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Anatomy and physiology of a vocal learning circuit in the Bengalese finch

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Abstract

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The basal ganglia are hypothesized to be involved in several aspects of motor control, including control of precise movement kinematics, motor sequencing, and motor learning. Chapter 1 of this dissertation provides a broad introduction to the structure and hypothesized functions of the basal ganglia, and discusses the strengths of the songbird as a model organism for studying the role of the basal ganglia in motor control and motor learning. In Chapter 2 we present new data on the neuroanatomy of the Bengalese finch dopamine system, a large population of putatively non-dopaminergic neurons spatially intermingled with dopaminergic neurons in the ventral tegmental area, the substantia nigra pars compacta, and the periaqueductal gray. In Chapter 3, we detail the results of *in vivo* electrophysiology experiments in Area X of the Bengalese finch. We first demonstrate that neurons in Area X of the Bengalese finch can be separated into striatal and pallidal subclasses. We next report for the first time that Area X single unit activity reflects information about song sequencing and phonology. Intriguingly, we find robust sequence representations and weak pitch representations. The implications of the results from Chapters 2 and 3, as well as future experiments, are then discussed at length in Chapter 4.

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List of Abbreviations

6-OHDA 6-hydroxydopamine.

AFP anterior forebrain pathway.

CTB cholera toxin subunit b.

DBS deep brain stimulation.

DLM dorsolateral nucleus of the anterior thalamus.

GPe globus pallidus external.

GPi globus pallidus internal.

HD Huntington's disease.

HVC Used as a proper name; songbird pallial nucleus.

IFR instantaneous firing rate.

L-DOPA L-3,4-dihydroxyphenylalanine.

LMAN lateral magnocellular nucleus of the anterior nidopallium.

LSt lateral striatum.

MP motor pathway.

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

MSNs medium spiny neurons.

MSt medial striatum.

nXIIts tracheosyringeal portion of the hypoglossal nucleus.

PAG periaqueductal gray.

PD Parkinson's disease.

RA the robust nucleus of the arcopallium.

RPE reward prediction error.

SNc substantia nigra pars compacta.

SNr substantia nigra pars reticulata.

SPE sensory prediction error.

STN subthalamic nucleus.

TH tyrosine hydroxylase.

TRITC tetramethylrhodamine.

VP ventral pallidum.

VTA ventral tegmental area.

Chapter 1

Background and literature review: The basal ganglia, motor control, and motor learning

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Throughout life, we acquire new motor skills, such as learning to speak, walk, ride a bike, play the piano, or dance the tango. Learning each of these activities requires the nervous system to activate our muscles in novel patterns, and with practice we become proficient; precisely performing tasks that once seemed nearly impossible quickly becomes second nature. Many circuits participate in some part of this process, from the periphery to the brainstem to the cortex.

The basal ganglia is a group of subcortical nuclei which are connected to the cortex and thalamus in partially segregated parallel loops (Parent and Hazrati, 1995a) with a long history of scholarship connecting it to motor control (Yanagisawa, 2018). But after over a century of research, the precise nature of its role in movement remains debated.

Broadly, hypotheses of basal ganglia function fall into two categories: Ongoing control of movement features, such as kinematics or sequencing (Desmurget and Turner, 2010), or higher-order organization of behavior, such as motor sequencing (Graybiel, 1998; Jin and Costa, 2015). The basal ganglia are also heavily implicated in learning (Schultz, 2019). None of these hypotheses are necessarily mutually exclusive, and all have supporting anatomical and physiological evidence to support them.

This chapter will first outline the major organizational principles of the basal ganglia (Chapter 1.1), and compare basal ganglia anatomy in mammals and songbirds (Chapter 1.2). It will next discuss the role of the basal ganglia in motor control, evaluating several major hypotheses (Chapter 1.3). Finally, the chapter will conclude with an examination of how dopaminergic signalling contributes to motor learning by evaluating several recent studies of songbirds in the context of both the literature on dopamine's role in associative learning, and the hypothesized neural computations underlying motor learning (Chapter 1.4).

1.1 Organizational principles of the basal ganglia

1.1.1 The direct and indirect pathways

One of the core organizational features of the basal ganglia is the presence of two parallel subcircuits: the direct pathway and the indirect pathway (Alexander and Crutcher, 1990). Striatal neurons in the direct pathway project directly to the globus pallidus internal (GPi), which is equivalent to the entopeduncular nucleus in rodents, and the substantia nigra pars reticulata (SNr). Striatal neurons in the indirect pathway project to the globus pallidus external (GPe). GPe projection neurons next project to the subthalamic nucleus (STN), which projects to the GPi/SNr. The discovery of these pathways revolutionized our understanding of the basal ganglia's role

in motor control, particularly with respect to the idea of action selection (discussed in detail in Chapter 1.3.2). (Alexander and Crutcher, 1990; Grillner and Robertson, 2015; Albin et al., 1989). The canonical circuitry of the cortico-basal ganglia-thalamic loop described above is found in species as distantly related as humans and lampreys, species whose lineages diverged over 500 million years ago (Kumar and Hedges, 1998), suggesting that the basal ganglia play fundamental role in the control of behavior (Ericsson et al., 2013; Grillner and Robertson, 2015; Ocaña et al., 2015; Stephenson-Jones et al., 2011).

The direct and indirect pathways are typically viewed as oppositional to each other in their function, competing for control of basal ganglia output. In the context of motor output they are sometimes characterized as "go" and "no go"/"stop" pathways. This view is supported by the respective effects of each pathway on the thalamus: the projection neurons of the striatum, GPe, GPi and SNr are all inhibitory and the projection neurons of the STN are excitatory. Consequently, activation of direct pathway medium spiny neurons (MSNs) should ultimately disinhibit the thalamus (promoting movement), while indirect pathway activity further inhibits it (suppressing movement) (Albin et al., 1989).

Striatal projection neurons are called (MSNs), and make up approximately 95% of striatal neurons (Kemp and Powell, 1971b). Direct and indirect pathway projection neurons in the striatum can be distinguished by their histochemical profiles. MSNs that are part of the direct pathway express D1-family dopamine receptors, and MSNs that are part of the indirect pathway express D2-family dopamine receptors (Gerfen et al., 1990). Both direct and indirect pathway MSNs are spatially intermingled throughout the striatum, and receive glutamatergic input from the cortex and thalamus and dopaminergic inputs from the midbrain (Kemp and Powell, 1971a; Hunnicutt et al., 2016; Kötter, 1994; Huerta-Ocampo et al., 2014). Because D1 receptor activation is excitatory and D2 receptor activity is inhibitory, dopamine release in the

striatum has opposite effects on the direct and indirect pathway.

A key hypothesized mechanism for dopamine-dependent learning is the modification of corticostriatal synaptic weights by concurrent excitatory and dopaminergic signaling (Reynolds and Wickens, 2002). Unlike the rest of the core basal ganglia nuclei, which have projection neurons with autonomous spontaneous activity (Surmeier et al., 2005), MSNs rarely show spontaneous activity and requires excitatory input from multiple axons to fire (Wilson et al., 1983; Wilson and Kawaguchi, 1996), suggesting that the striatum functions to integrate diverse cortical inputs, with dopamine-driven plasticity biasing the signal (the role of dopamine in the basal ganglia is discussed in detail in Chapter 1.4).

Direct pathway MSNs project to the GPi or SNr, the output nuclei of the basal ganglia. Both structures are highly similar, and some have proposed that it is better to conceptualize them as a structure 'accidentally' separated by the internal capsule (Nambu, 2007). However, significant differences exist as well. For example, motor, sensorimotor, and associative information appears to be processed through segregated parallel channels in the GPi, but not SNr (Parent and Hazrati, 1995b). The GPi/SNr can influence behavior via projections to the thalamus and brainstem, as well as respective cortical targets (Carpenter et al., 1976; Grofová and Rinvik, 1974; Kemel et al., 1988; Kemp and Powell, 1971a; Kultas-Ilinsky et al., 1978; Middleton and Strick, 2002). GPi/SNr projection neurons have high tonic firing rates and are thought to continuously inhibit their targets, punctuated by transient disinhibition when subsets of GPi/SNr cells decrease their firing rates. This inhibition is often conceptualized as either an inverse of the basal ganglia output, or as gating of excitatory cortical inputs (Albin et al., 1989; Deniau and Chevalier, 1985; Hikosaka and Wurtz, 1983; MacLeod et al., 1980).

The standard direct/indirect pathway model suggests that activation of direct pathway MSNs leads to inhibition of the GPi/SNr and disinhibition of the thalamus,

allowing it to drive movement via excitation of cortex. However, phenomena such as rebound spiking (Kim et al., 2017; Person and Perkel, 2005, 2007), and GPi and thalamic co-activation suggest a potentially more nuanced control of the thalamus by GPi/SNr than straightforward disinhibition (Goldberg and Fee, 2012; Goldberg et al., 2013; Kim et al., 2017).

The two intrinsic nuclei of the basal ganglia, the GPe and STN are thought to play an important role in inhibiting actions, broadly supporting the "no-go" function of the indirect pathway. The GPe receives projections from a subset of MSNs (Gerfen et al., 1990), and in turn sends inhibitory projections to the STN. The STN is excitatory and projects to the GPi/SNr and back to GPe. Simultaneous electrophysiological recordings of neurons in dorsolateral striatum, GPe, STN and SNr in rats is consistent with a model where behavior in a Go/No-Go task is governed by a "race" between striatum and STN for control of SNr (Schmidt et al., 2013). STN also receives direct excitatory input from pyramidal tract collaterals in the cortex, including a somatotopic projection from primary motor cortex, as part of the hyperdirect pathway (Giuffrida et al., 1985; Monakow et al., 1978; Nambu et al., 1997), making it a second basal ganglia input structure. One hypothesis of STN function, heavily influenced by the "action selection" hypothesis of basal ganglia function (discussed in Chapter 1.3.2) is that the hyperdirect pathway contributes to the surround inhibition in a center-surround model of action selection in the basal ganglia (Nambu et al., 2002). Others have interpreted STN's role as controlling the "decision threshold" for actions when conflicting premotor signals are propagated from cortex to the basal ganglia (Cavanagh et al., 2011; Frank, 2006).

The computational role of GPe is less well explored than that of STN. However, the GPe and STN are reciprocally connected (Shink et al., 1996; Carpenter et al., 1968), and have a strong influence on one another's firing patterns (Atherton et al., 2013; Hashimoto et al., 2003). Additionally, the GPe contains a subpopulation of

neurons projecting exclusively to the striatum (called arkypallidal neurons), which may play a role in quickly inhibiting actions before the final "decision" by the full cortico-basal ganglia-thalamic circuit (Mallet et al., 2016). This suggests that the indirect pathway may contain multiple mechanisms for inhibiting behavior: Inhibition of the thalamus, through the classic feed-forward indirect pathway (likely modulated by the hyperdirect pathway) and suppressing striatal neuron activity via arkypallidal activation.

1.1.2 The limbic-to-motor spiral

An important feature of the mammalian basal ganglia for understanding its role in behavior is the organization of reciprocal connections between the striatum and the midbrain dopaminergic regions, which provides a potential substrate for transforming sensory information and knowledge about the organism's internal state (hunger, for example) into motor action. The ventral, dorsomedial and dorsolateral striatum are often referred to as "limbic", "associative", and "motor"/"sensorimotor" striatum, based primarily on the cortical regions they receive input from (Nakano et al., 2000; Parent and Hazrati, 1995a). In primates, the caudate nucleus roughly corresponds to the dorsomedial striatum and the postcommisural putamen roughly corresponds to the dorsolateral striatum. The ventral striatum consists of both the nucleus accumbens and olfactory tubercle. Recent work in mice suggests that the ventral striatum may contain an additional subdivision, receiving inputs from a mixture of limbic, auditory, and visual areas (Hunnicutt et al., 2016). Rather than having discrete boundaries, these functional domains are a gradient (Haber, 2016).

The striatum is the primary input layer for the basal ganglia, and the canonical basal ganglia network is predominantly feed-forward through the direct and indirect pathways in segregated, parallel networks (Alexander et al., 1986). However, different subregions of the striatum communicate with each other through an ascending

spiral of connections with dopaminergic nuclei in the midbrain (Haber, 2016). The spiral begins in the nucleus accumbens shell, which both sends projections to and receives projections from the ventral tegmental area (VTA). However, it also sends a projection to medial substantia nigra pars compacta (SNc). The medial SNc then projects to the ventral portion of the nucleus accumbens core, which in turn projects to both medial SNc but also laterally to the dorsal tier of the SNc. The dorsal tier of the SNc projects to more dorsal regions in the nucleus accumbens core. This pattern continues, laterally through the dopaminergic midbrain and dorsally through the striatum, terminating in the dorsolateral motor regions of the striatum (Haber et al., 2000). This spiral allows ventral limbic regions to influence motor output via dorsolateral modulation of motor thalamus and M1 (Aoki et al., 2019). While the learning functions of dopamine are discussed in depth later (Chapters 1.4, 3.5), it is interesting to note here that dopaminergic activity in dorsomedial and dorsolateral striatum appear to reflect reward and upcoming motor actions, respectively (Parker et al., 2016; Lee et al., 2019).

Interpreting the role of the ascending spiral in behavior is not yet straightforward, in part because interpreting striatal function is not straightforward. The limbic-to-motor framework, which suggests information about goals in the ventral striatum is then transformed into an appropriate motor outcome to be executed by the dorsolateral striatum (Haber, 2016), breaks down somewhat when mapped to behavior. For example, pharmacological inactivation of the dorsolateral striatum leads to an increased sensitivity to action-outcome relationships in an instrumental lever press task (Yin et al., 2006). The latter result fits well with an alternative conceptualization of the striatal spiral not as a transition from reward to associative to motor striatum, but instead as a transition from goal-driven (instrumental) to habitual control of behavior, where rewarded behaviors are initially controlled by the ventral striatum and sensitive to devaluation (i.e. if the behavior is no longer associated with a reward

the animal will stop performing the behavior) but may later shift to dorsolateral control and become insensitive to devaluation (Graybiel and Grafton, 2015). In this framework, the connections between dorsolateral striatum and the motor system are best understood as a bypassing of cognitive systems for more streamlined stimulus-response associations. The limbic-to-motor spiral is also important for understanding the basal ganglia's diverse potential functions; different aspects of behavior could be controlled by distinct but interconnected basal ganglia loops.

1.2 Songbirds as a model system for motor control and motor learning

Several unique features make songbirds an attractive model organism for studying the motor system. Like human speech, bird song is an ethologically relevant and highly quantifiable complex motor skill primarily learned during an early critical period by copying an adult "tutor" (reviewed in Brainard and Doupe 2013), and stably maintained throughout life using sensorimotor error. Birdsong varies from trial to trial in both kinematics (Kuebrich and Sober, 2015; Price, 1979), and in some species contains variable sequencing (Honda and Okanoya, 1999; Okanoya, 2004). While birds are "experts" in their own songs, song is an actively maintained, rather than purely habitual or "hardwired" behavior (Kuebrich and Sober, 2015; Okanoya and Yamaguchi, 1997; Sober and Brainard, 2009). Birds can adaptively modify both kinematic and sequential features of their songs in response to reinforcement (Tumer and Brainard, 2007; Andalman and Fee, 2009; Warren et al., 2012), and when singing alone are thought to be an exploratory or "practice" state, suggesting that the behavior remains value-sensitive (Kao and Brainard, 2006). Thus, song shares many similarities with complex motor skills in humans. An additional advantage of using the songbird as a model system for studying vocal motor skills is the presence of song-

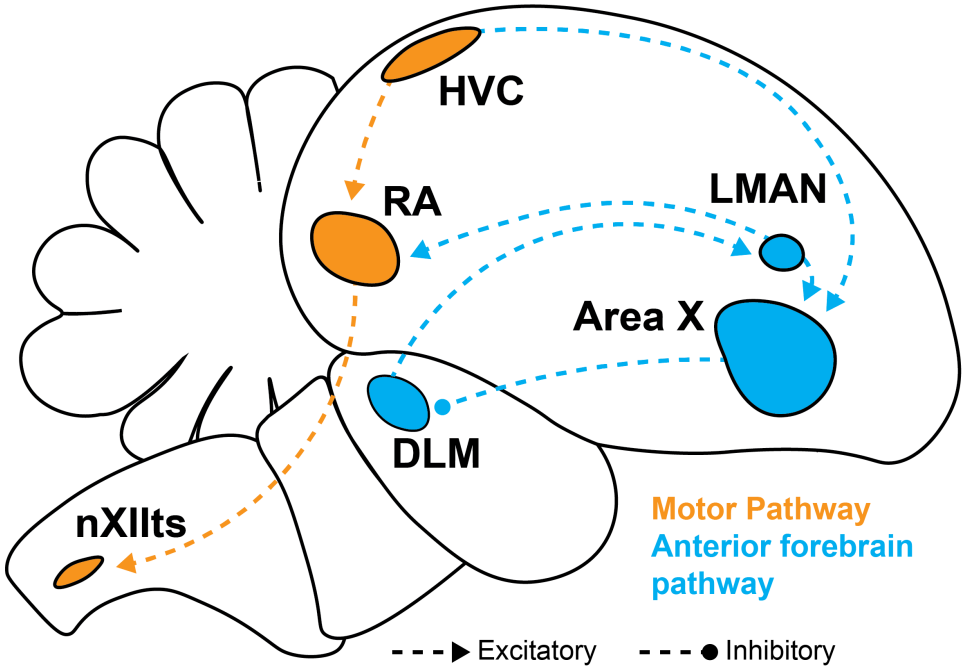

Figure 1.1: The two primary song song control pathways in the songbird brain. The motor pathway (orange) is necessary for song production, and the anterior forebrain pathway (blue) is necessary for song learning, but not production.

specialized, anatomically discrete nuclei throughout the pallium, thalamus, and basal ganglia, allowing for neural manipulations that affect song but not other behaviors (Nottebohm et al., 1976).

Two primary circuits underlie song control in songbirds (Brainard and Doupe, 2013). The motor pathway (MP) consists of two pallial nuclei, HVC and the robust nucleus of the arcopallium (RA), the latter of which projects to tracheosyringeal portion of the hypoglossal nucleus (nXIIts). Lesioning MP nuclei impairs song production (Nottebohm et al., 1976). In contrast, the nuclei of the anterior forebrain pathway (AFP) are required for song learning, but not production (Kao et al., 2005; Scharff and Nottebohm, 1991; Sohrabji et al., 1990). The AFP is a basal ganglia-thalamocortical circuit consisting of Area X, dorsolateral nucleus of the anterior thalamus (DLM) and lateral magnocellular nucleus of the anterior nidopallium (LMAN). The AFP and MP are connected via a projection from LMAN to RA. There is significant neuroanatom-

ical homology between songbirds and mammals (Figure 1.2) (Reiner et al., 2004), particularly with respect to the basal ganglia. However, there are also several notable differences, which may impact circuit function and need to be considered carefully when comparing results from songbirds to results from mammals.

The avian striatum is generally divided into lateral and medial subregions (Karten and Dubbeldam, 1973; Reiner et al., 2004). The organization of the basal ganglia circuit that flows through the lateral striatum (LSt) closely resembles the organization of the mammalian striatum. Striatal MSNs project to the globus pallidus, which contains a reciprocal connection with the STN. The LSt receives dopaminergic input from the SNc and projects to the SNc and SNr (Person et al., 2008). In contrast, the medial striatum (MSt) has its own intermingled population of pallidal neurons (Farries and Perkel, 2000), does not project to the globus pallidus outside of MSt, and is reciprocally connected to VTA and SNc. Unlike LSt, MSt does not contain a direct projection to or from STN (Person et al., 2008).

1.2.1 The song-specialized basal ganglia nucleus Area X

The MSt contains an anatomically discrete, song-specialized basal ganglia nucleus called Area X (Nottebohm et al., 1976; Sohrabji et al., 1990) (Figure 1.2). Like the rest of the MSt it contains both MSNs, striatal interneurons, and pallidal neurons (Carrillo and Doupe, 2004; Farries and Perkel, 2002; Goldberg and Fee, 2010; Goldberg et al., 2010). Unlike the rest of the MSt it does not contain a projection back to the dopaminergic midbrain regions (Person et al., 2008; Gale et al., 2008). However, Area X may be able to indirectly influence the activity via a pathway through the ventral pallidum (VP) via axon collaterals from DLM-projecting GPi neurons (Gale et al., 2008).

Area X is essential for song learning, but not song production (Sohrabji et al., 1990). However, while song remains broadly normal after Area X lesions, there is

an effect on the short-timescale modifications in pitch that occur during singing, suggesting a possible role in on-going kinematic modulation of song (Kojima et al., 2018). Area X lesions also cause disruptions in song sequence, primarily in the form of abnormal syllable repeats (Kubikova et al., 2014), pointing to a possible role in sequence modification.

There are two subpopulations of pallidal neurons within Area X, which have been characterized in zebra finches: The GPi-like population that has a high tonic firing rate and rarely has long bursts or pauses during singing, and projects to DLM. The GPe-like population has a high tonic firing rate punctuated by long bursts and pauses during singing, and projects to the GPi-like neurons (Goldberg and Fee, 2010; Farries et al., 2005). Interestingly, despite the notable physiological similarities between the two pallidal classes and mammalian GPi and GPe neurons, gene expression in both populations more closely resembles that of mammalian arkypallidal neurons (Xiao et al., 2020).

MSNs in Area X receive glutamatergic projections from the pallial regions HVC and LMAN and project to both types of pallidal neurons (Farries et al., 2005). *In vivo*, Area X MSNs are largely quiescent with temporally precise bursts during song (Goldberg et al., 2010). In mammals, direct pathway MSNs express D1 dopamine receptors, while indirect pathway MSNs express D2 dopamine receptors, leading to differential effects of dopamine on the direct and indirect pathway (Gerfen et al., 1990; Wichmann and DeLong, 1996). In songbirds, while there are both D1 and D2-expressing populations of MSNs, there is also a sizable third population expressing both receptor sub-types (Kubikova et al., 2010; Xiao et al., 2020). The response of individual MSNs to dopamine recorded in slice recordings is highly heterogeneous and does not fall into distinct clusters (Ding and Perkel, 2002), making the functional consequence of receptor co-expression unclear.

Like the rest of MSt, Area X does not contain a direct connection with STN (Per-

son et al., 2008). The absence of an STN connection is striking, given the otherwise strong homology of the core basal ganglia circuitry. While some primate GPe neurons do project directly to GPi (Parent and Hazrati, 1995b), the STN is considered to be a powerful regulator of activity in the rest of the basal ganglia (Bonnevie and Zaghloul, 2019). There is evidence for a population of glutamatergic neurons within Area X that modulate pallidal neuron activity, but it is not clear that they represent either a homologous or functionally analogous population to STN (Budzillo et al., 2017).

Despite several notable differences, the overall homology between Area X and mammalian basal ganglia is high: Striatal neurons receive input from cortical/pallial neurons, and project to either globus pallidus internal neurons (direct pathway) or globus pallidus external neurons (indirect pathway). Globus pallidus internal neurons output to the thalamus, and thalamic neurons project both back to the basal ganglia and to the cortex/pallium (Gale and Perkel, 2010) (Figure 1.2B).

1.2.2 Relationship between songbird pallium and mammalian cortex

In mammals, neocortex is a laminar structure that develops from the pallium. Birds do not have a laminar neocortex, but have several nucleated pallial regions that have similar molecular, anatomical, and physiological features to mammalian motor and sensory neocortical regions (Calabrese and Woolley, 2015; Dugas-Ford et al., 2012; Mello et al., 1998; Reiner et al., 2005). Interestingly, these similarities appear to be the product of convergent evolution rather than true homology (Colquitt et al., 2021).

