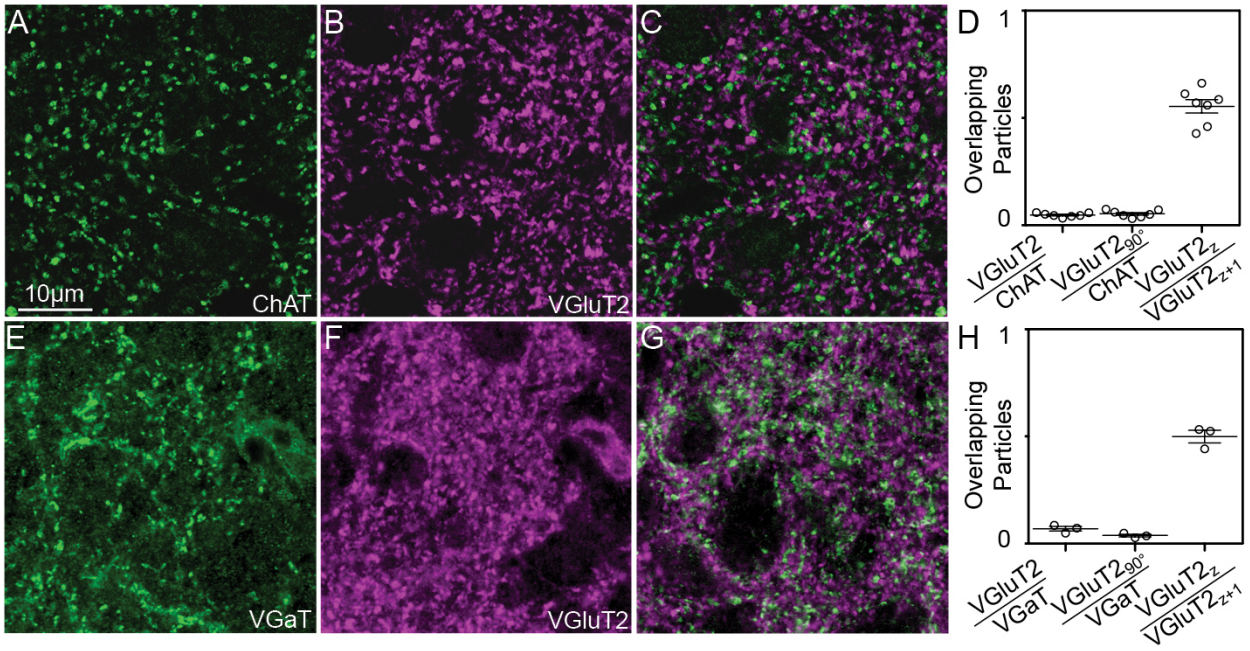

Figure 11. Glutamatergic, cholinergic and GABAergic synaptic terminals in area X

A, B. Sections of area X tissue immunolabeled with antibodies against ChAT (*A*) and VGluT2 (*B*). *C*. Overlay of *A* and *B*. *D*. Negligible overlap of VGluT2+ and ChAT+ puncta (n = 7 birds). *E, F.* Sections of area X tissue immunolabeled with antibodies against VGaT (*A*) and VGluT2 (*B*). *G.* Overlay of *E* and *F*. *H*, Negligible overlap of VGluT2+ and VGaT+ puncta (n = 3 birds).

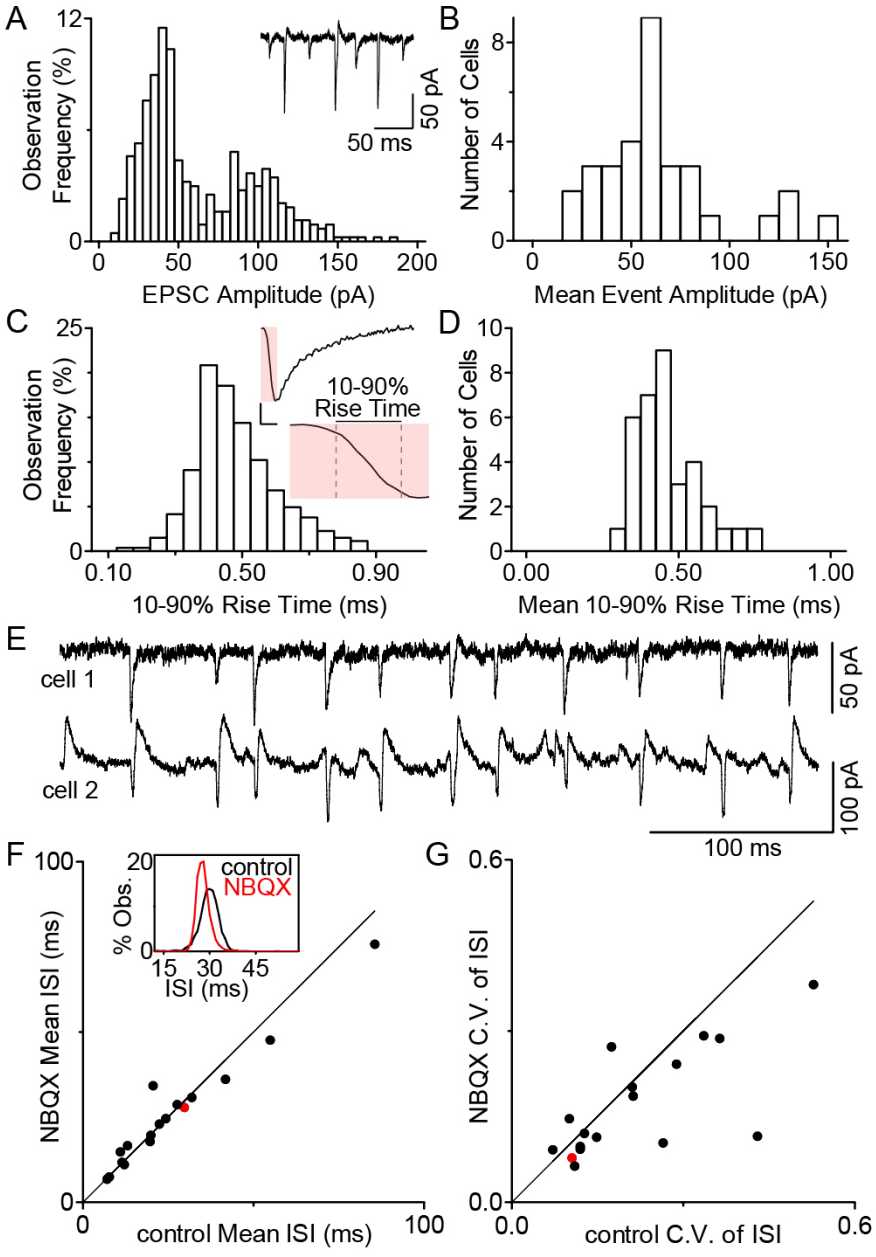

Figure 12. EPSCs are potent and introduce variability into pallidal neuron firing

A. Example pallidal EPSC amplitude histogram, with accompanying recording at a holding potential of -65 mV.
B. Distribution of mean EPSC amplitude (n = 32 pallidal neurons).
C. Example pallidal EPSC 10-90% rise time histogram. Insets contain an example EPSC from this cell, with its rising phase shaded and magnified to demonstrate 10-90% rise time. Scale bar = 1 ms/50 pA.
D. Distribution of mean EPSC 10-90% rise times (n = 35 pallidal neurons).This sample includes three additional neurons excluded from *B* because the recordings were made using Cs^+ internal solution and different holding potential.
E. Simultaneously recorded pallidal neurons, each at a holding potential of -60 mV.
F. Mean ISI before and after application of 10 μM NBQX (n = 17 pallidal neurons). Inset demonstrates change in ISI distribution after NBQX for neuron indicated by filled red circle.
G. Same as *F* for C.V. of ISI.

Excitatory Currents are Potent

Do spontaneous EPSCs exert a meaningful influence on pallidal neuron firing? EPSCs were often large (mean amplitude 64.18 ± 31.02 pA n = 32, ± *SD*), in some neurons exceeding 100 pA (Fig. 12*A-B*). EPSCs had a smooth rising phase with mean 10 - 90% rise time centered at 0.4 ms (Fig. 12*C-D*, n = 35), consistent with the time course for an event arising from a single presynaptic neuron. EPSCs are thus well-placed to deliver potent excitation to pallidal neurons, an idea underscored by observations in current-clamp mode, where EPSPs were frequently on the order of 3 - 5 mV (Fig. 8*E-F*). In a few cases simultaneous recordings from two nearby pallidal neurons revealed a high level of coincident EPSCs, suggesting that the glutamatergic neuron in area X is divergent, or alternatively, that the glutamatergic neuron population is synchronized (Fig. 12*E*).

Excitatory Synapses Introduce Variability into Pallidal Neuron Firing

One candidate role for these novel EPSCs is to contribute to variability in pallidal neurons. If so, glutamate receptor blockade should decrease the variability of pallidal neuron firing without an accompanying change in its global firing rate. NBQX significantly decreased the variability of pallidal firing (Fig. 12*G*, control CV = 0.22 ± 0.03 vs. NBQX CV = 0.17 ± 0.02, P = 0.045, ± *SE*), without altering mean ISI (Fig. 12*F*, control 25.9 ± 4.81 ms vs. NBQX 25.6 ± 4.12 ms, P = 0.78, n = 17, ± *SE*).

Excitatory Synaptic Activity May Drive Two Distinct Microcircuit States in Pallidal Neurons

Synaptic events in pallidal neurons could be simple EPSCs or IPSCs, but we also frequently encountered spontaneously occurring coupled excitatory-inhibitory events (Figs. 8-9). In evoked responses, such tight

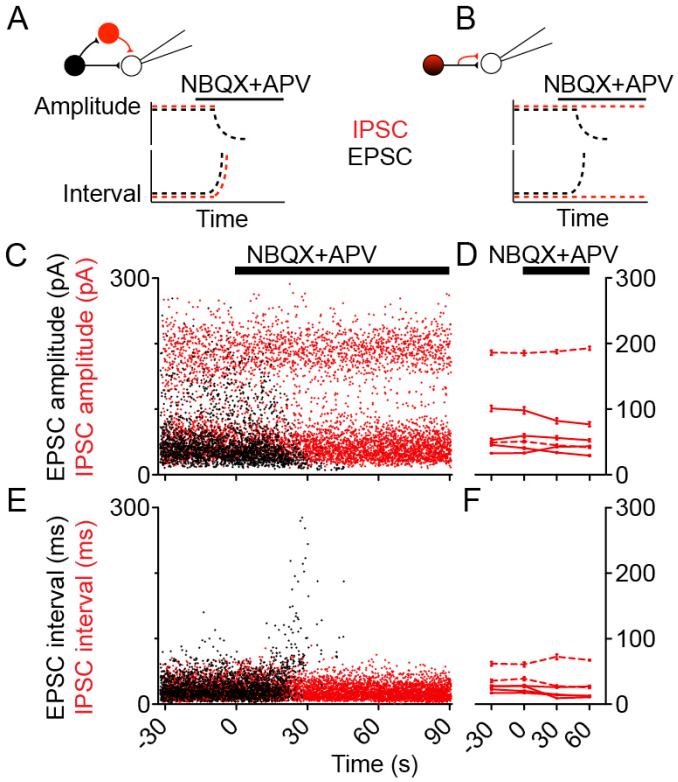

Figure 13. Origin of coupled EPSC-IPSCs

A. Expected effect of glutamatergic blockade on synaptic events resulting from disynaptic inhibition. In cartoons, inhibitory element is shown in red. *B.* Same as *A* for synaptic events resulting from glutamate/GABA co-release. *C.* Time course of synaptic event amplitude for one pallidal neuron during application of NBQX (20μM) and APV (100μM). *D.* Summary time course of IPSC amplitude for n = 6 datasets from 5 pallidal neurons. In one neuron, shown in *C*, two rhythmic IPSC populations of distinct sizes were analyzed separately. Example case marked by dashed lines. *E.* Time course of synaptic event intervals. Same neuron as *C*. EPSC intervals continued increasing beyond 300 ms, but were truncated for figure clarity. *F.* Summary time course of IPSC interval for n = 6 datasets from 5 pallidal neurons. Example case marked by dashed lines.

coupling between excitation and inhibition can play a powerful role in setting the window for temporal integration of excitatory input (Wehr and Zador, 2003; Gabernet et al., 2005). 23.07 ± 19.11% of EPSCs across all pallidal neurons were followed within 4 ms by an IPSC, (range 0 - 68.38%, n = 31, ± *SD*). Conversely, 17.06 ± 11.62% of all IPSCs were preceded by an EPSC within 4 ms (range 0 - 42.99%, n = 31). A Monte Carlo analysis involving shuffling IPSC times indicated that the rate of coupled events was much higher than expected by chance ($P < 0.0001$; shuffled % EPSC = 8.14 ± 5.87, shuffled % IPSC = 6.93 ± 4.62, ± *SD*). Coupled events could arise from a simple feedforward disynaptic microcircuit in which the presynaptic excitatory neuron synapses onto a pallidal neuron, but also recruits an inhibitory interneuron that synapses onto the same pallidal neuron (Fig. 13*A*). Alternatively, coupled events could result from co-release of glutamate and GABA from the presynaptic interneuron (Fig. 13*B*). These models make different predictions about synaptic activity after blockade of fast

glutamatergic transmission. In the disynaptic model, glutamatergic receptor blockade would prevent firing of the GABAergic interneuron, decreasing the frequency of IPSCs. In the co-release model, glutamatergic blockade would not alter the rate of IPSCs made by the spontaneously firing neuron. While we observed a sudden decrease in EPSC amplitude and simultaneous increase in EPSC interval during the application of NBQX and APV, neither IPSC amplitude nor interval was affected (Fig. 13*C-F*). These findings are consistent with the idea that synaptic release of GABA is not dependent on glutamate, but rather that the two neurotransmitters may be released simultaneously.

Dopaminergic Modulation Preferentially Amplifies One Microcircuit State

Pallidal neuron firing is modulated by dopamine release in area X during the production of stereotyped courtship song (Kao et al., 2005; Sasaki et al., 2006; Leblois et al., 2010). It is unclear how dopamine drives the D1 receptor mediated decrease in pallidal neuron variability underlying this behavioral change (Leblois et al., 2009; Woolley et al., 2014). Excitation through EPSCs alone or a balance of excitation and inhibition through coupled EPSCs with IPSCs could represent different microcircuit states, and affect pallidal neuron firing variability. Is the relative contribution of these inputs modifiable in this circuit, and if so, could it be a potential mechanism for enacting state transitions in the pallidal neuron network? We tested the hypothesis that D1 receptor activation with SKF-38393 could modulate the relative occurrence of the two microcircuit states driven by the putative excitatory interneuron. SKF-38393 did not significantly alter EPSC interval (Fig. 14*B*, 64.3 ± 5.47 vs. 46.9 ± 7.77 ms, P = 0.142, n = 11, ± *SE*). However, D1 receptor activation significantly increased both the proportion of EPSCs that were coupled to IPSCs (Fig. 14*C*, 26.7% ± 7.51% vs. 37.1% ± 7.46%, P = 0.003, n = 11, ± *SE*) and IPSCs that were

coupled with preceding EPSCs (Fig. 14*D*, 17.8 ± 4.06 vs. 26.6 ± 3.38, P = 0.024, n = 11, ± *SE*). These coupled events and their modulation by dopamine represent a novel mechanism for altering the area X microcircuit.

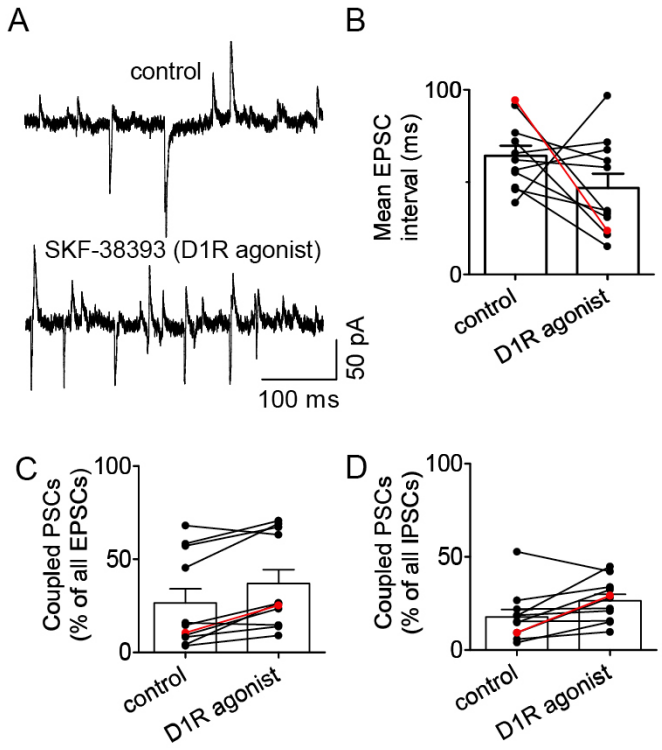

Figure 14. Modulation of coupled EPSC-IPSCs

A. Pallidal neuron in voltage-clamp configuration at a holding potential of -58 mV. Top, synaptic activity during control period; bottom, after co-application of 10 μM SKF-38393. *B-D*. Summary data for n = 11 pallidal neurons in 10 μM SKF-38393. Example in *A* is indicated in red. *B.* Mean EPSC interval. *C.* Percentage of all EPSCs that lead an IPSCs by at most 4 ms. *D.* Percentage of all IPSCs that are preceded by an EPSC within 4 ms. Error bars represent SEM.