Three songbird pallial nuclei are specialized for song-related behaviors: RA, HVC, and LMAN. HVC projects to both RA and Area X, and is important for song timing (Long and Fee, 2008). LMAN is the primary output nucleus of DLM, and thus is the only known pathway from Area X to RA. Lesions to both RA and HVC impair the bird's ability to sing (Nottebohm et al., 1976), while lesions to LMAN reduce song

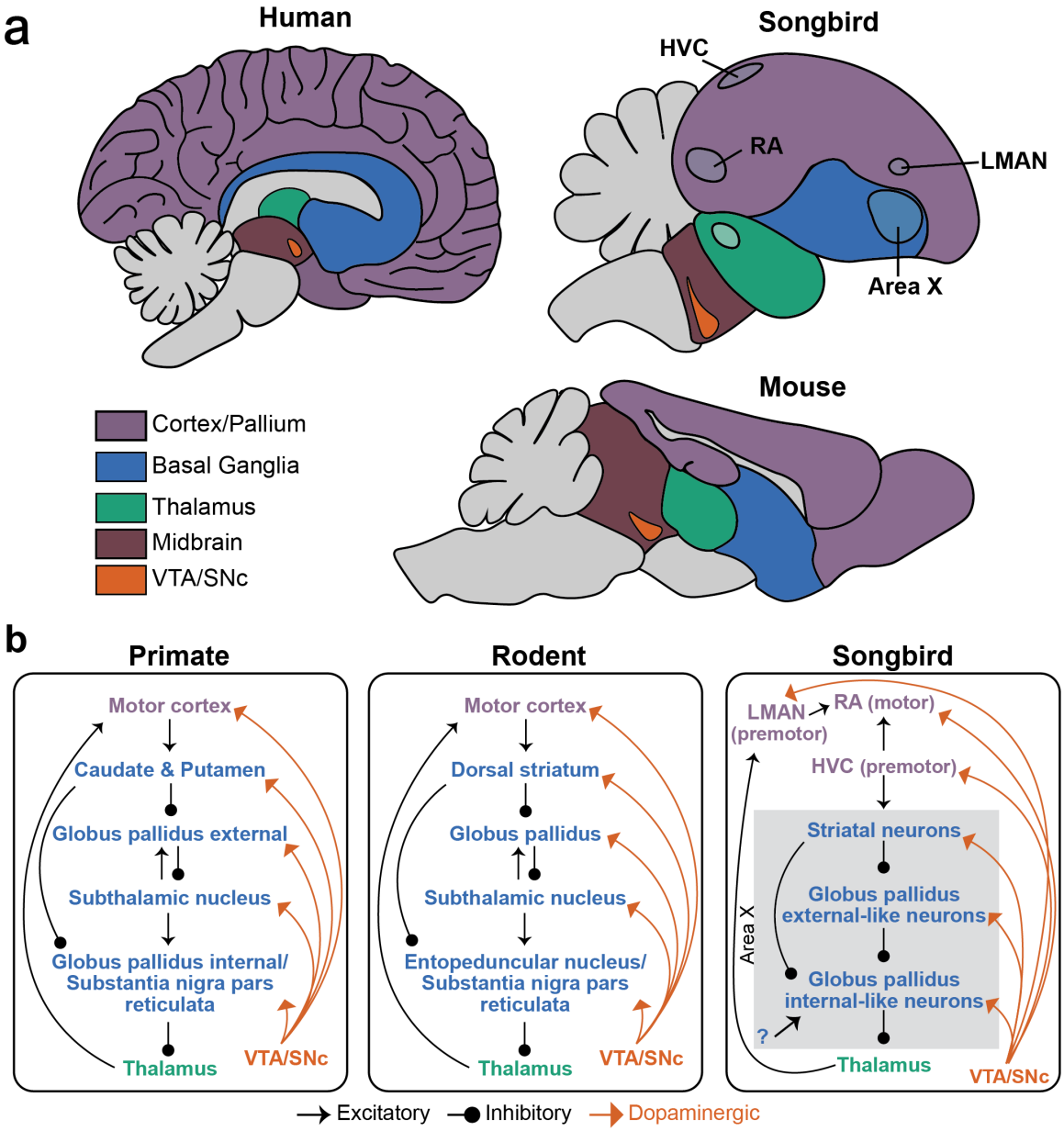

Figure 1.2: (a) Comparative basal ganglia neuroanatomy of humans, songbirds and rodents in the parasagittal plane (not to scale), with cortex/pallium, midbrain, basal ganglia, thalamus, and VTA/SNc highlighted. Song-specialized nuclei in the songbird are shown in lighter shades. (b) Comparison of primate and rodent basal ganglia motor circuits and the songbird vocal motor basal ganglia circuit (Area X indicated by gray box) with major excitatory, inhibitory, and dopaminergic projections. The songbird vocal motor pathway contains many of the same structures and neuroanatomical connections as its mammalian counterpart, but lacks a known subthalamic nucleus connection and has several specialized pallial motor nuclei instead of a laminar cortex. Adapted from Wood (2021).

variability and prevent learning, but leave the overall structure of the behavior intact (Bottjer et al., 1984; Kao et al., 2005).

1.3 What do the basal ganglia contribute to motor control?

1.3.1 Insights from movement disorders

How the normal functioning of the basal ganglia contributes to movement may be controversial, but the motor effects of basal ganglia dysfunction are profound. Though basal ganglia dysfunction is implicated in a wide range of disorders (Brown et al., 1997; Marchand, 2010; Obeso et al., 2014), three involving motor deficits are discussed below: Parkinson's disease (PD) (which involves the degeneration of striatonigral dopaminergic neurons), dystonia (a syndrome characterized by involuntary muscle spasms with co-contraction of antagonist muscles that is frequently linked with basal dysfunction), and Huntington's disease (HD) (a genetic disorder that leads to the degeneration of MSNs in the striatum). Each of these disorders give us insight into how the basal ganglia's role within the motor system.

Parkinson's disease

One of the most common disorders of the basal ganglia is PD, a progressive degenerative disease affecting the midbrain dopamine system. There were an estimated 680,000 people living with PD in the United States in 2010, with projections suggesting that number could rise to over a million by 2030 (Marras et al., 2018).

The primary symptoms of PD are bradykinesia (slowness of movement), akinesia (loss of voluntary movement) and tremor (this collection of symptoms is referred to as Parkinsonism and can arise from non-PD causes). Numerous studies also show

that patients have impairments across a range of motor learning tasks (Olson et al., 2019), as well as number of non-motor symptoms (Poewe, 2008). Both the breadth of the symptoms and the primacy of the motor symptoms are likely reflective of the pattern of degeneration in the dopaminergic midbrain: the SNc, which is the primary source of dopamine for the dorsolateral striatum, is most vulnerable to degeneration in PD (Hirsch et al., 1988; Kordower et al., 2013; McRitchie et al., 1997).

There is a large body of work examining the relationship between movement pathology and neurophysiological pathology in PD patients and in animal models of the disorder. However, the causal relationships between specific pathophysiologies and specific motor symptoms of PD are still debated. The influential "rate model" of PD posited that loss of dopamine leads to an imbalance in activity between the direct and indirect pathways leading to an overactive GPi and underactive thalamus, ultimately causing a hypokinetic motor state (Albin, 1995; Wichmann and DeLong, 1996). In nonhuman primates, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesions that lead to motor deficits are not always accompanied by clear GPi firing rate increases (Raz et al., 2000; Wichmann and Soares, 2006), and GPi lesions do not, by themselves, lead to hyperkinesia as the rate model would predict (Baron et al., 1996; Lozano et al., 1995). Other pathophysiologies of Parkinson's disease, such as abnormal oscillations, sometimes do not appear in MPTP-treated nonhuman primates until after the development of Parkinsonism (Leblois et al., 2007), in rodents dopaminergic manipulations can induce rigidity and akinesia without oscillations (Mallet et al., 2008).

While the motor effects of PD are severe, there is reason to believe that this is due to the propagation of pathological activity patterns from the basal ganglia to other motor regions, rather than the absence or disruption of normal basal ganglia activity (DeLong and Wichmann, 2010). Strikingly, lesions and inactivations to the STN or GP can alleviate the motor symptoms of MPTP treatment in nonhuman primates

(Baron et al., 2002; Bergman et al., 1990; Graham et al., 1990; Guridi et al., 1994) and STN inactivations can alleviate motor symptoms in PD rodent models (Levy et al., 2001). Thus, the absence of normal basal ganglia function may be responsible for some of the subtler deficits in PD rather than the Parkinsonian motor symptoms, such as sensorimotor learning (discussed in detail in Chapter 1.4.2). PD gives us insight into the potential control the basal ganglia can exert over the motor system, but is limited in what it tells us about the basal ganglia's normal contributions to motor behavior.

Dystonia

Dystonia is a heterogeneous syndrome of involuntary muscle contractions, which can be isolated to one part of the body (focal), involve multiple body parts, or affect the majority of the body, and be sustained or intermittent (Balint et al., 2018). It can be primarily genetic, or triggered by repetitive practice of a motor skill, such as writing or playing an instrument (Marsden and Sheehy, 1990; Rozanski et al., 2015). In line with the rate model of PD, dystonia was previously hypothesized to arise from insufficient inhibition of the thalamus from GPi and SNr, but is now generally believed to be the result of pathological firing patterns throughout the cortico-basal ganglia-cerebellar network (Jinnah et al., 2017).

Focal dystonia can be incredibly context-dependent, affecting only particular behaviors while sparing others that use the same body part (Zeuner and Volkmann, 2014; Amouzandeh et al., 2017). For example, someone with task-specific dystonia may struggle to write with a pen but have no problems playing the piano. Task-specific dystonias arrive from repetitive practice of a motor skill. This points to a specificity within basal ganglia circuit organization, allowing individual motor behaviors to be controlled in a very narrow and context-specific manner. In animal models, wile the ability to target and manipulate specific neural populations has improved

dramatically over the past several decades (e.g. Boyden et al., 2005; Alexander et al., 2009; Liu et al., 2012, current technology primarily targets neural subpopulations defined by gene expression and location rather than behavior. As a result, most studies aimed at using stimulation and inhibition are inadequate to investigate neural circuits controlling behavior at this level of specificity, though more precise tools are continually being developed (e.g. Gill et al. 2020)

Huntington's disease

HD is a neurodegenerative disease caused by a CAG trinucleotide repeat in the gene encoding the huntingtin protein, leading to neural degeneration primarily affecting striatal MSNs (Reiner et al., 1988; MacDonald et al., 1993). Symptoms include a mixture of cognitive and motor dysfunctions, the latter consisting primarily of chorea (involuntary irregular movements), incoordination, and the inability to maintain consistent muscle contraction (Walker, 2007).

HD patients also struggle with movement sequencing, particularly in the absence of visual aids (Georgiou et al., 1995). In pre-symptomatic and early stage HD patients (where neurodegeneration is more likely to be restricted to the striatum and chorea is absent or mild) switching between behavioral tasks is impaired (Aron et al., 2003; Migliore et al., 2019). This implicates the striatum in the organization of motor behavior.

In songbirds, expression of human mutant huntingtin in Area X leads to degeneration of MSNs, dysregulation of song sequencing and abnormal firing patterns in pallidal neurons. Specifically, firing patterns become more variable trial-to-trial rather than displaying consistent time-locked bursts and pauses. Inactivation of LMAN, the pallial nucleus that connects the AFP to the MP (Figure 1.1) restores normal song sequencing (Tanaka et al., 2016). This suggests that Area X MSNs can influence song sequencing via modulation of pallidal neuron firing patterns. A similar mechanism

has been proposed for Area X regulation of song kinematic variability (Woolley et al., 2014). A core question addressed in Chapter 3 of this thesis is whether or not Area X single unit neural activity is consistent with a role in online control of kinematics and sequence.

1.3.2 Hypotheses of basal ganglia motor function

Despite the profound motor deficits that can arise from damage to the basal ganglia, exactly what and how it contributes to motor control is debated. Broadly, one group of hypotheses posit a primarily premotor function for the basal ganglia, initiating or selecting high level actions (Albin et al., 1989; Wichmann and DeLong, 1996). Others suggest, in contrast, that the basal ganglia are key for the ongoing monitoring and modulation of movements as they are performed (Dudman and Krakauer, 2016). This chapter will review the evidence for both roles, and discuss if they can be reconciled.

Action selection

Lesions throughout the basal ganglia lead to profound hypokinesia, leading some researchers to hypothesize that the basal ganglia was primarily involved in the preparation or initiation of actions (Neafsey et al., 1978; Yanagisawa, 1996). This hypothesis was later expanded into the action selection hypothesis, which posited that the indirect pathway provided widespread inhibition to downstream motor areas, while the direct pathway selected actions through disinhibition of specific subcircuits for particular movements in a winner-take-all manner (Albin et al., 1989; Grillner and Robertson, 2015; Mink, 1996; Morita et al., 2016).

Another more recent variation of the action selection hypothesis posits that, rather than direct pathway selection on top of blanket inhibition, cortical initiation of a particular action leads to activation of both the direct and indirect pathway neuronal inputs for a particular basal ganglia output neuron are co-activated and "compete"

for control of the basal ganglia output (Bariselli et al., 2019). This version of the action selection hypothesis accounts for both results showing that optogenetic activation of mouse DMS direct pathway neurons increased locomotion while optogenetic activation of indirect pathway neurons induced bradykinesia and freezing (Kravitz et al., 2010), and results showing simultaneous activation of the direct and indirect pathway neurons during action initiation (Cui et al., 2013; Tecuapetla et al., 2016).

There is a wealth of computational work on how the basal ganglia could implement action selection (Frank 2011; Gurney et al. 2004), and many of the necessary ingredients, such as striatal neurons that correlate with action-specific values (Samejima et al. 2005), and correlations between the slowing/stopping of movement and increased firing in the GPe (Mallet et al., 2016). Additionally, electrophysiological activities in rats frequently show increases in striatal activity at both the beginning and end of movement bouts ("task-bracketing"), though these responses develop over the course of learning, suggesting a dynamic relationship with movement (Barnes et al., 2005; Jin and Costa, 2010). Overall the empirical results are mixed with respect to support for action selection and kinematic hypotheses of the basal ganglia. For example, some neurophysiological studies in the GPe and GPi of nonhuman primates show strong correlations with kinematic variables such as speed, but not movement initiation or direction (Thura and Cisek, 2017; Turner and Anderson, 1997), while others show direction selectivity but no correlations with speed or amplitude (Brotchie et al., 1991). Additionally, pallidal inactivations and microstimulations in primates have little effect on movement initiation or action selection (Horak and Anderson, 1984) and activation of both direct and indirect pathway MSNs has a highly heterogeneous effect on SNr activity (Freeze et al., 2013).

The action selection hypothesis has also been criticized on the grounds that movement-related changes in neural activity may not always begin early enough to be responsible for movement onset (DeLong and Wichmann, 2010; Mink and Thach,

1991; Nambu, 2008), occurring after the emergence of movement-related motor cortical activity (Arimura et al., 2013). This suggests that any role the basal ganglia plays in action selection needs to be divorced from a role in movement initiation. One possible interpretation is that the basal ganglia are not involved in action selection *per se*, but instead control action-specific kinematics (Desmurget and Turner, 2010; Becker et al., 2020; Dhawale et al., 2021). In this view, actions are represented in basal ganglia activity, but the function of the basal ganglia is to specify the parameters of actions rather than choose between competing potential actions.

Kinematics and vigor

An alternative hypothesis is that the basal ganglia do not control the selection or initiation of actions, but instead play a role in the ongoing modulation of movement kinematics. There is substantial evidence that the basal ganglia are involved in this process. In nonhuman primates, inactivation of the portion of the sensorimotor region of the GPi slow reaches (Desmurget and Turner, 2010), while microstimulations in the globus pallidus internal and external respectively slow down and speed up arm movements (Horak and Anderson, 1984). In mice, high and low velocity reaching movements can be reinforced by stimulating MSNs in the DMS (Yttri and Dudman, 2016).

Numerous electrophysiological studies find correlations between kinematics and activity within the basal ganglia (Dudman and Krakauer, 2016). For example, Rueda-Orozco and Robbe (2015) trained rats on a treadmill running task. After 7 seconds the rats could abort the trial and obtain a reward; aborting the trial before 7 seconds resulted in a 20 second time-out. Rats solved the task by learned stereotyped patterns of accelerations and deccelerations that lasted for roughly the minimum amount of time needed to obtain reward. Electrophysiological recordings from the dorsolateral striatum in rats that were already proficient in the task revealed a diverse array of

responses, including units selective to the "Go" cue, most units were continuously active throughout the task, and the majority of those units were correlated with either running speed, on the position on the treadmill, or both. Correlations between speed and firing rate could not be accounted for the transition between gaits, making it unlikely that these units were responsive to action selection in any traditional sense. Finally, pharmacological inactivation of DLS increased the trial-to-trial performance variability without interrupting the order of the sequence itself, suggesting a deficit in motor execution (Rueda-Orozco and Robbe, 2015).

A specific aspect of kinematics frequently linked to the basal ganglia is movement vigor, which is commonly quantified as a combination of movement speed and amplitude. In optimal control theory frameworks, the vigor of a particular movement arises from balancing speed-accuracy trade-off. In reinforcement learning frameworks, movement vigor is conceptualized as the balance of metabolic cost of a particular movement with its expected outcome (Niv et al., 2007), and is sometimes argued to be a quantitative proxy of subjective economic utility (Shadmehr et al., 2019). This is closely related to the concept of response vigor, which describes how often an animal will perform a particular action to receive a reward without reference to the speed or amplitude of the action itself (Niv et al., 2007).

Most studies of the basal ganglia's role in kinematics do not attempt to disambiguate general kinematic modulation from the more specific vigor modulation hypothesis. In many cases, the only kinematic variables measured are related to vigor (speed and amplitude). The kinematics and vigor-only hypotheses also make many of the same predictions. For example, in the vigor-only hypothesis we would still expect to see correlations between neural activity and non-vigor aspects of movement. That is because correctly modulating vigor requires that the basal ganglia continuously monitor (but not directly interfere with) movement kinematics (Dudman and Krakauer, 2016). Thus, while it is clear that the basal ganglia contribute to

the ongoing modulation of movement, the exact nature of that modulation remains unclear.

Motor sequencing

A third hypothesis that focuses specifically on the "motor" (or "habitual") basal ganglia circuits is that a primary function of the basal ganglia is motor sequencing. Specifically, the proposed role of the motor basal ganglia is chunking (or concatenating) the neural representations of movement sequences (Graybiel, 1998; Jin and Costa, 2015). This is related to the concept of instrumental-to-habitual organization of cortico-basal ganglia loops, where chunking is an aspect of motor habit formation.

There is some evidence from human studies that basal ganglia stroke may impair chunking motor sequences (Boyd et al., 2009; Vakil et al., 2000). In electrophysiology studies of nonhuman primates performing sequences of combined reaches and saccades, a substantial proportion of striatal units showed sequence-specific modulations: their firing rate was modulation depended not just on the current saccade and reach orientation, but also on what sequence was being performed (Kermadi and Joseph, 1995). One particularly compelling study comes from Jin et al. (2014). In a task where mice were trained to perform exactly four consecutive lever presses in exchange for reward. Over time, they were trained to execute four presses in smaller and smaller time windows. Mice with disrupted striatal plasticity (via selective deletion of the gene encoding NMDAR1 in MSNs) were able to learn to rapidly press the lever, but the number of times they pressed it each trial were highly variable and further from the target number (four) than control littermates. Electrophysiological recordings during sequence learning relieved that MSNs either had a task-bracketing pattern (firing at the beginning and end of the sequence) or were active throughout, almost none were active selectively for the second or third presses. The MSNs that were active throughout the task, but not the task-bracketing MSNs, showed firing

rate oscillations that matched the speed of the lever presses. Throughout training, the proportion of MSNs showing each of these patterns was relatively consistent. Both firing patterns were observed in GPe and SNr units, but with training the number of task-bracketing neurons decreased in GPe and increased in SNr (Jin et al., 2014). Task-bracketing activity is also present in SNr neurons at the beginning and end of naturally occurring syntactic grooming sequences in rats (Meyer-Luehmann et al., 2002).

An alternative view of task bracketing comes to the opposite conclusion: the absence of basal ganglia activity during the course of a sequence learning is due to an absence of basal ganglia engagement, possibly due to lack of motivation (Dudman and Krakauer, 2016). Whether task-bracketing is modulated by motivation or attention should be the topic of future studies.

1.3.3 Conclusion

Though the importance of the basal ganglia for motor control is clear, the precise nature of its involvement is not. Studying the songbird basal ganglia can help elucidate the contributions of the basal ganglia in kinematics and sequencing. Song is a precise, learned motor skill that varies from trial to trial in both kinematics (Kuebrich and Sober, 2015; Price, 1979), and in some species varies in sequence as well (Okanoya, 2004). While birds are "experts" in their own songs, there is reason to believe that it is not a habitual behavior. For example, song is continuously monitored and maintained via sensorimotor error correction (Kuebrich and Sober, 2015; Okanoya and Yamaguchi, 1997; Sober and Brainard, 2009). Birds can adaptively modify both kinematic and sequential features of their songs in response to reinforcement (Tumer and Brainard, 2007; Warren et al., 2012; Andalman and Fee, 2009), and the presence of song-specialized nuclei makes them a particularly tractable experimental system for elucidating the role of the basal ganglia. Chapter 3 of this thesis will discuss the

role of the songbird basal ganglia nucleus, Area X, and how it might contribute to motor sequencing and kinematics.

1.4 Motor learning: the role of dopamine

The neuromodulator dopamine is well-positioned to play a key role in motor learning. Dopamine is most famous for its dual roles in movement disorders (most notably Parkinson's disease) (Hening et al., 2004; Kordower et al., 2013; Shetty et al., 2019) and reward-related behaviors (Arias-Carrión et al., 2010; Schultz, 2019) and the activity of individual dopamine neurons appears to reflect information about both movement and reward (Coddington and Dudman, 2018; Engelhard et al., 2019; Syed et al., 2016).

Drawing conclusions about how dopamine contributes to motor learning requires contending with a number of debates about dopamine's computational role throughout the brain, particularly with regards to reinforcement learning. Dopamine is most commonly associated with reward-related error signaling (Glimcher, 2011; Schultz, 2015), though it has also been proposed that dopamine acts as a value-neutral sensory learning signal (Gardner et al., 2018; Langdon et al., 2018), motivational signal (Berke, 2018; Salamone and Correa, 2012; Berridge, 2012), or a credit-assignment signal (Coddington and Dudman, 2019). Understanding the predictions made by each of these hypotheses can aid in interpreting seemingly contradictory experimental results from studies of dopamine and motor learning. However, the study of motor learning can also in turn inform the broader study of dopaminergic function in other systems. There is extensive scholarship on the potential computational and neural mechanisms of reward-based and non-reward-based learning in the motor system, which provides a strong theoretical framework for testing the role of dopamine in different learning mechanisms (Haith and Krakauer, 2013; Wolpert et al., 2011).

1.4.1 Dopamine and associative learning: Computational hypotheses

In order to understand the role that dopaminergic signaling plays in motor learning, we must examine the computational frameworks used to understand dopamine's role in associative learning and how those frameworks can be applied to motor learning. This chapter will outline several major hypotheses of dopaminergic function, and the open questions that can be addressed in the context of motor learning.

Dopamine is frequently studied in the context of reinforcement learning. The idea that dopamine plays a role in reinforcing specific behaviors is decades old(German and Bowden, 1974; Olds, 1970). In computer science and computational neuroscience, reinforcement learning refers to a broad category of algorithms that seek to maximize reward by sampling from the environment and learning the values of different actions. The subject (or "agent") has a policy for choosing actions. Actions have values based on their likelihood of leading to reward, which are learned through trial and error (Sutton and Barto, 2018). In contrast with the multifaceted definition of reward used in psychology and neuroscience (Samson et al., 2010), in this case reward is simply a parameter that the subject is trying to maximize. As the subject's understanding of action values changes, the best action to take as dictated by the policy will change. Reinforcement learning algorithms provide a computational framework for understanding the behavioral phenomenon of reinforcement. This approach has been highly productive at generating testable hypotheses for the neurobiological underpinnings of behavioral reinforcement (Glimcher, 2011; Lee et al., 2019).

Most reinforcement learning algorithms can be separated into two classes: Model-based and model-free. Model-based reinforcement learning algorithms contain an explicit model of the environment and how the subject can transition between different positions (or "states"). The environment in question could be something physical, like the roads in a city, or conceptual, like the rules of chess. In the context of

motor learning, the "model" could be of the subject's own anatomy rather than the external environment. This model is constructed independently of any understanding the subject might have about the values of different positions within the environment. Separate reward function(s) dictate what outcomes are valuable, depending on the subject's current internal state or physiological needs. This separation allows for greater behavioral flexibility (since different reward functions can be applied to the same model of the environment as the subject's goals change), as well as the ability to learn through inference Daw et al. (2005); Sutton and Barto (2018); Wolpert and Miall (1996). In the context of motor learning, a model-based approach allows for the subject to use previously learned muscle activation patterns to achieve novel goals; a monkey does not need to relearn to extend its arm when the target changes from a piece of fruit to a toy. However, this approach has the downside of quickly becoming computationally expensive– imagine trying to choose an opening move in chess by mentally simulating every possible iteration of the game. In contrast to this, model-free algorithms do not contain explicit models of the environment. Instead, they have a list of different states the subject has experienced (without information about how the states are related to each other) and a separate list containing the current estimated value of each state, based on the subject's direct experience with the state Sutton and Barto (2018). This is much more computationally efficient to implement (in a model-free system, you choose your opening move in chess not based on an analysis of how it will influence future moves, but instead choose a move that has most frequently led to success in the past) (Keramati et al., 2011; Sutton and Barto, 2018). Habits are often thought of as model-free behaviors, in part because they persist for a long time after they cease to be rewarding, a feature re-captured in model-free reinforcement learning algorithms (Pauli et al., 2018). A model-based system will more easily adapt to new opponents deploying wildly different strategies, because at every step of the game it evaluates all possible options. A model-free

system will be slow to adapt to a new opponent, because it will need to learn through trial and error that its old moves and now low-value, and a different set of moves are high value. There is evidence that vertebrates engage in both model-free and model-based reinforcement learning (Daw et al., 2005; Dayan and Berridge, 2014; Doll et al., 2012; Kurdi et al., 2019; Shteingart and Loewenstein, 2014).

A key distinction between model-based and model-free learning is the type of prediction error used. Model-free algorithms use an reward prediction error (RPE), the difference between an expected reward and the one actually experienced. Model-based algorithms generally do not use RPEs, but instead generate errors when the experienced structure of the environment fails to match the model, called a state prediction error or sensory prediction error (SPE), with the latter referring to the sensory consequence of an action (Gläscher et al., 2010; Lerner et al., 2020; Tseng et al., 2007; Wolpert and Miall, 1996). An SPE can occur even if the subject is successful in the intended task (e.g. reaching for a rewarded target) if the sensory consequence (e.g. observed reach trajectory) deviates from the expected sensory consequence (e.g. intended reach trajectory given a particular motor command) (Figure 1.3).

Stimulation of mesencephalic dopamine neurons is behaviorally reinforcing (Corbett and Wise, 1980; Witten et al., 2011). There is a rich literature linking the dopaminergic projection from VTA to the nucleus accumbens (the "mesolimbic pathway"; nucleus accumbens is part of the ventral subdivision of the striatum) to reinforcement and reward-related behaviors (Pierce and Kumaresan, 2006), particularly the motivational aspects of reward-seeking (Berridge and Robinson, 1998; Salamone and Correa, 2012). The dopaminergic projection from the SNc to dorsal striatum (the "nigrostriatial pathway") is more commonly associated with motor control and habit formation. However, the framework of separate nigrostriatal and mesolimbic (and mesocortical) dopamine pathways is an oversimplification: the VTA, for exam-

ple, contains neurons that project to dorsal striatum, and SNc contains neurons that innervate cortex (Yetnikoff et al., 2014). There is also highly complex organization of dopaminergic projections within striatal subregions (Prager and Plotkin, 2019), and rather than existing as separate feed-forward channels the striatal subregions and mesencephalic dopamine regions are recurrently connected in a ventral-to-dorsal ascending loop (Haber et al., 2000; Haber, 2016). Thus, while studies of dopamine's motor functions understandably often focus on SNc and dorsal striatum, the role of VTA and ventral striatum also needs to be considered.

There is also substantial evidence that the activity of dopamine neurons in both the VTA and SNc correspond to model-free RPEs, reviewed at length in Glimcher (2011) and Schultz (2015). There is also some recent evidence that dopamine is involved in learning tasks that require model-based inferences (Sadacca et al., 2016; Sharpe et al., 2017). However, model-based and model-free behaviors can be challenging to disambiguate, particularly in complex learning tasks that likely rely on multiple learning mechanisms in parallel (Collins and Cockburn, 2020) making it difficult to draw strong conclusions about dopamine's computational role in these studies.

What, if any, role dopamine plays in model-based learning mechanisms is central to the question of dopamine's role in motor learning, because different motor learning mechanisms are thought to rely on internal model modification via SPEs and value-based learning via RPEs (Haith and Krakauer, 2013; Izawa and Shadmehr, 2011). Here, motor learning studies, particularly in human PD patients, offer insights into a possible role for dopamine and future directions for studies in model organisms.

Model-based and model-free learning mechanisms are not necessarily implemented in the brain as two dichotomous systems. Neurobiologically-plausible reinforcement learning algorithms integrate both model-based and model-free components (Dayan, 1993; Momennejad et al., 2017; Moran et al., 2019), and complex behaviors such as *de novo* skill acquisition likely make use of multiple learning mechanisms working

synergistically (Krakauer et al., 2019; Wolpert et al., 2011). However, the distinction is an important one for motor learning, where computational hypotheses of specific types of motor learning directly involve the modification of an internal model (Lalazar and Vaadia, 2008; Shadmehr et al., 2010). Additionally, the same dopaminergic regions in the midbrain have been shown to participate in both model-free associative learning and motor skill learning (Hosp et al., 2011; Schultz, 2015); asking the same dopaminergic mechanism can support both of these behaviors is an open question.

Thus, we see that the current framework for studying dopamine in associative learning provides a clear road map for one way to study dopamine's role in motor learning. There is a large body of literature suggesting that dopamine corresponds to model-free reinforcement learning RPEs. Several recent papers (reviewed in Langdon et al. 2018) raise the possibility of dopaminergic contributions to tasks believed to rely on model-based, in addition to model-free learning. However, disambiguation of model-based and model-free mechanisms is challenging. The motor system allows for the study of dopamine in a context in which the use of internal models are well-theorized (Wolpert and Miall, 1996; Wolpert et al., 2011), potentially shedding light on the issue of whether or not dopamine participates in learning that is purely model-based.

1.4.2 How do dopaminergic signals drive motor learning?

Dopaminergic reward prediction error and motor skill learning: Evidence from songbirds

The literature connecting dopamine and with model-free RPEs is expansive (Schultz, 2015), and there is evidence for model-free mechanisms of motor learning in humans (Haith and Krakauer, 2013; Huang et al., 2011; Izawa and Shadmehr, 2011). However, few studies in model organisms directly address whether motor learning can be driven by dopaminergic RPEs. An exception to this is the songbird literature, where several

recent studies provide evidence that dopaminergic RPEs drive vocal motor learning. This chapter will discuss how evidence from songbirds fits in to the broader literature on dopaminergic RPEs.

The hypothesis for how dopaminergic RPEs could lead to motor learning follows from the hypothesis for how dopaminergic RPEs drive associative learning. A particular movement could lead to an extrinsic reward (e.g. food, as is the case with many laboratory tasks) and corresponding RPE. Alternatively, execution of a movement as intended by the subject could be intrinsically rewarding (e.g. a musician correctly performing their favorite song). Regardless of whether the reward was extrinsically or intrinsically generated, the comparison between the expected and experienced outcome following a movement leads to a dopaminergic RPE signal, which then strengthens the synapses in regions such as motor cortex and striatum which are involved in the generation of the particular motor program. This alters the probability of the movement (or movement feature, e.g. speed) occurring in the future, in line with its expected value (Fee and Goldberg, 2011). Dopamine has a multifaceted role in synaptic plasticity, promoting either long term potentiation or long term depression depending on receptor subtype and activity of the postsynaptic neuron (Shen et al., 2008).

Recent advances in songbirds provide compelling evidence for RPE-driven motor learning. Of particular interest has been the dense dopaminergic projection to the song-specialized striatopallidal nucleus "Area X" (1.2B) (Bottjer, 1993; Person et al., 2008). Dopaminergic signaling in Area X is necessary for both intrinsically motivated and extrinsically motivated song learning. In juveniles, dopamine depletion in Area X during the critical period for learning leads to impaired *de novo* copying of the tutor song (Hisey et al., 2018). Tutor song learning does not require interaction with other birds during the "practice" stage of song learning (called the sensorimotor stage, not to be confused with the motor learning paradigm sensorimotor adaptation discussed

throughout the review), as long as the bird was previously exposed to conspecific song during the earlier sensory stage of learning (Brainard and Doupe, 2002; Marler, 1970b). Thus, dopamine is necessary for song learning that does not require an extrinsic reinforcement signal.

In adult songbirds, learning can be driven at one specific acoustic feature (e.g. pitch) at a temporally specific point in the song (the "target syllable"). This is done by playing a brief burst of white noise (an extrinsic negative reinforcer) every time the bird sings the target syllable at a pitch that is either above or below a set threshold. Over time, the bird learns to avoid the white noise by shifting the mean pitch of the syllable away from the threshold. When the white noise is discontinued, the bird's pitch gradually returns to the pre-experiment baseline (Tumer and Brainard, 2007). Lesioning the dopaminergic inputs to Area X leads to an impairment in learning from white noise (Hoffmann et al., 2016; Hisey et al., 2018). Interestingly, dopamine depletion does not impair the return back to baseline pitch when white noise is discontinued, suggesting that while dopamine is necessary for some vocal motor learning mechanisms, others may be dopamine-independent. However, the combined studies of *de novo* learning in juveniles and song modification in adults suggests that dopamine is necessary for both intrinsically and extrinsically-driven learning.

Dopaminergic signaling in the songbird pallium, which shares many functional, physiological, and molecular features with mammalian cortex (Calabrese and Woolley, 2015; Dugas-Ford et al., 2012; Mello et al., 1998; Reiner et al., 2005), is also important for motor learning. Though less dense than the projections to the striatum, songbird primary and supplementary pallial areas also contain dopaminergic terminals (Appeltants et al., 2000, 2002). Dopaminergic lesions to HVC, a song-specialized premotor pallial nucleus in songbirds that receives the majority of its dopaminergic input from the periaqueductal gray, disrupts song learning and learning-related changes in neural activity in juvenile birds (Tanaka et al., 2018). It is presently unknown whether

dopamine in the song-specialized primary motor pallium (nucleus RA) is necessary for learning in birds.

Striatal dopaminergic lesions in songbirds also tend to be incomplete, whether done via 6-OHDA or genetically-mediated ablation (Hoffmann et al., 2016; Hisey et al., 2018). This may explain the discrepancy between lesion studies in birds and primates, where dopamine lesions in motor striatum lead to severe movement deficits (Lane and Dunnett, 2008). If, as suggested by human PD patients, dopamine has distinct roles in permissively facilitating movement and actively driving learning, the partial lesions in birds may be sufficient to maintain movement facilitation while decreasing the strength of the learning signal. Notably, 6-OHDA lesions in birds do lead to subtle vocal motor changes over time, though it's not clear if these changes are consistent with a form of Parkinsonism (Saravanan et al., 2019).

Optogenetic studies point to an active role for dopamine in driving song learning. Stimulation and inhibition of dopaminergic Area X-projecting neurons can drive adaptive modifications of a song syllable's pitch in opposing directions in a manner consistent with a model-free RPE signal (Hisey et al., 2018; Xiao et al., 2018). Consistent with a role in learning rather than direct motor control or passive motor facilitation, optogenetic stimulation drove accumulated changes in behavior over the course of many trials, rather than directly causing an immediate change in the ongoing behavior(Xiao et al., 2018).

Neurophysiological evidence also supports the use of dopaminergic RPEs in song learning. In recordings of antidromically identified Area X-projecting dopaminergic VTA/SNc neurons, Gadagkar et al. (2016) reported similar activity patterns to those reported in nonhuman primates learning stimulus-reward associations. Specifically, once the bird had learned to expect to hear distorted auditory feedback when it sang a particular syllable (similar to the white noise reinforcement learning paradigm described earlier), the absence of distorted auditory feedback triggered a phasic burst of

activity from dopaminergic neurons, similar to studies in primates showing dopaminergic neurons burst after better-than-expected outcomes (Schultz, 2015).

These results are significant because Area X is a basal ganglia nucleus specialized for a motor behavior (song). In mice, the role of RPEs in motor basal ganglia circuits is less clear. Measuring the bulk activity of dopaminergic axons using fiber photometry revealed that signals in ventral ("limbic") striatum resembled RPEs, while dopaminergic signals in dorsal ("motor") striatum seemed to instead reflect choices about future movements (Lee et al. 2019; Parker et al. 2016). There is also evidence for a small population of reward-responsive dopaminergic axons in dorsal striatum (Howe and Dombeck, 2016), but it's not yet clear whether these represent a mechanism for RPE-driven motor learning. Interestingly, Area X-projecting dopaminergic neurons in birds show movement-related activity only outside of song, and RPE-like activity only during song (Chen et al. 2020). This suggests that, at least in birds, whether dopaminergic neurons are reward-responsive or movement-responsive may be dynamic depending on behavioral context.

Though the experiments described above were primarily done in adult birds learning to make subtle modifications to an already well-learned song, qualitative models inspired by model-free reinforcement learning algorithms propose how RPEs may contribute to *de novo* song learning as well. Similar to human babies, songbirds go through a stage of highly variable vocal "babbling" prior to learning to copy their adult tutor's song (Marler, 1970b). Juvenile babbling (and later more subtle song variability in adults) is driven by LMAN, a song-specialized nucleus that serves as the primary pathway between Area X (via the thalamus) and RA, a song-specialized pallial nucleus with functional similarities to mammalian motor cortex layer 5 (Aronov et al., 2008; Kao and Brainard, 2006; Ölveczky et al., 2005). Model-free dopaminergic RPEs in the basal ganglia could then reinforce "babbles" that sound more similar to the song memorized from the bird's adult tutor, biasing LMAN and upstream motor

circuits to perform the reinforced vocalizations more frequently, gradually refining the bird's song until it closely resembles the tutor song (Fee and Goldberg 2011). Chen and Goldberg 2020 extend this and show how a full model-free actor-critic reinforcement learning algorithm could be implemented within the song learning circuit, leading to a number of interesting observations. For example, in adult birds song learning is first implemented with neuroplastic changes in premotor circuits, but over the course of days is consolidated with neuroplastic changes in RA, the song-specialized pallial M1-analog (Andalman and Fee, 2009; Charlesworth et al., 2012). The authors propose that this two-step consolidation process mitigates "catastrophic forgetting", the tendency of reinforcement learning algorithms to forget old information as new information is learned (Chen and Goldberg, 2020). Whether or not proposed reinforcement learning neural circuits in other species also contain two-step consolidation processes is an open question worth exploring.

A few studies in humans also point to a role for dopaminergic RPEs in motor learning. One aspect of motor learning thought to be model-free is savings (Huang et al., 2011), the phenomenon of faster re-learning after extinction or washout. PD patients show impaired savings in sensorimotor adaptation compared to healthy controls, suggesting a possible role for dopamine (Bédard and Sanes, 2011; Leow et al., 2012). Whether or not PD patients are impaired in motor learning tasks specifically designed to rely only on RPEs instead of SPEs (Izawa and Shadmehr, 2011) should be a topic of future research.

In summary, the songbird model provides strong evidence for RPE-driven motor learning. Dopamine is both necessary and sufficient to drive learning in adults, and the neural activity of Area X-projecting dopaminergic VTA/SNc closely mimics RPEs. Though dopaminergic signaling during development is necessary for normal song learning, future studies need to firmly establish whether RPE-like signals drive *de novo* learning in birds. While it seems very plausible, given that the same

VTA/SNC-to-Area X dopaminergic pathway is necessary for both adult and juvenile learning, experiments in mice during *de novo* learning suggest that dopamine signals in the earliest stages of learning may not be consistent with RPE (Coddington and Dudman 2018), complicating the question of dopamine's role in learning generally. Whether or not RPE-driven motor learning exists in rodents is an open question, but seems plausible given the presence of both reward and movement related activity in dopaminergic neurons (Howe and Dombeck, 2016; Engelhard et al., 2019).

Model-based motor learning: Does dopamine play a role?

However, a present challenge with studying dopaminergic contributions to model-based learning using classical reinforcement learning paradigms is developing a strong theoretical conceptualization of the subject's internal model of the task structure. A potential solution to this problem is studying the role of dopamine in sensorimotor adaptation, a form of motor learning theorized to rely on internal models (Lalazar and Vaadia, 2008; Shadmehr et al., 2010). This chapter will evaluate the research on dopamine's role in model-based motor learning, primarily focusing on work in human patients.

Sensorimotor adaptation is the process by which a subject performing a repetitive motor task gradually reduces error after the introduction of a sensory perturbation. Here, the "internal model" refers not to the structure of a task or the external world, but instead refers to the relationship between motor commands and sensory feedback that occurs as a consequence of a particular motor command. The internal model hypothesis of sensorimotor adaptation posits that task error reduction occurs because the subject "re-maps" the sensorimotor internal model using SPEs. In the context of model-based motor learning, SPEs are typically thought to be generated by the cerebellum and modify the internal model with non-dopaminergic synaptic plasticity (Bostan and Strick, 2018; Boyden et al., 2004; Doya, 1999; Popa and Ebner, 2019).

In order to determine dopamine's computational role in motor learning, a critical re-evaluation of the literature on potential dopaminergic involvement in sensorimotor adaptation is warranted.

In the laboratory, sensorimotor adaptation is studied by perturbing a subject's sensory feedback while they perform a repetitive motor task. For example, the sensory perturbation may be a visuomotor rotation. In this task, subjects must use a joystick or mouse to move a cursor across a computer screen with the goal of hitting a particular target. The subjects' hand is hidden, so their only visual feedback is from the computer screen. But the direction that the cursor moves in is rotated relative to the direction of hand movement, leading to large performance errors in early trials (and large SPEs). Over time, the subject adapts to the perturbation and errors decrease to baseline levels.

Though any particular learning task (motor or otherwise) likely relies on both model-based and model-free mechanisms, the internal model hypothesis of sensorimotor adaptation makes several predictions that allow model-based and model-free aspects of motor learning to be dissociated (Shadmehr et al., 2010; Wolpert and Miall, 1996). specifically, sensorimotor re-mapping should rely on SPEs rather than RPEs (Figure 1.3), removing the sensorimotor perturbation after re-mapping should temporarily degrade performance ("after effect"), and re-mapping should lead to a generalizable effect on other movements using the same muscles. In a visuomotor rotation task where subjects were only given information about success or failure of their reach but were not shown the trajectory of the cursor on the screen (preventing subjects from generating SPEs during the reach), reduction in task error failed to produce the standard hallmarks of sensorimotor internal model remapping and was instead consistent with RPE-driven learning (Izawa and Shadmehr, 2011). This supports the hypothesis that sensorimotor adaptation relies on the modification of internal models via SPEs, and suggests that in the absence of sensory error-driven

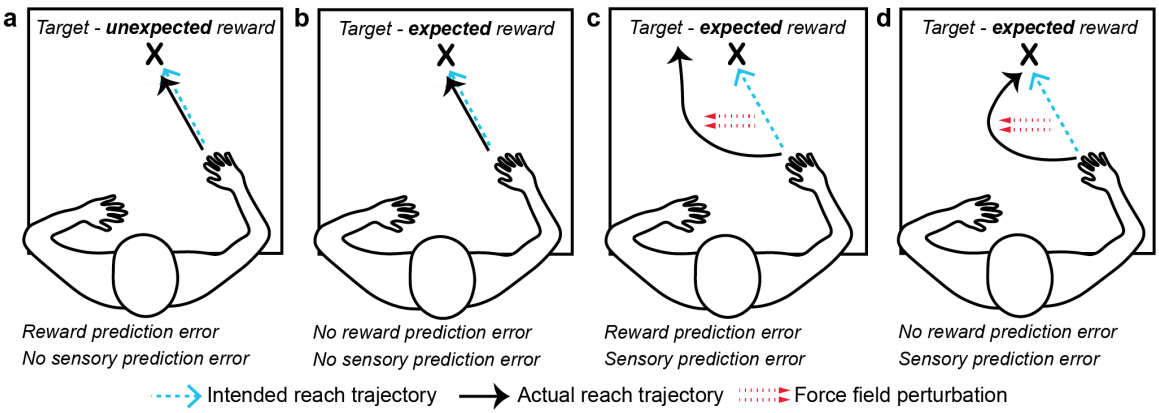

Figure 1.3: The same motor task can generate sensory prediction errors (SPEs) and reward prediction errors (RPEs) depending on the subject's expectations. (a) The subject reaches to a novel target for the first time. The selected motor program (intended reach trajectory) leads to the expected visual and somatosensory consequences (actual reach trajectory), so no SPE is generated. However, because reaching the target leads to an unexpected reward, a positive RPE is generated. (b) Actual and intended reach trajectories match, so no SPE is generated. The subject obtains the expected reward and no RPE is generated. (c) A force field perturbation leads to substantial deviation between the intended and actual reach trajectories, generating an SPE. Because the selected motor program failed to produce the expected reward, a negative RPE is generated. (d) A force field perturbation leads to substantial deviation between the intended and actual reach trajectories, generating an SPE. However, the subject still reaches the target and obtains the expected reward, so no RPE is generated. After sensorimotor adaptation occurs the subject returns to (a) despite the presence of a force field perturbation. Reproduced from Wood (2021)

learning the subject engages RPE learning mechanisms. Thus, sensorimotor adaptation provides a promising avenue through which to test dopamine's role in computing sensorimotor prediction errors specifically and model-based learning more generally.

To understand the role dopamine plays in sensorimotor adaptation, numerous studies have compared healthy controls to PD patients. The results of these studies are often contradictory, at least on the surface (Bédard and Sanes, 2011; Contreras-Vidal and Buch, 2003; Fernandez-Ruiz et al., 2003; Isaias et al., 2011; Leow et al., 2012; Marinelli et al., 2009; Mongeon et al., 2013; Paquet et al., 2008; Semrau et al., 2014; Venkatakrishnan et al., 2011). One possible source of this variability is the use of the dopamine replacement medication L-3,4-dihydroxyphenylalanine (L-DOPA),

which temporarily alleviates the motor symptoms of PD but does not completely return normal dopaminergic activity to the system (Whitfield et al. 2014). However, even studies directly comparing L-DOPA-on and L-DOPA-off groups report contradictory findings. Some studies of visuomotor adaptation find that patients in the L-DOPA-on state generally perform worse than those in the L-DOPA-off state (Mongeon et al., 2013; Semrau et al., 2014) while others have found the reverse (Paquet et al., 2008; Singh et al., 2019). Some find no impairment in PD in the initial sensorimotor adaptation, but impairment in the consolidation of adaptation when tested days (Bédard and Sanes, 2011; Leow et al., 2012; Marinelli et al., 2009) and weeks later (Isaias et al., 2011).

Two recurring issues with early PD-sensorimotor adaptation research are the use of different task designs in different studies, and the use of between-subjects study design to study the effect of L-DOPA. Singh et al. (2019) addressed the first of these concerns directly by confirming that cerebellar ataxia patients were impaired in their sensorimotor adaptation task, because the cerebellum is considered to be a likely neurobiological substrate for sensorimotor internal models and is required for sensory error-driven adaptation (Popa and Ebner, 2019). They next addressed the second issue by testing the same cohort of PD patients in both their L-DOPA-off and L-DOPA-on states, reasoning that the within-subjects design might allow them to detect effects that previous studies missed. They found substantial variability in the off-L-DOPA group, but that L-DOPA improved performance in nearly all subjects. Finally, they tested whether or not alleviating pathological activity in the basal ganglia had similar effects to L-DOPA treatment. To do this, they did the same visuomotor rotation task with a cohort of PD patients with (deep brain stimulation (DBS)) electrodes implanted in the subthalamic nucleus of the basal ganglia. Interestingly, patients performed better in the DBS-on state than DBS-off state. This suggests that it may not be the loss of specific dopaminergic signals that leads to

sensorimotor adaptation impairments in PD, but instead pathological network activity in the basal ganglia that interferes with adaptation. This opens the possibility of passive or facilitatory, rather than active/phasic, role for dopamine in sensorimotor model re-mapping. Future studies in animal models will be necessary to test this directly.

There is evidence that dopamine-dependent learning mechanisms are only engaged during sensorimotor adaptation when sensory errors are large. Mongeon et al. (2013) used a within-subjects design to test the effects of L-DOPA on visuomotor rotation in two very different contexts: Implicit adaptation in response to imperceptible sensory perturbations (what is more typically considered to be sensorimotor adaptation) and explicit adaptation in response to sensory perturbations large enough for the subject to notice. They found that PD patients off L-DOPA performed similarly to controls in the implicit task, but were impaired in the explicit task. They also found that L-DOPA had a variable effect from patient to patient, but on average impaired performance relative to the off-L-DOPA state. This is similar to a previous study that found PD patients on L-DOPA can adapt to gradually increasing perturbations but struggle to adapt to a single, large perturbation (Venkatakrishnan et al. 2011). The work discussed above indicates that learning that arises from the remapping of internal sensorimotor models relies on dopamine-independent SPEs computed by the cerebellum. However, when sensory errors are large dopamine and basal ganglia-dependent learning systems are engaged and may work in tandem with cerebellar mechanisms. In songbirds, dopaminergic signaling in the basal ganglia appears to play a role in sensorimotor adaptation. Saravanan et al. (2019) lesioned dopaminergic inputs to Area X, a manipulation previously shown to disrupt reinforcement-based learning (Hoffmann et al., 2016). To induce sensorimotor adaptation, birds were fitted with miniature headphones. Their song was processed and played back to them in close to real time (approximately 10ms delay) with the pitch artificially shifted either

upwards or downwards. As with previous studies of sensorimotor adaptation in songbirds (Sober and Brainard, 2009), control birds slowly shifted the pitch of their songs in the opposite direction of the pitch shift. Consistent with previous studies of vocal pitch shift adaptation in PD patients (Abur et al., 2018; Mollaei et al., 2013), birds with dopaminergic lesions showed an impaired sensorimotor adaptation, supporting the idea that dopaminergic signaling specifically in the basal ganglia plays a role in sensorimotor adaptation. However, because dopamine lesions in Area X also impair learning paradigms that use explicit reinforcement (Hoffmann et al., 2016; Hisey et al., 2018) and *de novo* song leaning (Hisey et al., 2018) (discussed in depth in the previous chapter), this suggests that dopaminergic signaling in this region may be involved in vocal plasticity independent of any specific learning modality, potentially functioning as a generalized prediction error (Gardner et al., 2018), or an entirely different type of learning mechanism, such a value-effort signal (Berke, 2018) or as an internal credit assignment mechanism (Coddington and Dudman, 2019). Recording Area X-projecting dopaminergic neurons or axons during sensorimotor adaptation could help illuminate the computational mechanism by which dopamine supports song learning.

Another important caveat to the human and songbird studies discussed above is that all rely on the chronic absence of dopamine (either due to PD in humans or lesions in birds), making it difficult to distinguish passive and active dopamine functions. The recent development of sensorimotor adaptation tasks in mice has the potential to address this gap by allowing for spatially and temporally specific dopaminergic manipulations during model-based motor learning. In a task using a joystick with directional force perturbation, mice show signs of sensorimotor adaptation, including after effects when the perturbation is removed. Models containing SPEs (state estimator and hybrid state estimator/actor-critic) fit the data better than a standard actor-critic model using model-free RPEs (Mathis et al., 2017). Combining paradigms like this one with genetic manipulations of subpopulations of dopaminergic neurons

in mice or songbirds could answer a number of questions about dopamine's role in model-based learning. For example, the hypothesis that dopamine is unnecessary for initial sensorimotor adaptation but required for long-term consolidation could be tested by disrupting dopaminergic signaling at different time points during and after learning.