Modeling the Effects of Microcircuit States on Pallidal Neuron Variability

We next explored how the novel excitatory drive to pallidal cells might affect firing variability using a simple model of a pallidal neuron with pacemaker properties. We used experimentally determined phase response curves (PRCs) to predict how periodic inputs from an excitatory neuron alone or a combination of excitatory and inhibitory inputs together affected the regularity of firing of the pallidal neuron, as measured by firing entropy. We then explored the robustness of this behavior to multiple parameters, and to observed changes in excitatory-inhibitory balance caused by dopamine. We used a mathematically tractable model pacemaker neuron whose

only state variable was the phase in its pacemaking cycle. To determine how synaptic inputs would affect firing phase, we first measured the infinitesimal PRC in real pallidal neurons by measuring the change in firing phase in response to small current injections at different phases of the firing cycle (Fig. 15*A-C*; Farries & Wilson, 2012). We then recorded spontaneous EPSC and coupled EPSC-IPSC waveforms and parameterized them (Fig 15*D*, *E*). We convolved the iPRC and the synaptic waveforms to produce synaptic PRCs that represent the overall effect of each type of synaptic input on pallidal neuron firing. Applying each microcircuit PRC to the pallidal neuron model at different phases resulted in different changes in phase. We thus built a map relating the phase of a given synaptic input to that of the following synaptic input, in units of pallidal phase (Fig. 15*F*, *G*, middle panels). We determined the model response to ongoing, periodic input of EPSCs alone or coupled EPSC-IPSC events, to simulate the experimentally recorded effect of dopamine (Fig. 14*I*, *J*). We found that, with some realistic parameters, EPSCs alone led to changing relative phase of the synaptic input from cycle to cycle. This ongoing change in relative phase implies irregular pallidal neuron firing (Fig. 15*F*, right, raster plot). Simulations from different initial phases led to distributed firing phase probability distributions (Fig. 15*F*, right, blue curve). With coupled EPSC-IPSC events, phase converged to a particular value, implying regular pallidal neuron firing (Fig. 15*G*, right). This consistent relative phase reflects the presence of a stable fixed point in the firing map (Fig. 15*G*, middle panel, filled red circle), indicating that the neuron's regular firing is robust to small perturbations in the phase of synaptic inputs. Note that firing regularity can be interpreted in two mathematically equivalent ways: as a measure of trial-to-trial variability for a single neuron, or as a measure of the variability of firing across a population of pallidal neurons on a single trial (Fig. 12*E*).

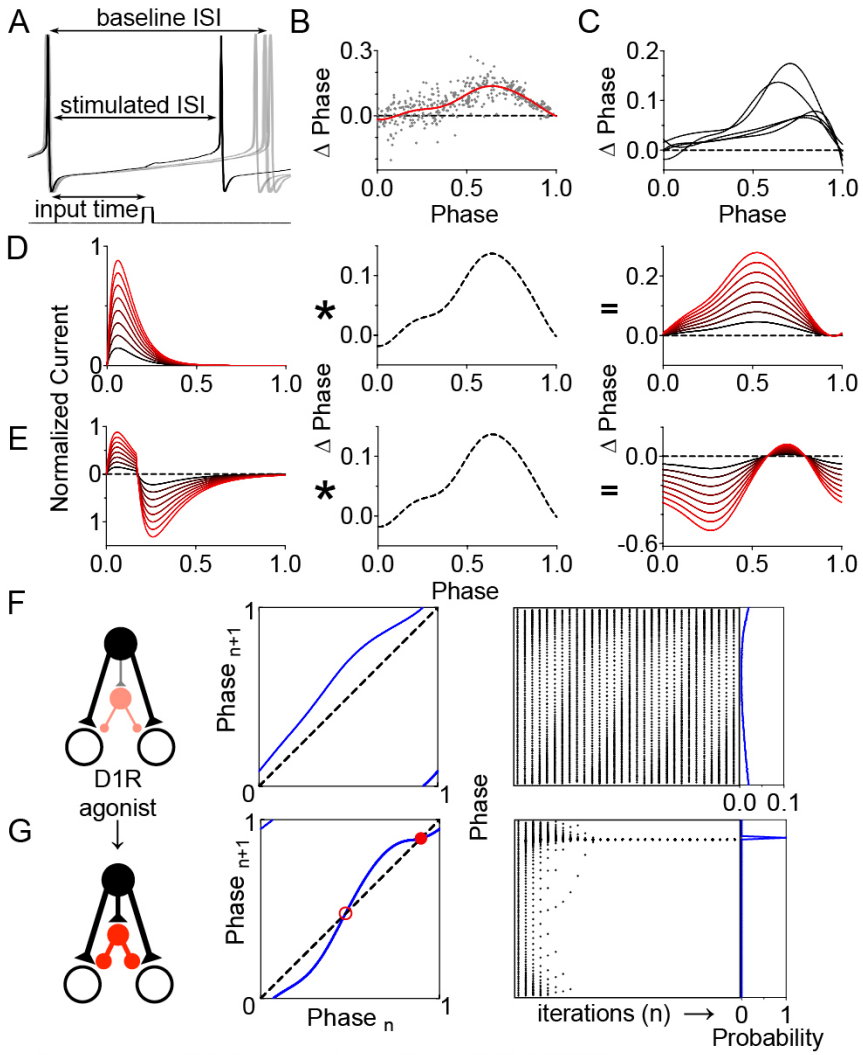

Figure 15. Generating a model firing map from the pallidal iPRC

A. Schematic of pallidal phase shift caused by current pulse (50 pA, 2 ms) in the presence of GABAzine and NBQX (each 10 μM). *B*. Each point represents the phase shift caused by a single current pulse in one neuron. Red line represents analytic fit ($R^2 = 0.52$). *C*. Fits to 5 neurons show qualitative resemblance. *D*. Synaptic PRC obtained by convolving the normalized synaptic current, synthesized using parameters constrained by fits to recorded PSCs, with the fitted pallidal iPRC. Left, normalized excitatory (E) synaptic current waveform at varying amplitudes; center, analytic fit to iPRC; right, resulting synaptic PRC. *E*. Same as in *D* but for linked excitatory-inhibitory (EI) synaptic input. *F*. Pallidal phase shift to E input. Left, schematic E input with no inhibitory element. Center, map relates the phase of the pallidal neuron at the onset of one input to its phase at the time of the next input. Right, pallidal phase across multiple initial conditions evolved over 200 iterations. Blue line at right plots phase probability distribution. Note lack of convergence to a single phase. *G*. Same as in *F* for EI input. Note convergence to a consistent phase. Filled red circle shows a stable fixed point; open red circle shows an unstable fixed point.

Further simulations showed that dopamine-induced alterations of the prevalence of coupled EPSC-IPSC events could rapidly reduce firing variability (Fig. 16*A*). To explore how robust these findings are to different synaptic strengths and periods, we varied the strength of the synaptic input, as well as its period relative to the pallidal neuron firing period. We measured firing variability as the entropy of the phase probability distribution after multiple simulations from different initial conditions. We found regions of parameter space where firing could be irregular (Fig. 16*B*; high entropy; white) or regular (Fig. 16*B*; low entropy; black). Large areas of parameter space showed stark differences in phase entropy for the two microcircuits, indicating that the observed synaptic changes caused by dopamine were likely to change firing variability. Because our experiments revealed that excitatory events couple to inhibitory events in a probabilistic manner, we explored systematically changing the ratio of isolated and coupled events. To simulate increased dopamine, we increased the probability of coupled events from 0 to 1, and asked how pallidal neuron periodic firing was perturbed when synaptic events were given at different phases in the pallidal neuron firing. We also added a small variance (C.V. = 0.05) in the intrinsic firing period of the pallidal neuron. We observed a complex landscape of behavior (Fig. 16*C*). The heat map illustrates the probability of relative phase at a given probability of coupled v. isolated events. As the probability of coupled events approached 1, phase distribution became more concentrated around a single value, resulting in a lower entropy. Insets illustrate sample phase distributions for different probabilities of coupled events coming from the input microcircuit. The white line indicates the entropy of the distribution as a function of the probability of coupled events, and shows a sharp decline in entropy with increased simulated dopamine level. Together, our simulation results show that a simple and highly constrained model microcircuit of area X can explain the observed effects of dopamine and social context on variability of pallidal neuron firing.

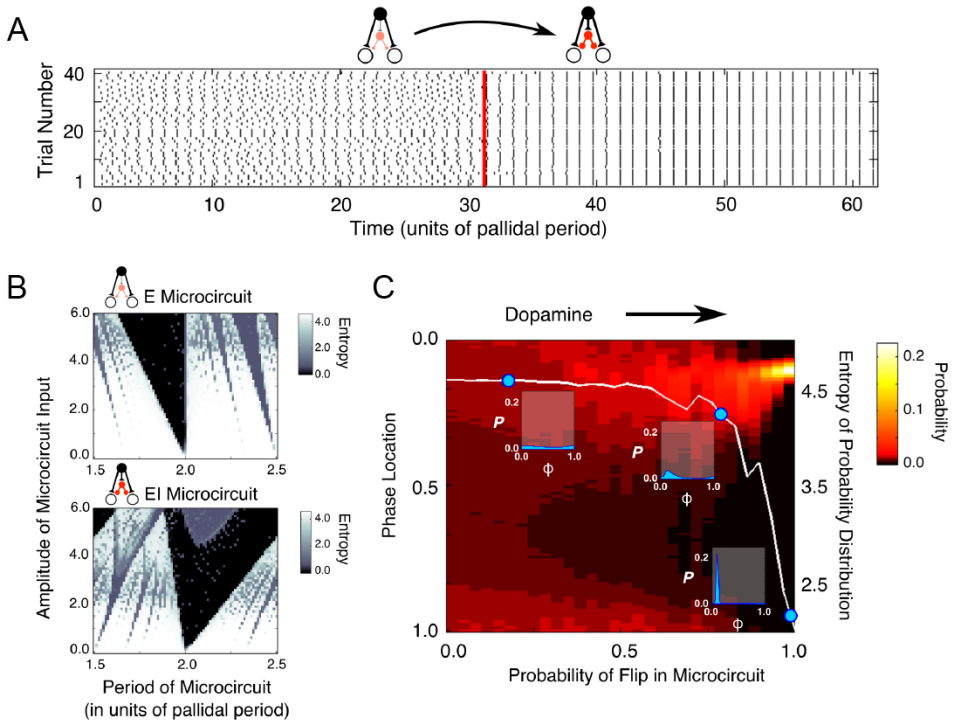

Figure 16. Effects of switching between E and EI microcircuits

A. Raster plot of pallidal firing during switch from E to EI microcircuit drive (vertical red line) illustrates rapid transition from desynchronized to synchronized state. *B*. The entropy of the distribution of pallidal phase varies with synaptic intensity and frequency of microcircuit drive. Upper panel, E microcircuit. Lower panel, EI microcircuit. Large regions of parameter space exhibit show dramatic differences in entropy between the two microcircuits. *C*. Effects of probabilistic inclusion of inhibitory element on firing phase distribution. Heat map shows the effect on the pallidal phase probability distribution as the probability of selecting the EI microcircuit varied from 0 to 1. The left ordinate indicates the phase location. For each probability, the resulting phase distribution is plotted as a column of heat values. The right ordinate indicates the entropy of the phase probability distribution, plotted as a white line. Insets show the probability distribution at each of three probability values, corresponding to blue circles. Small shifts in the probability of the microcircuit state can lead to large changes in entropy.

Discussion

Our main findings are that a regularly firing excitatory neuron type makes strong synaptic connections to multiple area X output neurons; this excitatory input is tightly coupled in time to inhibitory input; it contributes to pallidal firing variability; and these inputs are modulated by dopamine. Excitatory synaptic input with these properties connecting to very simple model output neurons leads to irregular firing, just as has been recorded during singing when a bird is alone and song is variable. Dopamine, which modulates the coupling of the excitatory and inhibitory events, shifts a modeled pallidal neuron from variability to stereotypy as a function of the degree of coupling. Such context-dependent changes in circuit dynamics are well placed to modulate behavioral variability to drive learning.

We have provided evidence for an excitatory neuron type that releases glutamate. It is not the cholinergic neuron type, as is the case in mammals (Higley et al., 2011; Gras et al., 2002), but may nonetheless have similar function. In mammals, neurons of the subthalamic nucleus (STN) fire rhythmically and excite pallidal output neurons (Beurrier et al., 1999). Loss of dopamine, as occurs in Parkinson's disease, leads to inappropriately synchronized and oscillatory firing of STN and pallidal neurons. Although an STN homolog has been identified in birds (Jiao et al., 2000), it is not connected to area X in songbirds (Person et al., 2008). Local glutamatergic activity in area X may thus be functionally analogous to that provided by the STN, packaged within the nucleus to address the temporal precision requirements inherent to song.

This glutamatergic cell type is likely rare. We did not record from cells firing spontaneously at 20 Hz. *In situ* hybridization for VGluT2 (SLC17a6) labels a sparse population of neuron somata in area X (ZEBrA database). To account for the ubiquity of spontaneous EPSCs in pallidal neurons, the rare excitatory neuron may have strong divergence. This is consistent with our observations of simultaneous EPSCs in pairs of pallidal neurons. Alternatively there may exist a synchronous population of excitatory neurons, in this way resulting in a widespread impact on its postsynaptic targets.

Our frequent observation of large, regular, coupled EPSCs and IPSCs in area X pallidal neurons suggests that they are an important feature of the circuitry in this nucleus. The tight temporal linkage is consistent with setting a narrow window in which the cell is sensitive to song-locked inputs. The output from area X to DLM is specialized for rapid and temporally precise signal propagation (Person and Perkel, 2007; Leblois et al., 2009; Goldberg et al., 2012). The modulation of the coupled synaptic events by dopamine thus provides a novel biophysical mechanism for the rapid changes in song-related firing that accompany changes in social context (Leblois et al., 2010; Woolley et al., 2014). While the precise source of the coupled events is not entirely clear, our finding that NBQX does not affect the rate of IPSCs argues strongly against a disynaptic origin. Corelease of GABA and glutamate has been observed in several locations in the adult nervous system, and this is the most likely explanation for the coupled events we observed. Another distinct possibility is that temporal coupling is the result of heterologous electrical coupling between the presynaptic excitatory neuron and an inhibitory neuron. The presence and function of electrical coupling is unknown in the songbird basal ganglia. Studying the mechanism and function of coupled excitation and inhibition in the song system can provide a testbed for understanding its role in regulating firing precision and social behavior.

We took a theoretical approach to understand the impact of regularly firing excitatory neurons on pallidal output neurons of area X. Because pallidal neurons are intrinsic pacemakers, we modeled them using a minimalist phase model, with no unconstrained parameters. We based synaptic input parameters on experimentally recorded values and modeled the effect of those inputs according to observed responses of pallidal neurons to experimental perturbation. We found a wide range of synaptic parameters that supported a regime of high pallidal firing variability. Changing parameters according to observed effects of dopamine could easily switch the circuit into a regime of low pallidal firing variability. Excitatory neurons intrinsic to area X are thus well placed to contribute to the rapid changes in network dynamics induced by different social contexts.

The model variability brings to mind the chaotic dynamics that balanced excitatory-inhibitory networks can produce (van Vreeswijk and Sompolinsky, 1996; Lajoie et al., 2014). This approach provides a theoretical foundation for understanding the complex interplay of excitation and inhibition in area X. It also provides a distinct perspective if viewed in light of how the ensemble of pallidal neurons throughout area X fires on a single song trial. If we assume that each pallidal neuron starts a trial in a different initial condition, and that excitatory neurons diverge widely to the ensemble of pallidal neurons, then our model predicts that dopamine will orchestrate a transition from a regime of asynchronous to synchronous firing. These assumptions and this prediction remain to be tested through simultaneous recordings from multiple pallidal neurons *in vitro* and *in vivo*.

We have shown one way in which area X may contribute to or regulate variability in the song learning circuit. A second likely source of variability is nucleus LMAN, which projects to nucleus RA in the song motor pathway

(Kao et al., 2005; Aronov et al., 2011; Goldberg and Fee, 2011; Ali et al., 2013). Area X can exert strong influence on LMAN via DLM (Leblois et al., 2009; Kojima et al., 2013). This could include transmitting variability directly, or gating any intrinsic variability created in LMAN. For example, any change in the degree of area X pallidal neuron synchrony may have a dramatic effect on LMAN firing. Area X projections to DLM neurons are thought to be one-to-one, but ensembles of DLM neurons converge on LMAN neurons (Boettiger, and Doupe, 1998). The momentary coherence of multiple area X pallidal neurons could thus enhance the activity of particular downstream neuron groups. Recurrent networks such as those within LMAN can undergo stimulus dependent suppression of their intrinsic, chaotic activity (Rajan et al., 2010). Temporarily pooling specific subsets of area X output neurons could be one way to strengthen a task-specific signal.

Conclusion

This work has shown the role of specific biophysical mechanisms in giving rise to behavioral variability that is important for learning precise skilled movements. Similar mechanisms could underlie action selection, a hypothesized function of the basal ganglia (Graybiel, 1995; Stocco et al., 2010). More broadly, outside the motor domain, neural variability could give rise to adaptive phenomena such as effective foraging or creativity, or to maladaptive phenomena such as intrusive thoughts or attention deficit disorder. The readily quantified song behavior and the discrete neural circuit underlying it offer a promising pathway for detailed mechanistic analysis of basal ganglia function in health and disease.

Experimental Procedures

Slice Preparation and Electrophysiology

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. 250 μm parasagittal brain slices were collected from 40 adult male zebra finches using procedures adapted from Farries and Perkel (2002). An ophthalmic micro-surgical blade was used to cut around area X in each slice, thereby removing the cell bodies of projections to area X. Isolated area X slices were stored for > 45 min in warm (30°C) ACSF (in mM: NaCl, 119; KCl, 2.5; $MgSO_4$, 1.3; $CaCl_2$, 2.5; NaH_2PO_4, 1; $NaHCO_3$, 26.2; D-glucose, 11) with 400 μM ascorbate (final pH of 7.4, osmolality of 300 mOsm, bubbled with 95% O_2 / 5% CO_2). Recordings were performed in ACSF at 30°C with internal solution containing in mM: $KMeSO_4$, 120; HEPES, 10; NaCl, 8; EGTA, 2; $MgSO_4$, 2; phosphocreatine,10; ATP-Mg, 2; GTP, 0.3; biocytin, 0.2-0.5%, at 290-295 mOsm and pH 7.3. In a subset of voltage-clamp recordings $KMeSO_4$ was replaced with $CsMeSO_4$ and 5 mM QX314 bromide (Tocris). Recordings were low-pass filtered (4 kHz for voltage-clamp, 10 kHz for current-clamp) and digitized at 20 kHz using Clampex 9.0 software (Axon Instruments). Cell-attached recordings were collected in voltage-clamp mode. See Supplementary Methods for data-inclusion criteria. The following drugs were bath applied: NBQX, muscimol, SKF 38393 hydrobromide, DL-APV (Tocris); GABAzine/SR-95531 (Sigma-Aldrich); TTX (Calbiochem).

Cell Type Identification

Aspiny fast firing neurons of area X (pallidal neurons) were identified based on previously described criteria (Farries and Perkel, 2002): 1) large soma size, 2) spontaneous activity > 10 Hz, 3) fast action potentials, and in a subset of cells that were reconstructed histologically, 4) morphological confirmation of large soma and aspiny

dendritic processes. Cells were subjected to a series of current pulses after whole-cell mode was established to measure intrinsic electrophysiological properties. In recordings using Cs^+ solution pallidal cells were identified based on soma size and spontaneous activity during seal formation.

Histology

Mouse anti-VGluT2 antibody (1:200; Millipore), goat anti-ChAT antibody (1:200; Millipore) and rabbit anti-VGaT antibody (1:200; Synaptic Systems) were used for immunohistochemistry, followed by Alexa conjugated secondary antibodies (1:200, Molecular Probes) for visualization. Degrees of VGluT2/ChAT and VGluT2/VGaT signal colocalization were determined using the threshold-based Particle Analyzer function in ImageJ, and a custom Matlab script. See Supplementary Methods for detailed information on histological sample preparation, confocal image acquisition and image analysis.

Electrophysiology Analysis

Holding potential was tuned individually for each cell (mean: -56.41 mV, range: -50 mV to -65 mV) to maximize EPSC/IPSC isolation while minimizing unclamped action potentials. Spontaneous currents within a minimum consecutive window of 25 s were analyzed with Mini Analysis (Synaptosoft). Event features (peak time, peak amplitude, 10-90% rise time, inter-event interval) were exported for further analysis in Matlab. Pallidal neurons with good isolation of EPSCs/IPSCs and a minimum spontaneous IPSC frequency of 2 Hz were included in analysis of coupled synaptic events. An EPSC was categorized as coupled when its peak time was followed by an IPSC peak time within 4 ms, with no other intervening events. Spike times were analyzed in Matlab by measuring action potential peak time.

iPRC measurement

iPRC experiments were conducted following previously described methods (Farries and Wilson, 2012) and explained in detail in Supplementary Methods. Briefly, 2 ms current pulses were injected at a frequency of 2 Hz, with 4 stimulus presentations per sweep, and repeated at different amplitudes (± 50/100/250 pA). Phase change was defined as the difference between the baseline ISI and the stimulated ISI (the time between the peak of the preceding spike and the peak of the spike following current injection) divided by the mean baseline ISI.

Firing map construction

The experimental iPRC was fit to an analytical form of sum of sines and cosines:

$$iPRC(\phi) = a_0 + \sum_{p=1}^{p=3} a_p \sin(p\omega\phi) + b_p \cos(p\omega\phi)$$

Because all cells measured showed qualitative similarity in their iPRC, we chose an analytic iPRC fit to a single representative cell (R^2 = 0.52). The PRC was calculated by convolving the iPRC with synaptic waveforms representing the two classes of synaptic input observed in our data: an excitatory synaptic input (E) and a coupled excitatory-inhibitory input (EI). Synaptic waveforms for E and EI inputs were drawn directly from fits to the observed data (see Supplementary Methods). The firing map was built using this PRC, according to:

$$\phi_{n+1} = \{\phi_n + PRC_{syn}(\phi_n) + T_{mc}\}_{mod\ T_p},$$

where T_{mc} is the period of the microcircuit inputs and T_p is the period of the pallidal cell. ϕ_n is the pallidal phase at which the 'nth' synaptic input arrives and the map computes the pallidal phase, ϕ_{n+1}, of the arrival of the next synaptic event.

Calculation of entropy

We calculated the entropy of phase distributions approximating the steady state probability density function of a cell ensemble. 1000 initial phases were drawn from a uniform distribution and then iterated 200 times under each realization of the firing maps. Phase (0-1) was divided into 100 bins, and a probability mass function was estimated by normalizing the counts of cells in each phase bin. Phase locations of the cell ensemble were summed over the last 5 iterations. Entropy was defined as:

$$S = -\sum_{i=1}^{M} p(\varphi_i)\ln\big(p(\varphi_i)\big),$$

where M = 100 is the total number of discrete phase partitions.

Modeling of noise in ISI distribution and likelihood of EI vs. E microcircuit

We model two aspects of noise: η represents variability in the pallidal ISI and is modeled as an independent Gaussian random variable with zero mean and variance, σ = 0.05, in units of pallidal phase; we model probabilistic jumps between microcircuit states as Bernoulli draws of firing maps f and g, the firing maps of the respective E and EI microcircuit drives:

$$\phi_{n+1} = f(\phi_n);$$

$$\phi_{n+1} = g(\phi_n);$$

$$f(\phi_n) = \{\phi_n + PRC_{EI}(\phi_n) + T_{mc} + \eta\}_{mod\ T_p};$$

$$g(\phi_n) = \{\phi_n + PRC_{E}(\phi_n) + T_{mc} + \eta\}_{mod\ T_p}.$$

The probability of either the E or EI microcircuit occurring at any one input is:

$$\mathrm{P}\{\phi_{n+1} = f(\phi_n)\} = 1 - \mathrm{P}\{\phi_{n+1} = g(\phi_n)\}\,.$$

Results in Fig. 16*C* were computed by varying the Bernoulli probability of the E firing map on a single draw from zero to one. To compute each phase location probability distribution, 1000 phases were drawn from a uniform distribution between zero and one. These were iterated 500 times for each Bernoulli probability of drawing the E firing map, switching probabilistically between the two firing maps at each iteration. We calculated the phase location probability distribution over the last 400 iterations.

Statistics

Calculations are reported as mean ± standard deviation or standard error, as specified. ISI variability was quantified using the coefficient of variation (standard deviation / mean). Pallidal firing statistic comparisons between isolated v. intact slices or cell-attached v. current-clamp recording configuration were quantified with unpaired, two-tailed t-tests. Synaptic event characteristics before and after applications of NBQX, APV, TTX, muscimol or SKF-38393 were quantified with paired two-tailed t-tests. For muscimol perfusion, experiments were conducted in a mix of pallidal and non-pallidal neurons; in all other cases n equals the number of pallidal neurons. Coupled events were quantified by the percentage of all synaptic events of the relevant type (EPSC or IPSC). IPSC times for each pallidal neuron (n = 31) were shuffled with replacement 1000 times, and the resulting percentage of coupled events for that cell was calculated for each individual permutation. A paired, one-tailed t-test was used to compare the mean observed percentage of linked EPSCs (out of all EPSCs) to the percentage resulting from shuffling. This procedure was repeated for the percentage of linked IPSCs out of all IPSCs.

Supplementary Methods

Slicing Procedure

Male zebra finches were either purchased from a local supplier or obtained from our breeding facility. Birds were communally housed in cages on a 14/10 hour light/dark cycle. Birds were anesthetized by isoflurane inhalation, and euthanized by decapitation. The brain was removed rapidly and submerged in ice cold cutting solution containing (in mM): sucrose, 205; KCl, 2.5; $MgCl_2$, 7.5; $CaCl_2$, 0.5; NaH_2PO_4, 1; $NaHCO_3$, 26.2; d-glucose, 11, at an osmolality of 310 mOsm, and bubbled with a mixture of 95% O_2 / 5% CO_2. Sections containing area X were sliced on a vibrating microtome (Oxford).

Electrophysiological Recording

Slices were transferred to a recording chamber perfused at approximately 2.5 ml/min with prebubbled ACSF heated to 30°C. Recording pipettes were pulled to a resistance of 3-5 MΩ and filled with internal solution containing (in mM) $KMeSO_4$, 120; HEPES, 10; NaCl, 8; EGTA, 2; $MgSO_4$, 2; phosphocreatine,10; ATP-Mg, 2; GTP, 0.3; biocytin, 0.2-0.5% (Sigma-Aldrich) for *post hoc* histological reconstruction, at a final osmolality of 290-295 mOsm and pH 7.3. In a subset of supporting voltage-clamp recordings $KMeSO_4$ was replaced with $CsMeSO_4$ and 5 mM QX314 bromide (Tocris) was included in the pipette for maintaining better voltage clamp. Cells were subjected to a series of current pulses after whole-cell mode was established to measure intrinsic electrophysiological properties. In the supporting voltage-clamp recordings using Cs^+ based solution it was not possible to confirm cell identity based on intrinsic electrophysiological properties. These cells were identified based on soma size and spontaneous activity during seal formation.

Electrophysiological signals were amplified with a MultiClamp 700B amplifier (Molecular Devices), and digitized at 20 kHz with a Digidata 1322A analog-digital converter (Molecular Devices). Recordings were collected and low-pass filtered (4 kHz for voltage-clamp, 10 kHz for current-clamp) in Clampex 9.0 software (Axon Instruments). Cell-attached recordings were collected in voltage-clamp mode. Input and series resistances were monitored in every recording sweep with a 250-ms hyperpolarizing test pulse. Mean series resistance was 15.5 MΩ across recordings, and ranged between 7.62 - 27.64 MΩ. Data were excluded from analysis if series resistance changed by more than ±25% during the recording or between conditions for pharmacological manipulations. In some muscimol perfusion experiments, washout condition was accompanied by a greater than 25% increase in series resistance from baseline. Nonetheless, we included these data in analysis of EPSC frequency, as this change in recording stability would be expected to reduce EPSC detection, resulting in an underestimation of their frequency. In two cases series resistance remained very stable after washout of 1 μM muscimol, allowing a second perfusion at 5 μM. Results at both drug concentrations are displayed in Fig. 10*D*, but only the higher concentration was included in statistical analysis of this experiment, so that each data point arose from an independent neuron. For cell-attached and current-clamp recordings cells were excluded from analysis if their spontaneous firing rate decreased by more than 20% over the course of the experiment. The following drugs were bath applied in these experiments: NBQX, Muscimol, SKF 38393 hydrobromide, DL-APV (Tocris); GABAzine/SR-95531 (Sigma-Aldrich); TTX (Calbiochem).

Histology and Immunohistochemistry

Slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C overnight, then cryoprotected in 30% sucrose in 0.1 M PB. Fixed slices were resectioned (50 μm) on a freezing microtome, and washed in 0.1 M PB. For VGluT2 / ChAT and VGluT2 / VGaT double immunostaining, sections were washed in 0.3% Triton-X in 0.1 M PB (PBT), incubated in PBT with 3% Bovine Serum Albumin for 30 min, then with mouse anti-VGluT2 antibody (1:200; Millipore) and either goat anti-ChAT antibody (1:200; Millipore) or rabbit anti-VGaT antibody (1:200; Synaptic Systems) in 0.1 M PB for 48 hours at 4°C. Slices were then washed with 0.1 M PB and incubated for two hours at 27°C with Alexa 488-conjugated donkey anti-goat and Alexa 568-conjugated donkey anti-mouse secondary antibodies (1:200, Molecular Probes). If sections contained neurons that had been used in electrophysiology experiments Alexa 633-conjugated streptavidin was also included (1:300, Molecular Probes). Stained slices were washed and mounted in 0.1 M PB on glass slides, coverslipped with Fluoromount-G (Southern Biotech). The same protocol was applied when sections were processed for morphological reconstruction - goat anti-ChAT antibody was used for enhanced visualization of the area X border, and Alexa 594-conjugated streptavidin (1:300) along with Alexa 488-conjugated donkey anti-goat antibody were used for the secondary incubation.

Confocal Microscopy

Confocal images were acquired with an Olympus FV-1000 microscope with a 100X objective, 2X optical zoom and a confocal aperture of 120 μm. Images collected were 64 x 64 μm in size, with a section depth of either 0.36 or 0.43 μm. Laser wavelengths used for excitation were 488, 561 and 633 nm. Image brightness and contrast for example figures were adjusted using ImageJ (National Institutes of Health). Filled cells were inspected in ImageJ

to confirm cell type based on previously described morphological characteristics (Farries and Perkel, 2002). Fluorescence image in Fig. 3 was collected on a Nikon Optiphot microscope with a 4x objective. A minimum of 5 sequential images from 1-2 locations in 7 birds (VGluT2/ChAT) and 3 birds (VGluT2/VGaT) were used to determine the degree of signal colocalization. The threshold-based Particle Analyzer function was applied to the images in ImageJ, and the centroid of each particle was exported for further analysis in Matlab where a custom script was used to quantify the proportion of centroids found in the ChAT or VGaT channel that could be matched with a nearby (< 0.4 μm), unique centroid in the VGluT2 channel. This quantification was compared with that for a 90° rotated version of the VGluT2 channel. Finally the proportion of overlap between sequential VGluT2 images in the stack was calculated.

Electrophysiology Analysis

Use of low chloride internal solution permitted simultaneous recording of opposite sign excitatory and inhibitory spontaneous synaptic activity in pallidal cells. Holding potential was tuned individually for each cell (mean: -56.41 mV, range: -50 mV to -65 mV) to maximize excitatory synaptic current (EPSC) / inhibitory synaptic current (IPSC) isolation while minimizing unclamped action potentials. Similarly, holding current was adjusted for each current-clamp recording. In 4 cases voltage-clamp recordings were performed with Cs^+ and QX314 in the internal solution, which permitted even better EPSC/IPSC isolation at a holding potential of -45 mV. Spontaneous currents were analyzed with Mini Analysis (Synaptosoft). A minimum consecutive window of 25 seconds was analyzed for each recording. Events found during the test pulse period were discarded from analysis. Event features (peak time, peak amplitude, 10-90% rise time, inter-event interval) for 36 pallidal neurons were exported for further analysis in Matlab. Voltage-clamp recordings performed with Cs^+ and QX314 in the internal solution

were included in summary data of inter-event interval and 10-90% rise time, but not amplitude. A subset of pallidal neurons (n = 31) with good isolation of EPSCs/IPSCs and a minimum spontaneous IPSC frequency of 2 Hz were included in analysis of coupled synaptic events. An EPSC was categorized as coupled when its peak time was followed by an IPSC peak time within 4 ms, with no other intervening events. Spike times were analyzed in Matlab by measuring action potential peak time.

iPRC Measurement

iPRC experiments were conducted in 5 pallidal neurons in the presence of GABAzine and NBQX (10 μM), following previously described methods, which are illustrated in Fig. 15*A* (Farries and Wilson, 2012). Briefly, 2 ms current pulses were injected at a frequency of 2 Hz, with 4 stimulus presentations per sweep, and repeated at different amplitudes (±50/100/250 pA). Each stimulated ISI was associated with a pool of spontaneous ISIs occurring within 30 seconds of the ISI in question. The baseline ISI for that stimulation was defined as the mean of ISIs in this pool longer than the stimulated ISI. If there were fewer than 10 ISIs from which to make this calculation the associated pool of spontaneous ISIs was approximated by a Gaussian distribution with mean and standard deviation equal to that of the spontaneous ISIs occurring within 30 seconds of the stimulation. Input phase for a given stimulus was determined by dividing its input time by the mean baseline ISI, where the input time was the time between the peak of the preceding spike and midpoint of the current pulse. Phase change was the difference between the baseline ISI and the stimulated ISI (the time between the peak of the preceding spike and the peak of the spike following current injection) divided by the mean baseline ISI.