Overall, the role of dopamine in model-based motor learning is much less clear than its role in model-free motor learning. Studies in PD patients suggest that dopamine may not play a direct role in modifying internal models ("pure" sensorimotor adaptation), but dopamine-dependent learning systems may be engaged when sensory errors are large, augmenting pure sensorimotor adaptation with other motor learning mechanisms to achieve the same ultimate task goal.

1.4.3 Conclusion

There is clear evidence for dopaminergic RPEs driving motor learning (Gadagkar et al., 2016; Hisey et al., 2018; Xiao et al., 2018). However, whether or not dopamine is fundamentally performing the same or different functions throughout the forebrain is an open question, and several lines of research suggest a previously-underappreciated heterogeneity in dopamine neuron gene expression, anatomy, and physiological responses (Collins and Saunders, 2020; Schultz, 2019). While much of this review has focused on how concepts of dopamine developed outside of the motor system may apply to motor learning, future studies that establish which specific populations of dopaminergic neurons are involved in motor learning will be invaluable for determining dopamine's computational role(s) in throughout the motor system. For example, dopaminergic signaling appears to be important for learning new motor skills in both motor cortex/pallium and striatum in both birds and rodents (Hosp et al., 2011; Tanaka et al., 2018), but whether or not dopaminergic projections in each area show similar activity during learning is unknown.

1.5 Dissertation Overview

1. Chapter 1 provides background and introduction on the role of the basal ganglia and dopamine in motor control and motor learning, and discusses the strengths and weaknesses of the songbird model system.

2. Chapter 2 details experimental results characterizing the dopaminergic projections to Area X in the Bengalese finch using retrograde tracer injections. We report large populations of spatially intermingled dopaminergic and non-dopaminergic neurons in the ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and periaqueductal gray (PAG) projecting to Area X. We also show that Area X and the motor nucleus RA receive distinct projections from VTA, SNc, and PAG. We then discuss the potential implications for these results, including the possible role of non-dopaminergic projection neurons in primarily dopaminergic regions.

3. Chapter 3 describes *in vivo* neurophysiology experiments in Area X of the Bengalese finch. We first show that Bengalese finch Area X neurons can be classified into pallidal and striatal subtypes based on firing properties. We then examine whether or not information about syllable sequencing or phonology is reflected in Area X single unit activity. We find strong representations of sequence, but not pitch. We discuss then discuss these results in the context of the basal ganglia's hypothesized roles in motor control and motor learning.

4. Chapter 4 details several outstanding questions raised by the data presented in Chapters 2 and 3, and discusses future experiments.

5. A comprehensive list of references in provided at the end of the document.

Chapter 2

Organization of the dopaminergic projections to a song-specialized region of the basal ganglia in the Bengalese finch

2.1 Abstract

One of the fundamental problems in neuroscience is understanding how the brain uses sensory feedback to correct errors in motor output. Several lines of evidence suggest that the neuromodulator dopamine plays a key role in motor learning. Songbirds provide an excellent model organism for studying motor learning due to the highly quantifiable nature of song and the presence of anatomically distinct circuits controlling song production and song learning (collectively called the 'song system'). However, there are significant gaps in our knowledge about the dopaminergic circuits that are thought to be important for shaping activity in subregions of the song system. Here, we characterize the dopaminergic projection to 'Area X', a song-specialized basal

ganglia nucleus in the Bengalese finch, a songbird. Our results show that there are dopaminergic Area X-projecting neurons throughout the Bengalese finch dopaminergic midbrain nuclei (the ventral tegmental area; VTA, substantia nigra pars compacta; SNc, and periaqueductal gray; PAG), but particularly concentrated in lateral VTA and ventral SNc. We also report large populations of putatively non-dopaminergic projection neurons to Area X. Finally, we also show that Area X and the motor cortical nucleus RA receive input from non-overlapping populations of projection neurons in the VTA, SNc, and PAG.

2.2 Introduction

Motor skill learning and the neurobiological mechanisms that underlie it are complex and multifaceted, involving multiple circuits and computational mechanisms (Wolpert et al., 2011). The neuromodulator dopamine has emerged as a key mediator of motor learning in rodents, primates, and birds (see Chapter 1.4 for detailed discussion; Matsumoto et al. 1999; Hosp and Luft 2013; Woolley 2019). Dopamine has a well-established role in associative learning, where it is thought to act as a prediction error signal (signalling the difference between experienced and expected outcomes; Schultz 2019). In the motor system, dopaminergic lesions impair learning in a variety of skilled motor tasks (Hosp et al., 2011; Yttri and Dudman, 2016), and the activity of dopaminergic neurons correlates with both movement and reward (Coddington and Dudman, 2018; Engelhard et al., 2019; Syed et al., 2016). However, the dopaminergic circuits supporting motor learning are still not well understood.

Songbirds (oscine passerines) are an excellent model organism for studying motor learning. Song is a motor skill with many parallels to human speech: it is socially learned by juveniles, actively maintained using auditory feedback in adults, and relies on a cortico-basal ganglia circuit (Doupe et al., 2005; Mooney, 2020). Song also has

highly quantifiable features, such as pitch (fundamental frequency), making it ideal for studying the relationship between neural activity and behavior. Further, the same basic midbrain dopamine structures appear to be conserved in both mammals and birds (Dahlstrom and Fuxe, 1964; Reiner et al., 1994, 2004; Kingsbury et al., 2011).

Songbird vocal learning is supported by a specialized motor circuit called the 'song system' consisting of two interconnected pathways: the motor pathway (MP) and anterior forebrain pathway (AFP) (Fig. 2.1a). The MP, which is necessary for the production of learned vocalizations, consists of the pallial nuclei analogous to mammalian primary and supplementary motor cortical areas. The AFP consists of a pallial-basal ganglia-thalamopallial loop (similar to cortico-basal ganglia-thalamocortical loops in mammals) which is necessary for vocal learning, but not production (Sohrabji et al., 1990; Reiner et al., 2004; Gale and Perkel, 2010).

In zebra finches, a commonly studied species closely related to the Bengalese finch, dopaminergic signalling in both the MP and AFP is essential for song learning. A particularly important projection is from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) to Area X, a song-specialized basal ganglia nucleus containing both pallidal and striatal neurons (Carrillo and Doupe, 2004; Farries and Perkel, 2002). Disrupted dopaminergic signaling in the Area X of zebra finches impairs learning in both juvenile and adult birds, and optogenetic stimulation of dopaminergic terminals is sufficient to drive learning (Hisey et al., 2018; Xiao et al., 2018). In the premotor nucleus HVC, dopaminergic signalling from the periaqueductal gray (PAG) is necessary for normal song learning and learning-associated changes in firing patterns in juvenile birds (Tanaka et al., 2018). Studies in both zebra finches and canaries also show that the motor nucleus the robust nucleus of the arcopallium (RA), a structure analogous to mammalian motor cortex, also receives dopaminergic input from VTA, SNc, and PAG, though the behavioral function of these projections are not known (Appeltants et al., 2002; Gale et al., 2008).

Among songbirds, the Bengalese finch (*Lonchura striata* var. *domestica*) is a particularly strong model organism for studying motor learning because it varies the sequencing of individual song elements from rendition to rendition ('syllables'). Like other aspects of song, syllable sequencing is socially learned and can be modified via reinforcement learning (Warren et al., 2012). The neurobiological basis for sequence learning is not known, but dopaminergic signalling in Area X is required for learned pitch changes in similar reinforcement-based experimental paradigms in the Bengalese finch (Hoffmann et al., 2016). However, while several studies have characterized the midbrain dopamine system in related species (e.g. Appeltants et al. 2000, 2002; Person et al. 2008), anatomy of the Bengalese finch dopamine system has only been grossly described (Hoffmann et al., 2016).

The primary goal of this study was to characterize the midbrain dopaminergic projections to Area X in the songbird Bengalese finch. Specifically, we used retrograde tracer injections (Fig. 2.1a) to assess the relative number of projection neurons to Area X from VTA, SNc, and PAG, and to determine the proportion of projection neurons in these structures expressing tyrosine hydroxylase (Fig. 2.1b-c). We also assessed the specificity of the dopaminergic projection to Area X by performing dual retrograde tracer injections in Area X and RA.

2.3 Materials and Methods

2.3.1 Subjects

Subjects (N=6) were adult (>100 days post hatch) male Bengalese finches. All animal procedures were approved by Emory University's Institutional Animal Care and Use Committee.

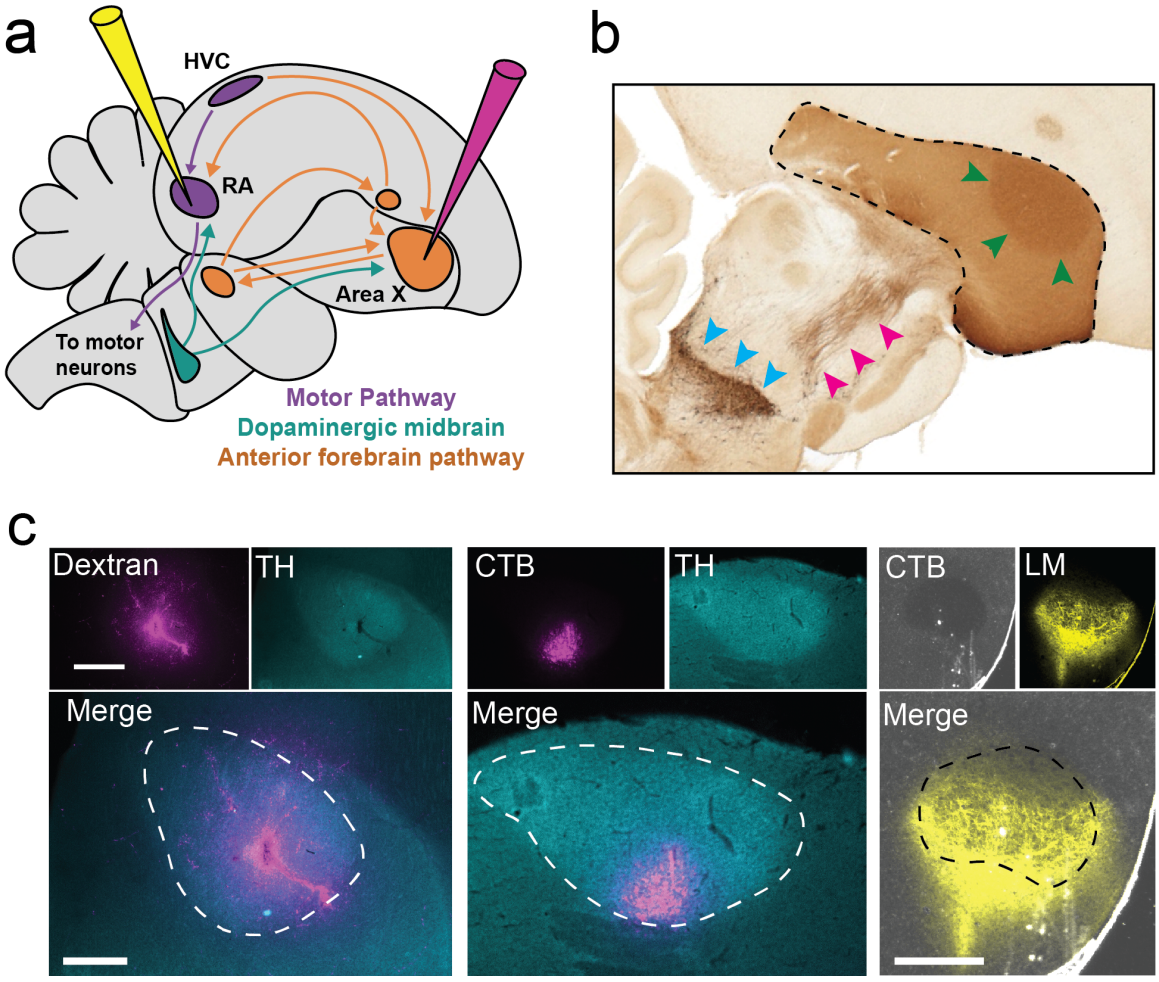

Figure 2.1: (a) Song system connectivity and neuroanatomical methods summary. Yellow and pink indicate tracer injections. The motor pathway and anterior forebrain pathway (AFP) are shown in orange and blue; dopaminergic projections are shown in turquoise. (b) Saggital section of 3,3'-diaminobenzidine immunostain for tyrosine hydroxylase (TH). Black dashed line indicates basal ganglia. Blue and magenta arrows indicate dopaminergic midbrain structures and projections. Green arrows indicate Area X. (c) Example Area X and robust nucleus of the arcopalilum (RA) injection sites. Area X injections of cholera toxin b (CTB) and dextran are shown in magenta. RA tracer (CTB) is shown in yellow. RA visualized using light microscopy (LM) with phase contrast. Dashed white lines indicate the borders of Area X (left, middle), dashed black line indicates borders of RA (right). Scale bar: 250 μm

2.3.2 Tracer injections

All tracer injections used either recombinant cholera toxin subunit b (CTB) conjugated to AlexaFluor™ 488 or 594 (Molecular Probes #C-34775/C-22841, and #C34777/C-22842), diluted to 1% in 0.1 M phosphate buffer (PB) or dextran conjugated to tetramethylrhodamine (TRITC) or AlexaFluor™ 488 (Invitrogen #D-1868 and #D-22910), diluted to 10% in 0.1 M PB. Tracer volumes are listed in Table 2.1. To inject the tracers, birds were anesthetized using either a mixture of ketamine, midazolam, and isoflurane or isoflurane alone (0.25 - 4%). Feathers around the head were removed, the scalp was anesthetized locally with 5% lidocaine, and the bird was placed into a stereotax. After performing three sterile scrubs, we made an incision along the midline of the head to reveal the top of the skull. The topmost layer of skull was removed, and we located Y0 (defined as the most posterior point visible at the junction of the midsagittal sinus and the two sinuses that run on either side of the cerebellum) to serve as the reference point for stereotactic coordinates. To perform injections, skull was removed over our target coordinates and pressure injections of tracer were done using a Nanoject II (Drummond Scientific Company) Area X coordinates were based on previous studies (Hoffmann et al., 2016; Nicholson et al., 2018). Because of its smaller size, coordinates for RA were located electrophysiologically in each subject using a 0.1 MΩ microelectrode (MicroProbes For Life Science #PI20030.1A3) connected to a differential amplifier (#69000 A-M Systems, Sequim, WA). After 7-14 days, birds were transcardially perfused using 4% paraformaldehyde in PB.

2.3.3 Histology

Brains were post-fixed overnight after perfusion in 4% paraformaldehyde and then allowed to cryoprotect in 30% sucrose. Tissue was cut coronally in 40 μm sections on a sliding freezing microtome. Fluorescent immunohistochemistry was performed

Subject	Coordinates	Tracer	Volume
Dextran1	X: 5.65/1.4/3.1	Dextran tetramethylrhodamine (TRITC) 10kDa	X: 55.2 nL
Dextran2	X: 5.65/1.4/3.2	Dextran TRITC 10kDa	X: 69.2 nL
Dextran3	X: 5.65/1.4/-3.1	Dextran AlexaFluor™-488 10kDa	X: 55.2 nL
CTB1	X: 5.65/1.4/3.1	Cholera Toxin B AlexaFluor™-488	X: 55.2 nL
CTB2	X: 5.65/1.4/3.2; RA: -1.1/2.2/-2.0	Cholera Toxin B AlexaFluor™-594, AlexaFluor™-488	X: 96.6 nL, RA: 96.6 nL
CTB3	X: 5.65/1.41/-2,9; RA: -0.99/2.0/-2.05	Cholera Toxin B AlexaFluor™-488, AlexaFluor™-594	X: 96.6 nL, RA: 96.6 nL

Table 2.1: Injection coordinates relative to Y0 (AP/ML/DV; mm), tracer, and total injection volume per region injected.

using a primary, secondary, and tertiary antibody to enhance immunofluorescence. Tissue was washed three times for five minutes each in 0.1 M PB after every step except for blocking, and all solutions were made in 0.1 molar phosphate buffer. First, tissue sections were incubated in 1% NaBH4 for 20 minutes to limit autofluorescence. Second, sections were incubated in block solution (2.5-5% normal serum corresponding to the secondary antibody host species and 0.5% Triton X-100) for 1 hour to limit non-specific antibody binding. Third, sections were incubated in primary antibody solution (1% normal horse serum, 0.1% Triton X-100) for 16-24 hours. Fourth, sections were incubated in a biotinylated secondary antibody solution (0.1% TX-100) for 4-16 hours. Fifth, sections were incubated in biotin-streptavidin tertiary solution (0.1% TX-100) for 4 hours to visualize the primary antibody. Tissue was then mounted on glass slides and coverslipped using Fluoro Gel with DABCOTM (#17985 Electron Microscopy Sciences). See Table 2.2 for antibody information and concentrations. All immunohistochemistry steps were performed at room temperature.

Immunogen/protein	Manufacturer, catalog #, RRID, species, mono/poly	Dilution
Tyrosine hydroxylase purified from PC12 cells	Millipore, MAB318, RRID:AB_2201528, mouse monoclonal	1:1000
Horse Anti-Mouse IgG, Biotinylated	Vector Laboratories, BP-2000, RRID:AB_2687893, polyclonal	1:400
Alexa Fluor 647-AffiniPure Donkey Anti-Mouse	Jackson ImmunoResearch Labs 715-605-151, RRID:AB_2340863, polyclonal	1:200
Streptavidin-AMCA	Vector Laboratories, SA-5008-1, biotin binding protein with fluorophore	1:200

Table 2.2: Antibodies and proteins used

2.3.4 Microscopy, digital photography, image processing, and quantification

Injection specificity was assessed by visualization of the injection sites using an Olympus IX51 widefield microscope. To assess retrograde labeling in the dopaminergic midbrain, two nonconsecutive sections at least 40 um apart from each of VTA, SNc, and PAG were imaged from the hemisphere ipsilateral to tracer injection sites using an Olympus FV1000 confocal microscope at 20x. Laser settings were determined by viewing signal intensity histograms and ensuring that the images were not under or over-saturated, and that the full dynamic range was used. Because of this, different subjects were imaged with different laser power settings to account for differences in fluorescent brightness. All confocal images were taken at a scan speed of 8μs per pixel with 2x Kalman averaging. Color channels were imaged sequentially for each line. Microscope images were analyzed in FIJI/ImageJ where brightness, contrast, and color balance were adjusted. Tile scans were stitched using the ImageJ Grid/Collection stitching plug-in (Preibisch et al., 2009).

Dopaminergic midbrain structures were visualized using tyrosine hydroxylase (TH)

immunohistochemistry. VTA, SNc, and PAG were identified by their characteristic shapes and locations in the midbrain (Bottjer, 1993; Reiner et al., 1994; Appeltants et al., 2000; Person et al., 2008; Kingsbury et al., 2011). Briefly, the VTA was identified as a large, dense oval-shaped TH+ structure in the anterior midbrain near the optic nerve. VTA extends caudally and laterally into SNc. PAG was identified based on its shape and proximity to the aqueduct and distinctive shape (Bottjer, 1993; Appeltants et al., 2001; Kingsbury et al., 2011; Haakenson et al., 2020). Because avian VTA and SNc form a continuous field without clear delineation in some sections we defined the boundary between the two as the narrowest point along the medial lateral axis, consistent with previous work (Person et al., 2008). The midbrain also contains a sparse tract of TH+ neurons that extends from the VTA/SNc complex to the PAG; these cells were not included in analysis.

Cells were counted manually in confocal z-stacks using the ImageJ CellCounter plug-in (2010). Only cells clearly within pre-defined borders of the brain region of interest were counted. Double labeling was assessed visually based on the presence of multiple fluorescent labels of the general shape in the same x,y, and z planes. Proportions of TH+ and TH neurons were analyzed using a z-test for proportions with a Bonferroni correction for multiple comparisons.

2.4 Results

We characterized the dopaminergic projections to Area X, a song-specialized basal ganglia nucleus required for song learning and implicated in vocal production (Sohrabji et al., 1990; Kojima et al., 2018). Using retrograde tracer injections (Fig. 2.1a,c), we analyzed the number of cell bodies containing tracer from Area X injections in five birds. One bird ('CTB3') was excluded from quantitative analysis due to inconsistent tyrosine hydroxylase (TH) staining. We observed robust retrograde labeling

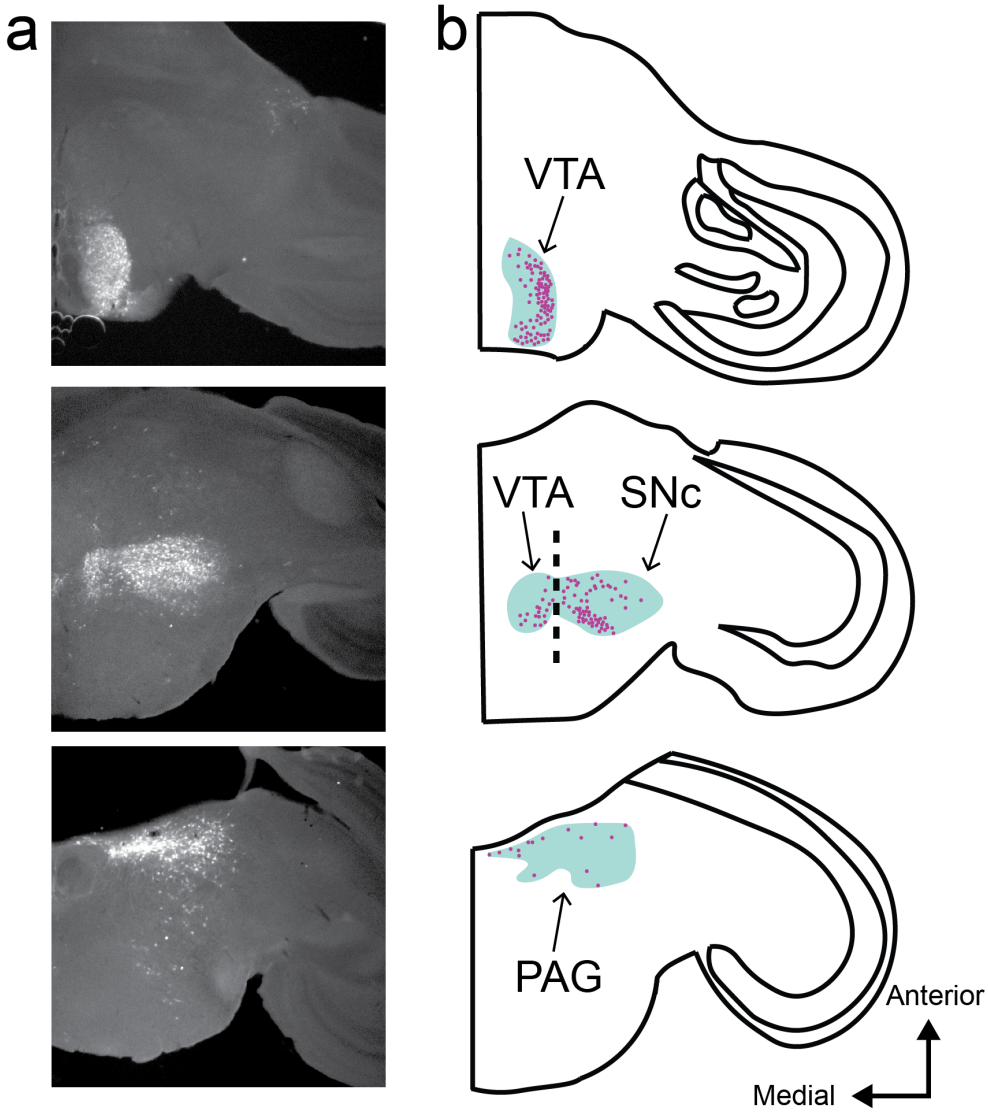

Figure 2.2: (a) Widefield images of tyrosine hydroxylase fluorescent immunostain illustrating the location and shape of VTA (top), VTA/SNC (middle) and PAG (bottom). (b) Distribution of tracer-labeled cells in dopaminergic midbrain nuclei, drawn based on representative images from one dextran-injected subject. Dopaminergic regions shown in light blue, tracer-labeled cells shown in magenta.

in the dopaminergic midbrain from both cholera toxin subunit b (CTB) and dextran injections in Area X (Figs. 2.2,2.3). Our analysis focused on three structures: The VTA and SNc, two compact and connected structures in the anterior portion of the midbrain, and PAG, a more diffuse structure in the posterior and dorsal part of the midbrain near the aqueduct (Fig. 2.2a). The majority of tracer-labeled cell bodies were in the dorsal, ventral, and lateral regions of VTA, ventral SNc and dorsal PAG (Fig. 2.2b).

Because our dataset contained both birds injected with CTB and birds injected with dextran, tracers which utilize different mechanisms for retrograde transport (Fritzsch, 1993; Wang et al., 1998), we next sought to quantify the tracer efficacy in each subject. Though labeling looked broadly similar between groups (Fig. 2.3a-f), our dextran injections yielded a consistently higher overall density of retrogradely labeled cells than did our CTB injections (Fig. 2.3g). Because of this, we chose to analyze the two tracer groups separately. It is unclear whether the differences in retrogradely labeled cells are due to differences in the efficacy of uptake or transport, or differences in injection parameters used: while a greater total volume of CTB solution was injected in each subject, the dextran solution was at a higher concentration (see 2.1 and Methods). One dextran-injected bird ('Dextran3') had a notably higher density of labeled cells than the other two, despite similar injection volumes (Fig. 2.3g, Table 2.1). This might indicate greater axonal damage during the tracer injection, which increases the axonal uptake of dextrans (Glover et al., 1986).