Firing map construction

The experimental iPRC was fit to an analytical form of sum of sines and cosines:

$$iPRC(\phi) = a_0 + \sum_{p=1}^{p=3} a_p \sin(p\omega\phi) + b_p \cos(p\omega\phi)$$

All cells measured showed qualitative similarity in their iPRC: almost entirely phase advancing with the biggest phase advance occurring approximately two thirds of the way into the oscillation cycle. Because of this similarity, we chose an analytic iPRC fit to a single representative cell. For this cell, the R^2 value of the fit parameters is 0.52. The parameters of the fit were:

Parameter	Value	Confidence interval (95%)
a_0	0.055601369321404;	(0.04054, 0.07066)
a_1	-0.061151688548318	(-0.09477, -0.02753)
a_2	-0.006863029049976	(-0.02946, 0.01574)
a_3	-0.005532332972041	(-0.01231, 0.001244)
b_1	-0.033316012078074	(-0.07761, 0.01098)
b_2	0.015454546227122	(-0.0007489, 0.03166)
b_3	-0.001488447238460	(-0.0116, 0.008619)
w	5.848805665250545	(4.72, 6.978)

Synaptic Waveforms

PRCs were generated by convolving a representative iPRC at +50 pA current injection with two classes of synaptic input observed in our data: an excitatory synaptic input and a coupled excitatory-inhibitory input. Excitatory synaptic input was modeled by a difference of exponentials:

$$Syn_E(t) = A_E * (e^{-\frac{t}{\tau_{E1}}} - e^{-\frac{t}{\tau_{E2}}}) + I$$

Coupled excitatory/inhibitory input was modeled by summing two such differences of exponentials, one representing the excitatory component and the other representing the inhibitory component of the event:

$$Syn_{EI}(t) = Syn_E(t) + Syn_I(t) - I$$

Coupled EPSCs from 6 pallidal neurons were collected and fit with the above equation in order to directly draw the distribution of parameters for events from the data. The following tau parameters yielded the best fit after examining both individual and coupled synaptic events: τ_{E1} = 1.2 ms; τ_{E2} = 0.7 ms; τ_{I1} = 2.5ms; τ_{I1} = 0.7 ms. These parameters were inserted as constants back into the above equations. Excitatory components of coupled events observed in our dataset precede inhibitory components, which could be explained by different activation and deactivation time courses of the underlying synaptic conductances, or simply a delay in the onset of the inhibitory component. We found that the best fits to our data occurred when activation time courses $\tau_{E2}$and τ_{I1} were equivalent, but a short delay was imposed on the inhibitory component (mean/SD delay = 2.25 ± 0.61 ms; n = 231). This step activation was approximated by a hyperbolic tangent function so that *x* in both rising and falling phases of the inhibitory component was replaced with $(t-d)*\frac{1}{2}(\tanh(c*(t-d))+1)$, where *d* = delay and *c* = 1000. The above constants, and allowing the inhibitory component of the equation to shift in time resulted in robust synaptic event fits (mean R^2= 0.96 ± 0.03; n = 231 coupled EPSCs). The synaptic event amplitudes and pauses between excitation and inhibition that were explored in the parameter space of our model were thus

drawn directly from fits to the observed data. The PRC was then calculated by convolving the iPRC with the synaptic waveform constructed from fits to observed data described in the previous methods section. The firing map was built using this PRC to be:

$$\phi_{n+1} = (\phi_n + PRC_{syn}(\phi_n) + T_{mc})_{mod\ T_p}$$

where T_{mc} is the period of the inputs and T_p is the period of the pallidal cell. ϕ_n is the pallidal phase at which the 'nth' synaptic input arrives and the map computes the pallidal phase, ϕ_{n+1}, of the arrival of the next synaptic event.

3. The Source and Role of Glutamate Signaling in the Songbird Basal Ganglia

As described in detail in Chapter 2, we have discovered a novel and unexpected excitatory element in the songbird basal ganglia. While we have made significant progress in characterizing this input in the projection neurons of the nucleus, there remain outstanding questions that warrant further investigation. One ongoing mystery is the identity of the putative glutamatergic interneuron. And while we have described one potential mechanism by which the oscillatory activity of this glutamatergic interneuron could impact pallidal projection neurons, we do not have a complete understanding of the functional contributions of this signal to information processing in area X signaling, or how it contributes to learning in the songbird. Here I describe potential experiments to address these two questions in the future, drawing on relevant preliminary data that did not fit into the scope of Chapter 2.

Does excitatory synaptic activity in area X arise from a known cell type?

Area X is known to contain at least 5 distinct cell types: spiny interneurons, fast-spiking interneurons, low-threshold spiking interneurons, cholinergic interneurons and pallidal projection *and* interneurons, which could have currently unidentified distinct intrinsic electrophysiological properties (Farries and Perkel, 2002; Farries et al., 2005). Local glutamate could be released from a rare cell type that had not been previously observed, or else from one of the above-mentioned neurons. Although glutamate release in the mammalian brain is controlled by three different vesicular glutamate transporter genes with distinct expression patterns, orthologs of only two of these - VGluT2 and VGluT3 - have been identified in avian genomes. In pigeon, VGluT3 mRNA has been detected in neurons of the caudal linear nucleus, and in the retina (Atoji and Karim, 2014). VGluT3's expression pattern in zebra finch has not been fully characterized, but it does appear to be present in area X (personal communication with Dr. Claudio Mello). VGluT2 mRNA is expressed most heavily outside of the basal ganglia in zebra finches, but upon closer inspection it can in fact be detected in a sparse population of cell bodies in area X (ZEBrA database).

We attempted to identify the excitatory neuron in area X using immunohistochemistry for VGluT2. One difficulty with this approach, however, was that anti-VGluT2 labeled only synaptic terminals in our preparation, with no cytoplasmic or cell membrane expression that could clearly identify the cell bodies of putative glutamatergic neurons. Since area X receives strong glutamatergic drive from the cortex-like regions HVC and

LMAN, we expected the majority of VGluT2+ signal to be expressed in the synaptic terminals of those extrinsic projections in the nucleus, with only a small fraction attributable to the synaptic terminals of putative local glutamatergic neurons. While this limited our efficiency in finding the glutamatergic neuron, we have gained some valuable insights that help to narrow down its identity.

First we looked for overlap of VGluT2-containing synaptic terminals with other synaptic markers. In mammals, striatal cholinergic interneurons co-release glutamate in a VGluT3-dependent manner (Higley et al., 2011; Guzman et al., 2011). Given the homologies between area X and mammalian striatum we reasoned that cholinergic interneurons in area X may also be releasing glutamate. Our immunohistochemistry data demonstrated that ChAT and VGluT2 are not co-localized in terminals in area X. (Fig. 11*A-D*). Barring the unlikely scenario that glutamate and acetylcholine are trafficked to non-overlapping populations of synaptic terminals, this finding strongly suggested that glutamate is not co-released from cholinergic neurons. Likewise, putative glutamatergic interneurons are estimated to fire action potentials spontaneously at approximately 20 Hz, much more rapidly than cholinergic interneurons encountered in the same *in vitro* preparation (Fig. 17) or in rat brain slices (Bennett and Wilson, 1999).

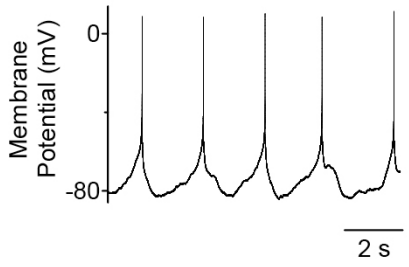

Figure 17. Spontaneous firing in area X cholinergic interneurons

Current-clamp recording of slowly firing cholinergic neuron in area X slice.

Next we investigated the possibility that glutamate is co-released from one of the GABA interneurons of area X. Glutamate/GABA co-transmission has been described for over a decade in the developing nervous system (Gillespie et al., 2005; Boulland et al., 2004). More recently it has also been reported in the adult mammalian brain in a number of regions including the cerebral cortex (Fattorini et al., 2009), hippocampus (Boulland et al., 2009; Zander et al., 2010), retina (Kao et al., 2004) and basal ganglia outputs to the habenula (Shabel et al., 2014; Root et al., 2014). We observed coupled excitatory-inhibitory synaptic events in pallidal neurons, as described in Chapter 2. While the fraction of coupled events varied between neurons and on average remained below half (Fig. 13), they were present in virtually all recorded pallidal neurons. Furthermore, it is possible that the calculated percentage of these events was underestimated: a weak inhibitory component may not result in a detectable outward current. Co-transmission of glutamate and GABA would be consistent with our observations of coupled synaptic events. We double-immunolabeled sections of isolated area X with antibodies targeted against VGluT2 and VGaT in order to address the hypothesis that glutamate is released from GABA interneurons, but found no significant overlap between the signals for these two proteins (Fig. 11*E-H*). However, even a small fraction of overlap could mean that glutamate is co-released from a GABAergic interneuron.

One way in which this question can be built on in the future is by combining confocal microscopy with electron microscopy (Root et al., 2014), in order to identify symmetric and asymmetric synapses which are typically inhibitory and excitatory, respectively (Colonnier, 1968). Notably, pallidal neurons are the only GABAergic cells in area X known to fire periodically. In our dataset we classified neurons firing action potentials above a threshold frequency of 10 Hz as pallidal neurons, based on criteria established by Farries and Perkel (2002). It is therefore possible that an unknown number of pallidal neurons in our data set are in fact the mystery

glutamatergic neuron. An alternative idea is that pallidal neurons are themselves releasing glutamate in area X. While most pallidal neurons project to the thalamus forming inhibitory synapses (Luo and Perkel, 1999b; Luo and Perkel, 1999a) a subset appear to form local connections onto other pallidal neurons, echoing the indirect pathway in mammalian basal ganglia (Farries et al., 2005; Goldberg et al., 2010; Pidoux et al., 2015). These two neuron types exhibit different activity profiles during the production of song, but in the *in vitro* preparation no difference in their electrical properties has thus far been identified. Although most literature is in agreement with the idea that basal ganglia output is GABAergic, a recent study in mice reports both excitatory and inhibitory outputs from Substantia Nigra neurons to the thalamus (Antal et al., 2014). This surprising finding raises the possibility that glutamate in area X could be released from locally branching pallidal neurons.

We systematically searched for the only known electrophysiological signature of the glutamatergic interneuron – an approximate spontaneous firing rate of 20 Hz. This was a surprisingly difficult feature to encounter given the universality of excitatory synaptic events in pallidal neurons. It is likely that these neurons are few in number, but that they are divergent, and thus able to contact many neurons at once (Fig. 12*E*). Nonetheless, we were able to fill a few candidate neurons with biocytin, and look for VGluT2 immunoreactivity in the processes of these recovered cells (Fig. 18). At this point we have not come across a cell fill that shows clear VGluT2 positive labeling in its processes. Occasionally we found varicosities on processes that showed encouraging proximity to VGluT2 positive puncta (Fig. 18*B*), but in these cases it was not possible to eliminate the possibility that was due to cell-to-cell apposition. We are conducting preliminary analyses of this data, constructing 3-dimensional masks of the neuron of interest in the analysis software package Amira (Fig. 18*D*), but in the future this method should be combined with a reliable presynaptic antibody (e.g. synapsin or synaptophysin) to eliminate contributions of

glutamatergic synapses onto the neuron of interest to the signal. This experiment could also be improved on by using ballistic labeling to deliver a dextran-conjugated fluorescent marker stochastically to a large number of neurons, in lieu of single cell biocytin fills (Morgan and Kerschensteiner, 2011).

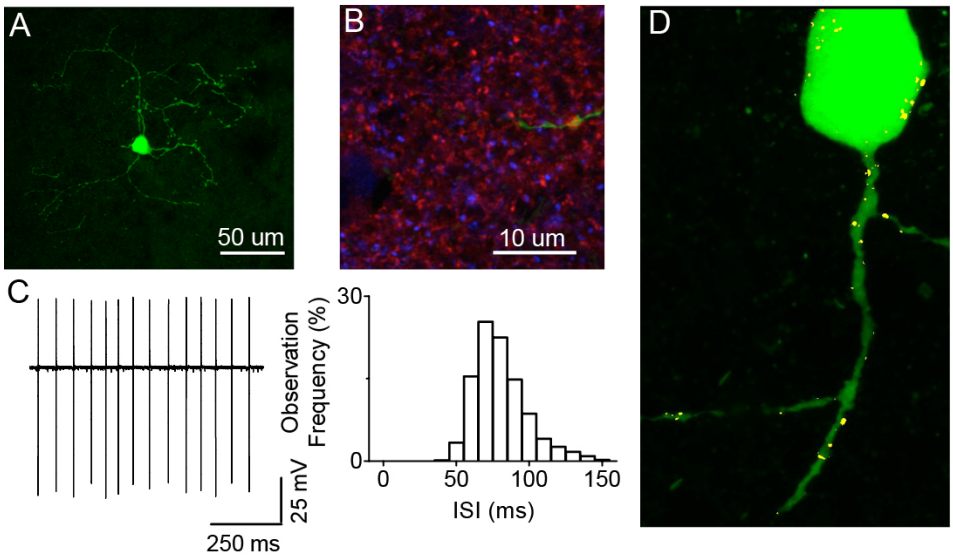

Figure 18. Search for the glutamatergic interneuron

A. Biocytin fill of a candidate 20 Hz firing neuron. *B*. Magnification of a process of neuron in *A*, overlayed with antibody label for ChAT (blue) and VGluT2 (red). *C*. Example cell-attached recording from a candidate low-frequency firing neuron, and accompanying interspike interval (ISI) histogram. *D*. Biocytin fill of neuron in *C*. Yellow denotes VGluT2 positive regions that fall within the volume of the neuron.

Finally, many of the difficulties that we have encountered while working with VGluT2 antibody could be eliminated by shifting approach to *in situ* hybridization. VGluT2 mRNA is sparsely expressed in area X (ZEBrA database). In theory, double *in situ* hybridization using probes for the SLC17A6 (VGluT2), SLC18A3 (VAChT), and SLC32A1 (VGaT) genes should determine whether these synaptic proteins are expressed in overlapping

populations of cell bodies. Currently, we still have very limited data on the identity of the glutamate-releasing neuron in area X. Although it is not likely to be the cholinergic interneuron, it may be one of the previously described GABAergic interneurons, or an entirely new cell type. Until we can properly target this neuron, we will not be able to identify its intrinsic properties and synaptic inputs, making it difficult to fully address its functional role in the songbird basal ganglia.

What is the broader impact of excitatory synaptic activity in area X?

We found evidence of a novel, spontaneously active, glutamatergic neuron in area X that contributes to variability of pallidal neuron firing. This effect on variability (as measured by the coefficient of variation of the interspike interval) was modest, but significant (Fig. 12), and is likely to represent a lower bound given variable preservation of glutamatergic inputs in the slice preparation. We were interested in exploring other analytical methods that could give further insight into how glutamatergic input shapes spontaneous pallidal firing statistics. Information theory provides another framework within which to quantify neural variability. Two relevant measures used in this field are entropy, or the hypothetical capacity that a given neural code has for transmitting information, and mutual information, or how much of that capacity is used in practice, given the neural responses to a stimulus (Dayan and Abbott, 2001). Entropy is calculated by multiplying the probability of a response by its logarithm, and then summating over all possible probabilities. For clarity, by definition a neural code associated with two different responses with equal likelihood has one bit of entropy. Practically, in neural data mutual information is typically acquired by computing the difference between total response entropy and

the noise entropy, which can be approximated by the average response entropy present in responses to an identical stimulus presentation.

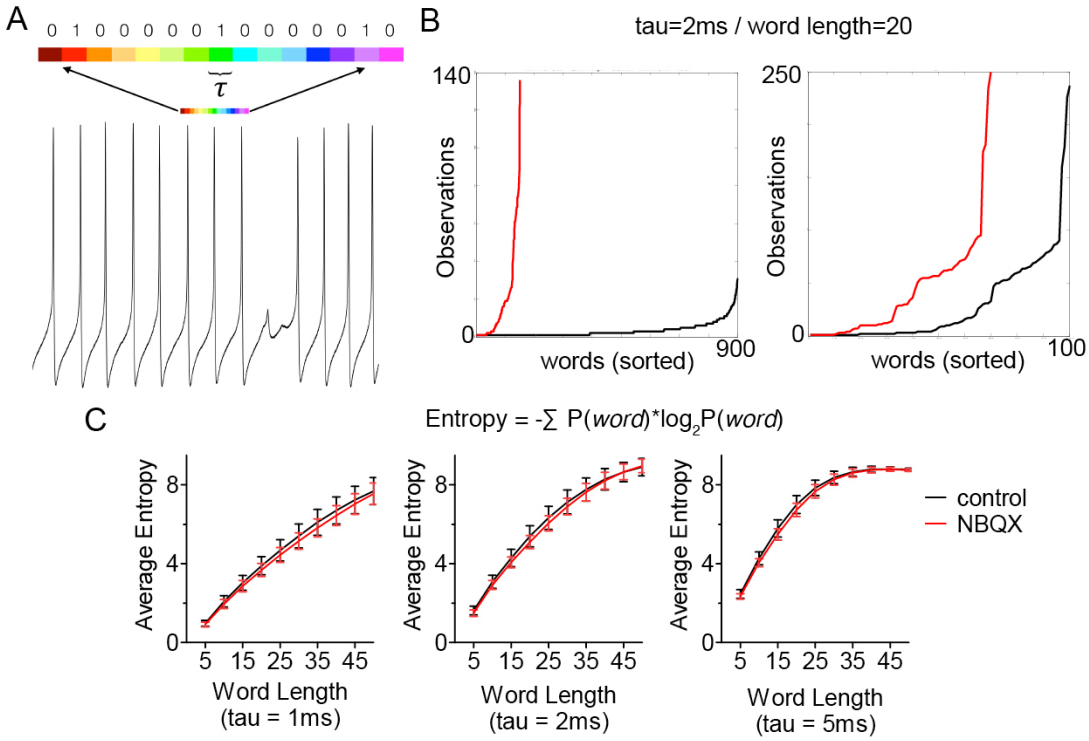

Figure 19. Entropy of spontaneous pallidal neuron firing

A. Each spike train is converted into "words" or integer strings representing the number of spikes that fall within a sliding bin of size tau. *B*. Examples of the 20-integer long word distributions in control (black) and NBQX (red) conditions for two neurons using a tau duration of 2 ms. *C*. Total entropy calculations averaged across all neurons (n = 12) as a function of word length and tau.

These measures could be adapted to our dataset in order to investigate variations and correlational structure in spontaneous pallidal neuron firing, even though the experiment has no such explicit stimulus presentations and

trials. Instead we can compare pallidal spike statistics across different binning strategies (Strong et al., 1998). In this scenario, the conditional response entropy is not in relation to a stimulus, but draws on the probability of a given "word" being formed by dividing the signal into bins of a given size (Fig. 19*A-B*). We chose bins of 1, 2 and 5 ms to encompass between one and a few pallidal neuron action potentials (Farries and Perkel, 2002; Goldberg et al., 2010). DLM neurons can fire rebound action potentials after short duration pauses in pallidal neurons that are on the order of 30 ms (Person and Perkel, 2005). We chose word lengths between 5 and 50 bins to straddle this relevant timescale. Our findings parallel those using the coefficient of variation, showing that total response entropy, or the overall capacity for pallidal spike trains to convey information, decreases in the presence of NBQX (10 μM) (Fig. 19*C*).

Ultimately, it would be desirable to compare the total mutual information present in control and NBQX conditions. The mutual information is the difference between this total response entropy and noise entropy. Noise entropy can be calculated by summating the product of the probability of a given tau being chosen and the conditional entropy given that tau, over all possible taus. To make this calculation, it may be necessary to make assumptions regarding the underlying probability of tau distributions.

Is the excitatory neuron functionally analogous to the STN?

The STN is the primary subcortical source of glutamatergic input to the basal ganglia in mammals. Its reciprocal connectivity with the GPe results in the presence of pathological oscillations in PD (Bevan et al., 2002b), suggesting that it plays a pivotal role in establishing network dynamics such as correlational structure. This

oscillatory activity is an emergent property of the STN-GPe network, persisting in the absence of cortical input (Plenz and Kital, 1999).

In Chapter 2 we identified one potential mechanism by which the regularly firing glutamatergic interneuron could locally modulate variability in its oscillating targets, the pallidal neurons. One possibility is that local glutamatergic activity in area X is a homologous or functionally analogous specialization akin to the STN, packaged within the nucleus to address the temporal precision requirements inherent to song. This hypothesis is particularly intriguing as an STN homolog has been identified in birds, but it is not reciprocally connected to the pallidum in songbirds, nor is there evidence of a projection to the song-specific portion of the basal ganglia, area X (Jiao et al., 2000; Person et al., 2008). There is no obvious cytochemical marker whose expression is enhanced in STN neurons as compared to other basal ganglia structures; thus we do not see an immediate immunocytochemical test for similarity between the glutamatergic area X neurons type and STN neurons.

However, another way of establishing analogy of putative glutamatergic neurons in area X to the mammalian STN is through the identification of similar functional mechanisms. Voltage-gated Na^+ channels underlie the depolarization phase of STN neurons' periodic firing, while an apamin-sensitive Ca^{2+}-activated K^+ current (SK) maintains their precise timing (Bevan and Wilson, 1999; Beurrier et al., 1999). First, the frequency of spontaneous EPSCs recorded in area X is consistent with what has been reported for STN neurons in rat brain slices (Beurrier et al., 1999). Additionally, preliminary data suggest that the SK channel blocker apamin can disrupt the regularity of low-frequency firing spontaneous neurons in area X (candidate glutamatergic interneurons) (Fig. 20), suggesting that these could be comparable cell types. Further exploration of burst generation and the resulting

network dynamics in the songbird basal ganglia would be a valuable avenue of research, in addition to establishing whether glutamatergic neurons are *reciprocally* connected to pallidal neurons.

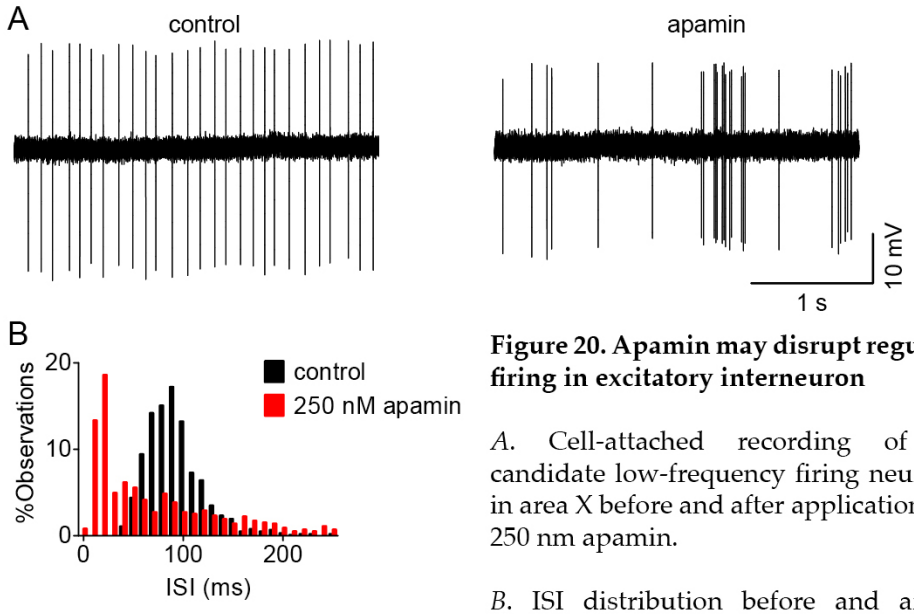

Figure 20. Apamin may disrupt regular firing in excitatory interneuron

A. Cell-attached recording of a candidate low-frequency firing neuron in area X before and after application of 250 nm apamin.

B. ISI distribution before and after apamin application.

Are glutamatergic neurons a synaptically-connected network?

In a few cases we were able to record simultaneous voltage-clamp activity from two nearby pallidal neurons (Fig. 12). This revealed a high level of coincident EPSCs, suggesting that the glutamatergic neuron is at least locally divergent. This may occur as a result of synaptic connections between excitatory neurons. In support of this idea we found that blocking GABAergic transmission in the isolated preparation of area X results not only in loss of IPSCs, but rapidly transitions into pathological bursting activity, with fairly regular inter-burst intervals (n > 10 neurons) (Fig. 21*A*). Although good voltage-clamp recordings could not be maintained under these conditions, it is likely that bursts are driven by barrages of excitatory synaptic activity (Fig. 21*B*). This epileptiform activity is

may be dependent AMPA receptors, as it is eliminated with application of NBQX (10 μM), but could involve other mechanisms as well. *In situ* hybridization and electrophysiology data alike suggest that excitatory neurons in area X are sparsely distributed (ZEBrA database). However, if these neurons are interconnected and acting as a synchronized network, they would have the ability to potently and rapidly affect area X cell dynamics, perhaps by synchronizing ensembles of pallidal neurons.

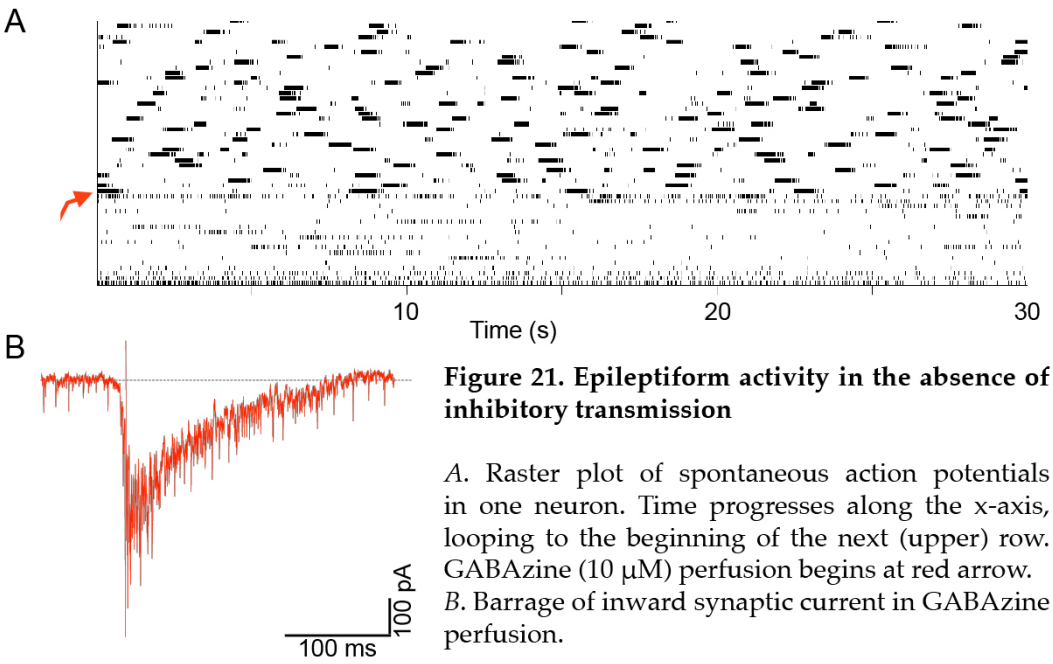

Figure 21. Epileptiform activity in the absence of inhibitory transmission

A. Raster plot of spontaneous action potentials in one neuron. Time progresses along the x-axis, looping to the beginning of the next (upper) row. GABAzine (10 μM) perfusion begins at red arrow. *B*. Barrage of inward synaptic current in GABAzine perfusion.

What is the role of local excitatory activity in area X in the behaving adult animal?

How might local excitation in area X contribute to social context dependent state transitions and juvenile learning alike? This first *in vivo* experiment that comes to mind to address this question is examining song variability in juvenile birds, and in directed song, while chronically infusing AMPA-type glutamate receptor blockers into area

X. However, the results would be difficult to interpret as area X receives excitatory drive from HVC and LMAN during song production, and in fact most excitatory synapses onto area X neurons are made by these afferent fibers. Optogenetics combined with cell-type specific targeting would be the ideal approach to this question (Tye and Deisseroth, 2012).

One future direction is to target the light-activated channels halorhodopsin or archeorhodopsin to area X neurons that normally express the VGluT2 promoter. Implanting a fiber optic cable under the skull and delivering light pulses during the production of song could provide valuable information about local excitation's role in the network. Silencing glutamate neurons while the bird is singing undirected song might decrease the variability that is normally present in the state, mimicking the directed state without the actual presence of a female bird. However, interpretation of even this experiment could be complicated if glutamate is in fact co-released from a GABAergic cell type in the nucleus. A less ambitious but nonetheless informative use of cell type-specific silencing, or activation channelrhodopsin, would be manipulation of glutamatergic neurons in the slice preparation, in order to address the potential ramifications of a synchronized glutamatergic network on pallidal neuron firing properties.

Unfortunately, while there are many advantages to the songbird system, there are also technical limitations. Transgenic zebra finches are just finally making their debut (Abe et al., 2015), and while the zebra finch genome is sequenced (Warren et al., 2010), it remains largely not annotated. Identification of cell type-specific promoters is thus a challenge. Beyond this there is limited knowledge about the infection efficiency of viral vector serotypes in different brain regions. While there is a long (and possibly endless) road ahead before these tools are accessible in

songbirds in the way that they are in rodents, there has been significant progress (Roberts et al., 2012; Abe et al., 2015). We have been conducting very preliminary experiments, expressing GFP-channelrhodopsin virally in various parts of the song circuit, and testing for blue light-evoked responses (Fig. 22).

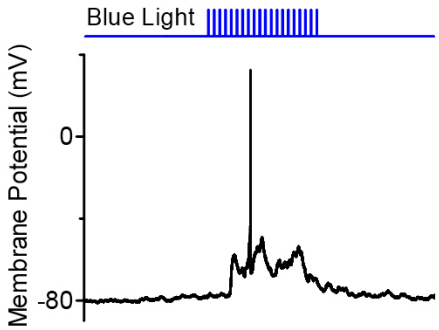

Figure 22. Preliminary light-evoked response in brain slice

HVC neuron showing blue light-evoked activation in current-clamp recording from brain slice.

Conclusions

Area X is striato-pallidal nucleus in the songbird integral to song learning that contributes to the modulation or perhaps generation of exploratory variability. We have contributed to understanding the circuit components of this complex nucleus by identifying a novel glutamatergic cell type. This putative glutamatergic neuron contributes to the variability of firing in area X projection neurons, a circuit feature that is modulated by dopamine mediated neural changes in social context. This putative interneuron can cause either excitatory events alone or excitatory events coupled with inhibition in pallidal neurons, and the probability of detecting either class of synaptic event is tunable through dopamine D1 receptor activation. Finally, we find that a simple phase model

with minimal assumptions can account for these two main findings and allows predictions of ensemble output from area X.

The putative local glutamatergic neuron is spontaneously active and thus may be playing an analogous role to the STN in mammals. Future work, possibly with the use of optogenetic targeting, will be necessary to determine the validity of this hypothesis by characterizing glutamatergic neuron cytochemical and intrinsic electrophysiological properties, as well as any sources of synaptic input. Independent of any specific similarities to the STN, the potential for dopamine to either synchronize or desynchronize a modeled population of area X output neurons through the modification of the putative glutamatergic input could provide important insight into the function and dysfunction of coupled neural oscillators in the basal ganglia.

Finally, glutamate may be co-released along with GABA in area X. While this has been reported in other systems, particularly in development, it is still a somewhat novel oddity of the nervous system. If glutamate/GABA co-release in area X is confirmed in the future, area X could be a fruitful location in which to study the functional role of co-release in an adult circuit whose activity can be correlated with a specific and measurable behavioral output.

References

Abe, K., Matsui, S., and Watanabe, D. (2015). Transgenic songbirds with suppressed or enhanced activity of CREB transcription factor. Proc Natl Acad Sci U S A 112, 7599-7604.

Albin, R.L., Young, A.B., and Penney, J.B. (1989). The functional anatomy of basal ganglia disorders. Trends Neurosci *12*, 366-375.

Ali, F., Otchy, T.M., Pehlevan, C., Fantana, A.L., Burak, Y., and Ölveczky, B.P. (2013). The basal ganglia is necessary for learning spectral, but not temporal, features of birdsong. Neuron *80*, 494-506.

Andalman, A.S., and Fee, M.S. (2009). A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. Proc Natl Acad Sci U S A *106*, 12518-12523.