2.4.1 Putative TH(-) neurons in primarily dopamine structures projecting to Area X

We used confocal microscopy to quantify the number of TH+ and TH- neurons in VTA, SNc, and PAG that project to Area X (five hemisphere analyzed from five birds). We found TH- projection neurons (cells labeled with retrograde tracer but

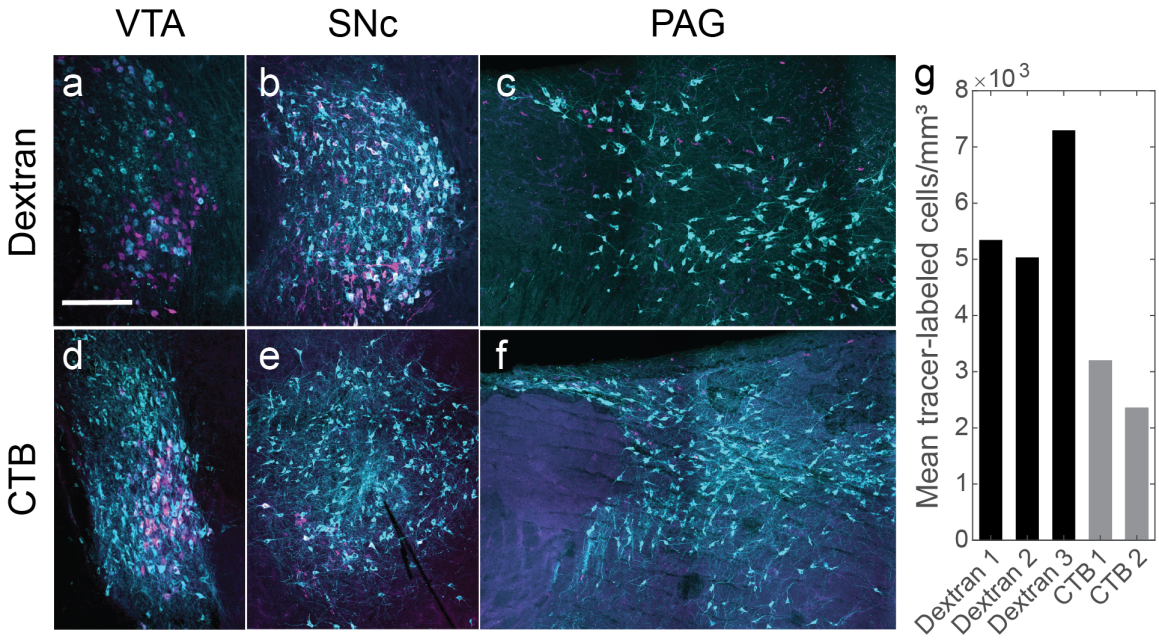

Figure 2.3: (a-f) Example images of Area X-projecting neurons labeled with either cholera toxin b (CTB) or dextran tracers. Magenta indicates tracer-labeled cells (a-c dextran, d-f CTB) and turquoise indicates tyrosine hydroxylase immunohistochemistry. Scale bar: 150 μm. (g) Tracer-labeled cells per um^3 for each subject, averaged across a total of six sections (two each of VTA, SNc, and PAG per bird). Black indicates dextran tracer injection, gray indicates CTB tracer injection.

not TH immunofluorescence) spatially intermingled with TH+ neurons (cells labeled with both retrograde tracer and TH immunofluorescence) in all three structures (Fig. 2.4a-i). In both dextran and CTB-injected birds we found significant differences ($p < 0.05$, z-test for proportions) in the ratio of TH+ to TH- neurons between PAG and each of VTA and SNc, but no difference in the ratio of TH+ to TH- neurons between VTA and SNc (Fig. 2.4m-n). The ratio of TH- to TH+ tracer-labeled neurons was qualitatively similar across both CTB and dextran groups, with a slightly larger proportion of retrogradely labeled neurons expressing TH in VTA than SNc, and the majority of retrograde labels apparently lacking TH in PAG. Across both tracer groups, we found 30-39% of tracer-labeled neurons in VTA and SNc and 65-87% of tracer-labeled neurons in PAG were putatively TH-.

2.4.2 Distinct populations of dopamine neurons project to RA and X

We next used dual injections of CTB conjugated to different fluorophores into Area X and RA (a pallial nucleus with similarities to mammalian motor cortex; Fig. 2.1a,c) to determine whether an overlapping population of dopaminergic neurons projected both regions (two hemispheres analyzed from two birds). Similar to previous results in canaries (Appeltants et al., 2002), we observed sparse labeling throughout the dopaminergic midbrain from our RA injections. Despite their often close proximity to each other, we did not identify any cells containing tracer from both RA and Area X in VTA, SNc, and PAG (Fig. 2.5a-i). This suggests that Area X and RA receive dopaminergic input from entirely separate populations of neurons.

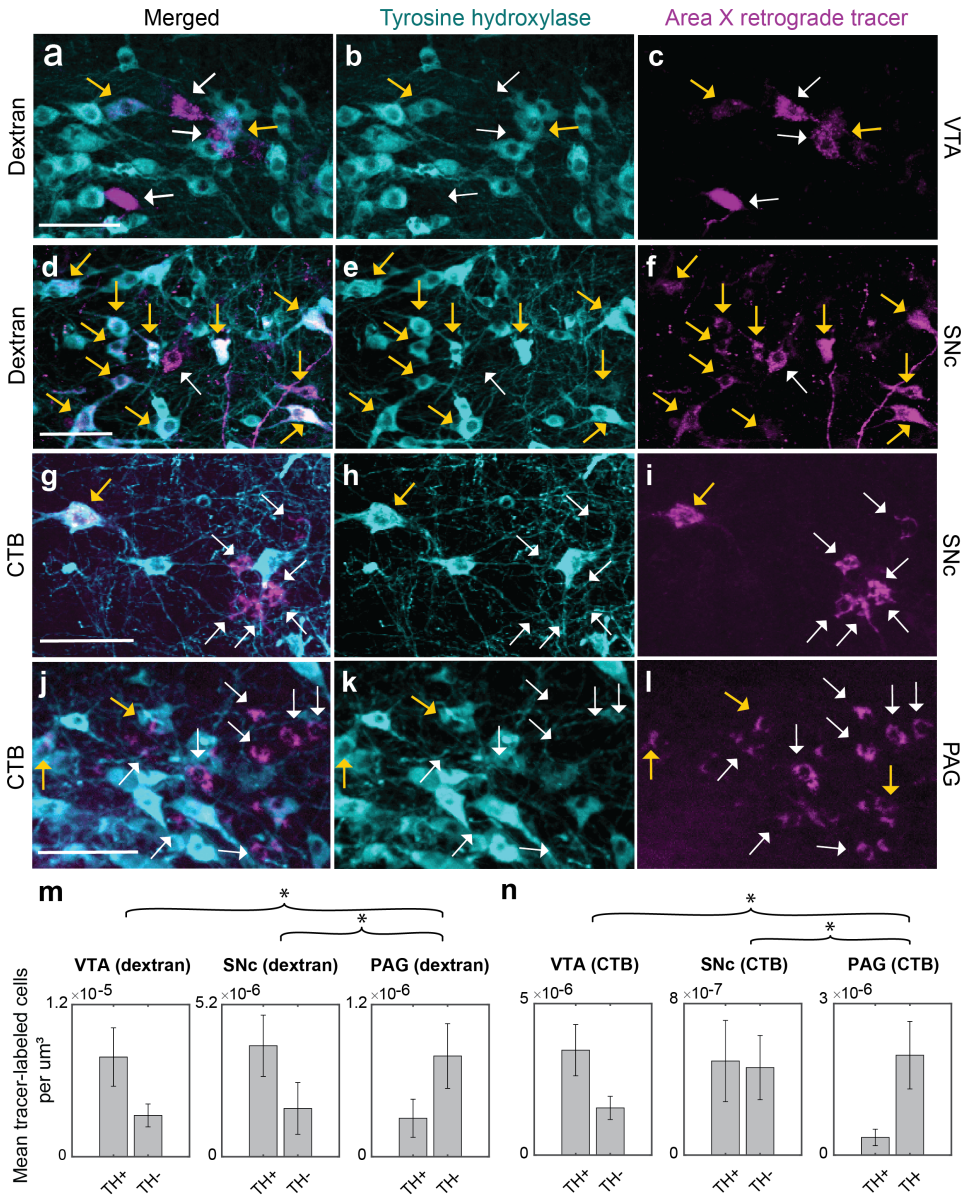

Figure 2.4: (a-l) Example images of Area X-projecting neurons retrogradely labeled with either cholera toxin b (CTB) or dextran tracers. Magenta indicates tracer-labeled cells (a-f dextran, g-l CTB) and turquoise indicates tyrosine hydroxylase (TH) immunohistochemistry. Yellow arrows indicate TH+ Area X-projecting neurons and white arrows indicate putative TH- Area X-projecting neurons. Scale: 50 μm. (m-n) Quantification of putative TH+ and TH- neurons in ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and periaqueductal gray (PAG). Mean +/- SEM shown. Asterisks indicate $p < .05$ in a z-test for proportions.

2.5 Discussion

Using retrograde tracer injections and immunofluorescent labeling of TH (Fig. 2.1a,c), we performed the first detailed characterization of the dopaminergic projections to Area X in the Bengalese finch. We show that Bengalese finch Area X receives dopaminergic input from VTA, SNc, and PAG (Figs. 2.2b,2.3). Surprisingly, we also find a large population of putative TH- projections to Area X in each of these primarily dopaminergic structures (Fig. 2.4). Finally, we find no evidence of neurons in the dopaminergic midbrain projecting to both Area X and the motor nucleus RA (Fig. 2.5).

2.5.1 Identification of putative non-dopaminergic neurons projecting to Area X

In our dataset, we identified 30-39% of Area X projection neurons in VTA and SNc as putatively TH-, suggesting a substantial non-dopaminergic projection (Fig. 2.4). This is in surprising contrast to previously published data in zebra finches, a closely related species, which found approximately 2-10% of Area X projection neurons were TH- (Person et al., 2008; Tanaka et al., 2018). In contrast to VTA/SNc, we found 65-87% of Area X projecting neurons in PAG were TH-, broadly similar to results from zebra finches (Tanaka et al., 2018). PAG, despite having a large dopaminergic neuron population, is not generally considered to be a primarily dopaminergic structure (Kingsbury et al., 2011).

Our results demonstrate a substantial difference between VTA and SNc Area X projectors between Bengalese and zebra finches, which could be reflective of the former's domestication. Few studies have directly compared Bengalese and zebra finch neuroanatomy. However, the Bengalese finch is a domesticated strain of the white-backed munia (*Lonchura striata*), and the two have some neurobiological and

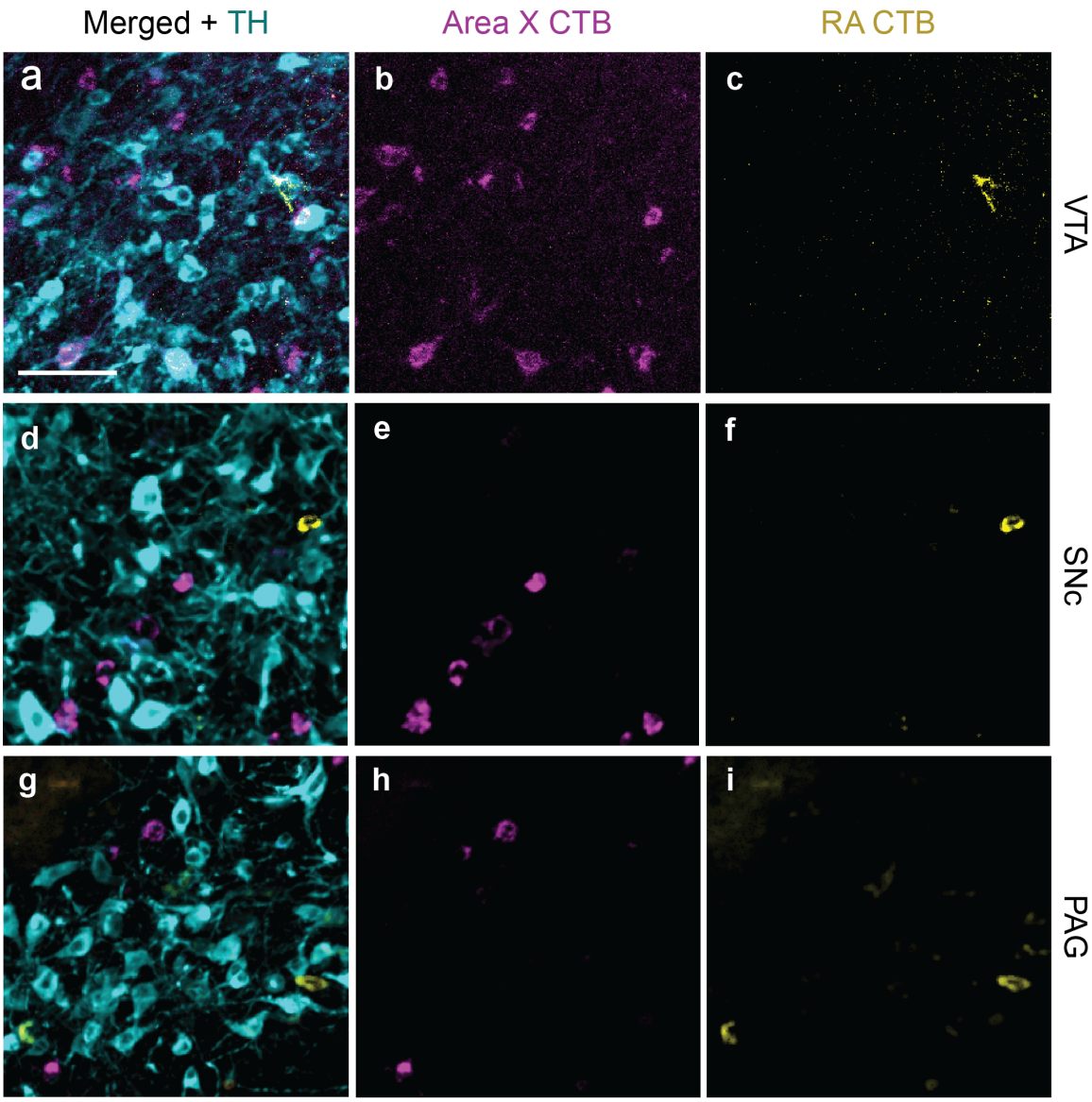

Figure 2.5: Separate populations of neurons project to the robust nucleus of the arcopallium (RA) and Area X. (a-c) ventral tegmental area (VTA), (d-f) substantia nigra pars compacta (SNc), (g-h) periaqueductal gray (PAG). Magenta indicates Area X projecting neurons labeled with cholera toxin b tracer (CTB), yellow indicates RA projecting neurons labeled with CTB, turquoise indicates tyrosine hydroxylase immunohistochemistry. Scale bar: 50 μm.

behavioral differences that are believed to be the result of domestication. Further, many of these behavioral differences are linked to dopaminergic signalling, though the dopaminergic systems of the two birds have not been directly compared (Okanoya, 2017; O'Rourke et al., 2021). Thus, it is possible that the Bengalese finch has domestication-linked differences from closely related but non-domesticated species more broadly. Follow-up experiments directly comparing domesticated and non-domesticated finch species under the same experimental conditions will be a crucial next step for expanding these results.

Though the large number of TH- projection neurons in VTA and SNc we report is unusual among studies of songbirds, VTA in particular is known to contain a substantial number of TH- projection neurons in other species. In rodents, estimated numbers of non-dopaminergic projection neurons in VTA and SNc vary among studies using similar methods to ours. Some studies report less than 5% of projection neurons from both SNc and VTA to striatum are TH- (Kooy et al., 1981; Farassat et al., 2019). In others, as many as 15% of all VTA projection neurons, 15-20% of VTA neurons projecting to nucleus accumbens, and 49% of VTA neurons projecting to prefrontal cortex were found to be TH- (Swanson, 1982; Yamaguchi et al., 2011). Large TH- projections have also been reported in rats from VTA to the deep cerebellar nuclei, and from VTA and lateral SNc to the amygdala (Ikai et al., 1992; Loughlin and Fallon, 1983; Avegno et al., 2021). Thus, while there is some disagreement in the literature, SNc projection neurons are generally reported to be almost entirely dopaminergic, while studies of VTA projection neurons frequently report a large non-dopaminergic population.

An important caveat to this work (and other studies using similar methods) is that putative TH- neurons might actually have low TH levels that our immunohistochemistry was not sensitive enough to detect. We believe the this is possible but unlikely, given that previous studies in rodents have shown comparable numbers of

TH+ and TH- VTA-amygdala projection neurons using both immunohistochemistry and RNAScope, a highly sensitive *in situ* hybridization method that is able to detect very low levels of messenger RNA (Wang et al., 2012; Avegno et al., 2021).

2.5.2 What is the function of VTA and SNc non-dopaminergic projection neurons?

The functional role of TH- VTA/SNc projection neurons in songbirds has not been established. From a computational perspective, VTA glutamatergic or GABAergic projection neurons could give dopaminergic signals a higher level of spatial and temporal precision. Dopaminergic signalling in the striatum acts largely through volume transmission and G protein-coupled receptors, making it a relatively slow and spatially nonspecific signalling mechanism (Beaulieu and Gainetdinov, 2011; Fuxe et al., 2013). But dopaminergic signalling has been shown to be an essential component of fast, temporally precise learning (Schultz, 2019). Coupling less precise dopaminergic signals with faster, more spatially restricted activation of ionotropic receptors could narrow the temporal and spatial range of dopamine-mediated plasticity via brief postsynaptic depolarization (Lapish et al., 2007; Fino et al., 2009; Yagishita et al., 2014). Because songbirds learn to change aspects of their song on extremely precise (submillisecond) timescales (Charlesworth et al., 2012), future studies in songbirds are well-suited to ask whether manipulation of TH- projections from the dopaminergic midbrain decrease the temporal specificity of learning.

Alternatively, it is possible that TH- neurons are involved in a specific form of reinforcement learning. For example, several studies in rodents show that glutamatergic projections from VTA to lateral habenula and both glutamatergic and GABAergic projections from VTA to nucleus accumbens are associated with aversive responses and aversive learning (Brown et al., 2012; Root et al., 2014; Qi et al., 2016). In reinforcement learning models of bird song learning, a poor performance (i.e. a syllable

sung at a higher pitch than intended) is believed to act as an internally-generated negative reinforcement signal. Further, robust experimental paradigms exist in songbirds for studying precisely timed learning from external aversive cues (Tumer and Brainard, 2007), which have already been shown to rely on dopaminergic signalling in Area X (Hoffmann and Sober, 2014; Hisey et al., 2018; Xiao et al., 2020). Thus, an important future experiment will be testing the role of Bengalese finch TH- neurons during aversive learning.

2.5.3 Separation of dopaminergic populations projecting to song-specialized basal ganglia and motor cortical nuclei

Using dual tracer retrograde tracer injections (Fig. 2.1a,c), we examined VTA, SNc, and PAG for evidence of collateralization between RA and Area X neurons. We did not find any evidence of double-labeled cells in our dataset (Fig. 2.5), suggesting that distinct populations project to each structure.

An important question is whether or not dopamine is fundamentally performing the same function throughout the forebrain (Berke, 2018; Schultz, 2019). Though they both receive dopaminergic input, RA and Area X have very different hypothesized functions. RA is a pallial structure with many functional and molecular similarities to mammalian motor cortex (Colquitt et al., 2021) which is necessary for song production (Nottebohm et al., 1976). Area X is a striatopallidal basal ganglia nucleus that is not necessary for song, but is necessary for song learning (Sohrabji et al., 1990). In particular, dopaminergic signalling in Area X is necessary for song learning, and Area X-projecting dopaminergic neurons display classic reward prediction error (RPE)-like activity (Hoffmann et al., 2016; Gadagkar et al., 2016; Hisey et al., 2018; Xiao et al., 2018). However, over longer timescales, learning that is initially dependent on Area X becomes 'consolidated' in RA (Andalman and Fee, 2009), suggesting a potential role for dopamine-mediated plasticity. The role of dopaminergic signalling in RA is

not well explored, though it has been demonstrated to modulate neuronal excitability in slice (Liao et al., 2013; Wang et al., 2020). Our results suggest that dopaminergic signals to RA and Area X have the potential to be highly functionally, as well as anatomically, distinct. Future studies to determine the role of dopaminergic signals in RA, and the neurophysiological patterns of dopaminergic RA projecting neurons will be crucial for establishing the function of dopamine in RA.

The separation of RA-projecting and Area X-projecting dopaminergic neurons is consistent with gross projection patterns of dopaminergic neurons in other species. In rodents, most SNc and VTA dopaminergic axons projecting to the striatum form wide dense arbors within the striatum, but do not project outside of the striatum. However, there is evidence for very sparse collateral projections from striatum-projecting dopaminergic neurons to globus pallidus external (GPe), cingulate cortex, and motor cortex (Takada and Hattori, 1986; Matsuda et al., 2009; Hosp et al., 2015). In addition to our results showing distinct RA and Area X projections, Area X and the immediately adjacent medial striatum also receive dopaminergic input from separate populations of neurons in VTA and SNc (Person et al., 2008). This supports the view that locally broad but regionally specific projection patterns for individual dopaminergic neurons represents a common organizational principle for dopaminergic neurons across species. This suggests that behavioral investigations of dopamine in the song system should be projection-specific.

Chapter 3

Neural activity in a song-specialized basal ganglia nucleus reflects syllable sequencing and phonology

3.1 Abstract

The basal ganglia are hypothesized to be involved in several aspects of motor control, including control of precise movement kinematics, motor sequencing, and motor learning. Songbirds provide an excellent model organism for studying motor learning due to the highly quantifiable nature of individual song syllables and the presence of anatomically distinct circuits controlling song production and song learning (collectively called the 'song system'). The songbird basal ganglia nucleus Area X has a well-established role in song learning, and increasing evidence suggests that it is involved in motor control as well. However, very little is known about the relationship between single unit neural activity in Area X and behavior. To address this

question, we perform Area X *in vivo* neurophysiology in Bengalese finches. We first demonstrate that Bengalese finch neurons can be classified into pallidal and striatal subtypes. We then show that Area X neurons represent both information about pitch and sequence. However, sequence representations are robust while pitch representations are relatively weak, consistent with reinforcement learning models of song learning which predict that Area X integrates dopaminergic reward signals with contextual information about recent motor commands (such as syllable transitions). These results provide insight into the potential role of Area X in vocal motor control and learning.

3.2 Introduction

Basal ganglia dysfunction leads to profound motor deficits (Yanagisawa, 2018). However, the specific role of the basal ganglia in motor control remains controversial. Broadly, hypotheses for basal ganglia function fall into three non-mutually exclusive groups: The macro or meso-level organization of behavior (e.g. action selection or motor sequencing) and kinematic control of ongoing movements (e.g. precise control of actions or modulation of action vigor; discussed at length in Chapter 1.3.2; Mink 1996; Morita et al. 2016; Graybiel 1998; Jin et al. 2014; Dudman and Krakauer 2016; Desmurget and Turner 2010; Dhawale et al. 2021); and motor learning (discussed at length in 1.4).

Songbirds (oscine passerines) have proven to be a powerful model system for studying the role of the basal ganglia in complex motor skills (Doupe et al., 2005; Ziegler and Ackermann, 2017; Mooney, 2020; Aamodt et al., 2020; Chen and Goldberg, 2020). Song is a complex motor behavior controlled by well-defined and anatomically distinct motor circuits (collectively called the "song system"; Fig. 3.2a) with strong similarities to mammalian cortical and corticothalamic-basal ganglia circuits, making

songbirds a uniquely tractable for studying the neurobiological basis of motor control and motor learning (Nottebohm et al., 1976; Sohrabji et al., 1990; Doupe et al., 2005).

Among songbirds, the Bengalese finch (*Lonchura striata* var. *domestica*) is a particularly useful model for studying the role of the basal ganglia in kinematics vs. higher level behavioral organization. Like other songbirds, their song is highly quantifiable with variable trial-to-trial kinematic (phonological) variation. Unlike other commonly studied species, however, they also have rich trial-to-trial variability in the sequencing of song elements (called 'syllables'; Fig. 3.1; Okanoya and K 1997; Okanoya 2004).

The song system contains a specialized basal ganglia nucleus called "Area X", which contains both striatal and pallidal neurons (Fig. 3.2b, Farries and Perkel 2002; Carrillo and Doupe 2004) and is required for vocal learning (Sohrabji et al., 1990). In mammals, the striatum serves as the input layer of the basal ganglia (Parent and Hazrati, 1995a). The globus pallidus internal (GPi) is one of the primary output nuclei of the basal ganglia and in mammals is believed to influence behavior via brief disinhibiton of motor thalamus (Albin et al., 1989; Parent and Hazrati, 1995b). The globus pallidus external (GPe) is an intrinsic basal ganglia nucleus in the indirect pathway between the striatum and GPi, and is thought to play an important role in inhibiting actions (Albin et al., 1989; Parent and Hazrati, 1995b; Mallet et al., 2016). Work in zebra finches (*Taeniopygia guttata*), a species closely related to Bengalese finches, has shown that Area X neurons can be classified *in vivo* based on firing properties (Goldberg and Fee, 2010; Goldberg et al., 2010), demonstrating that conserved features of GPi and GPe neural activity can be identified in both songbirds and mammals. However, while there are several studies of *in vivo* recordings in Bengalese finch Area X (Peng et al., 2013; Seki et al., 2014), it is unknown whether Bengalese finch Area X neurons can be classified based on homology to mammalian neurons. Thus, we first sought to determine whether Bengalese finch neurons could be classified into

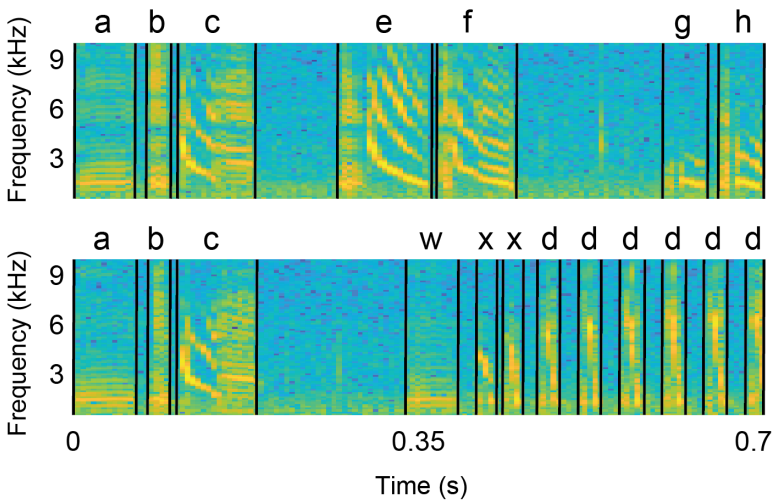

Figure 3.1: Example Bengalese finch song spectrograms of two different sequences sung by the same bird. Both sequences contain the common syllable motif 'abc', but then diverge into different sequences. Black lines indicate syllable onsets and offsets.

striatal, GPe and GPi subtypes.

Both phonological features of song and song sequence are actively maintained via sensorimotor learning. For example, deafening both zebra and Bengalese finches causes song to rapidly deteriorate (Nordeen and Nordeen, 1992; Woolley and Rubel, 1997), indicating that auditory feedback is necessary for the maintenance of normal vocal motor patterns. In laboratory settings, white noise bursts can be used to drive learned changes in syllable pitch in both zebra and Bengalese finches (Tumer and Brainard, 2007; Andalman and Fee, 2009; Hampton et al., 2009). It can also be used to change the frequency with which Bengalese finches sing different syllable sequences (Warren et al., 2012; Veit et al., 2021). Area X has a well-established role in learning the phonology of song (Sohrabji et al., 1990; Ali et al., 2013). Specifically, this learning is thought to be driven by dopaminergic projections to Area X (Hoffmann et al., 2016; Hisey et al., 2018; Xiao et al., 2018). There is also some evidence pointing to a role for Area X in real-time modulation of phonology in zebra finches. While lesions to Area X do not impact trial-to-trial variation in pitch, they decrease millisecond-scale pitch variations *within* individual syllables (Kojima et al., 2018). Interestingly, Area

X may be involved in transitioning between behavioral states associated with low and high variability in song pitch ('undirected' song sung in isolation and female-directed song, respectively ;Leblois et al. 2010). However, while some neural manipulations in Area X disrupt song sequence (e.g. Kubikova et al. 2014; Tanaka et al. 2016), its specific role in the control of sequence is much less well understood.

A key unanswered question is how song sequence and phonological song features such as pitch are represented in Area X neural activity. There is some behavioral evidence to suggest that when adult birds learn pitch changes (a form of learning which relies on Area X) in one sequential context, the learning partially generalizes to the same syllable in other non-targeted sequences (Hoffmann and Sober 2014; Tian and Brainard 2017; but see also Saravanan et al. 2020), suggesting the possibility of common neural representation of the same syllable in different sequential contexts. However, whether the neural representation of the same syllable in difference contexts is the same in Area X is unknown. Further, despite Area X's hypothesized role in learned pitch changes, to our knowledge no published studies attempt to correlate single unit neural activity with phonological features of song. This is notable because the highly recurrent nature of the anterior forebrain pathway (AFP) (illustrated in Fig. 3.2) makes both functional and lesion studies challenging to interpret. Thus, understanding Area X's role in song requires a better understanding of the neural representation of song in Area X.

In this study, we use *in vivo* neurophysiology in singing Bengalese finches to ask whether firing rate differs immediately prior to syllable onset for the same syllable in two different contexts. We hypothesize that firing rates of Area X neurons during a particular syllable would vary based on sequential context. Further, we hypothesized that we would find correlations between Area X neuron firing rates and pitch (an easily quantifiable phonological feature), but that the strength of correlation would be weaker than those observed in the robust nucleus of the arcopallium (RA), a

downstream motor nucleus (Fig. 3.2a; Sober et al. 2008), due to its distance from vocal motor neurons (approximately 4 synapses from Area X projection neurons).

3.3 Methods

3.3.1 Subjects

Subjects (N=4) were adult (>100 days post hatch) male Bengalese finches. For the duration of the experiment animals were isolated in sound-attenuated chambers. All animal procedures were approved by Emory University's Institutional Animal Care and Use Committee.

3.3.2 Microdrive implantation and data collection

For three subjects neural recordings were done using manual 16-channel movable microdrives (Innovative Neurophysiology, Inc., Durham, NC). In one subject a custom motorized microdrive (Okubo et al., 2014) was used.

Birds were anesthetized using either a mixture of ketamine, midazolam, and isoflurane or isoflurane alone (0.25 -4%). Feathers around the head were removed, the scalp was anesthetized locally with 5% lidocaine, and the bird was placed into a stereotax. After performing three sterile scrubs, we made an incision along the midline of the head to reveal the top of the skull. The topmost layer of skull was removed, and we located Y0 (defined as the most posterior point visible at the junction of the mid-sagittal sinus and the two sinuses that run on either side of the cerebellum) to serve as the reference point for stereotactic coordinates. Area X coordinates (5.65 anterior/posterior, 1.4 medial/lateral, -2.7 dorsal/ventral) were based on previous studies (Hoffmann et al., 2016; Nicholson et al., 2018). Recordings of both song and neural activity were done continuously in the home cage using either an Intan Technologies headstage and recording controller (#C3334 and #C3004) with the Innovative Neu-

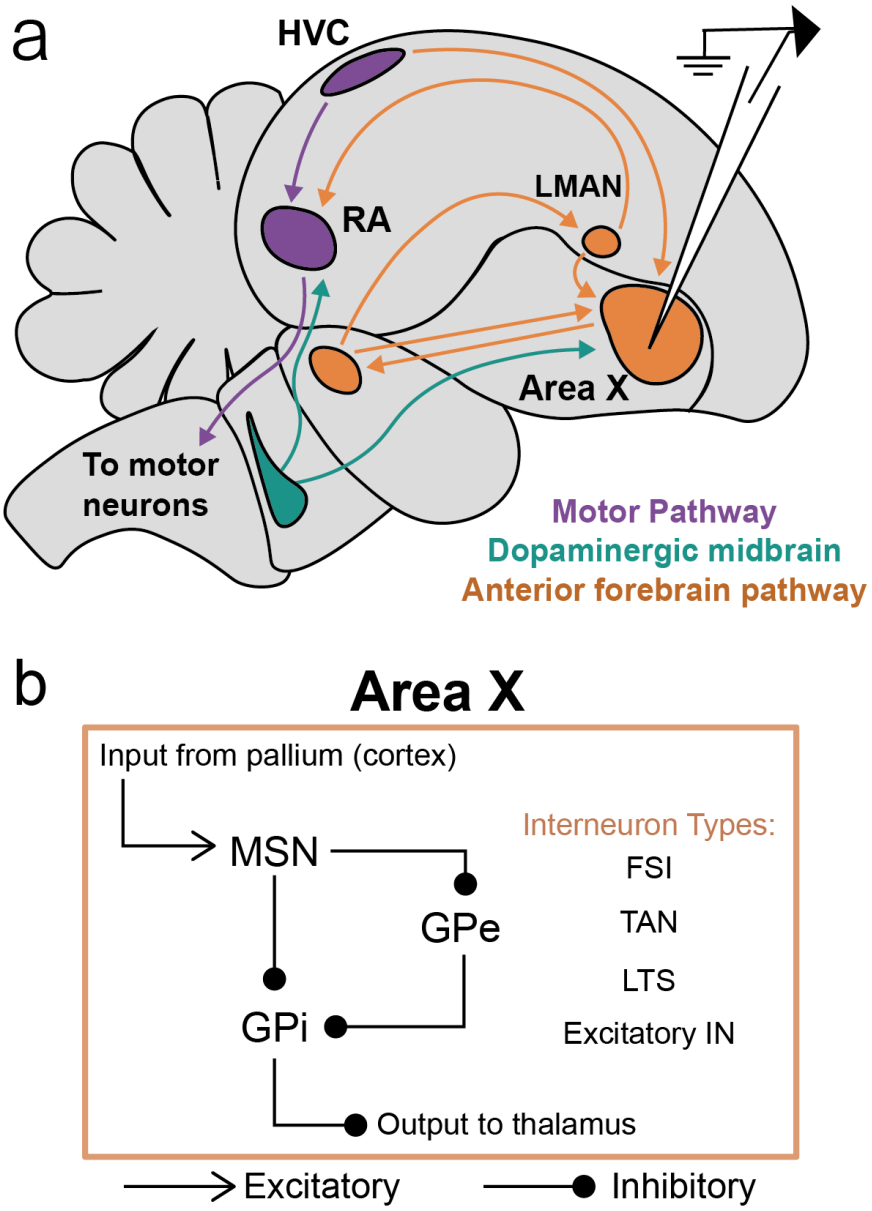

Figure 3.2: (a) Neurophysiology methods summary and diagram of primary neural pathways for song production and song learning. Unipolar electrodes (shown in white) were implanted in Area X to record single units *in vivo*. The motor pathway and anterior forebrain pathway (AFP) are shown in orange and navy; dopaminergic projections are shown in turquoise. (b) Area X is a striatopallidal nucleus containing medium spiny neurons (MSN), several classes of striatal interneurons including fast-spiking interneurons (FSIs), tonically active interneurons (TANs), and low-threshold spiking interneurons (LTS). It also contains two classes of pallidal neurons thought to correspond to globus pallidus internal (GPi) and globus pallidus external (GPe) neurons. Area X contains both a direct pathway from MSNs to GPi neurons, and an indirect pathway through GPe neurons. The precise connectivity of striatal interneurons within Area X has not been established.

rophysiology microdrives, or a connected to a differential amplifier (#69000, A-M Systems) using custom LabView software (National Instruments).

Recording locations were identified by the presence of characteristic changes in firing rate associated with the production of song and by *post hoc* histological confirmation of guide cannula placement above Area X (for birds implanted with Innovative Neurophysiology microdrives) or electrode tracts through Area X (for the bird implanted with a custom motorized microdrive).

3.3.3 Neural data analysis

Spike sorting

Spikes were sorted using custom Matlab (MathWorks) software using principal components analysis and k-means clustering (Sober et al., 2008). Units were classified as single units if there was less than 1% overlap in the spike and noise clusters and less than 1% of the interspike intervals were under 1 ms.

Neuron classification

For each single unit, summary statistics were computed from the first ten data files using custom Matlab software. If less than ten files were recorded for a unit all data was used. Units with less than two files were not included in the single unit dataset. Singing firing rates were calculated using song epochs lasting a minimum of 100 milliseconds. Non-singing firing rates were calculated from silent epochs lasting at least 100 milliseconds and separated from song epochs by at least 10 seconds.

To create a continuous estimate of firing rate, we took the instantaneous firing rate (IFR), defined as 1 over the interspike interval, and smoothed and filtered the signal as described in Goldberg et al. (2010). Briefly, we applied a finite impulse response equiripple 25-75 hz bandpass filter, and 80 dB stopband attenuation. IFRs were then mean-subtracted using an infinite impulse response 1 hz high-pass filter.

Bursts were defined as smoothed IFRs above 250 hz. Peak firing rate was defined as the 99th percentile smoothed IFR.

Spike half-width was measured from 1000 spike waveforms (or all waveforms in cases where fewer than 1000 waveforms were recorded), upsampling each waveform by a factor of 10 using cubic spline interpolation (Matlab 'spline' function). Peaks and half-widths were measured using the Matlab 'findpeaks' function, which defines peaks as local maxima and peak borders as the horizontal position of the lowest valley between peaks.

To quantitatively separate neurons classes we performed k-means clustering, a simple algorithm for separating groups based on minimization of the the sum of datapoint-to-cluster centroid distances. All k-means was performed with the Matlab 'kmeans' function using two-dimensional data and replicated 5 times from randomly selected centroids to find the smallest distance sum.

3.3.4 Behavioral analysis

Song was labeled by hand using custom Matlab software, except in one subjected where song was labeled using a convolutional recurrent neural network trained on hand-labeled data and spot-checked using custom Matlab software (Cohen et al., 2020; Nicholson and Cohen, 2021).

Pitch (fundamental frequency) was quantified at a fixed time point relative to syllable onset in harmonic stack syllables. Because the precise premotor latency for different Area X cell types is not known, we selected a large premotor window of 60 milliseconds based off the estimated premotor window of the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the AFP output nucleus (Giret et al., 2014).

3.3.5 Neuron-behavior correlations and significance testing

Sequence analysis

To determine whether the mean firing rates associated with a syllable in two different contexts were significantly different we performed a bootstrap analysis. For a given comparison, neural data from both branch points was combined into a single merged dataset, representing the null hypothesis (that neural activity for a given syllable is drawn from the same population, regardless of sequential context). We then created two individual datasets under the null distribution by resampling with replacement from the merged dataset (resampled dataset size for each branch matched the empirical dataset size). We then calculated the difference in mean firing rates between resampled datasets. We repeated this 10,000 times. This allowed us to create an estimated distribution of expected results under the null hypothesis. We then asked where on the null distribution our empirical difference in means fell; this percentile was our p-value. Any p-value less than .05/2 (two-tailed test) was considered statistically significant. Only neuron-branch point pairs where each branch had a minimum of ten trials were analyzed.

Effect size for sequence differences in neural firing rates was measured using discriminability index, or *d'*, defined as:

$$d' = \left| \frac{\hat{a} - \hat{b}}{\sqrt{\frac{\sigma_a^2 + \sigma_b^2}{2}}} \right| \tag{3.1}$$

Where a and b represent firing rates for a given sequence in the 60 millisecond premotor window. $\hat{a}$ and $\hat{b}$ represent the mean firing rates of a and b, and σ_a and σ_b represent the standard deviation (SD) of a and b. A *d'* of 1 would indicate that $\hat{a}$ and $\hat{b}$ were separated by a full standard deviation.

Pitch analysis

Pitch-firing rate relationships were analyzed using linear correlation. Correlation strength was estimated using the square of the Pearson correlation coefficient (r^2). Prior to computing pitch correlations, we discarded outliers with acoustic feature measurements lying >5 SDs from the mean. Visual inspection of audio spectrograms revealed that these were usually syllable iterations with highly irregular acoustics, which could potentially lead to errors in pitch quantification. Only neuron-syllable pairs with at least 50 trials were analyzed.

Controlling for multiple comparisons with a permutation test

Our analysis involved running numerous separate significance tests (69 and 42 for pitch and sequence, respectively). As a result, it was imperative to control for multiple comparisons. Because our conclusions do not depend on any one particular case having a significant difference, but instead on the overall proportion of significant cases we chose to do this using a permutation test. Briefly, for each case in the dataset we shuffled the behavioral variable (pitch or sequence) to simulate our data under the null hypothesis while still maintaining neural-neural correlations. We then re-ran our significance tests on the shuffled data. Next, we counted the total number of significant results from all shuffled cases. We then repeated this analysis for 1000 permutations, and created a histogram of significant p-values obtained for each permutation. We then compared the distribution of significant p-values under the null hypothesis to our empirically observed number of significant p-values, if the latter exceeded the 99th percentile of the former we considered it significantly different from the expected results under the null hypothesis (that the number of significant results was due to chance).

3.4 Results

3.4.1 Cell type identification

We first sought to determine whether Bengalese finch Area X neurons can be separated in striatal and pallidal subtypes based on *in vivo* firing properties. In mammals medium spiny neurons (MSNs) are the principal neuron type of the striatum. These neurons have characteristically sparse activity and low overall firing rates (Wilson and Groves, 1981) but display bursting activity 'tuned' to specific behaviors. This tuning is dynamic and changes with learning (Yin et al., 2009; Thorn et al., 2010). The striatum also contains tonically active cholinergic interneurons and several classes of GABAergic interneuron (Goldberg and Reynolds, 2011; Muñoz-Manchado et al., 2018). In contrast, the globus pallidus contains projection neurons with high tonic firing rates and few, if any, interneurons. GPe neurons have distinctive bursts and pauses, while GPi firing rate modulations are more shallow (DeLong, 1971; Kita, 2007; Nambu et al., 1997). Activity of neurons in both the GPe and GPi are correlated with movement (DeLong, 1971; Arkadir et al., 2004).

Songbird Area X contains both striatal and pallidal neurons, identified in zebra finches using both slice electrophysiology and molecular markers (Carrillo and Doupe, 2004; Farries et al., 2005; Xiao et al., 2021) and these neuron types are distinguishable based on firing properties from *in vivo* extracellular neural recordings. However, while several studies have recorded Bengalese finch Area X neural activity *in vivo* (Peng et al., 2013; Seki et al., 2014), none attempt to classify neurons based on their similarity to mammalian striatal or pallidal neurons. Because of the specific and oppositional organization of the direct and indirect pathways in Area X and the basal ganglia more broadly (Fig. 3.2c, also see Fig. 1.2b) this limits our ability to interpret electrophysiology results from Bengalese finches.

In our study, we recorded a total of 46 single unit neurons from 4 adult male

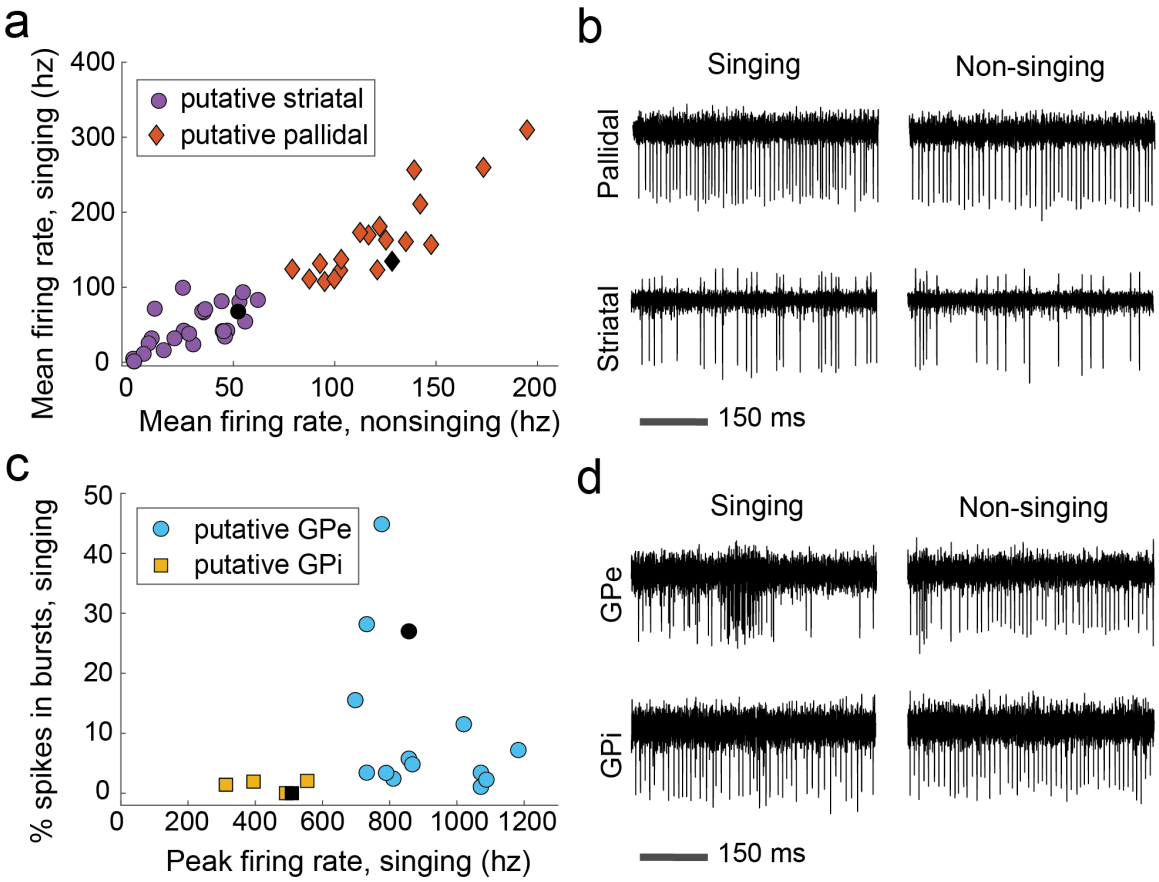

Figure 3.3: Classification of Bengalese finch Area X neuron subtypes (a) Separation of striatal and pallidal neurons based on firing rate. Black-filled markers indicate neurons shown in (b). (b) Example pallidal and striatal neural traces. (c) Separation of pallidal neurons into GPe and GPi clusters. Black-filled markers indicate neurons shown in (d). GPe neurons exhibit high-frequency bursts and long pauses, while GPi neurons exhibit slower rate modulations and brief pauses. All clustering done using k-means.

Bengalese finches during song. Our results show distinct clusters of low and high firing rate neurons (Fig. 3.3a-b) using two dimensional k-means clustering of singing and nonsinging firing rate, consistent with previous results in zebra finches (Goldberg and Fee, 2010; Goldberg et al., 2010; Woolley et al., 2014). The high firing rate group we have classified as putative pallidal neurons, while the low firing rate group we have classified as putative striatal neurons.

In mammals, the globus pallidus is separated into external and internal segments. Neurons in the GPe show characteristic high-frequency bursts and long pauses, while GPi neurons have tonic firing and only shallow rate modulations and brief pauses (DeLong, 1971). We found two distinct groups of putative pallidal neurons with these characteristics in our dataset using two-dimensional k-means clustering on peak firing rate and percent of total spikes occurring within a burst during song (Fig. 3.3c-d), similar to previous results in zebra finches (Goldberg et al., 2010; Woolley et al., 2014). These features were visible when observing song-aligned raster plots of neurons from the two groups, where GPe neurons had clear bursts and pauses at consistent times in song, and GPi neurons displayed more subtle rate changes (Fig. 3.4).

We next examined the firing properties of the putative striatal neurons in our dataset. Area X contains several types of striatal neurons (Farries et al., 2005). Due to the relatively small number of striatal neurons recorded we did not attempt to quantitatively separate these different cell types. However, within our group of putative striatal neurons we did observe diverse activity patterns consistent with MSNs, tonically active interneurons, and low-threshold spiking neurons (Fig. 3.5; Goldberg and Fee 2010; Woolley et al. 2014). Specifically, MSN-like neurons had sparse activity at specific, reliable points in song and otherwise were nearly silent. TAN-like neurons fire tonically with song-locked modulations. LTS-like neurons had long high-frequency bursts and long pauses. We did not record any units with the characteristic narrow waveforms of fast-spiking interneurons (Farries et al., 2005).

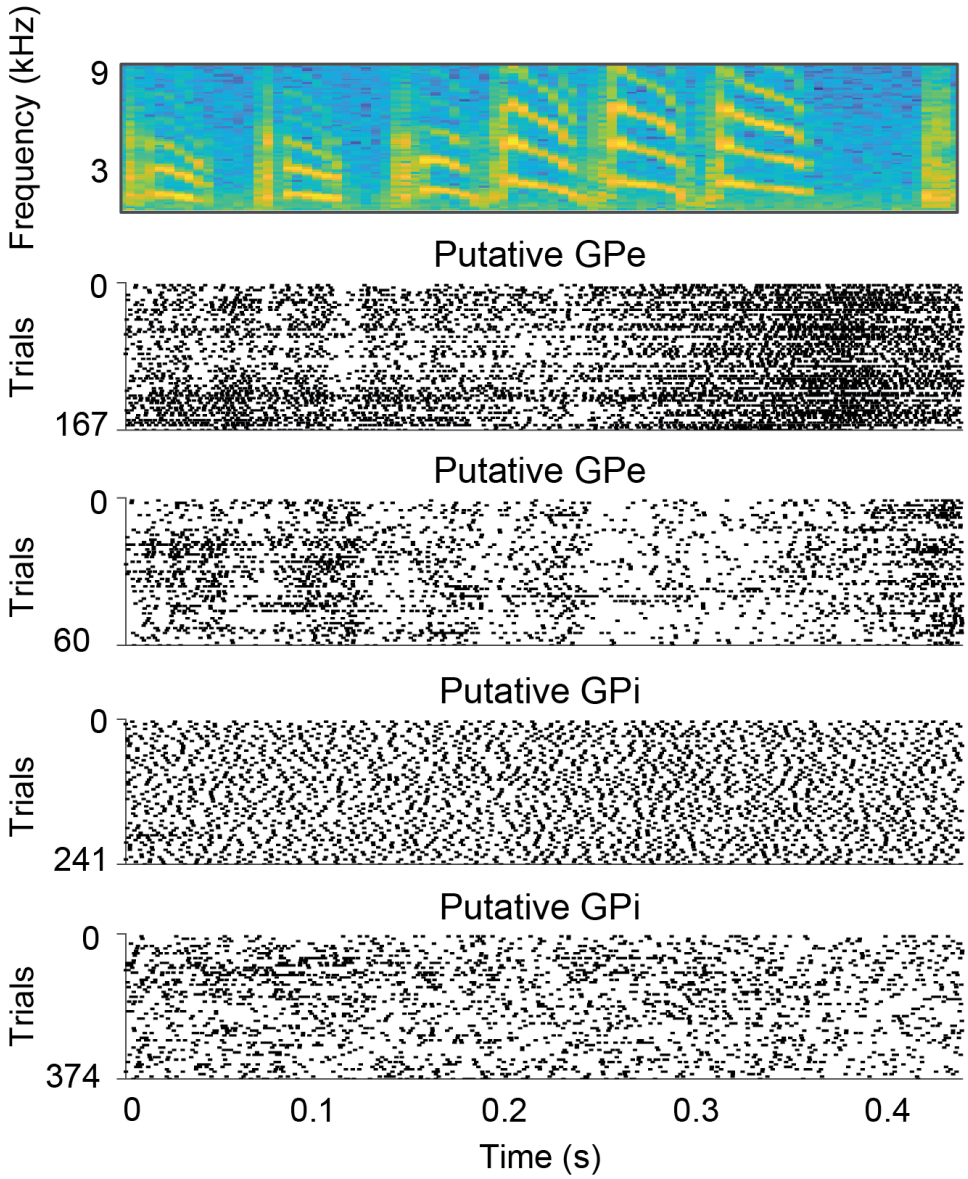

Figure 3.4: Raster aligned to song spectrogram for two putative globus pallidus external (GPe) and global pallidus external (GPi) neurons. GPe neurons exhibit characteristic high frequency bursts and long pauses. GPi neurons do not exhibit high frequency bursts for pauses. GPe and GPi neurons were classified using k-means clustering on peak firing rate and percent of spikes in burst during song.