Antal, M., Beneduce, B.M., and Regehr, W.G. (2014). The substantia nigra conveys target-dependent excitatory and inhibitory outputs from the basal ganglia to the thalamus. J Neurosci *34*, 8032-8042.

Aosaki, T., Tsubokawa, H., Ishida, A., Watanabe, K., Graybiel, A.M., and Kimura, M. (1994). Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. J Neurosci *14*, 3969-3984.

Apicella, P. (2007). Leading tonically active neurons of the striatum from reward detection to context recognition. Trends Neurosci *30*, 299-306.

Appeltants, D., Absil, P., Balthazart, J., and Ball, G.F. (2000). Identification of the origin of catecholaminergic inputs to HVc in canaries by retrograde tract tracing combined with tyrosine hydroxylase immunocytochemistry. J Chem Neuroanat *18*, 117-133.

Aronov, D., Andalman, A.S., and Fee, M.S. (2008). A specialized forebrain circuit for vocal babbling in the juvenile songbird. Science *320*, 630-634.

Aronov, D., Veit, L., Goldberg, J.H., and Fee, M.S. (2011). Two distinct modes of forebrain circuit dynamics underlie temporal patterning in the vocalizations of young songbirds. J Neurosci *31*, 16353-16368.

Atoji, Y., and Karim, M.R. (2014). Expression of vesicular glutamate transporter 3 mRNA in the brain and retina of the pigeon. J Chem Neuroanat *61-62*, 124-131.

Baptista, L.F., and Schuchmann, K.-L. (1990). Song learning in the Anna hummingbird (Calypte anna). Ethology *84*, 15-26.

Bar-Gad, I., Morris, G., and Bergman, H. (2003). Information processing, dimensionality reduction and reinforcement learning in the basal ganglia. Prog Neurobiol *71*, 439-473.

Barto, A.G. (1995). Adaptive critics and the basal ganglia. In Models of information processing in the basal ganglia, Houk J.C., Davis J.L., and Beiser D.G., ed. (Cambridge, Massachusetts: MIT press), pp. 215-232.

Barto, A.G., Sutton, R.S., and Anderson, C.W. (1983). Neuronlike adaptive elements that can solve difficult learning control problems. Systems, Man and Cybernetics, IEEE Transactions on , 834-846.

Beecher, M.D., Stoddard, P.K., Campbell L, E.S., and Horning, C.L. (1996). Repertoire matching between neighbouring song sparrows. Animal Behaviour *51*, 917-923.

Bennett, B.D., and Wilson, C.J. (1999). Spontaneous activity of neostriatal cholinergic interneurons in vitro. J Neurosci *19*, 5586-5596.

Bergman, H., Wichmann, T., and DeLong, M.R. (1990). Reversal of experimental parkinsonism by lesions of the subthalamic nucleus. Science *249*, 1436-1438.

Beurrier, C., Congar, P., Bioulac, B., and Hammond, C. (1999). Subthalamic nucleus neurons switch from single-spike activity to burst-firing mode. J Neurosci *19*, 599-609.

Bevan, M.D., and Wilson, C.J. (1999). Mechanisms underlying spontaneous oscillation and rhythmic firing in rat subthalamic neurons. J Neurosci *19*, 7617-7628.

Bevan, M.D., Magill, P.J., Hallworth, N.E., Bolam, J.P., and Wilson, C.J. (2002a). Regulation of the timing and pattern of action potential generation in rat subthalamic neurons in vitro by GABA-A IPSPs. J Neurophysiol *87*, 1348-1362.

Bevan, M.D., Magill, P.J., Terman, D., Bolam, J.P., and Wilson, C.J. (2002b). Move to the rhythm: oscillations in the subthalamic nucleus-external globus pallidus network. Trends Neurosci *25*, 525-531.

Bottjer, S.W. (1993). The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. J Neurobiol *24*, 51-69.

Bottjer, S.W., Miesner, E.A., and Arnold, A.P. (1984). Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science *224*, 901.

Boughey, M.J., and Thompson, N.S. (1981). Song variety in the brown thrasher (Toxostoma rufum). Zeitschrift für Tierpsychologie *56*, 47-58.

Boughman, J.W. (1998). Vocal learning by greater spear-nosed bats. Proc Biol Sci *265*, 227-233.

Boulland, J.L., Jenstad, M., Boekel, A.J., Wouterlood, F.G., Edwards, R.H., Storm-Mathisen, J., and

Chaudhry, F.A. (2009). Vesicular glutamate and GABA transporters sort to distinct sets of vesicles in a population of presynaptic terminals. Cereb Cortex *19*, 241-248.

Boulland, J.L., Qureshi, T., Seal, R.P., Rafiki, A., Gundersen, V., Bergersen, L.H., Fremeau, R.T., Edwards, R.H., Storm-Mathisen, J., and Chaudhry, F.A. (2004). Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. J Comp Neurol *480*, 264-280.

Böhner, J. (1990). Early acquisition of song in the zebra finch, Taeniopygia guttata. Animal Behaviour *39*, 369-374.

Börgers, C., and Kopell, N. (2003). Synchronization in networks of excitatory and inhibitory neurons with sparse, random connectivity. Neural Comput *15*, 509-538.

Brainard, M.S., and Doupe, A.J. (2000a). Auditory feedback in learning and maintenance of vocal behaviour. Nature Reviews Neuroscience *1*, 31-40.

Brainard, M.S., and Doupe, A.J. (2000b). Interruption of a basal ganglia-forebrain circuit prevents plasticity of learned vocalizations. Nature *404*, 762-766.

Bransford, J.D., Brown, A.L., Cocking, R.L., Committee on Developments in the Science of Learning and Committee on Learning Research and Educational Practice, Commission on Behavioral and Social Sciences and Education, National Research Council (2000). How people learn: mind, brain, experience, and school. National Academy Press.

Brenowitz, E.A., and Beecher, M.D. (2005). Song learning in birds: diversity and plasticity, opportunities and challenges. Trends Neurosci *28*, 127-132.

Brenowitz, E.A., and Zakon, H.H. (2015). Emerging from the bottleneck: benefits of the comparative approach to modern neuroscience. Trends Neurosci *38*, 273-278.

Britten, K.H., Shadlen, M.N., Newsome, W.T., and Movshon, J.A. (1992). The analysis of visual motion: a comparison of neuronal and psychophysical performance. J Neurosci *12*, 4745-4765.

Broussard, D.M., and Kassardjian, C.D. (2004). Learning in a simple motor system. Learn Mem *11*, 127-136.

Brown, P., Oliviero, A., Mazzone, P., Insola, A., Tonali, P., and Di Lazzaro, V. (2001). Dopamine dependency of oscillations between subthalamic nucleus and pallidum in Parkinson's disease. J Neurosci *21*, 1033-1038.

Butler, A.B., Reiner, A., and Karten, H.J. (2011). Evolution of the amniote pallium and the origins of mammalian neocortex. Ann N Y Acad Sci *1225*, 14-27.

Calabresi, P., Picconi, B., Tozzi, A., and Di Filippo, M. (2007). Dopamine-mediated regulation of corticostriatal synaptic plasticity. Trends Neurosci *30*, 211-219.

Carrillo, G.D., and Doupe, A.J. (2004). Is the songbird Area X striatal, pallidal, or both? An anatomical study. J Comp Neurol *473*, 415-437.

Chaiken, M., Böhner, J., and Marler, P. (1994). Repertoire turnover and the timing of song acquisition in European starlings. Behaviour *128*, 25-39.

Chuhma, N., Zhang, H., Masson, J., Zhuang, X., Sulzer, D., Hen, R., and Rayport, S. (2004). Dopamine neurons mediate a fast excitatory signal via their glutamatergic synapses. J Neurosci *24*, 972-981.

Churchland, M.M., Afshar, A., and Shenoy, K.V. (2006). A central source of movement variability. Neuron *52*, 1085-1096.

Cohen, D., and Nicolelis, M.A. (2004). Reduction of single-neuron firing uncertainty by cortical ensembles during motor skill learning. J Neurosci *24*, 3574-3582.

Colonnier, M. (1968). Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. Brain Res *9*, 268-287.

Cruz, A.V., Mallet, N., Magill, P.J., Brown, P., and Averbeck, B.B. (2011). Effects of dopamine depletion on information flow between the subthalamic nucleus and external globus pallidus. J Neurophysiol *106*, 2012-2023.

Dave, A.S., and Margoliash, D. (2000). Song replay during sleep and computational rules for sensorimotor vocal learning. Science *290*, 812.

Dayan, P., and Abbott, L.F. (2001). Theoretical neuroscience (Cambridge, MA: MIT Press).

DeLong, M.R. (1971). Activity of pallidal neurons during movement. J Neurophysiol *34*, 414-427.

DeLong, M.R. (1972). Activity of basal ganglia neurons during movement. Brain Res *40*, 127-135.

DeLong, M.R. (1990). Primate models of movement disorders of basal ganglia origin. Trends Neurosci *13*, 281-285.

Derégnaucourt, S., Mitra, P.P., Fehér, O., Pytte, C., and Tchernichovski, O. (2005). How sleep affects the developmental learning of bird song. Nature *433*, 710-716.

Ding, L., and Perkel, D.J. (2002). Dopamine modulates excitability of spiny neurons in the avian basal ganglia. J Neurosci *22*, 5210-5218.

Ding, L., and Perkel, D.J. (2004). Long-term potentiation in an avian basal ganglia nucleus essential for vocal learning. J Neurosci *24*, 488-494.

Ding, L., Perkel, D.J., and Farries, M.A. (2003). Presynaptic depression of glutamatergic synaptic transmission by D1-like dopamine receptor activation in the avian basal ganglia. J Neurosci *23*, 6086-6095.

Di Pino, G., Maravita, A., Zollo, L., Guglielmelli, E., and Di Lazzaro, V. (2014). Augmentation-related brain plasticity. Front Syst Neurosci *8*, 109.

Doupe, A.J., and Konishi, M. (1991). Song-selective auditory circuits in the vocal control system of the zebra finch. Proc Natl Acad Sci U S A *88*, 11339-11343.

Doupe, A.J., and Kuhl, P.K. (1999). Birdsong and human speech: common themes and mechanisms. Annu Rev Neurosci *22*, 567-631.

Doupe, A.J., and Solis, M.M. (1997). Song- and order-selective neurons develop in the songbird anterior forebrain during vocal learning. J Neurobiol *33*, 694-709.

Doupe, A.J., Perkel, D.J., Reiner, A., and Stern, E.A. (2005). Birdbrains could teach basal ganglia research a new song. Trends Neurosci *28*, 353-363.

Doya, K., and Sejnowski, T.J. (1995). A novel reinforcement model of birdsong vocalization learning. Advances in neural information processing systems , 101-108.

Faisal, A.A., Selen, L.P., and Wolpert, D.M. (2008). Noise in the nervous system. Nat Rev Neurosci *9*, 292-303.

Falls, J.B. (1988). Does song deter territorial intrusion in White-throated Sparrows (Zonotrichia albicollis)? Canadian Journal of Zoology *66*, 206-211.

Farabaugh, S.M., Linzenbold, A., and Dooling, R.J. (1994). Vocal plasticity in Budgerigars (Melopsittacus undulatus): evidence for social factors in the learning of contact calls. Journal of Comparative Psychology *108*, 81.

Farries, M.A., and Perkel, D.J. (2000). Electrophysiological properties of avian basal ganglia neurons recorded in vitro. J Neurophysiol *84*, 2502-2513.

Farries, M.A., and Perkel, D.J. (2002). A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J Neurosci *22*, 3776-3787.

Farries, M.A., and Wilson, C.J. (2012). Phase response curves of subthalamic neurons measured with synaptic input and current injection. J Neurophysiol *108*, 1822-1837.

Farries, M.A., Ding, L., and Perkel, D.J. (2005). Evidence for "direct" and "indirect" pathways through the song system basal ganglia. J Comp Neurol *484*, 93-104.

Fattorini, G., Verderio, C., Melone, M., Giovedì, S., Benfenati, F., Matteoli, M., and Conti, F. (2009). VGLUT1 and VGAT are sorted to the same population of synaptic vesicles in subsets of cortical axon terminals. J Neurochem *110*, 1538-1546.

Fee, M.S., and Goldberg, J.H. (2011). A hypothesis for basal ganglia-dependent reinforcement learning in the songbird. Neuroscience

Fehér, O., Wang, H., Saar, S., Mitra, P.P., and Tchernichovski, O. (2009). De novo establishment of wild-type song culture in the zebra finch. Nature *459*, 564-568.

Fiete, I.R., and Seung, H.S. (2008). Neural network models of birdsong production, learning, and coding. The New Encyclopedia of Neuroscience, L. Squire, T. Albright, F. Bloom, F. Gage, and N. Spitzer, eds.(New York: Elsevier)

Fiete, I.R., Fee, M.S., and Seung, H.S. (2007). Model of birdsong learning based on gradient estimation by dynamic perturbation of neural conductances. J Neurophysiol *98*, 2038-2057.

Fritschy, J.M., and Panzanelli, P. (2014). GABAA receptors and plasticity of inhibitory neurotransmission in the central nervous system. Eur J Neurosci *39*, 1845-1865.

Fujita, T., Fukai, T., and Kitano, K. (2012). Influences of membrane properties on phase response curve and synchronization stability in a model globus pallidus neuron. J Comput Neurosci *32*, 539-553.

Fukai, T. (1999). Sequence generation in arbitrary temporal patterns from theta-nested gamma oscillations: a model of the basal ganglia-thalamo-cortical loops. Neural Netw *12*, 975-987.

Gabernet, L., Jadhav, S.P., Feldman, D.E., Carandini, M., and Scanziani, M. (2005). Somatosensory integration controlled by dynamic thalamocortical feed-forward inhibition. Neuron *48*, 315-327.

Gale, S.D., and Perkel, D.J. (2005). Properties of dopamine release and uptake in the songbird basal ganglia. J Neurophysiol *93*, 1871-1879.

Gale, S.D., and Perkel, D.J. (2006). Physiological properties of zebra finch ventral tegmental area and substantia nigra pars compacta neurons. J Neurophysiol *96*, 2295-2306.

Gale, S.D., and Perkel, D.J. (2010). A basal ganglia pathway drives selective auditory responses in songbird dopaminergic neurons via disinhibition. J Neurosci *30*, 1027-1037.

Gale, S.D., Person, A.L., and Perkel, D.J. (2008). A novel basal ganglia pathway forms a loop linking a vocal learning circuit with its dopaminergic input. J Comp Neurol *508*, 824-839.

Garst-Orozco, J., Babadi, B., and Ölveczky, B.P. (2014). A neural circuit mechanism for regulating vocal variability during song learning in zebra finches. Elife *4*, e03697.

Gillespie, D.C., Kim, G., and Kandler, K. (2005). Inhibitory synapses in the developing auditory system are glutamatergic. Nat Neurosci *8*, 332-338.

Giret, N., Kornfeld, J., Ganguli, S., and Hahnloser, R.H. (2014). Evidence for a causal inverse model in an avian cortico-basal ganglia circuit. Proc Natl Acad Sci U S A *111*, 6063-6068.

Goldberg, J.H., Adler, A., Bergman, H., and Fee, M.S. (2010). Singing-related neural activity distinguishes two putative pallidal cell types in the songbird basal ganglia: comparison to the primate internal and external pallidal segments. The Journal of Neuroscience *30*, 7088.

Goldberg, J.H., and Fee, M.S. (2010). Singing-related neural activity distinguishes four classes of putative striatal neurons in the songbird basal ganglia. J Neurophysiol *103*, 2002-2014.

Goldberg, J.H., and Fee, M.S. (2011). Vocal babbling in songbirds requires the basal ganglia-recipient motor thalamus but not the basal ganglia. J Neurophysiol *105*, 2729-2739.

Goldberg, J.H., and Fee, M.S. (2012). A cortical motor nucleus drives the basal ganglia-recipient thalamus in singing birds. Nat Neurosci

Goldberg, J.H., Farries, M.A., and Fee, M.S. (2012). Integration of cortical and pallidal inputs in the basal ganglia-recipient thalamus of singing birds. J Neurophysiol *108*, 1403-1429.

Goldberg, J.H., Farries, M.A., and Fee, M.S. (2013). Basal ganglia output to the thalamus: still a paradox. Trends Neurosci *36*, 695-705.

Goldman, S.A., and Nottebohm, F. (1983). Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A *80*, 2390-2394.

Grace, A.A. (1991). Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. Neuroscience *41*, 1-24.

Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B., and El Mestikawy, S. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J Neurosci *22*, 5442-5451.

Graybiel, A.M. (1990). The basal ganglia and the initiation of movement. Rev Neurol (Paris) *146*, 570-574.

Graybiel, A.M. (1995). Building action repertoires: memory and learning functions of the basal ganglia. Curr Opin Neurobiol *5*, 733-741.