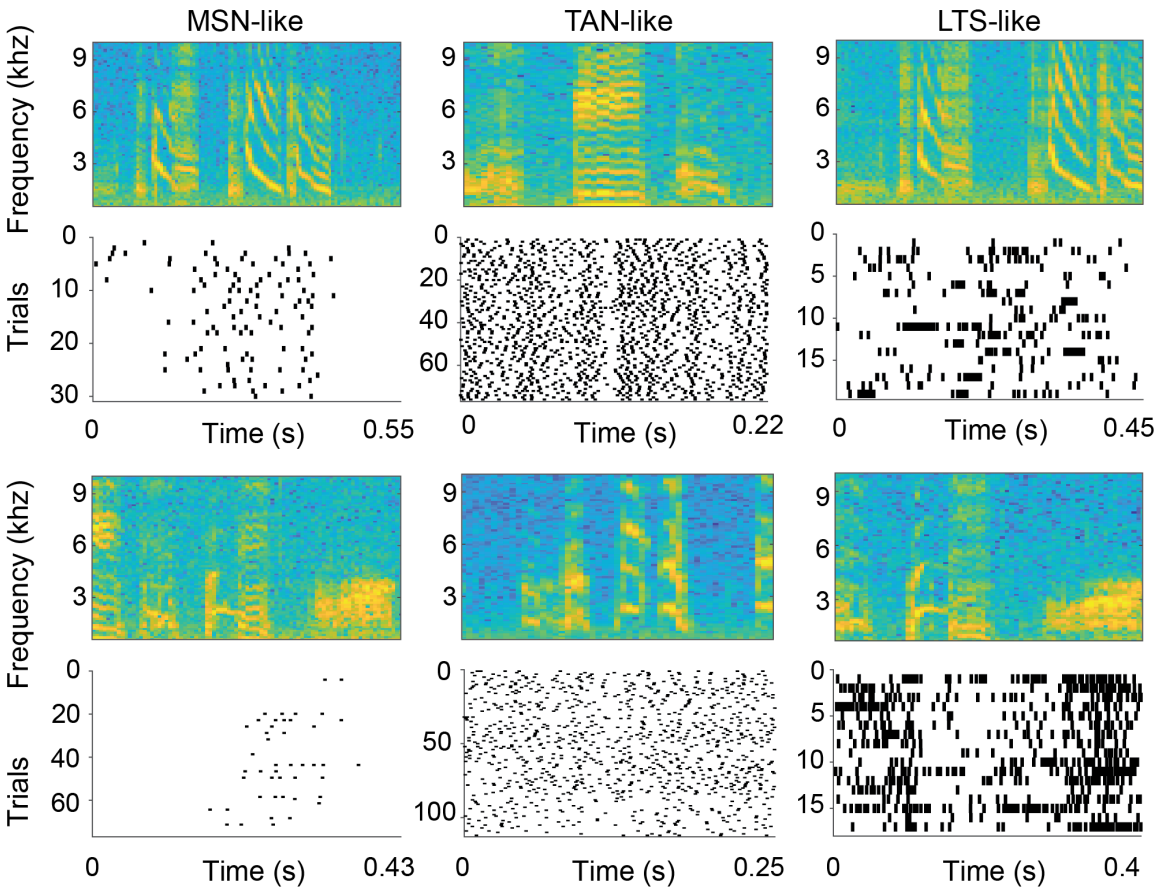

Figure 3.5: Example raster plots of putative striatal neuron activity aligned to song spectrograms. Though we did not attempt to quantitatively separate striatal subtypes, we observed neurons with diverse firing properties similar to medium spiny neurons (MSNs), tonically active neurons (TANs) and low-threshold spiking (LTS) neurons.

3.4.2 Area X neural activity varies based on sequential context

In order to understand how Area X might contribute to neural control of sequence control, we first need to determine how sequential information is represented in Area X neural activity. Unlike other commonly studied songbirds, Bengalese finch song can be sequentially complex (Okanoya, 2004). The point in song at which two or more different syllable sequences converge on to a common sequence or a common sequence diverges into two or more different sequences is called a branch point. We asked whether single unit firing rates in Area X for the same syllable differed when that syllable appeared in different sequences. To do this, we compared firing rates in a 60 millisecond premotor window for the syllable that appeared in all branched sequences but was closest to the branch point (examples highlighted with yellow boxes in Fig. 3.6a-b). For example, a bird might sing the syllable 'e' in the context 'daef' or in the context 'bbef'. In this case we compared the firing rate in the premotor window of 'e' between both contexts.

For this analysis we define a "sequence case" as one neuron-branch point pair. We ultimately analyzed 42 sequence cases from 20 neurons (14 putative pallidal, 6 putative striatal) and 6 song branch points from 4 birds. We found robust differences in premotor neural activity for the same syllable in different sequential contexts. Overall 23/42 sequence cases had significantly different ($p < .05$) firing rates depending on syllable context. In situations where a branch point had more than two possible sequence branches, we made pairwise comparisons and used a Bonferroni correction on the p-values (e.g. Fig. 3.6a,c). 14 neurons (including both striatal and pallidal neurons) and all six branch points analyzed had at least one significant sequence case. Examples from pallidal and striatal neurons are shown in Fig. 3.6. Cases were statistically significant for both sequence convergences and divergences, suggesting that the effects observed cannot be explained by residual representations of the previous

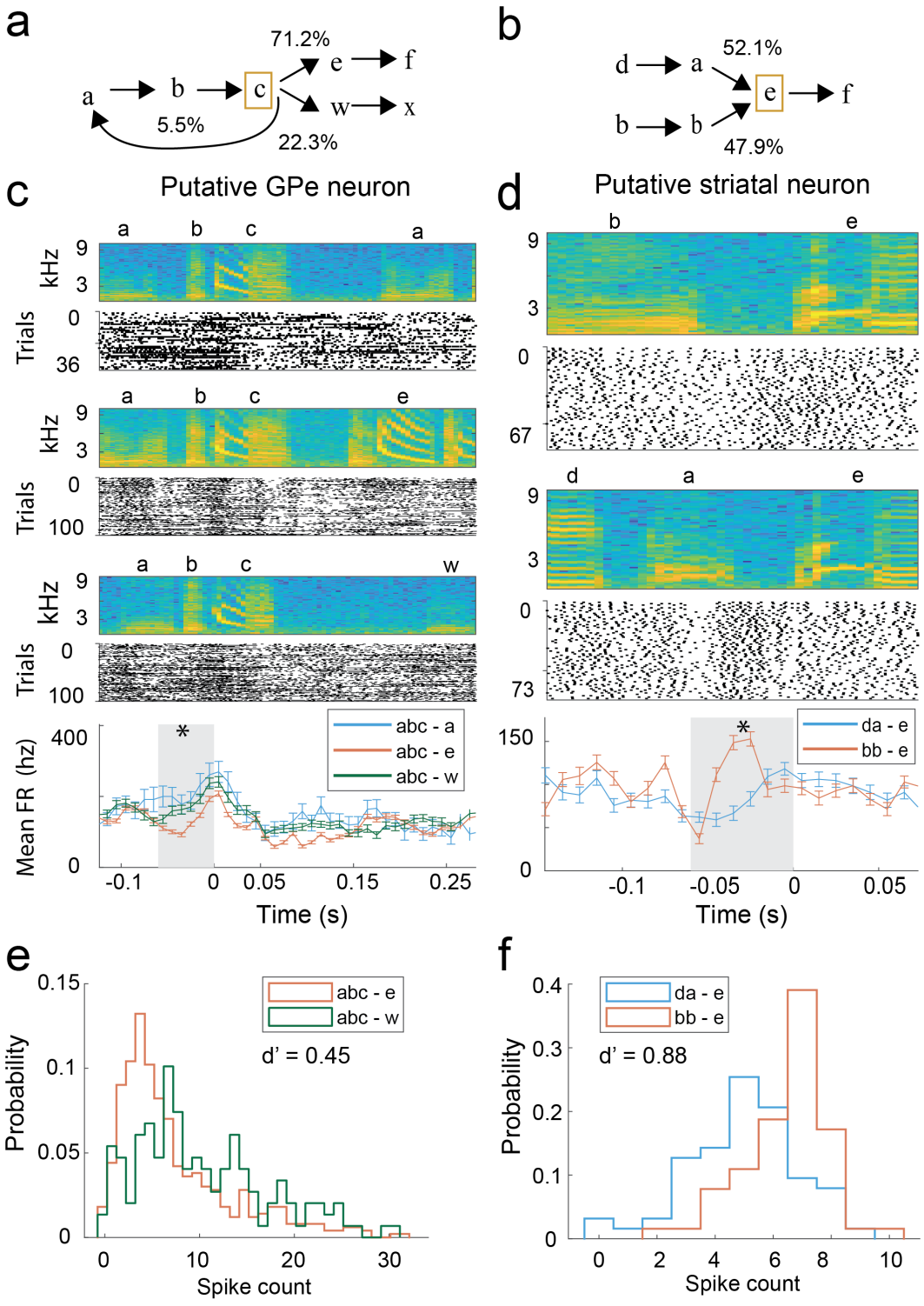
a
71.2%
a
b
c
e
f
w
x
5.5%
22.3%
b
52.1%
d
a
e
f
b
b
47.9%
c
Putative GPe neuron
d
Putative striatal neuron
a b c a
kHz
9
3
Trials
0
36
a b c e
100
a b c w
Mean FR (hz)
400
0
*
abc - a
abc - e
abc - w
-0.1
0.05
0.15
0.25
Time (s)
b e
67
d a e
73
150
da - e
bb - e
-0.05
e
Probability
0.15
0.1
0.05
d' = 0.45
Spike count
f
0.4
0.3
0.2
d' = 0.88
2
4
6
8
10

Figure 3.6: Examples of firing rate differences for the same syllable in different sequences. (a) Putative GPe neuron. Asterisk indicates significant difference ($p < .05$, two-tailed bootstrap test) in average firing rates during the shaded time window ('abc - e' vs 'abc - a' and 'abc - e' vs 'abc - w'; 'abc - w' vs 'abc - a' was not significantly different) after Bonferonni correction for multiple comparisons. Raster shows first 100 trials. (b) Putative striatal neuron. Asterisk indicates significant difference ($p < .05$, two-tailed bootstrap test) in average firing rates during the shaded time window. (c-d) Syllable transition diagrams for (a) and (b), respectively. Letters indicate individual syllables, percentages indicate transition probabilities at branch points. Yellow boxes indicate the syllable closest to the branch point that is in both sequences. (e-f) Example spike count histograms from sequence divergences shown in (a-c) and (b-d), respectively.

syllable.

We measured effect size using discriminability index (*d'*), the difference in means in units of standard deviation (Equation 3.1, see Methods for details). Among significant cases, the mean *d'* was 0.65 (Fig. 3.7a), indicating a robust effect.

To control for multiple comparisons, we performed a permutation test to quantify whether the observed proportion of significant sequence cases was greater than expected by chance (see 3.3.5 for more details). We found that our overall number of significant results (23 sequence cases) far exceeded the 99th percentile of the shuffled distribution (11 sequence cases; Fig. 3.7b), indicating that the observed proportion was significantly greater than that expected by chance.

3.4.3 Weak correlations between Area X activity and pitch

The AFP is required for learned changes in pitch in both Bengalese and zebra finches (Tumer and Brainard, 2007; Aronov et al., 2008). The primary mechanism through which the AFP drives learning is thought to be dopaminergic signalling in Area X (Hoffmann et al., 2016; Hisey et al., 2018; Xiao et al., 2018). There is also evidence that Area X is involved in short-timescale pitch modulations within syllables (Kojima et al., 2018). However, to our knowledge the relationship between single unit activity

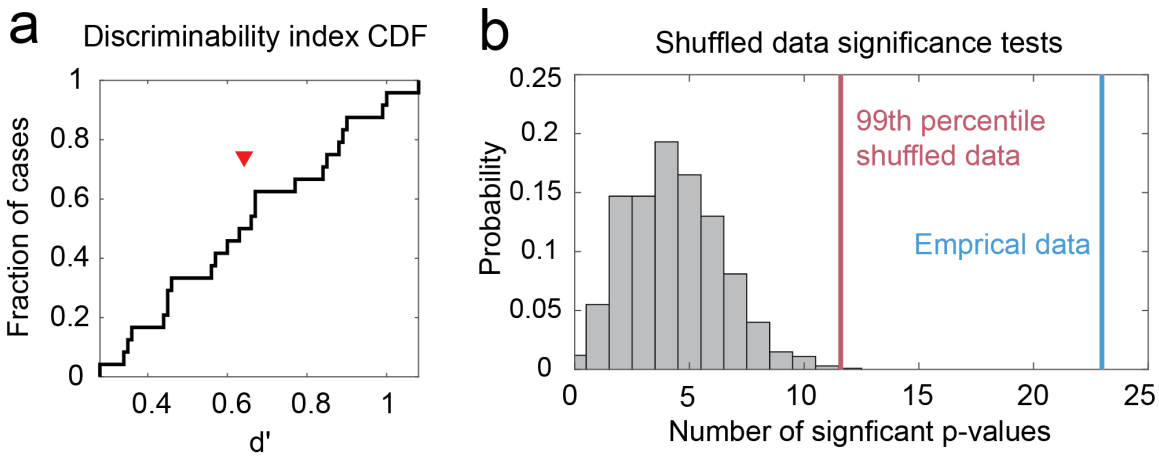

Figure 3.7: (a) Empirical cumulative distribution function plot of discriminability index (*d'*) values for statistically significant pitch cases. Red arrowhead indicates mean. (b) Permutation test results showing that the overall proportion of significant correlations was itself significantly greater than expected by chance.

in Area X and syllable phonology is completely unknown.

As in the analysis of sequence described above, firing rates were calculated for a 60 millisecond premotor window ending at the time of pitch quantification (illustrated in Fig. 3.8a). We measured the correlation between pitch and firing rate in 69 neuron-syllable pairs (referred to as "pitch cases") from 25 neurons (14 striatal, 11 pallidal) recorded across 4 birds. Example distributions of pitches, spike counts, and the associated pitch-spike count correlation are shown for one pitch-case in Fig. 3.8b-d. Overall, 16 pitch cases were significant ($p < .05$), and 11 neurons (4 striatal, 7 pallidal) had at least 1 significant pitch case.

Notably, the correlations we found between neural activity and pitch were very weak. In all but one pitch case, the r^2 values were less than 0.1 (see Fig. 3.8a for a representative example). The mean r^2 value for all significant pitch cases was 0.04 (i.e. only an average of 4% of the variance in pitch was accounted for by firing rate; Fig. 3.9a). Though not quite an "apples-to-apples" comparison, this is remarkably small compared to the effect size we observed in our sequence analysis (discussed in Chapter 3.5).

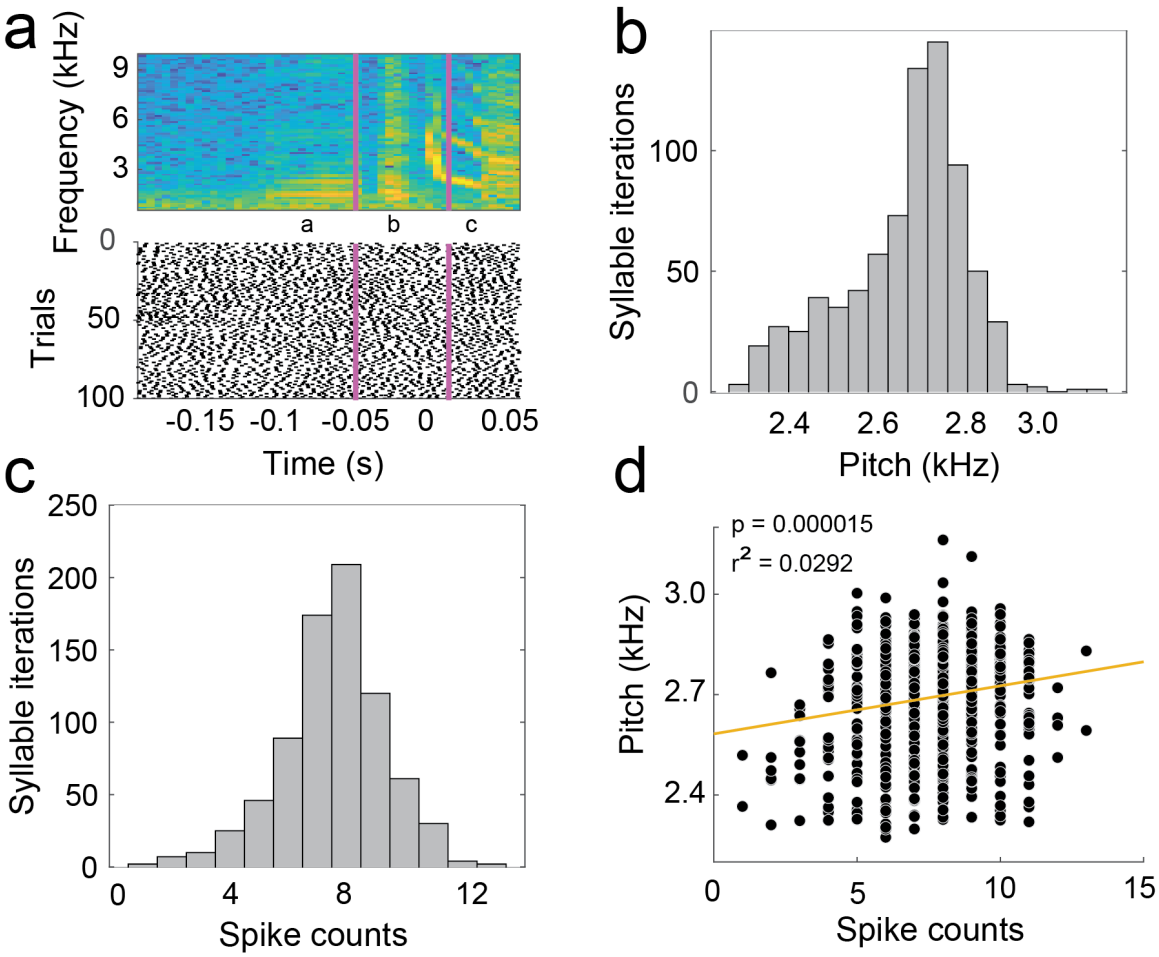

Figure 3.8: Representative weak correlation between Area X neuron firing rate and pitch. (a) Example spectrogram and raster plot (first 100 trials shown). Magenta lines indicate analysis window. (b) Pitch histogram. (c) Histogram of spike counts in analysis window. (d) Pitch vs spike count scatter plot in 60 millisecond window prior to the time of pitch analysis.

As with our analysis of sequence, we used a permutation test control for multiple comparisons (see 3.3.5 for more details). We found that our overall number of significant results (16) far exceeded the 99th percentile of the shuffled distribution (8; Fig. 3.9b), indicating that the observed proportions were significantly greater than those expected by chance.

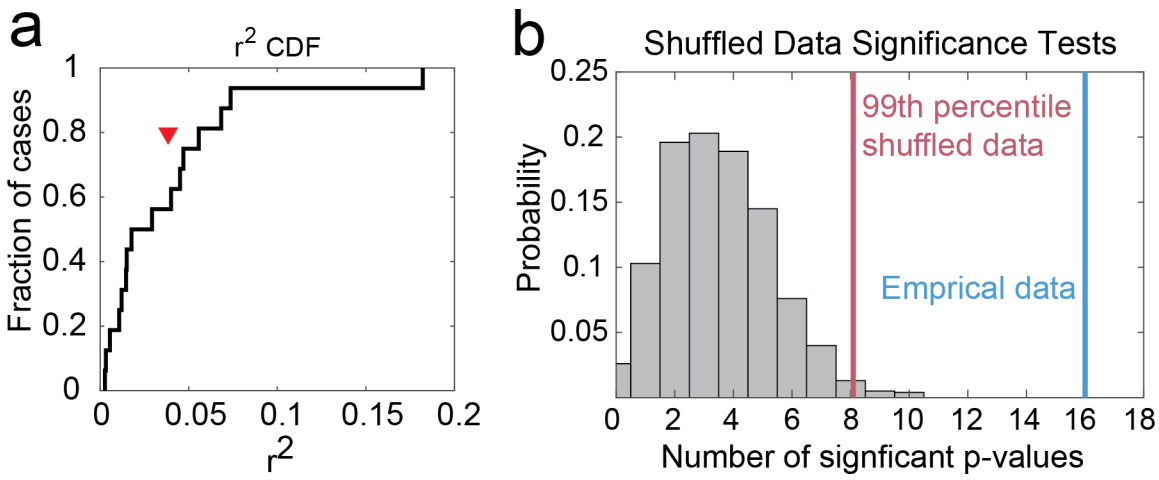

Figure 3.9: (a) Empirical cumulative distribution function plot of r^2 values for statistically significant pitch cases. Red arrowhead indicates mean. (b) Permutation test results showing that the overall proportion of significant correlations was itself significantly greater than expected by chance.

3.5 Discussion

In this study, we demonstrated that Bengalese finch Area X neurons can be separated in striatal, GPe and GPi subtypes, similar to zebra finches (Goldberg and Fee, 2010; Goldberg et al., 2010; Woolley et al., 2014). Area X neurons clustered into high and low firing rate groups, corresponding to putative pallidal and putative striatal neurons, respectively. We separated GPe and GPi neurons based on the former's high frequency bursts, using peak firing rate and percent spikes in burst during song (Fig. 3.3).

Our results also report for the first time a relationship between Area X neural activity and syllable sequencing (Figs. 3.6,3.7). Of all individual neuron-sequence cases analyzed, 23/42 were significantly different. The majority of neurons analyzed (14/20) had at least one syllable where firing rates were significantly different depending on sequential context. The mean *d'* value was 0.65, indicating that on average, the mean difference in firing rate for the branches in a sequence case was 0.65 standard deviations.

We also report for the first time correlations between Area X neural activity and syllable pitch (Figs. 3.8,3.9). We found significant correlations in 16/69 neuron-syllable pitch cases analyzed, and at least one significant correlation in 11/25 neurons included in this dataset. However, the correlations observed were weak ($r^2 < .1$ in all but one case). The mean r^2 was 0.04, suggesting very little variance in pitch could be explained by firing rate.

3.5.1 Comparisons between the songbird basal ganglia to the songbird vocal motor cortex

The only known pathway through which Area X can affect vocal motor output is by indirectly modulating RA activity through a thalamocortical circuit (Fig. 3.2a). RA is a pallial nucleus with functional and molecular similarities to mammalian motor cortex which projects directly to motor neurons in the brainstem (Nottebohm et al., 1976; Reiner et al., 2005). As we predicted, correlations between pitch and Area X neural activity were present but weaker than those found in RA. A previous study (Sober et al., 2008) in Bengalese finch RA using the same methods found a mean r^2 of .08 for neural activity and pitch. In contrast, we found a mean r^2 of .04 (Fig. 3.9a). However, in both RA and Area X a similar proportion of neuron-syllable cases had significant correlations (26.1 and 23.2 percent, respectively).

Why is the neuron-pitch relationship weaker in Area X than RA? Area X GPi neurons are three synapses away from RA, and neural signals from Area X must pass through the dorsolateral nucleus of the anterior thalamus (DLM) and LMAN to influence RA. Furthermore, LMAN neural activity is highly stochastic, and believed to function to inject biased variability into RA activity (Kao et al., 2005; Aronov et al., 2008; Ölveczky et al., 2011). Thus, any signal passing through LMAN likely becomes noisier prior to arriving at RA, potentially serving to generate the motor variability necessary for learning (Dhawale et al., 2017).