Graybiel, A.M., and Ragsdale, C.W. (1978). Histochemically distinct compartments in the striatum of human, monkeys, and cat demonstrated by acetylthiocholinesterase staining. Proceedings of the National Academy of Sciences *75*, 5723-5726.

Groves, P.M., Garcia-Munoz, M., Linder, J.C., Manley, M.S., Martone, M.E., and Young, S.J. (1995). Elements of the intrinsic organization and information processing in the neostriatum. In Models of information processing in the basal ganglia, Houk J.C., Davis J.L., and Beiser D.G., ed. (Cambridge, Massachusetts: MIT press), pp. 51-96.

Gutkin, B.S., Ermentrout, G.B., and Reyes, A.D. (2005). Phase-response curves give the responses of neurons to transient inputs. J Neurophysiol *94*, 1623-1635.

Guzman, M.S., De Jaeger, X., Raulic, S., Souza, I.A., Li, A.X., Schmid, S., Menon, R.S., Gainetdinov, R.R., Caron, M.G., Bartha, R., Prado, V.F., and Prado, M.A. (2011). Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS Biol *9*, e1001194.

Hahnloser, R.H., Kozhevnikov, A.A., and Fee, M.S. (2002). An ultra-sparse code underlies the generation of neural sequences in a songbird. Nature *419*, 65-70.

Hahnloser, R.H., Kozhevnikov, A.A., and Fee, M.S. (2006). Sleep-related neural activity in a premotor and a basal-ganglia pathway of the songbird. J Neurophysiol *96*, 794-812.

Hamaguchi, K., and Mooney, R. (2012). Recurrent Interactions between the Input and Output of a Songbird Cortico-Basal Ganglia Pathway Are Implicated in Vocal Sequence Variability. J Neurosci *32*, 11671-11687.

Harding, C.F. (2004). Brief alteration in dopaminergic function during development causes deficits in adult reproductive behavior. J Neurobiol *61*, 301-308.

Harding, C.F., Barclay, S.R., and Waterman, S.A. (1998). Changes in catecholamine levels and turnover rates in hypothalamic, vocal control, and auditory nuclei in male zebra finches during development. J Neurobiol *34*, 329-346.

Hebb, D.O. (2005). The organization of behavior: A neuropsychological theory (Psychology Press).

Herkenham, M., and Pert, C.B. (1981). Mosaic distribution of opiate receptors, parafascicular projections and acetylcholinesterase in rat striatum.

Hessler, N.A., and Doupe, A.J. (1999). Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. J Neurosci *19*, 10461-10481.

Higley, M.J., Gittis, A.H., Oldenburg, I.A., Balthasar, N., Seal, R.P., Edwards, R.H., Lowell, B.B., Kreitzer, A.C., and Sabatini, B.L. (2011). Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. PLoS One *6*, e19155.

Hikosaka, O., and Wurtz, R.H. (1989). The basal ganglia. Rev Oculomot Res *3*, 257-281.

Hikosaka, O., Nakamura, K., Sakai, K., and Nakahara, H. (2002). Central mechanisms of motor skill learning. Curr Opin Neurobiol *12*, 217-222.

Hoover, J.E., and Strick, P.L. (1993). Multiple output channels in the basal ganglia. Science *259*, 819-821.

Houk, J.C., Davis, J.L., and Beiser, D.G. (1995). Models of information processing in the basal ganglia (Cambridge, Massachusetts: MIT press).

Hultsch, H. (1992). Tracing the memory mechanisms in the song acquisition of nightingales. Netherlands journal of zoology *43*, 155-171.

Hultsch, H., and Todt, D. (1989). Memorization and reproduction of songs in nightingales (Luscinia megarhynchos): evidence for package formation. Journal of Comparative Physiology A *165*, 197-203.

Hunnicutt, B.J., Long, B.R., Kusefoglu, D., Gertz, K.J., Zhong, H., and Mao, T. (2014). A comprehensive thalamocortical projection map at the mesoscopic level. Nat Neurosci *17*, 1276-1285.

Iyengar, S., Viswanathan, S.S., and Bottjer, S.W. (1999). Development of topography within song control circuitry of zebra finches during the sensitive period for song learning. J Neurosci *19*, 6037-6057.

Izhikevich, E.M. (2007). Dynamical systems in neuroscience (MIT press).

Jaeger, D., Kita, H., and Wilson, C.J. (1994). Surround inhibition among projection neurons is weak or nonexistent in the rat neostriatum. J Neurophysiol *72*, 2555-2558.

Janik, V.M. (2000). Whistle matching in wild bottlenose dolphins (Tursiops truncatus). Science *289*, 1355-1357.

Jarvis, E.D., Güntürkün, O., Bruce, L., Csillag, A., Karten, H., Kuenzel, W., Medina, L., Paxinos, G., Perkel, D.J., Shimizu, T., Striedter, G., Wild, J.M., Ball, G.F., Dugas-Ford, J., Durand, S.E., Hough, G.E., Husband, S., Kubikova, L., Lee, D.W., Mello, C.V., Powers, A., Siang, C., Smulders, T.V., Wada, K., White, S.A., Yamamoto, K., Yu, J., Reiner, A., Butler, A.B., and Avian Brain Nomenclature Consortium (2005). Avian brains and a new understanding of vertebrate brain evolution. Nat Rev Neurosci *6*, 151-159.

Jarvis, E.D., Scharff, C., Grossman, M.R., Ramos, J.A., and Nottebohm, F. (1998). For whom the bird sings: context-dependent gene expression. Neuron *21*, 775-788.

Jiao, Y., Medina, L., Veenman, C.L., Toledo, C., Puelles, L., and Reiner, A. (2000). Identification of the anterior nucleus of the ansa lenticularis in birds as the homolog of the mammalian subthalamic nucleus. J Neurosci *20*, 6998-7010.

Johnson, F., Sablan, M.M., and Bottjer, S.W. (1995). Topographic organization of a forebrain pathway involved with vocal learning in zebra finches. J Comp Neurol *358*, 260-278.

Kao, M.H., and Brainard, M.S. (2006). Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability. J Neurophysiol *96*, 1441-1455.

Kao, M.H., Doupe, A.J., and Brainard, M.S. (2005). Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song. Nature *433*, 638-643.

Kao, M.H., Wright, B.D., and Doupe, A.J. (2008). Neurons in a forebrain nucleus required for vocal plasticity rapidly switch between precise firing and variable bursting depending on social context. J Neurosci *28*, 13232-13247.

Kao, Y.H., Lassová, L., Bar-Yehuda, T., Edwards, R.H., Sterling, P., and Vardi, N. (2004). Evidence that certain retinal bipolar cells use both glutamate and GABA. J Comp Neurol *478*, 207-218.

Karten, H.J. (1991). Homology and evolutionary origins of the'neocortex'. Brain, behavior and evolution *38*, 264-272.

Karten, H.J., and Dubbeldam, J.L. (1973). The organization and projections of the paleostriatal complex in the pigeon (Columba livia). J Comp Neurol *148*, 61-89.

Kawaguchi, Y. (1993). Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum. J Neurosci *13*, 4908-4923.

Kawaguchi, Y., Wilson, C.J., Augood, S.J., and Emson, P.C. (1995). Striatal interneurones: chemical, physiological and morphological characterization. Trends Neurosci *18*, 527-535.

Kemp, J.M. (1968). An electron microscopic study of the termination of afferent fibres in the caudate nucleus. Brain Res *11*, 464-467.

Kemp, J.M., and Powell, T.P.S. (1970). The cortico-striate projection in the monkey. Brain *93*, 525-546.

Kimpo, R.R., Theunissen, F.E., and Doupe, A.J. (2003). Propagation of correlated activity through multiple stages of a neural circuit. J Neurosci *23*, 5750-5761.

Kimura, M., Rajkowski, J., and Evarts, E. (1984). Tonically discharging putamen neurons exhibit set-dependent responses. Proc Natl Acad Sci U S A *81*, 4998-5001.

Klump, G.M., and Shalter, M.D. (1984). Acoustic behaviour of birds and mammals in the predator context; I. Factors affecting the structure of alarm signals. II. The functional significance and evolution of alarm signals. Zeitschrift für Tierpsychologie *66*, 189-226.

Kojima, S., and Doupe, A.J. (2009). Activity propagation in an avian basal ganglia-thalamocortical circuit essential for vocal learning. J Neurosci *29*, 4782-4793.

Kojima, S., and Doupe, A.J. (2011). Social performance reveals unexpected vocal competency in young songbirds. Proc Natl Acad Sci U S A *108*, 1687-1692.

Kojima, S., Kao, M.H., and Doupe, A.J. (2013). Task-related "cortical" bursting depends critically on basal ganglia input and is linked to vocal plasticity. Proc Natl Acad Sci U S A *110*, 4756-4761.

Konishi, M. (1965). The Role of Auditory Feedback in the Control of Vocalization in the White-Crowned Sparrow1. Zeitschrift für Tierpsychologie *22*, 770-783.

Koós, T., and Tepper, J.M. (1999). Inhibitory control of neostriatal projection neurons by GABAergic interneurons. Nat Neurosci *2*, 467-472.

Kosche, G., Vallentin, D., and Long, M.A. (2015). Interplay of inhibition and excitation shapes a premotor neural sequence. J Neurosci *35*, 1217-1227.

Kozhevnikov, A.A., and Fee, M.S. (2007). Singing-related activity of identified HVC neurons in the zebra finch. J Neurophysiol *97*, 4271-4283.

Krack, P., Benazzouz, A., Pollak, P., Limousin, P., Piallat, B., Hoffmann, D., Xie, J., and Benabid, A.L. (1998). Treatment of tremor in Parkinson's disease by subthalamic nucleus stimulation. Mov Disord *13*, 907-914.

Kreitzer, A.C. (2009). Physiology and pharmacology of striatal neurons. Annu Rev Neurosci *32*, 127-147.

Kreitzer, A.C., and Malenka, R.C. (2008). Striatal plasticity and basal ganglia circuit function. Neuron *60*, 543-554.

Kroodsma, D.E., and Parker, L.D. (1977). Vocal virtuosity in the brown thrasher. The Auk , 783-785.

Kroodsma, D.E., Liu, W.-C., Goodwin, E., and Bedell, P.A. (1999). The ecology of song improvisation as illustrated by North American Sedge Wrens. The Auk , 373-386.

Kubikova, L., and Kostál, L. (2010). Dopaminergic system in birdsong learning and maintenance. J Chem Neuroanat *39*, 112-123.

Kullmann, D.M., Ruiz, A., Rusakov, D.M., Scott, R., Semyanov, A., and Walker, M.C. (2005). Presynaptic, extrasynaptic and axonal GABAA receptors in the CNS: where and why? Prog Biophys Mol Biol *87*, 33-46.

Lajoie, G., Thivierge, J.P., and Shea-Brown, E. (2014). Structured chaos shapes spike-response noise entropy in balanced neural networks. Front Comput Neurosci *8*, 123.

Langmore, N.E. (1998). Functions of duet and solo songs of female birds. Trends in Ecology & Evolution *13*, 136-140.

Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E., and Seamans, J.K. (2005). Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. J Neurosci *25*, 5013-5023.

Leblois, A., Bodor, A.L., Person, A.L., and Perkel, D.J. (2009). Millisecond timescale disinhibition mediates fast information transmission through an avian basal ganglia loop. J Neurosci *29*, 15420-15433.

Leblois, A., Wendel, B.J., and Perkel, D.J. (2010). Striatal dopamine modulates basal ganglia output and regulates social context-dependent behavioral variability through D1 receptors. J Neurosci *30*, 5730-5743.

Leonardo, A., and Fee, M.S. (2005). Ensemble coding of vocal control in birdsong. J Neurosci *25*, 652-661.

Leonardo, A., and Konishi, M. (1999). Decrystallization of adult birdsong by perturbation of auditory feedback. Nature *399*, 466-470.

Lewis, J.W., Ryan, S.M., Arnold, A.P., and Butcher, L.L. (1981). Evidence for a catecholaminergic projection to area X in the zebra finch. J Comp Neurol *196*, 347-354.

Limousin, P., Krack, P., Pollak, P., Benazzouz, A., Ardouin, C., Hoffmann, D., and Benabid, A.L. (1998). Electrical stimulation of the subthalamic nucleus in advanced Parkinson's disease. N Engl J Med *339*, 1105-1111.

Limousin, P., Pollak, P., Benazzouz, A., Hoffmann, D., Le Bas, J.F., Broussolle, E., Perret, J.E., and Benabid, A.L. (1995). Effect of parkinsonian signs and symptoms of bilateral subthalamic nucleus stimulation. Lancet *345*, 91-95.

Long, M.A., and Fee, M.S. (2008). Using temperature to analyse temporal dynamics in the songbird motor pathway. Nature *456*, 189-194.

Long, M.A., Jin, D.Z., and Fee, M.S. (2010). Support for a synaptic chain model of neuronal sequence generation. Nature *468*, 394-399.

Luo, M., and Perkel, D.J. (1999a). A GABAergic, strongly inhibitory projection to a thalamic nucleus in the zebra finch song system. The Journal of neuroscience *19*, 6700.

Luo, M., and Perkel, D.J. (1999b). Long-range GABAergic projection in a circuit essential for vocal learning. J Comp Neurol *403*, 68-84.

Luo, M., and Perkel, D.J. (2002). Intrinsic and synaptic properties of neurons in an avian thalamic nucleus during song learning. J Neurophysiol *88*, 1903-1914.

Luo, M., Ding, L., and Perkel, D.J. (2001). An avian basal ganglia pathway essential for vocal learning forms a closed topographic loop. J Neurosci *21*, 6836-6845.

Mahrt, E.J., Perkel, D.J., Tong, L., Rubel, E.W., and Portfors, C.V. (2013). Engineered deafness reveals that mouse courtship vocalizations do not require auditory experience. J Neurosci *33*, 5573-5583.

Mandelblat-Cerf, Y., Las, L., Denisenko, N., and Fee, M.S. (2014). A role for descending auditory cortical projections in songbird vocal learning. Elife *3*

Mark, G.P., Shabani, S., Dobbs, L.K., and Hansen, S.T. (2011). Cholinergic modulation of mesolimbic dopamine function and reward. Physiol Behav *104*, 76-81.

Marler, P. (1955). Characteristics of some animal calls.

Marler, P. (1970). A comparative approach to vocal learning: song development in White-crowned Sparrows. J Comp Physiol Psychol *71*, 1.

Marler, P., and Tamura, M. (1964). Culturally transmitted patterns of vocal behavior in sparrows. Science *146*, 1483-1486.

Marler, P., and Waser, M.S. (1977). Role of auditory feedback in canary song development. J Comp Physiol Psychol *91*, 8-16.

Marler, P.R., and Slabbekoorn, H. (2004). Nature's music: the science of birdsong (Academic Press).

Martin, J.H., Leonard, M.E., and Radzyner, H. (1989). Neuroanatomy: text and atlas (Elsevier Amsterdam).

McCulloch, W.S., and Pitts, W. (1943). A logical calculus of the ideas immanent in nervous activity. The bulletin of mathematical biophysics *5*, 115-133.

McGregor, P.K. (2013). Playback and studies of animal communication (Springer Science & Business Media).

Mindlin, G.B., and Laje, R. (2006). The physics of birdsong (Springer Science & Business Media).

Montague, P.R., Dayan, P., and Sejnowski, T.J. (1996). A framework for mesencephalic dopamine systems based on predictive Hebbian learning. The Journal of neuroscience *16*, 1936-1947.

Mooney, R. (1992). Synaptic basis for developmental plasticity in a birdsong nucleus. J Neurosci *12*, 2464-2477.

Mooney, R., and Konishi, M. (1991). Two distinct inputs to an avian song nucleus activate different glutamate receptor subtypes on individual neurons. Proc Natl Acad Sci U S A *88*, 4075-4079.

Morgan, J.L., and Kerschensteiner, D. (2011). Shooting DNA, dyes, or indicators into tissue slices using the gene gun. Cold Spring Harb Protoc *2011*, 1512-1514.

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods *11*, 47-60.

Nordeen, K.W., and Nordeen, E.J. (1992). Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. Behav Neural Biol *57*, 58-66.

Nordeen, K.W., and Nordeen, E.J. (2010). Deafening-induced vocal deterioration in adult songbirds is reversed by disrupting a basal ganglia-forebrain circuit. J Neurosci *30*, 7392-7400.

Nottebohm, F. (1980). Testosterone triggers growth of brain vocal control nuclei in adult female canaries. Brain Res *189*, 429-436.

Nottebohm, F., and Arnold, A.P. (1976). Sexual dimorphism in vocal control areas of the songbird brain. Science *194*, 211-213.

Nottebohm, F., Kelley, D.B., and Paton, J.A. (1982). Connections of vocal control nuclei in the canary telencephalon. J Comp Neurol *207*, 344-357.

Nottebohm, F., Nottebohm, M.E., and Crane, L. (1986). Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. Behav Neural Biol *46*, 445-471.