With respect to sequence, we found that 55% (23/42) of sequence cases analyzed were statistically significant, slightly less than the 63% reported in RA using a broadly similar approach. However, our average *d'* value was higher: 0.65 (Fig. 3.7a), compared to approximately 0.25 in RA (Wohlgemuth et al., 2010). Though differences in method make it challenging to draw a conclusive comparison, our results show that sequence-related differences in firing rate are larger in Area X than RA. This has potential implications for our understanding of the computational role of Area X. Specifically, given that Area X's necessity for normal song sequencing is ambiguous (Hampton et al., 2009; Kubikova et al., 2014), we need to ask what other roles strong sequence-related activity in Area X could play.

3.5.2 Is Area X activity involved in online acoustic control?

Area X sits at the interface between between a cortico-basal ganglia circuit that drives dopaminergic learning signals but does not appear to be required for song production, and a premotor circuit that directly modulates vocal output via the projection from LMAN to RA (Chen et al., 2019; Kearney et al., 2019). To what extent Area X is a premotor structure that influences ongoing behavior vs a learning structure that influences behavior on longer timescales is not entirely clear. In the following sections we will discuss different hypotheses for how neural activity in Area X influences song.

Area X could broadly be involved in two aspects of motor control: Online control of movement (influencing actions as they happen), or motor learning (influencing downstream circuits in a way that will influence future actions). An example of online control would be selection of a particular action (e.g. which syllable to sing next at a branch point). In contrast, for a learning related function, the probability of selecting a particular action in the *future* is altered by inducing plastic changes in downstream circuits. In this section we will discuss the evidence for and against a role for Area X in online control of sequence and phonology.

Online control of syllable sequence

Area X, like the mammalian basal gangila, has been implicated in many aspects of motor control and motor learning (e.g. Sohrabji et al. 1990; Hessler and Doupe 1999; Leblois et al. 2007; Kubikova et al. 2014; Kojima et al. 2018; Xiao et al. 2021). One area of considerable disagreement in both mammals and birds is the specific role of the basal ganglia in motor sequencing (see Chapter 1.3.2 for a discussion of mammalian studies). Our results show large differences in average firing rates for the same syllable in different sequential contexts (fig 3.6), pointing to a potential role in control of sequence.

The only known path through which Area X could influence behavior goes through the premotor nucleus LMAN. Interestingly, however, LMAN lesions have little to no impact on syllable sequencing in Bengalese finches (Hampton et al., 2009). On the other hand, several studies in zebra finches find that more targeted Area X manipulations cause longer term changes to song sequence (Kubikova et al., 2014; Xiao et al., 2021). Most compellingly, loss of approximately 43% of Area X MSNs due to expression of mutant huntingtin protein leads to a loss of song-locked activity patterns in pallidal neurons and a drastic increase in syllable sequence variability (Tanaka et al., 2016). Thus, it seems that while neural activity from Area X is not necessary for normal song sequence, it does have the ability to powerfully affect it.

There is also some evidence that the role of Area X in song sequence may vary with age. In juvenile zebra finches, partial ablation of HVC-Area X projection neurons (a likely source of sequence modulation in Area X, Zhang et al. 2017) disrupts numerous aspects of song, including sequencing. In adults, however, the same manipulation had no effects on song performance (Sánchez-Valpuesta et al., 2019). This is consistent with the outsized influence the AFP has on RA during juvenile song learning, and its decreased influence compared to HVC-RA projections in adults (Ölveczky et al., 2011; Garst-Orozco et al., 2014).

Collectively, these studies point away from the hypothesis that Area X is a primary locus of control for syllable sequencing. However, it is able to affect sequence under certain conditions, and our results show large sequence-dependent changes in firing rate. This is more consistent with a role for Area X in sequence learning (discussed below in 3.5.3).

Online control of syllable phonology

Though Area X is not necessary for normal song production (Nottebohm et al., 1976; Sohrabji et al., 1990) it is hypothesized to play a role in the generation of pitch variability (Woolley and Kao, 2015; Kojima et al., 2018). Variability in Area X neural activity has been linked to behavioral states of high variability. When singing in isolation (directed song), song features and Area X neural activity are both more variable when compared to singing to a female (directed song) (Hessler and Doupe, 1999; Woolley et al., 2014; Woolley, 2016). However, the precise behavioral *consequence* of this activity may be quite nuanced: Lesions to Area X only transiently disrupt trial-by-trial song variability (Goldberg and Fee, 2011; Ali et al., 2013) but have a longer term effect on pitch modulations within individual syllables (Kojima et al., 2018). This is consistent with the weak nature of the trial-by-trial correlations we observed between Area X neural activity and pitch. Future studies will need to be done to determine whether or not Area X neural activity is more predictive of within-syllable modulations.

3.5.3 How Area X neural activity could contribute to learning

An alternative to the online control hypothesis outlined above is one where Area X functions as the center of a reinforcement learning circuit. Under this hypothesis, behavior-neural activity relationships in Area X are not primarily reflective of Area

X's influence on ongoing behavior; instead, they are related to different computational aspects of learning.

Sequence-related neural activity as temporal scaffolding for reinforcement learning

Numerous models of song learning are built around the framework of reinforcement learning (Doya and Sejnowski, 1996; Fiete and Seung, 2009; Fee and Goldberg, 2011; Teşileanu et al., 2017; Chen and Goldberg, 2020). In computational neuroscience, reinforcement learning refers to a broad category of learning algorithms that assign values to actions based on their likelihood of leading to reward, which are learned through trial and error (See Chapter 1.4.1 for further discussion of reinforcement learning algorithms; Sutton and Barto 2018). When applied to song learning, the value of a specific motor command could be derived from how well it matched the bird's intended vocalization, or an external cue.

In most reinforcement learning frameworks for song, Area X is posited to be the key region where values are updated (Doya and Sejnowski, 1996; Fee and Goldberg, 2011; Chen and Goldberg, 2020). A major reason for this is the presence of a large dopaminergic projection to Area X (Bottjer, 1993; Person et al., 2008). In mammals, a substantial body of literature suggests that dopamine functions as a reward prediction error (RPE)(Glimcher, 2011; Schultz, 2019). RPEs are a core part of a subclass of reinforcement learning algorithms called "model-free" algorithms. Briefly, RPEs are the difference between the experienced value of an action and the expected value of an action. If an animal performs an action (e.g. a bird initiates a motor program for singing a syllable at a particular pitch), and the outcome of that action matches expectations (the bird heard itself sing the syllable at the pitch it intended), no RPE will be generated. In contrast, if the animal performs an action and has an unexpected result (the bird intends to sing at a high pitch, but hears itself sing at a low pitch)

an RPE will be generated and used to update the perceived value of that motor command. Though primarily studied in the context of learning syllable phonology, the same process could be used to drive learnign of syllable sequences (Warren et al., 2012)

There is growing evidence that song learning is achieved through reinforcement learning. In songbirds, both experimenter-driven pitch learning in adults and natural tutor song learning in juveniles relies on dopaminergic signalling in Area X (Hoffmann et al., 2016; Hisey et al., 2018; Xiao et al., 2018). Further, Area X-projecting dopaminergic neurons increase activity in response to better-than-expected outcomes during song (Gadagkar et al., 2016), in a manner consistent with RPE signalling. Thus, there is good reason to ask how the neural signals we observe fit into this framework.

The role of sequence information in song reinforcement learning models

Syllable sequence is itself a flexible behavior that could be flexibly learned through reinforcement learning (Warren et al., 2012). However, sequence-related activity in Area X could function to ensure that *all* Area X-mediated learning (e.g. sequence or phonology) happens at the correct time in song.

A key issue in hypothesized biological implementations of reinforcement learning is the need for an eligibility trace to ensure that the correct synapses are modified by the dopaminergic RPE signal (Pan et al., 2005; Yagishita et al., 2014). There is strong evidence from studies in mammals that this requires coincident activity (Fino and Venance, 2010). Under the bird song reinforcement learning hypothesis, for the value of an action to be updated dopaminergic RPEs need to converge with contextual information in the form of an efference copy of the motor command transmitted from LMAN to RA, and temporal information about *when* in song the bird currently is, presumably from HVC-Area X projections (Reiner et al., 2005; Fee and Gold-

berg, 2011). In the Bengalese finch, where song sequencing is variable, rather than representing song time *per se*, this signal would need to include sequence-specific information. Thus, sequence-related activity in Area X could reflect an eligibility trace enabling dopamine-dependent plasticity in circuits responsible for transferring learned changes to song from the AFP to motor pathway (MP).

3.5.4 Conclusions

We define neuron types for the first time that Area X single unit neural activity contains information about both pitch and sequence (Figs. 3.6,3.8). A major challenge in the interpretation of this study is comparing the relative strength of the effect of phonology vs. sequence on neural activity. Because pitch is continuous and sequence is categorical, making direct statistical comparisons of effect size is fraught. However, in comparison to data from RA we find the relative effect of pitch on neural activity to be quite small, while the relative effect on sequence is robust. This is consistent with reinforcement learning models of song, which require a temporal signal about when in song an action is being reinforced. Future work will be required to assess whether neural signals in Area X reflecting sequence function as ongoing behavioral control, reflects an eligibility trace for temporally precise learning, or both (discussed in Chapter 4).

Chapter 4

Future Directions

In the studies described above, we characterized dopaminergic projections in the songbird Bengalese finch, including the surprising finding that a large population of neurons are putatively non-dopaminergic. We also report for the first time in songbirds changes in Area X single unit activity associated with syllable pitch and syllable sequence. Here, we propose a number of anatomical, electrophysiological, and behavioral studies that might expand on this work and further elucidate the role of dopamine and the basal ganglia in motor control and motor learning.

4.1 Which neurotransmitters are used by TH- Area X projectors?

We found a large population of putative TH- neurons in ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) projecting to Area X (Fig. 2.4). As a next step, future work might determine the neurotransmitter identities of these neurons to better understand the comparative neuroanatomy of the basal ganglia across species. Determining the neurotransmitter identity of TH- neurons will also be a crucial step for functional studies of their role in behavior.

Non-dopaminergic projection neurons in primarily dopaminergic regions are also present in mammals. In rodents, there are large populations glutamatergic and GABAergic projection neurons in VTA (Taylor et al., 2014), as well as populations of 'combinatorial' neurons that release more than one of dopamine, GABA, and glutamate (Morales and Margolis, 2017). While the main structures of the dopaminergic midbrain appear to be highly conserved between birds and mammals (Dahlstrom and Fuxe, 1964; Reiner et al., 1994, 2004; Kingsbury et al., 2011), it is unknown what neurotransmitters are released by TH- projection neurons in songbird VTA and SNc, or whether TH+ neurons can co-release other transmitters along with dopamine. Future studies could test this using a mixture of immunohistochemistry or *in situ* hybridization to determine if Area X projection neurons contain markers of glutamatergic, GABAergic, or other modulatory neurons.

After determining the neurotransmitters used by TH- projection neurons, an important next question will be asking on which Area X cell types they form synapses. For example, in rodents, VTA GABAergic projection neurons to NAc target tonically active cholinergic interneurons (Brown et al., 2012). Cholinergic interneurons in turn modulate local DA release (Threlfell et al., 2012), suggesting a possible role for VTA GABAergic projection neurons in driving learning by shaping dopaminergic signals to the striatum. Whether or not a similar local release control mechanism exists in birds is presently unknown, but once the identity of TH- VTA projection neurons is determined slice electrophysiology experiments could be used to identify cell-specific projection targets. For example, if TH- projection neurons are determined to be GABAergic, their axonal projections in Area X could be identified and selectively optogenetically activated during whole-cell patch clamp recordings of different Area X neuron subtypes. This would allow us to determine which cells types are directly inhibited by VTA-GABA neurons.

To assess the function of TH- VTA/SNc projection neurons, viral techniques could

be used to specifically excite, inhibit, or ablate populations of TH- neurons in birds during song learning. Previous studies in zebra finches have shown that when VTA-Area X terminals are stimulated during high pitch renditions of a syllable, mean pitch is driven up over time (or down when stimulation is targeted to low-pitch renditions; Hisey et al. 2018; Xiao et al. 2018), mirroring previous studies showing that learning can be driven when external white noise is contingent on pitch (Tumer and Brainard, 2007). However, previous studies targeting VTA-Area X projections did not attempt to differentiate TH+ and TH- populations. Future studies could use specific viral vectors to target sub-populations of TH- VTA-Area X projection neurons to assess whether (1) pitch-contingent activation of these neurons alone is sufficient to induce learning, and (2) whether inhibition of these terminals during pitch-contingent white noise enhances, impairs, or has no effect on learning compared to control birds without inhibitory opsins. Additionally, viral vectors could be used to introduce a caspase-3 construct to ablate specific populations of VTA-Area X neurons in order to determine whether they are necessary for learning. Further, our results suggest that Bengalese finches have a larger population of TH- neurons than zebra finches, making them a particularly interesting subject for studying the role of TH- neurons in behavior.

4.2 How does dopamine depletion affect neural activity in Area X?

Studies using chemical and viral techniques to lesion dopaminergic projections to Area X have found that dopaminergic signalling in Area X is necessary for song learning (Hoffmann et al., 2016; Hisey et al., 2018; Xiao et al., 2018). It is possible that learning impairments after loss of dopamine could be purely due to the loss of dopaminergic learning-related signals and that dopamine depletion may not affect typical song-locked firing patterns in Area X. However, other behavioral changes after 6-OHDA

lesions suggest there may be broader changes in Area X after chronic dopamine depletion. Dopamine depletion in Area X using 6-hydroxydopamine (6-OHDA) leads to changes in pitch over the course of several weeks, primarily in the form of a small but reliable downward shift (Saravanan et al., 2019). There is also some evidence that 6-OHDA reduces song variability (Miller et al., 2015), though other studies do not find this effect (Hoffmann et al., 2016). While the precise neurophysiological mechanism underlying these changes are unknown, it suggests the possibility of Area X dysfunction after dopamine depletion that extends beyond a simple loss of learning-related signals.

In mammals, dopamine depletion has a profound effect on neural activity throughout the basal ganglia, suggesting that the same might be true for dopamine depletion in Area X. For example, 6-OHDA lesions in the striatum of rodents lead to abnormal burst patterns and a decrease in movement-associated activity patterns in striatal neurons (Chen et al., 2018; Hernandez et al., 2013; Panigrahi et al., 2015). In both patients with Parkinson's disease and nonhuman primates with widespread dopamine depletion, activity in globus pallidus internal (GPi) and globus pallidus external (GPe) has highly abnormal bursting patterns, which are thought to contribute to Parkinsonian motor deficits (DeLong and Wichmann, 2010).

In songbirds, there are several factors that make it challenging to predict how chronic dopamine depletion affects Area X output from GPi neurons to the thalamus. Like mammalian basal ganglia circuits, Area X contains a direct pathway where inhibitory medium spiny neurons (MSNs) project to inhibitory GPi neurons (which in turn project to the thalamus) and an indirect pathway, where MSNs project to inhibitory GPe neurons, which project to GPi neurons (Reiner et al., 2005). MSNs, GPe, and GPi neurons all contain dopamine receptors. However, unlike the mammalian basal ganglia Area X does not have a known subthalamic nucleus (STN) homolog or projection to STN outside of Area X (Person et al., 2008). In mammals,

the reciprocal connections between STN and GPe are thought to play an important role in the generation and amplification of pathological neural activity after dopamine depletion (Nambu and Tachibana, 2014). Thus, depending on the particular effect dopamine depletion has on each of these neural subtypes, a variety of changes in GPi output to the thalamus are possible and may not match results from mammals.

There are also key differences in dopamine receptor expression patterns between birds and mammals that could influence the overall effect of dopamine on Area X neural activity. Like in mammals D1-type receptors are excitatory while D2-type receptors are inhibitory (Hernández-López et al., 1997, 2000; Ding and Perkel, 2002). Unlike in mammals, where direct pathway MSNs predominantly express D1 dopamine receptors and indirect pathway MSNs predominantly express D2 dopamine receptors (Gerfen et al., 1990), Area X contains a mixture of D1-expressing MSNs (likely, but not confirmed direct pathway MSNs), D2-expressing MSNs (likely, but not confirmed indirect pathway MSNs) and a third population of MSNs that expresses both D1 and D2 receptors (Kubikova et al., 2010; Xiao et al., 2021). Consistent with this, in slice preparations dopamine has a variable effect on MSN excitability that doesn't neatly cluster into inhibited and excited groups (Ding and Perkel, 2002). In mammals, pathological basal ganglia output from dopamine depletion is thought to arise from imbalanced activity between the D1-expressing direct pathway MSNs and D2-expressing indirect pathway MSNs, leading to hyperactivity in the GPi. However, it is not clear whether MSNs expressing both D1 and D2 receptors in birds belong to the direct or indirect pathway, or how they might affect GPi neuron output in either normal or dopamine-depleted states.

Electrophysiological recordings from Area X in dopamine depleted songbirds would address three pressing questions. First, it may be able to provide insight into the role of HVC-X projections in the generation of sequence-dependent activity patterns in Area X neurons. Dopamine decreases responses in Area X pallidal neurons to HVC

electrical stimulation in anesthetized birds (Leblois et al., 2010). If, as we hypothesize in Chapter 3.5.2, HVC-X projection neurons are the primary source of sequence-related changes in neural activity in Area X neurons, we may expect an increase in the fraction of recorded Area X neurons with significant sequence modulation and an increase in the average discriminability index for statistically significant neuron-branch cases. Second, electrophysiological recordings after dopamine depletion could provide insight into the role of Area X in controlling ongoing variations in pitch. Studies in awake, singing birds show striking modulation of firing patterns in both striatal and pallidal neurons in social contexts associated with high and low dopamine levels in Area X. The low dopamine state ('undirected song', performed without a female bird present) is associated with higher variability in pallidal neuron firing patterns (Woolley et al., 2014; Woolley, 2016). If we see similar changes after dopamine depletion, and those changes are correlated with changes in neuron-behavior relationships, that could help provide insight in to the role of Area X neural activity in regulating vocal acoustics. Finally, electrophysiological recordings in dopamine depleted birds could elucidate the mechanism of long term vocal changes after dopamine depletion with 6-OHDA (Saravanan et al., 2019). It is possible that these changes are due to widespread pathological changes to GPi output from the basal ganglia, similar to that observed in mammals. To assess this, electrophysiological recordings could be performed at different timepoints after dopamine depletion and features such as bursting patterns could be correlated to the onset of vocal changes.

4.3 What is the timescale of Area X neuron-behavior correlations?

We find a strong relationships between Area X single unit firing rate and sequence, and a weak relationship with pitch in a 60 ms premotor window (the time window

prior to syllable onset) using a means-difference bootstrap test and linear correlation, respectively (Figs. 3.6 and 3.8). One limitation of our analysis is that it assumes that the important feature of neural activity in Area X with respect to behavior is relatively slow (60 milliseconds) modulations in firing rate, and ignores spike patterns within the 60 millisecond window. However, there is evidence from several species (including songbirds) that the precise (millisecond-level) timing of spikes is an important feature of neural activity in both sensory and motor systems (Fairhall et al., 2012; Sober et al., 2018). Therefore, future studies should explicitly test the timescale of neural activity-behavior relationships in Area X.

Though very little is known about the neural coding scheme used for syllable sequence, there is evidence that precise spike timing is important for phonology elsewhere in the song system. In the robust nucleus of the arcopallium (RA), the songbird motor nucleus (analogous to mammalian motor cortex), precise spike timing (millisecond-level resolution spike patterns) is on average much more predictive of song phonology than firing rates binned over larger windows (up to 40ms; Tang et al. 2014). It is not currently known whether this is a common feature of neural coding throughout the song system for all vocal motor behavior, specific to just RA, or specific to just phonology.

There are reasons to think that Area X might not make use of precise spike timing. RA and Area X sit at very different places within the song system (Fig. 3.2a), have very different firing properties, and have very different hypothesized functions. RA is analogous to mammalian motor cortex and synapses directly on to motor neurons. It is the primary common path through which the entire song system influences song. Area X, by contrast, is a basal ganglia nucleus several synapses upstream from RA, and is not necessary for broadly normal song production, though it is necessary for song learning (Nottebohm et al., 1976; Sohrabji et al., 1990). While RA projection neurons are excitatory and fire in syllable-locked bursts, the output neurons of Area

X are pallidal neurons are inhibitory and have high tonic firing rates with subtle song-locked modulations. Therefore, it is not clear in principle that RA and Area X *should* use the same coding scheme.

On the other hand, there is reason to think that precise spike timing might matter for online control and learning in Area X. There is evidence that the anterior forebrain pathway (AFP) and Area X in particular are involved in sub-syllable phonological control (Kojima et al., 2018), potentially requiring equivalently short-timescale neural control. Further, birds can learn to modify the pitch of a syllable at millisecond-level timescales via reinforcement learning in a paradigm that requires an intact AFP (Charlesworth et al., 2011; Tumer and Brainard, 2007). Temporally precise learning requires temporally precise neural mechanisms for representing vocal motor commands and learning-related inputs. Precise spike timing in Area X is one possible way to achieve that.

It is possible that the timescale of neuron-behavior relationships may be different for phonology (Fig. 3.8) and sequence (Fig. 3.6). The timescale of *behavioral* variation for phonology and sequence are different: Phonological variables such as pitch can vary at sub-syllable timescales (as little as 1 millisecond), while sequence by definition varies based on the length of its constituent syllables, which can be greater than 50 milliseconds (Charlesworth et al., 2011). However, it is important to note that precise spike timing can still contribute to relatively slow behaviors; for example, in songbird respiratory motor neurons millisecond-level differences in spike timing affect breath cycles that last for hundreds of milliseconds (Srivastava et al., 2017). Because of this, future studies should examine the timescale of Area X neuron-behavior relationships for both pitch and phonology.

Testing the timescale of neuron-behavior correlations in Area X is important for understanding how information is propagated throughout the song system. This could be done using electrophysiological recordings from single neurons in Area X in

singing birds, and making use of analysis methods that determine the strength of the relationship between behavior spike times different time windows (e.g. Tang et al. 2014) or specific spike patterns (e.g. Hernández et al. 2019). If Area X single unit neural activity correlates with behavior at the same timescale as RA, that suggests that precise timing may be a common feature throughout basal ganglia and cortical networks for vocal control. If Area X is correlated with behavior on longer timescales than RA, it raises interesting questions about how the neural code is transformed from a less temporally precise signal in the AFP to a more precise one in the motor pathway (MP).

4.4 What is the role of Area X neural activity in vocal learning?

Area X is thought to play a role in both learning and vocal motor control (Fee and Goldberg, 2011; Woolley and Kao, 2015; Kojima et al., 2018). Our results show that two very different behavioral variables, pitch and syllable sequence, both correlate with the firing rate of Area X single units (Figs. 3.6, 3.8). However, it is not clear whether this represents a premotor signal, a learning signal, or some combination of the two. Several future studies taking advantage of both naturalistic learning in juveniles and experiment-driven learning in adults, combined with *in vivo* neurophysiology and manipulation of specific neural circuits could help answer the question of motor control vs learning.

Juveniles offer an avenue through which to test the potential premotor function of Area X neural activity. Song is primarily controlled by two pathways: The MP and AFP (Fig. 3.2a). In juveniles, the AFP drives early subsong (akin to human infant "babbling"; Marler 1970a; Aronov et al. 2008; Goldberg and Fee 2011). During plastic song, the AFP plays a crucial role in both learning and generating the

behavioral variability necessary for learning to occur (Woolley and Kao, 2015). Over time, as song begins to crystallize into its stable adult form, the AFP loses influence relative to HVC, a premotor nucleus in the MP. If Area X has a premotor function, we might expect single unit neural activity to be more correlated with behavior (pitch or sequence) during plastic song, reflecting the overall stronger relative influence of the AFP on vocal output during this time. We would expect the strength of the correlation to decline as song crystallized and HVC exerted greater control over RA and song. In contrast, if neuron-behavior correlations in Area X reflect part of a learning signal, we would not expect to see a change between plastic and adult song.

Adult song learning paradigms offer the opportunity for us to test the role of sequence-related neural activity in learning. In adults, the MP is required for normal production of song, while lesions to lateral magnocellular nucleus of the anterior nidopallium (LMAN), the output nucleus of the AFP, cause changes in trial-to-trial variability but leave the general structure of song intact (Kao et al., 2005). However, LMAN lesions do cause learning impairments (Tumer and Brainard, 2007). In Chapter 3.5.2, we described the hypothesis that Area X functions to compute the values of vocal motor actions as part of a reinforcement learning circuit (Fee and Goldberg, 2011; Chen and Goldberg, 2020). Under this framework, sequence information reflected in Area X neural activity could provide a song time/context signal, which is required for temporally-specific reinforcement learning to occur but does not necessarily play a premotor role in syllable sequencing. As described above, it is likely that sequence-related activity in Area X neurons originates from HVC, a premotor nucleus that projects to both Area X and RA and is important for controlling song tempo (Long and Fee, 2008; Zhang et al., 2017). If our hypothesis about the origin of sequence modulation is true, ablating HVC-X neurons using viral vector techniques (Sánchez-Valpuesta et al., 2019) should abolish sequence-dependent modulation of Area X single unit neural activity. Further, our hypothesis predicts that this neural

activity acts as a scaffold for learning, rather than a premotor signal, than adult birds with ablated HVC-X neurons should be impaired in learning paradigms that drive temporally precise learning (Charlesworth et al., 2011). Together, the experiments outlined above would provide invaluable information for elucidating the precise role of Area X neural activity in vocal behavior and give insight to the role of the basal ganglia more broadly.

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