Nottebohm, F., Stokes, T.M., and Leonard, C.M. (1976). Central control of song in the canary, Serinus canarius. J Comp Neurol *165*, 457-486.

Oh, S.W., Harris, J.A., Ng, L., Winslow, B., Cain, N., Mihalas, S., Wang, Q., Lau, C., Kuan, L., Henry, A.M., Mortrud, M.T., Ouellette, B., Nguyen, T.N., Sorensen, S.A., Slaughterbeck, C.R., Wakeman, W., Li, Y., Feng, D., Ho, A., Nicholas, E., Hirokawa, K.E., Bohn, P., Joines, K.M., Peng, H., Hawrylycz, M.J., Phillips, J.W., Hohmann, J.G., Wohnoutka, P., Gerfen, C.R., Koch, C., Bernard, A., Dang, C., Jones, A.R., and Zeng, H. (2014). A mesoscale connectome of the mouse brain. Nature *508*, 207-214.

Okanoya, K. (2004). The Bengalese finch: a window on the behavioral neurobiology of birdsong syntax. Ann N Y Acad Sci *1016*, 724-735.

Ölveczky, B.P., Andalman, A.S., and Fee, M.S. (2005). Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. PLoS Biol *3*, e153.

Ölveczky, B.P., Otchy, T.M., Goldberg, J.H., Aronov, D., and Fee, M.S. (2011). Changes in the neural control of a complex motor sequence during learning. J Neurophysiol *106*, 386-397.

Pascual-Leone, A., Amedi, A., Fregni, F., and Merabet, L.B. (2005). The plastic human brain cortex. Annu Rev Neurosci *28*, 377-401.

Person, A.L., and Perkel, D.J. (2005). Unitary IPSPs drive precise thalamic spiking in a circuit required for learning. Neuron *46*, 129-140.

Person, A.L., Gale, S.D., Farries, M.A., and Perkel, D.J. (2008). Organization of the songbird basal ganglia, including area X. J Comp Neurol *508*, 840-866.

Pidoux, M., Bollu, T., Riccelli, T., and Goldberg, J.H. (2015). Origins of basal ganglia output signals in singing juvenile birds. J Neurophysiol *113*, 843-855.

Pignatelli, M., and Bonci, A. (2015). Role of Dopamine Neurons in Reward and Aversion: A Synaptic Plasticity Perspective. Neuron *86*, 1145-1157.

Plenz, D., and Kital, S.T. (1999). A basal ganglia pacemaker formed by the subthalamic nucleus and external globus pallidus. Nature *400*, 677-682.

Rajan, K., Abbott, L.F., and Sompolinsky, H. (2010). Stimulus-dependent suppression of chaos in recurrent neural networks. Physical Review E *82*, 011903.

Reiner, A., Laverghetta, A.V., Meade, C.A., Cuthbertson, S.L., and Bottjer, S.W. (2004). An immunohistochemical and pathway tracing study of the striatopallidal organization of area X in the male zebra finch. J Comp Neurol *469*, 239-261.

Reiner, A., Medina, L., and Veenman, C.L. (1998). Structural and functional evolution of the basal ganglia in vertebrates. Brain research reviews *28*, 235-285.

Reiner, A., Yamamoto, K., and Karten, H.J. (2005). Organization and evolution of the avian forebrain. Anat Rec A Discov Mol Cell Evol Biol *287*, 1080-1102.

Roberts, T.F., Gobes, S.M., Murugan, M., Ölveczky, B.P., and Mooney, R. (2012). Motor circuits are required to encode a sensory model for imitative learning. Nat Neurosci *15*, 1454-1459.

Root, D.H., Mejias-Aponte, C.A., Zhang, S., Wang, H.L., Hoffman, A.F., Lupica, C.R., and Morales, M. (2014). Single rodent mesohabenular axons release glutamate and GABA. Nat Neurosci *17*, 1543-1551.

Rothwell, J.C. (2012). Control of human voluntary movement (Springer Science & Business Media).

Ruiz, A., Campanac, E., Scott, R.S., Rusakov, D.A., and Kullmann, D.M. (2010). Presynaptic GABAA receptors enhance transmission and LTP induction at hippocampal mossy fiber synapses. Nat Neurosci *13*, 431-438.

Sakata, J.T., and Brainard, M.S. (2008). Online contributions of auditory feedback to neural activity in avian song control circuitry. J Neurosci *28*, 11378-11390.

Sasaki, A., Sotnikova, T.D., Gainetdinov, R.R., and Jarvis, E.D. (2006). Social context-dependent singing-regulated dopamine. J Neurosci *26*, 9010-9014.

Scharff, C., and Nottebohm, F. (1991). A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J Neurosci *11*, 2896-2913.

Schäfer, M.K., Varoqui, H., Defamie, N., Weihe, E., and Erickson, J.D. (2002). Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. J Biol Chem *277*, 50734-50748.

Schultheiss, N.W., Prinz, A.A., and Butera, R.J. (2011). Phase response curves in neuroscience: theory, experiment, and analysis (Springer Science & Business Media).

Schultz, W. (1998). Predictive reward signal of dopamine neurons. J Neurophysiol *80*, 1-27.

Schultz, W., Dayan, P., and Montague, P.R. (1997). A neural substrate of prediction and reward. Science *275*, 1593-1599.

Shabel, S.J., Proulx, C.D., Piriz, J., and Malinow, R. (2014). Mood regulation. GABA/glutamate co-release controls habenula output and is modified by antidepressant treatment. Science *345*, 1494-1498.

Simpson, H.B., and Vicario, D.S. (1990). Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci *10*, 1541-1556.

Sizemore, M., and Perkel, D.J. (2011). Premotor synaptic plasticity limited to the critical period for song learning. Proc Natl Acad Sci U S A *108*, 17492-17497.

Smeets, W.J., Marín, O., and González, A. (2000). Evolution of the basal ganglia: new perspectives through a comparative approach. J Anat *196 (Pt 4)*, 501-517.

Soha, J.A., Shimizu, T., and Doupe, A.J. (1996). Development of the catecholaminergic innervation of the song system of the male zebra finch. J Neurobiol *29*, 473-489.

Sohrabji, F., Nordeen, E.J., and Nordeen, K.W. (1990). Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav Neural Biol *53*, 51-63.

Solis, M.M., and Doupe, A.J. (1997). Anterior forebrain neurons develop selectivity by an intermediate stage of birdsong learning. J Neurosci *17*, 6447-6462.

Solis, M.M., and Doupe, A.J. (1999). Contributions of tutor and bird's own song experience to neural selectivity in the songbird anterior forebrain. J Neurosci *19*, 4559-4584.

Sossinka, R., and Böhner, J. (1980). Song Types in the Zebra Finch Poephila guttata castanotis1. Zeitschrift für Tierpsychologie *53*, 123-132.

Stark, L.L., and Perkel, D.J. (1999). Two-stage, input-specific synaptic maturation in a nucleus essential for vocal production in the zebra finch. J Neurosci *19*, 9107-9116.

Stepanek, L., and Doupe, A.J. (2010). Activity in a cortical-basal ganglia circuit for song is required for social context-dependent vocal variability. J Neurophysiol *104*, 2474-2486.

Strausfeld, N.J., and Hirth, F. (2013). Deep homology of arthropod central complex and vertebrate basal ganglia. Science *340*, 157-161.

Stocco, A., Lebiere, C., and Anderson, J.R. (2010). Conditional routing of information to the cortex: a model of the basal ganglia's role in cognitive coordination. Psychol Rev *117*, 541-574.

Strong, S.P., Koberle, R., de van Steveninck, R.R.R., and Bialek, W. (1998). Entropy and information in neural spike trains. Physical review letters *80*, 197.

Sulzer, D., Joyce, M.P., Lin, L., Geldwert, D., Haber, S.N., Hattori, T., and Rayport, S. (1998). Dopamine neurons make glutamatergic synapses in vitro. J Neurosci *18*, 4588-4602.

Sutton, R.S., and Barto, A.G. (1998). Introduction to reinforcement learning (MIT Press Cambridge).

Tchernichovski, O., Mitra, P.P., Lints, T., and Nottebohm, F. (2001). Dynamics of the vocal imitation process: how a zebra finch learns its song. Science *291*, 2564-2569.

Tepper, J.M., Koós, T., and Wilson, C.J. (2004). GABAergic microcircuits in the neostriatum. Trends Neurosci *27*, 662-669.

Thorndike, E.L. (1911). Animal intelligence: Experimental studies (Macmillan).

Thorpe, W.H. (1954). The process of song-learning in the chaffinch as studied by means of the sound spectrograph.

Tramontin, A.D., and Brenowitz, E.A. (2000). Seasonal plasticity in the adult brain. Trends Neurosci *23*, 251-258.

Troyer, T.W., and Doupe, A.J. (2000a). An associational model of birdsong sensorimotor learning I. Efference copy and the learning of song syllables. J Neurophysiol *84*, 1204-1223.

Troyer, T.W., and Doupe, A.J. (2000b). An associational model of birdsong sensorimotor learning II. Temporal hierarchies and the learning of song sequence. J Neurophysiol *84*, 1224-1239.

Tumer, E.C., and Brainard, M.S. (2007). Performance variability enables adaptive plasticity of 'crystallized' adult birdsong. Nature *450*, 1240-1244.

Tunstall, M.J., Oorschot, D.E., Kean, A., and Wickens, J.R. (2002). Inhibitory interactions between spiny projection neurons in the rat striatum. J Neurophysiol *88*, 1263-1269.

Turing, A.M. (1950). Computing machinery and intelligence. Mind , 433-460.

Tye, K.M., and Deisseroth, K. (2012). Optogenetic investigation of neural circuits underlying brain disease in animal models. Nat Rev Neurosci *13*, 251-266.

Van Vreeswijk, C., Abbott, L.F., and Ermentrout, G.B. (1994). When inhibition not excitation synchronizes neural firing. J Comput Neurosci *1*, 313-321.

van Vreeswijk, C., and Sompolinsky, H. (1996). Chaos in neuronal networks with balanced excitatory and inhibitory activity. Science *274*, 1724-1726.

Vates, G.E., and Nottebohm, F. (1995). Feedback circuitry within a song-learning pathway. Proc Natl Acad Sci U S A *92*, 5139-5143.

Vates, G.E., Vicario, D.S., and Nottebohm, F. (1997). Reafferent thalamo-cortical loops in the song system of oscine songbirds. J Comp Neurol *380*, 275-290.

Venton, B.J., Zhang, H., Garris, P.A., Phillips, P.E., Sulzer, D., and Wightman, R.M. (2003). Real-time decoding of dopamine concentration changes in the caudate-putamen during tonic and phasic firing. J Neurochem *87*, 1284-1295.

Vicario, D.S. (1991). Organization of the zebra finch song control system: II. Functional organization of outputs from nucleus Robustus archistriatalis. J Comp Neurol *309*, 486-494.

Vicario, D.S., and Nottebohm, F. (1988). Organization of the zebra finch song control system: I. Representation of syringeal muscles in the hypoglossal nucleus. J Comp Neurol *271*, 346-354.

Vicario, D.S., and Simpson, H.B. (1995). Electrical stimulation in forebrain nuclei elicits learned vocal patterns in songbirds. J Neurophysiol *73*, 2602-2607.

Voorn, P., Vanderschuren, L.J., Groenewegen, H.J., Robbins, T.W., and Pennartz, C.M. (2004). Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci *27*, 468-474.

Vu, E.T., Mazurek, M.E., and Kuo, Y.C. (1994). Identification of a forebrain motor programming network for the learned song of zebra finches. J Neurosci *14*, 6924-6934.

Wade, J., and Arnold, A.P. (2004). Sexual differentiation of the zebra finch song system. Ann N Y Acad Sci *1016*, 540-559.

Wallhäusser-Franke, E., Collins, C.E., and DeVoogd, T.J. (1995). Developmental changes in the distribution of NADPH-diaphorase-containing neurons in telencephalic nuclei of the zebra finch song system. J Comp Neurol *356*, 345-354.

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Künstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., Heger, A., Kong, L., Ponting, C.P., Jarvis, E.D., Mello, C.V., Minx, P., Lovell, P., Velho, T.A., Ferris, M., Balakrishnan, C.N., Sinha, S., Blatti, C., London, S.E., Li, Y., Lin, Y.C., George, J., Sweedler, J., Southey, B., Gunaratne, P., Watson, M., Nam, K., Backström, N., Smeds, L., Nabholz, B., Itoh, Y., Whitney, O., Pfenning, A.R., Howard, J., Völker, M., Skinner, B.M., Griffin, D.K., Ye, L., McLaren, W.M., Flicek, P., Quesada, V., Velasco, G., Lopez-Otin, C., Puente, X.S., Olender, T., Lancet, D., Smit, A.F., Hubley, R., Konkel, M.K., Walker, J.A., Batzer, M.A., Gu, W., Pollock, D.D., Chen, L., Cheng, Z., Eichler, E.E., Stapley, J., Slate, J., Ekblom, R., Birkhead, T., Burke, T., Burt, D., Scharff, C., Adam, I., Richard, H., Sultan, M., Soldatov, A., Lehrach, H., Edwards, S.V., Yang, S.P., Li, X., Graves, T., Fulton, L., Nelson, J., Chinwalla, A., Hou, S., Mardis, E.R., and Wilson, R.K. (2010). The genome of a songbird. Nature *464*, 757-762.

Wehr, M., and Zador, A.M. (2003). Balanced inhibition underlies tuning and sharpens spike timing in auditory cortex. Nature *426*, 442-446.

West, M.J., and King, A.P. (1990). Mozart's starling. American Scientist , 106-114.

Wichmann, T., and DeLong, M.R. (1996). Functional and pathophysiological models of the basal ganglia. Curr Opin Neurobiol *6*, 751-758.

Wichmann, T., Bergman, H., and DeLong, M.R. (1994). The primate subthalamic nucleus. III. Changes in motor behavior and neuronal activity in the internal pallidum induced by subthalamic inactivation in the MPTP model of parkinsonism. J Neurophysiol *72*, 521-530.

Wickens, J.R., Reynolds, J.N., and Hyland, B.I. (2003). Neural mechanisms of reward-related motor learning. Curr Opin Neurobiol *13*, 685-690.

Wild, J.M. (1993). Descending projections of the songbird nucleus robustus archistriatalis. J Comp Neurol *338*, 225-241.

Wild, J.M. (1994). Visual and somatosensory inputs to the avian song system via nucleus uvaeformis (Uva) and a comparison with the projections of a similar thalamic nucleus in a nonsongbird, Columba livia. J Comp Neurol *349*, 512-535.

Wilkinson, R. (1980). Calls of nesting chaffinches Fringilla coelebs: The use of two sound sources. Zeitschrift für Tierpsychologie

Williams, H.W. (1969). Vocal behavior of adult California Quail. The Auk , 631-659.

Wilson, C.J. (2014). Oscillators and Oscillations in the Basal Ganglia. Neuroscientist

Wilson, C.J., and Kawaguchi, Y. (1996). The origins of two-state spontaneous membrane potential fluctuations of neostriatal spiny neurons. J Neurosci *16*, 2397-2410.

Wilson, C.J., Beverlin, B., and Netoff, T. (2011). Chaotic desynchronization as the therapeutic mechanism of deep brain stimulation. Front Syst Neurosci *5*, 50.

Winfree, A.T. (2001). The geometry of biological time (Springer Science & Business Media).

Wolpert, D.M., Diedrichsen, J., and Flanagan, J.R. (2011). Principles of sensorimotor learning. Nat Rev Neurosci *12*, 739-751.

Woolley, S.C., and Doupe, A.J. (2008). Social context-induced song variation affects female behavior and gene expression. PLoS Biol *6*, e62.

Woolley, S.C., Rajan, R., Joshua, M., and Doupe, A.J. (2014). Emergence of context-dependent variability across a basal ganglia network. Neuron *82*, 208-223.

Yu, A.C., and Margoliash, D. (1996). Temporal hierarchical control of singing in birds. Science *273*, 1871-1875.

Zander, J.F., Münster-Wandowski, A., Brunk, I., Pahner, I., Gómez-Lira, G., Heinemann, U., Gutiérrez, R., Laube, G., and Ahnert-Hilger, G. (2010). Synaptic and vesicular coexistence of VGLUT and VGAT in selected excitatory and inhibitory synapses. J Neurosci *30*, 7634-7645.

Zann, R.A. (1996). The zebra finch: a synthesis of field and laboratory studies (Oxford University Press).

ZEBrA database (Oregon Health & Science University, Portland, OR 97239; http://www.zebrafinchatlas.org)

Zhou, F.M., Wilson, C.J., and Dani, J.A. (2002). Cholinergic interneuron characteristics and nicotinic properties in the striatum. J Neurobiol *53*, 590-605.

Zuschratter, W., and Scheich, H. (1990). Distribution of choline acetyltransferase and acetylcholinesterase in the vocal motor system of zebra finches. Brain Res *513*, 193-201.